U.S. Food & Drug Administration

Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Table of Contents

BAM Media

BAM Reagents

About the Bacteriological Analytical Manual

FDA's Bacteriological Analytical Manual (BAM) presents the agency's preferred laboratory procedures for microbiological analyses of foods and cosmetics. AOAC International published previous editions of this manual in a loose-leaf notebook format, and, more recently, on CD-ROM. This online BAM is now available to the public. Some changes have been made to methods since the previous version. A listing of chapters updated since the last hard-copy version (Edition 8, Revision A /1998) can be found in About the Bacteriological Analytical Manual. Chapter numbers have been retained from the previous version, however, for this Table of Contents, chapters have been grouped by category. Please send comments to Ruth.Bandler@cfsan.fda.gov.

General Guidelines/Procedures

Methods for Specific Pathogens

19 A	Concentration and Preparation of *Cyclospora* from Berries for Polymerase Chain Reaction (PCR) and Microscopy	J.W. BIER, J.E. LeCLERC, and K.G. JINNEMAN Contact: P.A. ORLANDI
26	Detection and Quantitation of Hepatitis A Virus in Shellfish by the Polymerase Chain Reaction	B.B. GOSWAMI

Methods for Microbial Toxins

13 A	Staphylococcal Enterotoxins: Micro-slide Double Diffusion and ELISA-based Methods	R.W. BENNETT
13 B	Electrophoretic and Immunoblot Analysis of Staphylococcal Enterotoxins	A. RASOOLY
15	*Bacillus cereus* Diarrheal Enterotoxin	R.W. BENNETT

Gene Probe Methods for Foodborne Pathogens

24	Identification of Foodborne Bacterial Pathogens by Gene Probes	W.E. HILL, A.R. DATTA, P. FENG, K.A. LAMPEL, and W.L. PAYNE

Additional Methods

Appendixes

Introduction

To test for an organism or microbial toxin not covered by the BAM, or to analyze a sample that may require special handling or processing, the user is referred to the *Official Methods of Analysis* of the AOAC International; *Standard Methods for the Examination of Dairy Products*, *Recommended Procedures for the Examination of Seawater and Shellfish*, and *Compendium of Methods for the Microbiological Examination of Foods* of the American Public Health Association; also, *Standard Methods for Water Analysis* of the Environmental Protection Agency. FDA works closely with AOAC International, APHA, EPA, the International Dairy Federation (IDF/FIL), and, by way of participation in Codex Alimentarius, the International Organization for Standardization (ISO). However, not all methods appearing in the BAM have been collaboratively evaluated by one or more of these organizations.

Text for the BAM was peer-reviewed by scientists outside and within FDA.

Introduction to the 8th edition, Revision A (1998)

Innovations in methods for the microbiological analysis of food continue to appear at a rapid pace. Edition 8 (1995) of the Bacteriological Analytical Manual (BAM-8) contained numerous

refinements of procedures and updates of references from the 1992 edition. The list of commercially available test kits and the discussion of rapid methods in Appendix 1 were thoroughly revised. Three chapters were added: the use of reverse transcription (RT) and the polymerase chain reaction (PCR) to detect and quantify contamination of shellfish with hepatitis A virus (Chapter 26); new procedures for the alkaline phosphatase test to determine whether dairy foods were prepared with pasteurized milk (Chapter 27); and the use of PCR to detect toxigenic Vibrio cholerae in foods (Chapter 28). For this printing (BAM - 8A), the following has been revised or added: Campylobacter (Chapter 7), Yeast and Molds (Chapter 18), Cyclospora [Chapter 19 (Parasites)] and Staphylococcus enterotoxins (Chapter13). In addition, there are updated tables in Appendix 1 on Rapid Methods and revised and corrected tables in Appendix 2 on MPN. Appendix 3 reflects changes in media and corrects errors in the 8th Edition. A table summarizing changes from BAM-8 to BAM-8A is included.

The methods described in Chapters 1 to 28 are those preferred by FDA for the microbiological analysis of foods, drinks, and cosmetics as well as for their containers, contact materials, and the production environment. This is not necessarily the case for the rapid methods listed in Appendix 1: this appendix is a listing of different kits that are commercially available. These methods have not necessarily been evaluated by FDA, and listing of a method in this appendix does not constitute a recommendation.

To test for an organism or microbial toxin not covered by the BAM, or to analyze a sample that may require special handling or processing, the user is referred to the Official Methods of Analysis of the AOAC International; Standard Methods for the Examination of Dairy Products, Recommended Procedures for the Examination of Seawater and Shellfish, and Compendium of Methods for the Microbiological Examination of Foods of the American Public Health Association; also, Standard Methods for Water Analysis of the Environmental Protection Agency. FDA works closely with AOAC International, APHA, EPA, the International Dairy Federation (IDF/FIL), and, by way of participation in Codex Alimentarius, the International Organization for Standardization (ISO). However, not all methods appearing in the BAM have been collaboratively evaluated by one or more of these organizations.

Text for the BAM was peer-reviewed by scientists outside and within FDA. Outside reviewers included P. Entis, J. Smith, M. Doyle, N. Stern, R. Twedt, S. Tatini, R. Labbe, M. Eklund, M. Cousin, L. Eveland, R. Richter, J. Kabara, M. Curiale, and the staff of the National Food Processors Association. Reviews by FDA's field microbiologists, who made valuable suggestions concerning content and practicality, were coordinated by Meredith A. Grahn and her staff.

The 8th Edition of the BAM was prepared in the Technical Editing Branch, Center for Food Safety and Applied Nutrition, FDA by Lois A. Tomlinson with production assistance by Dorothy H. Hughley. This version (Revision A) of the 8th Edition, was prepared and produced by Dr. Robert I. Merker, Office of Special Research Skills, CFSAN, FDA.

Modified by: rim 2000-03-30 from Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Top

B A M | B A M Media | B A M Reagents | Bad Bug Book | Microbiological Methods

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

Media Index

(Return to Table of Contents)

No. in BAM 8, Rev. A	Name of Medium
M1	A-1 Medium
M29a	Abeyta-Hunt-Bark Agar
M2	Acetamide Medium
M3	Acetate Agar
M4	Acid Broth
M5	AE Sporulation Medium, Modified (for *C.perfringens*)
M6	Agar Medium P
M7	AKI Medium
M8	Alkaline Peptone Agar
M9	Alkaline Peptone Salt Broth (APS)
M10	Alkaline Peptone Water
M11	Anaerobe Agar
M12	Anaerobic Egg Yolk Agar

M13	Andrade's Carbohydrate Broth and Indicator
M14	Antibiotic Medium No.1 (Agar Medium A)
M15	Antibiotic Medium No.4 (Agar Medium B)
M16	Arginine-Glucose Slant (AGS)
M17	Baird-Parker Medium, pH 7.0
M18	Bile Esculin Agar
M19	Bismuth Sulfite Agar (Wilson and Blair)
M20	Blood Agar
M20a	Blood Agar Base
M21	Blood Agar Base (Infusion Agar)
M22	Blood Agar Base #2 (Difco)
M23	Brain Heart Infusion (BHI) Agar (0.7%) (for staphylococcal enterotoxin)
M24	Brain Heart Infusion (BHI) Broth and Agar
M25	Brilliant Green Lactose Bile Broth
M26	Bromcresol Purple Broth
M27	Bromcresol Purple Dextrose Broth (BCP)
M28a	Campylobacter enrichment broth (Bolton formula)
M31	Cary-Blair transport Medium
M32	Casamino Acids-Yeast Extract (CYE) Broth
M34	Casamino Acids-Yeast Extract-Salts (CA-YE) Broth (Gorbach)
M35	Cefsulodin-Irgasan Novobiocin (CIN) Agar
M36	Cell Growth Medium
M187	Cellulase Solution
M37	Cetrimide Agar
M38	Chopped Liver Broth
M39	Christensen Citrate Agar
M40	Christensen's Urea Agar
M41	Congo Red BHI Agarose (CRBHO) Medium*
M42	Cooked Meat Medium

M43	Cooked Meat Medium (Modified)
M44	Decarboxylase Basal Medium (Arginine, Lysine, Ornithine)
M184	Dichloran 18% glycerol (DG18) agar
M183	Dichloran rose bengal chloramphenicol (DRBC) agar
M45	Duncan-Strong (DS) Sporulation Medium, Modified (for *C. perfringens*)
M46	Eagle's Minimal Essential Medium (MEME) (with Earle's salts and nonessential amino acids)
M47	Earle's Balanced Salts (Phenol Red-Free)
M48	EB Motility Medium
M49	EC Broth
M50	EC-MUG Medium
M51	Egg Yolk Emulsion, 50%
M52	Enrichment Broth, pH 7.3 ± 0.1
M53	Esculin Agar, Modified (CDC)
M30b	Freezing medium.
M54	Gelatin Agar (GA)
M55	Gelatin Salt Agar (GS)
M57	Gentamicin Sulfate Solution
M58	Ham's F-10 Medium
M59	Heart infusion agar (HIA) (Difco)
M60	Heart Infusion (HI) Broth and Agar (HIA) (for *Vibrio*)
M61	Hektoen Enteric (HE) Agar
M62	HC (Hemorrhagic colitis *E. coli* strains) Agar
M63	Hugh-Leifson Glucose Broth (HLGB)
M64	Indole Medium
M65	Indole Medium (CDC)
M66	Indole Nitrite Medium (tryptic Nitrate)
M67	Irgasan-Ticarcillin-Chlorate (ITC) Broth
M68	Iron Milk Medium (Modified)

M69	King's B Medium
M70	King's O/F Basal Medium
M71	Kligler Iron Agar
M72	Koser's Citrate Broth
M73	L-15 Medium (Modified) Leibovitz
M74	Lactose Broth
M75	Lactose-Gelatin Medium (for *C. perfringens*)
M76	Lauryl tryptose (LST) Broth
M77	Lauryl tryptose MUG (LST-MUG) Broth
M78	Letheen Agar (Modified)
M79	Letheen Broth (Modified)
M80	Levine's Eosin-Methylene Blue (L-EMB) Agar
M81	Lithium Chloride-Phenylethanol-Moxalactam (LPM) Medium
M82	[Lithium Chloride-Phenylethanol-Moxalactam] LPM Plus Esculin and Ferric Iron
M83	Liver-Veal Agar
M84	Liver-Veal-Egg Yolk Agar
M85	Long-term Preservation Medium
M86	Lysine Arginine Iron Agar (LAIA)
M87	Lysine Decarboxylase Broth (Falkow) (for *Salmonella*)
M88	Lysine Decarboxylase (LDC) Medium (for Gram-negative nonfermentative bacteria)
M89	Lysine Iron Agar (Edwards and Fife)
M90	Lysozyme Broth
M91	MacConkey Agar
M92	Malonate Broth
M185	Malt Agar (MA)
M93	Malt Extract Agar (Cosmetics-General Microbiology)
M182	Malt Extract Agar (Yeasts and Molds) (MEAYM)
M94	Malt Extract Broth (Difco)

M95	Mannitol-Egg Yolk-Polymyxin (MYP) Agar
M96	Mannitol Maltose Agar
M97	Mannitol Salt Agar
M30a	Modified Campylobacter Blood-Free Selective Agar Base (CCDA)
M98	Modified Cellobiose-Polymyxin B-Colistin (mCPC) Agar
M99	Motility-Indole-Ornithine (MIO) Medium
M100	Motility Medium *(for B. cereus)*
M101	Motility Nitrate Medium (for Cosmetics)
M102	Motility-Nitrate Medium, Buffered (for *C. perfringens)*
M103	Motility Test Medium (Semisolid)
M104	MR-VP Broth
M105	Mucate Broth
M106	Mucate Control Broth
M107	Mueller-Hinton Agar
M108	Nitrate Broth
M109	Nitrate Broth, Enriched (CDC)
M110	Nitrate Reduction Medium and Reagents
M111	Nonfat Dry Milk (Reconstituted)
M112	Nutrient Agar
M113	Nutrient Agar (for B. cereus)
M114	Nutrient Broth
M115	Nutrient Gelatin (CDC) (for Gram-negative nonfermentative bacteria)
M116	OF Glucose Medium, Semisolid
M118	Oxford Medium
M117	Oxidative-Fermentative (OF) Test Medium
M118a	PALCAM Listeria Selective Agar
M56a	Papain Solution, 5%
M119	Penicillin-Streptomycin Solution
M120	Peptone Sorbitol Bile Broth

M121	Phenol Red Carbohydrate Broth
M122	Phenol Red Glucose Broth
M123	Phenylalanine Deaminase Agar
M124	Plate Count Agar (Standard Methods)
M125	PMP Broth
M126	Potassium Cyanide (KCN) Broth
M127	Potato Dextrose Agar
M128	Pseudomonas Agar F (for fluorescein production)
M129	Pseudomonas Agar P (for pyocyanine production)
M130	Purple Carbohydrate Broth
M130a	Purple Carbohydrate Fermentation Broth Base
M131	Pyrazinamidase Agar*
M132	Rappaport-Vassiliadis Medium
M133	Sabouraud's Dextrose Broth and Agar
M134	Selenite Cystine Broth
M30d	Semisolid Medium, modified, for Biochemical Identification
M30c	Semi-Solid Medium, modified, for Culture Storage
M135	Sheep Blood Agar
M136	Shigella Broth
M137	SIM Motility Medium
M138	Simmons Citrate Agar
M139	Sorbitol-MacConkey Agar
M140	Sporulation Broth (for C. perfringens)
M141	Spray's Fermentation Medium (for *C. perfringens*)
M142	Staphylococcus Agar No. 110
M143	Starch Agar
M144	T_1N_1 Medium
M145	Tetrathionate Broth
M146	Thioglycollate Medium (Fluid) (FTG)

M147	Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar
M148	Toluidine Blue-DNA Agar
M149	Triple Sugar Iron Agar (TSI)
M150	Trypticase Novobiocin (TN) Broth
M151	Trypticase-Peptone-Glucose-Yeast Extract Broth (TPGY)
M151a	Trypticase-Peptone-Glucose-Yeast Extract Broth with Trypsin (TPGYT)
M152	Trypticase (Tryptic) Soy Agar
M153	Trypticase Soy Agar with 0.6% Yeast Extract (TSAYE)
M154	Trypticase (Tryptic) Soy Broth
M156	Trypticase Soy Broth Modified (mTSB)
M186	Trypticase (Tryptic) Soy Broth (TSB) with Ferrous Sulfate
M155	Trypticase (Tryptic) Soy Broth (TSB) with Glycerol
M154a	Trypticase (Tryptic) Soy Broth with 10% NaCl and 1% Sodium Pyruvate
M157	Trypticase Soy Broth with 0.6% Yeast Extract (TSBYE)
M158	Trypticase Soy-Polymyxin Broth
M159	Trypticase Soy-Sheep Blood Agar
M160	Trypticase Soy-Tryptose Broth
M164	Tryptone (Tryptophane) Broth, 1%
M161	Tryptone Broth and Tryptone Salt Broths
M162	Tryptone Phosphate (TP) Broth
M163	Tryptone Salt (T_1N_1) Agar and T_1N_2 Agar
M165	Tryptone Yeast Extract Agar
M166	Tryptose Blood Agar Base
M167	Tryptose Broth and Agar (for serology)
M168	Tryptose Phosphate Broth (TPB)
M169	Tryptose-Sulfite-Cycloserine (TSC) Agar
M170	Tyrosine Agar

M188	Universal Preenrichment Broth
M171	Urea Broth
M172	Urea Broth (Rapid)
M173	Veal Infusion Agar and Broth
M174	Violet Red Bile Agar (VRBA)
M175	Violet Red Bile-MUG Agar (VRBA-MUG)
M176	Vogel-Johnson (VJ) Agar
M177	Voges-Proskauer Medium (Modified)
M178	Wagatsuma Agar
M179	Xylose Lysine Desoxycholate (XLD) Agar
M180	Y-1 Adrenal Cell Growth Medium
M181	Yeast Extract (YE) Agar
M35	*Yersinia* Selective Agar (YSA)

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

Hypertext updated by rim/cjm/kwg 2000-SEP-25

U.S. Food & Drug Administration

Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

Reagents Index

(Return to Table of Contents)

ID	Name
R1	Ammonium Acetate, 4 M
R2	Ammonium Acetate, 0.25 M
R3	Basic Fuchsin Staining Solution
R4	Bicarbonate Buffer (0.1 M ,pH 9.6)
R5	Bovine Serum Albumin (BSA) (1 mg/ml)
R6	Bovine Serum Albumin (1%) in Cholera Toxin ELISA Buffer
R7	Bovine Serum Albumin (1%) in PBS
R8	Brilliant Green Dye Solution. 1%
R9	Bromcresol Purple Dye Solution. 0.2%
R10	Bromthymol Blue Indicator. 0.04%
R11	Butterfield's Phosphate-Buffered Dilution Water
R12	Catalase Test

Rl2a	Chlorine Solution, 200 ppm, Containing 0.1% Sodium Dodecyl Sulfate
R13	Citric Acid (pH 4.0, 0.05 M)
Rl4	Clark's Flagellar Stain
R15	Coating Solution for V. vulnificus EIA
R16	Crystal Violet Stain (for Bacteria)
R17	Denhardt's Solution (50X)
R18	Disinfectants (for preparation of canned foods for microbiological analysis)
R19	Dulbecco's Phosphate-Buffered Saline (DPBS)
R20	EDTA, 0.5 M
R21	EIA (*V. vulnificus*) Wash Solution
R22	ELISA Buffer (for Cholera Toxin Test)
R32a	Endospore Stain (Schaeffer-Fulton)
R23	Ethanol Solution, 70%
R24.	Evans Blue Dye Solution (commercially available)
R25	Ferric Chloride, 10%
R26	Removed from Bacteriological Analytical Manual. 1988.
R27	Physiological Saline Solution
R28	Gel Diffusion Agar. 1.2%
R29	Gel-Phosphate Buffer
R30	Giemsa Stain
R31	Glycerin-Salt Solution (Buffered)
R32	Gram Stain (commercial staining solutions are satisfactory)
R33	Hippurate Solution, 1%
R34	Horseradish Peroxidase (color development solution)
R35	Hybridization Mixture (6X SSC, 5X Denhardt's, 1 mM EDTA, pH 8.0)
R36	Hydrochloric Acid, 1 N

R37	Kinase Buffer (10X)
R38	Kovacs' Reagent
R39	Lithium Hydroxide, 0.1 N
R40	Lugol's Iodine Solution
R41	May-Grunwald Stain
R42	McFarland Nephelometer
R43	Removed from Bacteriological Analytical Manual. 1988.
R44	Methyl Red Indicator
R45	Methylene Blue Stain (Loeffler's)
R46	Mineral Oil
R47	Ninhydrin Reagent (commercially available)
R48	Nitrite Detection Reagents
R49	North Aniline (Oil)-Methylene Blue Stain
R50	Novobiocin Solution (100 mg/ml)
R51	O/129 Disks (2,4-Diamino-6,7-diisopropyl pteridine)
R52	O157 Monoclonal Antibody Solution
R53	ONPG Test
R54	Oxidase Reagent
R55	Penicillinase (ß-Lactamase)
R56	Peptone Diluent, 0.1%
R57	Peroxidase Substrate for Membrane ELISA
R58	Peroxidase Substrate Solution (ABTS) (2,2''-Azino-di-^3-ethyl benzthiazoline sulfonate (6)|
R61	Phosphate Saline Buffer (0.02 M, pH 7.3-7.4)
R62	Phosphate Saline Solution (for Y-l LT Assay)
R60	Phosphate-Buffered Saline (0.01 M , pH 7.5)
R59	Phosphate-Buffered Saline (PBS), pH 7.4

R63	Physiological Saline Solution 0.85% (Sterile)
R64	Polymyxin B Disks, 50 Units
R65	Potassium Hydroxide Solution. 40%
R66	Saline Solution. 0.5% (Sterile)
R67	Salts-Phosphate Buffered Saline Solution (Salts-PBS)
R68	Scintillation Fluid
R69	Slide Preserving Solution
R70	Sodium Bicarbonate Solution. 10%
R71	Sodium Chloride Dilution Water, 2% and 3%
R72	Sodium Chloride, 0.2 M Solution
R73	Sodium Hydroxide Solution (1 N)
R74	Sodium Hydroxide Solution (10 N)
R75	Sonicated Calf-Thymus or Salmon-Sperm DNA
R76	Spicer-Edwards EN Complex Antibody Solution
R77	Standard Saline Citrate (SSC) Solution (20%)
R78	Tergitol Anionic 7
R79	Thiazine Red R Stain (for S. aureus enterotoxin gel diffusion technique)
R80	Tris (1.0 M, pH 8.0)
R84	Tris-Buffered Saline (TABS), 1% or 3% Gelatin, or Tween 20
R81	Tris-Buffered Saline (TBS) (PH 7.5)
R82	Tris-Buffered Saline (TBS), with Gelatin
R83	Tris-Buffered Saline (TBS)-Tween
R85	Tris-EDTA-Triton X-100™(TET) Buffer
R86	Triton X-100
R87	Trypsin-EDTA Solution, lX
R88	Verocytotoxin Antiserum

R89	Voges-Proskauer (VP) Test Reagents

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

Hypertext updated by rim/cjm/kwg 2000-SEP-25

U.S. Food & Drug Administration

Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

March 2001

About the Bacteriological Analytical Manual

General Microbiological Methods Information

About BAM and CFSAN's Use of Microbiological Methods

CFSAN Microbiological Methods Information

BAM online:
Table of Contents
Media
Reagents

Microbiological Detection Methods

Methods development has always been driven by the demand for tests that are faster, cheaper, easier, and more accurate. Pressure for improved procedures is particularly apparent in microbial food safety, because traditional tests may involve many steps -- resuscitation of stressed microbial cells, enrichment of the few cells that may be present in a sample, selection that leads to the isolation of pure cultures, followed by identification, which could require a combination of morphological, biochemical, immunological and genetic techniques and, possibly, tests for virulence or toxicity using animal inoculation. Often, such test protocols take longer than the shelf life of the food being analyzed. Ways proposed to accelerate the procedure included, initially, improved media and compacted culturing. Then, automation began to replace manual execution. Also, indirect identification, i.e., by biochemical (e.g., fatty acid profiles, nucleic acid sequences) or biophysical shortcuts (FT-IR) that reveal organisms' pertinent biomarkers or genetic fingerprints, began to make the isolation of viable microbes not as necessary. These newer tests -- known as "rapid methods" if they took hours rather than days and as "real-time" testing if they took minutes -- have not yet, however, made

traditional testing obsolete.

There are good reasons why analysts should continue to have the traditional skills to resuscitate, enrich, isolate, and identify microorganisms. Often, some culturing is necessary before there is enough material for the application of a rapid method or real-time test. Then, too, foods may contain substances that interfere with biochemical/molecular test shortcuts. Furthermore, having a viable microbial isolate may still provide quantitative and infectivity information not otherwise available, or be mandatory because of regulatory requirements and legal issues, or be useful later for retrospective investigations such as the characterization of new biomarkers. And, since no two types of test have the same sensitivity, the old ones serve as convenient standards for false positive and false negative rates. Kit versions of rapid methods are interpreted differently depending on whether the results are positive or negative: negative results are considered definitive but positive results require confirmation by another test.

The Bacteriological Analytical Manual

FDA's Bacteriological Analytical Manual (The BAM) is a collection of procedures preferred by analysts in U.S. Food and Drug Administration laboratories for the detection in food and cosmetic products of pathogens (bacterial, viral, parasitic, plus yeast and mold) and of microbial toxins. The manual's contents reflect the history of methods development described above. Except for some rapid methods listed in Appendix 1, all these methods have been used and peer reviewed by FDA scientists as well as by scientists outside FDA. However, not all of these methods have been fully validated by collaborative studies. In some instances, collaborative studies are not possible because uniform test samples can not be prepared (as with encysted parasites). In other instances, FDA needs to use a method before the time it takes to achieve full validation.

At first (1965, Edition 1), the BAM was intended to be only a vehicle for information and standardization within FDA. However, the manual's reputation as useful spread beyond the agency. Requests for copies proliferated and it was decided to make the BAM generally available. It has gone through 8 major editions, with, on occasion, revisions in between. Since 1976 (Edition 4), BAM has been published and distributed by AOAC International. In 1998, Edition 8, Revision A was issued not just as hard copy, but also in an electronic format (a CD-ROM

M28a. Campylobacter enrichment broth (Bolton formula) December 2000
29a. Abeyta-Hunt-Bark Agar December 2000
30d. Semisolid Medium, modified, for Biochemical Identification March 2001
52. Enrichment Broth, pH 7.3 ± 0.1 September 2000
79. Letheen Broth (Modified) August 2001
103. Motility Test Medium (Semisolid) September 2000
156. Trypticase Soy Broth Modified January 2002

Advisories for BAM Users

Cycloheximide Supply Update January 2001

version) by AOAC. Now, in 2000 AD, the BAM is being placed on the FDA/CFSAN internet site and is designated the BAM online. Continuous updating becomes possible, so editions as such will cease. Each section will bear the dates on which it was last reviewed and revised. AOAC will continue to publish a printed version on demand.

Microbiological Methods Information on the CFSAN Website

Advisory for BAM Users on Reported Supply Problems for Cycloheximide

There have been reports of limited supplies of this reagent used in the BAM to prevent growth of yeasts and molds in some media

Microbiological Methods Information on Selected Websites

USDA/FSIS Microbiological Laboratory Guidebook, 3rd Edition

Official Methods of Analysis of AOAC International

Date of final revision: 2001-MAR-05. Composed by: RIM, GJJ, RB.

Top

B A M | B A M Media | B A M Reagents | Bad Bug Book | Microbiological Methods

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

Chapter 1

Food Sampling and Preparation of Sample Homogenate

Authors

(Return to Table of Contents)

The adequacy and condition of the sample or specimen received for examination are of primary importance. If samples are improperly collected and mishandled or are not representative of the sampled lot, the laboratory results will be meaningless. Because interpretations about a large consignment of food are based on a relatively small sample of the lot, established sampling procedures must be applied uniformly. A representative sample is essential when pathogens or toxins are sparsely distributed within the food or when disposal of a food shipment depends on the demonstrated bacterial content in relation to a legal standard.

The number of units that comprise a representative sample from a designated lot of a food product must be statistically significant. The composition and nature of each lot affects the homogeneity and uniformity of the total sample mass. The proper statistical sampling procedure, according to whether the food is solid, semisolid, viscous, or liquid, must be determined by the collector at the time of sampling by using the *Investigations Operation Manual* (5). Sampling and sample plans are discussed in detail in ref. 6.

Whenever possible, submit samples to the laboratory in the original unopened containers. If products are in bulk or in containers too large for submission to the laboratory, transfer representative portions to sterile containers under aseptic conditions. There can be no compromise in the use of sterile sampling equipment and the use of aseptic technique. Sterilize one-piece stainless steel spoons, forceps, spatulas, and scissors in an autoclave or dry-heat oven. Use of a propane torch or dipping the instrument in alcohol and igniting is dangerous and may be inadequate for sterilizing equipment.

Use containers that are clean, dry, leak-proof, wide-mouthed, sterile, and of a size suitable for samples of the product. Containers such as plastic jars or metal cans that are leak-proof may be hermetically sealed. Whenever possible, avoid glass containers, which may break and contaminate the food product. For dry materials, use sterile metal boxes, cans, bags, or packets with suitable closures. Sterile plastic bags (for dry, unfrozen materials only) or plastic bottles are useful containers for line samples. Take care not to overfill bags or permit puncture by wire closure. Identify each sample unit (defined later) with a properly marked strip of masking tape. Do not use a felt pen on plastic because the ink might penetrate the container. Whenever possible, obtain at least 100 g for each sample unit. Submit open and closed controls of sterile containers with the sample.

Deliver samples to the laboratory promptly with the original storage conditions maintained as nearly as possible. When collecting liquid samples, take an additional sample as a temperature control. Check the temperature of the control sample at the time of collection and on receipt at the laboratory. Make a record for all samples of the times and dates of collection and of arrival at the laboratory. Dry or canned foods that are not perishable and are collected at ambient temperatures need not be refrigerated. Transport frozen or refrigerated products in approved insulated containers of rigid construction so that they will arrive at the laboratory unchanged. Collect frozen samples in pre-chilled containers.

Place containers in a freezer long enough to chill them thoroughly. Keep frozen samples solidly frozen at all times. Cool refrigerated samples, except shellfish and shell stock, in ice at 0-4°C and transport them in a sample chest with suitable refrigerant capable of maintaining the sample at 0-4°C until arrival at the laboratory. Do not freeze refrigerated products. Unless otherwise specified, refrigerated samples should not be analyzed more than 36 h after collection. Special conditions apply to the collection and storage of shucked, unfrozen shellfish and shell stock (1). Pack samples of shucked shellfish immediately in crushed ice (no temperature specified) until analyzed; keep shell stock above freezing but below 10C. Examine refrigerated shellfish and shell stock within 6 h of collection but in no case more than 24 h after collection. Further details on sample handling and shipment may be found in the *Investigations Operation Manual* (5) and the *Laboratory Procedures Manual* (3). The *Investigations Operation Manual* (5) contains sampling plans for various microorganisms. Some of those commonly used are presented here.

A. **Sampling plans**

1. *Salmonella* species

    a. Sample collection

    Because of the continuing occurrence of *Salmonella* in foods, sampling plans for these organisms have received the attention of committees of national and international organizations (6,7). Each of these committees has recommended varying the number of samples from a particular lot of food according to the sampling category to which a food is assigned. Generally, the assignment to a sampling or food category depends on 1) the sensitivity of the consumer group (e.g., the aged, the infirm, and infants); 2) the possibility that the food may have undergone a step lethal to *Salmonella* during the manufacturing process or in the home; and 3) the history of the food. The selection of a sampling plan depends mainly on the first 2 criteria cited. The history of the food would be important in deciding whether to sample, i.e., whether there was a past history of contamination. For the *Salmonella* sampling plan discussed here, 3 categories of foods are identified.

    Food Category I. - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption and are intended for consumption by the aged, the infirm, and infants.

    Food Category II. - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption.

    Food Category III. - Foods that would normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption.

    This sampling plan applies to the collection of finished products under surveillance and/or for determination of compliance for regulatory consideration. It also applies to the collection of factory samples of raw materials in identifiable lots of processed units and/or finished products where regulatory action is possible. It does not apply to the collection of in-line process sample units at various stages of manufacture since those samples do not necessarily represent the entire lot of food under production. The actual techniques involved in sampling are covered in the *Investigations Operation Manual* (5).

A sample unit consists of a minimum of 100 g and is usually a consumer-size container of product. Take sample units at random to ensure that a sample is representative of the lot. When using sample containers, submit a control consisting of one empty sample container that has been exposed to the same conditions as those under which the sample was collected. Collect more than one sample unit from large institutional or bulk containers when the number of sample units required exceeds the number of containers in the lot. A sample unit will consist of more than one container when containers are smaller than 100 g (e.g., four 25 g containers could constitute a sample unit).

The numbers of sample units to be collected in each food category are as follows: Food Category I, 60 sample units; Food Category II, 30 sample units; Food Category III, 15 sample units. Submit all samples collected to the laboratory for analysis. Advise the laboratory in advance of perishable sample shipments.

b. Sample analysis

The laboratory will analyze each sample for the presence of *Salmonella* according to methods described in this manual, or in *Official Methods of Analysis* (2). Take a 25 g analytical unit at random from each 100 g sample unit. When a sample unit consists of more than one container, aseptically mix the contents of each container before taking the 25 g analytical unit. To reduce the analytical workload, the analytical units may be composited. The maximum size of a composite unit is 375 g or 15 analytical units. The minimum number of composite units to be tested for each food category is as follows: Food Category I, 4 composite units; Food Category II, 2 composite units; Food Category III, one composite unit. For each 375 g composite, the entire amount of 375 g is analyzed for *Salmonella*.

Keep the remainder of the sample unit in a sterile container for compliance requirements as per section 702(b) of the Federal Food, Drug, and Cosmetic Act as amended through February, 1993. Refrigerate perishable samples and samples supporting microbial growth. An analytical control is required for each sample tested. The sampled lot is acceptable only if analyses of all composite units are negative for *Salmonella*. If one or more composite units are positive for *Salmonella*, the lot is rejected, provided that the analytical control is negative for *Salmonella*. A lot will not be resampled unless the environmental control for *Salmonella* is positive. For all samples positive for *Salmonella*, determine the somatic group. See Chapter 5 for information on further handling of these cultures.

Recommendations for regulatory action may be based on the identification of the *Salmonella* somatic group and will not require definitive serotyping before initiation of regulatory action.

c. Imports.

These sampling plans apply to imported food products intended for human consumption.

d. Classification of food products for sampling purposes

Foods that have been classified into the 3 categories described above for regulatory sampling are listed in the categories according to the Industry Product Code sequence and nomenclature (4). Listing does not necessarily mean that these products are probable sources of *Salmonella*. **Food Category I**. - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption and are intended for consumption by the aged, the infirm, and infants. **Food Category II**. - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption. Examples are as follows:

Industry Product Code

2	Milled grain products not cooked before consumption (bran and wheat germ)
3	Bread, rolls, buns, sugared breads, crackers, custard- and cream-filled sweet goods, and icings
5	Breakfast cereals and other ready-to-eat breakfast foods
7	Pretzels, chips, and other snack foods
9	Butter and butter products, pasteurized milk and raw fluid milk and fluid milk products for direct consumption, pasteurized and unpasteurized concentrated liquid milk products for direct consumption, dried milk and dried milk products for direct consumption, casein, sodium caseinate, and whey
12	Cheese and cheese products
13	Ice cream from pasteurized milk and related products that have been pasteurized, raw ice cream mix and related unpasteurized products for direct consumption

14	Pasteurized and unpasteurized imitation dairy products for direct consumption
15	Pasteurized eggs and egg products from pasteurized eggs, unpasteurized eggs and egg products from unpasteurized eggs for consumption without further cooking
16	Canned and cured fish, vertebrates, and other fish products; fresh and frozen raw shellfish and crustacean products for direct consumption; smoked fish, shellfish, and crustaceans for direct consumption
17	Meat and meat products, poultry and poultry products, and gelatin (flavored and unflavored bulk)
20-22	Fresh, frozen, and canned fruits and juices, concentrates, and nectars; dried fruits for direct consumption; jams, jellies, preserves, and butters
23	Nuts, nut products, edible seeds, and edible seed products for direct consumption
24	Vegetable juices, vegetable sprouts, and vegetables normally eaten raw
26	Oils consumed directly without further processing; oleomargarine
27	Dressings and condiments (including mayonnaise), salad dressing, and vinegar
28	Spices, flavors, and extracts
29	Soft drinks and water
30	Beverage bases
31	Coffee and tea
33	Candy (with and without chocolate; with and without nuts) and chewing gum
34	Chocolate and cocoa products
35	Pudding mixes not cooked before consumption, and gelatin products
36	Syrups, sugars, and honey
37	Ready-to-eat sandwiches, stews, gravies, and sauces
38	Soups
39	Prepared salads

54	Nutrient supplements, such as vitamins, minerals, proteins, and dried inactive yeast

Food Category III: Foods that would normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption. Examples are as follows:

Industry Product Code

2	Whole grain, milled grain products that are cooked before consumption (corn meal and all types of flour), and starch products for human use
3	Prepared dry mixes for cakes, cookies, breads, and rolls
4	Macaroni and noodle products
16	Fresh and frozen fish; vertebrates (except those eaten raw); fresh and frozen shellfish and crustaceans (except raw shellfish and crustaceans for direct consumption); other aquatic animals (including frog legs, marine snails, and squid)
18	Vegetable protein products (simulated meats) normally cooked before consumption
24	Fresh vegetables, frozen vegetables, dried vegetables, cured and processed vegetable products normally cooked before consumption
26	Vegetable oils, oil stock, and vegetable shortening
35	Dry dessert mixes, pudding mixes, and rennet products that are cooked before consumption

2. Aerobic plate counts, total coliforms, fecal coliforms, *Escherichiacoli* (including enteropathogenic strains), *Staphylococcus* spp., *Vibrio* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., *Bacilluscereus*, and *Clostridium perfringens*

    a. Sample collection

    From any lot of food, collect ten 8-oz subsamples (or retail packages) at random. Do not break or cut larger retail packages to obtain an 8-oz subsample. Collect the intact retail unit as the subsample even if it is larger than 8 oz.

    b. Sample analysis.

Analyze samples as indicated in current compliance programs.

B. Equipment and materials

1. Mechanical blender. Several types are available. Use blender that has several operating speeds or rheostat. The term "high-speed blender" designates mixer with 4 canted, sharp-edge, stainless steel blades rotating at bottom of 4 lobe jar at 10,000-12,000 rpm or with equivalent shearing action. Suspended solids are reduced to fine pulp by action of blades and by lobular container, which swirls suspended solids into blades. Waring blender, or equivalent, meets these requirements.

2. Sterile glass or metal high-speed blender jar, 1000 ml, with cover, resistant to autoclaving for 60 min at 121°C

3. Balance, with weights; 2000 g capacity, sensitivity of 0.1 g

4. Sterile beakers, 250 ml, low-form, covered with aluminum foil

5. Sterile graduated pipets, 1.0 and 10.0 ml

6. Butterfield's phosphate-buffered dilution water (Rll), sterilized in bottles to yield final volume of 90 ± 1 ml

7. Sterile knives, forks, spatulas, forceps, scissors, tablespoons, and tongue depressors (for sample handling)

C. Receipt of samples

1. **The official food sample is collected by the FDA inspector or investigator.** As soon as the sample arrives at the laboratory, the analyst should note its general physical condition. If the sample cannot be analyzed immediately, it should be stored as described later. Whether the sample is to be analyzed for regulatory purposes, for investigation of a foodborne illness outbreak, or for a bacteriological survey, strict adherence to the recommendations described here is essential.

2. **Condition of sampling container.** Check sampling containers for gross physical defects. Carefully inspect plastic bags and bottles for tears, pinholes, and puncture marks. If sample units were collected in plastic bottles, check bottles for fractures and loose lids. If plastic bags were used for sampling, be certain that twist wires did not puncture surrounding bags. Any cross-contamination resulting from one or

more of above defects would invalidate the sample, and the collecting district should be notified (**see** C-5, below)

3. **Labeling and records.** Be certain that each sample is accompanied by a completed copy of the Collection Report (Form FD-464) and officially sealed with tape (FD-415a) bearing the sample number, collecting official's name, and date. Assign each sample unit an individual unit number and analyze as a discrete unit unless the sample is composited as described previously in this chapter.

4. **Adherence to sampling plan.** Most foods are collected under a specifically designed sampling plan in one of several ongoing compliance programs. Foods to be examined for *Salmonella*, however, are sampled according to a statistically based sampling plan designed exclusively for use with this pathogen. Depending on the food and the type of analysis to be performed, determine whether the food has been sampled according to the most appropriate sampling plan.

5. **Storage.** If possible, examine samples immediately upon receipt. If analysis must be postponed, however, store frozen samples at -20°C until examination. Refrigerate unfrozen perishable samples at 0-4°C not longer than 36 h. Store nonperishable, canned, or low-moisture foods at room temperature until analysis.

6. **Notification of collecting district.** If a sample fails to meet the above criteria and is therefore not analyzed, notify the collecting district so that a valid sample can be obtained and the possibility of a recurrence reduced.

D. Thawing

Use aseptic technique when handling product. Before handling or analysis of sample, clean immediate and surrounding work areas. In addition, swab immediate work area with commercial germicidal agent. Preferably, do not thaw frozen samples before analysis. If necessary to temper a frozen sample to obtain an analytical portion, thaw it in the original container or in the container in which it was received in the laboratory. Whenever possible, avoid transferring the sample to a second container for thawing. Normally, a sample can be thawed at 2-5°C within 18 h. If rapid thawing is desired, thaw the sample at less than 45°C for not more than 15 min. When thawing a sample at elevated temperatures, agitate the sample continuously in thermostatically controlled water bath.

E. Mixing

Various degrees of non-uniform distribution of microorganisms are to be expected in any food sample. To ensure more even distribution, shake liquid samples thoroughly and, if

practical, mix dried samples with sterile spoons or other utensils before withdrawing the analytical unit from a sample of 100 g or greater. Use a 50 g analytical unit of liquid or dry food to determine aerobic plate count value and most probable number of coliforms. Other analytical unit sizes (e.g., 25 g for *Salmonella*) may be recommended, depending on specific analysis to be performed. Use analytical unit size and diluent volume recommended for appropriate *Bacteriological Analytical Manual* method being used. If contents of package are obviously not homogeneous (e.g., a frozen dinner), macerate entire contents of package and withdraw the analytical unit, or, preferably, analyze each different food portion separately, depending on purpose of test.

F. Weighing

Tare high-speed blender jar; then aseptically and accurately (± 0.1 g) weigh unthawed food (if frozen) into jar. If entire sample weighs less than the required amount, weigh portion equivalent to one-half of sample and adjust amount of diluent or broth accordingly. Total volume in blender must completely cover blades.

G. Blending and diluting of samples requiring enumeration of microorganisms

1. **All foods other than nut meat halves and larger pieces, and nut meal**. Add 450 ml Butterfield's phosphate-buffered dilution water to blender jar containing 50 g analytical unit and blend 2 min. This results in a dilution of 10^{-1}. Make dilutions of original homogenate promptly, using pipets that deliver required volume accurately. Do not deliver less than 10% of total volume of pipet. For example, do not use pipet with capacity greater than 10 ml to deliver 1 ml volumes; for delivering 0.1 ml volumes, do not use pipet with capacity greater than 1.0 ml. Prepare all decimal dilutions with 90 ml of sterile diluent plus 10 ml of previous dilution, unless otherwise specified. Shake all dilutions vigorously 25 times in 30 cm (1 ft) arc in 7 s. Not more than 15 min should elapse from the time sample is blended until all dilutions are in appropriate media.

2. **Nut meat halves and larger pieces**. Aseptically weigh 50 g analytical unit into sterile screw-cap jar. Add 50 ml diluent (G-l, above) and shake vigorously 50 times through 30 cm arc to obtain 10^{0} dilution. Let stand 3-5 min and shake 5 times through 30 cm arc to resuspend just before making serial dilutions and inoculations.

3. **Nut meal**. Aseptically weigh 10 g analytical unit into sterile screw-cap jar. Add 90 ml of diluent (G-l, above) and shake vigorously 50 times through 30 cm arc to obtain 10^{-1} dilution. Let stand 3-5 min and shake 5 times through 30 cm arc to resuspend just before making serial dilutions and inoculations.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

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Chapter 2

Microscopic Examination of Foods and Care and Use of the Microscope

Authors

Return to Table of Contents

Chapter Contents

If there is reason to suspect that a food has caused food poisoning or has undergone microbial spoilage, the original product or a low serial dilution of it should be used to prepare a slide for direct microscopic examination. The Gram stain reaction and cellular morphology of the bacteria on the slide may indicate the need for other types of examination. A microscopic examination must be made, even though the food may have undergone heat treatment and the microorganisms involved may no longer be viable. Large numbers of Gram-positive cocci on the slide may indicate the presence of staphylococcal enterotoxin, which is not destroyed by the

heat treatments that destroy enterotoxigenic *Staphylococcus aureus* strains. Large numbers of sporeforming, Gram-positive rods in a frozen food specimen may indicate the presence of *Clostridium perfringens*, an organism that is sensitive to low temperatures. Other Gram-positive, sporeforming rods such as *Clostridium botulinum* or *Bacillus cereus* may also be present in the food. When the microscopic examination of suspect food discloses the presence of many Gram-negative rods, consider the symptoms and incubation periods reported for the illness under investigation and select the specific examination method for isolating one or more of the following genera: *Salmonella*, *Shigella*, *Escherichia*, *Yersinia*, *Vibrio*, or *Campylobacter*.

Direct Microscopic Examination of Foods (Except Eggs)

A. Equipment and materials

1. Glass slides, 25 x 75 mm, with etched portion for labeling; 1 slide for each blended food sample (10^{-1} dilution)
2. Wire loop, 3-4 mm, platinum-iridium or nichrome, B&S gauge No. 24 or 26
3. Gram stain reagents (R32)
4. Microscope, with oil immersion objective lens (95-100X) and 10X ocular
5. Immersion oil
6. Methanol
7. Xylene

B. Procedure

Prepare film of blended food sample (10^{-1} dilution). Air-dry films and fix with moderate heat by passing films rapidly over Bunsen or Fisher burner flame 3 or 4 times. Alternatively, air-dry films and fix with methanol 1-2 min, drain excess methanol, and flame or air-dry (this is particularly helpful for foods with a high sugar content). Cool to room temperature before staining. De-fat films of food with high fat content by immersing films in xylene 1-2 min; then drain, wash in methanol, drain, and dry. Stain film by Gram-staining procedure (R32). Use microscope equipped with oil immersion objective (95-100X) and 10X ocular; adjust lighting systems to Koehlor illumination.

Examine at least 10 fields of each film, noting predominant types of organisms, especially clostridial forms, Gram-positive cocci, and Gram-negative bacilli.

Direct Microscopic Examination of Eggs (2)

A. Equipment and materials

1. Microscope, with 10X oculars and oil immersion objective (1.8 mm or 90-100X)
2. Microscope slides, 25 x 75 mm or 50 x 75 mm
3. Bacteriological pipet or metal syringe, to deliver 0.01 ml
4. North aniline (oil)-methylene blue stain (R49)
5. 0.1 N lithium hydroxide (R39)
6. Physiological saline solution, 0.85% (sterile) (R63)
7. Butterfield's phosphate-buffered dilution water (R11)
8. Xylene
9. Ethanol, 95%

B. Procedure for liquid and frozen eggs

1. Thaw frozen egg material as rapidly as possible to prevent increase in number of microorganisms present. Thaw below 45°C for 15 min with continuous agitation in thermostatically controlled water bath. Using bacteriological pipet or metal syringe, place 0.01 ml undiluted egg material on clean, dry microscope slide. Spread egg material evenly over area of 2 sq cm (circular area of 1.6 cm diameter is preferred). Add drop of water to each film for uniform spreading.
2. Let film dry on level surface at 35-40°C. Immerse in xylene up to 1 min; then immerse in 95% ethanol up to 1 min. Stain film 1 min in North aniline (oil)-methylene blue stain (10-20 min preferred; exposure to 2 h does not overstain). Wash slide by repeated immersions in beaker of water, and thoroughly air-dry before examining (do not blot). Count microorganisms observed in 10-60 fields. Multiply average number per field by microscopic factor and by 2, since 2 sq cm

area was used. Carry out subsequent operations and observe precautions as directed in "Direct Microscopic Method for Bacteria," *Standard Methods for the Examination of Dairy Products* (1). Express final results as number of bacteria (or clumps) per g of egg material.

C. **Procedure for dried egg products**

Thoroughly mix sample; prepare 1:10 dilution by aseptically weighing 11 g egg material into sterile, wide-mouth, glass-stoppered or screw-capped bottle. Add 99 ml diluent (R11) or sterile physiological salt solution and 1 sterile tablespoon of sterile glass beads (0.1 N lithium hydroxide may be used as diluent and is preferred for samples of whole egg and yolk products that are relatively insoluble). Thoroughly agitate 1:10 dilution to ensure complete solution or distribution of egg material by shaking each container rapidly 25 times, using up-and-down or back-and-forth movement of about 1 ft arc, within 7 s. Let bubbles escape.

Place 0.01 ml of 1:10 or 1:100 dilution dried egg material on clean microscope slide and spread evenly over 2 sq cm. Proceed as in Direct Microscopic Examination of Eggs, B-2, above. Multiply average number of microorganisms per field by twice the microscopic factor (since 2 sq cm area was used) and multiply by 10 or 100, depending on whether film was prepared from 1:10 or 1:100 dilution. Express final results as number of bacteria (or clumps) per g of egg material.

Care and Use of the Microscope

Caveats

- Never dust a lens by blowing on it. Saliva will inevitably be deposited on lenses and is harmful, even in minute amounts.
- If pressurized air is used for dusting, use an inline filter to trap oil and other contaminants.
- Do not use dry lens tissue on a lens.
- Never use facial tissues to clean lenses. They may contain glass filaments which can scratch lenses. Linen or chamois may be used for cleaning but may not be as convenient as lens tissue. Do not confuse lens tissue with bibulous paper, which should never be used to clean lenses. Follow the manufacturer's recommendation for using cleaning solvents other than water. Lens mounting glues are often soluble in alcohol. Xylene used sparingly is generally acceptable for serious stains, such as residual oils.

- Never leave microscope tubes open. Always keep them closed with dust plug, eyepiece, or objective, as appropriate.

- Avoid touching lenses. Even light fingerprints, especially on objectives, can seriously degrade image quality.

- Do not attempt to take optics apart for cleaning. Internal optics should not need routine cleaning and should be professionally serviced if needed.

- Use proper immersion liquids on immersion objectives as specified by the manufacturer. Avoid getting immersion liquid on non-immersion objectives; it can damage the lens mounting glue.

- Keep microscopes covered when not in use. Avoid extremes of temperature and high humidity. In work areas with consistently more than 60% relative humidity, store microscopes in circulating air if possible. In very high humidity, optical parts should be stored in tightly covered containers with desiccant and kept very clean to prevent mold growth on the optic coating.

- Do not use the substage diaphragm to control brightness.

Cleaning

1. Body of the microscope. Use alcohol or soapy water on cloth to wipe body. Lubricate sliding parts with a petroleum jelly, such as Vaseline, or use a manufacturer-recommended lubricant

2. Lenses and optics

    - Grit and dust can scratch lenses and coatings.

    - Blow away dust with rubber bulb.

    - For light cleaning, breathe on lens to fog it, and then use lens tissue as described below. The fogging is basically water and is not harmful.

    - For dirtier lenses, use lens cleaner solution, such as that manufactured by Kodak, available from any camera supply store. Remove stubborn stains by using xylene sparingly.

- Use the following procedure to properly clean lenses of microscopes or other optical equipment. Crumple a piece of lens tissue to create many folds to trap dirt without grinding it into the lens. Do not touch the part of the tissue that will be applied to the lens; excessive touching transfers natural oils from the fingers to the lens tissue. Apply a small amount of lens cleaning solution to the lens tissue and blot the tissue against absorbent material to prevent fluid from entering the lens mount. Wipe the lens very lightly to remove gross dirt that was not blown away by the rubber bulb. If necessary, repeat the cleaning process with a new piece of lens tissue and with more pressure to remove oily or greasy residue. Complete the process by using the light cleaning procedure (breath and lens tissue) described above.

3. Adjustments. Follow the manufacturer's recommendations for microscope adjustments that can be made by the user. Tension of the coarse focus mechanism can usually be adjusted by the user. Directions for adjusting the lighting and eyepiece follow.

Proper Set-up and Illumination of the Compound Microscope

Eyepieces (oculars) must be adjusted to the user's eyes. Only microscopes with binocular tubes are discussed here. Except for interpupillary distance, other adjustments hold true for microscopes with monocular tubes. To adjust interpupillary distance, lengthen or shorten the distance between centers of oculars to match the distance between centers of pupils of the eyes.

Adjust the microscope for each eye. One eyepiece, or the tube into which it fits, is usually adjustable. Place a specimen slide on the microscope stage, turn on the illumination, and focus at low magnification. Cover the eyepiece that has the focusing eye tube with a card and, with both eyes open, bring the specimen into focus for the other eye with the fine focus knob. It is important that vision be relaxed by looking up frequently to distant objects or to infinity by staring "through the wall." This will help prevent eyestrain caused by trying to "accommodate" the object, bringing it into focus with the eye at a point closer than infinity. When consistent sharp and relaxed focus has been obtained at one particular point on the slide, switch the card to cover the other eyepiece, but this time use the focusing ring on the open eyepiece to bring the same point on the slide into focus. Follow the same procedure for relaxed viewing.

The next important point is the distance from the eye to the eyepiece. If the eyes are too close or too far away, the field of view will be reduced and the specimen may appear less sharp. From a few inches away, move in slowly until the field appears the widest and sharpest. This distance, from the pupil of the eye to the lens, is the eye point or exit pupil of the microscope.

The focusing of the microscope and adjustment for each eye will correct for most conditions of

near or farsightedness, eliminating the need to wear corrective eyeglasses during microscope work. Even moderate astigmatism will not hamper most microscope use; however, for more serious astigmatism, for certain other conditions of the eyes, or if preferred, prescription eyeglasses should be worn. Correction for astigmatism in eyeglasses can easily be determined by holding the eyeglasses at arm's length and rotating them while looking at an object through one lens at a time. If the length or width of the object changes while doing this with either lens, then there is an astigmatism correction and wearing the glasses during microscope work may be recommended. Special high-point eyepieces are made with a longer exit pupil distance to easily accommodate the extra distance needed when eyeglasses are worn. These eyepieces are identified in some way by the manufacturer.

To take advantage of the optimal resolution and illumination of the microscope, a technique known as Koehlor illumination is used. NOTE: Some settings may have been preset by the manufacturer and will not be adjustable.

With a slide specimen on the stage, use a low power (2-10X) objective, and focus with the coarse and fine adjustments. Low magnification provides a larger field of view for easier searching of the specimen. It also provides a greater working distance than higher power objectives, offering more safety against focusing too low and breaking the slide.

The microscope may have an auxiliary swing-in (or swing-out) lens in the condenser. Follow manufacturer's recommendations for correct use of the swing-in/swing-out lens with various objectives. Use the coarse and fine adjustments to focus the specimen. To obtain Koehlor illumination, close the lamp (field) iris diaphragm, if present, at the base of the microscope, and bring into focus by vertical adjustment of the condenser. Use the centering screws or knobs on the condenser, if present, to center the focused circle in the field. If not on the condenser, centering screws or knobs for this purpose may be on the base near the lamp diaphragm. Open the lamp diaphragm until it is just past the field of view. This may not be possible with low power objectives of some microscopes until the auxiliary lens in the condenser is correctly adjusted.

If the microscope has no lamp diaphragm, place a piece of paper with a small hole cut in it over the lamp opening and make the same adjustments to focus on its inner edge. Microscopes with mirror and external light source are not discussed here, but the principles are similar. Follow the manufacturer's instructions for adjusting the lamp filament if possible.

Set the substage (aperture) diaphragm next. This adjustment has a crucial effect on the resolution and contrast of the image. The substage diaphragm is opened or closed to 2/3 the size of the field as seen by removing an eyepiece and looking down the tube. To approximate this setting without removing an eyepiece, open the substage diaphragm fully and gradually close it while looking through the microscope until the image gains a sudden increase in sharpness and detail. This should be close to the 2/3 open position; it can be achieved with a little practice and

double checking initially by removing the eyepiece and looking down the tube. Replace the eyepiece. If the lighting is too bright, use the rheostat, if provided, to turn it down, or add neutral density or other filters. Do not use the substage diaphragm to control brightness. Resolution will suffer if it is stopped down (closed) too far or opened too much. Although stopping down gives more contrast, it impairs resolution, and spurious details are formed by diffraction lines or fringes.

Repeat the procedures for Koehlor illumination with each objective used.

For phase-contrast microscopy, follow the same basic steps. Do not use the substage diaphragm but make adjustments to bring the phase annulus and annular diaphragm into coincidence. Refer to the manufacturer's instructions. Use of a green filter is recommended.

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1. American Public Health Association. 1985. Standard Methods for the Examination of Dairy Products, 16th ed. Chapter 10. APHA, Washington, DC.

2. *Official Methods of Analysis of AOAC International* (2000) 17th Ed., AOAC International, Gaithersburg, MD, USA , Official Method **940.37F**

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Bacteriological Analytical Manual

Online

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Chapter 3

Aerobic Plate Count

Authors

Return to Table of Contents

Chapter Contents

The aerobic plate count (APC) is intended to indicate the level of microorganism in a product. Detailed procedures for determining the APC of foods have been developed by the Association of Official Analytical Chemists (AOAC) (3) and the American Public Health Association (APHA) (1). The conventional plate count method for examining frozen, chilled, precooked, or prepared foods, outlined below, conforms to AOAC *Official Methods of Analysis*, sec. 966.23, with one procedural change (966.23C). The suitable colony counting range (10) is 25-250. The automated spiral plate count method for the examination of foods and cosmetics (5), outlined below, conforms to AOAC *Official Methods of Analysis*, sec. 977.27. For procedural details of the standard plate count, see ref. 2.Guidelines for calculating and reporting plate counts have been changed to conform with the anticipated changes in the 16th edition of *Standard Methods for the Examination of Dairy Products* (2) and the *International Dairy Federation* (IDF)

procedures (6).

Conventional Plate Count Method

A. Equipment and materials

1. Work area, level table with ample surface in room that is clean, well-lighted (100 foot-candles at working surface) and well-ventilated, and reasonably free of dust and drafts. The microbial density of air in working area, measured in fallout pour plates taken during plating, should not exceed 15 colonies/plate during 15 min exposure.

2. Storage space, free of dust and insects and adequate for protection of equipment and supplies

3. Petri dishes, glass or plastic (at least 15 x 90 mm)

4. Pipets with pipet aids (no mouth pipetting) or pipettors, 1, 5, and 10 ml, graduated in 0.1 ml units

5. Dilution bottles, 6 oz (160 ml), borosilicate-resistant glass, with rubber stoppers or plastic screw caps

6. Pipet and petri dish containers, adequate for protection

7. Circulating water bath, for tempering agar, thermostatically controlled to 45 ± 1°C

8. Incubator, 35 ± 1°C; milk, 32 ± 1°C

9. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate

10. Tally register

11. Dilution blanks, 90 ± 1 ml Butterfield's phosphate-buffered dilution water (R11); milk, 99 ± 2 ml

12. Plate count agar (standard methods) (M124)

13. Refrigerator, to cool and maintain samples at 0-5°C; milk, 0-4.4°C

14. Freezer, to maintain frozen samples from -15 to -20°C

15. Thermometers (mercury) appropriate range; accuracy checked with a thermometer certified by the National Institute of Standards and Technology (NIST)

B. Procedure for analysis of frozen, chilled, precooked, or prepared foods

Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate, of food homogenate (**see** Chapter 1 for sample preparation) by transferring 10 ml of previous dilution to 90 ml of diluent. Avoid sampling foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 s. Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes. Reshake dilution bottle 25 times in 30 cm arc within 7 s if it stands more than 3 min before it is pipetted into petri dish. Add 12-15 ml plate count agar (cooled to 45 ± 1°C) to each plate within 15 min of original dilution. For milk samples, pour an agar control, pour a dilution water control and pipet water for a pipet control. Add agar to the latter two for each series of samples. Add agar immediately to petri dishes when sample diluent contains hygroscopic materials, e.g., flour and starch. Pour agar and dilution water control plates for each series of samples. Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify. Invert solidified petri dishes, and incubate promptly for 48 ± 2 h at 35°C. Do not stack plates when pouring agar or when agar is solidifying.

C. Guidelines for calculating and reporting APCs in uncommon cases

Official Methods of Analysis (3) does not provide guidelines for counting and reporting plate counts, whereas *Standard Methods for the Examination of Dairy Products*, 16th ed. (2) presents detailed guidelines; for uniformity, therefore, use APHA guidelines as modified (6,8). Report all aerobic plate counts (2) computed from duplicate plates. For milk samples, report all aerobic plate (2) counts computed from duplicate plates containing less than 25 colonies as less than 25 estimated count. Report all aerobic plate counts (2) computed from duplicate plates containing more than 250 colonies as estimated counts. Counts outside the normal 25-250 range may give erroneous indications of the actual bacterial composition of the sample. Dilution factors may exaggerate low counts (less than 25), and crowded plates (greater than 250) may be difficult to count or may inhibit the growth of some bacteria, resulting in a low count. Report counts less than 25 or more than 250 colonies as estimated aerobic plate counts (EAPC). Use the following guide:

1. Normal plates (25-250). Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size, on selected plate(s). Record dilution(s) used and total number of colonies counted.

2. Plates with more than 250 colonies. When number of CFU per plate exceeds 250, for all dilutions, record the counts as too numerous to count (TNTC) for all but the plate closest to 250, and count CFU in those portions of plate that are representative of colony distribution. See ref. 2 for detailed guidelines. Mark calculated APC with EAPC to denote that it was estimated from counts outside 25-250 per plate range (*see* D-3).

3. Spreaders. Spreading colonies are usually of 3 distinct types: 1) a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacterial clump; 2) one that develops in film of water between agar and bottom of dish; and 3) one that forms in film of water at edge or on surface of agar. If plates prepared from sample have excessive spreader growth so that (a) area covered by spreaders, including total area of repressed growth, exceeds 50% of plate area, or (b) area of repressed growth exceeds 25% of plate area, report plates as spreaders. When it is necessary to count plates containing spreaders not eliminated by (a) or (b) above, count each of the 3 distinct spreader types as one source. For the first type, if only one chain exists, count it as a single colony. If one or more chains appear to originate from separate sources, count each source as one colony. Do not count each individual growth in such chains as a separate colony. Types 2 and 3 usually result in distinct colonies and are counted as such. Combine the spreader count and the colony count to compute the APC.

4. Plates with no CFU. When plates from all dilutions have no colonies, report APC as less than 1 times the corresponding lowest dilution used. Mark calculated APC with asterisk to denote that it was estimated from counts outside the 25-250 per plate range. When plate(s) from a sample are known to be contaminated or otherwise unsatisfactory, record the result(s) as laboratory accident (LA).

D. Computing and recording counts (see refs 6, 8)

To avoid creating a fictitious impression of precision and accuracy when computing APC, report only the first two significant digits. Round off to two significant figures only at the time of conversion to SPC. For milk samples, when plates for all dilutions have no colonies, report APC as less than 25 colonies estimated count. Round by raising the second digit to the next highest number when the third digit is 6, 7, 8, or 9 and use zeros for each successive digit toward the right from the second digit. Round down when the third digit is 1, 2, 3, or 4. When the third digit is 5, round up when the second digit is

odd and round down when the second digit is even.

Examples

Calculated Count	APC
12,700	13,000
12,400	12,000
15,500	16,000
14,500	14,000

1. Plates with 25-250 CFU.

    a. Calculate the APC as follows:

$$\frac{31 + 30 \text{ colonies}}{0.0015 \text{ ml}} = 4.1 \times 10^4$$

where N = Number of colonies per ml or g of product

SC = Sum of all colonies on all plates counted

n_1 = Number of plates in first dilution counted

n_2 = Number of plates in second dilution counted

d = Dilution from which the first counts were obtained

Example

1:100	1:1000
232, 244	33, 28

$$N = \frac{(232 + 244 + 33 + 28)}{[(1 \times 2) + (0.1 \times 2)]10^2}$$

$$= 537/0.022$$

$$= 24{,}409$$

$$= 24{,}000$$

b. When counts of duplicate plates fall within and without the 25-250 colony range, use only those counts that fall within this range.

2. All plates with fewer than 25 CFU. When plates from both dilutions yield fewer than 25 CFU each, record actual plate count but record the count as less than 25 x 1/d when d is the dilution factor for the dilution from which the first counts were obtained.

Example

	Colonies	
1:100	1:1000	EAPC/ml (g)
18	2	<2,500
0	0	<2,500

3. **All plates with more than 250 CFU**. When plates from both 2 dilutions yield more than 250 CFU each (but fewer than 100/cm^2), estimate the aerobic counts from the plates (EAPC) nearest 250 and multiply by the dilution.

Example

Colonies		
1:100	**1:1000**	**EAPC/ml (g)**
TNTC	640	640,000

TNTC, too numerous to count.

EAPC, estimated aerobic plate count.

4. All plates with spreaders and/or laboratory accident. Report respectively as Spreader (SPR), or Laboratory Accident (LA).

5. All plates with more than an average of 100 CFU per sq cm. Estimate the APC as greater than 100 times the highest dilution plated, times the area of the plate. The examples below have an average count of 110 per sq cm.

Example

Colonies/Dilution		
1:100	**1:1000**	**EAPC/ml (g)**
TNTC	7,150[(a)]	>6,500,000 EAPC[(b)]
TNTC	6,490	>5,900,000 EAPC

[a] Based on plate area of 65 cm^2.

[b] EAPC, estimated APC.

[c] Based on plate area of 59 cm^2.

Spiral Plate Method

The spiral plate count (SPLC) method for microorganisms in milk, foods, and cosmetics is an official method of the APHA (2) and the AOAC (3). In this method, a mechanical plater inoculates a rotating agar plate with liquid sample. The sample volume dispensed decreases as the dispensing stylus moves from the center to the edge of the rotating plate. The microbial concentration is determined by counting the colonies on a part of the petri dish where they are easily countable and dividing this count by the appropriate volume. One inoculation determines microbial densities between 500 and 500,000 microorganisms/ml. Additional dilutions may be made for suspected high microbial concentrations.

A. Equipment and materials

1. Spiral plater (Spiral Systems Instruments, Inc., 7830 Old Georgetown Road, Bethesda, MD 20814)
2. Spiral colony counter (Spiral Systems) with special grid for relating deposited sample volumes to specific portions of petri dishes
3. Vacuum trap for disposal of liquids (2-4 liter vacuum bottle to act as vacuum reservoir and vacuum source of 50-60 cm Hg)
4. Disposable micro beakers, 5 ml
5. Petri dishes, plastic or glass, 150 x 15 mm or 100 x 15 mm
6. Plate count agar (standard methods) (M124)
7. Calculator (optional), inexpensive electronic hand calculator is recommended
8. Polyethylene bags for storing prepared plates
9. Commercial sodium hypochlorite solution, about 5% NaOCl (bleach)
10. Sterile dilution water
11. Syringe, with Luer tip for obstructions in stylus; capacity not critical
12. Work area, storage space, refrigerator, thermometers, tally, incubator, as described for Conventional Plate Count Method, above.

13. Sodium hypochlorite solution (5.25%). Available commercially.

B. Preparation of agar plates.

Automatic dispenser with sterile delivery system is recommended to prepare agar plates. Agar volume dispensed into plates is reproducible and contamination rate is low compared to hand-pouring of agar in open laboratory. When possible, use laminar air flow hood along with automated dispenser. Pour same quantity of agar into all plates so that same height of agar will be presented to spiral plater stylus tip to maintain contact angle. Agar plates should be level during cooling.

The following method is suggested for prepouring agar plates: Use automatic dispenser or pour constant amount (about 15 ml/100 mm plate; 50 ml/150 mm plate) of sterile agar at 60-70°C into each petri dish. Let agar solidify on level surface with poured plates stacked no higher than 10 dishes. Place solidified agar plates in polyethylene bags, close with ties or heat-sealer, and store inverted at 0-4.4°C. Bring prepoured plates to room temperature before inoculation.

C. Preparation of samples.

As described in Chapter 1, select that part of sample with smallest amount of connective tissues or fat globules.

D. Description of spiral plater.

Spiral plater inoculates surface of prepared agar plate to permit enumeration of microorganisms in solutions containing between 500 and 500,000 microorganisms per ml. Operator with minimum training can inoculate 50 plates per h. Within range stated, dilution bottles or pipets and other auxiliary equipment are not required. Required bench space is minimal, and time to check instrument alignment is less than 2 min. Plater deposits decreasing amount of sample in Archimedean spiral on surface of prepoured agar plate. Volume of sample on any portion of plate is known. After incubation, colonies appear along line of spiral. If colonies on a portion of plate are sufficiently spaced from each other, count them on special grid which associates a calibrated volume with each area. Estimate number of microorganisms in sample by dividing number of colonies in a defined area by volume contained in same area. Studies have shown the method to be proficient not only with milk (4) but also with other foods (7,10).

E. Plating procedure

Check stylus tip angle daily and adjust if necessary. (Use vacuum to hold microscope

cover slip against face of stylus tip; if cover slip plane is parallel at about l mm from surface of platform, tip is properly oriented). Liquids are moved through system by vacuum. Clean stylus tip by rinsing for 1 s with sodium hypochlorite solution followed by sterile dilution water for 1 s before sample introduction. This rinse procedure between processing of each sample minimizes cross-contamination. After rinsing, draw sample into tip of Teflon tubing by vacuum applied to 2-way valve. When tubing and syringe are filled with sample, close valve attached to syringe. Place agar plate on platform, place stylus tip on agar surface, and start motor. During inoculation, label petri plate lid. After agar has been inoculated, stylus lifts from agar surface and spiral plater automatically stops. Remove inoculated plate from platform and cover it. Move stylus back to starting position. Vacuum-rinse system with hypochlorite and water, and then introduce new sample. Invert plates and promptly place them in incubator for 48 ± 3 h at 35 ± 1°C.

F. Sterility controls

Check sterility of spiral plater for each series of samples by plating sterile dilution water. CAUTION: Prepoured plates should not be contaminated by a surface colony or be below room temperature (water can well-up from agar). They should not be excessively dry, as indicated by large wrinkles or glazed appearance. They should not have water droplets on surface of agar or differences greater than 2 mm in agar depth, and they should not be stored at 0-4.4°C for longer than l month. Reduced flow rate through tubing indicates obstructions or material in system. To clear obstructions, remove valve from syringe, insert hand-held syringe with Luer fitting containing water, and apply pressure. Use alcohol rinse to remove residual material adhering to walls of system. Dissolve accumulated residue with chromic acid. Rinse well after cleaning.

G. Counting grid

1. **Description.** Use same counting grid for both 100 and 150 mm petri dishes. A mask is supplied for use with 100 mm dishes. Counting grid is divided into 8 equal wedges; each wedge is divided by 4 arcs labeled l, 2, 3, and 4 from outside grid edge. Other lines within these arcs are added for ease of counting. A segment is the area between 2 arc lines within a wedge. Number of areas counted (e.g., 3) means number of segments counted within a wedge. Spiral plater deposits sample on agar plate in the same way each time. The grid relates colonies on spiral plate to the volume in which they were contained. When colonies are counted with grid, sample volume becomes greater as counting starts at outside edge of plate and proceeds toward center of plate.

2. **Calibration.** The volume of sample represented by various parts of the counting grid is shown in operator's manual that accompanies spiral plater. Grid area

constants have been checked by the manufacturer and are accurate. To verify these values, prepare 11 bacterial concentrations in range of 10^6-10^3 cells/ml by making 1:1 dilutions of bacterial suspension (use a nonspreader). Plate all Incubate both sets of plates for 48 ± 3 h at 35 ± 1°C. Calculate concentrations for each dilution. Count spiral plates over grid surface, using counting rule of 20 (described in H, below), and record number of colonies counted and grid area over which they were counted. Each spiral colony count for a particular grid area, divided by aerobic count/ml for corresponding spirally plated bacterial concentrations, indicates volume deposited on that particular grid area. Use the following formula:

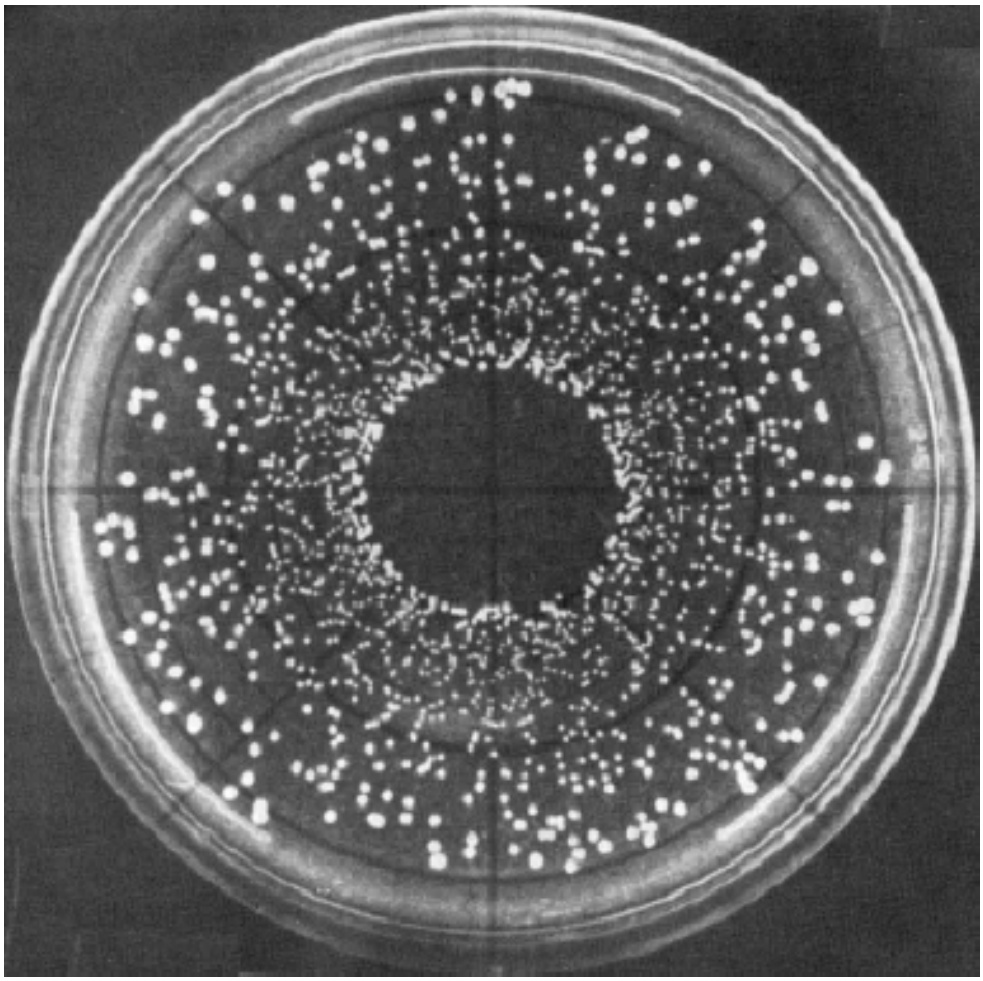
$$\text{Volume (ml) for grid area} = \frac{\text{Sprial colonies counted in area}}{\text{Bacterial count/ml (APC)}}$$

Example :

$$\text{Volume (ml)} = \frac{31 + 30 \text{ colonies}}{4.1 \times 10^4 \text{ bacteria/ml}}$$

$$= 0.0015 \text{ ml}$$

To check total volume dispensed by spiral plater, weigh amount dispensed from stylus tip. Collect in tared 5 ml plastic beaker and weigh on analytical balance (± 0.2 mg).



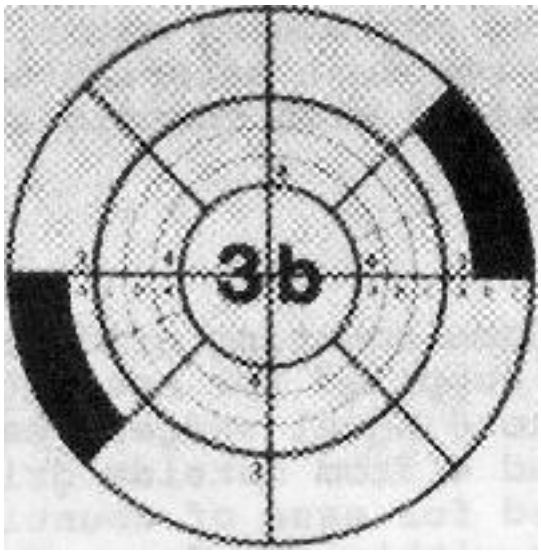


Figure 1. 10 cm plate, area (3b)

$$\frac{31 + 30 \text{ colonies}}{0.0015 \text{ ml}} = 4.1 \times 10^4$$

H. Examination and reporting of spiral plate counts.

Counting rule of 20. After incubation, center spiral plate over grid by adjusting holding arms on viewer. Choose any wedge and begin counting colonies from outer edge of first segment toward center until 20 colonies have been counted. Complete by counting remaining colonies in segment where 20th colony occurs. In this counting procedure, numbers such as 3b, 4c (Fig. l) refer to area segments from outer edge of wedge to designated arc line. Any count irregularities in sample composition are controlled by counting the same segments in the opposite wedge and recording results. Example of spirally inoculated plate (Fig. l) demonstrates method for determining microbial count. Two segments of each wedge were counted on opposite sides of plate with 31 and 30 colonies, respectively. The sample volume contained in the darkened segments is 0.0015 ml. To estimate number of microorganisms, divide count by volume contained in all segments counted. See example under Fig. l.

If 20 CFU are not within the 4 segments of the wedge, count CFU on entire plate. If the number of colonies exceeds 75 in second, third, or fourth segment, which also contains the 20th colony, the estimated number of microorganisms will generally be low because of coincidence error associated with crowding of colonies. In this case, count each circumferentially adjacent segment in all 8 wedges, counting at least 50 colonies, e.g., if the first 2 segments of a wedge contain 19 colonies and the third segment contains the 20th and 76th (or more), count colonies in all circumferentially adjacent first and second segments in all 8 wedges. Calculate contained volume in counted segments of wedges and divide into number of colonies.

When fewer than 20 colonies are counted on the total plate, report results as "less than 500 estimated SPLC per ml." If colony count exceeds 75 in first segment of wedge, report results as "greater than 500,000 estimated SPLC per ml." Do not count spiral plates with irregular distribution of colonies caused by dispensing errors. Report results of such plates as laboratory accident (LA). If spreader covers entire plate, discard plate. If spreader covers half of plate area, count only those colonies that are well distributed in spreader-free areas.

Compute SPLC unless restricted by detection of inhibitory substances in sample, excessive spreader growth, or laboratory accidents. Round off counts as described in I-D, above. Report counts as SPLC or estimated SPLC per ml.

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Top

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Bacteriological Analytical Manual *Online*

January 2001

Chapter 25

Investigation of Food Implicated in Illness

Authors

(Return to Table of Contents)

To investigate a food that has been implicated as the causative vehicle in an outbreak of illness, the microbiologist should make certain observations and perform certain tests as a matter of course; further analysis depends on the circumstances of the particular case. It is always crucial to note the general condition of the food sample, such as its consistency, color, and odor. As much information as possible should be obtained about its pre- and post-collection history (**see** Chapter l). Microscopic examination and Gram staining must be carried out, as described in Chapter 2.

To decide what treatments, enrichments, or other tests are needed, the microbiologist should evaluate the data in relation to two types of information: l) the causes epidemiologically associated with the type and condition of the implicated food, and 2) the clinical signs and symptoms observed in afflicted individuals. If possible, clinical microbial isolates (usually from stool specimens) and blood serum samples for serological and biochemical testing should be obtained from patients by way of their physicians.

Table l lists the major microbial or chemical agents of foodborne disease and their commonly associated food sources. Recently reported causative agents of foodborne outbreaks, cases, and deaths are given in Table 2. Clinical symptoms most often associated with specific microbial or chemical agents and their duration are listed in Table 3. Analysts should use these tables as an aid in deciding the most probable, less probable, and least likely associations. The tables should not be used to assume a single cause or to eliminate possibilities entirely.

The information in Tables 1-3 concerns mostly those infections designated as "reportable" in the United States by the Centers for Disease Control and Prevention (CDC). This agency, which is the principal source of epidemiologic data on reported foodborne disease outbreaks in the United States, periodically publishes summary surveillance reports of foodborne diseases in the *Morbidity and Mortality Weekly Report* series.

Most reports of foodborne illness are submitted to CDC by state health departments. CDC defines a foodborne disease outbreak as an incident in which at least two (or more) persons experience a similar illness after ingestion of a common food, and epidemiologic analysis implicates the food as the source of the illness. A few exceptions exist; for example, one case of botulism or chemical poisoning constitutes an outbreak. Although CDC's foodborne disease surveillance system has limitations (i.e., except for illnesses linked to chemicals or toxins, sporadic cases of foodborne illness are not reported), the system does provide helpful epidemiologic insights. The etiologic agent was confirmed in 909 (38%) of the 2397 outbreaks of foodborne disease reported to CDC from 1983 through 1987.

With new pathogens there is an inevitable lag before methods are installed and reporting by clinical and food laboratories becomes routine. Changes in food production or processing may make a food the vehicle or growth medium for microorganisms not previously associated with that product. For example, new varieties of tomatoes that are less acidic than the traditional types might support the growth and toxin production of *Clostridium botulinum*; freezing procedures improved to preserve taste may also preserve microorganisms that are killed in blast freezing. The food microbiologist should be aware that the clinical symptoms and diagnosis of the patient's illness, available when analysis of the food sample must begin, may be preliminary or incomplete. To proceed from the

generalities given in the tables to an analytical course of action, the microbiologist must use reason, imagination, and caution.

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General Reading

For more detailed information and instructions on the step-by-step procedures used in investigating foodborne illness, see the *Compendium of Methods for the Microbiological Examination of Foods*, published by the American Public Health Association of Washington, DC, USA.

Table 1. Number of food-implicated outbreaks in the USA reported to CDC from 1983 to l987, causative agents, and total and confirmed percentages

	FOOD SOURCE											Total	Total (%)	Confirmed (%)
Agent	Beef & pork	Poultry	Other meats	Seafood	Milk, eggs, cheese	Other dairy	Baked goods	Fruits&vegs	Salads	Other	Unknown			
Bacterial														
Bacillus cereus	1	0	0	1	1	0	0	1	0	9	4	16	0.7	1.8
Brucella	0	0	0	0	2	0	0	0	0	0	0	2	0.1	0.2
Campylobacter	0	1	0	0	12	0	0	1	1	4	9	28	1.2	.1
Clostridium botulinum	1	1	10	10	0	0	0	32	0	6	14	74	3.1	8.1
Clostridium perfringens	3	4	0	0	0	0	0	0	2	12	3	24	1.0	2.6
Escherichla coli	1	0	0	0	3	0	0	0	0	3	0	7	0.3	0.8
Salmonella	25	22	6	3	14	1	4	5	12	78	172	342	14.3	37.6
Shigella	0	2	1	2	0	0	0	3	7	9	20	44	1.8	4.8
Staphylococcus aureus	11	3	1	1	1	0	4	1	7	16	2	47	2.0	5.2
Streptococcus, Group A	0	0	0	0	0	0	0	0	2	2	3	7	0.3	0.8
Streptococcus, other	0	0	0	0	1	0	0	0	0	1	0	2	0.1	0.2
Vibrio cholerae	0	0	0	1	0	0	0	0	0	0	0	1	0.0	0.1
Vibrio parahaemolyticus	0	0	0	1	0	0	0	0	0	0	1	2	0.1	0.2
Other bacterial	0	0	0	1	2	0	0	0	0	0	1	4	0.2	0.4
Total	**42**	**33**	**18**	**20**	**35**	**1**	**8**	**43**	**31**	**140**	**229**	**600**	**25.2**	**66.0**
Chemical														
Ciguatoxin	0	0	0	86	0	0	0	0	0	0	1	87	3.6	9.6
Heavy metals	0	0	0	0	0	0	0	1	0	12	0	13	0.5	1.4
Monosodium glutamate	0	0	0	0	0	0	0	0	0	2	0	2	0.1	0.2

Mushrooms	0	0	0	0	0	0	0	0	0	14	0	14	0.6	1.5
Scombrotoxin	0	0	0	81	0	0	0	0	0	0	2	83	3.5	9.1
Shellfish	0	0	0	2	0	0	0	0	0	0	0	2	0.1	0.2
Other chemical	1	0	0	2	3	3	4	3	1	13	1	31	1.3	3.4
Total	**1**	**0**	**0**	**171**	**3**	**3**	**4**	**4**	**1**	**41**	**4**	**232**	**9.7**	**25.5**
Parasitic														
Giardia	0	0	0	0	0	0	0	1	0	1	1	3	0.1	0.3
Trichinella spiralis	24	0	8	0	0	0	0	0	0	0	1	33	1.4	3.6
Total	**24**	**0**	**8**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**2**	**36**	**1.5**	**4.0**
Viral														
Hepatitis A	1	0	0	0	0	0	0	1	2	2	22	28	1.2	3.1
Norwalk virus	0	0	0	1	1	0	0	1	1	4	4	12	0.5	1.3
Other viral	0	0	0	0	0	0	0	0	0	1	0	1	0.0	0.1
Total	**1**	**0**	**0**	**1**	**1**	**0**	**0**	**2**	**3**	**7**	**26**	**41**	**1.7**	4.5
Confirmed Total	**68**	**33**	**26**	**192**	**39**	**4**	**12**	**50**	**35**	**189**	**261**	**909**	**37.9**	
Unknown	**34**	**22**	**9**	**42**	**8**	**5**	**11**	**9**	**34**	**220**	**1094**	**1488**	**62.1**	
Total Outbreaks	**102**	**55**	**35**	**234**	**47**	**9**	**23**	**59**	**69**	**409**	**1355**	**2397**		

Table 2. Number and percent of confirmed foodborne disease outbreaks cases, and deaths in the USA reported to CDC from 1983 through 1987, listed by etiologic agent

	Outbreak		Cases		Deaths	
Etiologic agent	**No.**	**%**	**No.**	**%**	**No.**	**%**
Bacterial						
Bacillus cereus	16	1.8	261	0.5	0	0.0
Brucella	2	0.2	38	0.1	1	0.7
Campylobacter	28	3.1	727	1.3	1	0.7
Clostridium botulinum	74	8.1	140	0.3	10	7.3
Clostridium perfringens	24	2.6	2,743	5.0	2	1.5
Escherichia coli	7	0.8	640	1.2	4	2.9
Salmonella	342	37.6	31,245	57.3	39	28.5
Shigella	44	4.8	9,971	18.3	2	1.5
Staphylococcus aureus	47	5.2	3,181	5.8	0	0.0
Streptococcus, Group A	7	0.8	1,001	1.8	0	0.0
Streptococcus, other	2	0.2	85	0.2	3	2.2
Vibrio cholerae	1	0.1	2	0.0	0	0.0
Vibrio parahaemolyticus	3	0.3	11	0.0	0	0.0
Other bacterial	3	0.3	259	0.5	70	51.1
Total	**600**	**66.0**	**50,304**	**92.2**	**132**	**96.4**

Chemical						
Ciguatoxin	87	9.6	332	0.6	0	0.0
Heavy metals	13	1.4	176	0.3	0	0.0
Monosodium glutamate	2	0.2	7	0.0	0	0.0
Mushrooms	14	1.5	49	0.1	2	1.5
Scombrotoxin	83	9.l	306	0.6	0	0.0
Shellfish	2	0.2	3	0.0	0	0.0
Other chemical	31	3.4	371	0.7	1	0.7
Total	**232**	**25.5**	**1,244**	**2.3**	**3**	**2.2**
Parasitic						
Giardia	3	0.3	41	0.1	0	0.0
Trichinella spiralis	33	3.6	162	0.3	1	0.7
Total	**36**	**4.0**	**203**	**0.4**	**1**	**0.7**
Viral						
Hepatitis A	29	3.2	1,067	2.0	1	0.7
Norwalk virus	10	1.1	1,164	2.1	0	0.0
Other viral	2	0.2	558	1.0	0	0.0
Total	**41**	**4.5**	**2,789**	**5.1**	**1**	**0.7**
Confirmed Total	**909**	**100.0**	**54,540**	**100.0**	**137**	**100.0**

Source: Bean, N.H., P.M. Griffin, J.S. Golding, and C.B. Ivey. 1990. *Morbid. Mortal. Weekly Rep*. Special Supplement No. 1, Vol. 39.

Table 3. Onset, duration, and symptoms of foodborne illnesses[a1]

Onset and duration of illness	Predominant symptoms	Associated organism or toxin
Upper gastrointestinal tract symptoms (nausea, vomiting) occur first or predominate		
Less than 1 h	Nausea, vomiting, unusual taste,burning of mouth.	Metallic chemicals[a]
1-2 h	Nausea, vomiting, cyanosis, headache, dizziness, dyspnea, trembling, weakness, loss of consciousness.	Nitrites;[b] *Paragonimus* sp.
Onset 1-6 h, mean 2-4 h, duration 1-2 days	Nausea, vomiting, retching, diarrhea, abdominal pain, prostration.	*Staphylococcus aureus* and its enterotoxins; *Sarcocystis hominis*
8-16 h (1-4 h rarely)	Vomiting, abdominal cramps,diarrhea, nausea.	*Bacillus cereus*
6-24 h	Nausea, vomiting, diarrhea, thirst, dilation of pupils, collapse, coma.	*Amanita* species mushrooms;[c] *Sarcocystis suihominis*
Sore throat and respiratory symptoms occur		

12-72 h	Sore throat, fever, nausea, vomiting, rhinorrhea, sometimes a rash.	*Streptococcus pyogenes* (Lancefield Group A)
2-5 days	Inflamed throat and nose,spreading grayish exudate,fever, chills, sore throat,malaise, difficulty in swallowing,edema of cervical lymph node.	*Corynebacterium diphtheriae*
Lower gastrointestinal tract symptoms (abdominal cramps, diarrhea) occur first or predominate		
2-36 h, mean 6-12 h	Abdominal cramps, diarrhea, putrefactive diarrhea associated with *C. perfringens*, sometimes nausea and vomiting.	*Clostridium perfringens*, *Bacillus cereus*, *Streptococcus faecalis*, S. *faecium*
4-120 h, mean 18-36 h, duration 1-7 days	Abdominal cramps, diarrhea, vomiting, fever, chills, malaise, nausea, headache possible.Sometimes bloody or mucoid diarrhea, cutaneous lesions and hypotension associated with *V. vulnificus*; *V. cholerae* Ol may cause dehydration, shock; *Yersinia enterocoliticia* mimics flu and acute appendicitis.	*Salmonella* species (including *S. arizonae*), *Shigella*, enteropathogenic *Escherichia coli*, other *Enterobacteriacae*, *Vibrio parahaemolyticus*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa* (?), *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Campylobacter jejuni* (*coli*), *V. cholerae* (O1 and non-O1), *V. vulnificus*, *V. fluvialis* *V. hollisae*, *V. mimicus*
1-5 days	Diarrhea, fever, vomiting, abdominal pain, respiratory symptoms; often asymptomatic.	Enteroviruses, rotavirus, enteric adenovirus, Norwalk-like viruses; anisakid nematodes, *Nanophyetus salmincola*; *Cryptosporidium parvum*
1-6 weeks	Mucoid diarrhea (fatty stools), abdominal pain, weight loss.	*Giardia lamblia* *Nanophyetus salmincola*
1 to several weeks, mean 3-4 weeks	Abdominal pain, diarrhea, constipation, headache, drowsiness, ulcers, variable; often asymptomatic.	*Entamoeba histolytica* *Isospora belli*
3-6 months	Nervousness, insomnia, hunger pains, anorexia, weight loss, abdominal pain, sometimes gastroenteritis.	*Taenia saginata*, *T. solium*
Neurological symptoms (visual disturbances, vertigo, tingling, paralysis) occur		
Less than 1 h	Tingling and numbness, giddiness,staggering, drowsiness, tightness of throat, incoherent speech, respiratory paralysis.	Shellfish toxin[(d)]
	Gastroenteritis, nervousness,blurred vision, chest pain,cyanosis, twitching, convulsions.	Organic phosphate[(e)]
	Excessive salivation, perspiration, gastroenteritis, irregular pulse,pupils constricted, asthmatic breathing.	*Muscaria*-type mushrooms[(f)]
	Tingling and numbness, dizziness, pallor, gastroenteritis, hemorrhage, and desquamation of skin, fixed eyes, loss of reflexes, twitching, paralysis.	Tetraodon toxin[(g)]

1-6 h	Tingling and numbness, gastroenteritis, dizziness, dry mouth, muscular aches, dilated eyes, blurred vision, paralysis.	Ciguatera toxin[h]
	Nausea, vomiting, tingling, dizziness, weakness, anorexia,weight loss, confusion.	Chlorinated hydrocarbons[i]
2 h to 7 days, usually 12-36 h	Vertigo, double or blurred vision,loss of reflex to light, difficulty in swallowing, speaklng, and breathing, dry mouth, weakness, respiratory paralysis, death.	*Clostridium botulinum* and its neurotoxins
More than 72 h	Numbness, weakness of legs, spastic paralysis, impairment of vision, blindness, coma.	Organic mercury[j]
	Gastroenteritis, leg pain, ungainly high-stepping gait, foot and wrist drop.	Triorthocresyl phosphate[k]
Allergic symptoms (facial flushing, itching) occur		
Less than 1 h	Headache, dizziness, nausea, vomiting, peppery taste, burning of throat, facial swelling and flushing, stomach pain, itching of skin.	Histamine[1]
	Numbness around mouth, tingling sensation, flushing, dizziness, headache, nausea, vomiting.	Monosodium glutamate [m]
	Flushing, sensation of warmth,itching, abdominal pain, puffing of face and knees.	Nicotinic acid[n]
1-7 days	Coughing, asthma.	*Ascaris lumbricoides*
General infection symptoms (fever, chills, malaise, prostration, aches, swollen lymph nodes) occur		
4-28 days, mean 9 days	Gastroenteritis, fever, edema about eyes, perspiration, muscular pain, chills, prostration, labored breathing.	*Trichinella spiralis* *Gnathostoma* sp. *Paragonimus* spp. *Alaria* spp.
7-28 days, mean 14 days >10 months	Malaise, headache, fever, cough, nausea, vomiting, constipation, abdominal pain, chills, rose spots on abdomen, bloody stools	*Salmonella typhi* *Paragonimus westermani*
10-13 days	Fever, headache, myalgia, rash.	*Toxoplasma gondii*
10-50 days, mean 25-30 days	Fever, malaise, lassitude, anorexia, nausea, abdominal pain, jaundice.	Etiological agent not yet isolated--probably viral (especially Hepatitis A and E viruses)
Varying periods (depends on specific illness)	Fever, chills, head- or joint ache, prostration, malaise, swollen lymph nodes, and other specific symptoms of disease in question.	*Bacillus anthracis*, *Brucella melitensis*, *B. abortus*, *B. suis*, *Coxiella burnetii*, *Francisella tularensis*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Pasteurella multocida*, *Streptobacillus moniliformis*, *Campylobacter jejuni* *Leptospira* species

[a] Consider chemical tests for such substances as zinc, copper, lead, cadmium, arsenic, antimony.

[b] Consider nitrites, test for decoloration of blood.

[c] Consider *Amanita* species mushroom poisning. Identify mushrooms species eaten; test urine and blood for evidence of renal damage (SGOT, SGPT enzyme tests).

[d] Consider shellfish poisoning.

[e] Consider organic phosphate insecticide poisoning.

[f] Consider *Muscaria* species of mushrooms.

[g] Consider tetraodon (puffer) fish poisoning.

[h] Consider ciguatera fish poisoning.

[i] Consider chlorinated hydrocarbon insecticides.

[j] Consider organic mercury poisoning.

[k] Consider triorthocresyl phosphate.

[l] Consider scombroid poisoning. Examine foods for *Proteus* species or other organisms capable of decarboxylating histidine into histamine, and for histamine.

[m] Consider Chinese restaurant syndrome caused by monosodium glutamate, a flavor intensifier.

[n] Consider nicotinic acid.

(a1) Developed from *Compendium of Methods for the Microbiological Examination of Foods* (1984), pp. 454-457, American Public Health Association, Washington, DC, with permission of the publisher.

Hypertext Source: Bacteriological Analytical Manual, Edition 8, Revision A, 1998. Chapter 25.
*Authors: George J. Jackson, Joseph M. Madden, Walter E. Hill, and Karl C. Klontz

Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 4

Escherichia coli and the Coliform Bacteria

Authors

Return to Table of Contents

Chapter Contents

The methods in this chapter can be used to test for sanitary index bacteria (the coliforms, fecal coliforms, and *Escherichia coli* as a coliform) and for enterovirulent *E. coli* (EEC) strains, of which there are several major subgroups:

Enterotoxigenic *E. coli* (ETEC)--gastroenteritis, traveler's diarrhea

Enteropathogenic *E. coli* (EPEC)--infant diarrhea

Enterohemorrhagic *E. coli* (EHEC)--hemorrhagic colitis

Enteroinvasive *E. coli*--(EIEC)--bacillary dysentery

Enteroadherent *E. coli* (EAEC)--newly added category

The analyst's decision to perform a sanitary or pathogenic analysis should be based on whether the sample was implicated in an outbreak of enteric disease and whether the symptoms indicated enterovirulent *E. coli* involvement. The sanitary tests presented here include special tests for shellfish and a brief consideration of bottled water. Complete discussions of the definition and scope of the "coliforms" can be found elsewhere (5).

E. coli and the coliforms are Gram-negative, rod-shaped facultatively anaerobic bacteria. Identification criteria used are production of gas from glucose (and other sugars) and fermentation of lactose to acid and gas within 48 h at 35C (coliforms) and 45.5C (fecal coliforms and *E. coli* as a coliform). With all shellfish isolates, an incubation temperature of 44.5C (rather than 45.5C) is used (1,2). Some *E. coli* strains may be only weakly lactose-positive (delayed lactose fermentation) or even lactose-negative (5,17). Some properties of enterovirulent *E. coli* (EEC) subgroups are presented in Table 1, but for a full discussion, consult one of the recent reviews (13,25). Enteroadherent *E. coli* (EAEC), another proposed subgroup of EEC, is not fully characterized and so is not considered here. Some methods used herein correspond to AOAC's *Official Methods of Analysis* (6).

The standard weight of analytical portions of food samples examined for the presence of sanitary and enteropathogenic *E. coli* is 25 g. If desired, 50 g portions may be used with appropriate scale-up of the suspending medium. **See** Chapter 1 and current FDA field instructions on sampling and composing before proceeding with *E. coli* and coliform analyses.

Table 1. Some properties of the enterovirulent *E. coli* (EEC) subgroups[a]

Property	ETEC	EPEC	EHEC	EIEC
Toxin	LT/ST[b]	Verocytotoxin (endogenous)	Verocytotoxin	
Invasive				+
Stool	Watery	Watery, bloody	Watery, very bloody	Mucoid, bloody
Fever	Low	+		+
Fecal leukocytes				+
Intestine involved	Small	Small	Colon	Colon, lower small
Serology		Infantile types	O157:H7 & a few other serotypes	
I_D[c]	High	High	Low	Low

[a] Information on EAEC not yet available.
[b] LT, labile toxin; ST, stable toxin.
[c] I_D, infective dose.

Conventional Method for Determining Coliforms and *E. coli*

A. Equipment and materials

1. Covered water bath, with circulating system to maintain temperature of 45.5 +/- 0.2C. Water level should be above the medium in immersed tubes.

2. Immersion-type thermometer, 1-55°C, about 55 cm long, with 0.1 subdivisions, certified by National Institute of Standards and Technology (NIST), or equivalent

3. Incubator, 35 +/- 1°C

4. Balance with capacity of 2 kg and sensitivity of 0.1 g

5. Blender and blender jar (**see** Chapter 1)

6. Sterile graduated pipets, 1.0 and 10.0 ml

7. Sterile utensils for sample handling (**see** Chapter 1)

8. Dilution bottles made of borosilicate glass, with stopper or polyethylene screw caps equipped with Teflon liners

9. Quebec colony counter, or equivalent, with magnifying lens

10. Longwave UV light

11. pH meter

B. Media and reagents

1. Brilliant green lactose bile (BGLB) broth, 2% (M25)

2. Lauryl tryptose (LST) broth (M76)

3. EC broth (M49)

4. Levine's eosin-methylene blue (L-EMB) agar (M80)

5. Tryptone (tryptophane) broth (M164)

6. MR-VP broth (M104)

7. Koser's citrate broth (M72)

8. Plate count agar (PCA) (standard methods) (M124)

9. Butterfield's phosphate-buffered dilution water (R11) or equivalent diluent (except for shellfish)

10. Kovacs' reagent (R38)

11. Voges-Proskauer (VP) reagents (R89)

12. Gram stain reagents (R32)

13. Methyl red indicator (R44)

14. Violet red bile agar (VRBA) (M174)

15. VRBA-MUG agar (M175)

16. EC-MUG medium (M50)

17. Lauryl tryptose MUG (LST-MUG) broth (M77)

18. Peptone diluent, 0.1% (R56)

C. Presumptive test for coliform bacteria

1. Weigh 50 g (**see** comment on sample size on first page of this chapter) food (unthawed if frozen) into sterile high-speed blender jar. Add 450 ml Butterfield's phosphate-buffered dilution water and blend 2 min. Frozen sample can be softened by refrigerating 25 g portion for 18 h at 2-5°C. If necessary, the analytical sample may differ from 50 g in the range of 25-50 g -- depending on availability of the sample -- as long as the diluent is adjusted accordingly.

2. Prepare decimal dilutions with 90 ml sterile dilution water plus 10 ml from previous dilution. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm arc for 7 s. Do not use pipets to deliver <10% of their total volume. Transfer 1 ml portions to 3 LST tubes for each dilution for 3 consecutive dilutions. Hold pipet at angle so that its lower edge rests against tube. Let pipet drain 2-3 s. Not more than 15 min should elapse from time sample is blended until all dilutions are in appropriate media.

3. Incubate tubes 48 +/- 2 h at 35°C. Examine tubes at 24 +/- 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Reincubate negative tubes for additional 24 h. Examine a second time for gas. Perform a confirmed test on all presumptive positive (gassing) tubes.

D. Confirmed test for coliforms

Gently agitate each gassing LST tube and transfer loopful of suspension to tube of BGLB broth. Hold LST tube at angle and insert loop to avoid transfer of pellicle (if present). Incubate BGLB tubes 48 +/- 2 h at 35°C. Examine for gas production and record. Calculate most probable number (MPN) (**see** Appendix 2) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

E. Coliform group: solid medium method

1. Prepare violet red bile agar (VRBA) and pasteurize it by boiling for 2 min on day of use. **NOTE**: Overheating may result in decreased productivity (22). If autoclave is used to sterilize VRBA, heat small aliquots of about 100 ml no longer than 5 min at 121°C. Store sterile medium in the dark no longer than 2 weeks before use, and remelt agar in flowing steam, boiling water, or in a microwave oven. Cool to 48°C before use; pH, 7.0-7.2. Homogenize 25 g sample at high speed for 1 min in 225 ml Butterfield's phosphate-buffered dilution water or 0.1% peptone water. Prepare serial tenfold dilutions in Butterfield's diluent or 0.1% peptone water in accordance with anticipated level of coliforms. Transfer two 1 ml aliquots of each dilution to petri dishes.

2. Use either of two plating methods (1). For conventional method, pour 10 ml VRBA tempered to 48°C into plates. Swirl plates to mix, and let solidify. To prevent surface growth and spreading of colonies, overlay with 5 ml VRBA, and let solidify. If resuscitation is necessary, pour basal layer of 8-10 ml of tryptic soy agar tempered to 48°C. Swirl plates to mix, and incubate at room temperature for 2 +/- 0.5 h. Then overlay with 8-10 ml of melted, cooled VRBA and let solidify. To find *E. coli*among coliforms, use 100 g 4 methyl-umbelliferyl--D-glucuronide (MUG) per ml in the VRBA overlay and observe for fluorescent colonies under longwave UV light. (**See** LST-MUG section for theory and applicability.) Use aliquots of up to 4 ml of dilution when deeper plates are used and 15 ml VRBA is added.

3. Invert solidified plates and incubate 18-24 h at 35C. Incubate dairy products at 32°C (3). Examine plates with illumination under magnifying lens. Count purple-red colonies that are 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids. Plates should have 25-250 colonies. For confirmation, select colonies representing different types in accordance with their relative numbers, and transfer each to tube of BGLB broth. Incubate tubes at 35°C. Examine at 24 and 48 h for gas production.

4. Confirm colonies producing gas as coliform organisms. Perform Gram stain on sample from any tube showing a pellicle to exclude Gram-positive, lactose-fermenting bacilli. Determine number of coliforms per gram by multiplying

percentage of tubes confirmed as positive by original VRBA count, i.e., number of suspicious coliform colonies multiplied by dilution factor. A modification of this method has been commercialized (29); see later section on rehydratable dry-film method.

F. EC broth method for fecal coliforms and confirmed test for *E. coli*

The EC broth MPN method may be used for seawater and shellfish since it conforms to recommended procedures (1).

1. **Gently agitate** each gassing LST tube (or, less preferably, use gassing BGLB tubes) and transfer loopful of each suspension to tube of EC broth. Incubate EC tubes 48 +/- 2 h at 45.5 +/- 0.2°C. Examine for gas production at 24 +/- 2 h; if negative, examine again at 48 +/- 2 h. Use results of this test to calculate fecal coliform MPN. Streak loopful of suspension from each gassing tube to L-EMB agar. One portion of plate must exhibit well-separated colonies. Incubate 18-24 h at 35°C. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer 2 suspicious colonies from each L-EMB plate to PCA slants for morphological and biochemical tests. Incubate PCA slants 18-24 h at 35°C. If typical colonies are not present, transfer one or more colonies most likely to be *E. coli*. Pick one colony from every plate.

2. **Perform Gram stain**. Examine all cultures appearing as Gram-negative short rods or cocci for the following biochemical activities:

    a. **Indole production**. Inoculate tube of tryptone broth and incubate 24 +/- 2 h at 35°C. Test for indole by adding 0.2-0.3 ml of Kovacs' reagent. Appearance of distinct red color in upper layer is positive test. **NOTE**: Studies (17) indicate that indole-negative strains of *E. coli* are infrequent and probably belong to other species of *Enterobacteriaceae*. Examine these strains by using additional reactions suggested in ref. 17.

    b. **Voges-Proskauer (VP)-reactive compounds**. Inoculate tube of MR-VP broth and incubate 48 +/- 2 h at 35C. Transfer 1 ml to 13 x 100 mm tube. Add 0.6 ml a-naphthol solution and 0.2 ml 40% KOH, and shake. Add a few crystals of creatine. Shake and let stand 2 h. Test is positive if eosin pink color develops.

    c. **Methyl red-reactive compounds**. Incubate MR-VP tube additional 48 +/- 2 h at 35°C after VP test. Add 5 drops to methyl red solution to each tube. Distinct red color is positive test. Yellow is negative reaction.

d. **Citrate**. Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate 96 h at 35°C. Development of distinct turbidity is positive reaction.

e. **Gas from lactose**. Inoculate tube of LST broth and incubate 48 +/- 2 h at 35°C. Displacement of medium from inner vial or effervescence after gentle agitation is positive reaction.

f. **Interpretation**. All cultures that (a) ferment lactose with production of gas within 48 h at 35°C, (b) appear as Gram-negative nonsporeforming rods or cocci, and (c) give IMViC patterns ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*. Calculate MPN (**see** Appendix 2) of *E. coli* based on proportion of EC tubes in 3 successive dilutions that contain *E. coli*.

Rapid Method (RM-2) Using A-1 Medium for Recovery of Fecal Coliforms from Shellfish-Growing Waters

This rapid test may be used to enumerate fecal coliforms and as a presumptive test for *E. coli* in shellfish-growing waters but not, however, in shellfish tissues. Because geographical differences in these waters may affect the efficiency of this test, analysts should compare its results with those of the conventional method before using the A-1 medium method exclusively.

A. **Equipment, materials, and media**

1. Covered water bath, 44.5 +/- 0.2°C
2. A-1 medium (M1)
3. Levine's eosin-methylene blue (L-EMB) agar (M80)
4. Tryptone (tryptophane) broth (M164)
5. MR-VP broth (M104)
6. Koser's citrate broth (M72)
7. Butterfield's phosphate-buffered dilution water (R11)
8. Air incubator, 35 +/- 0.5°C

B. **Procedure for recovery of fecal coliforms from shellfish-growing waters**

Prepare samples as in presumptive test for coliform bacteria (**see** C, above). Incubate A-1 medium tubes 3 h at 35 +/- 0.5°C in air incubator. Transfer to water bath and incubate 21 +/- 2 h at 44.5 +/- 0.2C. Maintain water level in bath above level of liquid in inoculated tubes.

Presence of gas in inverted vial or of dissolved gas which can be removed by slight agitation is positive test. Report results as fecal coliform MPN/100 ml sample. **NOTE**: Fecal coliform counts tend to be greater than *E. coli* counts since no effort is made to obtain pure cultures and to identify them. Interpretation of data requires understanding of the microflorae of the specimen.

Bottled Water

For microbial quality of bottled water, FDA traditionally follows the guidelines of the Environmental Protection Agency (EPA) for *E. coli* and coliforms in drinking water. EPA's current methodology (16) is based partly on the APHA methodology (4) and partly, for coliforms only, on the Edberg et al. method (14). For information on the methodology used by FDA for water, contact A.D. Hitchins, FDA, Division of Microbiological Studies (HFS-516), Washington, DC 20204.

Examination of Shellfish and Shellfish Meats

The official bivalve molluscan FDA procedure for bacteriological analysis of domestic and imported shellfish is fully and properly described only in the 1970 edition of APHA's *Recommended Procedures of the Examination of Sea Water and Shellfish* (1). Methods, including the conventional 5-tube fecal coliform MPN test, are described for examining shell stock, fresh-shucked meats, fresh-shucked frozen shellfish, and shellfish frozen on the half shell. This procedure does not apply to the examination of crustaceans (crabs, lobsters, and shrimp) or to processed shellfish meats such as breaded, shucked, pre-cooked, and heat-processed products.

LST-MUG Method for Detecting *E. coli* in Chilled or Frozen Foods Exclusive of Bivalve Molluscan Shellfish

About 94% of *E. coli*, including many anaerogenic (non-gas-producing) strains, produce the enzyme -glucuronidase (GUD). Although some shigellae (44%) and salmonellae (29%) also produce GUD, its production by other *Enterobacteriaceae* is infrequent (19). EHEC strains, which are present in about 2% of beef, pork, lamb, and poultry samples, do not produce GUD

(12). The lack of GUD activity in enterohemorrhagic *E. coli* O157:H7 has been used as a selection criterion for this pathogen. Recently, about 34% of human fecal isolates of *E. coli* were reported to be GUD-negative (9). There is evidence, however, that GUD enzyme activity may be under catabolite repression control (9) and that the genetic sequences for the GUD enzyme (*uidA* gene) are present in most GUD-negative *E. coli* isolates (7,20).

The LST-MUG assay can presumptively identify *E. coli* within 24 h (19,33). The assay is based on the presence of GUD in *E. coli*, which cleaves the MUG substrate to release 4-methylumbelliferone (MU). When exposed to longwave (365 nm) UV light, MU exhibits a bluish fluorescence which is easily visualized. When MUG is incorporated into LST medium, coliforms can be enumerated on the basis of gas production from lactose. *E. coli* are presumptively identified by fluorescence in the medium under longwave UV light. The LST-MUG method described has been adopted as final action by the AOAC (33).

CAUTION: To observe for fluorescence, examine inoculated LST-MUG tubes under longwave (365 nm) UV light in the dark. A 6-watt hand-held UV lamp is satisfactory and safe. When using a more powerful UV source, such as a 15-watt fluorescent tube lamp, wear protective glasses or goggles (available from laboratory supply houses) if personal exposure exceeds a few minutes a day. Also, examine all glass tubes for fluorescence before use. Cerium oxide, which is sometimes added to glass as a quality control measure, will fluoresce under UV light and interfere with the MUG assay (21).

NOTE: Some foods, such as shellfish, contain natural GUD activity (36). In these instances the EC-MUG confirmatory test may be used. Recently, a hydrophobic grid membrane filter/MUG (HGMF/MUG) method for enumeration of total coliform and *E. coli* in foods was also adopted official first action by the AOAC (15).

A. Equipment and materials

1. Incubator, 35 +/- 1°C

2. Balance with capacity of 2 kg and sensitivity of 0.1 g

3. Blender and blender jar (**see** Chapter 1)

4. Sterile graduated pipets, 1.0 and 10.0 ml

5. Sterile utensils for sample handling (**see** Chapter 1)

6. Dilution bottles made of borosilicate glass, with stopper or polyethylene screw caps equipped with Teflon liners

7. UV lamp and protective eyewear

B. Media and reagents

1. Lauryl tryptose MUG (LST-MUG) broth (M77)
2. Lauryl tryptose (LST) broth (M76)
3. Levine's eosin-methylene blue (L-EMB) agar (M80)
4. MR-VP broth (M104)
5. Koser's citrate broth (M72)
6. Plate count agar (PCA) (standard methods) (M124)
7. Butterfield's phosphate-buffered dilution water (R11) or equivalent diluent
8. Kovacs' reagent (R38)
9. Voges-Proskauer (VP) reagents (R89)
10. Gram stain reagents (R32)
11. Methyl red indicator (R44)

C. Presumptive LST-MUG test for *E. coli*

1. Prepare food samples as described for Conventional Method for Determining Coliforms, C-1 above.
2. Prepare decimal dilutions as described for Conventional Method for Determining Coliforms, C-2 above, and inoculate 1 ml portions to 3 LST-MUG tubes for each dilution for 3 consecutive dilutions. Also inoculate one tube of LST-MUG with a known positive *E. coli* isolate as positive control.
3. Incubate tubes for 24 +/- 2 h at 35°C and examine each tube for growth (turbidity, gas, or fluorescence). To observe fluorescence, examine tubes in the dark under longwave UV lamp (365 nm). A bluish fluorescence is positive presumptive test

for *E. coli*. Studies by Moberg et al. (33) show that a 24 h fluorescence reading is an accurate predictor of *E. coli* and can identify 83-95% of the *E. coli*-positive tubes. After 48 h of incubation, 96-100% of *E. coli*-positive tubes can be identified (33). Perform a confirmed test on all presumptive positive tubes.

D. Confirmed LST-MUG test for *E. coli*

1. Streak loopful of suspension from each fluorescent tube to L-EMB agar and incubate 24 +/- 2 h at 35°C. Follow protocols outlined in F, above, for Gram stains, IMViC tests, and production of gas from lactose to confirm *E. coli*.

2. **Interpretation**. All cultures that (a) fluoresce, (b) ferment lactose with production of gas within 48 h at 35°C, (c) appear as Gram-negative nonsporeforming rods or cocci, and (d) give IMViC patterns of ++-- (biotype 1) or -+--- (biotype 2) are considered to be *E. coli*. Calculate MPN of *E. coli* based on proportion of fluorescent tubes in 3 successive dilutions that contain *E. coli*.

EC-MUG Method for Determining *E. coli* MPNs in Shellfish Meats

The EC-MUG method (36), like the LST-MUG method, uses MUG hydrolysis to detect *E. coli* (**see** LST-MUG section for theory and applicability). The very few other bacterial species that can hydrolyze MUG are seldom encountered in shellfish meats. Thus, by including MUG in EC broth at 44.5C and examining the incubated EC-MUG tubes for fluorescence under UV irradiation, an *E. coli* MPN can be readily obtained from a conventional 5-tube fecal coliform MPN determination for shellfish meats. The EC-MUG medium is inoculated from regular LST cultures of shellfish meats.

A. Equipment and materials

1. All those required for performing the conventional 5-tube MPN method for determining fecal coliforms

2. New, disposable borosilicate glass tubs (100 x 16 mm) for EC-MUG broths (5 ml)

3. New, disposable borosilicate glass Durham vials (50 x 9 mm) for inverted gas collection tubes in EC-MUG broths

4. Longwave UV lamp, 4 watt or equivalent

5. Positive control species: *E. coli*

6. Negative control species: *Klebsiella pneumoniae*

B. **Media and reagents**

1. All those required to determine fecal coliforms by the conventional MPN method

2. EC-MUG broth (M50)

C. **Determining fluorescence of EC-MUG broth cultures**

1. Use commercially prepared dehydrated EC-MUG, or prepare medium by adding MUG to EC broth (0.05 g/liter). Several sources of MUG compound are suitable: Marcor Development Corp., Hackensack, NJ; Biosynth International, Skokie, IL; and Sigma Chemical Co., St. Louis, MO. Sterilize EC-MUG broth at 121°C for 15 min; store up to 1 week at room temperature or refrigerate up to 1 month. Inoculate medium from LST cultures (24 h at 35°C) of shellfish meat homogenate. Determination of fluorescence in EC-MUG broth requires control cultures, which are examined with MPN EC-MUG tubes tested for fluorescence. Use 3 control tubes, 2 of which are inoculated and grown with bacterial species: *E. coli*, positive culture control; *K. pneumoniae*, negative culture control; and uninoculated, EC medium batch control. Inoculate the 2 culture control strains when EC-MUG broths are being inoculated from positive LST broths. Incubate all 3 control tubes at 44.5 +/- 0.2°C for 24 h with the other EC-MUG broths.

2. Determine fluorescence in darkened or partially darkened room. Ordinarily, turning off all room lights is sufficient. In some instances it may also be necessary to decrease light from windows by closing blinds or using shades.

3. The UV light source can affect the determination of fluorescence. Hold a 4 watt, longwave UV lamp 5-10 cm (2-4 inches) from EC-MUG cultures and shine UV light directly on sides of tubes. Use control EC-MUG broth tubes as references for judging whether tube is positive or negative for fluorescence.

4. A few isolates (<10%) of *E. coli* are MUG-positive, yet are anaerogenic (gas-negative). Include all tubes determined positive for fluorescence in *E. coli* MPN calculations. Obtain *E. coli* MPN from tables by determining tube code for EC-MUG broth cultures that fluoresce under UV irradiation.

Hydrophobic Grid Membrane Filter Methods for Rapid Enumeration of Total Coliforms, Fecal Coliforms, and *E. coli*

These methods are described in the APHA *Compendium of Methods for the Microbiological Examination of Foods* (5) and the AOAC *Official Methods of Analysis* (6).

Dry Rehydratable Film Method for the enumeration of total coliforms and *E.coli*

The Petrifilm™ Coliform Count plate and the Petrifilm™ E. coli Count plate methods (AOAC® Official Method 986.33, Bacterial and Coliform Counts in Milk - Dry Rehydratable Film Methods, AOAC® Official Method 989.10, Bacterial and Coliform Counts in Diary Products - Dry Rehydratable Film Methods, and AOAC® Official Method 991.14, Coliform and *Escherichia coli* Counts in Foods - Dry Rehydratable Film Methods) are described in the *APHA Standard Methods for the Examination of Dairy Products* and in the *Official Methods of Analysts of AOAC International*.

Isolation and Identification of Enterovirulent *E. coli* (EEC)

A. **Equipment and materials**

1. Balance, 50 g with 0.1 g sensitivity
2. Blender, Waring or equivalent, 2-speed standard model with low speed operation at 8000 rpm, with 1 liter glass or metal jar
3. Incubators, 22 +/- 2C and 35 +/- 2°C
4. Water bath, 44.0 +/- 0.2°C
5. Petri dishes, glass, clean, unscratched, 15 x 150 mm or 20 x 150 mm
6. Pipets, Pasteur
7. Pipet filler is recommended for distributing viable cultures
8. pH test paper, range 6.0-8.0

B. **Media**

1. Tryptone phosphate (TP) broth (M162)
2. Brain heart infusion (BHI) broth (M24)

3. Levine's eosin-methylene blue (L-EMB) agar (M80)
4. MacConkey agar (M91)
5. Triple sugar iron (TSI) agar (M149)
6. Blood agar base (BAB) (M21)
7. Tryptone (tryptophane) broth (M164)
8. Bromcresol purple both (M26) supplemented individually with the following carbohydrates, each at 0.5% (w/v): glucose, adonitol, cellobiose, sorbitol, arabinose, mannitol, and lactose
9. Urea broth (M171)
10. Decarboxylase basal medium (lysine, Falkow) (M44)
11. Potassium cyanide (KCN) broth (M126)
12. MR-VP broth (M104)
13. Indole nitrite medium (tryptic nitrate) (M66)
14. Acetate agar (M3)
15. Mucate broth (M105)
16. Mucate control broth (M106)
17. Malonate broth (M92)
18. Koser's citrate broth (M72)

C. **Reagents, inorganic, organic, and biological**

1. Sodium bicarbonate solution, 10%, aqueous (sterile) (R70)

2. ONPG (*o*-nitrophenyl--D-galactopyranoside) disks (R53)

3. Physiological saline solution, 0.85% (sterile) (R63)

4. Kovacs' reagent (R38)

5. VP reagents (R89)

6. Oxidase test reagent (R54)

7. Nitrite detection reagents (R48)

8. Mineral oil, heavy sterile (R46)

9. Gram stain reagents (R32)

D. Enrichment of EEC

Analyze samples promptly after they arrive. Do not freeze. If product is subject to microbiological alteration, refrigerate it before examination. **CAUTION**: Most pathogenic biotypes lose viability at 6°C. The approach recommended here permits qualitative determination of the presence of EEC. If enumeration is essential, consider either the dilution endpoint or MPN technique, depending on accuracy required and resources available.

Aseptically weigh 25 g sample into 225 ml BHI broth (see comment on sample size on first page of this chapter). If specimen is a large mass, aseptically cut slices 0.5 cm thick. Incubate 10 min at room temperature with periodic shaking. Decant medium into 500 ml Erlenmeyer flask and incubate 3 h at 35°C to resuscitate damaged cells. Transfer contents to 225 ml double strength TP broth in 1 liter flask. Incubate 20 h at 44.0 +/- 0.2°C.

E. Isolation of EEC

1. **Direct streak**. After resuscitation, streak BHI eluate to L-EMB and MacConkey agars. This approach is effective if *E. coli* is present at a level of 25,000 cells/g and if it constitutes at least 10% of microfloral growth on these agars. Incubate plates 20 h at 35°C.

2. **Enrichment**. After incubation at 44°C, streak to L-EMB and MacConkey agars. Incubate 20 h at 35°C.

3. **Selection**. Typical lactose-fermenting biotypes on L-EMB agar correspond to description given above in F-1, Method for Fecal Coliforms and Confirmed Test for *E. coli*. Typical colonies on MacConkey agar appear brick red. Lactose nonfermenting biotypes on both agars produce colorless or slightly pink colonies. Because of variety of biotypes based on pathogenic potential, 20 isolates (10 typical and 10 atypical), if possible, should be recovered for further characterization.

F. Retrieval and identification (17,24)

Because may species can grow in the enrichment, and non-(or slow) lactose fermenters must be considered, the standard procedure for biochemical and morphological recognition of coliforms described above is inadequate for identifying *E. coli*. Anaerogenic, nonmotile, slow lactose fermenters may be found in several genera of *Enterobacteriaceae*. Most tests are performed as described above. Thus, only new or modified reactions are discussed here.

1. **Primary screening**. Transfer suspicious colonies to TSI agar, BAB slant, tryptone broth, arabinose broth, and urease medium. Incubate 20 h at 35°C. Reject H_2S-positive, urease-positive, arabinose nonfermenters, and indole-negative cultures. Test ONPG reaction. Suspend growth from TSI in 0.85% saline to give detectable turbidity. Add ONPG-impregnated disk. Incubate 6 h at 35°C. Yellow color indicates positive reaction. Reject ONPG-negative, aerogenic cultures. Some Alkalescens-Dispar (i.e., anaerogenic *Escherichia*) strains are negative.

2. **Secondary screening** (48 h incubation at 35°C unless otherwise specified). To identify cultures, test additional reactions shown below. Use reactions in Table 2 to subdivide *E. coli* species. Since it is not known whether these additional species are of enteropathogenic significance to humans, organisms giving a typical reactions for *E. coli*should be further investigated. To differentiate *E. coli*from *Shigella*, examine anaerogenic, nonmotile, slow lactose fermenters for lysine decarboxylase, mucate, and acetate reactions.*E. coli*strains tend to give a positive response in one or more tests. *Shigellasonnei*, which may grow because of favorable enrichment conditions, is anaerogenic and nonmotile; it produces a negative indole reaction and slow or nonfermentation of lactose. The biochemical-physiological characteristics of *E. coli* are summarized in Table 3.

Serological Characterization (17)

Because of complex interrelationships among somatic (O), capsular (K, mainly B type), and

flagellar (H) antigens and the unknown specificity of sera available commercially, the serological analysis of *E. coli* is somewhat more difficult than that of other *Enterobacteriaceae* and should not be attempted on a routine basis. Likewise, virulence cannot be completely correlated with the presence of somatic, capsular, and colonization antigens. If serological identification of an isolate seems advisable, contact specialized laboratories, such as the Centers for Disease Control and Prevention, Atlanta, GA.

Table 4 lists serogroups and serotypes associated with major pathogenic groups of *E. coli*. Commercial sera are not available for all these pathogen-associated serogroups (Table 5). Along with the symptoms in disease cases, serotyping aids in suggesting which pathogenic attributes to test for, using tests described here or in other chapters.

Table 2. Differentiation of *Escherichia* species (17, 24)

Reaction	*E. coli* typical	*E. coli* inactive[a]	*E. hermanii*	*E. blattae*	*E. fergusoni*	*E. vulneris*
IMViC	++	++	++	++/-	+	+
KCN			+			+
Glucose, gas	+		+	+	+	
Lactose	+		+/			+
Cellobiose			+		+	
Adonitol					+	
Mannitol	+	+	+		+/	+
Malonate				+	+	+/

[a] Anaerogenic and lactose-negative.

Table 3. Biochemical-physiological behavior of *E. coli* (17)

Test	Reaction

Nitrate reduction	+
Cytochrome oxidase	-
Gram-negative, short rod	+
Fermentative (TSI)	+
Mannitol	+
Lactose	+
Malonate	-
H_2S	-
Urease	-
Citrate	-
Voges-Proskauer	-
Arabinose, acid	+
KCN	-
Indole	+
Acetate	+
Adonitol	-
Cellobiose	-
Glucose, gas	+
ONPG test	+
Mucate	90% +
Lysine decarboxylase	80% +
Methyl red	+

Table 4. Serogroups and serotypes of enterovirulent *E. coli* isolated from humans with intestinal infections (5)

Pathogenic type	**Serogroups and serotypes**		
Enteropathogenic (EPEC)	O18a,18c:H7	O20a,20b:H26	O26:NM(a)

	O26:H11	O28a,28c:NM	O44:H34
	O55:NM	O55:H6	O55:H7
	O86a:NM	O86a:H34	O111a,111b:NM
	O111a,111b:H2	O111a,111b:H12	
	O114:H10	O1114:H32	O119:NM
	O119:H6	O125a,125c:H21	O126:NM
	O126:H27	O127:NM	O127:H9
	O127:H21	O128a,128b:H2	O128a,128c:H12
	O142:H6	O158:H23	O159
Enterotoxigenic (ETEC)	O6:H16	O8:H9	O11:H27
	O15:H11	O20:NM	O25:H42
	O25:NM	O27:H7	O63
	O78:H11	O78:H12	O128:H7
	O148:H28	O149:H10	O159:H20
	O167		
Enteroinvasive (EIEC)	O28a,28c:NM	O112a,112c:NM	O124:NM
	O124:H30	O124:H32	O136:NM
	O143:NM	O144:NM	O152:NM
	O167:H4	O167:5	
Enterohemorrhagic (EHEC)	O157:H7 (and O26, O111, O113, O145, and O157:H$^-$serogroups of EPEC)		

[a] NM, nonmotile.

Table 5. *E. coli* O-serogroups recognizable with commercially available sera (5)

EPEC			ETEC	EIEC	A-D EHEC group[(a)]	
O26:K60	O86:K61	O18:K77	O6	O28:K73	O157:H7	O1

O55:K59	O119:K69	O20:K61	O8	O112:K66	O2
O111:K58	O124:K72	O20:K84	O11	O124:K72	O3
O127:K63	O125:K70	O28:K73	O78	O143:K[b]	O4
	O126:K71	O44:K74			
	O128:K67	O112:K66		O144:K[c]	

[a] Alkalescens-Dispar group; nonmotile anaerogenic *E. coli*.
[b] Detected with *Shigella boydii* 8 antiserum.
[c] Detected with *Shigella dysenteriae* 10 antiserum.

Tests for Enteroinvasive *E. coli* (EIEC)

A tissue culture (HeLa cell) test (30) is available to screen isolates for invasive potential before confirming invasiveness by the Sereny test. An in vitro staining technique using acridine orange to stain for intracellular (invasive) bacteria in HeLa cell monolayers is also an effective assay to determine invasiveness of pathogenic *E. coli* (31,32).

Sereny test for Confirmation of Invasive Potential (39)

A. **Equipment and materials**

1. Instruments for dissecting animals
2. Animal cages

B. **Media**

Veal infusion broth and agar (M173)

C. **Diagnostic reagents**

1. Guinea pigs (less than 6 months old)
2. May-Grunwald stain (R41)
3. Giemsa stain (R30)

4. Dulbecco's phosphate-buffered saline (DPBS) (R19)

D. Procedure

1. **Preparation of bacteria**. With needle, inoculate 30 ml veal infusion broth, using growth from veal infusion agar slant. Incubate 18-24 h at 35°C. Centrifuge culture (20 min at 1200 x *g* at 18°C). Resuspend cells in DPBS and recentrifuge. After last centrifugation, suspend total growth from 30 ml medium in 0.3 ml DPBS.

2. **Performance of test**. For each culture, use 3 guinea pigs, 1-6 months old. Examine the eyes for irritation or infection before use. With Pasteur pipet, transfer drop of bacterial suspension to left eye of each animal. Apply drop of uninoculated DPBS to right eye of each animal. Gently open and close eyes to spread fluids evenly over conjunctiva. Return animals to individual cages.

E. Interpretation of data

Examine animals daily for 5 days. A positive reaction is development of conjunctivitis ulceration (keratoconjunctivitis) and opacity in eye treated with bacteria, but not in control eye. Observation by veterinarian is advisable for differentiation of keratoconjunctivitis and conjunctivitis. Confirm by demonstrating intracellular location of bacteria in corneal epithelial cells, using May-Grunwald and Giemsa stains. Recovery of same culture from viruses, fungi, chlamydia, mycoplasma, and other bacteria. Consider bacteria invasive if test is positive in at least 2 of 3 trials.

Tests for Enterotoxigenic *E.coli* (ETEC)

ETEC strains produce two types of toxins: heat-labile toxin (LT) and heat-stable toxin (ST). LT can be detected by the Y-l tissue culture test (see below), and ST can be detected by the infant mouse test (see below). These toxins also can be detected by ELISA, and genes coding for them can be detected by gene probes (Chapter 24).

Y-1 Mouse Adrenal Cell Test for *E. coli* LT (37)

The validity of this method was established in a collaborative study (27). LT, which stimulates the enzyme adenylate cyclase with the production of cyclic adenosine monophosphate, is closely related to *V. cholerae* enterotoxin (CT) in molecular structure and mode of action. In this assay system, LT promotes conversion of elongated fibroblast-like cells into round, refractile cells.

A. Equipment and materials

1. Microtiter tissue culture plates, 96 flat-bottom wells, sterile, plastic with lid

2. Shaker incubator at 37°C

3. Swinnex™ filter holder, 25 mm, with 0.45 µm membrane filter

4. Microtiter pipet, 0.025 ml, sterile

5. Syringe, disposable, 1 ml; 5 ml, to accommodate Swinnex filter

6. Vertical laminar flow hood (biological contaminant hood equipped with HEPA™ filters) (Bellco Glass, Vineland, NJ 08360)

7. Freezer, -70 or -20°C.

B. **Media**

1. Ham's F-10 medium (with glutamine and $NaHCO_3$) (M58)

2. Y-1 adrenal cell growth medium (M180)

3. Y-1 adrenal cell maintenance medium. Same as above, except reduce FBS to 1 ml.

4. Trypticase soy-yeast extract (TSYE) broth (M157)

5. Trypticase (tryptic) soy agar (TSA) (M152)

6. Casamino acids-yeast extract-salts (CAYE) broth (M34)

C. **Diagnostic reagents**

1. Y-1 mouse adrenal tumor cell line, American Type Culture Collection-CCL79

2. Cholera enterotoxin. Available commercially from Schwarz-Mann, Inc., Division of Mediscience, 2 Ram Ridge Road, Spring Valley, NY 10977. Before use, dilute 1:1000 in 0.01 M phosphate-buffered saline.

3. Strains of *E. coli* producing LT and ST are available from laboratories actively

engaged in research on enteric illness.

4. Phosphate saline solution (for Y-1 assay) (R62)

D. Procedure

1. **Preparation of Y-1 cell culture.** Using standard cell culture techniques, grow Y-1 cells to confluence in 75 sq cm plastic culture flasks at 35°C in CO_2 incubator. To prepare microtiter plates, wash cell monolayer with 20 ml phosphate saline solution. Remove wash water with pipet and add 5 ml 0.25% trypsin. After 1 min exposure at room temperature, remove 4.5 ml trypsin and place flask in 35°C incubator. Observe at 5 min intervals for cell detachment. When cell sheet has detached, add 5 ml growth medium and pipet repeatedly to break clumps. Pipet this cell suspension to small beaker containing 35 ml growth medium (total volume now 40 ml). Agitate cell suspension while pipetting 0.2 ml portions of cell suspension to each well of 96-well microtiter plate. Cover plates and incubate 48 h at 35°C in CO_2 incubator.

2. **Preparation of test filtrates.** Inoculate TSYE broth (5 ml in 16 x 125 mm screw-cap tube) and TSA slant from each suspected *E. coli*colony on L-EMB agar. Examine at least 5 colonies from each subsample. Alternatively, inoculate TSYE broth from agar slant. Incubate both TSYE and TSA for 24 h at 37°C. Transfer 0.1 ml of each TSYE culture 10 ml CAYE broth in 50 ml Erlenmeyer flask. Incubate CAYE and TSYE cultures 24 h at 34°C in shaker incubator at 250 rpm. If growth occurs in CAYE, centrifuge culture 30 min at 1200 x *g*. If growth in CAYE is poor, substitute TSYE culture and treat as described for CAYE culture. Filter supernatant through 0.45 µm membrane. Heat 1 ml of each supernatant 30 min at 80°C. Store filtrates at 4°C.

3. **Assay.** Take microtiter plates prepare 48 h earlier and replace growth medium with maintenance medium. Add 0.025 ml each of heated and unheated filtrate to 4 wells of microtiter plate. Add 0.025 ml of cholera enterotoxin (CT) preparation (1 ng CT per ml) to 4 wells as positive control. Simultaneously, inoculate 4 wells with culture filtrates from known LT+ and LT- control cultures. Leave some wells uninoculated as medium controls. Incubate finished plates 30 min at 35°C in CO_2 incubator. After 39 min remove medium and replace with fresh maintenance medium. Re-incubate 18 h at 35°C in CO_2 incubator.

4. **Interpretation**. Examine all control inoculations first for proper response. A positive response is 50% or more rounded cells by visual estimate. Negative and medium controls should show 10% or less rounding. Results of this test can be

confirmed only in research centers with available resources. Confirmation models are rabbit ligated ileal loop and anti-CT or anti-LT serum neutralization in Y-1 cells. To confirm identity of LT-producing cultures as *E. coli*, use the approach recommended under retrieval and identification, F, above.

Infant Mouse Test for *E. coli* ST (10)

The validity of this method was demonstrated in a collaborative study (27).

A. Equipment and materials

1. Balance, accurate to 0.01 g
2. Forceps, dissecting, sharp point, 4-1/2 inch
3. Needle, 27 gauge
4. Needle, animal feeding, 24 gauge, 1 inch, straight
5. Tuberculin syringe, 1 ml, disposable
6. Scissors, dissecting, sharp point, 4-1/2 inch
7. Weighing boats

B. Media and reagents

1. Evans blue, 2% solution (R24)
2. Swiss albino mice, 3-5 days old

C. Procedure

1. **Preparation of host**. Suckling mice are commercially available from suppliers such as Charles River Breeding Laboratories, Wilmington, MA 01887.
2. **Preparation of bacterial filtrates**. **See** Y-1 mouse adrenal cell test, D-2, above. Add 2 drops 2% Evans blue to 1 ml sterile CAYE or TSYE culture broth filtrates. Use heated portion for ST assay.

3. **Assay**. Inject 0.1 ml culture filtrate intragastrically. Use 4 mice per filtrate. Include known ST+ and ST- controls. Keep mice 3 h at room temperature. Reject all mice not showing blue dye concentrated in the stomach or showing dye in peritoneal cavity. Sacrifice mice by CO_2 inhalation. Open abdomen and remove intestinal tract, excluding stomach and liver. Pool remaining carcasses in another tared weighing boat. Weigh both pools and compute ratio of intestinal weight to body weight.

4. **Interpretation**. A ratio of 0.083 or greater is considered positive; a ratio of 0.074 or less is considered negative. Filtrates giving ratios of 0.075-0.082 should be retested. Confirm identity of ST-producing cultures as *E. coli* by using the approach recommended under retrieval and identification, F, above.

Colonization Test

Virulence prerequisites for enterotoxigenic strains of *E. coli* include the ability to attach to the jejunal lining, to proliferate in situ, and to elaborate one or more toxins. Host specificity is manifested by possession of unique colonization factors, including antigens and lectins. At least 3 factors have been elucidated in strains of human significance: CFA I, CFA II, and 8755. Several types of mammalian cells have been proposed to show colonization: buccal, FLOW 11000, and HeLa.

Isolation Methods for Enterohemorrhagic *E. coli* O157:H7

Enterohemorrhagic *E. coli* was first recognized as an important foodborne pathogen in 1982 (35). The organism causes hemorrhagic colitis (35), which has characteristic symptoms of bloody diarrhea and abdominal cramps; however, it may progress into hemolytic uremic syndrome (23), a more severe complication that can result in kidney failure and death. Although there are many serotypes of EHEC, serotype O157:H7 has been most frequently implicated in foodborne diseases. In 1993, isolates of O157:H7 serotype caused numerous foodborne outbreaks, including a major outbreak in Washington state that infected about 500 persons. Most outbreaks of O157:H7 infections are caused by the consumption of contaminated ground beef; however, raw milk and other foods have also been implicated.

Several microbiological methods can be used to isolate *E. coli* O157:H7 from foods. Unlike typical *E. coli*, isolates of O157:H7 do not ferment sorbitol and are negative with the MUG assay; therefore, these criteria are commonly used for selective isolation. Sorbitol-MacConkey agar has been used extensively to isolate this organism from clinical specimens. Hemorrhagic colitis agar, a selective and differential medium, is used in a direct plating method to isolate O157:H7 from foods. A third procedure uses Sorbitol-MacConkey medium containing potassium tellurite and Cefixime. It includes an enrichment step and is a new method developed

as result of the recent foodborne outbreaks. This procedure has been highly effective in isolating O157:H7 from a variety of commonly contaminated foods (41).

A. **Isolation with sorbitol-MacConkey (SMAC) agar (28)** Homogenize 10 g of sample in 90 ml peptone water (R56) diluent. Prepare serial tenfold dilutions in peptone water diluent in accordance with anticipated level of contamination. Pipet 0.1 ml of each dilution in duplicate onto dried surface of SMAC (M139) agar and spread evenly across each plate. Incubate plates at 35°C and read after 18 h. Sorbitol-negative colonies are pale compared to bright pink sorbitol-positive colonies produced by *E. coli* and other enterics. As further confirmation, sorbitol-negative colonies can be tested for GUD activity by spotting culture on HC agar (with MUG; M62) and used to select for MUG-negative colonies (12,38). For definitive identification of O157:H7 serotype, test sorbitol-negative, MUG-negative colonies for agglutination with O157 and H7 antisera. **CAUTION**: high levels of contaminating coliforms in the sample may mask the presence of O157:H7 strains in this medium. Furthermore, isolates of *Escherichia hermanii* and other enterics may show similar biochemical phenotypes on SMAC and along with *Citrobacter freundii* may also agglutinate O157 antiserum; therefore, they may cause false-positive identifications (8,26).

B. **Isolation with HC agar (hemorrhagic colitis ,strains of *E. coli*|)(40)** Strains of O157:H7 may also be isolated from foods using the HC agar (M62). Because this medium contains sorbitol and the MUG reagent, distinguishing phenotypes based on reactions to these reagents can be determined simultaneously in the same medium. The fluorescence from the MUG reaction, however, is diffusible and may spread throughout the entire plate during extended incubation. A colorimetric substrate BCIG may also be used in HC agar instead of MUG (34). If low levels of O157:H7 are suspected, the food may be enriched first in modified trypticase soy broth (mTSB) (M156) containing novobiocin before it is plated on selective medium. Sorbitol-negative, MUG-negative colonies isolated on HC medium must be confirmed serologically with O157 and H7 antisera. The colonies may also be transferred to membranes for colony hybridization analysis for the presence of Shiga-like toxin genes. The procedures for isolation using HC agar and for enrichment in mTSB are described in Chapter 24, Identification of Foodborne Bacterial Pathogens by Gene Probes. **CAUTION**: Normal flora in foods may also proliferate in the mTSB enrichment medium and cause overgrowth or masking of O157:H7 colonies on the HC agar medium.

C. **Isolation with Tellurite-Cefixime-Sorbitol MacConkey (TC SMAC) agar** An enrichment/isolation procedure using the TC SMAC medium was recently introduced for detecting O157:H7 in foods. Both the enrichment and the selective media contain several antibiotics which effectively suppress the growth of normal flora. Comparative analysis of the TC SMAC procedure with the HC agar method using a variety of naturally contaminated and seeded foods showed that the TC SMAC procedure was superior to the

HC agar method in the recovery of O157:H7 bacteria (41). **CAUTION**: Although most *E. coli* are sorbitol fermenters, about 6% of the isolates will not ferment sorbitol. These atypical strains may be found in foods and will appear identical to O157:H7 colonies on the TC SMAC agar. The inclusion of MUG assay in the analysis procedure should distinguish these atypical *E. coli* strains from the O157:H7 isolates. For additional information on the TC SMAC procedure, contact Steve Weagant, FDA, Bothell, WA (206) 483-4874.

1. **Media Preparation**

   EHEC Enrichment Broth (EEB) - same as mTSB (M156) but with the following filter-sterilized antibiotics added after autoclaving:

Cefixime*	0.05 mg/liter
Cefsulodin	10.00 mg/liter
Vancomycin	8.00 mg/liter
*Available from Dynal Inc., Lake Success, NY (800) 638-9416	

   TC SMAC - Sorbitol-MacConkey agar (M139) with the following filter-sterilized additives after autoclaving and tempering:

Potassium tellurite	2.50 mg/liter
Cefixime*	0.05 mg/liter
*Available from Dynal Inc., Lake Success, NY (800) 638-9416	

2. **Enrichment**

   a. Weigh 25 g of food into 225 ml of EEB, blend or stomach briefly as necessary.

   b. Incubate at 37°C with shaking for 6 h; then after performing step "a" below, under "3. Isolation," reincubate the enrichment tube overnight

3. **Isolation**

   a. Spread plate 0.1 ml of 6-h EEB homogenate to a TC SMAC agar plate and

streak one loopful to a second TC SMAC plate.

b. Incubate agar plates at 37C overnight.

c. Sorbitol-fermenting normal flora bacteria appear as pink to red colonies. Typical O157:H7 colonies are neutral/gray with a smoky center and 1-2 mm in diameter. Pick several typical O157:H7 colonies from TC SMAC onto TSAYE (M153) slants and incubate at 37C overnight.

d. If plates do not show typical colonies, repeat steps 1-3 again, but from the 24 h enrichment tube.

4. **Confirmation**

a. Screen isolates by spotting growth from TSAYE slants to a filter wetted with Kovac's reagent (spot indole test). EHEC isolates are indole-positive.

b. If indole-positive, test for O157 antigen with commercial O157 antiserum. Both Prolex *E. coli* O157 Latex Test Reagent kit (Pro-Lab Diagnostics, Round Rock, TX, 800-522-7744) and RIM *E. coli* O157:H7 Latex Test (Remel, Lenexa, KS, 800-255-6730) give satisfactory results. From the TSAYE slant, also run an API or VITEK assay to identify the isolates as *E. coli*.

c. If indole-negative, do NOT perform latex test or further tests for Shiga-like toxin (SLT) production.

5. **Results**

a. If both the spot indole test and latex test kit results are positive, then confirm for the presence of SLT-I and II genes by colony hybridization (**see**, Chapter 24) or by polymerase chain reaction (**see** LIB 3811, Sept., 1993).

b. If the spot indole test is **positive**, but the latex test kit result is **negative**, confirmation for SLT production is not required.

Optional. Additional selective enrichment by use of immunomagnetic separation has been found useful for some samples (41). Anti-O157 immunomagnetic beads are available (Dynabeads; Dynal). Immunomagnetic separation is performed on 1 ml of EEB after 6 h of

incubation, following manufacturer's instructions. Beads are spread plated on TC SMAC and treated as outlined above. A partial list of commercially available rapid methods for detecting O157:H7 is given in Appendix 1.

D. **Identification of serotype O157:H7 isolates using an oligonucleotide probe**

As an alternative to serological typing, isolates of serotype O157:H7 may also be identified by using the serotype-specific DNA probe, PF-27. This 18-base oligonucleotide probe, developed at CFSAN, is directed at a unique region of the *uid*A gene in the O157:H7 isolate. Colony hybridization analysis of 280 bacterial isolates, including *E. coli*, several pathogenic enteric species, other Shiga-like toxin-producing EHEC and 42 isolates of O157:H7 implicated in a recent foodborne outbreak, showed that PF-27 is highly specific only for the isolates of O157:H7 serotype (18). For more information on PF-27, contact Peter Feng, CFSAN, FDA, Washington, DC. Phone (202) 205-4518. For protocols on using the probe, **see** Chapter 24.

Tests for Toxins of Enterohemorrhagic *E. coli* (EHEC)

EHEC isolates produce several toxins, but only a few have been well characterized. The major toxin is virtually identical to the Shiga toxin of *Shigelladysenteriae* type 1 and hence named Shiga-like toxin I (SLT-I). Another toxin, although only 60% homologous to SLT-I has been designated SLT-II. Since both toxins are cytotoxic to HeLa and Vero tissue culture cells, they are also known as verotoxin (VT) I and II. The toxins produced by EHECare detected by tissue culture assays. However, DNA probe and polymerase chain reaction assays have also been developed to detect the presence of SLT gene in EHEC isolates.

Tissue culture assay for Shiga-like toxins

A. Equipment and materials

1. CO_2 incubator maintained at 36°C
2. Sterile plastic plates containing 16 mm diameter wells
3. Cell counting chamber
4. Shaker incubator maintained at 37°C
5. Centrifuge
6. Sterile 0.45 µm membrane filters

7. Inverted stage microscope

B. **Media and reagents**

1. Cell growth medium (M36)
2. Dulbecco's phosphate-buffered saline (DPBS), pH 7.2 (R19)
3. Fetal bovine serum
4. Milk serum, 2%
5. Gentamicin sulfate solution (M57)
6. Eagle's minimal essential medium MEME-L15 (M46 and M73), mixed in equal proportions

Preparation of cytotoxin-VT

Inoculate culture into 20 ml trypticase soy broth (TSB) in 250 ml Erlenmeyer flask and incubate with agitation at 37°C for 20-24 h. Centrifuge culture at 7000 x *g* for 30 min to sediment bacteria. Filter supernatant through 0.45 µm membrane to remove residual bacteria. Store at 4°C. Dilute filtrate 1:5 in DPBS, pH 7.0, before use.

Preparation of Vero monolayers

Maintain Vero culture in MEME-L15 medium containing 2% milk serum, prepared as follows: Add dry milk to double distilled water to final concentration of 10%. Add concentrate of gentamicin sulfate to level of 50 µg/ml. Stir 1 h on magnetic stirrer at room temperature. With stirring, adjust pH to 4.5 with 1 N HCl. Filter through cheesecloth. Clarify by centrifugation at 2500 rpm for 15 min. Filter again through cheesecloth. Sterilize by filtration through 0.22 µm membrane. Add milk serum to give 2% concentration by volume in MEME-L15 medium. Incubate culture in 5% CO_2 incubator held at 36°C for 72 h. Examine culture for purity and appearance of cells. If cells are normal and not contaminated, treat with trypsin to remove monolayer. Suspend cells to density of 10^5 per ml in growth medium. With gentle agitation transfer 0.5 ml portions to 16 mm wells in sterile plastic dishes. Incubate 3-4 days at 36°C in CO_2 incubator. Examine for purity and appearance of cells. Remove spent medium and replace with 0.5 ml fresh medium.

1. **Toxicity test.**Add 0.05 ml diluted culture filtrate to well. For control, dilute TSB 1:5 in DPBS. Add 0.05 ml to well. Incubate 4 days at 36°C in CO_2 incubator.

2. **Examine daily for cytopathic effect,** i.e., rounding and shriveling of cells, including detachment. Potent preparations affect 50% of the cells in monolayer. There should be progressive increase in toxicity with increased incubation. Cytotoxicity in contrast to the cytotoxic effect of LT is not reversible if medium is changed and monolayers are re-incubated.

Detection of Shiga-like toxin genes in EHEC using DNA probes

The toxigenic potential of EHEC isolates may be determined by colony hybridization using oligonucleotide DNA probes that are specific for the genes that encode for SLT-I and SLT-II toxins. However, numerous other serotypes of EHEC also produce SLT. Therefore, serological or other assays are still required to identify isolates of O157:H7 serotype. For more information on these SLT probes, contact William L. Payne, CFSAN, FDA, Washington, DC, phone (202) 205-4361. For protocols on using the probes, **see** Chapter 24.

Detection of Shiga-like toxin genes in EHEC using polymerase chain reaction (PCR) assays

In addition to probes, two different sets of PCR primers specific for SLT genes have been developed at FDA, Bothell, WA, and at CFSAN. These primers can be used to determine potential toxigenicity of EHEC isolates. Both sets of primers have been tested extensively on reference and outbreak strains of EHEC and confirmed to be very effective. For more information on these SLT primers and for PCR methods and protocols **see** ref. 41; refer to FDA publication LIB 3811, Sept., 1993; or contact Walter E. Hill, SPRC, FDA, Bothell, WA, phone (206) 402-3176; or William L. Payne, CFSAN, FDA, Washington, DC, phone (202) 205-4361. Recently, a multiplex PCR procedure which can simultaneously identify the O157:H7 serotype as well as the toxin type has been developed at CFSAN and is currently under evaluation. For more information, contact Peter Feng, CFSAN, FDA, Washington, DC, phone (202) 205-4518.

CAUTION: Unlike the tissue culture assays which detect the toxins, reactivity of an EHEC isolate with SLT probes or with PCR primers merely indicates that genetic sequences for the toxins are present in that particular isolate. It does not, however, indicate that the toxins are actually produced.

Tests for Enteropathogenic *E. coli* (EPEC)

Enterovirulent *E. coli* strains that do not type as EHEC, EIEC, or ETEC strains are probably enteropathogenic *E. coli* (EPEC) strains, e.g., classical infantile diarrhea strains. Although there

are no specific tests for EPEC strains, some methods, which are still experimental, are available (see the review by Doyle and Padhye, ref. 11). Confirmation of a putative EPEC strain involves serogrouping and serotyping (Table 5) and consideration of case symptomology. Further complexity concerns the fact that some EPEC strains behave like EHEC strains (Tables 1 and 4).

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

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Center for Food Safety & Applied Nutrition

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Chapter 5

Salmonella

Authors

(Return to Table of Contents)

Chapter Contents

Introduction

Several changes are being introduced in this edition of BAM (8th Edition). The first change involves the expanded use of Rappaport-Vassiliadis (RV) medium for foods with both high and low levels of competitive microflora. In the previous edition, RV medium was recommended only for the analysis of shrimp. Based on the completion of AOAC precollaborative (5, 6) and collaborative (7, 8) studies, RV medium is now being recommended for the analysis of high

microbial and low microbial load foods. RV medium replaces selenite cystine (SC) broth for the analysis of all foods, except guar gum. In addition, RV medium replaces lauryl tryptose broth for use with dry active yeast. Tetrathionate (TT) broth continues to be used as the second selective enrichment broth. However, TT broth is to be incubated at 43°C for the analysis of high microbial load foods and at 35°C for the analysis of low microbial load foods, including guar gum.

The second change involves the option of refrigerating incubated preenrichments and selective enrichments of low-moisture foods for up to 72 h. With this option, sample analyses can be initiated as late as Wednesday or Thursday without weekend work being involved.

The third change involves reducing the period of incubation of the lysine iron agar (LIA) slants. In the former edition (BAM-7), triple sugar iron agar (TSI) and LIA slants were incubated at 35°C for 24 ± 2 h and 48 ± 2 h, respectively. Unpublished data have demonstrated that the 48 h reading of LIA slants is without diagnostic value. Of 193 LIA slants examined, all gave definitive results within 24 ± 2 h of incubation. No significant changes altered the final test result when the slants were incubated an additional 24 h. Thus, both the TSI and LIA slants are now incubated for 24 ± 2 h.

The fourth change involves the procedure for surface disinfection of shell eggs. In the previous edition (BAM-7), egg shells were surface-disinfected by soaking in 0.1% mercuric chloride solution for 1 h followed by soaking in 70% ethanol for 30 min. Mercuric chloride is classified as a hazardous waste, and is expensive to dispose of according to Environmental Protection Agency guidelines. In this edition (BAM-8), egg shells are now surface-disinfected by soaking for 30 min in a sodium hypochlorite solution (at a concentration of 200 ppm chloride) containing 0.1% sodium dodecyl sulfate.

UPDATED The fifth change involves the sample preparation of eggs. Egg contents (yolk and albumen) are thoroughly mixed before analysis. After mixing the egg contents, 25 g (ml) are added to 225 ml trypticase (tryptic) soy broth supplemented with ferrous sulfate.

A method for the analysis of guar gum has been included. When guar gum is preenriched at a 1:9 sample/broth ratio, a highly viscous, nonpipettable mixture results. Addition of the enzyme cellulase to the preenrichment medium, however, results in a readily pipettable mixture.

A method for orange juice (pasteurized and unpasteurized) has been included due to recent orange juice-related outbreaks.

The directions for picking colonies from the selective plating agars have been made more explicit to reflect the intent of the method. In the absence of typical or suspect colonies on the selective plating agars, it is recommended that atypical colonies be picked to TSI and LIA slants.

This recommendation is based on the fact that up to 4% of all *Salmonella* cultures isolated by FDA analysts from certain foods, especially seafoods, during the past several years have been atypical.

Finally, since the publication of BAM-7, a 6-way comparison was conducted of the relative effectiveness of the three selective plating agars recommended in the BAM (bismuth sulfite, Hektoen enteric, and xylose lysine desoxycholate agars) and three relatively new agars (EF-18, xylose lysine Tergitol 4, and Rambach agars). Our results (9) indicated no advantage in replacing any of the BAM-recommended agars with one or more of the newer agars. Thus, the combination of selective plating agars recommended in BAM-7 remains unchanged.

Return to Chapter Contents

A.

Equipment and materials

1. Blender and sterile blender jars (***see*** Chapter 1)
2. Sterile, 16 oz (500 ml) wide-mouth, screw-cap jars, sterile 500 ml Erlenmeyer flasks, sterile 250 ml beakers, sterile glass or paper funnels of appropriate size, and, optionally, containers of appropriate capacity to accommodate composited samples
3. Sterile, bent glass or plastic spreader rods
4. Balance, with weights; 2000 g capacity, sensitivity of 0.1 g
5. Balance, with weights; 120 g capacity, sensitivity of 5 mg
6. Incubator, 35 ± 2 °C
7. Refrigerated incubator or laboratory refrigerator, 4 ± 2°C
8. Water bath, 49 ± 1°C
9. Water bath, circulating, thermostatically-controlled, 43 ± 0.2°C
10. Water bath, circulating, thermostatically-controlled,42 ± 0.2°C
11. Sterile spoons or other appropriate instruments for transferring food samples
12. Sterile culture dishes, 15 x 100 mm, glass or plastic
13. Sterile pipets, 1 ml, with 0.01 ml graduations; 5 and 10 ml, with 0.1 ml graduations
14. Inoculating needle and inoculating loop (about 3 mm id or 10 µl), nichrome, platinum-iridium, chromel wire, or sterile plastic
15. Sterile test or culture tubes, 16 x 150 mm and 20 x 150 mm; serological tubes, 10 x 75 mm or 13 x 100 mm
16. Test or culture tube racks

17. Vortex mixer
18. Sterile shears, large scissors, scalpel, and forceps
19. Lamp (for observing serological reactions)
20. Fisher or Bunsen burner
21. pH test paper (pH range 6-8) with maximum graduations of 0.4 pH units per color change
22. pH meter
23. Plastic bags, 28 x 37 cm, sterile, with resealable tape. (Items 23-25 are needed in the analysis of frog legs and rabbit carcasses.)
24. Plastic beakers, 4 liter, autoclavable, for holding plastic bag during shaking and incubation
25. Mechanical shaker, any model that can be adjusted to give 100 excursions/min with a 4 cm (1-1/2 inches) stroke, such as the Eberbach shaker with additional 33 and 48 cm clamp bars.

Return to Chapter Contents

B. Media and reagents

For preparation of media and reagents, refer to Methods 967.25-967.28 in *Official Methods of Analysis* (1).

1. Lactose broth (M74)
2. Nonfat dry milk (reconstituted) (M111)
3. Selenite cystine (SC) broth (M134)
4. Tetrathionate (TT) broth (M145)
5. Rappaport-Vassiliadis (RV) medium (M132)
6. Xylose lysine desoxycholate (XLD) agar (M179)
7. Hektoen enteric (HE) agar (M61)
8. Bismuth sulfite (BS) agar (M19)
9. Triple sugar iron agar (TSI) (M149)
10. Tryptone (tryptophane) broth (M164)
11. Trypticase (tryptic) soy broth (M154)
12. Trypticase soy broth with ferrous sulfate (M186) New!
13. Lauryl tryptose (LST) broth (M76)
14. Trypticase soy-tryptose broth (M160)
15. MR-VP broth (M104)
16. Simmons citrate agar (M138)
17. Urea broth (M171)
18. Urea broth (rapid) (M172)
19. Malonate broth (M92)
20. Lysine iron agar (LIA) (Edwards and Fife) (M89)

21. Lysine decarboxylase broth (M87)
22. Motility test medium (semisolid) (M103)
23. Potassium cyanide (KCN) broth (M126)
24. Phenol red carbohydrate broth (M121)
25. Purple carbohydrate broth (M130)
26. MacConkey agar (M91)
27. Nutrient broth (M114)
28. Brain heart infusion (BHI) broth (M24)
29. Papain solution, 5% (M56a)
30. Cellulase solution, 1% (M187)

New!

31. Tryptose blood agar base (M166)
32. Universal preenrichment broth(M188)

New!

33. Potassium sulfite powder, anhydrous
34. Chlorine solution, 200 ppm, containing 0.1% sodium dodecyl sulfate (R12a)
35. Ethanol, 70% (R23)
36. Kovacs' reagent (R38)
37. Voges-Proskauer (VP) test reagents (R89)
38. Creatine phosphate crystals
39. Potassium hydroxide solution, 40% (R65)
40. 1 N Sodium hydroxide solution (R73)
41. 1 N Hydrochloric acid (R36)
42. Brilliant green dye solution, 1% (R8)
43. Bromcresol purple dye solution, 0.2% (R9)
44. Methyl red indicator (R44)
45. Sterile distilled water
46. Tergitol Anionic 7 (R78)
47. Triton X-100 (R86)
48. Physiological saline solution, 0.85% (sterile) (R63)
49. Formalinized physiological saline solution (R27)
50. *Salmonella* polyvalent somatic (O) antiserum
51. *Salmonella* polyvalent flagellar (H) antiserum
52. *Salmonella* somatic group (O) antisera: A, B, C_1, C_2, C_3, D_1, D_2, E_1, E_2, E_3, E_4, F, G, H, I, Vi, and other groups, as appropriate
53. *Salmonella*Spicer-Edwards flagellar (H) antisera

Return to Chapter Contents

C.

Preparation of foods for isolation of *Salmonella*

The following methods are based on the analysis of a 25 g analytical unit at a 1:9 sample/broth ratio. Depending on the extent of compositing, add enough broth to maintain this 1:9 ratio unless otherwise indicated. For samples not analyzed on an exact weight basis, e.g., frog legs, refer to the specific method for instructions.

1. **Dried egg yolk, dried egg whites, dried whole eggs, liquid milk (skim milk, 2% fat milk, whole, and buttermilk), and prepared powdered mixes (cake, cookie, doughnut, biscuit, and bread), infant formula, and oral or tube feedings containing egg**.

   Preferably, do not thaw frozen samples before analysis. If frozen sample must be tempered to obtain analytical portion, thaw suitable portion as rapidly as possible to minimize increase in number of competing organisms or to reduce potential of injuring *Salmonella* organisms. Thaw below 45°C for 15 min with continuous agitation in thermostatically controlled water bath or thaw within 18 h at 2-5°C. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. For nonpowdered samples, add 225 ml sterile lactose broth. If product is powdered, add about 15 ml sterile lactose broth and stir with sterile glass rod, spoon, or tongue depressor to smooth suspension. Add 3 additional portions of lactose broth, 10, 10, and 190 ml, for total of 225 ml. Stir thoroughly until sample is suspended without lumps. Cap jar securely and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1 N NaOH or 1 N HCl. Cap jar securely and mix well before determining final pH. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11 , below.
2. Eggs

   a. **Shell eggs**. Wash eggs with stiff brush and drain. Soak eggs in 200 ppm Cl^- solution containing 0.1% sodium dodecyl sulfate (SDS) for 30 min. Prepare the 200 ppm Cl^-/0.1% SDS solution by adding 8 ml commercial bleach (5.25% sodium hypochlorite) to 992 ml distilled water containing 1 g SDS. This disinfectant should be prepared immediately before analysis. Crack eggs aseptically into a sterile container and thoroughly mix egg yolks and egg whites with a sterile spoon or other sterile instrument. Aseptically weigh 25 g into sterile 500 ml Erlenmeyer flask or other appropriate container. Add 225 ml trypticase (tryptic) soy broth (TSB) supplemented with ferrous sulfate (35 mg ferrous sulfate added to 1000 ml TSB) and mix well by swirling. Let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if

necessary, to 6.8 ± 0.2. Incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

b. **Liquid whole eggs (homogenized)**. Weigh 25 g into sterile 500 ml Erlenmeyer flask or other appropriate container. Add 225 ml TSB supplemented with ferrous sulfate as described above and mix well by swirling. Continue as described above.

c. **Hard-boiled eggs (chicken, duck, and others)**. If the egg shells are still intact, disinfect the shells as described above and aseptically separate the shells from the eggs. Pulverize the eggs (egg yolk solids and egg white solids) aseptically and weigh 25 g into a sterile 500 ml Erlenmeyer flask or other appropriate container. Add 225 ml TSB (without ferrous sulfate) and mix well by swirling. Continue as described above.

3. **Nonfat dry milk**
   a. **Instant**. Aseptically weigh 25 g sample into sterile beaker (250 ml) or other appropriate container. Using sterile glass or paper funnel (made with tape to withstand autoclaving), pour 25 g analytical unit gently and slowly over surface of 225 ml brilliant green water contained in sterile 500 ml Erlenmeyer flask or other appropriate container. Alternatively, 25 g analytical units may be composited and poured over the surface of proportionately larger volumes of brilliant green water. Prepare brilliant green water by adding 2 ml 1% brilliant green dye solution per 1000 ml sterile distilled water. Let container stand undisturbed for 60 ± 5 min. Incubate loosely capped container, without mixing or pH adjustment, for 24 ± 2 h at 35°C. Continue as in D, 1-11, below.
   b. **Non-Instant**. Examine as described for instant nonfat dry milk, except that the 25 g analytical units may not be composited.
4. **Dry whole milk**. Examine as described for instant nonfat dry milk, except that the 25 g analytical units may not be composited.
5. **Casein** UPDATED
   a. **Lactic casein and rennet casein.** Aseptically weigh 25 g sample into sterile beaker (250 ml) or other appropriate container. Using sterile glass or paper funnel (made with tape to withstand autoclaving), pour 25 g analytical unit gently and slowly over the surface of 225 ml lactose broth contained in sterile 500 ml Erlenmeyer flask or other appropriate container. Analytical units (25 g) may be composited. Let container stand undisturbed 60 ± 5 min. Incubate loosely capped container, without mixing or pH adjustment, for 24 ± 2 h at 35 °C. Continue as in D, 1-11, below.
   b. **Sodium caseinate**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml

sterile lactose broth and mix well. Analytical units may be composited. Let stand 60 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Loosen jar about 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

6. **Soy flour**. UPDATED Examine as described for lactic casein and rennet casein, except 25 g analytical units (25 g) may not be composited.
7. **Egg-containing products (noodles, egg rolls, macaroni, spaghetti), cheese, dough, prepared salads (ham, egg, chicken, tuna, turkey), fresh, frozen, or dried fruits and vegetables, nut meats, crustaceans (shrimp, crab, crayfish, langostinos, lobster), and fish**. Preferably, do not thaw frozen samples before analysis. If frozen sample must be tempered to obtain analytical portion, thaw below 45°C for ≤15 min with continuous agitation in thermostatically controlled water bath or thaw within 18 h at 2-5°C.

   Aseptically weigh 25 g sample into sterile blending container. Add 225 ml sterile lactose broth and blend 2 min. Aseptically transfer homogenized mixture to sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container and let stand 60 ± 5 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Mix well and loosen jar cap about 1/4 turn. Incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

8. **Dried yeast (active and inactive yeast)**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile trypticase soy broth. Mix well to form smooth suspension. Let stand 60 ± 5 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2, mixing well before determining final pH. Loosen jar cap 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.
9. **Frosting and topping mixes**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml nutrient broth and mix well. Cap jar securely and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.
10. **Spices**
    a. **Black pepper, white pepper, celery seed or flakes, chili powder, cumin, paprika, parsley flakes, rosemary, sesame seed, thyme, and vegetable flakes**.

Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile trypticase soy broth (TSB) and mix well. Cap jar securely and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Loosen jar cap about l/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

b. **Onion flakes, onion powder, garlic flakes**.

Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Preenrich sample in TSB with added K_2SO_3 (5 g K_2SO_3 per 1000 ml TSB, resulting in final 0.5% K_2SO_3 concentration). Add K_2SO_3 to broth before autoclaving 225 ml volumes in 500 ml Erlenmeyer flasks at 121°C for 15 min. After autoclaving, aseptically determine and, if necessary, adjust final volume to 225 ml. Add 225 ml sterile TSB with added K_2SO_3 to sample and mix well. Continue as in C-10a.

c. **Allspice, cinnamon, cloves, and oregano**.

At this time there are no known methods for neutralizing the toxicity of these 4 spices. Dilute them beyond their toxic levels to examine them. Examine allspice, cinnamon, and oregano at 1:100 sample/broth ratio, and cloves at 1:1000 sample/broth ratio. Examine leafy condiments at sample/broth ratio greater than 1:10 because of physical difficulties encountered by absorption of broth by dehydrated product. Examine these spices as described in C-10a, above, maintaining recommended sample/broth ratios.

11. **Candy and candy coating (including chocolate)**. Aseptically weigh 25 g sample into sterile blending container. Add 225 ml sterile, reconstituted nonfat dry milk and blend 2 min. Aseptically transfer homogenized mixture to sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container and let stand 60 ± 5 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Add 0.45 ml 1% aqueous brilliant green dye solution and mix well. Loosen jar caps 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.
12. **Coconut**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile lactose broth, shake well, and let stand 60 ± 5 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8

± 0.2. Add up to 2.25 ml steamed (15 min) Tergitol Anionic 7 and mix well. Alternatively, use steamed (15 min) Triton X-100. Limit use of these surfactants to minimum quantity needed to initiate foaming. For Triton X-100 this quantity may be as little as 2 or 3 drops. Loosen jar cap about l/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

13. **Food dyes and food coloring substances**. For dyes with pH 6.0 or above (10% aqueous suspension), use method described for dried whole eggs (C-l, above). For laked dyes or dyes with pH below 6.0, aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml tetrathionate broth without brilliant green dye. Mix well and let stand 60 ± 5 min at room temperature with jar securely capped. Using pH meter, adjust pH to 6.8 ± 0.2. Add 2.25 ml 0.1% brilliant green dye solution and mix thoroughly by swirling. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 3-11, below.
14. **Gelatin**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile lactose broth and 5 ml 5% aqueous papain solution and mix well. Cap jar securely and incubate at 35°C for 60 ± 5 min. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.
15. **Meats, meat substitutes, meat by-products, animal substances, glandular products, and meals (fish, meat, bone)**. Aseptically weigh 25 g sample into sterile blending container. Add 225 ml sterile lactose broth and blend 2 min. Aseptically transfer homogenized mixture to sterile wide-mouth, screw-cap jar (500 ml) or other appropriate container and let stand 60 ± 5 min at room temperature with jar securely capped. If mixture is powder or is ground or comminuted, blending may be omitted. For samples that do not require blending, add lactose broth and mix thoroughly; let stand for 60 ± 5 min at room temperature with jar securely capped.

    Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Add up to 2.25 ml steamed (15 min) Tergitol Anionic 7 and mix well. Alternatively, use steamed (15 min) Triton X-100. Limit use of these surfactants to minimum quantity needed to initiate foaming. Actual quantity will depend on composition of test material. Surfactants will not be needed in analysis of powdered glandular products. Loosen jar caps 1/4 turn and incubate sample mixtures 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

16. **Frog legs**. (This method is used for all domestic and imported frog legs.) Place 15 pairs of frog legs into sterile plastic bag and cover with sterile lactose broth (**see** A, 23-25, above). If single legs are estimated to average 25 g or more, examine

only one leg of each of 15 pairs. Place bag in large plastic beaker or other suitable container and shake 15 min on mechanical shaker set for 100 excursions/min with stroke of 4 cm (1-1/2 inches). Pour off lactose broth from bag into another sterile plastic bag and add more lactose broth to total volume of 3500 ml. Mix well and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Place plastic bag containing the lactose broth into plastic beaker or other suitable container. Incubate 24 ± 2 h at 35°C. Continue examination as in D, 1-11, below.

17. **Rabbit carcasses.** (This method is used for all domestic and imported rabbit carcasses.) Place each of 3 rabbit carcasses into sterile plastic bag and cover with sterile lactose broth (see A, 23-25, above). Place bag in large plastic beaker or other suitable container and shake 15 min on mechanical shaker set for 100 excursions/min with stroke of 4 cm (1-1/2 inches). Composite lactose broth rinsings by pouring into another sterile container and add more lactose broth to total volume of 3500 ml. Mix well and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Incubate 24 ± 2 h at 35°C. Continue examination as in D, 1-11, below.
18. **Guar gum**. Aseptically weigh 25 g sample into sterile beaker (250 ml) or other appropriate container. Prepare a 1.0% cellulase solution (add 1 g cellulase to 99 ml sterile distilled water). Dispense into 150 ml bottles. (Cellulase solution may be stored at 2-5°C for up to 2 weeks). Add 225 ml sterile lactose broth and 2.25 ml sterile 1% cellulase solution to sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. While vigorously stirring the cellulase/lactose broth with magnetic stirrer, pour 25 g analytical unit quickly through sterile glass funnel into the cellulase/lactose broth. Cap jar securely and let stand 60 ± 5 min at room temperature. Incubate loosely capped container without pH adjustment, for 24 ± 2 h at 35°C. Continue as in D, 1-11, below.
19. **Orange juice (pasteurized and unpasteurized), apple cider (pasteurized and unpasteurized), and apple juice (pasteurized)** Aseptically add 25 ml sample to 225 ml Universal preenrichment broth in a sterile, wide mouth, screw-capped jar (500 ml) or other appropriate container. Swirl the flask contents thoroughly. Cap jar securely and let stand 60 ± 5 min at room temperature. Do not adjust pH. Incubate loosely capped container for 24 ± 2 h at 35°C. Continue as in D, 1-11, below (treat as a low microbial load food).

Return to Chapter Contents

D.

Isolation of *Salmonella*

1. Tighten lid and gently shake incubated sample.

Guar gum. Transfer 1 ml mixture to 10 ml selenite cystine (SC) broth and another 1 ml mixture to 10 ml TT broth . Vortex.

All other foods. Transfer **0.1** ml mixture to 10 ml Rappaport-Vassiliadis (RV) medium and another 1 ml mixture to 10 ml tetrathionate (TT) broth. Vortex.

2. Incubate selective enrichment media as follows:

   Foods with a high microbial load. Incubate RV medium 24 ± 2 h at 42 ± 0.2°C (circulating, thermostatically-controlled, water bath). Incubate TT broth 24 ± 2 h at 43 ± 0.2°C (circulating, thermostatically-controlled, water bath).

   Foods with a low microbial load (except guar gum). Incubate RV medium 24 ± 2 h at 42 ± 0.2°C (circulating, thermostatically controlled, water bath). Incubate TT broth 24 ± 2 h at 35 ± 2.0°C.

   Guar gum. Incubate SC and TT broths 24 ± 2 h at 35°C.

3. Mix (vortex, if tube) and streak 3 mm loopful (10 µl) incubated TT broth on bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar. **Prepare BS plates the day before streaking and store in dark at room temperature until streaked.**
4. Repeat with 3 mm loopful (10 µl) of RV medium (for samples of high and low microbial load foods) and of SC broth (for guar gum).
5. Refer to 994.04 in *Official Methods of Analysis* (1) for option of refrigerating incubated sample preenrichments and incubated sample selective enrichments (SC and TT broths only) of low moisture foods. This option allows sample analyses to be initiated as late as Thursday while still avoiding weekend work.
6. Incubate plates 24 ± 2 h at 35°C.
7. Examine plates for presence of colonies that may be *Salmonella*.

TYPICAL *Salmonella* COLONY MORPHOLOGY

Pick 2 or more colonies of *Salmonella* from each selective agar after 24 ± 2 h incubation. Typical *Salmonella* colonies are as follows:

a. **Hektoen enteric (HE) agar**. Blue-green to blue colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.

b. **Xylose lysine desoxycholate (XLD) agar**. Pink colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.
c. **Bismuth sulfite (BS) agar. Brown, gray, or black colonies; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the so-called halo effect.**

If typical colonies are present on the BS agar after 24 ± 2 h incubation, then pick 2 or more colonies. Irrespective of whether or not BS agar plates are picked at 24 ± 2 h, reincubate BS agar plates an additional 24 ± 2 h. After 48 ± 2 h incubation, pick 2 or more typical colonies, if present, from the BS agar plates, only if colonies picked from the BS agar plates incubated for 24 ± 2 h give atypical reactions in triple sugar iron agar (TSI) and lysine iron agar (LIA) that result in culture being discarded as not being *Salmonella* . **See** sections D.9 and D.10, below, for details in interpreting TSI and LIA reactions.

ATYPICAL *Salmonella* COLONY MORPHOLOGY

In the absence of typical or suspicious *Salmonella* colonies, search for atypical *Salmonella* colonies as follows:

a. **HE and XLD agars**. Atypically a few *Salmonella* cultures produce yellow colonies with or without black centers on HE and XLD agars. In the absence of typical *Salmonella* colonies on HE or XLD agars after 24 ± 2 h incubation, then pick 2 or more atypical *Salmonella* colonies.
b. **BS agar.** Atypically some strains produce green colonies with little or no darkening of the surrounding medium. If typical or suspicious colonies are not present on BS agar after 24 ± 2 h, then do not pick any colonies but reincubate an additional 24 ± 2 h. If typical or suspicious colonies are not present after 48 ± 2 h incubation, then pick 2 or more atypical colonies.

SUGGESTED CONTROL CULTURES

In addition to the positive control cultures (typical *Salmonella*), 3 additional *Salmonella* cultures are recommended to assist in the selection of atypical *Salmonella* colony morphology on selective agars. These cultures are a lactose-positive, H_2S-positive *S. diarizonae* (ATCC 12325) and a lactose-negative, H_2S-negative *S. abortus equi* (ATCC 9842); **OR** a lactose-positive, H_2S-negative *S. diarizonae* (ATCC 29934). These cultures may be obtained from the American

Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209.

8. Lightly touch the very center of the colony to be picked with sterile inoculating needle and inoculate TSI slant by streaking slant and stabbing butt. Without flaming, inoculate LIA slant by stabbing butt twice and then streaking slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm). Store picked selective agar plates at 5-8°C.
9. Incubate TSI and LIA slants at 35°C for 24 ± 2 h. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H_2S production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar) in TSI. In LIA, *Salmonella* typically produces alkaline (purple) reaction in butt of tube. Consider only distinct yellow in butt of tube as acidic (negative) reaction. Do not eliminate cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce H_2S in LIA. Some non- *Salmonella* cultures produce a brick-red reaction in LIA slants.

10. All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates and submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an alkaline slant and acid butt in TSI should also be considered potential *Salmonella* isolates and should be submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella* . Test retained, presumed-positive TSI cultures as directed in D-11, below, to determine if they are *Salmonella* species, including *S. arizonae*. If TSI cultures fail to give typical reactions for *Salmonella* (alkaline slant and acid butt) pick additional suspicious colonies from selective medium plate not giving presumed-positive culture and inoculate TSI and LIA slants as described in D-8, above.
11. Apply biochemical and serological identification tests to:
    a. Three presumptive TSI cultures recovered from set of plates streaked from RV medium (or SC broth for guar gum), if present, and 3 presumptive TSI agar cultures recovered from plates streaked from TT broth, if present.
    b. If 3 presumptive-positive TSI cultures are not isolated from one set of agar plates, test other presumptive-positive TSI agar cultures, if isolated, by biochemical and serological tests. Examine a minimum of 6 TSI cultures for each 25 g analytical unit or each 375 g composite.

Return to Chapter Contents

Identification of *Salmonella*

1. **Mixed cultures**. Streak TSI agar cultures that appear to be mixed on MacConkey agar, HE agar, or XLD agar. Incubate plates 24 ± 2 h at 35°C. Examine plates for presence of colonies suspected to be *Salmonella*.
    a. **MacConkey agar**. Typical colonies appear transparent and colorless, sometimes with dark center. Colonies of *Salmonella* will clear areas of precipitated bile caused by other organisms sometimes present.
    b. **Hektoen enteric (HE) agar**. **See** D-7a, above.
    c. **Xylose lysine desoxycholate (XLD) agar**. **See** D-7b, above. Transfer at least 2 colonies suspected to be *Salmonella* to TSI and LIA slants as described in D-7, above, and continue as in D-9, above.
2. Pure cultures
    a. **Urease test (conventional)**. With sterile needle, inoculate growth from each presumed-positive TSI slant culture into tubes of urea broth. Since occasional, uninoculated tubes of urea broth turn purple-red (positive test) on standing, include uninoculated tube of this broth as control. Incubate 24 ± 2 h at 35°C.
    b. **Optional urease test (rapid)**. Transfer two 3-mm loopfuls of growth from each presumed-positive TSI slant culture into tubes of rapid urea broth. Incubate 2 h in 37 ± 0.5°C water bath. Discard all cultures giving positive test. Retain for further study all cultures that give negative test (no change in color of medium).
3. Serological polyvalent flagellar (H) test
    a. Perform the polyvalent flagellar (H) test at this point, or later, as described in E-5, below. Inoculate growth from each urease-negative TSI agar slant into either 1) BHI broth and incubate 4-6 h at 35°C until visible growth occurs (to test on same day); or 2) trypticase soy-tryptose broth and incubate 24 ± 2 h at 35°C (to test on following day). Add 2.5 ml formalinized physiological saline solution to 5 ml of either broth culture.
    b. Select 2 formalinized broth cultures and test with *Salmonella* polyvalent flagellar (H) antisera. Place 0.5 ml of appropriately diluted *Salmonella* polyvalent flagellar (H) antiserum in 10 x 75 mm or 13 x 100 mm serological test tube. Add 0.5 ml antigen to be tested. Prepare saline control by mixing 0.5 ml formalinized physiological saline solution with 0.5 ml formalinized antigen. Incubate mixtures in 48-50°C water bath. Observe at 15 min intervals and read final results in 1 h.

Positive--agglutination in test mixture and no agglutination in control.

Negative--no agglutination in test mixture and no agglutination in control.

Nonspecific--agglutination in both test mixture and control. Test the cultures giving such results with Spicer-Edwards antisera.

4. **Spicer-Edwards serological test**. Use this test as an alternative to the polyvalent flagellar (H) test. It may also be used with cultures giving nonspecific agglutination in polyvalent flagellar (H) test. Perform Spicer-Edwards flagellar (H) antisera test as described in E, 3b, above. Perform additional biochemical tests (E, 5a-c, below) on cultures giving positive flagellar test results. If both formalinized broth cultures are negative, perform serological tests on 4 additional broth cultures (E, 3a, above). If possible, obtain 2 positive cultures for additional biochemical testing E, 5a-c, below). If all urease-negative TSI cultures from sample give negative serological flagellar (H) test results, perform additional biochemical tests E, 5a-c, below).
5. Testing of urease-negative cultures
    a. **Lysine decarboxylase broth**. If LIA test was satisfactory, it need not be repeated. Use lysine decarboxylase broth for final determination of lysine decarboxylase if culture gives doubtful LIA reaction. Inoculate broth with small amount of growth from TSI slant suspicious for *Salmonella* . Replace cap tightly and incubate 48 ± 2 h at 35°C but examine at 24 h intervals. *Salmonella* species cause alkaline reaction indicated by purple color throughout medium. Negative test is indicated by yellow color throughout medium. If medium appears discolored (neither purple nor yellow) add a few drops of 0.2% bromcresol purple dye and re-read tube reactions.
    b. **Phenol red dulcitol broth or purple broth base with 0.5% dulcitol**. Inoculate broth with small amount of growth from TSI culture. Replace cap loosely and incubate 48 ± 2 h at 35°C, but examine after 24 h. Most *Salmonella* species give positive test, indicated by gas formation in inner fermentation vial and acid pH (yellow) of medium. Production of acid should be interpreted as a positive reaction. Negative test is indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromcresol purple as indicator) color throughout medium.
    c. **Tryptone (or tryptophane) broth**. Inoculate broth with small growth from TSI agar culture. Incubate 24 ± 2 h at 35°C and proceed as follows:

        - **1) Potassium cyanide (KCN) broth.** Transfer 3 mm loopful of 24 h tryptophane broth culture to KCN broth. Heat rim of tube so that good seal is formed when tube is stoppered with wax-coated cork. Incubate 48 ± 2 h at 35°C but examine after 24 h. Interpret growth (indicated by turbidity) as positive. Most *Salmonella* species do not grow in this medium, as indicated by lack of turbidity.

- **2) Malonate broth**. Transfer 3 mm loopful of 24 h tryptone broth culture to malonate broth. Since occasional uninoculated tubes of malonate broth turn blue (positive test) on standing, include uninoculated tube of this broth as control. Incubate 48 ± 2 h at 35°C, but examine after 24 h. Most *Salmonella* species cultures give negative test (green or unchanged color) in this broth.

- **3) Indole test.** Transfer 5 ml of 24 h tryptophane broth culture to empty test tube. Add 0.2-0.3 ml Kovacs' reagent. Most *Salmonella* cultures give negative test (lack of deep red color at surface of broth). Record intermediate shades of orange and pink as ±.

- **4) Serological flagellar (H) tests for *Salmonella* .** If either polyvalent flagellar (H) test (E-3, above) or the Spicer-Edwards flagellar (H) test tube test (E-4, above) has not already been performed, either test may be performed here.

- **5)** Discard as not *Salmonella* any culture that shows either positive indole test and negative serological flagellar (H) test, or positive KCN test and negative lysine decarboxylase test.

- Serological somatic (O) tests for *Salmonella*.

(Pre-test all antisera to *Salmonella* with known cultures.)

a. **Polyvalent somatic (O) test**. Using wax pencil, mark off 2 sections about 1 x 2 cm each on inside of glass or plastic petri dish (15 x 100 mm). Commercially available sectioned slides may be used. Emulsify 3 mm loopful of culture from 24-48 h TSI slant or, preferably, tryptose blood agar base (without blood) with 2 ml 0.85% saline. Add 1 drop of culture suspension to upper portion of each rectangular crayon-marked section. Add 1 drop of saline solution to lower part of one section only. Add 1 drop of *Salmonella* polyvalent somatic (O) antiserum to other section only. With clean sterile transfer loop or needle, mix culture suspension with saline solution for one section and repeat for other section containing antiserum. Tilt mixtures in back-and-forth motion for 1 min and observe against dark background in good illumination. Consider any degree of agglutination a positive reaction. Classify polyvalent somatic (O) test results as follows:

Positive--agglutination in test mixture; no agglutination in saline control.

Negative--no agglutination in test mixture; no agglutination in saline control.

Nonspecific--agglutination in test and in control mixtures. Perform further biochemical and serological tests as described in *Edwards and Ewing's Identification of Enterobacteriaceae* (2).

b. **Somatic (O) group tests**. Test as in E-6a, above, using individual group somatic (O) antisera including Vi, if available, in place of *Salmonella* polyvalent somatic (O) antiserum. For special treatment of cultures giving positive Vi agglutination reaction, refer to sec. 967.28B in *Official Methods of Analysis* (1). Record cultures that give positive agglutination with individual somatic (O) antiserum as positive for that group. Record cultures that do not react with individual somatic (O) antiserum as negative for that group.

- **Additional biochemical tests**. Classify as *Salmonella* those cultures which exhibit typical *Salmonella* reactions for tests 1-11, shown in Table 1. If one TSI culture from 25 g analytical unit is classified as *Salmonella,* further testing of other TSI cultures from the same 25 g analytical unit is unnecessary. Cultures that contain demonstrable *Salmonella* antigens as shown by positive *Salmonella* flagellar (H) test but do not have biochemical characteristics of *Salmonella* should be purified (E-l, above) and retested, beginning with E-2, above.

Perform the following additional tests on cultures that do not give typical *Salmonella* reactions for tests 1-11 in Table 1 and that consequently do not classify as *Salmonella*.

a. **Phenol red lactose broth or purple lactose broth**.

- **1)** Inoculate broth with small amount of growth from unclassified 24-48 h TSI slant. Incubate 48 ± 2 h at 35°C, but examine after 24 h.

  Positive--acid production (yellow) and gas production in inner fermentation vial. Consider production of acid only as positive reaction. Most cultures of *Salmonella* give negative test result, indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromcresol purple as indicator) throughout medium.

- **2)** Discard as not *Salmonella,* cultures that give positive lactose tests, except cultures that give acid slants in TSI and positive reactions in LIA, or cultures that give positive malonate broth reactions. Perform further tests on these cultures to determine if they are *S. arizonae.*

b. **Phenol red sucrose broth or purple sucrose broth**. Follow procedure described in E,7a-1, above. Discard as not *Salmonella,* cultures that give positive sucrose tests, except those that give acid slants in TSI and positive reactions in LIA.

c. **MR-VP broth**. Inoculate medium with small amount of growth from each unclassified TSI slant suspected to contain *Salmonella*. Incubate 48 ± 2 h at 35°C.

- **1)** Perform Voges-Proskauer (VP) test at room temperature as follows: Transfer 1 ml 48 h culture to test tube and incubate remainder of MR-VP broth an additional 48 h at 35°C. Add 0.6 ml α -naphthol and shake well. Add 0.2 ml 40% KOH solution and shake. To intensify and speed reaction, add a few crystals of creatine. Read results after 4 h; development of pink-to-ruby red color throughout medium is positive test. Most cultures of *Salmonella* are VP-negative, indicated by absence of development of pink-to-red color throughout broth.

- **2)** Perform methyl red test as follows: To 5 ml of 96 h MR-VP broth, add 5-6 drops of methyl red indicator. Read results immediately. Most *Salmonella* cultures give positive test, indicated by diffuse red color in medium. A distinct yellow color is negative test. Discard, as not *Salmonella*, cultures that give positive KCN and VP tests and negative methyl red test.

d. **Simmons citrate agar**. Inoculate this agar, using needle containing growth from unclassified TSI agar slant. Inoculate by streaking slant and stabbing butt. Incubate 96 ± 2 h at 35°C. Read results as follows:

Positive--presence of growth, usually accompanied by color change from green to blue. Most cultures of *Salmonella* are citrate-positive.

Negative--no growth or very little growth and no color change.

- **Classification of cultures**. Classify, as *Salmonella*, cultures that have reaction patterns of Table l. Discard, as not *Salmonella*, cultures that give results listed in any subdivision of Table 2. Perform additional tests described in *Edwards and Ewing's Identification of Enterobacteriaceae* (2) to classify any culture that is not clearly identified as *Salmonella* by classification scheme in Table l or not eliminated as not being *Salmonella* by test reactions in Table 2. If neither of 2 TSI cultures carried through biochemical tests confirms the isolate as *Salmonella*, perform biochemical tests, beginning with E-5, on remaining urease-negative TSI cultures from same 25 g analytical unit.

Table 1. Biochemical and serological reactions of *Salmonella*

Test or substrate	Result		*Salmonella* species reaction[(a)]
	Positive	Negative	

1. Glucose (TSI)	yellow butt	red butt	+
2. Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3. H_2S (TSI and LIA)	blackening	no blackening	+
4. Urease	purple-red color	no color change	-
5. Lysine decarboxylase broth	purple color	yellow color	+
6. Phenol red dulcitol broth	yellow color and/or gas	no gas; no color change	+[b]
7. KCN broth	growth	no growth	-
8. Malonate broth	blue color	no color change	-[c]
9. Indole test	violet color at surface	yellow color at surface	-
10. Polyvalent flagellar test	agglutination	no agglutination	+
11. Polyvalent somatic test	agglutination	no agglutination	+
12. Phenol red lactose broth	yellow color and/or gas	no gas; no color change	-[c]
13. Phenol red sucrose broth	yellow color and/or gas	no gas; no color change	-
14. Voges-Proskauer test	pink-to-red color	no color change	-
15. Methyl red test	diffuse red color	diffuse yellow color	+
16. Simmons citrate	growth; blue color	no growth; no color change	v

[a] +, 90% or more positive in 1 or 2 days; -, 90% or more negative in 1 or 2 days; v, variable.

[b] Majority of *S. arizonae* cultures are negative.

[c] Majority of *S. arizonae* cultures are positive.

Table 2. Criteria for discarding non-*Salmonella* cultures

Test or substrate	Results

1. Urease	positive (purple-red color)
2. Indole test and Polyvalent flagellar (H) test;	positive (violet color at surface) negative (no agglutination)
or Indole test and Spicer-Edwards flagellar test	positive (violet color at surface) negative (no agglutination)
3. Lysine decarboxylase and KCN broth	negative (yellow color) positive (growth)
4. Phenol red lactose broth	positive (yellow color and/or gas)[a], [b]
5. Phenol red sucrose broth	positive (yellow color and/or gas)[b]
6. KCN broth, Voges-Proskauer test, and Methyl red test	positive (growth) positive (pink-to-red color) negative (diffuse yellow color)

[a] Test malonate broth positive cultures further to determine if they are *S. arizonae*.

[b] Do not discard positive broth cultures if corresponding LIA cultures give typical *Salmonella* reactions; test further to determine if they are *Salmonella* species.

- **Presumptive generic identification of *Salmonella*** . As alternative to conventional biochemical tube system, use any of 5 commercial biochemical systems (API 20E, Enterotube II, *Enterobacteriaceae* II, MICRO-ID, or Vitek GNI) for presumptive generic identification of foodborne *Salmonella* . Choose a commercial system based on a demonstration in analyst's own laboratory of adequate correlation between commercial system and biochemical tube system delineated in this identification section. Commercial biochemical kits should not be used as a substitute for serological tests (l). Assemble supplies and prepare reagents required for the kit. Inoculate each unit according to Method 978.24 (API 20E, Enterotube II, and *Enterobacteriaceae* II), sec. 989.12 (MICRO-ID), and Method 991.13 (Vitek GNI) in *Official Methods of Analysis* (1), incubating for time and temperature specified. Add reagents, observe, and record results. For presumptive identification, classify cultures, according to ref. 1, above, as *Salmonella* or not *Salmonella*.

For confirmation of cultures presumptively identified as *Salmonella*, perform the *Salmonella* serological somatic (O) test (E-6, above) and the *Salmonella* serological flagellar (H) test (E-3, above) or the Spicer-Edwards flagellar (H) test (E-4, above), and classify cultures according to the following guidelines:

a. Report as *Salmonella* those cultures classified as presumptive *Salmonella* with commercial biochemical kits when the culture demonstrates positive *Salmonella* somatic (O) test and positive *Salmonella* (H) test.
b. Discard cultures presumptively classified as not *Salmonella* with commercial biochemical kits when cultures conform to AOAC criteria (1) for classifying cultures as not *Salmonella* .
c. For cultures that do not conform to a or b, classify according to additional tests specified in E, 2-7, above, or additional tests as specified by Ewing (2), or send to reference typing laboratory for definitive serotyping and identification.

- **Treatment of cultures giving negative flagellar (H) test**. If biochemical reactions of certain flagellar (H)-negative culture strongly suggest that it is *Salmonella*, the negative flagellar agglutination may be the result of nonmotile organisms or insufficient development of flagellar antigen. Proceed as follows: Inoculate motility test medium in petri dish, using small amount of growth from TSI slant. Inoculate by stabbing medium once about 10 mm from edge of plate to depth of 2-3 mm. Do not stab to bottom of plate or inoculate any other portion. Incubate 24 h at 35°C. If organisms have migrated 40 mm or more, retest as follows: Transfer 3 mm loopful of growth that migrated farthest to trypticase soy-tryptose broth. Repeat either polyvalent flagellar (H) (E-3, above) or Spicer-Edwards (E-4 , above) serological tests. If cultures are not motile after the first 24 h, incubate an additional 24 h at 35°C; if still not motile, incubate up to 5 days at 25°C. Classify culture as nonmotile if above tests are still negative. If flagellar (H)-negative culture is suspected of being a species of *Salmonella* on the basis of its biochemical reactions, FDA laboratories should submit the culture to the Arkansas Regional Laboratory 3900 NCTR Road, Building 17, Jefferson AR 72079-9502 for further identification and/or serotyping. Laboratories other than FDA should make arrangements with a reference laboratory for the serotyping of *Salmonella* cultures.
- **Submission of cultures for serotyping**. Submit 2 isolates of each somatic group recovered from each analytical unit, unless otherwise instructed. Submit cultures on BHI agar slants in screw-cap tubes (13 x 100 mm or 16 x 125 mm) with caps secured tightly. Label each tube with sample number, subsample (analytical unit) number, and code, if applicable. Submit copies of the following records for each sample: 1) Collection Report, FD-464, or Import Sample Report, FD-784; 2) Analyst's Worksheet, FD-431; and 3) *Salmonella* Record Sheet, FD-431g. Place cultures in culture container with official FDA seal. Place accompanying records (E-11, above) inside shipping carton but not within officially sealed culture container. Submit memo or cover letter for each sample number to expedite reporting of results. Prepare cultures for shipment according to requirements for shipment of etiological agents (3). Label secondary shipping container according to ref. 4. Send container by most rapid mail service available. Maintain

duplicate cultures of those submitted for serotyping only on those samples under consideration for legal action.

Return to Chapter Contents

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October 25, 2001 Extension of the applicability of the orange juice method in section C.19 to apple juice and apple cider.

Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Rapid Methods [Appendix 1]

Hypertext updated by cjm/rim/kwg 2001-OCT-30

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

Chapter 6
Shigella

Authors

(Return to Table of Contents)

Contents

- References

Shigellosis, although commonly regarded as waterborne, is also a foodborne disease restricted primarily to higher primates, including humans. It is usually spread among humans by food handlers with poor personal hygiene. Foods most often incriminated in the transmission have been potato salad, shellfish, raw vegetables, and Mexican dishes.

The genus *Shigella* consists of four species: *S. dysenteriae* (subgroup A), *S. flexneri* (subgroup B), *S. boydii* (subgroup C), and *S. sonnei* (subgroup D). *Shigella* organisms may be very difficult to distinguish biochemically from *Escherichia coli*. Brenner (1) considers *Shigella* organisms and *E. coli* to be a single species, based on DNA homology. Nonetheless, *Shigella* species are Gram-negative, facultatively anaerobic, nonsporulating, nonmotile rods in the family *Enterobacteriaceae*. They do not decarboxylate lysine or ferment lactose within 2 days. They utilize glucose and other carbohydrates, producing acid but not gas. However, because of their affinity to *E. coli*, frequent exceptions may be encountered, e.g., some biotypes produce gas from glucose and mannitol. Neither citrate nor malonate is used as the sole carbon source for growth, and the organisms are inhibited by potassium cyanide.

A. Equipment and Materials

1. Same as for *Salmonella*, Chapter 5
2. Water baths; circulating, thermostatically-controlled, maintained at 42.0 ± 0.2°C and 44.0 ± 0.2°C
3. Anaerobic jar with catalyst

B. Media

1. *Shigella* broth with novobiocin (M136)
2. Trypticase soy-yeast extract (TSYE) broth (M157)
3. MacConkey agar (M91)
4. Triple sugar iron (TSI) agar (M149)
5. Urea broth (M171)
6. Motility test medium (semisolid) (M103)
7. Potassium cyanide (KCN) broth (M126)
8. Malonate broth (M92)
9. Tryptone (tryptophane) broth, 1% (M164)
10. MR-VP broth (M104)
11. Christensen citrate agar (M39)

12. Veal infusion agar (M173)
13. Bromcresol purple broth (M26) supplemented with the following carbohydrates, each at a level of 0.5%: adonitol, salicin, rhamnose, glucose, inositol, lactose, mannitol, raffinose, sucrose, xylose, dulcitol, and glycerol.
14. Acetate agar (M3)
15. Mucate broth (M105)
16. Mucate control broth (M106)
17. Decarboxylase basal medium (lysine, Falkow)(M44)
18. Decarboxylase basal medium (ornithine)(M44)

Return to Chapter Contents

C. Reagents and stains

1. Kovacs' reagent (R38)
2. Voges-Proskauer test reagents (R89)
3. 1 N Sodium hydroxide solution (R73)
4. 1 N Hydrochloric acid (R36)
5. Methyl red indicator (R44)
6. Physiological saline solution, 0.85% (sterile) (R63)
7. Novobiocin
8. Polyvalent *Shigella* antisera for groups A, A_1, B, C, C_1, C_2, D and Alkalescens-Dispar biotypes 1-4
9. Gram stain reagents(R32)

Return to Chapter Contents

D. Enrichment

Two approaches are provided for the recovery of *Shigella*. The first approach is a conventional culture method that involves the use of a specially formulated medium,

Shigella broth. Novobiocin is added to provide a selective environment. Sample enrichments are incubated as described below, and streaked to MacConkey agar. Typical colonies are biocehmically and serologically confirmed as *Shigella* spp.

The second approach uses DNA hybridization. The enzyme DNA gyrase induces negative supercoiling into closed circular DNA. It has been reported, however, that novobiocin inhibits DNA gyrase (3). Thus, the use of novobiocin in *Shigella* broth may cause this medium to be incompatible with DNA hybridization for detecting *Shigella*. Because DNA hybridization can detect *Shigella* in the presence of overwhelming numbers of competitors, a selective agent such as novobiocin is not needed in the enrichment medium and may actually be counterproductive. Thus, the use of tryptic soy broth with yeast extract added (TSYE) to a final concentration of 0.6% is the recommended enrichment, if DNA hybridization is being used.

1. **Conventional culture method**

    a. **Enrichment of *Shigella sonnei***. Aseptically weigh 25 g sample into 225 ml *Shigella* broth to which novobiocin (0.5 µg/ml) has been added. Hold suspension 10 min at room temperature and shake periodically. Pour supernatant into sterile 500 ml Erlenmeyer flask. Adjust pH, if necessary, to 7.0 ± 0.2 with sterile 1 N NaOH or 1 N HCl. Place flask in anaerobic jar with fresh catalyst, insert GasPak and activate by adding water. Because of high humidity within jar, heat catalyst as recommended after each use. Incubate jars in 44.0°C water bath for 20 h. Agitate enrichment culture suspension and streak on a MacConkey agar plate. Incubate 20 h at 35°C.

    b. **Enrichment of other *Shigella* species.** Proceed as above, but use novobiocin at 3.0 µg/ml and incubate anaerobically in 42.0°C water bath.

2. **DNA hybridization method**

    Aseptically weigh 25 g sample into 225 ml TSYE. Hold suspension 10 min at room temperature and shake periodically. Pour supernatant into sterile 500 ml Erlenmeyer flask and adjust pH, if necessary, to 7.0 ± 0.2 with sterile 1 N NaOH or 1 N HCl. Incubate sample enrichment 20-24 h at 35-37°C.

Return to Chapter Contents

E. Isolation of *Shigella* species

1. Conventional culture method

Examine MacConkey agar plates. *Shigella* colonies are slightly pink and translucent, with or without rough edges. Inoculate suspicious colonies into the following media: glucose broth, TSI agar slant, lysine decarboxylase broth , motility agar, and tryptone. Incubate at 35°C for 48 h, but examine at 20 h. Discard all cultures showing motility, H_2S, gas formation, lysine decarboxylation, and fermentation of sucrose or lactose. With respect to indole formation, discard positive cultures from 44.0°C enrichment. All suspicious isolates from 42°C enrichment may be either positive or negative and consequently should be retained.

2. DNA hybridization method.

Proceed as described in Chapter 24.

Return to Chapter Contents

F. Physiological characterization

Perform Gram stain and inoculate cultures giving satisfactory screening reactions to the other recommended biochemicals. The characteristics of *Shigella* are summarized as follows: Gram-negative rods; negative for H_2S, urease, glucose (gas), motility, lysine decarboxylase, sucrose, adonitol, inositol, lactose (2 days), KCN, malonate, citrate, and salicin; positive for methyl red. Pick isolates having positive reactions for *Shigella* to veal infusion agar slants. Use antisera for identification of serotype or compare with physiological behavior of the 32 serotypes presented in Table 1. If serotype cannot be identified by these tests, two explanations are possible: 1) Several provisional serotypes have not been accepted by an international commission on the taxonomy of *Shigella* species. Resolve by referral to the U.S. Centers for Disease Control and Prevention

(CDC), Atlanta, GA, or to the World Health Organization (WHO), *Shigella* spp. Reference Laboratories. 2) The cultures may be *E. coli*. Proper interpretation of the mucate and acetate reactions should help. *Shigella* species tend to be negative in all these reactions, whereas anaerogenic *E. coli* tend to be positive in at least one of the reactions (Table 2) (2).

Table 2. Reactions of *Shigella* and *Escherichia coli* in acetate, citrate, and mucate media[a,b]

Genera and species	Sodium acetate	%+	(%+)	Christensen's citrate	%+	(%+)	Sodium mucate	%+	(%+)
S. dysenteriae	-	0	0	-	0	0	-	0	0
S. flexneri	-	0	0	-	0	0	-	0	0
S. boydii	-	0	0	-	0	0	-	0	0
S. sonnei	-	0	0	-	0	0	D	6.4	(30.3)
E. coli	+ or (+)	83.8	(9.7)	D	15.8	(18.4)	+	91.6	(1.4)
Alkalescens-Dispar biotypes	+ or (+)	89.6	(4.7)	D	75	(12.5)	D	29.5	(27.9)

[a]+, 90% or more positive in 1 or 2 days; -, 90% or more negative; + or -, majority positive; - or +, majority negative; (+) delayed positive; D, different reactions [+,(+), -].

[b]From ref. 2. Reproduced with permission.

G. Serological characterization

Suspend growth from 24 h veal infusion agar slant in 3 ml 0.85% saline to McFarland Turbidity Standard No. 5. Mark nine 3 x 1 cm rectangles on clear glass petri dish with wax pencil. Add drops of suspension, antisera, and saline in accordance with the following protocol.

Rectangle	Suspension	Polyvalent antiserum								Saline
		A	A_1	B	C	C_1	C_2	D	A - D	
1	+	+								
2	+		+							
3	+			+						
4	+				+					

5	+					+				
6	+						+			
7	+							+		
8	+								+	
9	+									+

Mix contents of each rectangle with a needle, taking care that no mixing between rectangles occurs. Rock petri dish 3-4 min to accelerate agglutination. Read extent of agglutination as follows: 0 = no agglutination; 1+ = barely detectable agglutination; 2+ = agglutination with 50% clearing; 3+ = agglutination with 75% clearing; 4+ = visible floc with suspending fluid totally cleared. Re-examine suspension in monovalent sera belonging to each polyvalent in which a distinct positive reaction (2+, 3+, 4+) has occurred. In the event of a negative reaction, heat suspension in steamer 30 min to hydrolyze interfering capsular antigen. Re-examine in polyvalent, and, if positive, in corresponding monovalent sera. Because of tentative serotypes, a negative reaction may occur with the available sera. Consequently, it is advised that cultures retrieved from an outbreak and suspect foods giving *Shigella*-like reactions in physiological tests be referred to the CDC or to a WHO *Shigella* laboratory for confirmation.

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Bad Bug Book:
Shigella

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Top

B A M | B A M Media | B A M Reagents | Rapid Methods [Appendix 1] | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

Hypertext updated by rim/kwg/cjm/ear 2001-NOV-26

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual *Online*

March 2001

Chapter 7
Campylobacter

Authors

(Return to Table of Contents)

Isolation of *Campylobacter* Species from Food and Water

Campylobacter is considered by many to be the leading cause of enteric illness in the United States (20,26). *Campylobacter* species can cause mild to severe diarrhea, with loose, watery stools often followed by bloody diarrhea (7,20). *C. jejuni*, *C. coli*, and *C. lari* account for more than 99% of the human isolates (*C. jejuni* 90%). Other species have been associated with human illness in recent years (7,17,18,23,26,27).

Campylobacter species are highly infective. The infective dose of *C. jejuni* ranges from 500 to 10,000 cells, depending on the strain, damage to cells from environmental stresses, and the susceptibility of the host (4,6,7,20,27). Only the mesophilic *C. fetus* is normally invasive. Thermophilic species (optimum 42°C) such as *C. jejuni* are occasionally invasive. The infections are manifested as meningitis, pneumonia, miscarriage, and a severe form of Guillain-Barré syndrome (6,20). Thermotolerant strains of *C. fetus* that grow at 42°C have been isolated from patients (17).

Campylobacters are carried in the intestinal tract of a wide variety of wild and domestic animals, especially birds. They can establish a temporary asymptomatic carrier state, as well as illness, in humans. This is especially prevalent in developing countries (20). Consumption of food and water contaminated with untreated animal or human waste accounts for 70% of *Campylobacter*-related illnesses each year. The foods include unpasteurized milk, meats, poultry, shellfish, fruits, and vegetables, (1,8-11,17,19,20,22, 25,26).

C. jejuni can survive 2-4 weeks under moist, reduced-oxygen conditions at 4°C, often outlasting the shelf life of the product (except in raw milk products). They can also survive 2-5 months at -20°C, but only a few days at room temperature (5,8-11,20). Environmental stresses, such as exposure to air, drying, low pH, heating, freezing, and prolonged storage, damage cells and hinder recovery to a greater degree than for most bacteria. Older and stressed organisms gradually become coccoidal and increasingly difficult to culture (5,20). Oxygen quenching agents in media such as hemin and charcoal as well as a microaerobic atmosphere and preenrichment can significantly improve recovery (2,14-16,21,25,28).

Campylobacters are microaerophilic, very small, curved, thin, Gram-negative rods (1.5-5 µm), with corkscrew motility. They often join to form zigzag shapes (20,24). *Campylobacter* spp. are currently identified by tests described by Harvey (13) and Barret et al.(3). PCR genus and species identification methods have been published (12,18,30).

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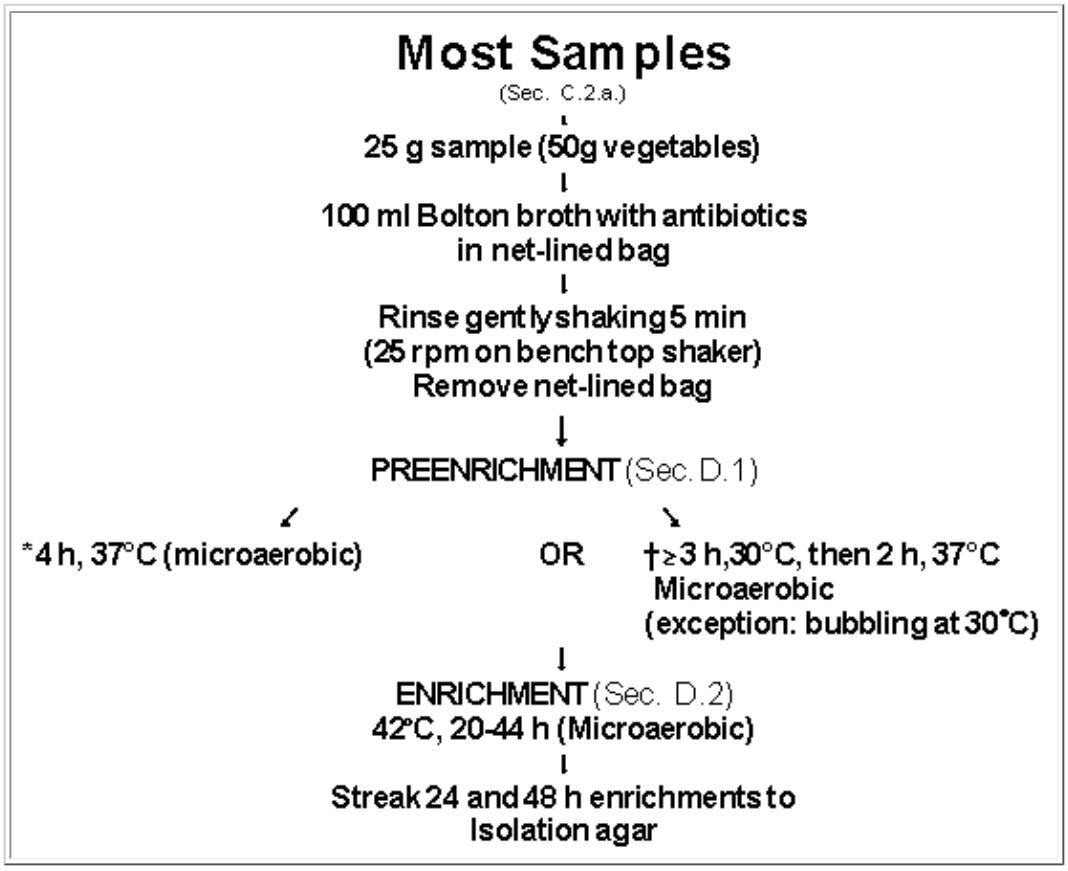
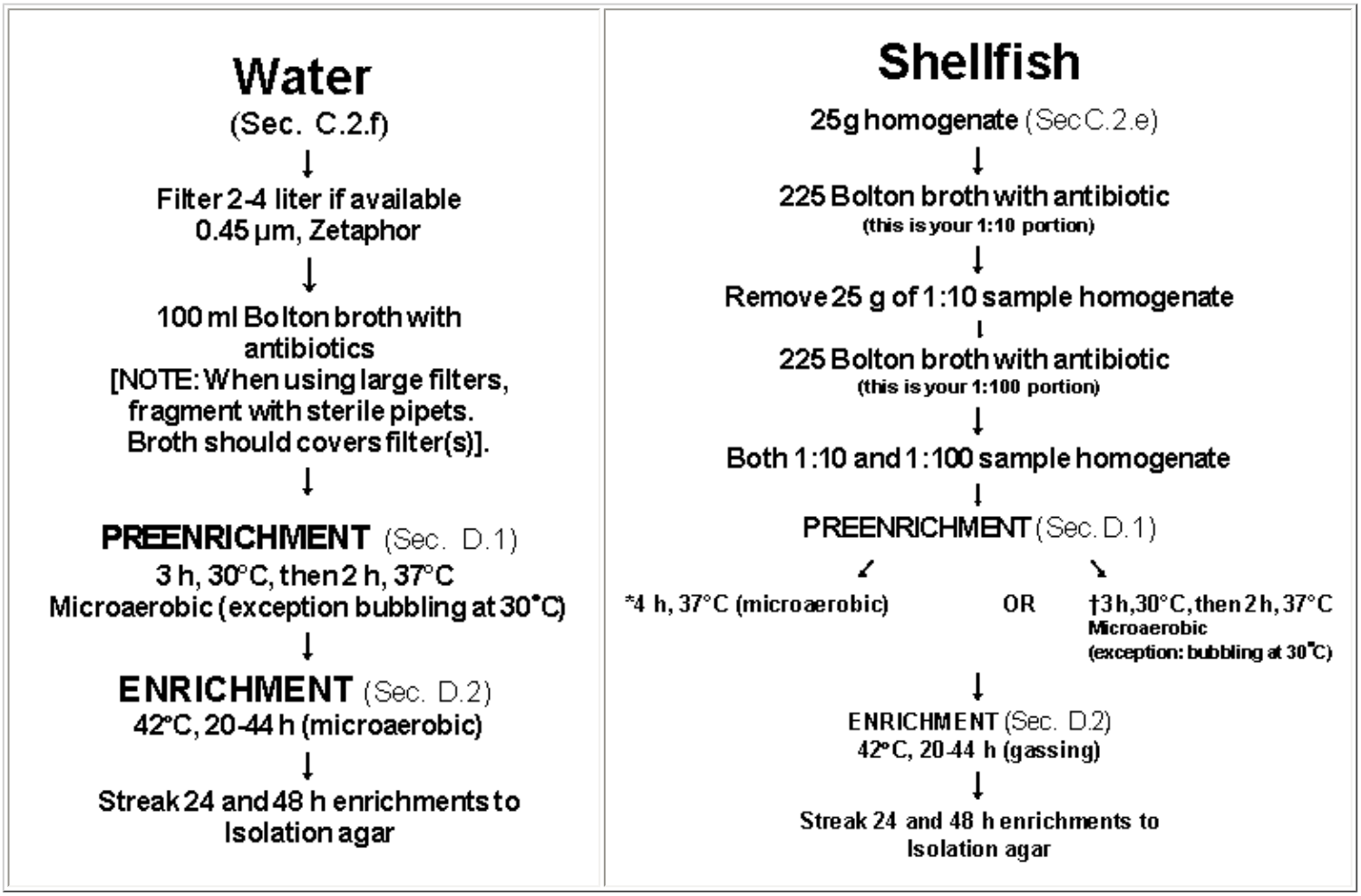
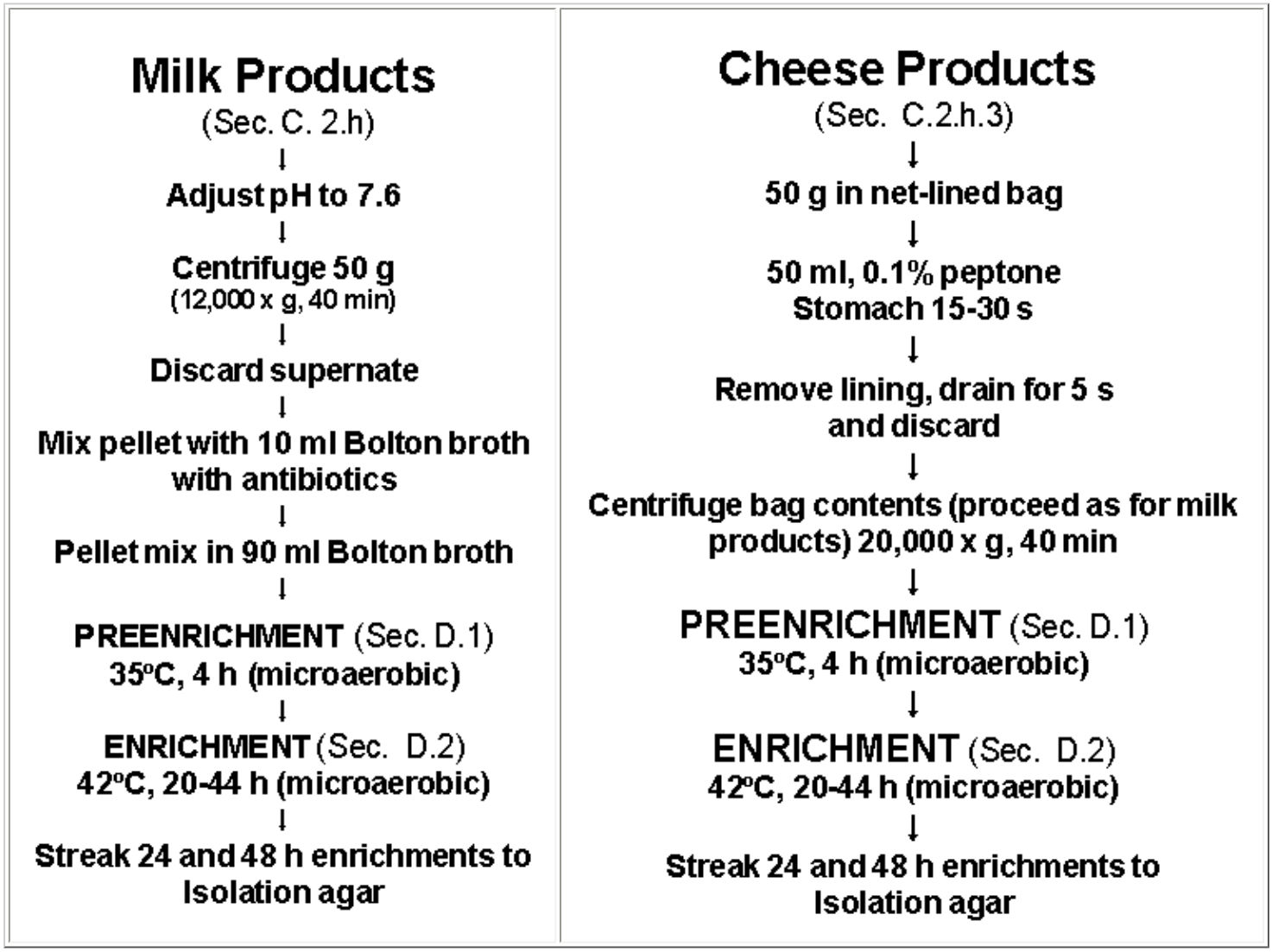
SAMPLE PREPARATION FLOWCHART FOR VARIOUS FOODS AND WATER (SECTION C.2.a-h)





***for samples produced or processed <10 days previously.**
†for frozen samples or samples produced or processed > 10 days previously.



A. Equipment and materials

1. Balances, 6000 g capacity, accurate to 0.1 g; and 200 g capacity, accurate to 0.0001 g
2. Sterile stomacher bags, 400 and 3500 ml bags and 400 ml filter bags (other bag types and sizes described in refs. 16, 21, and 22)
3. Whirl-pak bag racks and stainless steel baskets
4. Bench top shaker
5. Centrifuge, refrigerated, capable of 20,000 x *g*
6. Polypropylene or stainless steel 250 ml centrifuge bottles and 50 ml centrifuge tubes, sterile
7. Large funnels with cheese cloth linings, sterile (for whole seafood and meat samples or if filter bags are unavailable)
8. White or orange grease pencils to mark blood-free agar plates
9. 50 ml sterile conical centrifuge tubes
10. Plastic 5-10 ml tubes with screw cap lids, sterile
11. Cryotubes, 1 ml, sterile
12. Phase-contrast microscope, with 100X oil immersion objective or dark-field microscope with 63X objective or light microscope with 1000X objective
13. Microscope slides, 1 cm sq cover slips and immersion oil
14. Gas tank assembly (5% O_2, 10% CO_2, 85% N_2) and vacuum source (Fig. 1)

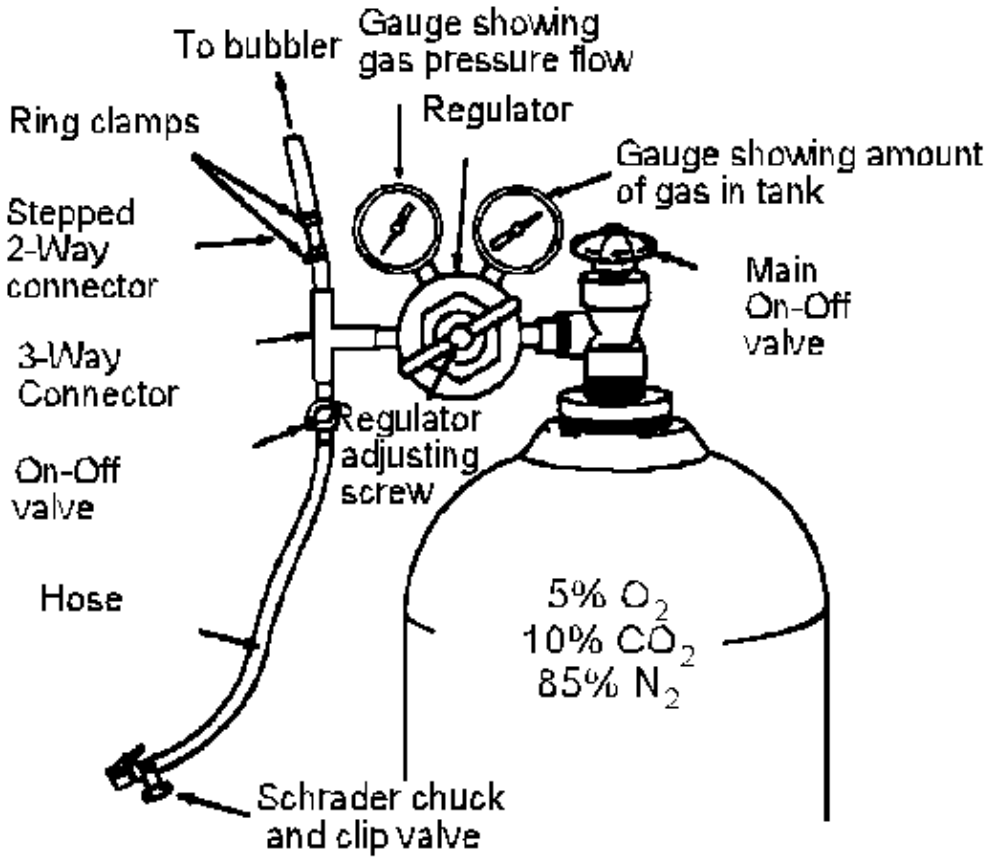


Figure 1. Gas Tank System

15. Microaerobic container system

    a. Anaerobe jars and bags:

        1. Jars with vacuum-pressure gauge and Schrader valves. They may be used with either the gas tank/vacuum assembly or with gas-generating envelopes. 3.4 L, (Difco 1950-30-2 or Oxoid HP11) may be used with either the gas tank/vacuum assembly or with gas-generating envelopes.
        2. Jars without a gauge or valves (2.5 liter BBL or EM Diagnostics [Remel, Lenexa, KS 66215] and 9.5 liter BBL) anaerobe jars. These are used with gas-generating envelopes (2.5 liter type such as Oxoid N025A or the BBL and EM gas paks).
        3. Rectangular jars 2.5 and 5.5 liter anaerobe rectangular jars (International Bioproducts, 800-729-7611 or Mitsubishi Gas Chemical America, 212-752-4620).
        4. Anaerobe pouches or rectangular jars, 0.4 L, for 2-plate incubation (International Bioproducts or Mitsubishi). Pouches for 1-plate incubation are available from EM Diagnostics.
        5. Air-tight plastic bags (4 mil wt), 12" X 16" or larger that can be closed by heat sealing or tape can be used as an incubation chamber.

    b. Campy gas-generating envelopes or pouches: for 3.4 liter jars, Oxoid BR56 or CN035A; for 2.5 liter and 9.5 liter jars, Oxoid CN025A, Difco 1956-24-4, BBL 71040 or 71034 or EM Diagnostics 53013678; for rectangular jars, Mitsubishi 10-04; for 2-plate, Mitsubishi 20-04 and 1-plate, EM Diagnostics, 53-13699. The Oxoid CNO25A and the Mitsubishi and EM envelopes are used without water.

§ CAUTION: ONLY GAS PACS USED WITHOUT WATER ARE COMPATIBLE WITH THE ITEMS IN A.3) AND A.4). THE RECTANGULAR JARS CAN EXPLODE IF USED WITH THE OXOID BR56, BBL OR DIFCO ENVELOPES.

c. An **Anerobe gas pak (1 only)** can be used with a **9.5 liter BBL anaerobe jar.** A single pak in a large container reduces the oxygen level to ~ 5% and produces other gases promoting Campylbacter growth. Either gas pak type (does/does not require water) can be used.

16. Air incubators, 25 ± 2, 30 ± 2, 37 ± 2, and 42 ± 1°C.
17. Water bath, preferably shaker type, range 30-42°C or coliform bath set to 37 and 42°C. Shaker water bath should have flask clamps, 250 or 500 ml if gassed flasks are used. Shaker water bath may be used either with bubbler system or gassed flask system. Static water bath can be used only with the bubbler system.
18. Shaker air incubator or air incubator with shaker platform (alternative to shaker water bath)

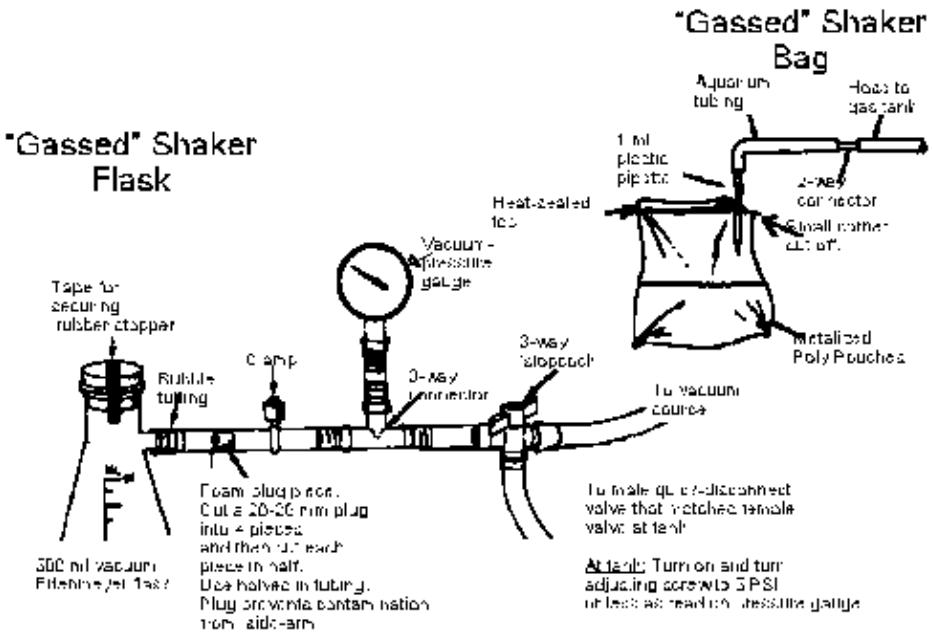


Figure 2. Shaking gas flask system

19. Shaking gassed flask or bag system (Fig. 2)

    a. Bags, see Fig. 2. Metalized poly pouches, Associated Bag Company, Milwaukee, Wi., 800-926-6100. Use 6" X 8" for 100 ml enrichments and 8" X 10" bags for 250 ml enrichments. Larger bags also are available. Bags are not sterile but can be radiation sterilized. If using non-sterile bags, include a bag control using Bolton broth or Listeria Enrichment broth without antibiotics.
    b. Vacuum flasks, 250 or 500 ml, with rubber stopper and foam-plugged vacuum tubing on the side arm, sterilized (see Fig.2). Further information on assembly and use is contained in BAM, 7th ed., 1992, chapter 7.

20. Bubbler system (Fig. 3). Two gas delivery valve systems are available.

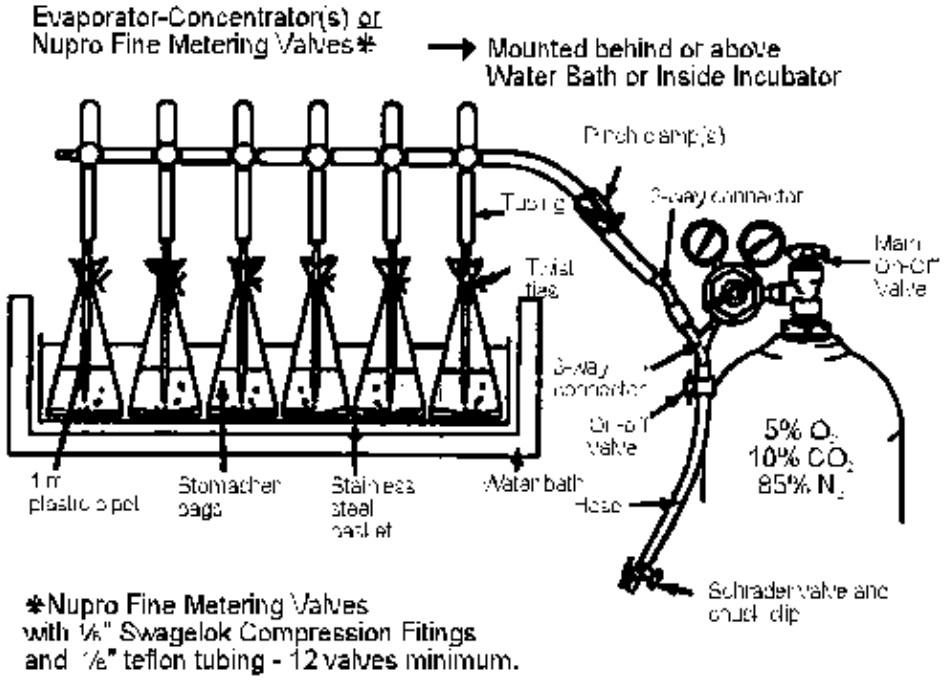


Figure 3. Bubbler System

a. Evaporator/concentrator manifold with Y-connector, available in 6 or 12 position sets (AFC International, Inc., Downers Grove, IL; 800-952-3293) See sections D-3 for use and F-1 for assembly instructions.

    1. Plastic luer-lock stopcocks. Stopcocks lock into the outlet valves.
    2. Plastic aquarium tubing, 3/16" inner diameter

b. Nupro S-series fine metering valves with 1/8" Swage-lok compression fittings and 1/8" teflon or poly-flo tubing. Two to four inch long pieces of 3/16" inner-diameter aquarium tubing (equal to number of valves). See sects. D-3 & F-2 for instructions on operating and assembling this bubbler unit. These components are available from local valve suppliers or contact Indianapolis Valve and Fitting Co, Indianapolis, IN; 317-248-2468, for information on the nearest supplier.

c. Enrichment broth container (use either):

    1. 400 ml or larger stomacher or stomacher filter bags, twist ties and stainless steel baskets
    2. 250 or 500 ml Erlenmeyer flasks, foam plugged with foil wrap and sterilized. Two-inch sq Parafilm pieces and weighted rings or a platform with clamps. For instructions for assembly and use, see fig. 2 and BAM, 7th ed., 1992, chapt. 7.

d. Plastic 1 ml sterile pipets.

21. Water analysis apparatus

a. Zetapor filters, 45 µm (Cuno, Meriden, CT, 800-243-6894; no substitutions), 47-293 mm (depending on filtering unit size), autoclaved separately from filtering unit
b. For 47 mm filter apparatus:

    1. Teflon-faced borosilicate glass 47 mm holder(s) and filter clamp forceps, sterilized
    2. Manifold, 6-12 place, for multiple subsamples
    3. Vacuum flask, 1-4 L, and if a manifold is used, a rubber stopper with a 6 to 8-inch plastic tube inserted and a hose connecting the plastic tube to the manifold. Another hose connects the flask side arm to a vacuum source.

c. For 90, 142, or 293 mm filter apparatus

    1. 90, 142, or 293 mm filtering unit with 3 ft hose attached at top, sterilized

2. Vacuum flask, 4-6 L, set up as in b-3 above, except the hose attached to the vacuum flask's stopper tube connects to the filtering unit outlet port

B. Media, biochemicals and reagents (section G, except where otherwise indicated)

1. Media

a. Campylobacter Enrichment Broth (Bolton formula, Oxoid AM7526 or Malthus Diagnostics LAB-135, Malthus Diagnostics, North Ridgeville, OH; 216-327-2585) with lysed horse blood and antibiotic supplement (Oxoid NDX131 or Malthus Diagnostics X131). Alternatively, antibiotic supplement may be prepared from individual components (G-1).[M28a]
b. *Campylobacter* isolation agars (use either)

1. Abeyta-Hunt-Bark (AHB) agar (G-2)[M29a]
2. Modified Campy blood-free agar (mCCDA)(G-2)[M30a]

c. Abeyta-Hunt-Bark agar (G-2), without antibiotics
d. Heart infusion agar (HIA) slants (M59)
e. 0.1% Peptone diluent (R56)
f. Semi-solid medium, modified, for **biochemical identification** (G-5)

1. Neutral red (NR) solution, glycine, NaCl, cysteine HCl, KNO_3

g. Triple sugar iron (TSI) agar slants (M149)
h. O-F glucose (M116), modified; prepare half the tubes with glucose and half without.
i. MacConkey agar (M91)
j. Culture shipping media
k. Cary-Blair medium (M31) or A-H slants with 5% filtered fetal bovine serum or lysed horse blood, w/o antibiotics (G-2)
l. Culture storage media

1. Semi-solid medium, modified, for short-term culture storage (G-4)[M30c]
2. Culture freezing media (G-3) for long-term storage (M30b)

2. Biochemicals and reagents

a. Hippurate (R33) and ninhydrin (R47) reagents
b. Nalidixic acid and cephalothin antibiotic disks (Difco)
c. Hydrogen peroxide, 3%
d. Fetal bovine serum (FBS), filtered (0.22 µm)
e. Oxidase reagent, liquid type preferred (R54)
f. Gram stain reagents (R32); counterstain with 0.5% carbol fuchsin (Difco)
g. Nitrate detection reagents A and B (R48)
h. Lead acetate strips (Difco)
i. UPDATED Dryspot Campy Test (Oxoid, DR150 available from Hardy Diagnostics 800-266-2222 [www.hardydiagnostics.com]) or Alert for Campylobacter (Cat. No. 9800 [94 tests] or 9801 [22 tests], Neogen Corporation, 800-234-5333 or www.neogen.com).
j. Sterile water, 1-2 liters; 70% ethanol or 1000 ppm hypochlorite solution (G-6)

C. Sample preparation.

1. Background information

Campylobacter spp. can survive, but not multiply, in food at refrigeration temperatures for 1-3 weeks, especially if foods (except raw milk) are in airtight containers. Their numbers decrease 2 logs upon freezing at -20°C, but the surviving organisms can be recovered ≥5 months. Samples should be analyzed for *Campylobacter* as soon as a sample package is opened; introduction of fresh oxygen adds significant stress to already weakened organisms.

Production of oxygen-neutralizing enzymes is decreased in microaerophiles, especially when cells are under stress. To

combat this problem oxygen-quenching compounds, such as FBP, hemin, blood and/or charcoal, are added to the media. Prepared media absorb oxygen during storage; use freshly prepared media whenever possible. Alternatively, if prepared broth base is stored in tightly closed containers away from light (hemin is light sensitive), it can be used for up to 2 months. Protect agar containing FPB from light and refrigerate when not in use.

Both the initial sample preparation and a 1:10 dilution are often needed for enrichment when high numbers of background flora (with broad species diversity) are present. With the sample dilution, antibiotics perform more effectively and campylobacter cells can utilize the low-oxygen atmosphere more efficiently. If heavy background contamination is suspected, add 1:10 dilution enrichment. The following instructions include mandatory dilution enrichments for shellfish and eggs.

2. Preparation of Samples

Add 2 rehydrated vials of Bolton antibiotic additives and 50 ml lysed horse blood to 1000 ml Bolton broth base. Alternatively, antibiotic additives can be prepared from individual components (G-1).

a. All sample types except those listed in following sect. 2(b-h)

Place filter bag in wire petri dish holder (type used in anaerobe jars). Hold bag lining in place with metal binder clip to prevent collapse during filling. Weigh 25 g sample (50 g if fruit or vegetables) into bag, and add 100 ml enrichment broth. Remove bag from holder, keeping clip attached and wrap twist-tie around top. Place bag(s) in basket or whirl-pak rack. Shake gently for 5 min. or place on a table-top shaker set at 25 rpm.

After the rinsing step, hold 5 min. Remove filter lining and allow it to drain a few seconds. If filter bag is not available, rinse sample in a sterile bag, and pour contents through a sterile, cheesecloth-lined funnel into the incubation bag or flask. When using metalized poly pouches for the gassed bag incubation, place filter liner from a stomacher bag into the pouch before weighing in the sample. **Note**: When analyzing acidic foods, such as chicken salad, adjust broth pH to 7.4 with 2N NaOH after the rinsing step.

b. Lobster tail or crab claws. Weigh 50-100 g into a filter-lined bag and rinse as in a, above.

c. Whole meat carcass or sample that cannot be easily reduced to 25 g (e.g., whole rabbit, lobster or larger piece of game meat)

Place sample into 3500 ml stomacher or other sterile bag. Add 200 ml 0.1% peptone water. Twist bag to seal, and swirl contents for 2-3 min. Tilt bag, and hold back food pieces to let rinse liquid flow to one corner. Sanitize a bottom bag corner with 1000 ppm hypochlorite solution or 70% ethanol; then rinse with sterile water. Aseptically cut corner of bag, and pour rinse through sterile cheesecloth-lined funnel into a 250 ml centrifuge bottle. Centrifuge at 16,000 x *g* for 15 min. Discard supernatant, and resuspend pellet in **10** ml 0.1% peptone water. Transfer 3 ml pellet mixture to 100 ml broth.

d. Liquid egg yolk or whole egg mixture

Divide sample into composites of two subsamples per composite, 25 g per sub. Weigh 25 g of each composite into 125 ml broth. After gently mixing, transfer 25 ml to another 100 ml broth. Analyze both the 1:6 and 1:48 dilutions.

e. Shellfish, shucked

In general, a minimum of 12 shellfish shall be taken in order to obtain a representative sample (*APHA 1970, Recommended Procedures for the Examination of Sea Water and Shellfish*). Depending on the size of the species, this will yield an approximately 100 to 200 g composite of shell liquor and meat. Collect the appropriate quantities of shell liquor and meats in a sterile blender or other suitable sterile container. Blend at low speed or stomach for 60 s. Remove 25 g shellfish homogenate for sample analysis to a Stomacher bag or 500 ml flask.

Add **225** ml enrichment broth. Transfer 25 ml of the mixture to a second 225 ml enrichment broth. Analyze both the 1:10 and 1:100 enrichments.

If enrichments are bubbled during incubation, leave them in bags or 500 ml flasks. If incubating in gassed bag or flask shaker system, use 6 x 10-inch metalized poly pouches or 500 ml vacuum flasks. **If incubating in anaerobe**

jars, reduce volume/flask or bag to 125 ml by dividing each enrichment into two parts. The gas does not penetrate into a larger volume sufficiently to provide proper growth of campylobacters.

f. Water

Request investigators collect 2-4 liters for analysis. When collected, 5 ml of 1 M sodium thiosulfate should be added per liter of chlorinated water sample.

Filter smaller volume samples through 45 µm Zetapor filters, 47 mm diameter. These filters have a positive charge. The negatively charged Gram-negative organisms are more effectively retained in the filter. Filter larger volumes, especially those that are turbid, through 90 mm or larger diameter filters.

Place filter unit into autoclavable pan. If filter clogs, wear sterile gloves and open filter holder unit to aseptically remove filter with sterile forceps. Place filter into enrichment broth (**see** below). Place another sterile filter in unit, reassemble, and continue filtering. Use as many filters as needed per subsample. When analyzing sea or other salt water, flush excess salt off filter by running 100-1,000 ml (depending on filter size) sterile phosphate buffer through the filter as the last of the sample is going through the filter. Do this with every filter used for salt water analyses. **Do not let filter become completely dry**. **Immediately transfer finished filter to broth**. **Campylobacters are very sensitive to drying and high salt concentrations**.

Place filter(s) in broth in the enrichment container. When using large filters, fragment with a sterile pipet. Be sure the broth covers the filter(s).

Enrichments incubated in Campy gas in anaerobe jars should be 125 ml or less. Larger volumes should be divided into smaller amounts, aseptically dividing the filters.

g. Swabs

Pipet 10 ml enrichment broth into sterile 50 ml Erlenmeyer flasks with foil tops. Place one swab into each flask, aseptically breaking off the sticks below the top of the flask. Replace covers loosely. Place flasks in anaerobe jar. To fit two layers of flasks in jar, place a cardboard circle over bottom layer, leaving space around the cardboard's edge for gas circulation.

h. Milk, frozen dairy products

1. **Raw milk**. Instruct the investigator to test raw milk at the collection site by using a sterile pipette to place test portion onto pH test paper (pH 6-8 range). If the pH is below 7.6, add sterile 1-2 N NaOH and gently to adjust it to 7.5 ± 0.2. Immediately upon receipt in the laboratory, test the pH of the dairy sample with pH test paper and adjust to pH 7.5 ± 0.2 with sterile 1-2 N NaOH if necessary. Centrifuge a 50 g portion at 20,000 x *g* for 40 minutes. Discard supernatant and dissolve pellet (not fat layer) in 10 ml enrichment broth. Transfer pellet to 90 ml enrichment broth.

2. **Other milk types and ice cream**. Adjust pH as in raw milk. Centrifuge a 50 g portion at 20,000 x g for 40 minutes. Discard supernatant and dissolve pellet (not fat layer) in 10 ml enrichment broth. Transfer pellet to 90 ml enrichment broth.

   Ice cream and other frozen dairy products: melt and aseptically remove any candy or other solids before weighing out.

3. **Cheese**. Weigh 50 g into a filter bag and add **50** ml 0.1% peptone. Stomach 15-30 s. Remove lining, letting it drain 5 s, and discard. Centrifuge and remove pellet to broth as in raw milk (h,1).

4. **"Milk sock" or strainer (gauze piece used to filter out solids during milking)**. Place 50 g piece in 100 ml broth.

D. Preenrichment and enrichment (modified Park and Humphrey methods)

1. Pre-enrichments

a. **4 h pre-enrichment** If the age of the sample is known to be within 10 days of production or time of contamination, or if the sample is a dairy product, pre-enrich at 37°C for 4 h. The pre-enrichment should be incubated under microaerobic conditions.

b. **5 h pre-enrichment** Use the 5 h method if any product has been refrigerated for ≥10 days. All water or shellfish samples are pre-enriched by the 5 h method.

Incubate at 30°C for 3 h, then at 37°C for 2 h. **NOTE: Incubate microaerobically at 30°C unless using a non-shaking bath-bubbler system (D-3). *Bubbling* static enrichments at 30°C fosters growth of anaerobes (D-3)**. Perform the 37°C incubation under microaerobic conditions. This method yields greater recovery for severely stressed organisms.

c. **General information concerning both methods**. Set the shaker speed for bubbling enrichments to 50-60 rpm and to 175-200 rpm for gassed bags or flasks.

2. Enrichment (microaerobic, D-3)

After pre-enrichment, raise the temperature in the water bath or move to a 42°C incubator. If analyzing for *C. fetus*, keep the temperature at **37°C**, even if a thermotolerant strain (growth at 42°C) was associated with the sample. Incubate **shaking** enrichments 23-24 h, except shellfish samples, which are incubated an extra 4 h. Dairy samples are incubated 48 hrs total. Incubate **non-shaking** enrichments 28-29 h, except shellfish, which are incubated 48 h. Incubate samples for *C. fetus* at 48 h (shaking) or 52 h (non-shaking).

3. Incubation and atmosphere modification methods for enrichments

Analysts may choose from three methods for generating microaerobic conditions in enrichment broth. These are: bubbling the gas mixture through broth, shaking enrichments to incorporate the gas, or incubating in anaerobe jars with a modified atmosphere.

The first method uses the bubbler system that also can incorporate shaking the enrichments during incubation. The second uses heat-sealed, gassed, metalized poly pouches or evacuated and gassed flasks.

The third method is the evacuated and gassed anaerobe jar (or a jar that uses a Campy gas envelope). Choose this when other systems are not available. Exception: incubation of enrichments in 50 ml Erlenmeyer flasks (i.e., swabs), which can be accomplished only in the jar system.

The systems are described as follows:

a. **Bubbler system**

Double-bag enrichments to prevent bags from leaking (bags can tear during shaking). Add about10 ml water to the outer bag for optimum heat trasfer to the broth. Place stomacher bags into stainless steel baskets (4-6/basket). Fill excess space in basket with water-filled dilution bottles. Place 1 ml plastic pipet tip end into each bag and fasten tightly with a twist-tie. Insert the plugged end of each pipet into the tubing connected to the bubbler valves.

Open the main gas tank valve and set the pressure to 4-6 lb with the regulator adjusting screw. This will give a flow rate of 2-3 bubbles per sec into each bag (figs. 1 & 3). Ensure that the pipet tip in each bag is inserted 2/3 into the broth. Tie bubbler tubing for each enrichment loosely together above the baskets to keep the bags standing upright. Bring the water level of the bath up slightly higher than the level of the broths in the bags. Replenish water as needed during the incubation period.

Refer to the BAM, 7th ed., 1992, chap. 7. for instructions on using Erlenmeyer flasks with the bubbler.

b. **Shaking flask or bag system (use bags with an air shaker incubator)**

1. Shaker bag system. Loosen the ring clamp holding the Schrader chuck and clip valve on the hose at the gas tank, and remove the valve. Insert the two-way connector attached to a length of 3/16" inner-diameter aquarium tubing. Open the main gas tank valve and set the regulator to 2 lb with the regulator adjusting screw. Place a sterile 1 ml plastic pipet in other end of the aquarium tubing, and keep pipet tip sterile by placing the

tip end in a sterile bag (figures 1 & 2). Use a new pipet for each bag.

Heat seal each filled bag (metalized poly pouch) with a bag sealer. Cut a very small corner from the top of the sealed end. Squeeze air from bag by pressing area above liquid until the area is flat. Insert pipet into open corner of bag and open on-off valve on the gas hose. Fill area above the broth with gas. Repeat squeezing and gassing each bag 2 more times, ending with a gassing step. Quickly heat seal or tape the corner of the bag shut. Place gassed bags into baskets lined with plastic bags. Set the basket(s) onto a shaker incubator platform. Set shaker speed to 175-200 rpm.

2. **Shaker flask system**. Refer to BAM, 7th ed., 1992, chap. 7.

c. **Gassed jar system**. Place stomacher bags with the tops loosely closed with a twist tie in a gas jar. Amount of broth in each bag should not be over 125 ml. When using the 5.5 liter rectangular jar, prepare a deep tray from foil and tape to contain the bottoms of the bags inside the jar.

1. **Gas pak envelopes.** Use 3 BBL Campy pak, Pack Plus or EM Anaerocult C gas-generating envelopes per 9.5 liter BBL jar and 1 per small jar. With the 3.4 liter Difco and Oxoid jars, use Difco or Oxoid gas pak envelopes, which are designed for use with a 3.4 liter jar. Gas paks requiring water need to be used with a catalyst. With a 2.5 liter rectangular jar use 1 gas pak **(type not used with water);** 3 for a 5.5 liter jar. **Or use 1 anaerobe** gas pak in a 9.5 liter BBL jar.

2. **Gas tank and vacuum source.** After tightening jar lid, attach chuck and clip valve of vacuum hose to valve indicated for vacuum on jar lid. Turn on vacuum and evacuate jar to 15-20 inches of Hg. Detach hose and tighten lid slightly if needed. Open the main valve on the gas tank. Adjust the pressure to 6-8 lb with regulator adjusting screw (fig. 1). Attach chuck and clip valve on the tank hose to the other valve on the lid. Open the regulator on-off valve and fill jar to 5-10 lb. Disconnect the hose and close the regulator valve. Repeat the evacuation and gassing twice more, ending with a gassing step. When opening the jars, first release pressure by pressing down on one of the valve stems with an inoculating loop handle or similar object.

3. **Guidelines for storing and maintaining jars.** If a jar lid with gauges is knocked against a hard surface, a gauge can become misaligned. Mark new "0" place on gauge and adjust vacuum and gas readings accordingly.

Store jars with screw clamps placed in jars so that one end is lying over lip of jar bottom. Prop lid against clamp to allow free flow of air and prevent mold build-up from damp jar. Or clean jars between uses with 70% alcohol and dry before storing.

If a jar will not hold vacuum or gas pressure, check for the following: cracks in the jar bottom, cracked or missing rubber rings or seals in the lids or a faulty valve stem. Replacement valve stems and a Schrader extractor tool are available from the jars' distributors or bicycle shops. To replace stems, place prongs of extractor over valve stem and turn counter-clockwise until stem is removed. Drop new valve stem (pin-head side up) into valve and turn clockwise until meeting resistance.

4. Positive controls

Store *Campylobacter* cultures in freezing medium (G-) at -70°C. If cultures are used often, they can be kept at room temperature in semi-solid storage media (G-4). Control cultures can be ordered from ATCC. Labs should stock *C. jejuni* (ATCC 33560), *C. coli* (ATCC 33559), *C. lari* (ATCC 358221), and *C. fetus* (ATCC 27374).

Inoculate broth or agar positive controls from a frozen culture by rubbing a moistened sterile swab against the culture and breaking off the swab end into broth or swabbing agar plate. Incubate microaerobically.

To freeze a culture, grow it first on Abeyta-Hunt-Bark (AHB) agar without antibiotics. Inoculate plates generously and incubate under microaerobic conditions, 42°C, 24 h. Incubate *C. fetus* cultures at 37°C, 48 h. Mix enough freezing media to allow 1 ml/plate. Wearing gloves, pipet 1.0 ml onto each plate. Use a sterile hockey stick to wash the growth to one end of each plate. Transfer washings to a sterile test tube. Pipet 0.5 ml of culture washings to cryotubes or sterile polypropylene test tubes. Freeze at -70°C. Freezer shock can be reduced by freezing the cultures in an alcohol-dry ice bath. . Alcohol will remove most markers' identification, so mark tubes with tape labels on lids or use marker that will not be affected by alcohol.

When storing cultures in semi-solid medium, inoculate the medium **at the surface** and incubate loosely-capped tubes under microaerobic conditions, 24 h. See previous paragraph for proper incubation temperatures. After incubation, tighten caps and place away from direct light. Cultures can be stored up to 2 months with subsequent transfer.

Note: To ship cultures, grow the culture on AHB agar plates w/o antibiotics and swab off growth. Place swab in a sterile polypropylene screw-cap shipping tube filled with Cary-Blair media. Aseptically break off the excess swab stem and tighten the tube cap. Alternatively, grow culture and ship on AHB agar slants (in shipping test tube) w/wo 5% lysed horse blood, w/o antibiotics.

E. Isolation, identification and confirmation

1. Isolation procedure

After 24 and 48 h, streak enrichments onto **either** Abeyta-Hunt-Bark or modified CCDA agars. Make a 1:100 dilution (0.1ml to 9.9 ml 0.1% peptone) of each enrichment and streak undiluted and diluted portions. For shellfish, eggs, and other enrichments prepared as dilutions, streak from the broths only. Transfer two loopfuls of enrichment broth to each plate and then streak for isolation. Protect plates from light.

UPDATED Place plates in anaerobe jars (1/2 full if possible) or air tight plastic bag (4 mil wt). Heat seal or roll close and tape the mouth of the bag. Do not delay bringing jars or bags to microaerobic conditions. For jars, use either the evacuation/gassing method, Campy gas paks or 1 anaerobe pak with a 9.5 liter BBL large jar (see D,3,c-gassed jar system). If using bags, attach a pipet to both the gassing and evacuation tubing. With the vacuum set very low, evacuate through a cut corner, then gas, repeat 2X and tape the corner to close. Bags can be used with Incubate at 42°C, 24-48 h. Check for growth at 24 h. If analyzing for *C. fetus*, incubate at 37°C for 48-72 h..

The inoculated agars may be incubated at a range of 37-42°C, but thermophilic campylobacters show more rapid growth at higher temperature. **NOTE**: When preparing agar in plates, dry plates overnight on bench. If plates must be used the same day, place them in 42°C incubator for several hours. Do not dry in a hood with lids open. Even very brief drying of surface will inhibit campylobacter growth.

2. Identification

Campylobacter colonies on agar are round to irregular with smooth edges. They can show thick translucent white growth to spreading, film-like transparent growth. Pick one typical colony per plate and prepare wet mount slide. To prepare, emulsify pick in drop of saline or buffer on slide. Cover each with 22 x 22 mm cover slip and examine without oil under dark-field optics at 63X or with oil under phase-contrast at 1000X. Store plates to be picked at 4°C under microaerobic conditions if analysis is not begun quickly.

If neither type of scope is available, prepare wet mounts as follows: Emulsify a colony pick in 0.1 ml of contrast stain (50-50 mix of Gram's crystal violet to saline or buffer). After 3-5 min., prepare a wet mount and view under a 1000X oil-immersion light microscope. Compare with a positive control culture. *Campylobacter* cells are curved, 1.5-5 m long, usually in chains resembling zigzag shapes (any length). Cells picked from agar often demonstrate only "wiggly" motility, whereas those from broth swim rapidly in corkscrew motion. About 10% of strains are nonmotile. Older or stressed cells have decreased motility and may show coccoid forms. Wear gloves or wash hands and disinfect microscope stage and lens after completing wet mounts. An infective dose can be acquired from cell suspensions that leak from slides.

If organisms appear typical, restreak to Abeyta-Hunt-Bark agar without antibiotics, two colonies/sub. Confirm only one plate/sub. Choose the plate with the least background growth. Refrigerate isolation agar plates microaerobically in case repicking is necessary. Incubate restreaked picks at 42°C, 24-48 h, microaerobically (37°C for *C. fetus*). Continue to restreak as necessary to obtain a pure culture. One or two plates can be incubated using the pouch-bag or pouch-jar systems. (A.15a)

3. Confirmation

UPDATED Perform catalase and oxidase tests from growth on a restreaked AHB plate. Place a loopful of growth in a drop of 3% H_2O_2. Bubbles indicate positive catalase test. Rub a loopful of growth on filter dampened with oxidase reagent. If the reagent turns purple, it is oxidase-positive. All Campylobacter spp. are oxidase-positive. Note: Colonies grown on charcoal agar plates can give a false-negative reaction.

4. Biochemical tests (Table 1)

All tests should include the following controls: *C. jejuni* (for hippurate and other tests) and *C. lari* (for antibiotic resistance and hippurate). If testing for *C. fetus*, also include *C. fetus* as a positive control.

Table 1. Biochemical Tests

Characteristic	*C. jejuni*	*C. jejuni* subsp. *doylei*	*C. coli*	*C. lari*	*C. fetus* subsp. *fetus*	*C. hyo-intestinalis*	*"C. upsaliensis"*[b]
Growth at 25°C	-	±	-	-	+	D	-0
Growth at 35-37°C	+	+	+	+	+	+	+
Growth at 42°C	+	±	+	+	D	+	+
Nitrate reduction	+	-	+	+	+	+	+
3.5% NaCl	-	-	-	-	-	-	-
H_2S, lead acetate strip	+	+	+	+	+	+	+
H_2S, TSI	-	-	D	-	-	+[c]	-
Catalase	+	+	+	+	+	+	-
Oxidase	+	+	+	+	+	+	+
MacConkey's agar	+	+	+	+	+	+	-
Motility (wet mount)	+(81%)	+	+	+	+	+	+
Growth in 1% glycine	+	+	+	+	+	+	+
Glucose utilization	-	-	-	-	-	-	-
Hippurate hydrolysis	+	+	-	-	-	-	-
Resistance to naladixic acid	S[d]	S	S	R	R	R	S
Resistance to cephalothin	R	R	R	R	S[e]	S	S

[a] Symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative; D, 11-89% of strains are positive; R, resistant; S, susceptible.

[b] Proposed species name.

[c] Small amount of H_2S on fresh (<3 days) TSI slants.

[d] Nalidixic acid-resistant *C. jejuni* have been reported.

[e] Cephalothin-resistant *C. fetus* subsp. *fetus* strains have been reported.

NOTE: *C. hyointestinalis* requires H_2 for vigorous growth and grows very poorly in O_2, CO_2, N_2 gas mixture. Use Campy Pak gas generating envelopes without catalyst for all incubations if analyzing samples for this species. "*C. upsaliensis*" does not grow in the FDA medium because of its sensitivity to antibiotics. Please call for more information.

Chart information adapted from T.J. Barret, C.M. Patton, and G.K. Morris (1988). Lab. Med. 19:96-102.

Gram stain. Use 0.5% carbol fuchsin as counterstain. *Campylobacter* spp. are Gram negative.

Hippurate hydrolysis. Emulsify generous 2 mm loopful of growth from the restreaked pick on the nonselective or antibiotic inhibition plate to 0.4 ml 1% hippurate solution in 13 x 100 mm tube. Incubate 2 h in 37°C water bath. Add 0.2 ml ninhydrin reagent (R47), agitate, and reincubate 10 min. Violet (not medium or pale purple) color is positive reaction. Only *C. jejuni* is hippurate-positive. Refrigerate hippurate solution up to 1 month and ninhydrin solution up to 3 months.

TSI reaction. Generously inoculate slant and stab butt of TSI (M149) slant from blood plate. Incubate under microaerobic atmosphere at 35-37°C for 5 days. Eighty percent of *C. coli* and a few *C. lari* produce H_2S at stab; *C. jejuni* does not produce

H_2S. All *Campylobacter* spp. produce alkaline/alkaline reactions. Prepare slants no more than 7 days before use.

Glucose utilization test. Stab 2 tubes of O-F media (M116), 3 times in each tube from blood plate. One tube contains glucose and one contains base alone. Incubate 4 days under microaerobic atmosphere at 35-37°C. *Campylobacter* spp. do not utilize glucose or other sugars and show no change in either tube.

UPDATED **Dryspot Campy Test or Alert for Campylobacter (see B, 2.i.)**. Follow manufacturer's instructions to test 1-2 colonies from an isolation agar plate (presumptive identification only), or a restreaked AHB plate. These kits produce a presumptive identification and are not a substitute for biochemical identification. They are not serotyping kits. If the kits do not produce a positive test, the culture might be another species of *Campylobacter* if other tests indicate *Campylobacter*.

Tests using diluted inoculum. Emulsify growth from colony into 5 ml 0.1% peptone and adjust turbidity to McFarland No. 1. Use this suspension to inoculate the following tests.

1. **Antibiotic inhibition**. Swab an Abeyta-Hunt-Bark agar plate without antibiotics with the suspension and drop nalidixic acid and cephalothin disks onto opposite sides of plate. Incubate microaerobically, 24-48 h, 37°C. Any size zone indicates sensitivity.

2. **Growth temperature tolerance**. Using loopful of diluted culture, streak a line across each of 3 plates of Abeyta-Hunt-Bark agar. Inoculate up to 4 cultures or lines per plate. Incubate one plate at 25°C, one at 35-37°C, and one at 42°C under microaerophilic atmosphere for 3 days. More growth than the initial inoculum is a positive test.

3. **Growth on MacConkey agar (M91)**. This alternative test is not necessary to identify *C. jejuni*, *C. coli*, or *C. lari*, but is useful to identify other species. Streak loopful from diluted culture across MacConkey agar plate, 4 cultures per plate. Incubate under microaerophilic atmosphere, at 37°C for 3 days. Record positive or negative growth. Agar plates should be not more than 3 days old.

4. **Growth in modified semisolid media (G-5)**. Inoculate surfaces of the following biochemicals with 0.1 ml diluted culture. Incubate microaerobically all semisolid media at 35-37°C for 3 days, except nitrate media, which are incubated 5 days. **Growth will be in a narrow band pattern just under the surface.**

   1% glycine. Record ± growth.

   3.5% NaCl. Record ± growth.

   H_2S from cysteine. Inoculate cysteine medium and hang a lead acetate strip from top, keeping cap loose. Do not let strip touch medium. Blackening of strip, even slightly, is positive reaction.

   Nitrate reduction. After 5 days, add nitrate reagents A and B (R48). Red color is positive reaction.

   Send identified cultures to Jan Hunt or Carlos Abeyta, at the address in the chapter introduction.

F. Bubbler apparatus assembly (Two systems available)

1. Concentrator/evaporator apparatus

   Insert and twist the luer stopcocks into the outlet valves. Connect the intake valves to the Y-connector with two 3/16"-diameter aquarium tubing pieces; then connect the Y-connector to the gas tank (Fig. 1) with a longer length of tubing. Determine the length of aquarium tubing pieces needed to reach from the unit's outlet valves to the enrichment bags or flasks. Cut one piece/valve and attach to each outlet port. Fasten the unit to a board or rack. Bubbler flow is determined by screws on the intake valves. Close stopcocks on unused outlet valves. More concentrator/evaporator units can be added by splitting the gas line to the tank with connectors. It can also be mounted in a 42°C air incubator with a shaking platform placed inside and the gas line connected through the wall of the incubator. A second line should go to a bubbler in a 37°C incubator.

2. Fine metering S-series Nupro valve/Swage-lok apparatus

   Determine how the valves should be arranged and connect with appropriate lengths of teflon tubing. Leave one outlet port/valve. Determine the length of the teflon tubing pieces needed to reach from the valves (when mounted) to the

enrichment containers. Cut one piece/valve and attach to each outlet port. Place 2" long pieces of 3/16" aquarium tubing over the end of each outlet valve tubing piece. This enables insertion of 1 ml pipets. Mount the assembled bubbler unit to a board placed at the back of the water bath or on a rack suspended over the bath. It can also be mounted in a 42°C air incubator with a shaking platform placed inside and the gas line connected through the wall of the incubator. A second line should go to a bubbler in a 37°C incubator.

G. Media

1. Campylobacter enrichment broth (Bolton formula), Oxoid AM-7526 (manufactured by Med-Ox Chemicals Ltd.)or Malthus Diagnostics Lab-135.(M28a)

   a. Enrichment Broth Base

Meat Peptone	10 g
Lactalbumin Hydrolysate	5 g
Yeast Extract	5 g
NaCl	5 g
Haemin	0.01 g
Sodium Pyruvate	0.5 g
α-Ketoglutaric Acid	1 g
Sodium Metabisulphite	0.5 g
Sodium Carbonate	0.6 g
Distilled Water	1000 ml

   Final pH, 7.4 ± 0.2.

   Prepare the broth base in screw-capped bottles, if possible. Mix 27.61 g in 1liter water and soak approximately 10 min. Once the powder is dissolved, adjust to pH 7.4 and autoclave 15 min at 121°C. Tighten the caps after the broth has cooled. Before use, add 50 ml lysed horse blood and 2 rehydrated [5 ml per vial 50:50 sterile filtered H_20-Ethanol solution] vials of Campylobacter enrichment broth (Bolton formula) supplement (Oxoid NDX131 or Malthus Diagnostics X-131). If supplement is not available add 4 ml each of antibiotic concentrates (formulas below, solutions made separately).

   UPDATED Note: Substitute solubilized amphotericin B (Sigma Cat. No. A9528) for cycloheximide if cycloheximide not available. (See G.2.a.3.)

   Store powdered media in a tightly fastened container in a cool, dry area to reduce oxygen infusion and peroxide formation, which can inhibit recovery of microaerophiles. Use prepared broth within 1 month of preparation (preferably less than 2 weeks).

   b. Lysed Horse Blood

   Use fresh blood and freeze to lyse upon receipt. To freeze, resuspend blood cells gently and pour ~40 ml portions into sterile 50 ml disposable centrifuge tubes. Freeze at -20°C. Thaw and refreeze once more to complete lysis. Store blood up to 6 months. Unused portions can be refrozen several times.

   c. **Campylobacter Enrichment Broth Supplements** (Prepare each solution separately. Na cefoperazone, vancomycin and FBP have very short shelf-lives. Prepare only the amount needed for your analysis. Smaller volumes can be sterilized using a 0.22 µm syringe filter.)

      1. **Sodium cefoperazone** (Sigma Cat. No. C4292) Dissolve 0.5 g in 100 ml distilled water in a volumetric flask. Filter-sterilize, using a 0.22 µm filter. Store the solution 5 days at 4°C, 14 days at -20°C, and 5 months at -70°C. Freeze in sterile plastic tubes or bottles. Add 4 ml to each liter of medium for a final concentration of 20 mg/liter.

         i. **Trimethoprim lactate** (Sigma Cat. No. T0667). Dissolve 0.66 g in 100 ml distilled water, and filter. May be stored 1 year at 4°C. Add 4 ml/liter to yield a final concentration of 20 mg/liter Trimethoprim.

or

ii. **Trimethoprim** (Sigma Cat. No. T7883) [TMP-HCl is a low cost alternative]. Add 0.5 g TMP to 30 ml 0.05N HCl at 50°C (stir until dissolved using hot plate with magnetic stirrer). Adjust volume to 100 ml with distilled water. Add 4 ml/liter to yield a final concentration of 20 mg/liter Trimethoprim.

3. **Vancomycin** (Sigma). Dissolve 0.5 g in 100 ml distilled water and filter. Store up to 2 months at 4°C. Because of short shelf life, prepare smaller amounts. Add 4 ml/liter for a final concentration of 20 mg/liter.

4. UPDATED **Cycloheximide** Dissolve 1.25 g in 20-30 ml ethanol in a 100 ml volumetric flask and bring to line with water. Filter-sterilize. Store at 4°C up to 1 year. Add 4 ml for final a concentration of 50 mg/liter. Use amphotericin B as in 2.a.3 below if cycloheximide is not available.

2. Isolation agars

a. Abeyta-Hunt-Bark Agar (M29a)

Heart infusion agar (Difco)	40 g
Yeast extract	2 g
Distilled water	1000 ml

Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Cool and add the selective agents listed below.

After pouring plates, dry plates overnight on bench.If plates must be used the same day, place them in 42°C incubator for several hours. Do not dry in a hood with lids open. Even very brief surface drying will inhibit campylobacter growth.

1. **Sodium cefoperazone** Dissolve 0.8 g in 100 ml d. water in a 100 ml volumetric and filter sterilize. Add **4 ml/liter** agar, or 6.4 ml of the preparation for the Bolton broth. Final concentration is 32 mg/liter.

2. **Rifampicin** Dissolve 0.25 g **slowly** into 60-80 ml alcohol in a 100 volumetric, swirling repeatedly. When powder is dissolved completely, bring to the line with distilled water. Store up to 1 year at -20°C. Add 4 ml per liter. Final concentration is 10 mg/liter.

3. UPDATED **Amphotericin B**, **solubilized** (Sigma Cat. No. A9528). Dissolve 0.05 g in water in a 100 ml volumetric flask and bring to the line. Filter sterilize and store at -20°C for 1 year. Final concentration is 2 mg/liter. Add 4 ml per liter.

4. **FBP** Dissolve 6.25 g Sodium pyruvate in 10-20 ml distilled water. Pour into a 100 ml volumetric. Add 6.25 g Ferrous sulfate and 6.25 g Sodium metabisulfite. Bring to the line with distilled water and filter sterilize. Add 4 ml/liter agar. **FBP is light sensitive and absorbs oxygen rapidly. Only prepare the amount needed. 10-25 ml amounts can be filtered with a 0.22 u syringe filter. Freeze unused portions in 5 ml amounts at -70°C as soon as possible after preparation. It can be stored at -70° for 3 mos or -20° for 1 mo.**

b. Modified Campylobacter Blood-Free Selective Agar Base (CCDA) (M30a)

CCDA agar base (OXOID)	45.5 g
Yeast extract	2 g
Distilled water	1 liter

Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Cool and add of sodium cefoperazone (**6.4 ml** of the concentration used in Bolton broth (M28a) or **4 ml** of the solution added to AHB agar(M29a)), 4 ml rifampicin, and 4 ml amphotericin B. See AHB directions for precautions when drying plates.

3. Freezing medium.(M30b) Bolton broth base (9.5 ml), 1 ml fetal bovine serum (filtered, 0.22 µm) and 1 ml glycerol (10%). Mix well before use.

4. Semi-Solid Medium, modified, for Culture Storage (M30c)

Campylobacter Enrichment Broth (Bolton)(Oxoid AM-7526)	27.6 g
Agar	1.8 g
Sodium Citrate	0.1 g
Distilled water	1000 ml

Mix ingredients, pH to 7.4 ± 0.2, boil and dispense 10 ml per 16 X 125 screw-cap tube. Autoclave 121°C, 15 min. Keep tubes tightly capped during storage. Do not add horse blood or antibiotics.

5. Semisolid Medium, modified, for Biochemical Identification

Base Medium

Campylobacter Enrichment Broth (Bolton)(Oxoid AM-7526) without blood or antibiotics	27.6 g
Agar	1.8 g
Distilled water	1000 ml

Biochemicals (see below)

Neutral red solution, 0.2% Dissolve 0.2 g neutral red in 10 ml EtOH in a 100 ml volumetric and bring to line with d. water.

Mix base medium ingredients, then boil. Divide into four 250 ml portions. Add 2.5 ml neutral red to **3** of the 4 portions. Add glycine, NaCl and cysteine-HCl to the 3 portions containing neutral red. Add potassium nitrate in the portion without neutral red. Adjust the pH of each portion to 7.4 ± 0.2. Dispense 10 ml per 16 X 125 mm screw-cap tube. Autoclave 121°C, 15 min.

a. **Potassium nitrate** (for Final Concentration of 1% (w/v) . Add **2.5 g to 250 ml** (10 g/liter) semi-solid mixture **without** neutral red.

b. **Glycine**(for Final Concentration of 1% (w/v) . Add **2.5 g to 250 ml** (10 g/liter) semi-solid mixture with neutral red.

c. **NaCl**(for Final Concentration of 3.5% (w/v). Add **7.5 g to 250 ml** (30 g/liter) semi-solid mixture with neutral red.

d. **Cysteine-HCl** (for Final Concentration of 0.02% (w/v). Add 0.05 g to 250 ml (0.2 g/liter) semi-solid mixture with neutral red.

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Note: For information on cycloheximide availability problems, see Advisory for BAM Users on Reported Supply Problems for Cycloheximide

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

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Chapter 8

Yersinia enterocolitica and *Yersinia pseudotuberculosis*

Authors

(Return to Table of Contents)

Yersinia enterocolitica and bacteria that resemble it are ubiquitous, being isolated frequently from soil, water, animals, and a variety of foods. They comprise a biochemically heterogeneous group that can grow at refrigeration temperatures (a strong argument for use of cold enrichment). Based on their biochemical heterogeneity and DNA relatedness, members of this group were separated into four species: *Y. enterocolitica*, *Y. intermedia*, *Y. frederiksenii*, and *Y. kristensenii* (8). Through additional revisions, the genus *Yersinia* has grown to include eleven species (2,8,9,50), three of which are potentially pathogenic to humans: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. Of these, *Y. enterocolitica* is most important as a cause of foodborne illness.

Y. enterocolitica strains and related species can be separated serologically into groups based on their heat-stable somatic antigens. Wauters (48) described 54 serogroups for *Y. enterocolitica* and related species. Aleksic and Bockemuhl (1) proposed simplifying this to 18 serogroups within the *Y. enterocolitica* species. Presently, pathogenic strains belonging to serogroups O:1, 2a, 3; O:2a,3; O:3; O:8; O:9; O:4,32; O:5,27; O:12,25; O:13a,13b; O:19; O:20; and O:21 have been identified. Therefore, pathogenic strains can belong to diverse serogroups. Serogroups that predominate in human illness are O:3, O:8, O:9, and O:5,27.

The association of human illness with consumption of *Y. enterocolitica*-contaminated food, animal wastes, and unchlorinated water is well

documented (4,5). Refrigerated foods are potential vehicles. because contamination is possible at the manufacturing site (4) or in the home (5). This organism may survive and grow during refrigerated storage.

A number of virulence tests have been proposed to distinguish potentially pathogenic *Y. enterocolitica*. Some strains of *Y. enterocolitica* and related species produce an in vitro heat-stable enterotoxin (ST) that can be detected by intragastric injection of cultural filtrates in suckling mice and is very similar to *Escherichia coli* ST (11). However, *Yersinia* spp. produce ST only at temperatures below 30°C. Many environmental strains of *Yersinia* produce this protein, whereas some otherwise fully virulent strains of *Y. enterocolitica* do not. The role of ST in the disease process of *Yersinia* remains uncertain.

Yersinia spp. that cause human yersiniosis carry a plasmid (41-48 Mdal) (17,27,53) that is associated with a number of traits related to virulence: autoagglutination in certain media at 35-37°C (6,28); inhibition of growth in calcium-deficient media (17) and binding of crystal violet dye (10) at 35-37°C; increased resistance to normal human sera (35); production of a series of outer membrane proteins at 35-37°C (37); ability to produce conjunctivitis in guinea pig or mouse (Sereny test) (46,53); and lethality in adult and suckling mice by intraperitoneal (i.p.) injection of live organisms (7,12,39,41). The plasmid associated with virulence can be detected by gel electrophoresis or DNA colony hybridization (20). Recent evidence, however, indicates that presence of plasmid alone is not sufficient for the full expression of virulence in *Yersinia* (19,38,44). The intensity of some plasmid-mediated virulence properties such as mouse lethality and conjunctivitis is variable, depending on the genes carried on the bacterial chromosome (35-37,42) and the serogroup, suggesting that chromosomal genes also contribute to *Yersinia* virulence.

Virulent strains of *Yersinia* invade mammalian cells such as HeLa cells in tissue culture (29). However, strains that have lost other virulent properties retain HeLa invasiveness, because the invasive phenotype for mammalian cells is encoded by chromosomal loci. Two chromosomal genes of *Y. enterocolitica*, *inv* and *ail*, which encode the phenotype for mammalian cell invasion, have been identified (33,34). Transfer of these genetic loci into *E. coli* confers the invasive phenotype to the *E. coli* host (33). The *inv* gene allows high level *Yersinia* invasion of several tissue culture cell lines (33). However, Southern blot analyses show that *inv* gene sequences are present on both tissue culture invasive and noninvasive isolates (34,42). Although this suggests that the *inv* gene in *Y. enterocolitica* may not be directly correlated with invasiveness, genetic evidence shows that *inv* genes are nonfunctional in the noninvasive isolates (36). The *ail* gene shows greater host specificity with regard to cell invasion and appears to be present only on pathogenic *Yersinia*. In disease-causing strains, all virulent *Y. enterocolitica* isolates were shown to be tissue culture-invasive and to carry the *ail* gene (33,37). The *ail* locus, therefore, may be an essential chromosomal virulence factor in *Y. enterocolitica* (34,42).

Y. pseudotuberculosis is less ubiquitous than *Y. enterocolitica*, and although frequently associated with animals, has only rarely been isolated from soil, water, and foods (16,47). Among *Y. pseudotuberculosis* strains there is little or no variation in biochemical reactions, except with the sugars melibiose, raffinose, and salicin. Serologically (based on a heat-stable somatic antigen), the *Y. pseudotuberculosis* strains are classified into six groups, each serogroup containing pathogenic strains. Gemski et al. (18) reported that serogroup III strains harbor a 42-Mdal plasmid as do serogroup II strains that are lethal to adult mice. The association of yersiniosis in humans with the presence of a 42-Mdal plasmid in *Y. pseudotuberculosis* has been established (45).

Virulence genes present on the chromosome of *Y. pseudotuberculosis* have also been identified (22,23). The *inv* gene of *Y. pseudotuberculosis* is

homologous with that of *Y. enterocolitica*, and encodes for an invasion factor for mammalian cells. Transfer of *inv* gene into *E. coli* K-12 resulted in the expression of the invasive phenotype in *E. coli* (23). The *inv* gene is thermoregulated (21,25); it encodes for a 103 Kdal protein, invasin, which binds to specific receptors on mammalian cells and facilitates the entry of *Y. pseudotuberculosis* into tissue (24). Tests for *Y. pseudotuberculosis* virulence are not as abundant as those for *Y. enterocolitica*; however, tissue cell-invasive and plasmid-carrying isolates of *Y. pseudotuberculosis* may be identified by DNA colony hybridization.

A. Equipment and materials

1. Incubators, maintained at 10 ± 1°C and 35-37°C
2. Blender, Waring or equivalent, 8000 rpm, with 500 ml-1 liter jar
3. Sterile glass or plastic petri dishes, 15 x 100 mm
4. Microscope, light 900X and illuminator
5. Sterile syringes, 1 ml; 26-27 gauge needle
6. Disposable borosilicate tubes, 10 x 75 mm; 13 x 100 mm
7. Wire racks to accommodate 13 x 100 mm tubes
8. Vortex mixer
9. Mouse racks and mouse cages
10. Laminar flow animal isolator
11. Anesthetizing jar
12. CO_2, compressed
13. Minitek (BBL, Division of Bioquest, Cockeysville, MD). System includes disk dispenser, humidor, pipet and tips, Minitek 20-well plates, and color comparator.

B. Media

1. Peptone sorbitol bile broth (PSBB) (M120)
2. MacConkey agar (M91) (use mixed bile salts; BBL Mac agar and DIFCO Mac CS are acceptable)
3. Celfsulodin-irgasan-novobiocin (CIN) agar (M35)
4. Bromcresol purple broth (M26) supplemented individually with the following carbohydrates, each at 0.5%: mannitol, sorbitol, cellobiose, adonitol, inositol, sucrose, rhamnose, raffinose, melibiose, salicin, xylose, and trehalose
5. Christensen's urea agar (M40) (plated media or slants)
6. Phenylalanine deaminase agar (M123) (plated media or slants)
7. Motility test medium (M103). Add 5 ml of 1% 2,3,5-triphenyl tetrazolium chloride per liter before autoclaving.
8. Tryptone broth, 1% (M164)
9. MR-VP broth (M104)

10. Simmons citrate agar (M138)
11. Veal infusion broth (M173)
12. Bile esculin agar (M18)
13. Anaerobic egg yolk agar (M12)
14. Minitek enteric and nonfermenter broth (BBL)
15. Trypticase (tryptic) soy agar (TSA) (M152)
16. Lysine arginine iron agar (LAIA) (M86)
17. Decarboxylase basal medium (Falkow) (M44) supplemented individually with 0.5% arginine, 0.5% lysine, or 0.5% ornithine
18. Congo Red-brain heart infusion agarose (CRBHO) (M41)
19. Irgasan-ticarcillin-chlorate (ITC) broth (M67)
20. Pyrazinamidase agar slants (M131)
21. PMP broth (M125)
22. β-D-glucosidase test (**see** instructions at end of chapter)

C. Reagents

1. Gram stain reagents (R32)
2. Voges-Proskauer (VP) test reagents (R89)
3. Ferric chloride, 10% in distilled water (R25)
4. Oxidase test reagent (R54)
5. Saline, 0.5% (sterile) (R66)
6. Kovacs' reagent (R38)
7. 0.5% Potassium hydroxide in 0.5% NaCl, freshly prepared
8. Minitek biochemical discs for the following substrates: esculin, V-P, H_2S-indole, citrate, lysine, arginine, ornithine, mannitol, sorbitol, cellobiose, adonitol, inositol, sucrose, rhamnose, raffinose, melibiose, salicin, xylose, and trehalose
9. Mineral oil, heavy grade, sterile (R46)
10. API 20E system
11. 1% Ferrous ammonium sulfate

D. Enrichment

The following simplified procedure for isolating *Yersinia* from food, water, and environmental samples is recommended.

1. Analyze samples promptly after receipt, or refrigerate at 4°C. (Freezing of samples before analysis is not recommended, although *Yersinia* have been recovered from frozen products.) Aseptically weigh 25 g sample into 225 ml PSBB. Homogenize 30 s and

incubate at 10°C for 10 days.

2. If high levels of *Yersinia* are suspected in product, spread-plate 0.1 ml on MacConkey agar (13,52) and 0.1 ml on CIN agar (43,45) before incubating broth. Also transfer 1 ml homogenate to 9 ml 0.5% KOH in 0.5% saline (3), mix for several seconds, and spread-plate 0.1 ml on MacConkey and CIN agars. Incubate agar plates at 30°C for 1 day. Transfer colonies from one of these plates onto sterile Whatman 541 filter paper for direct examination for *Yersinia* virulence gene by DNA colony hybridization (20) (**see** also Chapter 24). If high levels of *Yersinia* contamination are not suspected, omit this step.
3. On day 10, remove enrichment broth from incubator and mix well. Transfer 0.1 ml enrichment to 1 ml 0.5% KOH in 0.5% saline and mix for 5-10 s (3). Successively streak one loopful to MacConkey plate and one loopful to CIN plate. Transfer additional 0.1 ml enrichment to 1 ml 0.5% saline and mix 5-10 s before streaking, as above. Incubate agar plates at 30°C for 1 day.
4. (Optional) An alternative enrichment technique (49), although unconfirmed, gives promising preliminary results. Aseptically weigh 25 g sample into 225 ml ITC broth. Homogenize for 30 s and incubate at room temperature (RT) for 2 days. Continue as in D-2, above.

E. Isolation of *Yersinia*

Examine MacConkey agar plates after 1 day incubation. Reject red or mucoid colonies. Select small (1-2 mm diameter) flat, colorless, or pale pink colonies. Examine CIN plates after 1 day incubation. Select small (1-2 mm diameter) colonies having deep red center with sharp border surrounded by clear colorless zone with entire edge. Inoculate each selected colony into LAIA slant (51), Christensen's urea agar plate or slant, and bile esculin agar plate or slant by stabbing with inoculation needle. Incubate 48 h at RT. Isolates giving alkaline slant and acid butt, no gas and no H_2S (KA--) reaction in LAIA, which are also urease-positive, are presumptive *Yersinia*. Discard cultures that produce H_2S and/or any gas in LAIA or are urease-negative. Give preference to typical isolates that fail to hydrolyze (blacken) esculin.

F. Identification

Using growth from LAIA slant, streak culture to one plate of anaerobic egg yolk (AEY) agar and incubate at RT. Use growth on AEY to check culture purity, lipase reaction (at 2-5 days), oxidase test, Gram stain, and inoculum for biochemical tests. From colonies on AEY, inoculate the following biochemical test media and incubate all at RT for 3 days (except one motility test medium and one MR-VP broth, which are incubated at 35-37°C for 24 h).

1. Decarboxylase basal medium (Falkow) (M44), supplemented with each of 0.5% lysine, arginine, or ornithine; overlay with sterile mineral oil
2. Phenylalanine deaminase agar (M123)
3. Motility test medium (semisolid) (M103), 22-26°C and 35-37°C
4. Tryptone broth (M164)

5. Indole test (**see** instructions at end of chapter)
6. MR-VP broth (M104). RT for autoagglutination test (**see** G-2, below), followed by V-P test (48 h) (**see** instructions at end of chapter); 35-37°C for autoagglutination test (**see** G-l)
7. Bromcresol purple broth (M26) with 0.5% of the following filter-sterilized carbohydrates: mannitol, sorbitol, cellobiose, adonitol, inositol, sucrose, rhamnose, raffinose, melibiose, salicin, trehalose, and xylose
8. Simmons citrate agar (M138)
9. Veal infusion broth (M173)
10. In lieu of Nos. 1,2,4,7, and 8, Minitek (BBL) Minikit biochemical test kit can be used with biochemical disks for esculin, V-P, H_2S, indole, citrate, phenylalanine deaminase, lysine, arginine, ornithine, mannitol, sorbitol, cellobiose, adonitol, inositol, sucrose, rhamnose, raffinose, melibiose, salicin, trehalose, and xylose. Set up and inoculate according to manufacturer's instructions. Incubate in humidor at RT for 48 h.
11. Use API 20E system for biochemical identification of *Yersinia*. Follow manufacturer's instructions.
12. Pyrazinamidase agar slants (48 h) (**see** instructions at end of chapter)
13. β-D-glucosidase test (30°C, 24 h) (**see** instructions at end of chapter)
14. Lipase test. Positive reaction is indicated by oily, iridescent, pearl-like colony surrounded by precipitation ring and outer clearing zone.

G. Interpretation

Yersinia are oxidase-negative, Gram-negative rods. Use Tables 1 and 2 to identify species and biotype of *Yersinia* isolates. Currently only strains of *Y. enterocolitica* biotypes 1B, 2, 3, 4, and 5 are known to be pathogenic. These biotypes and *Y. enterocolitica* biotype 6 and *Y. kristensenii* do not rapidly (within 24 h) hydrolyze esculin or ferment **salicin** (Tables 1 and 2). However, *Y. enterocolitica* biotype 6 and *Y. kristensenii* are relatively rare; they can be distinguished by failure to ferment sucrose, and they are pyrazinamidase-positive (26). Hold *Y. enterocolitica* isolates which are within biotypes 1B, 2, 3, 4, and 5 for further pathogenicity tests.

H. Pathogenicity testing

1. **Autoagglutination test**. The MR-VP tube incubated at RT for 24 h should show some turbidity from bacterial growth. The 35-37°C MR-VP should show agglutination (clumping) of bacteria along walls and/or bottom of tube with clear supernatant fluid. Isolates giving this result are presumptive positive for the virulence plasmid. Any other pattern for autoagglutination at these two temperatures is considered negative.
2. **Freezing cultures**. Plasmids that determine traits related to pathogenicity of *Yersinia* can be spontaneously lost during culture above 30°C or with lengthy culture and passage below 30°C in the laboratory. It is important, therefore, to immediately freeze presumptive positive cultures to protect plasmid content. Inoculate into veal infusion broth and incubate 48 h at RT. Add 10% sterile glycerol (e.g., 0.3 ml in 3 ml veal infusion broth) and freeze immediately. Storage at -70°C is recommended.

3. **Low calcium response Congo Red agarose virulence test**. Inoculate test organism into BHI broth. Incubate overnight at 25-27°C. Make decimal dilutions in physiologic saline to obtain 10 cells/ml (10^{-6}). Spread-plate 0.1 ml of appropriate dilution on each of two Congo Red agarose plates. Incubate one at 35°C and one at 25°C. Examine at 24 and 48 h. Presumptive plasmid-bearing *Y. enterocolitica* will appear as pinpoint, round, convex, red, opaque colonies. Plasmidless *Y. enterocolitica* will appear as large, irregular, flat, translucent colonies.

Table 1. Biochemical Characteristics[a] of *Yersinia* species (2,8,9,50)

	Yersinia species										
Reaction	*Y. pestis*	*Y. pseudotuberculosis*	*Y. enterocolitica*	*Y. intermedia*	*Y. frederiksenii*	*Y. kristensenii*	*Y. aldovae*	*Y. rohdei*	*Y. mollaretii*	*Y. bercovieri*	*Y. ruckeri*
Lysine	-	-	-	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	-	-	-	-	-
Ornithine	-	-	+[c]	+	+	+	+	+	+	+	+
Motility at RT (22-26°C)	-	+	+	+	+	+	+	+	+	+	+
35-37°C	-	-	-	-	-	-	-	-	-	-	-
Urea	-	+	+	+	+	+	+	+	+	+	-
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+/-	-	+	+	+	+	+	+	+	+	-
Cellobiose	-	-	+	+	+	+	-	+	+	+	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	+/-(+)	+/-(+)	+/-(+)	+/-(+)	+	-	+/-	-	
Sucrose	-	-	+[c]	+	+	-	-	+	+	+	-
Rhamnose	-	+	-	+	+	-	+	-	-	-	-
Raffinose	-	+/-	-	+	-	-	-	+/-	-	-	-

Melibiose	-	+/-	-	+	-	-	-	+/-	-	-	-
Simmons citrate	-	-	-	+/-	+/-	-	-	+	-	-	+
Voges-Proskauer	-	-	+/-(+)	+	+	-	+	-	-	-	-
Indole	-	-	+/-	+	+	+/-	-	-	-	-	-
Salicin	+/-	+/-	+/-	+	+	-(+/-)	-	-	+/-	(+)	
Esculin	+	+	+/-	+	+	-	+	-	(+)	(+)/-	-
Lipase	-	-	+/-	+/-	+/-	+/-	+/-	-	-	-	
Pyrazinamidase	-	-	+/-	+	+	+	+	+	+	+	

[a] = positive after 3 days, (+) = positive after 7 days.
[b] Some strains of *Y. intermedia* are negative for either Simmons citrate, rhamnose, and melibiose, or raffinose and Simmons citrate.
[c] Some biotype 5 strains are negative.

Table 2. Biotype scheme[(a)] for *Y. enterocolitica*

	Reaction for biotypes[(b)]						
Biochemical test	**1A**	**1B**	**2**	**3**	**4**	**5**	**6**
Lipase	+	+	-	-	-	-	-
Esculin/salicin (24 h)	+/-	-	-	-	-	-	-
Indole	+	+	(+)	-	-	-	-
Xylose	+	+	+	+	-	V	+
Trehalose	+	+	+	+	+	-	+
Pyrazinamidase	+	-	-	-	-	-	+
β-D-Glucosidase	+	-	-	-	-	-	-

Voges-Proskauer	+	+	+	+/-(c)	+	(+)	-

[a] Based on Wauters (48).

[b] ( ) = Delayed reaction; V = variable reactions.

[c] Biotype of serotype O:3 found in Japan.

4. **Crystal violet binding test (10)**. This rapid screening test differentiates potentially virulent *Y. enterocolitica* cultures. Grow suspect cultures 18 h at 22-26°C in BHI broth with shaking. Dilute each culture to 1000 cells/ml in physiological saline. Spread-plate 0.1 ml of each culture to each of two BHI agar plates. Incubate one plate at 25°C and the other at 37°C for 30 h. Gently flood each plate with 8 ml of 85 µg/ml crystal violet (CV) solution for 2 min and decant the CV. Observe colonies for binding of CV. Plasmid-containing colonies grown at 37°C will bind CV, but not when grown at 25°C. Plasmidless colonies grown at either temperature should not bind CV.

5. **DNA colony hybridization**. Currently, three oligonucleotide probes are available from FDA's Center for Food Safety and Applied Nutrition for the detection of virulence factors in *Yersinia*. The probe INV-3 is specific only for the *inv* gene of *Y. pseudotuberculosis*. All INV-3 reactive isolates of *Y. pseudotuberculosis* examined are also invasive for HeLa cells. The PF13 oligo probe is specific only for the invasion gene, *ail*, of *Y. enterocolitica*. Comparison of colony and Southern blot analysis using PF13 vs HeLa cell invasion studies shows close correlation between probe and cell invasion. The oligo probe, SP12, developed under FDA contract by the Maryland Center for Vaccine Development (32,42), is specific for the 41-48 Mdal virulence plasmid in both *Y. enterocolitica* and *Y. pseudotuberculosis*. For more information on probes and protocols, **see** Chapter 24.

Table 3. Calculation of LD_{50} by Reed-Muench method (40)

A	B	C	D	E	F	G	H
					Cumulative values [a]		
			No. of mice		No. of mice		
Dilution	Bacterial cells/ml	Cells/mouse $\log_{10}$	dead	live	dead	live	% Mortality
10^0	3 x 10^8	7.477	3	0	15	0	100

10^{-1}	3 x 10^{7}	6.477	3	0	12	0	100
10^{-2}	3 x 10^{6}	5.477	3	0	9	0	100
10^{-3}	3 x 10^{5}	4.477	3	0	6	0	100
10^{-4}	3 x 10^{4}	3.477	2	1	3	1	75
10^{-5}	3 x 10^{3}	2.477	1	2	1	0 3	25[c]
10^{-6}	3 x 1O^{2}	1.477	0	3	0	6	0
10^{-7}	3 x 10^{1}	0.477	0	3	0	9	0

[a] Cumulative values in columns F and G are determined by adding values vertically in columns D and E, respectively. Arrows indicate direction of addition for accumulated values.

[b] % Mortality (H) is determined by dividing the number from column F by the total of the number from column F and column G for each dilution.

[c] The 50% endpoint lies between 10^{-4} and 10^{-5} dilutions.

$$\textit{Fractional titer} = \frac{(50\ \%) - (\%\ \textit{mortality below 50\%})}{(\textit{mortality above 50\%}) - (\textit{mortality below 50\%})}$$

$$= \frac{(50\% - 25\%)}{75\% - 25\%} = \frac{0.25}{0.50} = 0.50$$

$$LD_{10}\ \textit{titer} = \log_{10}(\textit{cells/mouse at dilution below 50 \% mortality} + \textit{fractional titer})$$

$$= 2.477 + 0.50 = 2.977$$

$$LD_{50}\ \textit{titer} = \textit{antilog}\ 2.977 = 948\ \textit{cells}$$

6. **Intraperitoneal infection of adult mice pretreated with iron dextran and desferrioxamine B**. A positive result from any test (H, 1-5 above) is presumptive evidence of pathogenicity and should be confirmed by a biological test. Inoculate presumptively virulent *Y. enterocolitica* isolates into BHI broth and incubate overnight at RT with agitation. This will result in broth cultures at approximately 10^9 bacterial cells per ml. Make decimal dilutions in sterile physiologic saline to use for mouse infections. Spread-plate 0.1 ml of appropriate dilutions (usually 10^6) to two plates each of TSAYE and CRBHO. Incubate TSAYE at RT for 48 h and CRBHO at 35°C for 24 h. Count TSAYE colonies to determine inoculum level and CRBHO for ratio of plasmid to non-plasmid cells in inoculum.

   One day before infection, inject Swiss Webster adult mice i.p. with 0.2 ml physiologic saline solution containing 25 mg/ml each of iron dextran (Fermenta Animal Health Co., Kansas City, MO 64153) and desferrioxamine B (Desferal mesylate, Ciba Geigy, Greensboro, NC 27409). Inject 0.1 ml of decimally diluted bacterial cells i.p. to each of five mice per dilution. Observe mice for 7 days. If possible, maintain infected mice in a laminar flow isolator. Deaths occurring within 7 days, especially preceded by signs of illness, are specific for *Y. enterocolitica* virulence and are used to calculate LD_{50} titer by method of Reed and Muench (40) as outlined in Table 3. Calculated LD_{50} titer of less than 10^4 cell is typical of virulent *Y. enterocolitica* regardless of biotype or serotype. A screening test may be performed by inoculating five pretreated mice at the 10^{-4} dilution only. A virulent *Yersinia* culture will kill at least 4 of 5 mice.

7. **Invasiveness**. An in vitro HeLa cell assay is available for screening *Yersinia* isolates for invasive potential (30,31). Acridine orange is used to stain infected HeLa cell monolayers, which are then examined under fluorescence microscope for the presence of intracellular *Yersinia* (30,31). This in vitro staining technique can be used to determine invasiveness in both *Y. enterocolitica* and *Y. pseudotuberculosis* (14).

I. *Yersinia pseudotuberculosis*

Generally, all *Y. pseudotuberculosis* strains are biochemically homogeneous except for production of acid from melibiose, raffinose, and salicin. *Y. pseudotuberculosis* heat-stable somatic antigens are also used to subgroup the species. At present there are six serogroups represented by Roman numerals I-VI. Serogroups I, II, III, and IV have subtypes, but antiserum to one serogroup type will cross-react with the subtype strain and vice versa. Strains belonging to serogroups II and III are lethal when fed to adult mice even though these strains do not elaborate lipase. HeLa cell-invasive strains are esculin-positive, which is contrary to findings with *Y. enterocolitica*. *Y. pseudotuberculosis* strains harbor a 41-48 Mdal plasmid and will autoagglutinate at 37°C. Association of yersiniosis in humans with the presence of a plasmid has been established (45).

1. **Enrichment**. Aseptically weigh 25 g sample into 225 ml PMP broth (15). Homogenize for 30 s and incubate at 4°C for 3 weeks. At 1, 2, and 3 weeks, mix enrichment well. Transfer 0.1 ml enrichment to 1 ml 0.5% KOH in 0.5% NaCl and mix for 5-10 s. Successively streak one loopful to MacConkey agar plate and one loopful to CIN agar plate. Streak one additional loopful directly

from enrichment broth to one MacConkey and one CIN agar plate. Incubate agars at RT.

2. **Isolation and identification**. Continue as in E-H, above, noting biochemical differences (Table 1). Notably, *Y. pseudotuberculosis* strains are ornithine-, sorbitol-, and sucrose-negative.

Instructions for *Yersinia* Identification Tests

Phenylalanine deaminase agar test: Add 2-3 drops 10% ferric chloride solution to growth on agar slant. Development of green color is positive test.

Indole test: Add 0.2-0.3 ml Kovacs' reagent. Development of deep red color on surface of broth is positive test.

V-P test: Add 0.6 ml **alpha**-naphthol and shake well. Add 0.2 ml 40% KOH solution with creatine and shake. Read results after 4 h. Development of pink-to-ruby red color in medium is positive test.

Pyrazinamidase test: After growth of culture on slanted pyrazinamidase agar at RT, flood 1 ml of 1% freshly prepared ferrous ammonium sulfate over slant. Development of pink color within 15 min is positive test, indicating presence of pyrazinoic acid formed by pyrazinamidase enzyme.

Beta-D-Glucosidase test: Add 0.1 g 4-nitrophenyl-**beta**-D-glucopyranoside to 100 ml 0.666 M NaH_2PO_4 (pH 6). Dissolve; filter-sterilize. Emulsify culture in physiologic saline to McFarland Turbidity Standard No. 3. Add 0.75 ml of culture to 0.25 ml of test medium. Incubate at 30°C overnight. A distinct yellow color indicates a positive reaction.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 9

Vibrio cholerae, V. parahaemolyticus, V. vulnificus, and Other *Vibrio* spp.

Authors

(Return to Table of Contents)

The genus *Vibrio* includes Gram-negative, oxidase-positive (except two species), rod- or curved rod-shaped facultative anaerobes. Many *Vibrio* spp. are pathogenic to humans and have been implicated in foodborne disease (Table 1). *Vibrio* spp. other than *V. cholerae* and *V. mimicus* do not grow in media that lack added sodium chloride, and are referred to as "halophilic."

V. cholerae was first described as the cause of cholera by Pacini in 1854. Pathogenic *V. cholerae* produces a heat-sensitive enterotoxin that causes the characteristic cholera symptoms, including "rice water stool." The species comprises several somatic (O) antigen groups, including O-group-1, which is associated with classical and El Tor biotypes. *V. cholerae* Ol may have several serotypes, including Inaba, Ogawa, and Hikojima. *V. cholerae* non-O1 (referred to in older literature as nonagglutinable or NAG vibrios) also can cause gastrointestinal disease, though typically less severe than that caused by *V. cholerae* O1 (35). Serotype O139 is an exception, and produces classic cholera symptoms. This serotype was first identified in 1992 (4a) as the cause of a new epidemic of cholera in India and Bangladesh. Non-O1 *V. cholerae* is found more readily in estuarine waters and seafood in the United States than is the Ol serogroup; however, the 0139 serogroup has not yet been found here. Because this species can grow in media lacking sodium chloride, it is not considered a halophilic vibrio, although traces of sodium ion are required for growth. The standard FDA method for recovery of *V. cholerae* is qualitative (presence/absence). Testing *V. cholerae* O1 and non-O1 isolates for production of cholera toxin is recommended.

Table 1. Association of Vibrio spp. with different clinical syndromes [a], [b]

	Clinical Syndrome				
Species	**Gastroenteritis**	**Wound Infection**	**Ear Infection**	**Primary Septicemia**	**Secondary Septicemia**
***V. cholerae* O1**	+++	+			
***V. cholerae* non-O1**	+++	++	+	+	+
V. mimicus	++		+		
V. fluvialis	++				
V. parahaemolyticus	+++	+	+		+
V. alginolyticus	(+)	++	++	+	
V. cincinnatiensis			+		
V. hollisae	++			+	
V. vulnificus	+	++		++	++
V. furnissii	(+)				
V. damsela		++			
V. metschnikovii	(+)			(+)	
V. carchariae		+			

[a] +++ = frequently reported, ++ = less common (6-100 reports); + = rare (1-5 reports), and (+) = association is unclear.

[b] Table taken from A.T. Pavia et al. (22).

Some diarrheal and otitis isolates, once thought to be atypical *V. cholerae* non-O1 (sucrose-negative), are now recognized as a separate species, *V. mimicus* (5,25). Members of the species may produce cholera-like enterotoxins. *V. mimicus* can be identified by biochemical procedures used for the identification of *V. cholerae*.

V. parahaemolyticus is a halophilic bacterium found naturally in estuarine waters and animals. It was first described as the cause of gastroenteritis in Japan (9) and was first found in the United States by Baross and Liston (1) in the estuarine waters of Puget Sound. It has a worldwide distribution in estuarine and coastal environments and has been isolated from many species of fish, shellfish, and crustaceans. *V. parahaemolyticus* has been implicated in numerous outbreaks of seafood-borne gastroenteritis in the United States. Between 1971 and 1978, crab, oyster, shrimp, and lobster were implicated in 14 outbreaks, which may have resulted from the consumption of raw or insufficiently heated seafood or properly cooked seafood contaminated after cooking. The FDA method of enumeration uses an MPN format.

V. vulnificus is a halophilic bacterium found in the estuarine environment and is similar phenotypically to *V. parahaemolyticus* (21). The species was first described as "lactose-positive" because most strains ferment lactose and are **o**-nitrophenyl-β-D-galactosidase (ONPG) positive. It causes foodborne and wound disease, either of which may progress to rapidly fatal septicemia in individuals with liver disease (cirrhosis) or other underlying illnesses such as diabetes. Raw oysters are the major source of foodborne disease caused by *V. vulnificus*. The FDA method of enumeration uses an MPN series confirmed by biochemical testing or an immunological test, such as the ELISA, with monoclonal antibody to a species-specific intracellular antigen.

Other halophilic *Vibrio* spp., including *V. fluvialis*, *V. hollisae*, *V. alginolyticus*, *V. furnissii*, and *V. metschnikovii*, have been associated with gastroenteritis and are present in estuarine environments along with other pathogenic and nonpathogenic species of *Vibrio*. *V. cincinnatiensis*, *V. damsela*, and *V. carchariae* have not been associated with gastroenteritis, but on rare occasions are pathogenic to humans (Table 1). *V. anguillarum*, *V. damsela*, and *V. carchariae* are pathogenic t o fish. Biochemical testing is required for taxonomic speciation.

NOTE: Although many of the same media are used for the enrichment and identification of various *Vibrio* spp., procedures have been optimized for detection of *V. cholerae* and for enumeration of *V. parahaemolyticus* and *V. vulnificus*. *Vibrio* species other than these may be encountered on thiosulfate-citrate-bile salts-sucrose (TCBS) agar and can be identified by the characteristics given in Tables 2-4 and in supplementary literature such as *Bergey's Manual of Systematic Bacteriology* (3). All of the cultural and serological tests should be performed in conjunction with known strains for comparative purposes.

V. hollisae does not grow readily on TCBS agar and a selective agar has not been developed. If *V. hollisae* is to be detected, a differential medium such as blood agar flooded with oxidase reagent after incubation (13) or mannitol-maltose agar (20) may be used.

A. Equipment and materials

1. Same as for *Salmonella*, Chapter 5
2. Water baths, 35-37°C
3. Incubator, 39-40°C and 42°C
4. Commercial bacterial identification strips
5. VET-RPLA TD20 kit (Oxoid) for cholera toxin
6. V-bottom (conical) microwell plates for VET-RPLA kit assay
7. Low protein-binding 0.2 µm filters (Millipore SLGV)
8. Micropipettors or micro-repeating pipettors
9. Tissue culture cluster plates, 96-well (CoStar, Cambridge, MA)
10. Flat-bottomed ELISA (EIA) plates, 96-well (CoStar or Immulon 2, Dynatech Laboratories, Alexandria, VA)
11. ELISA plate washer (optional)
12. ELISA (EIA) plate reader
13. Shaker incubator, 35-37°C
14. Sterile syringes, 1 ml
15. Centrifuge
16. For gene probes, **see** Chapter 24
17. For tissue culture: cell culture flasks; cell culture 96-well plates; CO_2 incubator, 37°C; inverted phase-contrast microscope
18. 10,000 M.W. cutoff ultrafiltration membranes (PM10 or YM10; Amicon, Inc., Danvers, MA) and apparatus
19. Glass petri dishes for membrane ELISA
20. Nitrocellulose membranes, 82 mm (Bio-Rad Labs or Schleicher and Schuell) or nylon membranes (Magnagraph), MSI (Micron Separations, Inc.)

B. Media and reagents

NOTE: Halophilic *Vibrio* spp. require added NaCl (2-3% final concentration). *V. cholerae* grow well in media with 0-3% NaCl. Add NaCl to media listed in Appendix 3 to achieve a final 2-3% concentration of NaCl, with the following exception. Do not add NaCl to gelatin agar (M54) or alter the NaCl concentrations of salt tolerance testing broths (M161 and M164).

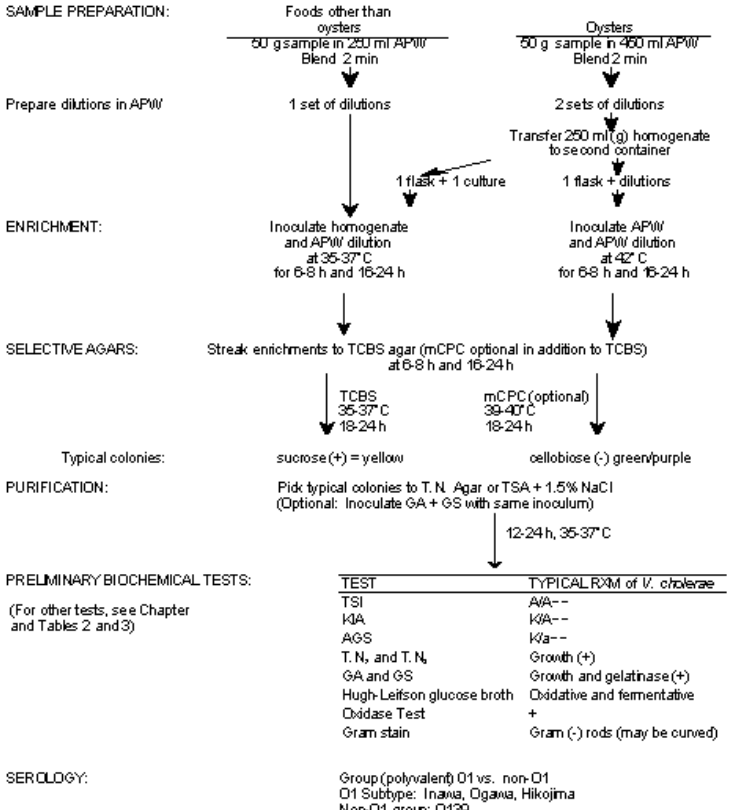
1. AKI broth (M7)
2. Alkaline peptone salt (APS) broth (M9)
3. Alkaline peptone water (APW) (M10)
4. Arginine glucose slant (AGS) (M16)
5. Bicarbonate buffer, 0.1 M, pH 9.6 (R4)
6. Blood agar (M20)
7. 1% Bovine serum albumin (BSA) in ELISA buffer (R6) and in PBS (R7)
8. Brain heart infusion (BHI) agar (M24)
9. Bromcresol purple broth (M26) supplemented individually with sucrose, lactose, cellobiose, arabinose, D-mannitol, or D-mannose
10. Cary-Blair transport medium (M31)
11. Casamino acids-yeast extract (CYE) broth (M32)
12. Chicken red blood cells, 2.5%, in physiological saline
13. Cholera enterotoxin (Becton-Dickinson Immunodiagnostics, Orangeburg, NY 10962; Sigma Chemical Co., St. Louis, MO 63178; or List Biological Laboratories, Inc., Campbell, CA 95008)
14. Christensen's urea agar (M40)
15. Citric acid, 0.05 M, pH 4.0 (R13)
16. Coating solution for *V. vulnificus* EIA (R15)
17. Decarboxylase basal medium (M44), unsupplemented, and supplemented individually with arginine, lysine, or ornithine
18. ELISA buffer for cholera toxin assay (R22)
19. El Tor phage V (ATCC, Rockville, MD)
20. Enzyme immunoassay (EIA) wash solution (R21) for *V. vulnificus*
21. Fetal calf serum
22. Gelatin agar (GA) (M54) and gelatin salt (GS) agar (M55)
23. Goat antiserum to cholera toxin (CT) (List Biological Laboratories)
24. Gram stain dyes (R32)
25. Ham's F-10 medium with glutamine (M58), commercial preparation preferred
26. Heart infusion broth and agar (M60)
27. Hugh-Leifson glucose broth (M63)
28. 1 N hydrochloric acid (HCl) (R36)
29. Kligler iron agar (KIA) (M71)
30. Kovacs' reagent (R38)
31. Long-term preservation medium (M85)
32. Mannitol-maltose agar (M96)
33. Methyl red indicator (R44)
34. Mineral (paraffin) oil, sterile (R46)
35. Modified cellobiose-polymyxin B-colistin (mCPC) agar (M98)
36. Monoclonal antibody to *V. vulnificus* [Available from FDA Gulf Coast Seafood Laboratory, P.O. Box 158, Dauphin Island, AL 36528-0158]
37. Motility test medium, semisolid (M103)
38. MR-VP broth (M104)
39. Mueller-Hinton agar (M107)
40. Mukerjee phage IV
41. O/129 (2,4-diamino-6,7-diisopropyl pteridine) disks, 10 and 150 µg (R51)
42. OF medium, semisolid (M116), supplemented individually with glucose, sucrose, lactose, cellobiose, arabinose, D-mannitol, or D-mannose
43. ONPG test reagents (R53) or commercially available disks
44. Oxidase test reagent (R54)
45. Penicillin-streptomycin (Pen-strep) solution (Mll9)
46. Peroxidase-conjugated goat immunoglobulin G (anti-mouse; anti-rabbit)
47. Peroxidase substrate solution (4-chloro-1-naphthol) for membrane ELISA (R57)
48. Peroxidase substrate solution (ABTS) (R58)
49. Phosphate-buffered saline (PBS), pH 7.4 (R59)
50. Physiological saline solution, 0.85% (R63)
51. Polymyxin B antibiotic disks, 50 units (R64) or commercially prepared
52. Poly, Inaba, and Ogawa antisera for *V. cholerae* (Difco; Burroughs Wellcome). Monoclonal antibody reagents are available from Denka Seiken Co., Tokyo, Japan, through Nichimen America, Inc., 1185 Avenue of the Americas, New York, NY 10036. Monoclonal antibody reagents to *V. cholerae* Ol antigen are available from Global Diagnostics, One Progress Blvd., Box 33, Room N-112, Alachua, FL (904)-462-7997)

and from OXOID.

53. Rabbit antiserum to cholera toxin (contact M. Kothary)
54. Reagents for gene-probe testing, **see** Chapter 24)
55. Reference strains for *V. cholerae* classical and El Tor biotypes, serogroup O1; cytotoxin-producing strains of *V. cholerae* serogroup non-O1; reference strains of Kanagawa phenomenon-positive *V. parahaemolyticus*; cytotoxin-hemolysin-positive strains of *V. vulnificus*, and *V. mimicus* (contact M.Kothary)
56. Sera for O and K antigen testing of O (somatic) and K (capsular) antigens of *V. parahaemolyticus* (Denka Seiken Co., through Nichimen America, Inc.or through OXOID or from Charles Kaysner (phone: 206-483-8788)
57. Sodium chloride dilution water, 2% and 3% (R71)
58. 1 N sodium hydroxide (NaOH) solution (R73)
59. Sheep red blood cells, 5%, in physiological saline
60. Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (M147)
61. Triple sugar iron (TSI) agar (M149)
62. Tris-buffered saline, pH 7.5 (TBS) (R81); with 1% or 3% gelatin (R82); with 0.05% Tween 20 (TBS-Tween (R83)
63. Triton X-100 (R86)
64. Trypsin-EDTA solution, 1X (R87)
65. Trypticase (tryptic) soy agar (TSA) (M152) and broth (TSB) (M154)
66. Tryptic soy broth (TSB) without dextrose, supplemented with 1% NaCl and 24% glycerol (M155) (total 1.5% NaCl), and with 2.5% NaCl (total 3% NaCl)
67. 1% Tryptone, 1% NaCl (T_1N_1) agar (M163) and T_1N_2 agar
68. Tryptone broth (1%), unsupplemented (0% NaCl) (M164), or supplemented with 1, 3, 6, 8, or 10% NaCl (M161)
69. Voges-Proskauer test reagents (R89)
70. Wagatsuma agar (M178)
71. Y-1 adrenal cell growth medium (M180)
72. Y-l mouse adrenal cells or Chinese hamster ovary (CHO) cells (ATCC)

C. Procedure for enrichment and isolation of *V. cholerae* and *V. mimicus* from foods (Fig. 1). For stool, rectal swab, or vomitus specimens, **see** D-l, below, for transport and initial inoculation procedures.

Figure 1. SCHEMATIC DIAGRAM FOR *Vibrio cholerae* ISOLATION METHODS



Hugh-Leifson glucose broth	Oxidative and fermentative
Oxidase Test	+
Gram stain	Gram (-) rods (may be curved)
SEROLOGY:	Group (polyvalent) O1 vs. non-O1 O1 Subtype: Inawa, Ogawa, Hikojima Non-O1 group: O139
BIOCHEMICAL TESTS including BIOTYPE:	See method
TOXIGENICITY	Tissue culture, ELISA, RPLA or gene probe

1. **Sample preparation**. Aseptically weigh 25 g sample into 500 ml tared sterile blender jar or Stomacher. Cut large samples into smaller pieces before blending. Add 225 ml alkaline peptone water (APW) to jar and blend for 2 min at top speed. **For oysters only** (especially oysters freshly harvested from warm waters), prepare composite of 10-12 animals including shell liquor; blend to mix. Blend 50 g of this composite with 450 ml of APW. Pour 250 ml (g) of this mixture into another sterile container. Replicates for samples of oysters are incubated at different temperatures (**see** 2, below).

   NOTE: Isolating specific *Vibrio* spp. from samples containing high concentrations of enteric bacteria may be difficult because of overgrowth. For vegetables, estuarine waters, and other environmental samples expected to have high numbers of bacteria, dilute the blended samples to a final 1:100 dilution and proceed as usual. For example, take 25 ml of blended sample and add to 225 ml APW.

   For seafood samples, especially oysters, also prepare tenfold dilutions of the blended seafood sample in 9 (or 90) ml APW blanks (1:100 and 1:1000 dilutions) and proceed as usual. Prepare 2 sets of dilution tubes for oysters. Dilutions are made to decrease competition from other vibrios.

   Dilutions may also be used to analyze for *V. parahaemolyticus* and *V. vulnificus*. If sample is to be tested for all three *Vibrio* species (and others), use a sample large enough to inoculate all required media, and prepare the homogenate in APW or PBS, pH 7.2-7.5. For example, if sample is to be analyzed for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, homogenize a 50 g sample with 450 ml APW. Place 250 ml (g) of APW homogenate in sterile container and follow the method for *V. cholerae*. (If PBS is used during homogenation, transfer 250 ml (g) of PBS homogenate to 2250 ml APW.) If an MPN is to be determined with the remainder, prepare dilutions in PBS, pH 7.2-7.5, inoculate MPN tubes of APW, and incubate tubes at 35-37°C. These tubes will serve as MPN enrichment tubes for *V. parahaemolyticus* and *V. vulnificus*, as well as *V. cholerae* in materials that may have high background microflora. From APW, inoculate selective plating media at 6-8 h for *V. cholerae* and at 18-24 h for *V. cholerae, V. parahaemolyticus*, and *V. vulnificus*. **See** part D for identification of halophilic *Vibrio* spp. For oyster samples to be tested for the three *Vibrio* species, use a sample of at least 75 g since two 250 ml (g) test portions of APW homogenate are incubated for *V. cholerae* analyses (one at 35-37°C and one at 42°C).

2. Leave blended solutions of samples, including frozen or otherwise processed food homogenate and dilutions, in jars or pour into loosely stoppered sterile 500 ml Erlenmeyer flasks and incubate jars, flasks, and dilutions 6-8 h at 35-37°C. Plate inocula to isolation agar (**see** 3, below), and reincubate enrichment broths for total incubation time of 18-24 h. Plate the 18-24 h enrichment broths to isolation agar.

   a. **Exception**. Incubate second jar or flask of **oyster homogenate** and one set of dilutions at 42°C for 6-8 h (6).

3. After incubation, and without shaking flask, transfer 3-5 mm loopful of inoculum from pellicle (surface growth) onto at least one plate ofselective plating medium: TCBS agar. (mCPC agar is optional; it may be used in addition to TCBS. Classical biotype *V. cholerae* is inhibited on mCPC agar by polymyxin B.) Incubate TCBS agar for 18-24 h at 35-37°C and mCPC agar for 18-24 h at 39-40°C.

4. Examine plates for colony characteristics described below. Carefully pick 3 or more suspect colonies from each plate, streak each for isolation on T_1N_1, T_1N_2, or tryptic soy agar (2% total NaCl concentration), and incubate for 18-24 h at 35-37°C. Streaking for isolation on nonselective medium may be necessary to ensure colonial purity before biochemical testing. Gelatin agar (GA) and gelatin salt (GS) agar (**see** 5b, below) may also be inoculated with the same inoculum.

   a. **Thiosulfate-citrate-bile salts-sucrose (TCBS) agar**. On TCBS agar, *V. cholerae* (El Tor and classical) are large, smooth, yellow (sucrose-positive), and slightly flattened colonies with opaque centers and translucent peripheries. **NOTE**: *Vibrio* spp. **do not** produce tiny, creamy yellow colonies on TCBS agar. Colonies of closely related *V. mimicus* are green (sucrose-negative). Most other *Vibrio* spp. grow on TCBS agar and produce yellow or green colonies.
   b. **Modified cellobiose-polymyxin B-colistin (mCPC) agar (30)**. Colonies of *V. cholerae* El Tor are green-to-purple (cellobiose fermentation-negative). *V. vulnificus* produces flattened yellow colonies with opaque centers and translucent peripheries. Most other *Vibrio* spp. do not grow readily on CPC agar (19) or mCPC agar.

5. Distinguish suspect vibrios from non-vibrios

   a. **TSI, KIA, and arginine glucose slant (AGS)**. Inoculate individual colonies into TSI or KIA, and AGS media by stabbing butt and streaking slant. Incubate loosely stoppered or capped inoculated slants 18-24 h at 35-37°C. These media are recommended because the reactions permit early presumptive differentiation between most *Vibrio* spp., *Aeromonas* spp., *Plesiomonas shigelloides*, and other bacteria (Tables 2 and 3).
   b. **1% Tryptone (tryptophane) broth (T_1N_0)** and broth containing 3% NaCl (T_1N_3). Inoculate individual olonies into T_1N_0 and T_1N_3 broths and incubate 18-24 h at 35-37°C. Reincubate growth-negative tubes an additional 18-24 h. *V. cholerae* and *V. mimicus* will grow in T_1N_0 and T_1N_3. Some non-vibrio bacterial species producing reactions similar to those of *V. cholerae* in TSI and KIA media will not grow in T_1N_3. Most *Vibrio* spp., including some *V. cholerae* non-O1, will grow in T_1N_3 only.
      Alternatively, gelatin agar (GA) and gelatin agar containing 3% NaCl (GS) can be used to screen isolates for salt tolerance (29). Divide plates into 8 sectors. Inoculate a straight line in the center of one sector of both GA and GS plates with each isolate. Incubate 18-24 h at 35°C. *V. cholerae* and *V. mimicus* will grow on both plates because they do not require salt. Halophilic *Vibrio* spp. will grow only on the GS plate. To read the gelatinase reaction, hold plate above a black surface. An opaque halo will be present around growth of gelatinase-positive organisms.
   c. **Oxidation-fermentation test.** Inoculate 2 tubes of Hugh-Leifson glucose broth or OF glucose medium (semisolid) with growth from an isolated colony. Overlay medium in one tube with sterile mineral oil or liquid Vaspar (50% petrolatum, 50% paraffin) to depth of 1-2 cm and incubate 1-2 days or more at 35-37°C. Acid causes dye to change from purple to yellow in Hugh-Leifson broth, and from green to yellow in OF medium, semisolid. *Vibrio* spp. ferment glucose and produce acid from glucose oxidatively. *Pseudomonas* spp., commonly isolated from seafood by enrichment methods used for *Vibrio* spp., utilize glucose oxidatively only.
   d. **Oxidase test**. Perform oxidase test on 18-24 h growth from TSA or other medium containing no fermentable carbohydrate such as GA or GS. An easy rapid method for testing large numbers of isolates is to

place a filter paper circle in a petri plate and moisten the entire filter paper with a few drops of oxidase reagent. With a sterile wooden applicator stick, toothpick, or platinum loop, pick growth from the plate and touch the moistened paper. Oxidase positive organisms will turn the paper dark purple or blue within 10 seconds. Pathogenic *Vibrio* spp. are oxidase-positive (except for *V. metschnikovii*).

6. Identification and confirmation of *V. cholerae* O1, *V. cholerae* non-O1, and *V. mimicus*.

   a. **Read results** of TSI, KIA, AGS, T_1N_0 and T_1N_3 or GA and GS, and oxidation-fermentation tests.
   b. **Perform Gram stain** on 18-24 h broth or agar culture.

   NOTE: Isolates to be carried through the remaining *V. cholerae* serological and biochemical tests are sucrose-positive (yellow) on TCBS agar]sucrose-negative (green) for *V. mimicus*)(or cellobiose-negative (green-purple) on mCPC agar. They grow in T_1N_0 and T_1N_3 broths or on GA and GS plates; show characteristic reactions (see Table 2) in TSI, KIA, and AGS; are gelatinase and oxidase-positive; are Gram-negative rods or curved rods; and produce acid from glucose both oxidatively and fermentatively in Hugh-Leifson glucose broth or OF glucose medium, semisolid.

Table 2. Reactions[(a)] of certain *Vibrio* spp. and related microorganisms in differential tube agar media

Microorganism	KIA	TSI		AGS		
	Slant	Butt	Slant	Butt	Slant	Butt
V. cholerae	K	A	A(K rare)	A	K	a
V. mimicus	K	A	K(A rare)	A	K	A
V. parahaemolyticus	K	A	K	A	K	A
V. alginolyticus	K	A	A	A	K	A
V. vulnificus	K or A	A	K(A rare)	A	K	A
A. hydrophila	K or A	A	K or A	A	K	K
P. shigelloides	K or A	A	K or A	A	ND	ND

[a] K, alkaline; A, acid; a, slightly acid; ND, not determined. None of the listed *Vibrio* spp. produce hydrogen sulfide gas in KIA, TSI, or AGS media, or gas from glucose in detectable quantities in KIA, TSI, or AGS media. Some *Aeromonas* spp. may produce gas from glucose in these media.

   c. **Serological agglutination test**. Use diagnostic antisera of Group O1 and subgroup Inaba (factors AC) and Ogawa (factors AB) to serotype Ol antigen and antisera or monoclonal antibodies to serotype O139 antigen to identify serogroup O139. Use 16-24 h cultures from TSA. Include positive and negative cultures and saline controls for each antiserum used. Follow directions included with antisera. Drops of 10 µl are sufficient for the test. Because antigens in different species may be related, biochemical tests must be completed before isolate is confirmed as *V. cholerae* O1 or non-O1.

   NOTE: Monoclonal antibodies are available, but anti-B and anti-C cross-react with bacteria of other species (26). Use polyclonal sera and/or monoclonal antibodies to the A antigen of the O1 complex.

   Cultures that agglutinate in group Ol antiserum and not in plain physiological saline are *V. cholerae* group O1 if biochemical reactions confirm the isolate as *V. cholerae*. Cultures that agglutinate in this group-specific antiserum may be subtyped with Inaba and Ogawa antibodies.

   Notify Dr. Joseph Madden, FDA, telephone (202) 205-4197, or Dr. Mahendra Kothary (202) 205-4454, of *V. cholerae* O1 isolates. Continue with biochemical characterization and toxigenicity determination of the isolates. Direct questions about methodology to Dr. LeeAnne Jackson (202) 205-4231.

   Cultures that agglutinate in poly (group O1) antiserum and in **both** Inaba and Ogawa antisera have all 3 factors (A, B, and C) and are serotype Hikojima.

   Cultures that agglutinate in poly antiserum but not in Inaba or Ogawa antisera cannot be typed using these antisera.

   Cultures confirmed biochemically as *V. cholerae* that do not agglutinate in Group Ol antiserum are *V. cholerae* non-O1. Test such cultures with O139 antiserum.

   Cultures that agglutinate in Group O1 antiserum and in saline cannot be typed. However, using a richer growth medium, such as heart infusion (HI) agar or BHI agar, may eliminate this autoagglutination.

   d. **Biochemical reactions** (Table 3). For specific directions for biochemical tests, including O/129 sensitivity, growth at 42°C, and ONPG tests, **see** D-3, below. Formulations for all biochemical media should

include at least 2% NaCl. API strips may be used in lieu of conventional media, with 2% NaCl as diluent. For *V. cholerae*, use physiological saline (0.85% NaCl) as diluent.

Table 3. Biochemical characteristics of several of the *Vibrionaceae*

	V. alginolyticus	*V. alguilllaum*	*V. carchariae*	*V. cholerae*	*V. cincinnatiensis*	*V. damsela*	*V. fluvialis*	*V. furnissii*	*V. harveyi*	*V. hollisae*	*V. metchnikovii*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*	*A. hyrdophila*	*P. shigelloides*	*Photobacterium* spp.
TCBS agar	Y	Y	Y	Y	Y	G	Y	Y	Y/G	NG	Y	G	G	G	Y	G	NG/G
mCPC agar	NG	NG	nd	P	nd	NG	NG	NG	nd	NG	NG	NG	NG	Y	NG	NG	NG
AGS medium	KA	nd	nd	Ka	nd	nd	KK	KK	nd	Ka	KK	KA	KA	KA	KK	nd	nd
Growth in:																	
O% NaCl	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+	-
3% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	+	+	-	+	V	+	+	+	+	+	-	+	+	+	-	V
8% NaCl	+	-	+	-	-	-	V	+	V	-	V	-	+	-	-	-	-
10% NaCl	+	-	-	-	-	-	-	-	V	-	-	-	-	-	-	-	-
Growth at 42°C	+	-	nd	+	-	-	V	-	V	nd	V	+	+	+	V	+	-
Acid from:																	
Sucrose	+	+	+	+	+	-	+	+	V	-	+	-	-	-	V	-	V
D-Cellobiose	-	+	+	-	+	+	+	-	nd	-	-	-	V	+	+	-	-
Lactose	-	-	-	-	-	-	-	-	V	-	-	-	-	+	V	-	-
Arabinose	-	V	-	-	+	-	+	+	-	+	-	-	+	-	V	-	-
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	-	+
D-Mannitol	+	+	+	+	-	-	+	+	+	-	+	+	+	V	+	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	V
ONPG	-	+	-	+	+	-	+	+	V	-	+	+	-	+	+	-	+
Voges-Proskauer	+	+	-	V	+	+	-	-	-	-	+	-	-	-	+	-	V
Arginine dihydrolase	-	+	-	-	-	+	+	+	-	-	+	-	-	-	+	+	+
Lysine decarboxylase	+	-	+	+	+	V	-	-	+	-	+	+	+	+	V	+	V
Ornithine decarboxylase	+	-	+	+	-	-	-	-	+	-	-	+	+	+	-	+	-
Sensitivity to:																	
10 µg 0/129	R	S	R	S	R	S	R	R	R	nd	S	S	R	S	R	S	V
150 µg 0/129	S	S	S	S	S	S	S	S	S	nd	S	S	S	S	R	S	S
Gelatinase	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	-	-
Urease		-	-	+	-	-	+	-	-	V	-	-	-	V	-	-	--

Abbreviations: TCBS, thiosulfate-citrate-bile salts-sucrose; mCPC, modified cellobiose-polymyxin B-colistin; AGS, arginine-glucose slant; Y, yellow; G, green; P, purple; NG, no growth; nd, not determined; K, alkaline; A, acid; a, slightly acid; +, 80% or more of strains positive; -, 80% or more of strains negative (fewer than 20% of strains positive); V, variable reaction depending on species or strain; S, sensitive; R, resistant. Arginine glucose slant (AGS) reactions: slant, butt; all strains tested were hydrogen sulfide and gas negative. ONPG: **o**-nitro-β-D-galactopyranoside hydrolysis by β-galactosidase. Biochemical reactions from refs 4,7,10,11,18,21,23,24,33,34.

e. **Determination of classical and El Tor biotypes**. Two biotypes of *V. cholerae* serogroup O1 (classical and El Tor) may be distinguished by the following methods (**see** Table 4). Use more than one test to differentiate biotypes. The easiest methods are polymyxin B sensitivity, hemolysin test, and Voges-Proskauer test.

Table 4. Differentiation of biotypes of *V. cholerae* O1[a], [b]

Test	El Tor	Classical
Sensitivity to El Tor phage V	+	-
Sensitivity to classical phage IV	-	+
Sensitivity to polymyxin B, 50 units	-	+
Hemolysis (sheep erythrocytes)	v	-
Hemagglutination (chicken erythrocytes)	+	-
Voges-Proskauer	+	-

[a] From Baumann and Schubert (3) and Madden et al. (18).

[b] +, Positive; -, negative; v, strains vary.

Bacteriophage susceptibility. This method is a modification of that described by Finkelstein and Mukerjee (8). Inoculate HI broth with strain to be tested and incubate at 35-37°C for 4 h. Swab surface of Mueller-Hinton agar plate with 4 h broth culture to obtain confluent bacterial growth. Let plates absorb inoculum, and place 1 loopful of appropriate test dilution of phage IV onto agar surface with 3 mm platinum loop. Observe plate after overnight incubation at 35-37°C. Classical biotype strains are usually sensitive to this bacteriophage and will lyse on plate where phage was placed (indicated by clear plaque). El Tor biotype strains are resistant to this bacteriophage and will not be lysed (indicated by confluent growth).

Use this same method to test for sensitivity to El Tor phage V.

Polymyxin B sensitivity. This procedure is a modification of technique described by Han and Khie (12). Swab surface of Mueller-Hinton agar plate with 4 h HI broth culture (35-37°C) to obtain confluent growth. Let plates absorb inoculum and place 50 unit polymyxin B antibiotic disk on medium surface. Invert plates and incubate for 18-24 h at 35-37°C. Classical biotype strains will demonstrate zone of inhibition around disk (10-15 mm diameter). E1 Tor biotype strains will grow to edge of disk or will be inhibited slightly (7-8 mm diameter). Alternatively, use TSA, GA, or GS agar in place of Mueller-Hinton agar.

NOTE: If isolate was picked from mCPC, it is polymyxin B-resistant.

Hemolysin test. Mix equal volumes (0.5 or 1 ml) of 24 h HI broth culture and 5% saline suspension of sheep red blood cells. Set up similar mixtures with portion of culture that has been heated for 30 min at 56°C. Use known hemolytic and nonhemolytic strains of *V. cholerae* as controls. Incubate mixtures for 2 h in 35-37°C water bath, then refrigerate overnight at 4-5°C. Examine tubes for hemolysis. Low speed centrifugation may aid in detection of cell lysis. Most El Tor strains will lyse red blood cells. Heated portion of culture should produce no hemolysis because hemolysin is thermolabile.

Classical biotypes of *V. cholerae* and some strains of biotype El Tor will not lyse red blood cells. Alternatively, spot inoculum onto blood agar plates containing 5% sheep red blood cells, as for Kanagawa phenomenon (**see** below). Incubate at 35°C for 24 h and check for β -hemolysis surrounding colonies.

Chicken red blood cell agglutination. Prepare thick, milky bacterial suspension in physiological saline from 18 to 24 h TSA culture. On clean glass slide, mix 1 loopful of washed chicken red blood cells (2.5% in physiological saline) with suspension of bacterial culture to be tested. Visible clumping of red blood cells indicates El Tor biotype. Classical strains usually do not agglutinate red blood cells. Perform positive and negative controls.

Voges-Proskauer (VP) test. Perform test in MR-VP broth after 18-24 h incubation at 22°C. El Tor biotype strains are usually positive; classical strains are negative.

f. Minimal characters for biochemical identification of *V. cholerae*. The following characteristics are presumptive of *V. cholerae*:
   - Morphology: Gram-negative asporogenous rod or curved rod TSI or KIA appearance: Acid slant/acid butt, gas production- negative, H_2S-negative
   - Hugh-Leifson test: Glucose fermentation- and oxidation-positive
   - Cytochrome oxidase: Positive

- Arginine dihydrolase test: Negative
- Lysine decarboxylase test: Positive
- Voges-Proskauer test: El Tor biotype-positive, classical biotype-negative; *V. mimicus*-negative
- Growth at 42°C: Positive
- Halophilism test: 0% NaCl-positive; 3% NaCl-positive; 6% NaCl- usually negative. Some strains of *V. cholerae* non-O1 may not grow in 0% NaCl.
- Sucrose fermentation: Positive (negative for *V. mimicus*)
- ONPG test: Positive
- Arabinose fermentation: Negative
- O/129 sensitivity: Sensitive to 10 and 150 µg O/129

7. Detection of cholera enterotoxin (CT) and cytotoxin

Test isolate determined to be *V. cholerae* (including O1, O139, and other non-O1 serogroups) or *V. mimicus* biochemically and/or serologically for CT by direct or immunological test. Direct tests include the effect of toxin in vitro on Y-l mouse adrenal cells or Chinese Hamster Ovary (CHO) cells, and the in vivo suckling mouse assay (2). Immunological methods include ELISAs and latex agglutination tests. DNA probes are available for CT-like enterotoxin gene sequences (**see** Chapter 24).

a. Y-l Adrenal cell or Chinese Hamster Ovary (CHO) cell assay for enterotoxin (CT) and cytotoxin

Cell culture flasks. Using standard cell culture techniques, grow Y-l or CHO cells on surface of 25 cm sq plastic cell culture flasks, using 5 ml Y-l cell growth medium at 37°C in 5% CO_2 incubator. Replace medium after 48 and 96 h and observe appearance of cells, using inverted phase-contrast microscope. Test cells before they become totally confluent in flask. Before preparing fresh flasks or monolayers in wells of microwell plates, wash monolayer with 5 ml sterile 0.85% saline or PBS. Add 0.5 ml lX trypsin-EDTA solution and incubate at 37°C for 15 min. Cells can be dislodged from surface by tapping flask against hand. To stop trypsin activity, add 4.5 ml Y-l growth medium (total volume in flask, 5 ml), wash cells from surface of flask, and transfer cell suspension to sterile tubes. Let large clumps of cells settle for 2 min. To prepare new flask of cells, add 1 ml cell suspension and 4 ml Y-l growth medium to new 25 cm sq tissue culture flask.

Preparation of microwell plates. Add 15 ml Y-l growth medium to 5 ml cell suspension prepared above. Transfer 0.2 ml of this diluted cell suspension to each of 96 wells. Incubate plates in CO_2 incubator at 37°C. When cell monolayers are confluent (usually within 3 days), plate is ready for use.

Preparation and concentration of test filtrates. Use AKI medium to enhance CT production by *V. cholerae* O1 El Tor (15). Inoculate 15 ml AKI tubes and incubate 4 h without shaking at 35-37°C. Then transfer entire volume to 250 ml flask and incubate 16 h with shaking (200 rpm) at 35-37°C. Centrifuge culture at 900 x **g** for 30 min in refrigerated centrifuge. Discard cells. Filter-sterilize supernatant, using 0.22 µm low protein-binding membrane, before testing. If large volumes of supernatant are collected, concentrate by ultrafiltration through 10,000 M.W. exclusion membrane, such as Amicon PM10 or YM10.

Optional broth medium: Inoculate CYE broth with isolate and incubate for 18-24 h at 35-37°C. Transfer 0.1-0.2 ml of this culture into 125 ml flask containing 25 ml CYE broth. Incubate with shaking (100-200 rpm) for 24 h at 35°C.

Prepare supernatant as described above.

Assay. Remove old Y-l medium and drain microwell plate by inverting on sterile towel. Add 0.1 ml fresh medium per well. Add 0.05 ml of each filter-sterilized bacterial culture supernatant to microtiter plate wells. For CT assay, use 0.05 ml of cholera enterotoxin (5 ng/ml) for positive control. For cytotoxin assay, use 0.05 ml of *V. cholerae* 2194C culture supernatant for positive control. Use sterile CYE medium for negative control. For second negative control well, boil culture supernatant from *V. cholerae* 2194C for 5 min. Incubate overnight at 35-37°C in CO_2 incubator.

Interpretation. Examine wells at 100 or 200X magnification, using inverted stage phase microscope. Compare test wells to positive control wells. For CT assay, a positive well contains more than 10% rounded Y-l cells. For cytotoxin assay, a positive well contains 50% or more dead, lysed, and/or detached cells. El Tor strains are typically hemolytic (cytotoxic). Consequently, CT cannot be detected unless its concentration exceeds that of cytotoxin, and the sample is titered (various dilutions are used in assay).

Chinese hamster ovary (CHO) cell alternative. CHO cells may be used rather than Y-l cells. CHO cells elongate when exposed to CT.

b. Immunological detection of cholera enterotoxin (CT): microwell plate and membrane ELISAs.

Screen suspect colonies for production of these toxins with appropriate dilutions of specific antibodies against CT, using membrane ELISA, a 96-well microELISA, or reversed-passive-latex agglutination (RPLA) assay kit in 96-well format. As with all immunological tests, when suppliers or stocks of antibody or conjugate change, the new reagents must be titered to determine optimum test dilutions.

Grow isolates in AKI medium at 35-37°C, as described for the Y-l assay (CYE medium optional). Centrifuge at 900 x *g* for 20 min in refrigerated centrifuge. Decant supernatant into sterile flask and discard cells. To obtain clear supernatants, filter through low protein-binding membrane. Supernatants need not be sterilized and may be frozen at -20°C before use.

To detect low amounts of toxin in culture supernatant, concentrate toxin using method of Yamamoto et al. (35).

1. **Micro ELISA**

Coat each well of a flat-bottomed, 96-well ELISA plate with 50 µl of appropriate dilution (e.g., 1:1000) of goat anti-cholera toxin diluted in 0.1 M bicarbonate buffer, pH 9.6. Cover and place at 4°C

overnight. Wash plates 3 times with ELISA buffer. To reduce nonspecific binding, block wells with 200 µl 1% bovine serum albumin (BSA) in ELISA buffer for 30 min at 25°C. Wash 3 times with ELISA buffer.

Add 100 µl test substance to each well. Run positive controls (enterotoxin-producing bacterial strains) and negative controls (uninoculated growth medium). Cover and incubate plates for 1 h at 37°C. Wash plates 3 times with ELISA buffer.

Add 100 µl of appropriately diluted (in 1% BSA in ELISA buffer) rabbit anti-CT to each well. Cover and incubate plates at 37°C for 1 h. Wash plates 3 times with ELISA buffer. **NOTE**: Titrate rabbit anti-CT before use. A 1:500 dilution has been used successfully with some preparations.

Add 100 µl of appropriately diluted goat anti-rabbit peroxidase conjugate (e.g., 1:1000 in 1% BSA in ELISA buffer) solution to each well. Cover plates and incubate at 37°C for 1 h. Wash plates 3 times with ELISA buffer.

Add 100 µl of ABTS solution to each well. Cover and incubate plates at 37°C for 10-30 min. If necessary, reincubate plates at 37°C to obtain darker reactions.

Results. Read optical density of each well at 410 nm on spectrophotometer]ELISA (EIA) reader(. Culture supernatants yielding optical density 0.1 unit greater than background are positive for production of either CT or cytotoxin.

2. Reversed-passive-latex agglutination (RPLA) assay (Oxoid VET-RPLA TD20). This assay tests supernatants after simple centrifugation or filtration of 24 h, 35-37°C AKI or CYE medium culture. **See** kit for instruction on assay set-up following supernatant preparation.

3. Membrane ELISA

   Fill large petri dish with 50 ml TBS. Handle nitrocellulose membrane with forceps and gloves. Mark membrane with pencil for future orientation. Place membrane at angle into TBS buffer to wet thoroughly. Remove after 10 min and place on filter paper for 5 min. Displace 2 µl of each culture supernatant, including positive and negative control cultures and uninoculated medium, to tip of micropipet and touch to membrane surface. Place each sample 6-8 mm apart. To avoid increasing spot size, let membrane dry completely before applying additional 2 µl aliquots to spots. Let membrane dry completely for 5 min after application of last sample. About 75 cultures can be analyzed on a single membrane 9 cm in diameter. Immerse membrane in 50 ml TBS-3% gelatin solution for 1 h. Agitate solution intermittently or place on rotary shaker.

   Remove membrane from TBS-3% gelatin and transfer to 50 ml solution of rabbit anti-CT antiserum diluted 1:100 (or other appropriate dilution) in TBS-1% gelatin. Incubate 2-3 h at 25°C with gentle agitation. Briefly rinse membrane in 50 ml double distilled water. Wash membrane 5 min with 50 ml TBS-Tween-20. Repeat washing procedure 3 more times.

   Transfer membrane from wash solution to 50 ml goat anti-rabbit IgG-peroxidase conjugate diluted 1:3000 (or other appropriate dilution) with TBS-1% gelatin. Incubate at 25°C for 2 h with gentle agitation. Remove and wash membrane as above.

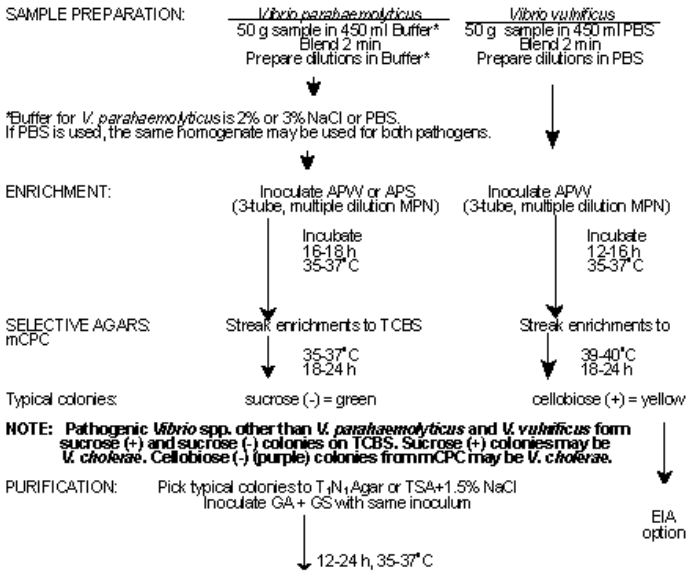
   Prepare R57 peroxidase color development solution immediately before use. Transfer membrane from wash solution into color development solution. CT-positive spots will appear as purple dots within 5 min. Avoid prolonged color development beyond 15-30 min. If precipitate forms in color development solution, prepare fresh solution and use immediately. Immerse membrane in distilled water to stop color development.

   Interpretation of data. Spots containing 1 ng or greater concentration of CT become visible as purple dots within 2-5 min. Negative control cultures may give a very faint spot if color development proceeds beyond 5 min. High titer sera should be diluted (1:200 or 1:400) to minimize background color changes that may be observed with CT-negative control cultures.

8. Report. The following should appear in the final report: identification of isolate based on biochemical test results; serological results (Ol, Inaba, Ogawa, Hikojima, Non-O1, O139); biotype of *V. cholerae* Ol (El Tor or classical); and toxigenicity results (toxigenic or nontoxigenic, and cytotoxic or noncytotoxic if tissue culture was used). Notify Dr. Joseph Madden, FDA, telephone (202) 205-4197 and Dr. Mahendra Kothary, FDA, telephone (202) 205-4454, of *V. cholerae* O1 and O139 isolates.

D. Procedure for enrichment, isolation, and enumeration of *V. parahaemolyticus*, *V. vulnificus*, and other halophilic *Vibrio* spp. (**see** Fig. 2)

Figure 2. SCHEMATIC DIAGRAM FOR *Vibrio parahaemolyticus* and *Vibrio vulnificus* METHODS



NOTE: Halophilic *Vibrio* spp. do not grow on GA. They grow and are gelatinase (-) on GS. Sucrose (+) strains that grow on GA may be *V. cholerae*.

PRELIMINARY BIOCHEMICAL TESTS:

(For other tests, see Chapter and Tables 2 and 3)

TEST	TYPICAL REACTIONS OF *V. parahaemolyticus*	*V. vulnificus*
Oxidase	+	+
Motility	motile	motile
AGS	K/A–	K/A–
TSI	K/A–	K(A rare)/A–
O/129 (10 µg)	Resistant	Sensitive
O/129 (150 µg)	Sensitive	Sensitive
ONPG	-	+
Gram stain	Gram (-) rods	Gram (-) rods

SEROLOGY: O and K, optional EIA

PATHOGENICITY TESTS: Kanagawa phenomenon or gene probe (may correlate with pathogenicity)

1. **Stool sample**. The importance of obtaining blood, tools, rectal swabs, or vomitus specimens in outbreaks cannot be overemphasized. Obtain specimens at the earliest opportunity because the carrier state is short-lived. Transport specimens in Cary-Blair medium, or inoculate immediately into APW enrichment broth, or streak onto TCBS and mCPC agar plates, depending on transit time. Incubate TCBS agar at 35°C and mCPC agar at 39-40°C for 18-24 h.

   If transit time will be longer than 8 h, place stool specimen in Cary-Blair transport medium. In the laboratory, streak loopful onto TCBS and mCPC agars.

   If transit time is 8 h or less, place stool specimen in APW. After 12-16 h incubation at 35-37°C, streak enrichment broth on both TCBS and mCPC agars. Incubation times longer than 16 h result in progressively lowered bacterial viability.

   Sample rectal swab specimen (preferably with polyester fiber-tipped swab), contained in 7 ml Cary-Blair transport medium or APW, by streaking onto both TCBS and mCPC agars.

   Examine plates and proceed to biochemical identification.

2. **Food sample**

   a. Sample composition

      Fish: surface tissues, gut, or gills

      Shellfish: entire interior contents of animal; pool 10-12 animals, homogenize, and remove 50 g from composite for test sample

      Crustaceans: entire animal, if possible, or central portion of animal, including gills and gut

b. Test sample preparation

V. parahaemolyticus. Aseptically prepare 1:10 dilution of 50 g seafood in 2 or 3% NaCl dilution water or PBS, pH 7.2-7.5, in sterile, tared blender jar. Blend 2 min at high speed. For example, blend 50 g seafood with 450 ml of 2 or 3% NaCl dilution water or PBS, OR blend 50 g seafood with 50 ml of dilution liquid (1:2); then make 1:5 dilution (20 g of 1:2 dilution in 80 ml dilution liquid) of the homogenate for a 1:10 total dilution. Prepare tenfold dilutions in 2 or 3% NaCl dilution water or PBS, pH 7.2-7.5. Inoculate 3-tube, multiple dilution, alkaline peptone water (APW) or alkaline peptone salt (APS) broth MPN series (i.e., add 1 ml portions of each 1:10 and higher dilution to sets of 3 tubes containing 10 ml APW or APS). Incubate tubes 16-18 h at 35-37°C. **NOTE**: Inoculations of MPN tubes must be completed within 15-20 min of dilution preparation.

V. vulnificus. Prepare APW original and 10-fold dilutions in PBS, pH 7.2-7.5. Inoculate MPN series as described for *V. parahaemolyticus* and incubate at 35-37°C for 12-16 h.

A rapid, specific enumeration technique for *V. vulnificus*, using a nonradioactive probe (Wright et al., 1993, Appl. Environ. Microbiol. 59:541-546) is currently being evaluated.

NOTE: If enrichment is for both *V. parahaemolyticus* and *V. vulnificus*, use PBS, pH 7.2-7.5 and APW enrichment broth.

c. After incubation do not shake tubes. For isolation of *V. parahaemolyticus*, for all dilutions containing a turbid tube, and at least one dilution higher, streak TCBS agar with 1 loopful from top 1 cm of each enrichment broth. For isolation of *V. vulnificus* streak on mCPC (30) agar. Also streak APW broth cultures of verified *V. parahaemolyticus* and *V. vulnificus* strains on TCBS and mCPC agar plates as controls for subsequent tests. Incubate TCBS at 35-37°C and mCPC at 39-40°C for 18-24 h. Optional: Use blood agar or mannitol-maltose agar, incubated at 35-37°C for 18-24 h, to detect *V. hollisae*.

d. Examine TCBS and mCPC agars for typical *V. parahaemolyticus* and *V. vulnificus* colonies. Pick 3 or more typical or suspicious colonies from each medium, inoculate sectors of GA and GS plates and streak T_1N_2 agar or TSA + 1.5% NaCl (final 2% NaCl concentration) for isolation. Incubate at 35-37°C for 18-24 h. AGS or other screening media may be inoculated at this time.

TCBS agar. On TCBS agar, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, and *V. harveyi* are round, 2-3 mm diameter, green or blue-green colonies. *V. alginolyticus*, *V. fluvialis*, *V. cholerae*, *V. metschnikovii*, and some *V. vulnificus* colonies are larger and yellow (acid from sucrose fermentation).

mCPC agar. On mCPC agar, *V. vulnificus* colonies are flat and yellow (acid from cellobiose fermentation) with opaque centers and translucent peripheries, about 2 mm in diameter. This is a presumptive identification of *V. vulnificus*. Non-cellobiose fermenters, such as *V. cholerae* El Tor, appear as purple or green, raised colonies. *V. parahaemolyticus* rarely grows on mCPC. Other species of *Vibrio* do not grow readily on mCPC agar. Pseudomonads produce purple or green colonies and are frequently observed at low dilutions of sample.

For rapid confirmed identification of *V. vulnificus*, transfer colonies from mCPC agar to APW for the monoclonal antibody-EIA (30) (**see** F.2, Serology, below). Use isolates confirmed by EIA to compute MPN of *V. vulnificus* in sample.

A specific gene probe method, available for detection of cytotoxin-hemolysin gene of *V. vulnificus*, may be used as an additional presumptive identification procedure (**see** Chapter 24).

Blood agar. Flood 18-24 h plate with oxidase reagent and pick oxidase-positive colonies (13). Because *V. hollisae* does not grow on TCBS or mCPC agars, this nonselective method may isolate the organism. However, overgrowth by other bacteria may be a problem.

Mannitol-maltose agar. On this nonselective medium, *V. hollisae* colonies are round, shiny, and purple (non-mannitol, non-maltose fermenting), whereas other *Vibrio* spp. are yellow (acid from mannitol and/or maltose fermentation) (20). Overgrowth by other bacteria may be a problem.

Enumeration. After suspect colonies are identified biochemically or serologically with EIA, apply MPN tables (Appendix 2) for final enumeration of species.

3. Procedure for biochemical identification

a. **Read GS and GA plates**. Halophilic *Vibrio* spp. will grow only on GS plates. Most *Vibrio* spp. are gelatinase-positive and will form an opaque halo around growth.

b. **Oxidase test**. Use growth from GS plate (or other medium with no fermentable carbohydrate) for oxidase test. Place 2 or 3 drops of oxidase test reagent on bacterial growth, or transfer small amount of growth with sterile toothpick or platinum loop to filter paper moistened with oxidase reagent. (Do not use nickel chromium loops.) Dark blue color should develop rapidly (within 2 min) for positive reaction. *V. metschnikovii* is the only oxidase-negative, pathogenic, halophilic *Vibrio* spp.

c. From isolated colonies, inoculate motility test medium, AGS, TSI, TSB, TSA slant, and TSA plate for O/129 sensitivity test (all with final 2% NaCl concentration) and incubate 18-24 h at 35-37°C. Use various tests in Tables 2 and 3 for identification.

NOTE: Before proceeding, make sure culture does not grow on GA, is gelatinase-positive, and is pure. *Vibrio* spp. cultures often have 2 colony morphologies, which may or may not be stable.

Motility test medium. Stab inoculum in center and to 2/3 the depth of motility test medium. Diffuse circular bacterial growth from line of stab is a positive test. *V. vulnificus*, *V. parahaemolyticus*, and related *Vibrio* spp. are motile. After 24 h, tightly cap tube and store at 20-25°C to preserve culture.

Arginine-glucose slant. Streak slant and stab butt of AGS, modified from Kaper et al. (16). *Vibrio* spp. do not produce H_2S or gas. Typical reactions of *V. parahaemolyticus* and *V. vulnificus* are alkaline (purple) slant and acidic butt (yellow) (Tables 2 and 3).

Triple sugar ron. Streak slant and stab butt of TSI agar. *Vibrio* spp. produce acidic butt (yellow) and do not produce gas or H_2S. *V. parahaemolyticus* produces alkaline slant (red). *V. vulnificus* usually produces an alkaline slant (red) (Table 2). Use this or other medium containing lactose as source of inoculum for ONPG test.

Inoculate tubes of TSA and TSB or motility test medium as source of inoculum for further testing.

O/129 Vibriostat sensitivity. Use the disk diffusion method described above for polymyxin B sensitivity of *V. cholerae* O1 or place disks on densely streaked area of an isolation plate (TSA with 2% NaCl final concentration). Use disks containing 10 and 150 µg of vibriostat O/129. *Vibrio* spp. are sensitive to 150 µg of O/129, but some are resistant to 10 µg of O/129. **See** Table 3 for differentiation based on sensitivity to 10 µg of O/129. Disks are commercially available or can be prepared in the lab. Alternatively, use TSA agar containing 10 or 150 µg of O/129 per ml.

d. Continue identification tests after finding typical reactions in media inoculated previously (Tables 2 and 3). Compute MPN of *V. parahaemolyticus* (**see** Appendix 2), based on number of tubes containing *V. parahaemolyticus*.

ONPG test. Perform ONPG test using portion of culture from TSI or other medium containing lactose. Use conventional tube test (preferred) in fume hood, or commercially available disks. Strip tests for ONPG are sometimes unreliable for *Vibrio* spp. *V.vulnificus* is ONPG-positive; *V. parahaemolyticus* is ONPG-negative.

Rapid test strips. Use multiwell (e.g., API 20E) strips as alternative to conventional tube format for biochemical tests. However, some *Vibrio* spp. will not grow in commercial test strip media when physiological saline (0.85% NaCl) is used as diluent. Use 2% NaCl as diluent, since most halophilic *Vibrio* spp. require higher concentration of NaCl (17). If commercial test strips do not allow identification, continue with conventional tests.

Hugh-Leifson glucose broth or OF glucose medium, semisolid.
Inoculate 2 tubes of medium with growth from TSA. Overlay medium in 1 tube with sterile mineral oil to a depth of 1-2 cm, and incubate 2 days at 35 ± 2°C. *Vibrio* spp. ferment glucose and produce acid oxidatively. Acid causes dye to change from purple to yellow in Hugh-Leifson glucose broth and from green to yellow in OF glucose medium.

Arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase. Inoculate 1 tube of each of the 3 media containing amino acid and 1 tube lacking amino acid. (The arginine reaction can also be read from the AGS tube: acid butt (yellow) from glucose fermentation means isolate is negative for arginine dihydrolase.) Overlay each tube with sterile mineral oil 1-2 cm thick, and incubate 4 days at 35-37°C. Examine tubes every day. Alkaline pH resulting from decarboxylation of amino acids turns medium purple (positive). Yellow color results from acid production from glucose fermentation (negative). Control tubes containing no amino acid should be yellow. Purple color medium in control tubes indicates no growth. Most *V. parahaemolyticus* and *V. vulnificus* strains are arginine dihydrolase-negative, lysine decarboxylase-positive, and ornithine decarboxylase-positive. Some *V. vulnificus* and *V. parahaemolyticus* are ornithine decarboxylase-negative. Rare strains of *V. vulnificus* are lysine decarboxylase-negative.

Salt tolerance. From TSB culture, inoculate 1 tube each of 1% tryptone broth containing 0, 1, 3, 6, 8, or 10% NaCl (T_1N_0, T_1N_1, T_1N_3, T_1N_6, T_1N_8, or T_1N_{10}), and incubate 18-24 h at 35-37°C. Consider only profuse growth as positive. Halophilic *Vibrio* spp. do not grow in broth containing 0% NaCl, but all *Vibrio* spp. grow in broth containing 3% NaCl. Various species have different salt tolerances that can be used for identification (Table 3).

Growth at 42°C. Inoculate prewarmed tube of TSB containing 2% NaCl with small loopful of 24 h TSB-2% NaCl culture. Incubate in 42°C water bath for 24 h. Consider only profuse growth as positive. *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus* grow at 42°C.

Voges-Proskauer (VP) test. Inoculate MR-VP broth containing NaCl with growth from TSA slant and incubate 2 days at 35-37°C. Perform VP test. *V. parahaemolyticus*, *V. vulnificus*, and *V. fluvialis* are VP-negative.

Carbohydrate fermentation. From growth on TSA slant, inoculate 1 tube each of bromcresol purple broth or OF medium containing NaCl, semisolid, containing sucrose, lactose, D-mannitol, mannose, arabinose, or cellobiose. Overlay medium with sterile mineral oil to depth of 1-2 cm and incubate at 35-37°C for 4-5 days. Acidic fermentation turns medium yellow. Check tubes every 24 h and compare reactions to those in Table 3. Occasional strains of *V. vulnificus* are mannitol-negative.

Urea hydrolysis. Test presumptive *V. parahaemolyticus* for urea hydrolysis by inoculating Christensen's urea agar tubes or plates and incubating at 35-37°C for 18 h. *V. parahaemolyticus* strains vary in ability to hydrolyze urea. Urea hydrolysis may be correlated with certain somatic (O-antigen) groups.

NOTE: Urease-positive strains give API codes not found in the ID book. Call API for confirmation of strain.

Culture preservation. Inoculate semisolid, long-term preservation medium or motility test medium by stabbing deeply into agar. Incubate 24 h at 35-37°C. Tighten caps after 24 h to prevent dehydration. Alternatively, add layer of sterile mineral oil to 24 h cultures in motility test medium. **Store cultures at room temperature after initial growth**. **DO NOT REFRIGERATE**. For long-term preservation, place 1 ml of 6-12 h TSB-2% NaCl culture and 0.1 ml sterile glycerol into sterile cryotubes. Freeze immediately at -70°C or in liquid nitrogen.

e. **Kanagawa phenomenon**. The Kanagawa reaction demonstrates the presence of a specific thermostable direct hemolysin (TDH) on Wagatsuma agar. A positive reaction correlates closely with pathogenicity of *V. parahaemolyticus* isolates. Strains recovered from seafood are usually Kanagawa-negative.

Some clinical isolates of *V. parahaemolyticus* produce related hemolysins but not TDH. Two other hemolysins, having sequence homology with TDH but exhibiting no hemolysis on Wagatsuma agar, were recently identified and purified (14).

Fresh human or rabbit red blood cells (within 24 h of draw) are necessary for preparation of Wagatsuma agar.

Spot droplet from 18 h TSB-3% NaCl culture on duplicate plates of well-dried Wagatsuma agar. Spot several cultures including verified positive and negative controls in circular pattern on plate. Incubate at 35-37°C and observe results in 24 h.

A positive test is zone of **beta**-hemolysis, i.e., sharply defined zone of transparent clearing of red blood cells around colony, without multiple concentric rings or greening.

Measure zone of hemolysis from edge of colony to outer edge of zone. Isolates that produce clear zone of hemolysis 3 mm or larger are considered Kanagawa phenomenon-positive and are presumed to be pathogenic. Isolates that produce clear zones of hemolysis of less than 3 mm may be weakly pathogenic and should be tested in rabbit ileal loop assay (32). **No observation of plate beyond 24 h is valid**.

A gene probe method for detecting TDH of *V. parahaemolyticus* is available (**See** Chapter 24). *V. hollisae* is positive for TDH by gene probe, but its hemolysin cannot be detected on Wagatsuma agar. Request gene probe sequence tdh-3 from servicing laboratory.

Table 5. Antigenic scheme of *V. parahaemolyticus*[a]

O Group	K Antigen
1	1, 25, 26, 32, 38, 41, 56, 58, 64, 69
2	3, 28
3	4, 5, 6, 7, 29, 30, 31, 33, 37, 43, 45, 48, 54, 57, 58, 59, 65
4	4, 8, 9, 10, 11, 12, 13, 34, 42, 49, 53, 55, 63, 67
5	15, 17, 30, 47, 60, 61, 68
6	18, 46
7	19
8	20, 21, 22, 39, 70
9	23, 44
10	19, 24, 52, 66, 71
11	36, 40, 50, 51, 61

[a] From R.M. Twedt (31), personal communication from R. Sakazaki in 1986.

E. Characteristics for biochemical identification of *V. parahaemolyticus* and *V. vulnificus*

The following characteristics are presumptive of *V. parahaemolyticus* or *V. vulnificus*:

- Morphology: Gram-negative asporogenous rod
- TSI appearance: *V. parahaemolyticus*, alkaline slant/acid butt, gas production-negative, H_2S-negative; *V. vulnificus*, alkaline slant (rarely acidic)/acid butt, gas production-negative, H_2S-negative
- Hugh-Leifson test: Glucose oxidation and fermentation-positive
- Cytochrome oxidase: Positive
- Arginine dihydrolase test: Negative
- Lysine decarboxylase test: Positive (rare *V. vulnificus* are lysine decarboxylase-negative)
- Voges-Proskauer test: Negative
- Growth at 42°C: Positive
- Halophilism test: *V. parahaemolyticus*: 0% NaCl-negative; 3, 6, and 8% NaCl-positive; 10% NaCl-negative or poor. *V. vulnificus*: 0% NaCl-negative; 3, 6% NaCl-positive; 8% NaCl-negative
- Sucrose fermentation: Negative (rare *V. vulnificus* are positive)
- ONPG test: *V. parahaemolyticus*, negative; *V. vulnificus*, positive
- Arabinose fermentation: *V. parahaemolyticus*, usually positive (variable); *V. vulnificus*, negative
- Sensitivity to O/129: *V. parahaemolyticus*: sensitive to 150 µg, resistant to 10 µg; *V. vulnificus*: sensitive to 10 and 150 µg.

F. Serology

1. **Serological identification of *V. parahaemolyticus***. Determination of somatic (O) and capsular (K) serotypes of *V. parahaemolyticus* is not required for identification. Serotyping antisera are expensive and few, if any, FDA labs have the complete set. Contact Charles Kaysner (206) 483-8788 for possible referral of isolates. For those who wish to determine the O and K antigens (Table 5), the following protocol is offered.

   a. Inoculate 2 slants of TSA-2% NaCl; incubate at 35-37°C for 18-24 h.

   b. Somatic (O) antigen

      Preparation. Wash growth from one TSA-2% NaCl slant with solution containing 2% NaCl and 5% glycerol; transfer to autoclavable centrifuge tube. Autoclave suspension at 121°C for 1 h. Centrifuge suspension at 4000 rpm for 15 min. Resuspend the packed cells in 2% NaCl. A heavy suspension is best for this slide agglutination test.

      Determination. With wax pencil, divide microscope slide into 12 equal compartments. Place small drop of heavy suspension into each compartment. Add 1 drop of the 11 O-group antisera to separate compartments. Add 1 drop of 2% NaCl to 12th compartment (autoagglutination control). Tilt slide gently to mix all components, and rock slide back and forth for 1 min. Positive agglutination may be read immediately.

      If no agglutination occurs with any of the 11 O antisera, autoclave the suspension at 121°C again for 1 h and retest. If agglutination is still negative, the O antigens of the culture are unknown.

   c. **Capsular (K) antigen**

      Preparation. Capsular (K) antigen. Wash growth from one TSA-2% NaCl slant with 2% NaCl solution to make a smooth heavy suspension of cells.

      Determination. Test first with pooled K antisera (I-IX), and then with each of the monovalent K antisera within the pool showing agglutination. (Each pool consists of 8-10 flagellar agglutinins.)

      On slide, mark off appropriate number of compartments plus control compartment. Place small drop of heavy cell suspension and add 1 drop of appropriate K antiserum to individual compartments. Add 1 drop of 2% NaCl to autoagglutination control. Tilt slide gently to mix components, and rock slide back and forth for 1 min. Positive agglutination may be read immediately.

2. ***V. vulnificus* EIA (30).** Use EIA specific for intracellular antigen to confirm identity of *V. vulnificus* isolates directly from mCPC agar (yellow translucent colonies with opaque centers).

   Prepare log phase cultures. Transfer 2 typical *V. vulnificus* colonies from each inoculated plate and confirmed culture of *V. vulnificus*, using sterile wooden sticks, toothpicks, or inoculating loop, to individual wells of 96-well plate (tissue culture cluster plate) containing 100 µl APW per well. Incubate 3-4 h, or until turbid, at 35-37°C.

   Coat enzyme immunoassay (EIA) plates. After incubating microtiter plates, transfer 25 µl from each cluster plate well to one well of a 96-well EIA plate. Add 25 µl EIA coating solution (0.02% Triton X-100) to each well. Place EIA plates in dry 35°C incubator overnight to evaporate samples in wells.

   Optional: To store isolates after transfer to EIA plates, add equal volume sterile TSB supplemented with 1% NaCl and 24% glycerol to each well of tissue culture plate. Isolates can be stored indefinitely at -70°C.

   Block binding sites. Remove dried EIA plates from incubator. To reduce nonspecific binding of reagents, add 200 µl of 1% BSA in PBS to each well. Incubate at room temperature for 1 h.

   Discard BSA. Remove BSA solution by firmly slapping plates onto countertop covered with absorbent towels.

   Add monoclonal antibody. Prepare diluted (e.g., 1:4) monoclonal antibody specific for *V. vulnificus* in PBS. Add 50 µl to test wells. Control wells receive antibody with specificity other than *V. vulnificus*, tissue culture media, or PBS. Incubate at room temperature for 1 h. Wash plate 3 times with wash solution.

   Add conjugate. Dilute peroxidase-conjugated goat anti-mouse IgG with PBS. Add 50 µl to each well and incubate in dark at room temperature for 1 h. Wash 5 times.

   Add substrate. Add 100 µl freshly prepared ABTS substrate solution to each well. Incubate about 10 min at room temperature, or until maximum color develops (usually less than 30 min). Compare negative controls to respective test wells for positive reactions. A well is usually considered positive if its optical density is 0.200 above that of negative control. An EIA plate reader is normally not required to differentiate reactions, but if used, read optical density at 410 nm.

G. Gene probes

Gene probes (oligonucleotides) for *V. cholerae* enterotoxin (CTX All), *V. parahaemolyticus* thermostable direct hemolysin (TDH-3), and *V. vulnificus* cytotoxin-hemolysin are available from Dr. Joseph Madden, FDA, 200 C St., SW, Washington, DC 20204, or from Fannie Harrell, HFR-MW460, MCI, MIN-DO, 240 Hennepin Ave., Minneapolis, MN 55401.

These probes are for genes associated with pathogenicity or species specificity. **See** Chapter 24 for gene probe methods.

H. Fatty acid analysis

Vibrio spp. may be identified by gas chromatographic analysis of cellular fatty acids. Warren Landry, FDA, Dallas District Office (214) 655-5308, has developed a computer library for identification of many bacterial species, including *Vibrio* spp., using the Hewlett-Packard Microbial Identification System. The equipment is not available in all FDA laboratories, but unusual *Vibrio* spp. isolates may be sent to the Dallas laboratory for

study and confirmation.

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Bad Bug Book:
Vibrio cholerae O1
Vibrio cholerae non-O1
Vibrio parahaemolyticus and other *vibrios*
Vibrio vulnificus

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

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Chapter 28

Detection of Enterotoxigenic *Vibrio cholerae* in Foods by the Polymerase Chain Reaction

Authors

(Return to Table of Contents)

Recent epidemics of cholera in various parts of the world have emphasized the urgent need for rapid and reliable detection methods for *Vibrio cholerae*, especially in food and water. Classical microbiological methods are sensitive and specific; however, they require several days to complete (**see** Chapter 9) and may result in considerable loss of perishable foods. Since cholera toxin production (encoded by the *ctxAB* genes) is the major factor in the pathogenesis of cholera, a polymerase chain reaction (PCR) method that selectively amplifies a DNA fragment within the *ctxAB* operon of *V. cholerae* has been developed and applied to various foods (19).

The PCR was first described by Mullis et al. in 1985, and since then has revolutionized most of the biological sciences (25). In this technique, double-stranded target DNA is denatured to provide single-stranded templates to which specific oligonucleotide primers are hybridized, followed by primer extension with a thermostable DNA polymerase (26). Primer pairs complementary to opposite strands of a DNA region are chosen so that 5' to 3' directional

extensions are toward one another. Thus repetitive denaturation, annealing, and primer extension cycles exponentially amplify a unique DNA fragment bordered by the primers. The process is extremely rapid (as little as 30 min for 25 cycles with certain thermocyclers) and sensitive (amplification of gene sequences from a single cell is possible). Further, the process can be designed to be specific for genus, species, or allele.

Use of the PCR as a detection method for microbial pathogens in foods has been documented in approximately two dozen PCR-based, detection procedures published by early 1994 (Table 1). PCR-based methods have been developed to detect a wide variety of foodborne pathogens, including *Listeria monocytogenes*, enterotoxigenic *Escherichia coli* (**see** Chapter 4), *V. vulnificus*, *V. cholerae*, *Shigella flexneri*, *Yersinia enterocolitica*, various *Salmonella* and *Campylobacter* species, and the Hepatitis A (15) (**see** Chapter 26) and Norwalk viruses.

Table 1. PCR methods developed for the detection of pathogenic microbes in foods

Organism	Target gene/gene product	Reference
Campylobacter spp.	16S rRNA	14
C. jejuni	*flaA-flaB* intergenic sequences	32
C. coli		
Escherichia coli	*malB*, LT1, ST1	5
	LT	34
	SLTI, SLTII	13
	VT1, VT2 and VTE	23
	plasmid invasion gene (*ial*)	1,20
Listeria monocytogenes	listeriolysin O (*hly*)	3,4,9,10,12,21,22,24,29,34
	16S rRNA	30
	Listeria cell surface protein	31
Salmonella spp.	*oriC*, chromosomal origin of replication	11
Shigella flexneri	plasmid invasion gene	20
Vibrio cholerae	*ctxAB*	19
V. vulnificus	cytotoxin-hemolysin	16
Yersinia enterocolitica	virulence plasmid *virF* gene	35
	yadA	17
Hepatitis A virus[(a)]	polymerase gene	2,15
Norwalk virus[(a)]	polymerase gene	2

[a] Reverse transcription PCR (RT-PCR).

Many of these PCR methods have relied on extraction of DNA from contaminated foods, an additional step which adds several hours to the procedure and often requires modification of each diverse food matrix tested. An advantage of PCR is that the amplification reaction often proceeds well with crude lysates of cells, in some cases requiring only brief boiling of a bacterial suspension.

Although the PCR method, in principle, can detect a single bacterial cell with extended cycle regimens (50-60), the detection limit of direct PCR is effectively confined to about 10^4 bacteria per gram of food. This limitation is due to reaction volume constraints (25-100 µl), the increased propensity to amplify nonspecific products at high cycle numbers, and the inhibitory effect of many food components on *Taq* polymerase. Thus, the coupling of enrichment procedures with the PCR has effectively served two purposes: 1) It increases the sensitivity of detection to as few as 0.1 organism per gram of food; 2) it demonstrates by comparison of pre- and post-enrichment inocula that the food contains **viable** organisms. The added sensitivity and information regarding cell viability warrants the additional 4-24 hours (depending on the growth characteristics of the organism) required for such a procedure, and overcomes an early criticism that PCR would give false-positive results because it amplifies any DNA, including that of dead or nonviable organisms.

Results of amplification reactions are usually obtained by the resolution of products based on size via agarose gel electrophoresis and visualization by UV-induced fluorescence after staining with ethidium bromide. The complete PCR amplification and agarose gel analysis generally requires only about 2-4 hours after enrichment. In terms of sensitivity, specificity, and analysis time, selective enrichment followed by PCR is clearly a powerful, rapid, and robust methodology for detecting foodborne bacterial pathogens.

V. cholerae of the Inaba and Ogawa serotypes which lack the cholera toxin genes have been isolated; however, such strains are generally nonpathogenic. Since the presence of the cholera toxin operon is a prerequisite for pathogenicity, various PCR methods for the detection of pathogenic *V. cholerae* have all used the *ctxAB* genes as a target for amplification (8,18,28); these and the PCR method described here, will not detect nontoxigenic *V. cholerae*. As a practical matter, this PCR detection method allows one to define food samples as negative for the presence of toxigenic *V. cholerae* much more quickly than by following the complete microbiological identification scheme. However, it is recommended that alkaline peptone water (APW) enrichment broths used for PCR analysis also be plated onto selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar (**see** Chapter 9) for isolation and direct confirmation of the

presence of *V. cholerae* in samples that give positive PCR results.

A. **Equipment and materials**

1. For APW enrichment of *V. cholerae* (**see** Chapter 9).
2. Programmable automatic thermocyler
3. Horizontal gel electrophoresis apparatus
4. Electrophoresis constant-voltage power supply
5. Heating plate
6. Microcentrifuge tubes, 1.5 and 0.6 ml
7. Variable digital micropipettors (e.g., 0.5-20 µl, 20-200 µl)
8. Aerosol-resistant pipet tips
9. Microcentrifuge
10. UV transilluminator
11. Polaroid camera
12. Polaroid film

B. **Media and reagents**

1. Alkaline peptone water (APW) (**see** Chapter 9)
2. Cholera toxin PCR primers, 10 pmol/µl stock solutions (5'-TGAAATAAAGCAGTCAGGTG-3', 5'-GGTATTCTGCACACAAATCAG-3'; **see** ref. 19)
3. *Taq* DNA polymerase (native available from various vendors) or Amplitaq® (Perkin-Elmer)
4. 2'-Deoxynucleoside-5'-triphosphates (dATP, dCTP, dGTP, dTTP); stock solution 1.25 mM of each dNTP
5. 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$)
6. Light mineral oil
7. Sterile deionized water
8. 10X TBE (0.9 M Tris-borate, 0.02 M EDTA, pH 8.3)
9. Agarose (nucleic acid electrophoresis grade)
10. Ethidium bromide solution, 10 mg/ml
11. 6X sample loading buffer (**see** ref. 27)
12. DNA molecular weight markers (e.g., 123 bp ladder, Bethesda Research Laboratories, Gaithersburg, MD)

C. **Procedure for amplification of cholera toxin gene sequences from *V. cholerae* using APW enrichment broth**

Food sample preparation and APW enrichment (**see** Chapter 9).

APW enrichment lysate preparation. Prepare APW washes or blends (**see** Chapter 9). Sample and freeze **immediately** (about 1 ml). After enrichment (6-24 h), prepare crude APW lysates for PCR by boiling 1 ml samples in 1.5 ml microcentrifuge tubes for approximately 5 min. Lysates may be used for PCR immediately or stored in a -20°C freezer until use. **NOTE:** Due to the enormous amplification possible with the PCR, minute levels of contamination can result in false positives. It is recommended that sample preparation, PCR reaction set-up, and PCR product analysis be physically separated from one another to minimize contamination. For an excellent discussion of considerations in setting up a PCR laboratory, see *PCR Methods and Applications* **3**(2):S1-S14, (1993) A Manual Supplement, Section 1: Establishing PCR in the Laboratory (6,7). Minimally, use of aseptic technique in handling all PCR reagents and solutions is absolutely necessary. Use aerosol-resistant pipet tips for preparing samples and reagents for PCR reactions, and, if possible, a separate set of pipettors for analysis of PCR reaction products.

PCR reaction preparation. To minimize cross contamination of PCR reagents, it is recommended that master mix solutions be prepared, aliquoted, and stored frozen. Master mixes contain all necessary reagents except *Taq* polymerase and the lysates being amplified. The final reaction contains 10 mM Tris-HCl, pH 8.3; 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP, and dTTP; 2 to 5% (v/v) APW lysate; 0.5 µM of each primer and 2.5 U *Taq* polymerase per 100 µl; reaction volumes of 25-100 µl may be used. Add *Taq* polymerase to the master mix and add APW lysate upon distribution to 0.6 ml microcentrifuge tube reaction vessels. Cover with approximately 50-70 µl of mineral oil.

Temperature cycling. While there is some variability in the heating and cooling dynamics of thermocylers from different manufacturers, use of the following temperature cycling regimen should yield efficient amplification of the *ctx* gene fragment: Denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and primer extension 72°C for 1 min, repeated for no more than 35 cycles. Increasing the cycle number beyond 35 cycles often leads to the formation of nonspecific amplification products, including primer dimers.

Agarose gel analysis of PCR products. Mix 10-20 µl portions of PCR reactions with 6X gel loading buffer (choose one of four common buffers from *Molecular Cloning: A Laboratory Manual* by Sambrook et al. (27) and load into sample wells of 1.5-1.8% agarose gel submerged in 1X TBE containing 1 µg/ml ethidium bromide. After appropriate migration with a constant voltage of 5-10 V/cm, illuminate the agarose gel with a UV transilluminator and visualize bands relative to molecular weight marker migration. The primers listed above give rise to a 777 bp fragment (19). Take Polaroid photographs of gels to document results. Further details regarding gel electrophoresis analyses may be found in the above-mentioned *Molecular Cloning Laboratory Manual*

(27).

Proper controls. The need for a number of control reactions to ensure accurate interpretation of PCR results cannot be overemphasized. Minimally, for PCR analysis of food types previously optimized for this method (e.g., vegetable washes, oyster, crab and shrimp blends; **see** ref. 19), include a master mix contamination control containing no lysate and a toxigenic *V. cholerae* APW positive control in **every** analysis. For every **new** food blend to be analyzed by this PCR method, determine the potential inhibitory effects of that food. Minimally, this entails spiking 1 ml of a 1:10 and 1:100 APW food blend **post-enrichment** with about 5×10^6 organisms per ml (or an equivalent amount of positive control lysate). A direct comparison of these spiked samples with the APW (no food) lysate containing identical numbers of ctx^+ cells, allows one to determine if any inhibition occurs at either of the two food concentrations and prevents the occurrence of false negatives. It is unlikely that food washes (e.g., fruits and vegetables) will inhibit the PCR reaction unless the fruits are bruised and washing releases excessive acidity to the APW wash.

For additional information on this PCR method, contact Walter H. Koch at FDA, CFSAN, Division of Molecular Biological Research and Evaluation, 200 C St., S.W., Washington, DC 20204. Telephone: (202) 205-4172 or (202) 205-5060; FAX: 205-4183; E-Mail: WHK@VAX8.CFSAN.FDA.GOV.

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Top

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Bacteriological Analytical Manual

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Chapter 10

Listeria monocytogenes

Author

(Return to Table of Contents)

Bergey's Manual of Systematic Bacteriology, 9th edition (15), lists eight species in the genus *Listeria*: *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. ivanovii, L. grayi, L. murrayi, L. denitrificans*. Of these, the species *L. grayi* and *L. murrayi* are considered here as subspecies of a single redefined species, *L. grayi* (9,14); *L. denitrificans* is now in the genus *Jonesia* (8). Thus, six species need to be differentiated (Table 1). Differentiation of the newly recognized subspecies of *L. ivanovii* is not needed in *Listeria* analysis (4). *Brocothrix,* which is closely related phylogenetically to *Listeria,* is distinguished by its inability to grow at 35°C and its lack of motility. Distinguishing features of the Gram-positive asporogenic rods *Erysipelothrix* (3) and *Kurthia,* which occur rarely in *Listeria* analysis, can be found in Bergey's manual (15).

Both *L. ivanovii* and *L. monocytogenes* are pathogenic for mice, but only *L. monocytogenes* is consistently associated with human illness. In the methodology for isolating *L. monocytogenes,* suspected foods are sampled and the samples are composited, if required, according to compliance instructions. Analytical portions (25 g) are enriched for *Listeria* species in selective enrichment broth at 30°C for 48 h. The enrichment culture is streaked at 24 and 48 h on two different, but complementary, differential selective agars to isolate *Listeria* species. Isolates are purified on nonselective agar and speciated directly by a battery of conventional tests or by such tests in kit form, or are preliminarily identified as members of the genus *Listeria* by genus-

specific rapid test ELISA or DNA-probe kits. Alternatively, isolate cultures are rapidly speciated, within 1 h, as *L. monocytogenes* (or not *L. monocytogenes*) by a chemiluminescent-labeled DNA probe kit or by equivalent *L. monocytogenes*-specific DNA probes or probe kits. Serotyping, virulence testing, and enumeration of *Listeria* by direct plating on selective agar or by MPN enrichment and selection are optional methods.

In this version of the *Listeria* methodology several important modifications have been made, but note that further major revisions are anticipated:

The AOAC Official First Action enrichment formula for milk and dairy products (2) has been modified by increasing its buffering strength. Preliminary collaborative and other studies showed that the degree of buffering is not crucial in a 2-day (as opposed to a 7-day) enrichment when conventional isolation and identification techniques are used. Nevertheless, rapid DNA probe methods require more stringent control of pH to counteract effects of competitors. With this addition, the methodology is better positioned for future incorporation of rapid DNA probe and other methods that may be less sensitive than conventional cultural methodology. The nondairy food enrichment has also been modified to make it identical to the milk and dairy product enrichment by reducing the acriflavin concentration from 15 to 10 mg/ml Furthermore, the addition of sodium pyruvate and the delayed addition of selective agents until the fourth hour of enrichment will speed the recovery of damaged cells.

Table 1. Differentiation of *Listeria* species

			Acid produced from			
Species	**Hemolytic (Beta)[(a)]**	**Nitrate reduced**	**Mannitol**	**Rhamnose**	**Xylose**	**Virulent (mouse)**
L. monocytogenes	+	-	-	+	-	+
L. ivanovii	+	-	-	-	+	+
L. innocua	-	-	-	V[(b)]	-	-
L. welshimeri	-	-	-	V[(b)]	+	-
L. seeligeri	+	-	-	-	+	-
***L. grayi*[(c)]**	-	V	+	V	-	-

[a] Sheep blood stab.

[b] V, variable.

[c] *L. grayi* now includes the former nitrate-reducing, rhamnose-variable species *L. murrayi*.

The differential selective agar PALCAM (17) has been introduced as a companion selective agar to Oxford agar (OXA), optionally replacing LPM plus esculin and Fe^{3+} or plain LPM as the required second selective agar. This substitution brings the methodology closer to that used outside the U.S. and decreases reliance on the delicate Henry illumination technique now used only optionally to confirm the purity of *Listeria* isolates. Other selective agar media are currently under consideration as alternative substitutes for LPM. The normal mouse pathogenicity test has been replaced by the more sensitive and slightly faster immunocompromised mouse pathogenicity test of Stelma et al. (16).

Several rapid DNA probe and ELISA kits have been sanctioned for use in the generic or specific identification of *Listeria* isolates on selective isolation or purification agars and for screening enrichments (see FDA/CFSAN field instructions). In line with the recent taxonomic evidence and the redefinition of *L. grayi*, use of the nitrate reduction test is now optional.

A. Equipment and materials

1. Balance for weighing sample
2. Cover slip, glass
3. Erlenmeyer flask, 500 ml
4. Fermentation tubes (Durham tubes)
5. Grease pencil or magic marker
6. Incubators, 30 and 35°C
7. Immersion oil
8. Inoculating loops
9. Inoculating needle
10. Microscope slides
11. Needle, 26 gauge, 3/8 inch
12. Phase-contrast microscope with oil immersion phase objective (100X)
13. Petri plates
14. Pipets, 25, 10, and 1 ml
15. Tubes, 16 x 125 mm or other appropriate sizes, screw-cap
16. Blender and jars or Stomacher and Stomacher bags
17. Tuberculin syringe, sterile, disposable
18. Dissecting or low power microscope with illuminator (Fig. 1)

B. Media and reagents **NOTE**: Alternative companies may be used when the products are equivalent.

1. Acetic acid, 5 N
2. Acriflavin HCl
3. Agar (Difco)
4. N-(1-naphthyl) ethylene diamine (R48)
5. α-naphthol reagent (R48)
6. Blood agar base No. 2 (Unipath)
7. Cycloheximide (Advisory for BAM Users on Reported Supply Problems for Cycloheximide)
8. Sheep blood, defibrinated; sheep blood agar (M135)
9. Ethanol, absolute
10. Fluorescent antibody (FA) buffer (Difco)
11. Glycine anhydride
12. Gram stain kit
13. Hydrogen peroxide solution, 3% for catalase test (R12)
14. KOH, 40% solution (R65)
15. *Listeria*-typing sera set (Difco)
16. Lithium chloride-phenylethanol-moxalactam (LPM) agar (M81); LPM + esculin/iron (M82)
17. Nalidixic acid (sodium salt)
18. Nitrate broth (M108) and nitrite detection reagents (R48)
19. Nutrient broth (M114)
20. Physiological saline solution, 0.85% (R63)
21. Purple carbohydrate fermentation broth base (M130a), containing 0.5% (w/v) solutions of dextrose, esculin, maltose, rhamnose, mannitol, and xylose
22. SIM medium (Becton-Dickinson Microbiology Systems:BDMS) (M137) or motility test medium (MTM, Difco) (M103)
23. Sulfanilic acid reagent (R48)
24. Trypticase soy agar with 0.6% yeast extract (TSAYE) (M153)
25. Trypticase soy broth with 0.6% yeast extract (TSBYE) (M157)
26. Tryptose broth and agar (Difco) (M167)
27. Oxford medium (OXA) (M118)
28. Buffered enrichment broth (M52)
29. PALCAM agar (M118a)
30. Carrageenan (Sigma type II)

C. Pre-enrichment and enrichment procedure

See current compliance instructions for information about sampling plans and compositing in *Listeria* analysis. Be sure the sample represents the outer surface as well as the interior of the food. Refrigeration at 4°C is recommended for handling, storing, and shipping materials to be analyzed for *L. monocytogenes*, which will grow, although slowly, at this temperature. However, if the laboratory sample is already frozen, it should be kept frozen until analysis.

Add 25 ml liquid or 25 g cream or solid test material to 225 ml enrichment broth (EB) without selective agents in blender or stomacher. Blend or stomach as required for thorough mixing. Place enrichment culture in blender jar or stomacher bag, or transfer to 500 ml Erlenmeyer flask; incubate 4 h at 30°C, add selective agents acriflavin, nalidixic acid, and cycloheximide and continue incubating another 44 h, for a total of 2 days, at 30°C.

D. Isolation procedure

At 24 and 48 h, streak EB culture onto both OXA (5) and LPM (11) or LPM plus esculin/Fe^{3+} agars. PALCAM agar (17) may be substituted for LPM agars. Incubate OXA and PALCAM plates (use of a CO_2-air atmosphere is optional) at 35°C for 24-48 h, and LPM plates at 30°C for 24-48 h. Examine LPM plates for suspect colonies by using beamed white light powerful enough to illuminate plate well, striking plate bottom at 45 angle (Fig. 1). When examined in this oblique-transmitted light (Henry illumination) from an eye position directly above the plate (i.e., at 90 to the plate) either directly or via low power microscope or dissecting microscope (with mirror detached), *Listeria* spp. colonies on LPM agar appear sparkling blue (bluish crushed glass) or white. The use of positive and negative control colonies (not the test plate) to attune the observer's eyes is strongly recommended. Although components of the optical systems used may vary, the important points are the 45 angle of incident light and the 90 angle of emergent light.

Esculin and ferric iron salt may be added to LPM (M82) to eliminate the need for Henry illumination. In this case, proceed as with OXA isolates. With OXA, the Henry illumination method is inapplicable because on OXA, *Listeria* colonies have a black halo. Certain other bacteria can form black haloes, but color development takes longer than 2 days. *Listeria* behaves similarly on OXA and PALCAM.

Transfer 5 or more typical colonies from OXA and PALCAM or LPM to TSAYE, streaking for purity and typical isolated colonies. Purification on TSAYE is mandatory in the conventional analysis because isolated colonies on OXA, PALCAM, and LPM may still be in contact with an invisible weak background of partially inhibited competitors. At least 5 isolates are necessary because more than one species of *Listeria* may be isolated from the same sample. Incubate TSAYE plates at 30°C for 24-48 h. The plates may be incubated at 35°C if colonies will not be used for a wet-mount motility observation (**see** E-2, below).

E. Identification procedure

Identify purified isolates by the following classical tests (E, 1-10). Rapid kits are available to facilitate biochemical testing to genus or species level (**see** E-11 and E-12).

1. Examine TSAYE plates for typical colonies. With the oblique Henry illumination system, already described, colonies appear blue-gray to blue. The use of known controls on TSAYE is recommended.

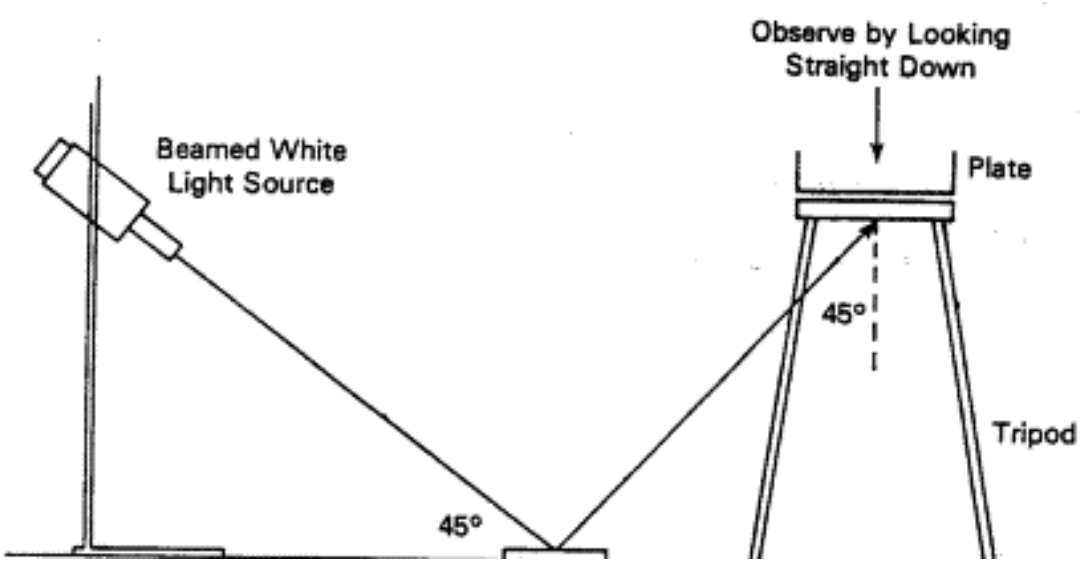

Figure 1. Examination of plates by Henry illumination for suspect colonies of Listeria spp. Light source used, Bausch & Lomb Nicholas Illuminator; see current Fisher Catalog. Reference: H.P.R. Seeliger, Listeriosis. Hafner Publishing Co., New York (1961). NOTE: Some observers prefer a concave mirror.

2. Pick typical colony from culture plate incubated at 30°C or lower and examine in a wet mount, using 0.85% saline for suspending medium, with the oil immersion objective of a phase-contrast microscope. Choose a colony with enough growth to make a fairly heavy suspension; emulsify thoroughly. If too little growth is used, the few cells present will stick to the glass slide and appear nonmotile. *Listeria* spp. are slim, short rods with slight rotating or tumbling motility. Always compare with known culture. Cocci, large rods, or rods with rapid, swimming motility are not *Listeria* spp. Alternatively, use the 7-day motility test medium (**see** E-9).
3. Test typical colony for catalase. *Listeria* species are catalase-positive.
4. Gram stain 16-h to 24-h cultures. All *Listeria* spp. are short, Gram-positive rods; however, with older cultures the Gram stain reaction can be variable and cells may appear coccoidal. The cells have a tendency to palisade in thick-stained smears, leading to false rejection as a diphtheroid.
5. Pick typical colony to a tube of TSBYE for inoculating carbohydrate fermentation and other test media. Incubate at 35°C for 24 h. This culture may be kept at 4°C several days and used repeatedly as inoculum.

6. Inoculate heavily (from TSAYE colony) 5% sheep blood agar (or horse blood agar) by stabbing plates that have been poured thick and dried well (check for moisture before using). Draw grid of 20-25 spaces on plate bottom. Stab one culture per grid space. Always stab positive controls (*L. ivanovii* and *L. monocytogenes*) and negative control (*L. innocua*). Incubate for 48 h at 35°C. Stabs should pass right through the agar layers. Try to avoid hitting bottom of plate too hard and fracturing agar.
7. Examine blood agar plates containing culture stabs with bright light. *L. monocytogenes* and *L. seeligeri* produce slightly cleared zone around stab. *L. innocua* shows no zone of hemolysis, whereas *L. ivanovii* produces well-defined clear zone around stab. Do not try to differentiate species at this point, but note nature of hemolytic reaction. Resolve doubtful reactions by the CAMP test.
8. Nitrate reduction test (optional). For this test, use TSBYE culture to inoculate nitrate broth (M108). Incubate at 35°C for 5 days. Add 0.2 ml reagent A, followed by 0.2 ml reagent B (R48). A red-violet color indicates presence of nitrite, i.e., nitrate has been reduced. If no color develops, add powdered zinc and let stand 1 h. A developing red-violet color indicates that nitrate is still present and has not been reduced. Only *L. grayi* ssp. *murrayi* reduces nitrates, and the test is only necessary to distinguish it from *L. grayi* ssp. *grayi*.

   As an alternative procedure (R48), add 0.2 ml reagent A followed by 0.2 ml reagent C. An orange color indicates reduction of nitrate. If no color develops, add powdered zinc as above. Development of an orange color indicates unreduced nitrate.
9. Inoculate SIM or MTM from TSBYE. Incubate for 7 days at room temperature. Observe daily. *Listeria* spp. are motile, giving a typical umbrella-like growth pattern. MTM gives better defined umbrellas. Alternatively, observe 30°C TSBYE cultures by phase-contrast microscopy (1000X) for tumbling motility.
10. From TSBYE culture, inoculate the following carbohydrates as 0.5% (w/v) solutions in purple carbohydrate broth (the use of Durham tubes is optional): dextrose, esculin, maltose, rhamnose, mannitol, and xylose. Incubate 7 days at 35°C. Positive-reacting *Listeria* spp. produce acid with no gas. Consult Table 1 for xylose-rhamnose reactions of *Listeria* spp. All species should be positive for dextrose, esculin, and maltose. All *Listeria* spp. except *L. grayi* should be mannitol-negative. If pigmentation of the isolate on OXA, PALCAM or LPM plus esculin/Fe^{3+} is unequivocal, the esculin test may be omitted.
11. Purified isolates can be rapidly identified by conventional tests (additional tests may be needed to speciate completely) using commercial kits: Vitek Automicrobic Gram Positive and Gram Negative Identification cards (BioMerieux, Hazelwood, MO); or API Listeria (BioMerieux sa Marcy-l'Etoile, France), which does not require an additional CAMP test. The MICRO-ID™ kit (Organon Teknika Corp., Durham, NC) permits speciation of *Listeria* isolates if

their CAMP reactions are known. AOAC INTERNATIONAL, Arlington, VA, has adopted the Micro-ID and the Vitek Automicrobic System as Official First Action Methods.

12. Several nonconventional rapid methods are available for identifying *Listeria* isolates to genus or species (*L. monocytogenes*) level. These methods, particularly the *L. monocytogenes* specific methods, accelerate identification. Isolates in pure broth culture may be identified to genus level by using commercial ELISA kits (Organon; Bioenterprises Pty Ltd, Roseville, NSW, Australia) or nonradiolabeled DNA probe kits (GeneTrak, Framingham, MA) (10,12). If such kits are used to screen enrichment cultures for *Listeria* spp., cultures should still be streaked on selective agars regardless of screening results.

Nonradioactive DNA probe tests specific for identification of *L. monocytogenes* at the isolation and purification culture steps are available (6,7,13; **see** chapter 24). These methods are highly recommended, but with the indicated provisos. Identification of colonies from isolation or purification agar or in pure broth cultures with the Accuprobe™ (Gen-Probe, Inc., San Diego, CA) *Listeria* culture confirmation test (1) or the *L. monocytogenes* assay (GeneTrak) is recommended. Culture-positive enrichments will sometimes test positive with these kits, especially at 48 h. These rapid nonradioactive probe tests are specific for *L. monocytogenes*. Purified isolates identified as *L. monocytogenes* should be retained for regulatory reference.

F. Serology (optional)

Table 2 shows serological relationships of *Listeria* spp. Most *L. monocytogenes* isolates obtained from patients and the environment are type 1 or 4, and more than 90% can be serotyped with commercially available sera. All nonpathogenic species, except *L. welshimeri*, share one or more somatic antigens with *L. monocytogenes*. Serotyping alone without thorough characterization, therefore, is not adequate for identification of *L. monocytogenes*.

Table 2. Serology of *Listeria* species[15]

Species	Serotypes
L. monocytogenes	1/2A, 1/2B, 1/2C, 3A, 3B, 3C, 4A, 4AB, 4B, 4C, 4D, 4E, "7"

L. ivanovii	5
L. innocua	4AB, 6A, 6B, Un[(a)]
L. welshimeri	6A, 6B
L. seeligeri	1/2B, 4C, 4D, 6B, Un

[a] Un, undefined.

Serology is useful when epidemiological considerations are crucial. Use TSBYE culture to inoculate tryptose broth. Make 2 successive transfers of cultures incubated in tryptose broth for 24 h at 35°C. Make a final transfer to 2 tryptose agar slants and incubate 24 h at 35°C. Wash both slants in a total of 3 ml Difco fluorescent antibody (FA) buffer and transfer to sterile 16 x 125 mm screw-cap tube. Heat in water bath at 80°C for 1 h. Centrifuge at 1600 x *g* for 30 min. Remove 2.2-2.3 ml of supernatant fluid and resuspend pellet in remainder of buffer. Follow manufacturer's recommendations for sera dilution and agglutination procedure. If flagellar (H) and sub-factor (O) serotyping is required, **see** Chapter 11 on serological methods. Phage typing, DNA restriction fragment analysis, and enzyme allele analysis are also very helpful in epidemiological studies of listeriosis outbreaks.

G. Immunocompromised mouse pathogenicity (optional). The classical tests for *Listeria* pathogenicity are the Anton conjunctivitis test (rabbits) and inoculation of mice and of embryonated eggs (3). An immunocompromised mouse test, using intraperitoneal (i.p.) injection is recommended because of its greatly improved sensitivity (16). Confirmation of *L. monocytogenes* animal pathogenicity is not routine for clinical isolates and is optional for regulatory isolates. An isolate should be identified as *L. monocytogenes* if it meets all the other criteria outlined in this chapter.

   Carrageenan (Sigma type II) dissolved in distilled water (40 mg/ml) is injected i.p. into 18-20 g mice (200 mg/kg) 24 h before the *Listeria* challenge. Grow isolate for 24 h at 35°C in TSBYE. Transfer to 2 tubes of TSBYE for another 24 h at 35°C. Place a total of 10 ml culture broth from both tubes into 16 x 125 mm tube and centrifuge at 1600 x *g* for 30 min. Discard supernatant and resuspend pellet in 1 ml phosphate-buffered saline (PBS). This suspension will contain approximately 10^{10} bacteria/ml; dilute to 10^5 bacteria per ml and determine actual concentration by a pour- or spread-plate count. Inject (i.p.) 16- to 18-g immunocompromised Swiss white mice (5 mice/culture) with 0.1 ml of the concentrated suspension, i.e., about 10^4 bacteria per mouse. Observe for death over 5-day period. Nonpathogenic strains will not kill, but 10^4 pathogenic cells will kill, usually within 3 days. Use known pathogenic and nonpathogenic strains and carrageenan-treated uninoculated mice as controls. Use 5 mice per control group. Carrageenan controls should be challenged with 0.1 ml PBS.

H. CAMP test (optional). The Christie-Atkins-Munch-Peterson (CAMP) test (Table 3 and

Fig. 2) is useful in confirming species, particularly when blood agar stab test results are equivocal. To perform the test, streak a -hemolytic *Staphylococcus aureus* and a *Rhodococcus equi* culture in parallel and diametrically opposite each other on a sheep blood agar plate. Streak several test cultures parallel to one another, but at right angles to and between the *S. aureus* and *R. equi* streaks. After incubation at 35°C for 24-48 h, examine the plates for hemolysis. Hemolysis is more easily read when the blood agar is thinner than usual. The *L. monocytogenes* reaction is often optimal at 24 h rather than 48 h. To obtain enough *R. equi* to give a good streak of growth, incubate the inoculum slant culture longer than 24 h. Use of known control *Listeria* spp. on a separate sheep blood agar plate is recommended. Sheep blood agar plates should be as fresh as possible.

Streak weakly β-hemolytic *S. aureus* FDA strain ATCC 49444 (CIP 5710; NCTC 7428) or strains ATCC 25923 and *R. equi* (ATCC 6939; NCTC 1621) vertically on sheep blood agar. Separate vertical streaks so that test strains may be streaked horizontally between them without quite touching the vertical streaks (1mm gap). After 24- and 48-h incubation at 35°C, examine plates for hemolysis in the zone of influence of the vertical streaks. CAMP test cultures are available from several national culture collections, including the American Type Culture Collection (ATCC), Manassas, VA.

Figure 2 shows arrangement of streak cultures on a CAMP plate. Hemolysis of *L. monocytogenes* and *L. seeligeri* is enhanced near *S. aureus* streak; *L. ivanovii* hemolysis is enhanced near *R. equi* streak. Other species are nonhemolytic and do not react in this test. The CAMP test differentiates *L. ivanovii* from *L. seeligeri* and can differentiate a weakly hemolytic *L. seeligeri* (that may have been read as nonhemolytic) from *L. welshimeri*. Isolates giving reactions typical for *L. monocytogenes* except for hemolysin production should be CAMP-tested before they are identified as nonhemolytic *L. innocua*. A factor easily prepared from *S. aureus* cultures may be used to enhance hemolysis by *L. monocytogenes* and *L. seeligeri* in sheep blood agar plates. β-lysin disks (REMEL, Lenexa, KS) may be used for the same purpose.

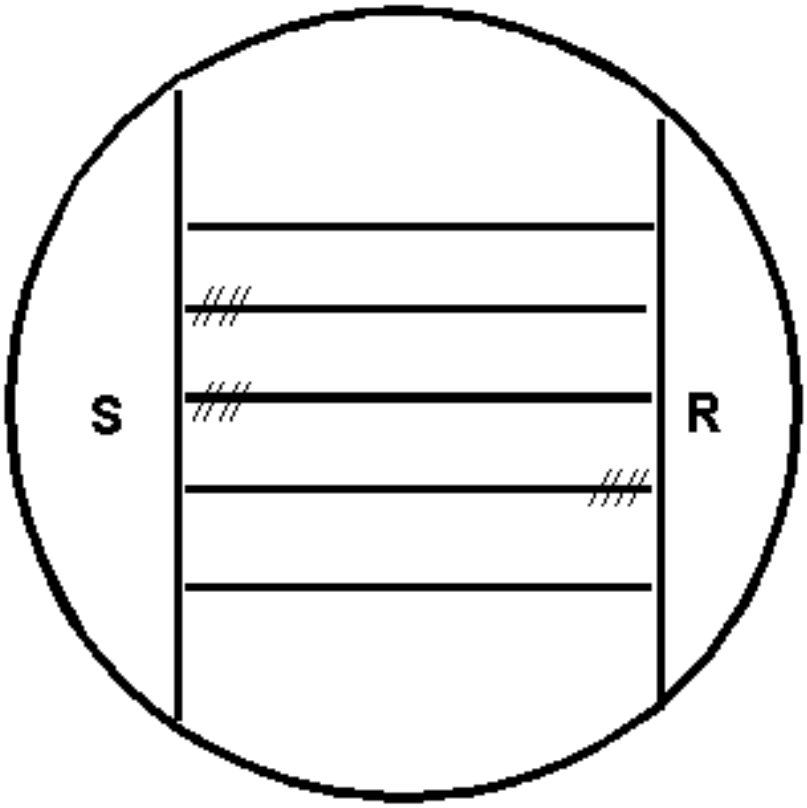

Figure 2. CAMP test for *L. monocytogenes*: Inoculation pattern of sheep blood agar plate. Horizontal lines represent streak inoculations of 5 test strains. Vertical lines represent streak inoculations of *S. aureus* (S) and *R. equi* (R). Hatched lines indicate (diagrammatically only) locations of hemolysis enhancement regions. Horizontal culture streaks should not touch the vertical culture streaks. There should be uninoculated gaps of not more than about 1mm width.

Table 3. CAMP test reactions of *Listeria* species

Species	Hemolytic interaction	
	***Staphylococcus aureus* (S)**	***Rhodococcus equi* (R)**
L. monocytogenes	+	-(a)
L. ivanovii	-	+
L. innocua	-	-
L. welshimeri	-	-
L. seeligeri	+	-

[a] Rare strains are S+ and R+, but the R+ reaction is much less pronounced than that of *L. ivanovii*.

I. Interpretation of analyses data for speciation

The importance of completely characterizing each isolate cannot be overemphasized. Partial characterization, even if accurate, may be misleading. Since all *Listeria* spp. are negative for indole, oxidase, urease, and H_2S production, and are positive for methyl red and Voges-Proskauer, these tests are discretionary.

All *Listeria* spp. are small, catalase-positive, Gram-positive rods that are motile in wet mounts and in SIM. They utilize dextrose, esculin, and maltose; some species utilize mannitol, rhamnose, and xylose with production of acid. *L. grayi* utilizes mannitol with acid production. *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* produce hemolysis in sheep blood stabs and consequently are CAMP test-positive. Of the three, only *L. monocytogenes* fails to utilize xylose and is positive for rhamnose utilization. The difficulty in differentiating *L. ivanovii* from *L. seeligeri* can be resolved by the CAMP test. *L. seeligeri* shows enhanced hemolysis at the *S. aureus* streak. *L. ivanovii* shows enhanced hemolysis at the *R. equi* streak. Of the nonhemolytic species, *L. innocua* may give the same rhamnose-xylose reactions as *L. monocytogenes* but is negative in the CAMP test. *L. innocua* is sometimes negative for utilization of both rhamnose and xylose. *L. welshimeri*, which is rhamnose-negative, may be confused with a weakly hemolytic *L. seeligeri* unless resolved by the CAMP test.

After all other results are available, the serotyping of *Listeria* isolates becomes meaningful. Biochemical, serological, and pathogenicity data are summarized in Tables 1-3. All data collection must be completed before species are determined.

J. Enumeration (optional)

Enumeration data are essential for estimating the infectious dose in an outbreak and for estimating the degree of contamination by *Listeria* spp. *Listeria* should be quantitated before enrichment by direct plate count (on OXA) and/or by a 3-tube MPN culture procedure on 10, 1, 0.1, and 0.01 g samples in EB (30°C, 48 h) followed by streaking on OXA. Presence of *L. monocytogenes* among the *Listeria* colonies on the OXA plates may be ascertained by pooling 5 colonies and performing the Accuprobe test (**see** section E-12). Not less than 10 µl from each enrichment tube of the MPN enrichments broths, at each dilution level, may be tested by the Accuprobe method (**see** E-12) at 48 h to estimate the end point dilution level of *L. monocytogenes* relative to the total *Listeria* count. (When the Oxford plating result is becoming apparent and if 48 h enrichment subsamples have been kept frozen, pooling may be done more selectively and economically with

esculin-positive samples as guides.) Since the probe method requires a threshold level of about 10^6 per 50 µl, a negative result would not necessarily mean absence of *L. monocytogenes*. Instead it may mean that *L. monocytogenes* was not a substantial component of the total *Listeria* count. For further guidance on this procedure contact the author. FDA's CFSAN DNA probe may also be used to enumerate *L. monocytogenes* (**see** Chapter 24). Other MPN and direct plating enumeration methods are currently being considered for adoption.

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Bad Bug Book:
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Top

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B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 11

Serodiagnosis of *Listeria monocytogenes*

Authors

(Return to Table of Contents)

Although serological confirmation is not necessary for regulatory identification of *Listeria monocytogenes*, it is useful for determining the prevalence of specific serotypes in epidemiological studies and for environmental recontamination tracking. Early attempts to confirm *L. monocytogenes* serologically were generally not successful because of cross-reactions with other organisms (1). Serology, therefore, should always follow cultural and biochemical identification (**see** Chapter 10). Serological assays of *Listeria*, reviewed by Gray and Killinger (4), include agglutination, precipitation, and complement fixation assays as well as automated fluorescent antibody techniques, using flow cytometry (3) and rapid method ELISA kits, which are based on monoclonal and polyclonal antibodies (5). However, these serological systems were developed to detect all *Listeria* spp. They have been used successfully to screen foods for the genus (**see** Chapter 10), but they do not differentiate *L. monocytogenes* from other *Listeria* species.

This chapter presents procedures for the serological identification of *L. monocytogenes* by flagellar and somatic antigenic profiles (2), using agglutination as the serological tool.

A. Equipment and materials

1. Centrifuge
2. Refrigerator (4-6°C)
3. Steamer
4. Inoculating needle
5. Tubes, 6 x 50 mm

6. Test tube rack (for 6 x 50 mm tubes)
7. Dispenser, 25, 50, 100 µl, or comparable
8. Water bath (48°C)
9. Microscope slides

B. Media and reagents

1. EB motility medium (M48)
2. Tryptose phosphate broth (TPB) (M168)
3. Formaldehyde, 37% solution
4. Formal saline, 0.5%
5. Physiological saline, 0.85% (R63)
6. McFarland No. 3 turbidimetric standard (R42)
7. Somatic (O) antisera
8. Flagellar (H) antisera

C. Preparation of materials and media

1. **EB motility medium**. Prepare as specified. Distribute 10 m1 amounts of medium in 18 x 125 mm or comparable size screw-cap tubes. Refrigerate medium until used.

2. **Tryptose phosphate broth (TPB)**. Prepare as directed. Distribute 8.0 ml amounts in 16 x 125 mm or comparable size screw-cap tubes.

3. **Formal saline (0.5%)**. Add formaldehyde in a concentration of 0.5% to 0.85% physiological saline.

4. **No. 3 McFarland standard**. Prepare turbidity standard No. 3 of the McFarland nephelometer scale. Mix 3.0 ml 1% $BaCl_2$ solution with 97.0 ml 1% H_2SO_4 solution.

5. **H and O antisera**. Dilute according to manufacturer's directions.

6. **Cells (antigens to be tested)**. Prepare *L. monocytogenes* cells as specified under procedures.

D. Procedures

The uniform approach to serotyping is first to determine the flagellar (H) serotype, and then to determine the somatic (O) serotype and/or subserovar, depending on the refinement of the antisera. Specific somatic types are associated with specific flagellar serogroups. The relationship of somatic and flagellar antigenic factors for *L. monocytogenes* is shown in Table 1.

Table 1. Diagnostic scheme showing relationship of somatic (O) and flagellar (H) antigenic factors of *L. monocytogenes*

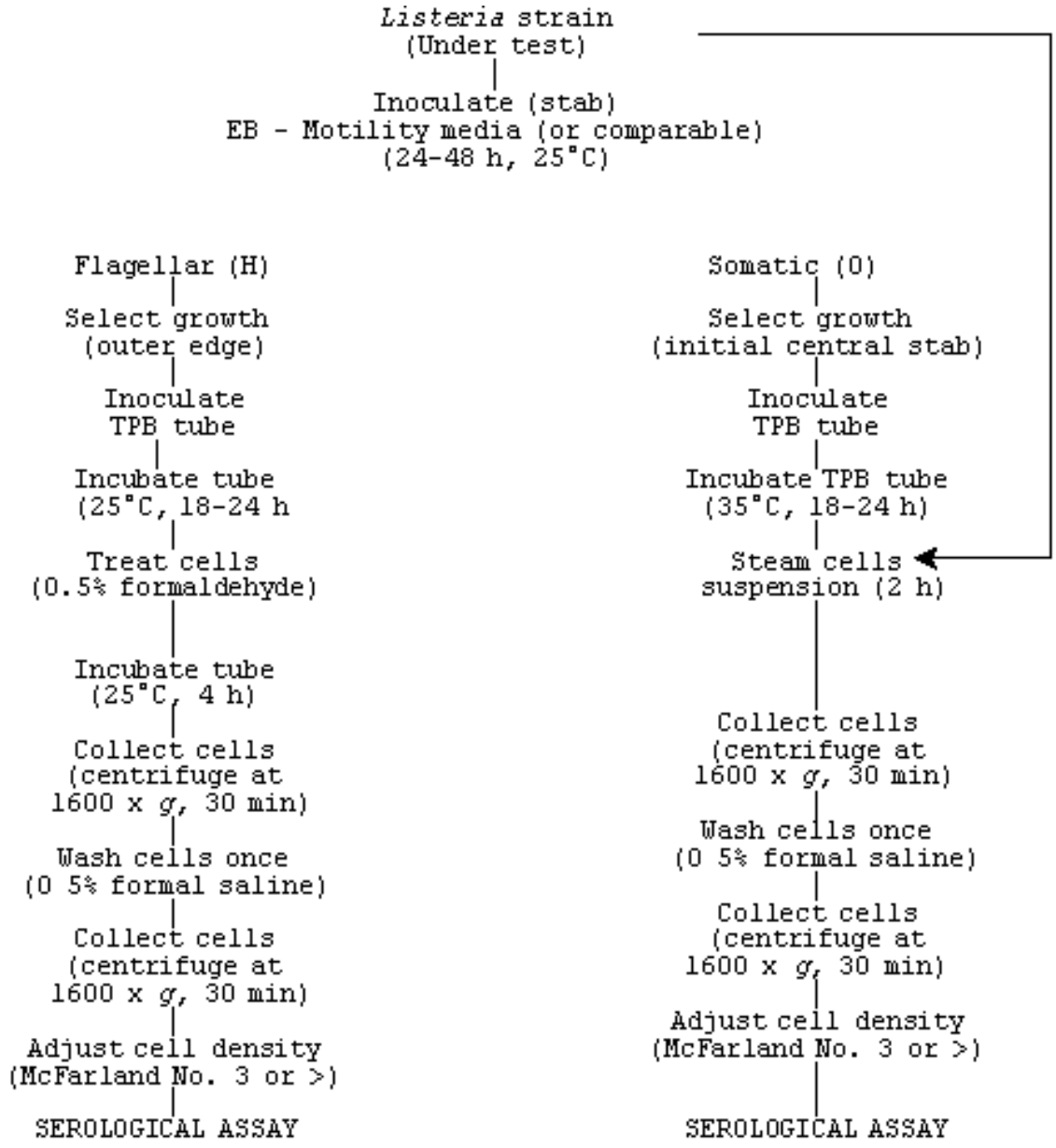
H factors	O factors
A	1a (1/2a) 3a [1a(1); 1a(1,2);3a (4)][b]
C	1b (1/2b)[a];3b [4a(7,9); 4b(5,6); 4b(6); 4d(8)][b]
D	2 (1/2c)[a] 3C

[a] Seeliger and Donker-Voet designations.

[b] Brackets indicate that antisera to somatic antigens are available.

Antisera to H antigens are also available.

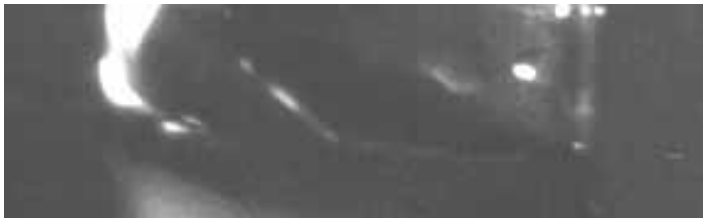
Scheme for routine serodiagnosis of L. monocytogenes, based on H and O antigenic factors.



1. **Routine serological typing for flagellar antigens**

   Remove growth from agar slant with straight inoculating needle and stab tube of EM motility agar if both flagellar and somatic antigens are required for serodiagnosis. **See** scheme for growth and treatment events. Incubate inoculated motility agar at 25°C for 24-48 h. Pick colonies from outer edge of motile growth on EB motility agar (Fig. 1) and inoculate tube of TPB or similar agar. Incubate inoculated TPB for 18-24 h at 25°C.

   Fig. 1. Typical umbrella-like growth of *L. monocytogenes* on motility agar.

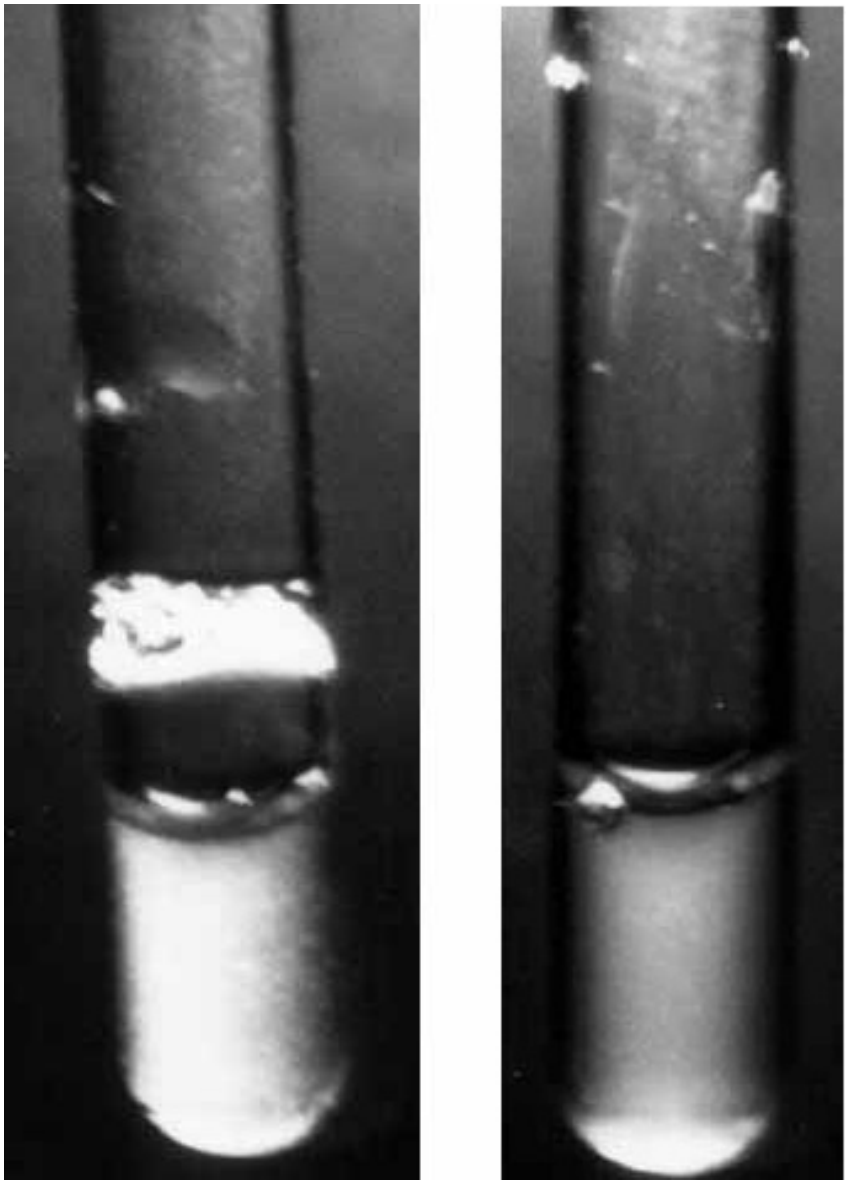


Add formaldehyde solution (37%) for final volume of 0.5% to broth culture (0.04 ml of formaldehyde to 8.0 ml of broth culture). Allow formaldehyde-treated broth to incubate 4 h at 25°C or treat as live culture. Collect cells by centrifugation (1600 x *g*, 30 min); then resuspend cells in 0.5% formal saline to turbidity equal to McFarland No. 3 standard. The broth cell suspension works equally as well as the washed standardized cells for the agglutination test.

In 6 x 50 mm tubes, mix cells (antigen) to be tested with equal volumes (100 l) of predetermined dilutions of H antisera with serological factors, A, C, and D. To prepare negative control, place 100 l of test cells in equal volume of saline. Incubate tubes containing bacterial cells and antisera as well as negative control in water bath preset at 48°C.

Observe tubes for agglutination after 1 h incubation. If agglutination occurs, a sediment will form and the supernatant will be as clear as the negative control (or clearer). Agitate tubes slightly (execute gently with finger) to resuspend sediment. Typical tube agglutination reaction is shown in Fig. 2.

Fig. 2. Comparative tube agglutination test showing positive agglutination reaction (tube on left with granular appearance) and typical negative reaction (tube on right with smooth, homogenous appearance in the serodiagnosis of *L. monocytogenes*.



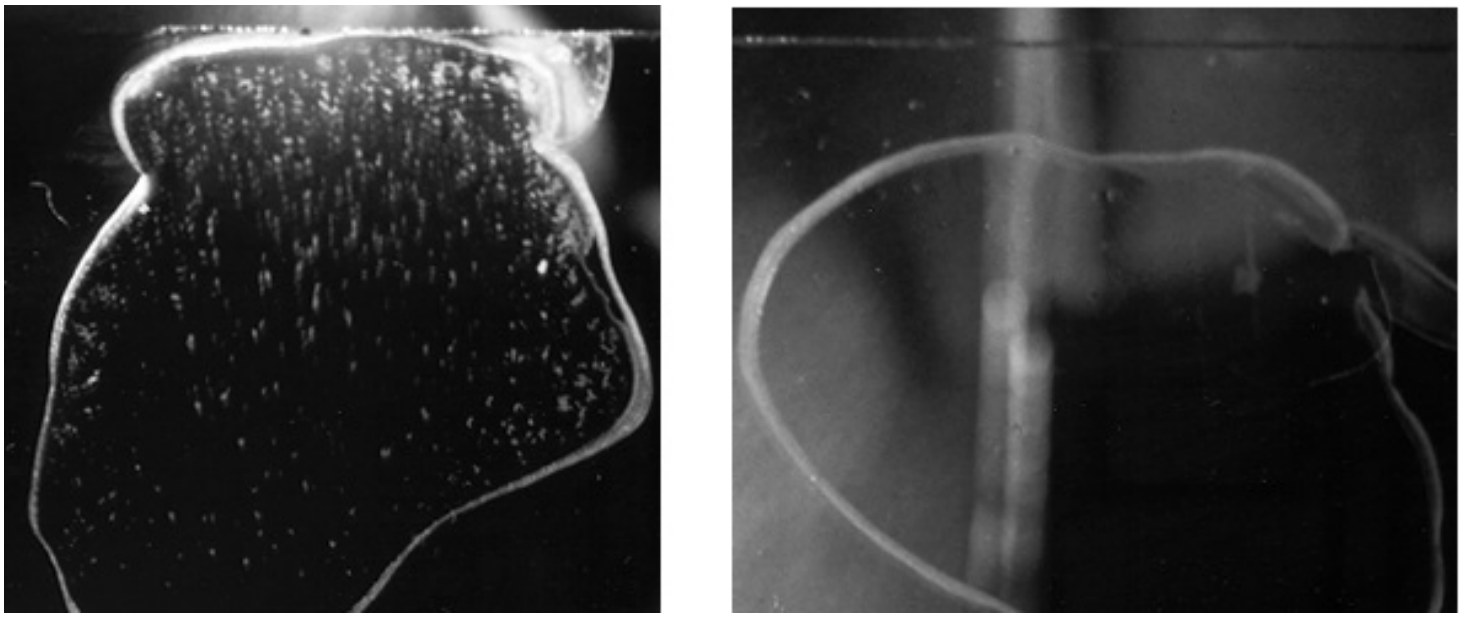
2. **Routine serological typing for somatic antigens**

   Remove growth from agar slant or comparable media with inoculating needle and inoculate tube(s) of TPB if flagellar serodiagnosis is not being done. If both flagellar and somatic antigen profiles are being determined, **see** scheme for routine serodiagnosis of *L. monocytogenes* based on H and O factors. Collect cells (antigen) by centrifugation (1600 x *g*, 30 min). Wash cells once in TPB for slide test or once with 0.5% formal saline for tube agglutination testing.

   For slide testing, resuspend cells in minimal amount of 0.5% formal saline to prepare heavy cell suspension (cell turbidity equal to or greater than that of McFarland No. 3). Place 25 µl of factor serum on slide with equal volume of cells (antigen). To prepare negative control, mix 25 µl of cell suspension with 25 µl of saline. Mix antiserum and cells together while rocking slide back and forth.

To observe agglutination, hold slide against black background near a desk lamp. Typical positive (agglutination) and negative (smooth) reactions are shown in Fig. 3. If reaction is not smooth or fails to give good observable agglutination, test cells by tube agglutination, which is considerably more sensitive. The tube test for routine serotyping of O antigens is the same as that used for testing H antigen factors. For somatic serotyping, incubate tubes for 2 h in 48°C water bath and refrigerate overnight, or incubate overnight in 48°C water bath.

Fig. 3. Comparative slide agglutination test showing typical positive agglutination reaction (left section of slide) and negative serological reaction (right section of slide) in the serological typing of *L. monocytogenes*.



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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

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Chapter 12

Staphylococcus aureus

Authors

(Return to Table of Contents)

Staphylococcus aureus is highly vulnerable to destruction by heat treatment and nearly all sanitizing agents. Thus, the presence of this bacterium or its enterotoxins in processed foods or on food processing equipment is generally an indication of poor sanitation. *S. aureus* can cause severe food poisoning. It has been identified as the causative agent in many food poisoning outbreaks and is probably responsible for even more cases in individuals and family groups than the records show. Foods are examined for the presence of *S. aureus* and/or its enterotoxins to confirm that *S. aureus* is the causative agent of foodborne illness, to determine whether a food is a potential source of "staph" food poisoning, and to demonstrate post-processing contamination, which is generally due to human contact or contaminated food-contact surfaces. Conclusions regarding the significance of *S. aureus* in foods should be made with circumspection. The presence of a large number of *S. aureus* organisms in a food may indicate poor handling or sanitation; however, it is not sufficient evidence to incriminate a food as the cause of food poisoning. The isolated *S. aureus* must be shown to produce enterotoxins. Conversely, small staphylococcal populations at the time of testing may be remnants of large populations that produced enterotoxins in sufficient quantity to cause food poisoning. Therefore, the analyst should consider all possibilities when analyzing a food for *S. aureus*.

Methods used to detect and enumerate *S. aureus* depend on the reasons for testing the food and on the past history of the test material. Processed foods may contain relatively small numbers of debilitated viable cells, whose presence must be demonstrated by appropriate means. Analysis of food for *S. aureus* may lead to legal action against the party or parties responsible for a contaminated food. The methods of analysis for *S. aureus* that have been studied collaboratively and found suitable for use in providing the type of information necessary for FDA requirements are presented in this chapter.

There has been considerable controversy about the significance and correct method of reading the coagulase test. Research results have indicated that the weak coagulase activity represented by 1+, 2+, and 3+ reactions seldom corresponds with other criteria associated with *S. aureus* (4). A consensus of peers has established that a 4+ coagulase reaction is necessary for unquestioned identification of *S. aureus*. Those strains suspected of being *S. aureus* on the basis of coagulase reactions of less than 4+ should be confirmed by other tests, such as anaerobic glucose fermentation, lysostaphin sensitivity, and thermonuclease production. Studies of colonial morphology on Baird-Parker agar, lysostaphin sensitivity, coagulase and thermonuclease production, and glucose and mannitol fermentation were conducted on 100 enterotoxigenic and 51 nonenterotoxigenic strains of *S. aureus* (3). In all cases, the reactions of enterotoxigenic and nonenterotoxigenic strains varied by 12% or less. This research indicates that none of these tests can be relied upon to differentiate toxic and nontoxic staphylococci.

Direct Plate Count Method

This method is suitable for the analysis of foods in which more than 100 *S. aureus* cells/g may be expected. It conforms to the method in ref. 1.

A. Equipment and materials

1. Same basic equipment as for conventional plate count (Chapter 3).
2. Drying cabinet or incubator for drying surface of agar plates
3. Sterile bent glass streaking rods, hockey stick or hoe-shaped, with fire-polished ends, 3-4 mm diameter, 15-20 cm long, with an angled spreading surface 45-55 mm long

B. Media and reagents

1. Baird-Parker medium (M17)
2. Trypticase (tryptic) soy agar (TSA) (M152)
3. Brain heart infusion (BHI) broth (M24)
4. Coagulase plasma (rabbit) with EDTA

5. Toluidine blue-DNA agar (M148)
6. Lysostaphin (Schwartz-Mann, Mountain View Ave., Orangeburg, NY 10962)
7. Tryptone yeast extract agar (M165)
8. Paraffin oil, sterile
9. 0.02 M phosphate-saline buffer (R61), containing 1% NaCl
10. Catalase test (R12)

C. Preparation of sample (**see** Chapter 1).

D. Isolation and enumeration of *S. aureus*

1. For each dilution to be plated, aseptically transfer 1 ml sample suspension to 3 plates of Baird-Parker agar, distributing 1 ml of inoculum equitably to 3 plates (e.g., 0.4 ml, 0.3 ml, and 0.3 ml). Spread inoculum over surface of agar plate, using sterile bent glass streaking rod. Retain plates in upright position until inoculum is absorbed by agar (about 10 min on properly dried plates). If inoculum is not readily adsorbed, place plates upright in incubator for about 1 h. Invert plates and incubate 45-48 h at 35°C. Select plates containing 20-200 colonies, unless only plates at lower dilutions (>200 colonies) have colonies with typical appearance of *S. aureus*. Colonies of *S. aureus* are circular, smooth, convex, moist, 2-3 mm in diameter on uncrowded plates, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasionally from various foods and dairy products, nonlipolytic strains of similar appearance may be encountered, except that surrounding opaque and clear zones are absent. Strains isolated from frozen or desiccated foods that have been stored for extended periods frequently develop less black coloration than typical colonies and may have rough appearance and dry texture.

2. Count and record colonies. If several types of colonies are observed which appear to be *S. aureus* on selected plates, count number of colonies of each type and record counts separately. When plates of the lowest dilution contain <20 colonies, these may be used. If plates containing >200 colonies have colonies with the typical appearance of *S. aureus* and typical colonies do not appear at higher dilutions, use these plates for the enumeration of *S. aureus*, but do not count nontypical colonies. Select > 1 colony of each type counted and test for coagulase production. Add number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of *S. aureus*/g of food tested.

E. Coagulase test

Transfer suspect *S. aureus* colonies into small tubes containing 0.2-0.3 ml BHI broth and emulsify thoroughly. Inoculate agar slant of suitable maintenance medium, e.g., TSA, with loopful of BHI suspension. Incubate BHI culture suspension and slants 18-24 h at 35°C. Retain slant cultures at room temperature for ancillary or repeat tests in case coagulase test results are questionable. Add 0.5 ml reconstituted coagulase plasma with EDTA (B-4, above) to the BHI culture and mix thoroughly. Incubate at 35°C and examine periodically over 6 h period for clot formation. Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*. Partial clotting, formerly 2+ and 3+ coagulase reactions, must be tested further (4). Test known positive and negative cultures simultaneously with suspect cultures of unknown coagulase activity. Stain all suspect cultures with Gram reagent and observe microscopically. A latex agglutination test (AUREUS TEST™, Trisum Corp., Taipei, Taiwan) may be substituted for the coagulase test if a more rapid procedure is desired.

F. Ancillary tests

1. **Catalase test**. Use growth from TSA slant for catalase test on glass slide or spot plate, and illuminate properly to observe production of gas bubbles.

2. **Anaerobic utilization of glucose**. Inoculate tube of carbohydrate fermentation medium containing glucose (0.5%). Immediately inoculate each tube heavily with wire loop. Make certain inoculum reaches bottom of tube. Cover surface of agar with layer of sterile paraffin oil at least 25 mm thick. Incubate 5 days at 37°C. Acid is produced anaerobically if indicator changes to yellow throughout tube, indicating presence of *S. aureus*. Run controls simultaneously (positive and negative cultures and medium controls).

3. **Anaerobic utilization of mannitol**. Repeat 2, above, using mannitol as carbohydrate in medium. *S. aureus* is usually positive but some strains are negative. Run controls simultaneously.

4. **Lysostaphin sensitivity**. Transfer isolated colony from agar plate with inoculating loop to 0.2 ml phosphate-saline buffer, and emulsify. Transfer half of suspended cells to another tube (13 x 100 mm) and mix with 0.1 ml phosphate-saline buffer as control. Add 0.1 ml lysostaphin (dissolved in 0.02 M phosphate-saline buffer containing 1% NaCl) to original tube for concentration of 25 µg lysostaphin/ml. Incubate both tubes at 35°C for not more than 2 h. If turbidity clears in test mixture, test is considered positive. If clearing has not occurred in 2 h, test is negative. *S. aureus* is generally positive.

5. **Thermostable nuclease production**. This test is claimed to be as specific as the

coagulase test but less subjective, because it involves a color change from blue to bright pink. It is not a substitute for the coagulase test but rather is a supportive test, particularly for 2+ coagulase reactions. Prepare microslides by spreading 3 ml toluidine blue-deoxyribonucleic acid agar on the surface of each microscope slide. When agar has solidified, cut 2 mm diameter wells (10-12 per slide) in agar and remove agar plug by aspiration. Add about 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures used for coagulase test to well on prepared slide. Incubate slides in moist chamber 4 h at 35°C. Development of bright pink halo extending at least 1 mm from periphery of well indicates a positive reaction.

G. Some typical characteristics of 2 species of staphylococci and the micrococci, which may be helpful in their identification, are shown in Table 1.

Table 1. Typical characteristics of *S. aureus*, *S. epidermidis*, and micrococci[(a)]

Characteristic	*S. aureus*	*S. epidermidis*	Micrococci
Catalase activity	+	+	+
Coagulase production	+	-	-
Thermonuclease production	+	-	-
Lysostaphin sensitivity	+	+	-
Anaerobic utilization of			
glucose	+	+	-
mannitol	+	-	-

[a] +, Most (90% or more) strains are positive; -, most (90% or more) strains are negative.

Most Probable Number Method for *Staphylococcus* spp.

The most probable number (MPN) method (2) is recommended for routine surveillance of products in which small numbers of *S. aureus* are expected and in foods expected to contain a large population of competing species.

A. Equipment and materials--Same as for Direct Plate Count Method, above.

B. Media and reagents--Same as for Direct Plate Count Method, above. In addition: Trypticase (tryptic) soy broth (TSB) containing 10% NaCl and 1% sodium pyruvate (M154a).

C. Preparation of sample--Same as for Direct Plate Count Method, above.

D. Determination of MPN

Inoculate 3 tubes of TSB containing 10% NaCl and 1% sodium pyruvate (B, above) with 1 ml portions of decimal dilutions of each sample. Highest dilution must give negative endpoint. Incubate tubes 48 ± 2 h at 35°C. Using 3 mm loop, transfer 1 loopful from each tube showing growth (turbidity) to plate of Baird-Parker medium with properly dried surface. Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak inoculum to obtain isolated colonies. Incubate plates 48 h at 35°C. From each plate showing growth, transfer at least 1 colony suspected to be *S. aureus* to BHI broth (**see** D and E of Direct Plate Count Method, above). Continue procedure for identification and confirmation of *S. aureus* (E and F, Direct Plate Count, above). Report *S. aureus*/g as MPN/g, according to tables in Appendix 2, MPN Determination.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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U.S. Food & Drug Administration
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Bacteriological Analytical Manual *Online*

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Chapter 14

Bacillus cereus

Authors

(Return to Table of Contents)

Food poisoning caused by *Bacillus cereus* may occur when foods are prepared and held without adequate refrigeration for several hours before serving. *B. cereus* is an aerobic sporeforming bacterium that is commonly found in soil, on vegetables, and in many raw and processed foods. Consumption of foods that contain $>10^6$ *B. cereus*/g may result in food poisoning. Foods incriminated in past outbreaks include cooked meat and vegetables, boiled or fried rice, vanilla sauce, custards, soups, and raw vegetable sprouts. Two types of illness have been attributed to the consumption of foods contaminated with *B. cereus*. The first and better known is characterized by abdominal pain and diarrhea; it has an incubation period of 4-16 h and symptoms that last for 12-24 h. The second, which is characterized by an acute attack of nausea and vomiting, occurs within 1-5 h after consumption of contaminated food; diarrhea is not a common feature in this type of illness.

Examination of Foods for *B. cereus*

A. Sampling

If the quantity of food to be examined is large, take representative samples of 50 g each from different parts of the suspect food because contamination may be unevenly distributed.

B. Transporting and storage of samples

Transport and examine samples promptly without freezing, if possible. If samples must be shipped to the laboratory, pack them in insulated shipping containers with enough gel-type refrigerant to maintain them at 6°C or below. Upon receipt in the laboratory, store the samples at

4°C and analyze as soon as possible. If analysis cannot be started within 4 days after collection, freeze samples rapidly and store at -20°C until examined. Thaw at room temperature and proceed with analysis as usual. Dehydrated foods may be stored at room temperature and shipped without refrigeration.

Enumeration and Confirmation of *B. cereus* in Foods

A. Equipment and materials

1. **See** Chapter 1 for preparation of food homogenate
2. Pipets, 1, 5, and 10 ml, graduated in 0.1 ml units
3. Glass spreading rods (e.g., hockey stick) 3-4 mm diameter with 45-55 mm spreading area
4. Incubators, 30 ± 2°C and 35 ± 2°C
5. Colony counter
6. Marking pen, black felt type
7. Large and small Bunsen burners
8. Wire loops, No. 24 nichrome or platinum wire, 2 mm and 3 mm id
9. Vortex mixer
10. Microscope, microscope slides, and cover slips
11. Culture tubes, 13 x 100 mm, sterile
12. Test tubes, 16 x 125 mm, or spot plate
13. Bottles, 3 oz, sterile
14. Anaerobic jar, BBL GasPak, with H_2 + CO_2 generator envelopes and catalyst
15. Water bath, 48-50°C
16. Culture tube racks
17. Staining rack
18. Petri dishes, sterile, 15 x 100 mm

B. Media and reagents

1. Mannitol-egg yolk-polymyxin (MYP) agar plates (M95)
2. Egg yolk emulsion, 50% (M51)
3. Trypticase soy-polymyxin broth (M158)
4. Polymyxin B solutions for MYP agar (0.1%) and trypticase soy-polymyxin broth (0.15%) (**see** M95 and M158)
5. Phenol red glucose broth (M122)
6. Tyrosine agar (M170)
7. Lysozyme broth (M90)
8. Voges-Proskauer medium (M177)
9. Nitrate broth (M108)
10. Nutrient agar for *B. cereus* (M113)
11. Motility medium (*B. cereus*) (M100)
12. Trypticase soy-sheep blood agar (M159)
13. Nitrite detection reagents (R48)
14. Butterfield's phosphate-buffered dilution water (R11) sterilized in bottles to yield final volumes of 450 ± 5 ml and 90 ± 2 ml

15. Voges-Proskauer test reagents (R89)
16. Creatine crystals
17. Gram stain reagents (R32)
18. Basic fuchsin staining solution (R3)
19. Methanol

C. Sample preparation

Using aseptic technique, weigh 50 g of sample into sterile blender jar. Add 450 ml Butterfield's phosphate-buffered dilution water (1:10 dilution) and blend for 2 min at high speed (18,000-21,000 rpm). Using the 1:10 dilution, make serial dilutions of sample for enumeration of *B. cereus* as described in D or E, below.

D. Plate count of *B. cereus*

Prepare serial dilutions from 10^{-2} to 10^{-6} by transferring 10 ml homogenized sample (1:10 dilution) to 90 ml dilution blank, mixing well with vigorous shaking, and continuing until 10^{-6} dilution is reached. Inoculate duplicate MYP agar plates with each dilution of sample (including 1:10) by spreading 0.1 ml evenly onto surface of each plate with sterile glass spreading rod. Incubate plates 24 h at 30°C and observe for colonies surrounded by precipitate zone, which indicates that lecithinase is produced. *B. cereus* colonies are usually a pink color which becomes more intense after additional incubation.

If reactions are not clear, incubate plates for additional 24 h before counting colonies. Select plates that contain an estimated 15-150 eosin pink, lecithinase-producing colonies. Mark bottom of plates into zones with black felt pen to facilitate counting and count colonies that are typical of *B. cereus*. This is the presumptive plate count of *B. cereus*. Pick 5 or more presumptive positive colonies from the MYP agar plates and transfer to nutrient agar slants for confirmation as *B. cereus*. Confirm isolates as *B. cereus* as described in F and G, below. Calculate number of *B. cereus* cells/g of sample, based on percentage of colonies tested that are confirmed as *B. cereus*. For example, if average count obtained with 10^{-4} dilution of sample was 65 and 4 of 5 colonies tested were confirmed as *B. cereus*, the number of *B. cereus* cells/g of food is 65 x 4/5 x 10,000 x 10 = 5,200,000. (**NOTE**: Dilution factor is tenfold higher than sample dilution because only 0.1 ml was tested).

E. Most probable number (MPN) of *B. cereus*

The MPN technique is recommended for enumerating *B. cereus* in foods that are expected to contain fewer than 10 *B. cereus* organisms/g. It may also be preferred for examining certain dehydrated starchy foods for which the plate count technique is inappropriate.

Inoculate 3-tube MPN series in trypticase soy-polymyxin broth, using 1 ml inoculum of 10^{-1}, 10^{-2}, and 10^{-3} dilutions of sample with 3 tubes at each dilution. (Additional dilutions should also be tested if *B. cereus* population is expected to exceed 10^3/g.) Incubate tubes 48 ± 2 h at 30°C and observe for dense growth, which is typical of *B. cereus*. Streak cultures from positive tubes onto

separate MYP agar plates and incubate plates 24-48 h at 30°C. Pick one or more eosin pink, lecithinase-positive colonies from each MYP agar plate and transfer to nutrient agar slants for confirmation as *B. cereus*. Confirm isolates as *B. cereus* as described in F and G, below, and calculate MPN of *B. cereus* cells/g of sample (**see** Appendix 2) based on the number of tubes at each dilution in which the presence of *B. cereus* was confirmed.

F. Confirmation of *B. cereus*

Pick 5 or more eosin pink, lecithinase-positive colonies from MYP agar plates and transfer to nutrient agar slants. Incubate slants 24 h at 30°C. Prepare Gram-stained smears from slants and examine microscopically. *B. cereus* will appear as large Gram-positive bacilli in short-to-long chains; spores are ellipsoidal, central to subterminal, and do not swell the sporangium. Transfer 3 mm loopful of culture from each slant to 13 x 100 mm tube containing 0.5 ml of sterile phosphate-buffered dilution water and suspend culture in diluent with Vortex mixer. Use suspended cultures to inoculate the following confirmatory media:

1. **Phenol red glucose broth**. Inoculate 3 ml broth with 2 mm loopful of culture. Incubate tubes anaerobically 24 h at 35°C in GasPak anaerobic jar. Shake tubes vigorously and observe for growth as indicated by increased turbidity and color change from red to yellow, which indicates that acid has been produced anaerobically from glucose. A partial color change from red to orange/yellow may occur, even in uninoculated control tubes, due to a pH reduction upon exposure of media to CO_2 formed in GasPak anaerobic jars. Be sure to use appropriate positive and negative controls so that a distinction can be made between positive and "false-positive" reactions.

2. **Nitrate broth**. Inoculate 5 ml broth with 3 mm loopful of culture. Incubate tubes 24 h at 35°C. To test for nitrite, add 0.25 ml each of nitrite test reagents A and C to each culture. An orange color, which develops within 10 min, indicates that nitrate has been reduced to nitrite.

3. **Modified VP medium**. Inoculate 5 ml medium with 3 mm loopful of culture and incubate tubes 48 ± 2 h at 35°C. Test for production of acetylmethyl-carbinol by pipetting 1 ml culture into 16 x 125 mm test tube and adding 0.6 ml **alpha**-naphthol solution (R89) and 0.2 ml 40% potassium hydroxide (R89). Shake, and add a few crystals of creatine. Observe results after holding for 1 h at room temperature. Test is positive if pink or violet color develops.

4. **Tyrosine agar**. Inoculate entire surface of tyrosine agar slant with 3 mm loopful of culture. Incubate slants 48 h at 35°C. Observe for clearing of medium near growth, which indicates that tyrosine has been decomposed. Examine negative slants for obvious signs of growth, and incubate for a total of 7 days before considering as negative.

5. **Lysozyme broth**. Inoculate 2.5 ml of nutrient broth containing 0.001% lysozyme with 2 mm loopful of culture. Also inoculate 2.5 ml of plain nutrient broth as positive control. Incubate tubes 24 h at 35°C. Examine for growth in lysozyme broth and in nutrient broth

control. Incubate negative tubes for additional 24 h before discarding.

6. **MYP agar**. This test may be omitted if test results were clear-cut with original MYP agar plates and there was no interference from other microorganisms which were present. Mark bottom of a plate into 6-8 equal sections with felt marking pen, and label each section. Inoculate premarked 4 cm sq area of MYP agar plate by gently touching surface of agar with 2 mm loopful of culture. (Six or more cultures can be tested in this manner on one plate.) Allow inoculum to be fully absorbed before incubating for 24 h at 35°C. Check plates for lecithinase production as indicated by zone of precipitation surrounding growth. Mannitol is not fermented by isolate if growth and surrounding medium are eosin pink. (Yellow color indicates that acid is produced from mannitol.) *B. cereus* colonies are usually lecithinase-positive and mannitol-negative on MYP agar.

7. **Record results obtained with the different confirmatory tests**. Tentatively identify as *B. cereus* those isolates which 1) produce large Gram-positive rods with spores that do not swell the sporangium; 2) produce lecithinase and do not ferment mannitol on MYP agar; 3) grow and produce acid from glucose anaerobically; 4) reduce nitrate to nitrite (a few strains may be negative); 5) produce acetylmethylcarbinol (VP-positive); 6) decompose L-tyrosine; and 7) grow in the presence of 0.001% lysozyme.

   These basic characteristics are shared with other members of the *B. cereus* group, including the rhizoid strains *B. mycoides*, the crystalliferous insect pathogen *B. thuringiensis*, and the mammalian pathogen *B. anthracis*. However, these species can usually be differentiated from *B. cereus* by determining specific characteristics typical of each species or variety. The tests described in G, below, are useful for this purpose and can easily be performed in most laboratories. Strains that produce atypical results from these tests require additional analysis before they can be classified as *B. cereus*.

G. Tests for differentiating members of the *B. cereus* group (Table 1)

The following tests are useful for differentiating typical strains of *B. cereus* from other members of the *B. cereus* group, including *B. mycoides*, *B. thuringiensis*, and *B. anthracis*.

1. **Motility test**. Inoculate BC motility medium by stabbing down the center with 3 mm loopful of 24 h culture suspension. Incubate tubes 18-24 h at 30°C and examine for type of growth along stab line. Motile organisms produce diffuse growth out into the medium away from the stab. Nonmotile organisms produce growth only in and along stab. Alternatively, add 0.2 ml sterile distilled water to surface of nutrient agar slant and inoculate slant with 3 mm loopful of culture suspension. Incubate slant 6-8 h at 30°C and suspend 3 mm loopful of liquid culture from base of slant in a drop of sterile water on microscope slide. Apply cover glass and examine immediately with microscope for motility. Report whether or not isolates tested were motile. Most strains of *B. cereus* and *B. thuringiensis* are motile by means of peritrichous flagella. *B. anthracis* and all except a few strains of *B. mycoides* are nonmotile. A few *B. cereus* strains are also nonmotile.

Table 1. Differential characteristics of large-celled Group I *Bacillus* species

Feature	*B. cereus*	*B. thuringiensis*	*B. mycoides*	*B. anthracis*	*B. megaterium*
Gram reaction	+[a]	+	+	+	+
Catalase	+	+	+	+	+
Motility	+/-[b]	+/-	-[c]	-	+/-
Reduction of nitrate	+	+/	+	+	-[d]
Tyrosine decomposed	+	+	+/-	-[d]	+/-
Lysozyme-resistant	+	+	+	+	-
Egg yolk reaction	+	+	+	+	-
Anaerobic utilization of glucose	+	+	+	+	-
VP reaction	+	+	+	+	-
Acid produced from mannitol	-	-	-	-	+
Hemolysis (Sheep RBC)	+	+	+	-[d]	-
Known pathogenicity[e]/characteristic	produces enterotoxins	endotoxin crystals pathogenic to insects	rhizoidal growth	pathogenic to animals and humans	

[a] +, 90-100% of strains are positive.

[b] +/-, 50-50% of strains are positive.

[c] -, 90-100% of strains are negative.

[d] -, Most strains are negative.

[e] See Section H, Limitations of method for *B. cereus*.

2. **Rhizoid growth**. Pour 18-20 ml nutrient agar into sterile 15 x 100 mm petri dishes and allow agar to dry at room temperature for 1-2 days. Inoculate by gently touching surface of medium near center of each plate with 2 mm loopful of 24 h culture suspension. Allow inoculum to be absorbed and incubate plates 48-72 h at 30°C. Examine for development of rhizoid growth, which is characterized by production of colonies with long hair or root-like structures that may extend several centimeters from site of inoculation. Rough galaxy-shaped colonies are often produced by *B. cereus* strains and should not be confused with typical rhizoid growth, which is the definitive characteristic of *B. mycoides*. Most strains of this species are also nonmotile.

3. **Test for hemolytic activity**. Mark bottom of a plate into 6-8 equal sections with felt

marking pen, and label each section. Inoculate a premarked 4 cm sq area of trypticase soy-sheep blood agar plate by gently touching medium surface with 2 mm loopful of 24 h culture suspension. (Six or more cultures can be tested simultaneously on each plate.) Incubate plates 24 h at 35°C. Examine plates for hemolytic activity. *B. cereus* cultures usually are strongly hemolytic and produce 2-4 mm zone of complete (β) hemolysis surrounding growth. Most *B. thuringiensis* and *B. mycoides* strains are also β-hemolytic. *B. anthracis* strains are usually nonhemolytic after 24 h incubation.

4. **Test for protein toxin crystals**. Inoculate nutrient agar slants with 3 mm loopfuls of 24 h culture suspensions. Incubate slants 24 h at 30°C and then at room temperature 2-3 days. Prepare smears with sterile distilled water on microscope slides. Air-dry and lightly heat-fix by passing slide through flame of Bunsen burner. Place slide on staining rack and flood with methanol. Let stand 30 s, pour off methanol, and allow slide to air-dry. Return slide to staining rack and flood completely with 0.5% basic fuchsin or TB carbolfuchsin ZN stain (Difco). Heat slide gently from below with small Bunsen burner until steam is seen.

   Wait 1-2 min and repeat this step. Let stand 30 s, pour off stain, and rinse slide thoroughly with clean tap water. Dry slide without blotting and examine under oil immersion for presence of free spores and darkly stained tetragonal (diamond-shaped) toxin crystals. Crystals are usually somewhat smaller than spores. Toxin crystals are usually abundant in a 3- to 4-day-old culture of *B. thuringiensis* but cannot be detected by the staining technique until lysis of the sporangium has occurred. Therefore, unless free spores can be seen, cultures should be held at room temperature for a few more days and re-examined for toxin crystals. *B. thuringiensis* usually produces protein toxin crystals that can be detected by the staining technique either as free crystals or parasporal inclusion bodies within the exosporium. *B. cereus* and other members of the *B. cereus* group do not produce protein toxin crystals.

5. **Interpreting test results**. On the basis of the test results, identify as *B. cereus* those isolates which are actively motile and strongly hemolytic and do not produce rhizoid colonies or protein toxin crystals. Nonmotile *B. cereus* strains are also fairly common and a few strains are weakly hemolytic. These nonpathogenic strains of *B. cereus* can be differentiated from *B. anthracis* by their resistance to penicillin and **gamma** bacteriophage. **CAUTION**: Nonmotile, nonhemolytic isolates that are suspected to be *B. anthracis* should be submitted to a pathology laboratory such as the Centers for Disease Control and Prevention, Atlanta, GA, for identification or destroyed by autoclaving. Acrystalliferous variants of *B. thuringiensis* and nonrhizoid strains derived from *B. mycoides* cannot be distinguished from *B. cereus* by the cultural tests.

H. Limitations of method for *B. cereus*

B. *cereus* from culturally similar organisms that could occasionally be encountered in foods. These organisms include 1) the insect pathogen *B. thuringiensis*, which produces protein toxin crystals; 2) *B. mycoides*, which characteristically produces rhizoid colonies on agar media; and 3) *B. anthracis*, which exhibits marked animal pathogenicity and is nonmotile. With the exception of *B. thuringiensis*, which is currently being used for insect control on food and forage crops, these

organisms are seldom encountered in the routine examination of foods. The tests described in G, above, are usually adequate for distinguishing the typical strains of *B. cereus* from other members of the *B. cereus* group. However, results with atypical strains of *B. cereus* are quite variable, and further testing may be necessary to identify the isolates. Although a few diarrheal toxin detection kits are commercially available, none are recommended, pending further evaluation. At present, no practical tests for detecting the emetic toxin are available. Until reliable tests are available, cultural tests such as those described in this method must be relied upon for confirming isolates from foods as *B. cereus*.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Bacteriological Analytical Manual

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Chapter 16

Clostridium perfringens

Authors

(Return to Table of Contents)

Food poisoning caused by *Clostridium perfringens* may occur when foods such as meat or poultry are cooked and held without maintaining adequate heating or refrigeration before serving. The presence of small numbers of *C. perfringens* is not uncommon in raw meats, poultry, dehydrated soups and sauces, raw vegetables, and spices. Because the spores of some strains are resistant to temperatures as high as 100°C for more than l h, their presence in foods may be unavoidable. Furthermore, the oxygen level may be sufficiently reduced during cooking to permit growth of the clostridia. Spores that survive cooking may germinate and grow rapidly in foods that are inadequately refrigerated after cooking. Thus, when clinical and epidemiological evidence suggests that *C. perfringens* is the cause of a food poisoning outbreak, the presence of hundreds of thousands or more of these organisms per gram of food substantiates the diagnosis.

Illness typically occurs 8-15 h after ingestion of the contaminated food. The symptoms, which include intense abdominal cramps, gas, and diarrhea (nausea and vomiting are rare), have been attributed to a protein enterotoxin produced during sporulation of the organism in the intestine. The enterotoxin can be detected in sporulating cultures, and a method for this purpose is included. A high correlation has been established between the ability of *C. perfringens* strains to

produce enterotoxin and their ability to cause food poisoning. However, it is difficult to obtain consistent sporulation with some strains.

C. perfringens cells lose their viability when foods are frozen or held under prolonged refrigeration unless special precautions are taken. Such losses may make it difficult to establish *C. perfringens* as the specific cause of a food poisoning outbreak. It is recommended that samples which cannot be examined immediately be treated with buffered glycerin-salt solution and stored or shipped frozen to the laboratory as described below.

A. Sampling

Sample the entire portion of food (whole roast, chicken, gravy, etc.) or take representative samples of 25 g each from different parts of the suspect food because contamination may be unevenly distributed.

B. Transporting and storage of samples

Transport and examine samples promptly without freezing, if possible, and store at about 10°C until examined. If analysis cannot be started within 8 h or if the sample must be shipped to the laboratory for analysis, treat it with sterile buffered glycerin-salt solution, store immediately at -70 to -90°F, and transport it to the laboratory with dry ice, as described below.

Use aseptic technique to prepare sample for storage or shipment. Transfer 25 g portion of sample (sliced beef, turkey, hash, etc.) to sterile 150 ml container, such as plastic Whirl-Pak bag. Add 25 ml buffered glycerin-salt solution, exclude air from bag, and mix the sample well with glycerin solution. Liquid samples such as gravy or beef juice should be mixed well with equal volume of double strength buffered glycerin-salt solution.

Store glycerin-treated samples immediately at -70 to -90°F in low temperature freezer or with dry ice so that freezing occurs as quickly as possible. Maintain samples at this temperature until analysis. Thaw samples at room temperature and transfer sample and glycerin-salt solution to sterile blender jar. Add 200 ml peptone dilution fluid to blender jar and proceed with examination.

If sample must be shipped to the laboratory, follow procedures above and pack frozen sample in contact with dry ice to maintain temperature as low as possible during shipment. Pack sample in a container such as a paint can or Nalgene bottles which are impervious to CO gas, because absorption of CO_2 by the sample could lower the pH and diminish the viability of *C. perfringens*. Store sample at -70 to -90°F on receipt and keep

at this temperature until examined, preferably within a few days.

Cultural Methods for Enumeration and Identification of *Clostridium perfringens* in Foods

A. Equipment and materials

1. Pipets, 1.0 ml with 0.1 ml graduations, and 10.0 ml with 1.0 ml graduations
2. Colony counter
3. High speed blender, Waring or equivalent, and 1 L glass or metal blender jars with covers; 1 jar required for each sample
4. Anaerobic jars, BBL GasPak, or Oxoid anaerobic jars equipped with GasPak H_2 + CO_2 generator envelopes and catalyst
5. Incubator, 35°C
6. Petri dishes, sterile 15 x 100 mm
7. Platinum loop, 3 mm id
8. Water bath, 46 ± 0.5°C
9. Reversed passive latex agglutination (RPLA) test kit for *C. perfringens* enterotoxin (Oxoid USA, Columbia, MD)

B. Media and reagents

1. Tryptose-sulfite-cycloserine (TSC) agar (M169)
2. Egg yolk emulsion, 50% (M51)
3. Chopped liver broth (M38) or cooked meat medium (modified) (M43) (chopped liver is preferred)
4. Thioglycollate medium (fluid) (M146)
5. Iron milk medium (modified) (M68)
6. Lactose-gelatin medium (for *C. perfringens*) (M75)
7. Sporulation broth (for *C. perfringens*) (M140)
8. Motility-nitrate medium, buffered (for *C. perfringens*) (M102)
9. Spray's fermentation medium (for *C. perfringens*) (M141)
10. AE sporulation medium, modified (M5)
11. Duncan-Strong sporulation medium, modified (M45)
12. Peptone diluent (R56)
13. Nitrite detection reagents (R48)
14. Glycerin-salt solution (buffered) (R31)
15. Gram stain reagents (R32)
16. Fermentation test papers. Saturate 15 cm Whatman No. 31 filter paper disks with 0.2% aqueous bromthymol blue solution adjusted to pH 8-8.5 with ammonium hydroxide. Air-dry the disks and store for later use.

17. Bromthymol blue, 0.04% aqueous solution (R10).

C. Cultural and isolation procedures

Prepare Gram stain of sample and examine for large Gram-positive rods.

Plate count of viable *C. perfringens*. Using aseptic technique, place 25 g food sample in sterile blender jar. Add 225 ml peptone dilution fluid (1:10 dilution). Homogenize 1-2 min at low speed. Obtain uniform homogenate with as little aeration as possible. Using 1:10 dilution prepared above, make serial dilutions from 10^{-1} to 10^{-6} by transferring 10-90 ml peptone dilution fluid blanks. Mix each dilution thoroughly by gently shaking before each transfer. Pour 6-7 ml TSC agar without egg yolk into each of ten 100 x 15 mm petri dishes and spread evenly on bottom by rapidly rotating dish. When agar has solidified, label plates, and aseptically transfer 1 ml of each dilution of homogenate to the center of duplicate agar plates. Pour additional 15 ml TSC agar without egg yolk into dish and mix with inoculum by gently rotating dish.

An alternative plating method preferred for foods containing other types of sulfite-reducing organisms is to spread 0.1 ml of each dilution with sterile glass rod spreader over previously poured plates of TSC agar containing egg yolk emulsion. After inoculum has been absorbed (about 5 min), overlay plates with 10 ml TSC agar without egg yolk emulsion. When agar has solidified, place plates in upright position in anaerobic jar. Establish anaerobic conditions and place jar in 35°C incubator for 20-24 h. (TSC agar containing egg yolk is incubated 24 h.) After incubation, remove plates from anaerobic jar and select those containing 20-200 black colonies for counting. *C. perfringens* colonies in egg yolk medium are black with a 2-4 mm opaque white zone surrounding the colony as a result of lecithinase activity. Using Quebec colony counter with white tissue paper over counting area, count black colonies and calculate number of clostridia cells/g food. Save plates for identification tests (**see** D, below).

Prepare chopped liver broth (or cooked meat medium) for inoculation by heating 10 min in boiling water or flowing steam and cooling rapidly without agitation. Inoculate 3 or 4 broth tubes with 2 ml of 1:10 homogenate as back-up for preceding plating procedure. Incubate these tubes 24-48 h at 35°C in standard incubator. Disregard if plate counts for viable *C. perfringens* are positive.

D. Presumptive confirmation test

Select 10 typical *C. perfringens* colonies from TSC or TSC-egg yolk agar plates and inoculate each into a tube of freshly deaerated and cooled fluid thioglycollate broth. Incubate in standard incubator 18-24 h at 35°C. Examine each culture by Gram stain and check for purity. *C. perfringens* is a short, thick, Gram-positive bacillus. If there is

evidence of contamination, streak contaminated culture(s) on TSC agar containing egg yolk and incubate in anaerobic jar 24 h at 35°C. Surface colonies of *C. perfringens* are yellowish gray with 2-4 mm opaque zones caused by lecithinase activity. This procedure is also used for isolating *C. perfringens* from chopped liver broth whenever the organism is not detected by direct plating on TSC agar.

Iron-milk presumptive test. Inoculate modified iron-milk medium with 1 ml of actively growing fluid thioglycollate culture and incubate medium at 46°C in a water bath. After 2 h, check hourly for "stormy fermentation." This reaction is characterized by rapid coagulation of milk followed by fracturing of curd into spongy mass which usually rises above medium surface. Remove positive tubes to prevent spilling over into water bath. For this reason, do not use short tubes for the test. Cultures that fail to exhibit "stormy fermentation" within 5 h are unlikely to be *C. perfringens*. An occasional strain may require 6 h or more, but this is a questionable result that should be confirmed by further testing. Some strains of *C. baratii* react in this manner, but this species can be differentiated by its inability to liquefy gelatin in lactose-gelatin medium. The rapidity with which the "stormy fermentation" occurs depends on the strain and the initial population. Therefore, only actively growing cultures are appropriate for this test. The presumptive test in iron-milk medium may be sufficient for some purposes. However, the completed test must always be performed with isolates associated with food poisoning outbreaks. The following tests must be included for the completed test.

E. Completed confirmation test

Stab-inoculate motility-nitrate (buffered) and lactose-gelatin media with 2 mm loopfuls of pure fluid thioglycollate medium culture or portion of isolated colony from TSC agar plate. Stab lactose-gelatin repeatedly to ensure adequate inoculation, and then rinse loop in beaker of warm water before flaming to avoid splattering. Incubate inoculated media 24 h at 35°C. Examine lactose-gelatin medium cultures for gas production and color change from red to yellow, which indicates acid production. Chill tubes 1 h at 5°C and examine for gelatin liquefaction. If medium gels, incubate an additional 24 h at 35°C and examine for gelatin liquefaction.

Inoculate sporulation broth with 1 ml fluid thioglycollate medium culture and incubate 24 h at 35°C. Prepare Gram stain of sporulation broth and examine microscopically for spores. Store sporulated cultures At 4° if further testing of isolates is desired.

C. perfringens is nonmotile. Examine tubes of motility-nitrate medium for type of growth along stab line. Nonmotile organisms produce growth only in and along stab. Motile organisms usually produce diffuse growth out into the medium, away from the stab.

C. perfringens reduces nitrates to nitrites. To test for nitrate reduction, add 0.5 ml reagent A and 0.2 ml reagent B (R48) to culture in buffered motility-nitrate medium. Violet color which develops within 5 min indicates presence of nitrites. If no color develops, add a few grains of powdered zinc metal and let stand a few minutes. A negative test (no violet color) after zinc dust is added indicates that nitrates were completely reduced. A positive test after addition of zinc dust indicates that the organism is incapable of reducing nitrates.

Tabulate results. *C. perfringens* is provisionally identified as a nonmotile, Gram-positive bacillus which produces black colonies in TSC agar, reduces nitrates to nitrites, produces acid and gas from lactose, and liquefies gelatin within 48 h. Some strains of *C. perfringens* exhibit poor sporulation in sporulation medium or weak lecithinase reactions on TSC agar containing egg yolk. Organisms suspected to be *C. perfringens* which do not meet the stated criteria require additional testing for confirmation.

Subculture isolates which do not meet all criteria for *C. perfringens* into fluid thioglycollate medium. Incubate 24 h at 35°C, prepare Gram stain, and examine for purity and typical cell morphology.

Inoculate 0.1 ml pure fluid thioglycollate culture into 1 tube of freshly deaerated Spray's fermentation medium containing 1% salicin, 1 tube containing 1% raffinose, and 1 tube of medium without carbohydrate. Incubate media 24 h at 35°C and examine medium containing salicin for acid and gas. Test for acid by transferring a 2 mm loopful of culture to bromthymol blue test paper. Use only a platinum loop. No color change or development of a slight green color indicates that acid was produced. Alternatively, transfer 1.0 ml of culture to test tube or spot plate and add 1 or 2 drops of 0.04% bromthymol blue. A light green or yellow color indicates that acid was produced. Incubate media for another 48 h and test for acid production. Salicin is rapidly fermented with production of acid and gas by culturally similar species but usually is not fermented by *C. perfringens*. Acid is usually produced from raffinose within 3 days by *C. perfringens* but is not produced by culturally similar species. A slight change in pH can occur in the medium without fermentation of carbohydrates.

Some species of *Clostridium* occasionally isolated from foods have characteristics which differentiate them from *C. perfringens*.

C. paraperfringens and *C. baratii*--slender cells frequently in filamentous chains with large spherical bodies in cooked meat or other media containing carbohydrate; nitrite weak or absent after 18 h; very weak lecithinase production; gelatin never liquefied.

C. absonum or *C. sardiniensis*--young cultures may exhibit weak motility; gelatin slowly liquefied; strong lecithinase production; nitrite production weak or absent after 18 h.

C. celatum--similar to *C. paraperfringens*, except that cells form large mass in bottom of tube; usually grows very slowly; all reported isolates of *C. celatum* are from feces. *C. celatum* differs from *C. paraperfringens* by the absence of lecithinase activity and by the production of acid from starch.

Calculate number of *C. perfringens* cells in sample on the basis of percent of colonies tested that are confirmed as *C. perfringens*. Example: If average plate count of 10^{-4} dilution was 85, and 8 of 10 colonies tested were confirmed as *C. perfringens*, the number of *C. perfringens* cells/g food is 85 x (8/10) x 10,000 = 680,000. **NOTE:** The dilution factor with plates containing egg yolk is tenfold higher than that of the sample dilution because only 0.1 ml was plated.

F. Culturing procedures for sporulation and enterotoxin production

If isolates are to be tested immediately for sporulation and enterotoxin production, subculture in fluid thioglycollate broth as described above. Cultures to be stored or shipped to another laboratory for testing should be subcultured in Difco cooked meat medium and incubated for 24 h at 35°C, followed by an additional 24 h at room temperature. Store cooked meat culture at 4°. To subculture for sporulation and enterotoxin production, mix cooked meat culture with Vortex mixer and transfer 0.5 ml of the mixture to each of two tubes containing 10 ml of freshly steamed fluid thioglycollate medium. Heat one tube in a beaker of water or in a water bath at 75°C for 10 min, and incubate at 35°C for 18 h. Incubate the second tube at 35°C for 4 h, and use this culture to inoculate modified AE sporulation medium. For best results use 0.75 ml of 4 h thioglycollate culture to inoculate 15 ml of modified AE or modified Duncan-Strong sporulation media. Incubate inoculated spore broth at 35°C in anaerobic jar or incubator for 18-24 h.

Check resulting culture for spores by using a phase-contrast microscope or by examining stained smears. Fewer than 5 spores per microscopic field is not considered good sporulation.

Centrifuge a portion of the sporulated culture for 15 min at 10,000 x **g** and test cell-free culture supernatant for enterotoxin by using reversed passive latex agglutination (RPLA) test kit.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Bacteriological Analytical Manual

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Chapter 17

Clostridium botulinum

Authors

(Return to Table of Contents)

Clostridium botulinum is an anaerobic, rod-shaped sporeformer that produces a protein with characteristic neurotoxicity. Botulism, a severe food poisoning, results from ingestion of food containing botulinal toxin produced during the growth of these organisms in food. Although this food poisoning is rare, the mortality rate is high; the 962 recorded botulism outbreaks in the United States from 1899 to 1990 (2) involved 2320 cases and 1036 deaths. In outbreaks in which the toxin type was determined, 384 were caused by type A, 106 by type B, 105 by type E, and 3 by type F. In two outbreaks the foods implicated contained both types A and B toxins. The limited number of reports of C and D toxins as the causative agent of human botulism have not been generally accepted. However, all types except F and G, which have not been as thoroughly studied, are important causes of animal botulism.

Antigenic types of *C. botulinum* are identified by complete neutralization of their toxins by the homologous antitoxin; cross-neutralization by heterologous antitoxins does not occur or is minimal. There are seven recognized antigenic types: A, B, C, D, E, F, and G. Cultures of five of these types apparently produce only one type of toxin but all are given type designations corresponding to their toxin production. Types C and D cross-react with antitoxins to each other because they each produce more than one toxin and have at least one common toxin component.

Type C produces predominantly C_1 toxin with lesser amounts of D and C_2, or only C_2, and type D produces predominantly type D toxin along with smaller amounts of C_1 and C_2. Mixed toxin production by a single strain of *C. botulinum* may be more common than previously realized. There is a slight reciprocal cross-neutralization with types E and F, and recently a strain of *C. botulinum* was shown to produce a mixture of predominantly type A toxin, with a small amount of type F.

Aside from toxin type, *C. botulinum* can be differentiated into general groups on the basis of cultural, biochemical, and physiological characteristics. Cultures producing types C and D toxins are not proteolytic on coagulated egg white or meat and have a common metabolic pattern which sets them apart from the others. All cultures that produce type A toxin and some that produce B and F toxins are proteolytic. All type E strains and the remaining B and F strains are nonproteolytic, with carbohydrate metabolic patterns differing from the C and D nonproteolytic groups. Strains that produce type G toxin have not been studied in sufficient detail for effective and satisfactory characterization.

C. botulinum is widely distributed in soils and in sediments of oceans and lakes. The finding of type E in aquatic environments by many investigators correlates with cases of type E botulism that were traced to contaminated fish or other seafoods. Types A and B are most commonly encountered in foods subjected to soil contamination. In the United States, home-canned vegetables are most commonly contaminated with types A and B, but in Europe, meat products have also been important vehicles of foodborne illness caused by these types.

Measures to prevent botulism include reduction of the microbial contamination level, acidification, reduction of moisture level, and whenever possible, destruction of all botulinal spores in the food. Heat processing is the most common method of destruction. Properly processed canned foods will not contain viable *C. botulinum*. Home-canned foods are more often a source of botulism than are commercially canned foods, which probably reflects the commercial canners' great awareness and better control of the required heat treatment.

A food may contain viable *C. botulinum* and still not be capable of causing botulism. If the organisms do not grow, no toxin is produced. Although many foods satisfy the nutritional requirements for the growth of *C. botulinum*, not all of them provide the necessary anaerobic conditions. Both nutritional and anaerobic requirements are supplied by many canned foods and by various meat and fish products. Growth in otherwise suitable foods can be prevented if the product, naturally or by design, is acidic (of low pH), has low water activity, a high concentration of NaCl, an inhibitory concentration of $NaNO_2$ or other preservative, or two or more of these conditions in combination. Refrigeration will not prevent growth and toxin formation by nonproteolytic strains unless the temperature is precisely controlled and kept below 3°C. Foods processed to prevent spoilage but not usually refrigerated are the most common vehicles of botulism.

Optimum temperature for growth and toxin production of proteolytic strains is close to 35°C; for nonproteolytic strains it is 26-28°C. Nonproteolytic types B, E, and F can produce toxin at refrigeration temperatures (3-4°C). Toxins of the nonproteolytics do not manifest maximum potential toxicity until they are activated with trypsin; toxins of the proteolytics generally occur in fully (or close to fully) activated form. These and other differences can be important in epidemiological and laboratory considerations of botulism outbreaks. Clinical diagnosis of botulism is most effectively confirmed by identifying botulinal toxin in the blood, feces, or vomitus of the patient. Specimens must be collected before botulinal antitoxin is administered to the patient. Identifying the causative food is most important in preventing additional cases of botulism. **See** Examination of Canned Foods, Chapter 21.

Botulism in infants 6 weeks to 1 year of age was first recognized as a distinct clinical entity in 1976. This form of botulism results from growth and toxin production by *C. botulinum* within the intestinal tract of infants rather than from ingestion of preformed toxin. It is usually caused by *C. botulinum* types A or B, but a few cases have been caused by other types. Infant botulism has been diagnosed in most U.S. states and in every populated continent except Africa (1).

Constipation almost always occurs in infant botulism and usually precedes characteristic signs of neuromuscular paralysis by a few days or weeks. There is a broad range of severity of illness. Some infants show only mild weakness, lethargy, and reduced feeding and do not require hospitalization. Many have shown more severe symptoms such as weakened suck, swallowing, and cry; generalized muscle weakness; and diminished gag reflex with a pooling of oral secretions. Generalized muscle weakness and loss of head control in some infants reaches such a degree of severity that the patient appears "floppy." In some hospitalized cases, respiratory arrest has occurred, but most were successfully resuscitated, and with intense supportive care have ultimately recovered. As a result, the case-fatality rate (2%) for this form of botulism is low. Recovery usually requires at least several weeks of hospitalization (1).

Honey, a known source of *C. botulinum* spores, has been implicated in some cases of infant botulism. In studies of honey, up to 13% of the test samples contained low numbers of *C. botulinum* spores (3). For this reason, the FDA, the Centers for Disease Control and Prevention (CDC), and the American Academy of Pediatrics recommend not feeding honey to infants under the age of 1 year.

A. Equipment and materials

1. Refrigerator
2. Clean dry towels
3. Bunsen burner
4. Sterile can opener (bacteriological or puncture type)
5. Sterile mortar and pestle
6. Sterile forceps

7. Sterile cotton-plugged pipets
8. Mechanical pipetting device (**NEVER** pipet by mouth)
9. Sterile culture tubes (at least a few should be screw-cap tubes)
10. Anaerobic jars (GasPak or Case-nitrogen replacement)
11. Transfer loops
12. Incubators, 35 and 28°C
13. Sterile, reserve sample jars
14. Culture tube racks
15. Microscope slides
16. Microscope, phase-contrast or bright-field
17. Sterile petri dishes, 100 mm
18. Centrifuge tubes
19. Centrifuge, refrigerated, high-speed
20. Trypsin (1:250; Difco Laboratories, Detroit, MI)
21. Syringes, 1 and or 3 ml, sterile, with 25 gauge, 5/8 inch needles for injecting mice
22. Mice, 16-24 g (for routine work, up to 34 g)
23. Mouse cages, feed, water bottles, etc.
24. Millipore filters: 0.45 µm pore size

B. Media and reagents

1. Alcoholic solution of iodine (4% iodine in 70% ethanol) (R18)
2. Chopped liver broth (M38) or cooked meat medium (M42)
3. Trypticase-peptone-glucose-yeast extract (TPGY) (M151) broth or with trypsin (TPGYT) (M151a)
4. Liver-veal-egg yolk agar (M84) or anaerobic egg yolk agar (M12)
5. Sterile, gel-phosphate buffer, pH 6.2 (R29)
6. Absolute ethanol
7. Gram stain reagents (R32), crystal violet (R16), or methylene blue (R45) solutions
8. Sterile physiological saline solution (R63)
9. Monovalent antitoxin preparations, types A-F (obtain from CDC)
10. Trypsin solution (prepared from Difco 1:250)
11. 1 N Sodium hydroxide solution (R73)
12. 1 N Hydrochloric acid solution (R36)

C. Sample preparation

Preliminary examination. Refrigerate samples until testing, except unopened canned foods, which need not be refrigerated unless badly swollen and in danger of bursting. Before testing, record product designation, manufacturer's name or home canner, source of sample, type of container and size, labeling, manufacturer's batch, lot or production

code, and condition of container. Clean and mark container with laboratory identification codes.

Solid and liquid foods. Aseptically transfer foods with little or no free liquid to sterile mortar. Add equal amount of gel-phosphate buffer solution and grind with sterile pestle before inoculation. Alternatively, inoculate small pieces of product directly into enrichment broth with sterile forceps. Inoculate liquid foods directly into enrichment broth with sterile pipets. Reserve sample; after culturing, aseptically remove reserve portion to sterile sample jar for tests which may be needed later. Refrigerate reserve sample.

Opening of canned foods (**see** Chapter 21).

Examine product for appearance and odor. Note any evidence of decomposition. **DO NOT TASTE** the product under any circumstances. Record the findings.

D. Detection of viable *C. botulinum*

1. **Enrichment**. Remove dissolved oxygen from enrichment media by steaming 10-15 min and cooling quickly without agitation before inoculation.

   Inoculate 2 tubes of cooked meat medium with 1-2 g solid or 1-2 ml liquid food per 15 ml enrichment broth. Incubate at 35°C.

   Inoculate 2 tubes of TPGY broth as above. Incubate at 28°C. Use TPGYT as alternative only when organism involved is strongly suspected of being a nonproteolytic strain of types B, E, or F.

   Introduce inoculum slowly beneath surface of broth to bottom of tube. After 5 days of incubation, examine enrichment cultures. Check for turbidity, gas production, and digestion of meat particles. Note the odor.

   Examine cultures microscopically by wet mount under high-power phase contrast, or a smear stained by Gram reagent, crystal violet, or methylene blue under bright-field illumination. Observe morphology of organisms and note existence of typical clostridial cells, occurrence and relative extent of sporulation, and location of spores within cells. A typical clostridial cell resembles a tennis racket. At this time test each enrichment culture for toxin, and if present, determine toxin type according to procedure in F, below. Usually, a 5-day incubation is the period of active growth giving the highest concentration of botulinal toxin. If enrichment culture shows no growth at 5 days, incubate an

additional 10 days to detect possible delayed germination of injured spores before discarding sample as sterile. For pure culture isolation save enrichment culture at peak sporulation and keep under refrigeration.

2. **Isolation of pure cultures.** *C. botulinum* is more readily isolated from mixed flora of enrichment culture or original specimen if sporulation has been good.

   Pre-treatment of specimens for streaking. Add equal volume of filter-sterilized absolute alcohol to 1 or 2 ml of enrichment culture in sterile screw-cap tube. Mix well and incubate 1 h at room temperature. To isolate from sample, take 1 or 2 ml of retained portion, and add an equal volume of filter-sterilized absolute alcohol in sterile screw-cap tube. Mix well and incubate 1 h at room temperature. Alternatively, heat 1 or 2 ml of enrichment culture or sample to destroy vegetative cells (80°C for 10-15 min). **DO NOT** use heat treatment for nonproteolytic types of *C. botulinum*.

   Plating of treated cultures. With inoculating loop, streak 1 or 2 loopfuls of ethanol or heat-treated cultures to either liver- veal-egg yolk agar or anaerobic egg yolk agar (or both) to obtain isolated colonies. If necessary, dilute culture to obtain well-separated colonies. Dry agar plates well before use to prevent spreading of colonies. Incubate streaked plates at 35°C for about 48 h under anaerobic conditions. A Case anaerobic jar or the GasPak system is adequate to obtain anaerobiosis; however, other systems may be used.

E. Selection of typical *C. botulinum* colonies

Selection. Select about 10 well-separated typical colonies, which may be raised or flat, smooth or rough. Colonies commonly show some spreading and have an irregular edge. On egg yolk medium, they usually exhibit surface iridescence when examined by oblique light. This luster zone, often referred to as a pearly layer, usually extends beyond and follows the irregular contour of the colony. Besides the pearly zone, colonies of *C. botulinum* types C, D, and E are ordinarily surrounded by a wide zone (2-4 mm) of yellow precipitate. Colonies of types A and B generally show a smaller zone of precipitation. Considerable difficulty may be experienced in picking toxic colonies since certain other members of the genus *Clostridium* produce colonies with similar morphological characteristics but do not produce toxins.

Inoculation. Use sterile transfer loop to inoculate each selected colony into tube of sterile broth. Inoculate *C. botulinum* type E into TPGY broth. Inoculate other toxin types of *C. botulinum* into chopped liver broth or cooked meat medium. Incubate as described in D-1, above, for 5 days. Test for toxin production as described in F, below. To determine toxin type, **see** F-3, below.

Isolation of pure culture. Restreak toxic culture in duplicate on egg yolk agar medium. Incubate one plate anaerobically at 35°C. Incubate second plate aerobically at 35°C. If colonies typical of *C. botulinum* are found only on anaerobic plate (no growth on aerobic plate), the culture may be pure. Failure to isolate *C. botulinum* from at least one of the selected colonies means that its population in relation to the mixed flora is probably low. Repeated serial transfer through additional enrichment steps may increase the numbers sufficiently to permit isolation. Store pure culture in sporulated state either under refrigeration, on glass beads, or lyophilized.

F. Detection and identification of botulinal toxin

1. **Preparation of food sample**. Culture one portion of sample for detection of viable *C. botulinum*; remove another portion for toxicity testing, and store remainder in refrigerator. Centrifuge samples containing suspended solids under refrigeration and use supernatant fluid for toxin assay. Extract solid foods with equal volume of gel-phosphate buffer, pH 6.2, by macerating food and buffer with pre-chilled mortar and pestle. Centrifuge macerated sample under refrigeration and use supernatant fluid for toxin assay. Rinse empty containers suspected of having held toxic foods with a few milliliters of gel-phosphate buffer. Use as little buffer as possible to avoid diluting toxin beyond detection. To avoid or minimize nonspecific death of mice, filter supernatant fluid through a millipore filter before injecting mice. For non-proteolytic samples or cultures, trypsinize after filtration.

2. **Determination of toxicity in food samples or cultures**

   Trypsin treatment. Toxins of nonproteolytic types, if present, may need trypsin activation to be detected. Therefore, treat a portion of food supernatant fluid, liquid food, or TPGY culture with trypsin before testing for toxin. Do not treat TPGYT culture with trypsin since this medium already contains trypsin and further treatment may degrade any fully activated toxin that is present. Adjust portion of supernatant fluid, if necessary, to pH 6.2 with 1 N NaOH or HCl. Add 0.2 ml aqueous trypsin solution to 1.8 ml of each supernatant fluid to be tested for toxicity. (To prepare trypsin solution, place 0.5 g of Difco 1:250 trypsin in clean culture tube and add 10 ml distilled water, shake, and warm to dissolve. Analysts who are allergic to trypsin should weigh it in a hood or wear a face mask.) Incubate trypsin- treated preparation at 35-37°C for 1 h with occasional gentle agitation.

   Toxicity testing. Conduct parallel tests with trypsin-treated materials and untreated duplicates. Dilute a portion of untreated sample fluid or culture to 1:5,

1:10, and 1:100 in gel-phosphate buffer. Make the same dilutions of each trypsinized sample fluid or culture. Inject each of separate pairs of mice intraperitoneally (i.p.) with 0.5 ml untreated undiluted fluid and 0.5 ml of each dilution of untreated test sample, using a 1 or 3 ml syringe with 5/8 inch, 25 gauge needle. Repeat this procedure with trypsin-treated duplicate samples. Heat 1.5 ml of untreated supernatant fluid or culture for 10 min at 100°C. Cool heated sample and inject each of a pair of mice with 0.5 ml undiluted fluid. These mice should not die, because botulinal toxin, if present, will be inactivated by heating.

Observe all mice periodically for 48 h for symptoms of botulism. Record symptoms and deaths. Typical botulism signs in mice begin usually in the first 24 h with ruffling of fur, followed in sequence by labored breathing, weakness of limbs, and finally total paralysis with gasping for breath, followed by death due to respiratory failure. Death of mice without clinical symptoms of botulism is not sufficient evidence that injected material contained botulinal toxin. On occasion, death occurs from other chemicals present in injected fluid, or from trauma.

If after 48 h of observation, all mice except those receiving the heated preparation have died, repeat the toxicity test, using higher dilutions of supernatant fluids or cultures. It is necessary to have dilutions that kill and dilutions that do not kill in order to establish an endpoint or the minimum lethal dose (MLD) as an estimate of the amount of toxin present. The MLD is contained in the highest dilution killing both mice (or all mice inoculated). From these data, the number of MLD/ml can be calculated.

3. **Typing of toxin**. Rehydrate antitoxins with sterile physiological saline. **Do not use glycerin water**. Dilute monovalent antitoxins to types A, B, E, and F in physiological saline to contain 1 international unit (IU) per 0.5 ml. Prepare enough of these antitoxin solutions to inject 0.5 ml of antitoxin into each of 2 mice for each dilution of toxic preparation to be tested. Use the toxic preparation that gave the higher MLD, either untreated or trypsinized. Prepare dilutions of the toxic sample to cover at least 10, 100, and 1000 MLD below the previously determined endpoint of toxicity if possible (**see** 2, above). The untreated toxic preparation can be the same as that used for testing toxicity. If a trypsinized preparation was the most lethal, it will be necessary to prepare a freshly trypsinized fluid. The continued action of trypsin may destroy the toxin.

   Inject the mice with the monovalent antitoxins, as described above, 30 min to 1 h before challenging them with i.p. injection of the toxic preparations. Inject pairs of mice (protected by specific monovalent antitoxin injection) i.p. with each dilution of the toxic preparation. Also inject a pair of unprotected mice (no injection of antitoxin) with each toxic dilution as a control. The use of 4

monovalent antitoxins (types A, B, E, and F) for the unknown toxic sample prepared at 3 dilutions requires a total of 30 mice--6 mice for each antitoxin (24 mice) plus 2 unprotected mice for each of the 3 dilutions (6 mice) as controls. Observe mice for 48 h for symptoms of botulism and record deaths. If test results indicate that toxin was not neutralized, **repeat** test, using monovalent antitoxins to types C and D, plus polyvalent antitoxin pool of types A through F.

Screening Procedure for *Clostridium botulinum* Type E Spores in Smoked Fish

A. Equipment and materials

1. 12 mice (16-24 g, or up to 34 g) per subsample (24 or more required for positives)
2. Types A, B, E antisera
3. Saline, sterile, 0.85% NaCl (R63)
4. Trypsin (Difco); 1:250, 5% solution
5. Syringes, 1 and 3 ml, 25 gauge, 5/8 inch needle
6. Incubator 28°C
7. TPGY medium (M151)
8. Water bath, 37°C
9. Gel-phosphate diluent (R29)
10. Centrifuge, refrigerated
11. Plastic bags, strong and water-tight

B. Procedure

Incubation. Place each smoked fish subsample (which may consist of 1 or more fish, depending on size, and may be either vacuum-packed or bulk-smoked fish) in a strong water-tight plastic bag. Add freshly steamed and cooled TPGY broth to subsample. **NOTE**: Add enough TPGY broth to completely cover fish. Squeeze bag to expel as much air as possible and seal it with hot-iron bag sealer or other air-tight closure device. Incubate at 28°C for 5 days. Precautions should be taken during incubation period since bag may swell and split from gas formation.

Cultures. At end of incubation period, centrifuge 20 ml of TPGY culture from each subsample at 7500 x **g** rpm for 20 min. Use refrigerated centrifuge. Determine pH of TPGY. If above 6.5, adjust to 6.0-6.2 with HCl. Refrigerate for overnight storage.

Trypsinization. To 3.6 ml of culture, adjusted to pH 6.0-6.2, add 0.4 ml of 5% solution

of trypsin. Incubate at 35-37°C for 1 h. Remove culture and let cool to room temperature before injecting mice. Trypsinized extract cannot be stored overnight.

Toxicity screening. Dilute trypsinized and nontrypsinized broth cultures to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (**NOTE**: Do not store trypsinized material overnight.) Inject mice i.p. with 0.5 ml of each dilution. Inject 2 mice per dilution, i.e., trypsinized and nontrypsinized (total 12 mice per subsample). Observe mice for botulism symptoms and record condition of mice at frequent intervals for 48 h. If no deaths occur, no further tests are indicated. Deaths are presumptive evidence of toxin and should be confirmed.

Confirmation with protected mice. Dilute new portion of nontrypsinized or trypsinized culture (whichever showed the highest titer) to 1:5, 1:10, and 1:100 in gel-phosphate diluent. (Do not store trypsinized material overnight.) Inject 6 mice i.p. with 0.5 ml of 1:5 saline dilution of type E antiserum. These will be compared to 6 mice without this protection (controls). After 30 min, inject 0.5 ml of each dilution into 2 mice protected with antiserum and into 2 mice not so protected. Record their condition at intervals up to 48 h. If unprotected mice die and protected mice live, the presence of type E toxin is indicated. If all protected mice die, repeat confirmation with higher dilutions of toxic culture in type E-protected mice and with mice protected against *C. botulinum* types A and/or B antiserum. If all antiserum-protected mice die, send toxic culture media on dry ice to Division of Microbiological Studies (HFS-516), FDA, 200 C Street, S.W., Washington, DC 20204, for further tests. Isolate and identify cultures from samples containing toxin of type E, if possible.

Obtain *C. botulinum* antisera from Centers for Disease Control and Prevention, Atlanta, GA 30333, USA. Reconstitute lyophilized antisera with sterile saline. Dilute sera 1:5 with sterile saline for mouse injection.

If you have questions about the method, contact Haim Solomon, FDA. Telephone (202) 205-4469; FAX (202) 401-7740.

General Hints Regarding *C. botulinum* Toxin Analysis

1. The first 24 hours are the most important time regarding symptoms and death of mice: 98-99% of animals die within 24 hours. Typical symptoms of botulism and death may occur within 4 to 6 hours.

2. If deaths occur after 24 hours, be very suspicious, unless typical botulism symptoms are clearly evident.

3. If deaths occur in mice injected with the 1:2 or 1:5 dilution but not with any higher dilution, be very suspicious. Deaths may have been from nonspecific causes.

4. Mice can be marked on tails with dye to represent various dilutions. Dye does not come off easily.

5. Mice injected with botulinal toxin may become hyperactive before symptoms occur.

6. Food and water may be given to the mice right away; it will not interfere with the test.

7. Rehydrated antitoxin may be kept up to 6 months under refrigeration, and may be frozen indefinitely.

8. TPGY medium is relatively stable and can be kept 2-3 weeks under refrigeration.

9. With cooked meat medium, vortex tubes completely; toxin may adhere to meat particles.

10. Trypsin is not filtered. Use 0.5 g in 10 ml of distilled water. It can be kept up to 1 week under refrigeration.

Interpretation of Data (NOTE: Laboratory tests are designed to identify botulinal toxin and/or organisms in foods)

1. Toxin in a food means that the product, if consumed without thorough heating, could cause botulism.

2. Viable *C. botulinum* but no toxin in foods is not proof that the food in question caused botulism.

3. The presence of toxin in food is required for an outbreak of botulism to occur.

4. Ingested organisms may be found in the alimentary tract, but are considered to be unable to multiply and produce toxin in vivo, except in infants.

5. Presence of botulinal toxin and/or organisms in low-acid (i.e., above pH 4.6) canned foods means that the items were underprocessed or were contaminated through post-processing leakage.

- Swollen cans are more likely than flat cans to contain botulinal toxin since the organism produces gas during growth.

- Presence of toxin in a flat can may imply that the seams were loose enough to allow gas to escape.

- Botulinal toxin in canned foods is usually of a type A or a proteolytic type B strain, since spores of the proteolytics can be among the more heat-resistant.

- Spores of nonproteolytics, types B, E, and F, generally are of low heat resistance and would not normally survive even mild heat treatment.

6. The protection of mice from botulism and death with one of the monovalent botulinal antitoxins confirms the presence of botulinal toxin and determines the serological type of toxin in a sample.

7. The following reasons may explain why deaths occur in mice that are protected by one of the monovalent antitoxins:

    - There may be too much toxin in the sample.

    - More than one kind of toxin may be present.

    - Deaths may be due to some other cause.

    Retesting at higher dilutions of toxic fluids is required, and mixtures of antitoxins must be used in place of monovalent antiserum. Some other toxic material, which is not heat-labile, could be responsible if both heated and unheated fluids cause death. The heat-stable toxic substance could possibly mask botulinal toxin.

Safety Precautions for the *Clostridium botulinum* Laboratory

1. Place biohazard signs on doors to restrict entrance and keep the number of people in the laboratory to a minimum.

2. All workers in the laboratory should wear laboratory coats and safety glasses.

3. Use 1% hypochlorite solution to wipe laboratory table tops before and after work.

4. **NEVER PIPETTE ANYTHING BY MOUTH. USE MECHANICAL PIPETTORS.**

5. Use a biohazard hood for transfer of toxic material, if possible.

6. Centrifuge toxic materials in a hermetically closed centrifuge with safety cups.

7. Personally take all toxic material to the autoclave and see that it is sterilized immediately.

8. Do not work alone in the laboratory or animal rooms after hours or on weekends.

9. Have an eye wash fountain and foot-pedaled faucet available for hand washing.

10. No eating and drinking in the laboratory when someone works with toxins.

11. In a very visible location, list phone numbers where therapeutic antitoxin can be obtained in case of emergency. **THIS IS VERY IMPORTANT!**

12. Reduce clutter in the laboratory to a minimum and place equipment and other materials in their proper place after use.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 18

Yeasts, Molds and Mycotoxins

Authors

(Return to Table of Contents)

The large and diverse group of microscopic foodborne yeasts and molds (fungi) includes several hundred species. The ability of these organisms to attack many foods is due in large part to their relatively versatile environmental requirements. Although the majority of yeasts and molds are obligate aerobes (require free oxygen for growth), their acid/alkaline requirement for growth is quite broad, ranging from pH 2 to above pH 9. Their temperature range (10-35°C) is also broad, with a few species capable of growth below or above this range. Moisture requirements of foodborne molds are relatively low; most species can grow at a water activity (a_w) of 0.85 or less, although yeasts generally require a higher water activity.

Both yeasts and molds cause various degrees of deterioration and decomposition of foods. They can invade and grow on virtually any type of food at any time; they invade crops such as grains, nuts, beans, and fruits in fields before harvesting and during storage. They also grow on processed foods and food mixtures. Their detectability in or on foods depends on food type, organisms involved, and degree of invasion; the contaminated food may be slightly blemished, severely blemished, or completely decomposed, with the actual growth manifested by rot spots of various sizes and colors, unsightly scabs, slime, white cottony mycelium, or highly colored sporulating mold. Abnormal flavors and odors may also be produced. Occasionally, a food appears mold-free but is found upon mycological examination to be contaminated. Contamination of foods by yeasts and molds can result in substantial economic losses to producer, processor, and consumer.

Several foodborne molds, and possibly yeasts, may also be hazardous to human or animal health because of their ability to produce toxic metabolites known as mycotoxins. Most mycotoxins are stable compounds that are not destroyed during food processing or home cooking. Even though the generating organisms may not survive food preparation, the preformed toxin may still be present. Certain foodborne molds and yeasts may also elicit allergic reactions or may cause infections. Although most foodborne fungi are not infectious, some species can cause infection, especially in immunocompromised populations, such as the aged and debilitated, HIV-infected individuals, and persons receiving chemotherapy or antibiotic treatment.

The dilution plating and the direct plating methods may be used to detect fungi in foods. The direct plating method is more efficient than the dilution plating method for detecting individual mold species, including most of the toxin producers, but it is less effective in detecting yeasts. It is also used to determine whether the presence of mold is due to

external contamination or internal invasion. Methodology for testing the ability of isolates of toxigenic mold species to produce mycotoxins on sterile rice water substrate is included here.

Enumeration of Yeasts and Molds in Food--Dilution Plating Technique

A. Equipment and materials

1. Basic equipment (and appropriate techniques) for preparation of sample homogenate, see Chapter 1
2. Equipment for plating samples, see Chapter 3
3. Incubator, 25°C
4. Arnold steam chest
5. pH meter
6. Water bath, 45 ± 1° C

B. Media and reagents

Media

1. Dichloran rose bengal chloramphenicol (DRBC) agar (M183)
2. Dichloran 18% glycerol (DG18) agar (M184)
3. Plate count agar (PCA), standard methods (M124); add 100 mg chloramphenicol/liter when this medium is used for yeast and mold enumeration. This medium is not efficient when "spreader" molds are present.
4. Malt agar (MA)(M185)
5. Malt extract agar (Yeasts and Molds) (MEAYM)(M182)
6. Potato dextrose agar (PDA), dehydrated; commercially available (M127)

Antibiotic solutions

Antibiotics are added to mycological media to inhibit bacterial growth. Chloramphenicol is the antibiotic of choice, because it is stable under autoclave conditions. Therefore, media preparation is easier and faster due to the elimination of the filtration step. The recommended concentration of this antibiotic is 100 mg/liter medium. If bacterial overgrowth is apparent, prepare media by adding 50 mg/liter chloramphenicol before autoclaving and 50 mg/liter filter-sterilized chlortetracycline when the media have been tempered, right before pouring plates.

Prepare stock solution by dissolving 0.1 g chloramphenicol in 40 ml distilled water; add this solution to 960 ml medium mixture before autoclaving. When both chloramphenicol and chlortetracycline are used, add 20 ml of the above chloramphenicol stock solution to 970 ml medium before autoclaving. Then, prepare chlortetracycline stock solution by dissolving 0.5 g antibiotic in 100 ml distilled water and filter sterilize. Use 10 ml of this solution for each 990 ml of autoclaved and tempered medium. Refrigerate in the dark and re-use remaining stock solutions for up to a month. Stock solutions should be brought to room temperature before adding to tempered medium.

C. Procedures

Sample preparation

Analyze 25-50 g from each subsample; generally, larger sample sizes increase reproducibility and lower variance compared with small samples. Test individual subsamples or composite according to respective Compliance Program for the food under analysis. Add appropriate amount of 0.1% peptone water to the weighed sample to

achieve 10^{-1} dilution, then homogenize in a stomacher for 2 min. Alternatively, blending for 30-60 sec can be used but is less effective. Make appropriate 1:10 (1+9) dilutions in 0.1% peptone water. Dilutions of 10^{-6} should suffice.

Plating and incubation of sample

Spread-plate method. Aseptically pipet 0.1 ml of each dilution on pre- poured, solidified DRBC agar plates and spread inoculum with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

Pour-plate method. Use sterile cotton-plugged pipet to place 1.0 ml portions of sample dilution into prelabeled 15 x 100 mm Petri plates (plastic or glass), and immediately add 20-25 ml tempered DG18 agar. Mix contents by gently swirling plates clockwise, then counterclockwise, taking care to avoid spillage on dish lid. After adding sample dilution, add agar within 1-2 min; otherwise, dilution may begin to adhere to dish bottom (especially if sample is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate.

From preparation of first sample dilution to pouring or surface-plating of final plate, no more than 20 min (preferably 10 min) should elapse. **Note:** Spread plating of diluted sample is considered better than the pour plate method. When the pour plate technique is used, fungal colonies on the surface grow faster and often obscure those underneath the surface, resulting in less accurate enumeration. Surface plating gives a more uniform growth and makes colony isolation easier. DRBC agar should be used for spread plates only.

Incubate plates in the dark at 25°C. Do not stack plates higher than 3 and do not invert. **Note:** Let plates remain undisturbed until counting.

Counting of plates

Count plates after 5 days of incubation. If there is no growth at 5 days, re-incubate for another 48 h. Do not count colonies before the end of the incubation period because handling of plates could result in secondary growth from dislodged spores, making final counts invalid. Count plates containing 10-150 colonies. If mainly yeasts are present, plates with 150 colonies are usually countable. However, if substantial amounts of mold are present, depending on the type of mold, the upper countable limit may have to be lowered at the discretion of the analyst. Report results in colony forming units (CFU)/g or CFU/ml based on average count of triplicate set. Round off counts to two significant figures. If third digit is 6 or above, round off to digit above (e.g., 456 = 460); if 4 or below, round off to digit below (e.g., 454 = 450). If third digit is 5, round off to digit below if first 2 digits are an even number (e.g., 445 = 440); round off to digit above if first 2 digits are an odd number (e.g., 455 = 460). When plates from all dilutions have no colonies, report mold and yeast counts (MYC) as less than 1 times the lowest dilution used.

Isolate individual colonies on PDA or MA, if further analysis and species identification is necessary.

Enumeration of Molds in Foods--Direct Plating Technique for Foods That Can Be Handled with Forceps (Dried Beans, Nuts, Whole Spices, Coffee and Cocoa Beans, etc.)

A. **Equipment and materials**

1. Freezer, -20° C
2. Beakers, sterile, 300 ml

3. Forceps, sterile
4. Arnold steam chest
5. Water bath, 45 ± 1° C
6. Incubator, 25° C

B. **Media and reagents**

1. Dichloran rose bengal chloramphenicol (DRBC) agar (M183)
2. Dichloran 18% glycerol (DG18) agar (M184)
3. Antibiotic solutions (see previous section)
4. NaOCl (commercial bleach) solution, 10%
5. Sterile distilled water

C. **Analysis of non-surface-disinfected (NSD) foods**

Sample and media preparation

Before plating, hold sample at -20° C for 72 h to kill mites and insects that might interfere with analysis.

Prepare DRBC agar as described in the appendix. If DRBC is not available, or the water activity of the analyzed sample is less than 0.95, use DG18 agar. Media should be prepared no more than 24 h prior to use.

Plating and incubation of sample

From each sample, transfer about 50 g into a sterile 300 ml beaker. Using 95% ethanol-flamed forceps place intact food items on surface of solidified agar, 5-10 items per plate (depending on size of food item) 50 items total per sample.

Flame forceps between plating of each item. Use several forceps alternately to avoid overheating. Do not plate visibly moldy or otherwise blemished items.

Align 3-5 plates in stacks and identify with sample number plus date of plating. Incubate stacks, undisturbed in the dark at 25°C for 5 days. If there is no growth at 5 days of incubation, re-incubate for another 48 h to allow heat- or chemically-stressed cells and spores enough time to grow.

Reading of plates

Determine occurrence of mold in percentages. If mold emerged from all 50 food items, moldiness is 100%; if from 32 items, moldiness is 64%. Determine percent occurrence of individual mold genera and species in like manner. Experienced analysts may identify *Aspergillus, Penicillium* and most other foodborne mold genera directly on medium with low power (10-30X) magnification.

D. **Analysis of surface-disinfected (SD) foods**

Perform disinfection in clean laboratory sink, not stainless steel, free from any acid residues, with tap water running (precautions against chlorine gas generation). Wear rubber gloves and transfer about 50 g of sample into a sterile 300 ml beaker. Cover with 10% chlorine (commercial bleach) solution for 2 min, while swirling beaker contents gently but constantly in clockwise-counterclockwise motion. Decant 10% chlorine solution and give beaker contents two 1-min rinses with sterile distilled water. Prepare plates; plate sample, incubate, and read plates as in non-surface disinfected direct plating method, above. Compare NSD and SD results from the same sample to determine if moldiness was due mainly to surface contamination or to internal invasion and growth.

Isolate individual colonies on PDA or MA.

Fluorescence Microscopy Procedure for Quantitation of Viable and Nonviable Yeasts in Beverages

Methods for counting viable yeasts by plating are described above. A direct microscopic procedure for counting nonviable and viable yeasts in beverages and other liquid samples is presented here. Quantitating yeast cells by microscopy eliminates the need for extended incubation, thus reducing the analytical time required. All yeasts can be counted, and living and dead yeast cells can be differentiated.

A. Equipment and materials

1. Millipore disk filter holders for standard syringes
2. Millipore filters: AABG, 0.8 µm, black, gridded; 25 mm diameter
3. Syringes, disposable
4. Pipets
5. Forceps
6. Bibulous paper
7. Microscope slides and 24 x 24 mm coverslips
8. Fluorescence microscope: blue excitation; l0X eyepieces with Howard mold count or other eyepiece grid; 20X or 40X objective

B. Reagents

1. Aniline blue; 1% in M/15 K_2HPO_4 (M/15 is equivalent to 11.6 g/L), adjusted to pH 8.9 with K_3PO_4. A stock solution can be made; age improves fluorescence.
2. NaOH; 25 g in 100 ml water

C. Sample preparation for filterable liquids (e.g. water and grape juice)

Filter aliquot (usually 10 ml) of sample through Millipore filter (AABG, 0.8 µm, black, gridded).(Portion size can be increased or decreased, depending on level of contamination). Use Millipore disk filter holder which attaches to standard syringe. Make sure that syringe is accurate. If not, remove plunger, attach syringe to filter holder, and pipette 10 ml into syringe. Press all of sample through filter. Do this with air cushion of about 3 ml between plunger and sample. Keep filter holder vertical to ensure even distribution of sample over filter. Remove filter from filter holder and place on microscope slide; grid should be parallel to edges of slide to facilitate counting.

D. Sample preparation for non-filterable liquids that clog the filter (e.g. orange juice)

To suppress background interference in fluorescence microscope, mix 4 ml sample with 1 ml sodium hydroxide (25 g in 100 ml water). Shake well and wait 10 min. Place Millipore filter (AABG, 0.8 µm, black, gridded) on a piece of bibulous paper and spread 0.1 or 0.01 ml (depending on level of contamination) of sample over filter. When filter surface is dry, place filter on microscope slide, keeping grid parallel to edges of slide to facilitate counting.

E. Microscopic counting procedure

Cover filter with a drop of aniline blue, 1% in M/15 (11.6 g/L) K_2HPO_4, adjusted to pH 8.9 with K_3PO_4. Spread aniline blue stain over whole filter with glass rod or coverslip without touching filter itself. Wait about 5 min;

then cover filter with 24 x 24 mm coverslip.

Count yeasts, using fluorescence microscope with blue excitation. Use 10X eyepiece with Howard mold count or other eyepiece grid, and 20X (or 40X) objective. Count 3 squares of eyepiece grid in each field of filter not covered by gasket. Count budding yeasts as 1 cell if daughter cell is obviously smaller than mother cell. If they are approximately equal in size, count them as 2 cells. Count all yeasts located completely within an eyepiece square and all yeasts touching left and lower border of eyepiece square. Do not count yeasts touching right and upper borders.

This method also differentiates dead (heat- or formaldehyde-killed) and living yeast cells. Dead cells show fairly uniform fluorescence, and plasma may be granular. In living cells, the cell wall stains brighter and is more defined than the plasma, which is less prominent and uniformly stained.

F. **Calculations to determine number of yeasts per ml**

Determine area of filter covered by 1 square of eyepiece grid, using objective (stage) micrometer. For filtered samples, the working area of the Millipore filter (portion not covered by the gasket) is 380 mm^2. For nonfiltered samples, it is the entire filter, or 491 mm^2, since no gasket is used.

$$\text{No. of yeasts per ml} = \frac{\text{No. of yeasts counted}}{\text{No. of eyepiece squares examined}} \times \frac{\text{working area of filter}}{\text{area of one eyepiece square}} \times \frac{1}{\text{volume of liquid}}$$

NOTE: For non-filterable liquids, volume includes only net amount used and not volume of NaOH added (i.e., 80% of total volume applied to filter).

For background information on the method, including photographs of dead and living yeast cells, **see** Koch et al., ref. 8, below.

Methods for Determining Toxin Production by Molds

A. **Equipment and materials**

1. Erlenmeyer flasks, 300 ml, wide-mouth
2. Cotton, nonabsorbent
3. Funnels, short-stem glass, 90-100 mm diameter
4. Filter paper, 18 cm diameter, folded (Schleicher & Schuell No. 588)
5. Boiling chips, silicon carbide
6. Fume hood equipped with steam bath; air-flow rate, 100 cubic ft/min
7. Blender, high speed, explosion-proof
8. Thin layer chromatographic apparatus or high-performance liquid chromatograph
9. Incubator, 22-25°C

B. **Media and reagents**

1. Long or short grain polished rice
2. Chloroform for extraction of aflatoxins, ochratoxins, sterigmatocystin, xanthomegnin, luteoskyrin, patulin, penicillic acid, citrinin, T-2 toxin, zearalenone
3. Methanol for extraction of deoxynivalenol
4. Appropriate mycotoxin standards
5. NaOCl solution, 5%

C. **Toxin production**

Into 300 ml wide-mouth Erlenmeyer flask, add 50 g rice and 50 ml distilled water. Plug flasks with cotton and autoclave 20 min at 121°C and 15 psi. Aseptically multispore-inoculate separate cooled flasks with individual mold isolates. Incubate inoculated flasks at 22-25°C until entire surface is covered with growth, and mycelium has penetrated to bottom of flask (15-20 days). To each flask, add 150 ml chloroform (150 ml methanol if toxin in question is deoxynivalenol), using short-stem glass funnel inserted alongside unremoved cotton plug (to minimize mold spore dissemination). Heat flask contents in fume hood on steam bath until solvent begins to boil. (Conduct all subsequent steps in fume hood.) With spatula, break up moldy rice cake and transfer flask contents into explosion-proof blender and blend at high speed for 1 min. Filter blender contents through filter paper inserted into short-stem glass funnel. Collect filtrate in 300 ml Erlenmeyer flask. Return rice cakes to blender, add 100 ml unheated solvent and blend 1 min at high speed. Filter as above and combine filtrates. Add boiling chips to flask containing filtrates and evaporate with steam to 20-25 ml. If analysis is not to follow immediately, evaporate to dryness and store flask in the dark. Rinse all glassware, etc., used for extraction in 5% NaOCl solution before soap and water cleansing. Submerge rice cake in 5% NaOCl solution for 72 h before autoclaving and disposal.

D. **Toxin analysis**

Appropriate mycotoxin standards are required for both qualitative and quantitative analysis of toxin. Use either thin layer chromatography as described in references 16 or 17 or high performance liquid chromatography,as described in reference 15a, to determine mycotoxins extracted from mold cultures. Naturally occurring mycotoxins in foods or feeds can best be determined by methods described in *Official Methods of Analysis* (16).

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Media

Dichloran 18% glycerol (DG18) agar(M184)

Glucose	10.0 g
Peptone	5.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Dichloran (2,6-dichloro-4-nitroaniline) solution (0.2% (w/v) in ethanol)	1.0 ml
Chloramphenicol	0.1 g
Agar	15.0 g
Distilled water	800 ml

Mix above items and steam to dissolve agar, then bring volume to 1000 ml with distilled water. Add 220 g glycerol (analytical reagent grade), and sterilize by autoclaving at 121°C for 15 min. Temper medium to 45° C and pour plates under aseptic conditions. The final a_w of this medium is 0.955. DG18 agar is used as a general purpose mold enumeration medium and is preferred when the a_w of the analyzed food is less than 0.95. The low water activity of this medium reduces interference by bacteria and fast-growing fungi. When both yeasts and molds must be enumerated,

DRBC agar should be used.

Dichloran rose bengal chloramphenicol (DRBC) agar (M183)

Glucose	10.0 g
Bacteriological peptone	5.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Rose bengal (5% aqueous soln., w/v)	0.5 ml
Dichloran (0.2% in ethanol, w/v)	1.0 ml
Chloramphenicol	0.1 g
Agar	15.0 g
Distilled water	1.0 liter

Final pH should be 5.6

Mix ingredients, heat to dissolve agar and sterilize by autoclaving at 121°C for 15 min. Temper to 45 ± 1° C in a water bath and pour plates.

Notes: DRBC agar is especially useful for analyzing samples containing "spreader" molds (e.g. *Mucor*, *Rhizopus*, etc.), since the added dichloran and rose bengal effectively slow down the growth of fast-growing fungi, thus readily allowing detection of other yeast and mold propagules, which have lower growth rates.

Media containing rose bengal are **light-sensitive**; relatively short exposure to light will result in the formation of inhibitory compounds. Keep these media in a dark, cool place until used. DRBC agar should be used for spread plates only.

Malt Agar (MA)(M185)

Malt extract, powdered	20.0 g
Agar	20.0 g
Distilled water	1.0 liter

Mix ingredients, steam to dissolve agar and sterilize for 15 min at 121° C. Temper medium to 45° C and pour plates under aseptic conditions. To prepare slants dispense 5-6 ml of steamed medium (before autoclaving) into each of several 16 x 125 mm screw-cap tubes, loosely cap tubes and sterilize as above. After autoclaving lay tubes in a slanting position and let them cool. This medium is recommended as a general maintenance medium.

Malt Extract Agar (Yeasts and Molds)(MEA) (M182)

Malt extract, powdered	20.0 g
Glucose	20.0 g
Peptone	1.0 g
Agar	20.0 g
Distilled water	1.0 liter

Mix ingredients, heat to dissolve agar and sterilize at 121° C for 15 min. Temper media to 45° C and pour plates under

aseptic conditions. Dehydrated MEA is commercially available, but since more than one MEA formula exists, check for the appropriate composition. This medium is recommended for the identification of *Aspergillus* and *Penicillium*.

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*Authors: Valerie Tournas, Michael E. Stack, Philip B. Mislivec, Herbert A. Koch and Ruth Bandler
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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Center for Food Safety & Applied Nutrition

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Chapter 19

Parasitic Animals in Foods

Authors

(Return to Table of Contents)

Humans unknowingly consume microscopic and small macroscopic animals with their food. The intestinal tract is inhospitable to most of these organisms, which are either digested or evacuated in the feces. However, some obligate or facultative parasites may become established in the human body. Although a number of parasites produce no symptoms and are not associated with disease, others may cause mild, moderate, or severely acute illness and even permanent damage. The following methods are used to examine foods and food-contact materials for the presence of parasites. For the most part, these techniques are labor-intensive and tedious; work continues to refine them and to develop additional techniques and rapid methods. Several alternative ways to examine fish and shellfish are presented. However, candling is the only method currently used for regulatory purposes with finfish.

I. Digestion to Select Mammalian Parasites in Edible Flesh

By mimicking the chemical and temperature conditions of the mammalian stomach, this method frees parasites from the surrounding flesh and reduces the background of nonparasitic organisms.

A. Equipment and materials

1. Balance, at least 250 g capacity
2. Stirrer or rotating incubator shaker
3. Water bath, 37 ± 0.5°C
4. Beakers, 100 and 1500 ml
5. Sedimentation cone and support, 1 L, plastic with removable plug, e.g., Imhoff cone
6. Tubing, amber gum, 2.4 and 9.5 mm diameter
7. Tubing clamp
8. Microscopes, dissecting and inverted
9. Culture dishes, plastic, various sizes
10. Sieve No. 18 (U.S. standard sieve series), 1 mm mesh, 204 mm diameter, 51 mm high; other sizes optional
11. Tray, rectangular, polypropylene, about 325 x 260 x 75 mm
12. Cylinder, graduated, 1 L
13. pH meter
14. Pasteur pipets, or polypropylene eyedroppers
15. Rubber bulb, about 2 ml capacity
16. Spoon
17. Spatula
18. Optional materials: blender, meat grinder, food processor, negative pressure hood, foil, plastic wrap, tweezers, and dissecting needles

B. Reagents

1. Physiological saline (R63)
2. Pepsin, laboratory grade
3. pH reference solutions
4. HCl, concentrated
5. Optional reagents: papain, ethanol, glacial acetic acid, glycerine, lactophenol, phenol, formalin, Lugol's iodine (R40), ether

C. Sampling and sample preparation

From a sample weighing 1 kg, take a subsample (100 g) of beef, pork, or poultry, or 250 g of fish. Subsamples of most mammalian meat, poultry, or fish require no further preparation. They may be torn or separated into 5 or more pieces to increase the surface area. Samples with relatively large amounts of connective tissue are not readily digested; snail meat, for example, is digested very poorly. The following methods improve digestion. A 100 g sample is blended in 750 ml saline. Ten intermittent, instantaneous bursts in a blender will destroy some macroscopic organisms but usually will not affect

microscopic organisms. A meat grinder is less destructive, although not suitable for some foods such as snails. Least destructive is initial digestion with papain followed by pepsin digestion.

CAUTION: Pathogens that are easily disseminated may be contained in samples and will be liberated by digestion. Of special concern are macroscopic tapeworm cysts and microscopic cysts of protozoa. When the presence of such pathogens is suspected, carry out the digestion and subsequent sample handling in a negative pressure hood until the suspect digest is placed in a safely closed dish. Handle all utensils as if contaminated, and autoclave or incinerate after use.

D. Digestion, sedimentation, and examination

Adjust incubator-shaker or water bath to 37 ± 0.5°C. Prepare digestion fluid in 1500 ml beaker by dissolving 15 g pepsin in 750 ml saline, add sample, and adjust to pH 2 with concentrated HCl (about 3 ml). Place in incubator or water bath and stir (about 100 rpm) after equilibration for about 15 min; check and adjust pH again. Cover beaker with aluminum foil (if using stirrer, pierce hole for stirring rod) and continue incubating until digestion is complete. The time required for digestion will vary but should not exceed 24 h.

Carefully pour beaker contents through sieve into tray. Rinse remains with 250 ml saline and add to digest. Examine rinsed contents of sieve and record results. Larger parasites will remain on sieve. Replace plug of sedimentation tube with rubber tubing and clamp folded tubing. Carefully transfer contents of tray to sedimentation cone. Transfer undigested sample or parasites to a petri dish, using spoon, tweezers, or dissecting needle.

After 1 h of sedimentation, remove bottom 50 ml of sediment by releasing clamp and collecting in 100 ml beaker. Transfer fluid to petri dish(es) with eyedropper. (Digests vary in their clarity; if digest is dense, dilute with saline until it is translucent.) Cover dish and examine macroscopically for parasites; then examine with dissecting microscope and finally with inverted microscope (a contrasting background can be helpful). Count, tentatively identify, and record observations. Count total number of organisms and differentiate those that are living (motile) and dead (nonmotile), if possible. Examine complete contents of beaker. Light infections may require repeated sampling to detect parasites.

E. Interpretation and further identification

Further information about recovered organisms is usually required, both to classify them and to decide whether the criterion of movement is valid for determining viability. For

example, the eggs of *Ascaris* must be "embryonated," i.e., allowed to incubate so that moving embryos develop inside. Cysts of some protozoa must be excysted to detect motion; those of *Toxoplasma gondii* can be judged viable only by the outcome of experimental inoculation into the peritoneal cavity of mice. Brief summaries of fixation and staining methods for frequently recovered parasites are given below and in the following references: invertebrates in general parasitology (2); animals' parasites (11); medical aspects of parasitology (10); food parasitology: methods, references, expert consultants (4,7); immunology and serology of parasitic diseases (8); protozoa (9); nematodes (3,16); trematodes (1,14); cestodes (15); arthropods (5).

F. Fixation and staining

Protozoan cysts and helminth eggs. Fix and stain fresh material with Lugol's iodine solution (R40) or use fluorescent antibody stain (if available) on formalin-fixed material.

Nematodes. Fix in glacial acetic acid overnight and store in 70% ethanol with 10% glycerin. Study nematode morphology in temporary mounts by removing from alcohol and clearing in glycerin, lactophenol, or phenol ethanol. Before returning to storage, rinse away clearing fluid(s) with 70% ethanol. Sectioning and staining may be necessary for detailed identification of nematodes.

Trematodes and cestodes. Before fixation, relax both trematode and cestode flatworms in cold distilled water for 10 min. Fix flukes (trematodes) in hot (60°C) 10% formalin. Fix tapeworms (cestodes) by adding 10X volume of 70°C fixative to the relaxing fluid, or dip them in 70°C water repeatedly; then fix in a mixture of ethanol, glacial acetic acid, and formalin (85:10:5) overnight. Store in 70% ethanol. Flatworms are usually stained and mounted as permanent slides, but some may require sectioning and staining for detailed identification.

Acanthocephala. Place in water to evert the proboscis. (Some proboscises may evert almost immediately; others require several hours. Do not extend over 8 h.) Fix in steaming 70% ethanol with a few drops of glacial acetic acid. Store in fixative or 70% ethanol. Acanthocephala may be stained and mounted as permanent slides or, like the nematodes, cleared in phenol or glycerol.

Arthropoda. Fix fleas, lice, mites, copepods, fly larvae, and other parasitic or food-inhabiting arthropods in hot water. Store in 70% ethanol.

Keep parasite specimens in tightly capped vials with identifying data (anatomic location of source in host, geographic origin, date of sample collection, date of parasite collection, collector's name, presumed identification) written in indelible pencil or ink on slip of hardened paper. Place paper in liquid-filled vial with parasite. Assistance in

identification may be obtained from Dan-My Chu, CFSAN, FDA, 200 C St., S.W., Washington, DC 20204; Ann M. Adams, FDA, 11510 W. 80th St., Lenexa, KS 66214; or Marie Chaput, FDA, 109 Holton St., Winchester, MA 01890. Please send a minimum of three whole parasites of each type found and all head/tail fragments as well as all pertinent information to the nearest of the three individuals named above.

G. Viability determination

The major criterion for the viability of helminths is spontaneous movement. Observe organisms for 10 min to see if spontaneous movement occurs. If autonomous movement is not observed, touch with a dissecting needle and observe to see if movement has been stimulated. Allow specimens from salted products to equilibrate in at least 20X volumes of physiological saline for 3 h before viability determination; osmotic pressure may cause apparent movement. Culturable protozoa should be cultured in vitro to determine viability. If culture is not feasible, dye exclusion is the method of choice for viability determination.

II. Candling to Detect Parasites in Finfish

The following procedures are used to determine parasites in finfish. The candling procedure is applicable to fresh or frozen fish with white flesh processed as fillets, loins, steaks, chunks, or minced fish. The ultraviolet (UV) light procedure is for fish with dark flesh and for breading removed from fish portions.

NOTE: This method is not applicable to dried fish or fish in the round.

A. Equipment and materials

Sharp knife

Candling table. Rigid framework to hold light source below rigid working surface of white, translucent acrylic plastic or other suitable material with 45-60% translucency. Length and width of working surface should be large enough to permit examination of entire fillet, e.g., 30 x 60 cm sheet, 5-7 mm thick.

Light source. "Cool white" with color temperature of 4200 K. At least two 20-watt fluorescent tubes are recommended. Tubes and their electrical connections should be constructed to prevent overheating of light source. Average light intensity above working surface should be 1500-1800 lux, as measured 30 cm above center of acrylic sheet. Distribution of illumination should be in ratio of 3:1:0.1, i.e., brightness directly above

light source should be 3 times greater than that of outer field, and brightness of outer limit of visual field should be not more than 0.1 that of inner field. Illumination in examining room should be low enough not to interfere with detection of parasites, but not so dim as to cause excessive eye fatigue.

B. Reagents (for preservation reagents, **see** section I. B-5, above)

C. Sample preparation

Weigh entire sample and record weight on analytical reporting form.

Fillets. If fillets are large (200 g or larger), use one fillet for each of the 15 subsamples. If fillets are small (less than 200 g), randomly select fillets to prepare 15 subsamples of approximately 200 g each. Record actual weight analyzed for each subsample. If fillets are more than 30 mm thick, cut with a sharp knife into 2 pieces of approximately equal thickness (not to exceed 30 mm per fillet). Examine both pieces as described below. If fillets have a thickness of 20 mm or less, examine whole.

Fish blocks. Analyze 15 subsamples randomly selected from 2 thawed and drained blocks. Prepare the subsamples as described for fillets, above. Note separately any parasites observed in minced fish added to block around subsamples.

Steaks, loins, chunks. Prepare as for fillets.

Minced fish. If frozen in blocks, analyze 15 subsamples randomly selected from 2 thawed and drained blocks. Prepare subsamples as described for fillets, above. Select portions from different parts of block. If not in blocks, analyze 15-200 g portions. Do not further shred or chop minced fish.

Breaded fish portions. Thaw frozen products at room temperature in a beaker of appropriate size. After thawing, pour hot (50°C) solution of 2% sodium lauryl sulfate in water over fish in increments of 100 ml per 300 g of product. Stir with glass rod for 1 min. Let stand for at least 10 min or until breading separates from flesh. Transfer individual portions to No. 10 sieve nested over No 40 sieve. Wash breading through No. 10 sieve with gentle stream of warm tap water. Periodically examine No. 40 sieve containing the breading, using UV light. Parasites will appear fluorescent under this light. Note any parasites detected and record on the analytical reporting form. Discard breading by backflushing the No. 40 sieve with tap water. Examine fish portions by candling, using white light. If the flesh is pigmented, use UV light.

D. Examination

Parasites near the surface will appear red, tan, cream-colored, or chalky white. Parasites deeper in the flesh will appear as shadows. Remove representative types of parasites or other defects found. Record general location, size, identification, and other observations as outlined below. For minced fish, spread portion on light table to depth of 20-30 mm for examination. Select representative parasites for descriptive analysis.

E. Ultraviolet examination of dark-fleshed fish

Visually examine each portion (de-breaded or de-skinned, as necessary) on both sides under a desk lamp or similar light source. A magnifying desk lamp may be used. Report findings as described below. Conduct UV examination in darkened room. Examine each portion on both sides with reflected longwave UV light (366 nm wavelength). Parasites should fluoresce blue or green under light of this wavelength. Fish bones and connective tissues, which also fluoresce blue, may be differentiated by their regular distribution and shape. Bone fragments will be rigid when probed (6).

CAUTION: Never expose unprotected eyes to UV light from any source either direct or reflected. Always wear appropriate eye protection such as goggles with uranium oxide lenses, welder's goggle, etc., when such radiations are present and unshielded. Keep skin exposure to UV radiations to a minimum.

F. Parasite identification. Fix parasites as described in section I.F, above.

III. Compression Candling: Detection of Parasites in Molluscs and other Translucent Foods

Parasites may be detected visually in such translucent foods as white-fleshed fish and shellfish by observing the outline of the organism or its capsule in transmitted light. The method described was developed to examine the viscera and muscle of the surf clam *Spisula solidissima* for the presence of *Sulcascaris* sp. nematodes, but is also applicable to other foods and parasites. However, not all parasites are detected (12), probably because they are obscured by the shadows produced by connective tissue. The method was compared with two other visual methods for detecting nematodes in the calico scallop, *Argopectin gibbus*. Compression candling detected more nematodes and yielded fewer false positives than the other two methods.

A. Equipment and materials

1. Hinged Plexiglas plates 305 x 305 mm. To construct plates, attach two 305 mm (about 12 inch) squares of 3/8 inch Plexiglas stacked plates to a piano hinge so

that they are separated by 3 mm with six 32-5/8 inch machine screws. If proper size piano hinge is not available, a nominal 1-inch hinge can be retapped to give proper spacing. Attach a 3-mm spacer to each end of the surface of one plate at the edge opposite the piano hinge.
2. Light box
3. Knife
4. Specimen vials or jars
5. Dissecting needles
6. Petri dish

B. Reagents

1. Physiological saline solution, 0.85% (R63)
2. Glacial acetic acid
3. 70% Ethanol

C. Method

1. Distribute portion of sample on inside of plastic plate. Quantity to be examined at one time depends on size and thickness of sample. Samples over 100 g cannot be compressed. Cut cylindrical samples (e.g., scallops) in half longitudinally to facilitate compression.
2. Close plate and squeeze outer edges firmly.
3. Examine each side of plate for parasites by placing on light table. Parasites in flesh appear as shadows.
4. Record number of parasites. To confirm that parasites are present, mark plate with wax pencil, open, and check by dissection.
5. Fix representative sample to confirm identity (**see** section I.F, above).

IV. Mechanical Disruption and Sedimentation for Detection of Larval Parasites in Fish Flesh

This method detects larval anisakids in the flesh of fillets. It is not applicable to fish that have been treated with salt without being de-boned, such as pickled herring, and, in general, may not be applicable to species such as herring. Subsamples should not exceed 200 g in the food processor, but may be pooled for final analysis.

A. Materials

1. Food processor; Cuisinart Model DLC 10, Moulinex Model 663, or equivalent.

2. Glass tray, 350 x 25 x 60 mm
3. Beaker, 1000 ml
4. White fluorescent lamp
5. UV lamp, <365 nm, or similar, light box
6. Appropriate eye protection
7. Glass rod
8. Forceps
9. Vials or jars
10. Fixative

B. Method

Fillet and skin fish before weighing; then place in food processor with plastic dough hook in place. Add 35°C water equal to twice the weight of the fish. Activate food processor intermittently until flesh is dissociated (1-2 min). Pour into beaker and wait 30-60 s before decanting all but 100 ml of the supernatant fluid. Add water and stir; then wait 30-60 s, and decant to 100 ml again (2X).

*Place about 25 ml of sediment in glass tray; dilute until quite translucent or until depth of 10 mm is reached (about 375 ml). Examine, collect, or count parasites. Agitation of sediment with forceps may aid in recovery, and forceps will be useful in collecting parasites. Record parasite movement. Collect and fix a representative portion of the parasites present for identification (**see** section I.F, above). Examine under high intensity (>500 µW/cm^2) shortwave (about 365 µm) light. Parasites will fluoresce blue or yellow-green. Count and record. Repeat from *, above, until sample is complete.

V. Concentration of Helminths and Protozoa from Vegetables

Vegetables may become contaminated with parasitic organisms through contact with animal or human fecal material or through application of sewage-derived fertilizer to croplands (13). The method outlined below can be used to examine fresh vegetables for parasites. A similar method recovered *Cryptosporidium* sp. from 1% of water samples examined. Recovery from vegetables would be estimated at 1% or less. (A sample consists of five 1-kg subsamples.)

A. Equipment and materials

1. Balance
2. Polypropylene beakers, 1 L
3. Sonic bath, about 2 L capacity
4. Centrifuge, large capacity, low speed with swinging bucket

5. Polypropylene centrifuge tubes, 50 ml
6. Eye dropper, polypropylene
7. Culture dish with 2 mm grid
8. Microscope slides

B. Reagents

1. Lugol's iodine (R40)
2. Sheather's fluid (500 g sucrose, 320 ml deionized water, 6.5 g phenol)
3. Detergent solutions Nos. 1, 2, and 3

   No. 1- 2.5% formaldehyde, 0.1% sodium dodecyl sulfate (SDS), 0.1% Tween 80
   No. 2- 1% Tween 80, 1% SDS
   No. 3- 1% Tween 80
4. Fluorescent antibody kit

C. Procedure

Store vegetables in refrigerator before analysis. Separate vegetables into units: **tight head type** (cabbage), remove outer 3 layers of leaves; **loose head type** (leaf lettuce), separate individual leaves; **root type** (carrot), no preparation; **floret type** (cauliflower), separate into florets of about 50 g. *Pour 1-1.5 liters of detergent solution No. 1 into sonic bath and add vegetables loosely to about 250 g; operate bath for 10 min. Remove vegetables individually and drain well. Repeat from *, above, until subsample is completely sonicated.

Transfer detergent to beaker; then dispense all of the material into 50 ml centrifuge tubes. Centrifuge at 1200 x *g* for 10 min. Remove supernatant to 1.5-2 ml and consolidate sediment into one tube with eyedropper or plastic pipet. Rinse each tube twice with 1.5 ml detergent No. 2, and add to consolidation tube. Rinse and centrifuge sediment twice with detergent No. 2. Dilute to 10 ml with detergent No. 3 and sonicate for 10 min. Add 25 ml Sheather's fluid to clean centrifuge tube and layer on detergent suspension from sonic bath. Centrifuge at 1200 x *g* for 30 min. Remove 7 ml of fluid from interface and transfer to centrifuge tube; fill tube with detergent; then centrifuge at 1200 x *g* for 10 min. *Remove supernatant and dilute with detergent No. 3. Then centrifuge for 10 min at 1200 x *g*. Repeat from *, above, 2 times.

For helminth eggs, transfer sediment to gridded petri plate and add 1 ml Lugol's iodine. Dilute and examine entire plate with inverted microscope. **For protozoa**, dilute sediment sufficiently with detergent No. 3 to make translucent 100 µl thin smears on cleaned polylysine-coated microscope slide cleaned with acid alcohol.

Let slides air-dry. Add positive and negative control samples to separate well or slide and let air-dry. Follow manufacturer's instructions for fluorescent antibody staining. Examine each slide at 200-300X with fluorescent microscope. Record results. If sample is positive, calculate number of cysts present per kg of food specimen by measuring remaining suspension and estimating number. If sample is negative, stain and examine remaining sediment.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 19 A.

Concentration and Preparation of *Cyclospora* from Berries for the Polymerase Chain Reaction (pcr) and Microscopy

Authors

(Return to Table of Contents)

A. **Reagents & Materials**

1. **Water**
   a. Deionized Water (for washing produce)
   b. Sterile Deionized Water (for PCR)

2. **Glass Beads** (Sigma #G-9139, TMS siliconized, 140-270 mesh)

3. **Sterile 1X TE, pH 7.4**. (Digene catalog #3400-139 or equivalent)

4. **50 ml Centrifuge Tubes** (e.g., 50 ml polypropylene centrifuge tubes with caps,

Gibco #925-4900XT) or **250 ml centrifuge tubes** (Sorvall Instruments #03939 or equivalent) depending on the centrifuge and rotor used. Reusable tubes are thoroughly washed in detergent, rinsed 5X (including at least 2X in deionized water) and drained.

5. **Epi-illuminated Fluorescence Microscope** equipped with a UV 1A filter block (Excitation Filter, EX 365/10; Dichroic mirror, DM 400; Barrier Filter, BA-400; or equivalent)

6. **GeneAmp PCR Core Reagent Kit II** (Perkin Elmer Catalog #N808-0009) (no $MgCl_2$)

   Components: AmpliTaq polymerase, 250 Units, 5U/µl

   GeneAmp deoxynucleoside triphosphates (dNTPs), set of four; consists of dATP, dTTP, dGTP and dCTP, each vial containing 320 µl of a 10 mM solution of the specified dNTP.

   GeneAmp 10X PCR Buffer II, 1.5 ml, consisting of 500 mM KCl and 100 mM Tris-HCl, pH 8.3.

   $MgCl_2$ solution, 1.5 ml, 25 mM

7. **Primers**: CYCF1E, CYCR2B, CYCF3E and CYCR4B are described in Relman et. al., (J. Infect. Dis. 173:440-445, 1996) and in Table 1. Primers may be commercially prepared (e.g., Midland Certified Reagent Co.). Store primers at -20°C as a stock solution of 100 M in deionized water. Prepare a working solution of 10 µM in sterile deionized water and store at -20°C (in a non-self-defrosting freezer, if possible).

Table 1: PCR Primer Sequences

Primer	**Sequence (5' - 3')**	**Use**
CYCF1E	GGAATTCCTACCCAATGAAAACAGTTT	1st PCR - forward
CYCR2B	CGGGATCCAGGAGAAGCCAAGGTAGG	1st PCR - reverse
CYCF3E	GGAATTCCTTCCGCGCTTCGCTGCGT	2nd PCR - forward
CYCR4B	CGGGATCCCGTCTTCAAACCCCCTACTG	2nd PCR - reverse

8. **Agarose** (SeaKem LE Agarose FMC BioProducts catalog #50004 or equivalent)

9. **Ethidium Bromide** (Sigma Chemical Co. catalog #E-8751 or equivalent)

10. **1X TBE**

    100 ml 10X TBE (Digene catalog #3400-1036 or equivalent product).
    900 ml deionized water.

11. **Gel Loading Buffer**.

    1 ml Reconstituted Bromphenol blue-Xylene Cyanole Dye Solution (Sigma catalog #B-3269 or equivalent)

    0.6 ml Sterile Glycerol (J.T. Baker Inc. catalog #4043-00 or equivalent)

    qs to 2 ml with sterile deionized water

12. **100 bp Molecular Weight Ladder** (BioMarker Low, BioVentures 101, catalog #M-1 or equivalent)

13. **Polaroid Type 667 and Type 665 film**. Polaroid Corporation, Cambridge, MA.

14. **Restriction Endonuclease Enzyme *Mnl*I**. (Amersham Life Sciences Inc., Arlington Heights, IL) comes with **Buffer M** (catalog #E0215Y)

15. **4% NuSieve 3:1 agarose** or **GTG agarose**, FMC, Rockland, ME (catalog #50091 or #50081).

B. **Recovery of Oocysts**

Wash Method for Fresh Produce (Berries, Lettuce, etc.) or Puree (For Vinaigrette, Proceed to Step 4)

1. Prepare a ziplock or Stomacher bag containing 250 ml of deionized water (Reagent #1).

2. Add 250-500 g of produce (berries +/- 1 berry; USE ONLY INTACT BERRIES, because the juice from cut or broken berries may be inhibitory to PCR and debris may interfere with microscopy). Use 250 g of puree (because this consists of mashed berries, juice and debris are unavoidable).

3. Agitate gently for 20 min by placing on a platform shaker set at 60 to 150 cycles per min, depending on the condition of the produce (especially the berries). Invert the bag at 10 min to ensure thorough washing.

4. Pour suspension into the centrifuge tubes.

5. Centrifuge 50 ml tubes at 1500 X *g* for 10 min. Centrifuge 250 ml tubes at 1500 X *g* for 20 min.

6. Decant or aspirate supernatant and discard, leaving approximately 1 ml of supernatant and pellet fraction. If the 50 ml tubes are used for centrifugation, combine all pellet material and residual supernatant into one tube. The volume of the residual supernatant and pellet fraction varies, depending on the type and condition of samples, and the volume should be recorded. Appropriate aliquots will be removed from this tube for microscopy (Section C) and PCR (Section D). Store the remaining portion at 4°C for up to one month. After one month, dilute the remaining material with an equal volume of 2.5% potassium dichromate, mix, and store at 4°C.

NOTE: If the sediment for the puree is too gelatinous, dilute with equal volume of deionized water. When decanting or aspirating samples with small pellets (e.g. from lettuce), adjust the volume of supernatant to less than one ml. Leaving more than one ml of supernatant on top of small pellets may make microscopy difficult.

C. **Slide Preparation and Microscopic Analysis**

Cyclospora oocysts autofluoresce cobalt blue with the UV-1A emission filter or blue-green with broader emission spectra filters under ultraviolet illumination. Prepare slides in duplicate, and examine slides under ultraviolet illumination as described below.

Laboratories should use a microscope reticle capable of measuring 8-10 µm to check on cyst size when organisms are recovered. Compare presumptive oocysts to those in a known standard.

Slide Preparation

1. Apply silicone vacuum grease to edge of cover slip.

2. Place 10 µl sediment to a clean glass slide and prepare a wet mount using pre-greased cover slip.

Microscopy

1. Examine slide under UV light at 400 X. *Cyclospora* oocysts fluoresce cobalt blue. Determine cyst size and compare to a standard.

2. Switch from epi-fluorescence microscopy to bright field microscopy or differential interference contrast microscopy to confirm internal structures of presumptive *Cyclospora* oocysts. Recheck size and again compare to a standard.

3. Seal cover slips to the glass slides of presumptive positives with fingernail polish, slide compound or paraffin wax.

4. Document presumed positive samples with photographs taken at multiple planes.

D. **PCR Analysis**

Template Extraction and Preparation:

1. Remove 100 µl of produce sediment prepared in Section B, Step 6 and transfer to a sterile cryo-microcentrifuge tube (Sarstedt #72.694.006 or equivalent tube [should be screw-capped and durable enough to with-stand the freeze/thaw procedure]). Trim the ends from small bore pipet tips or use wide-bore pipet tips (Rainin #HR-250WS or equivalent)to facilitate pipetting of produce sediment.

2. Centrifuge at 14,000 RPM (15,800 X *g*) for 3 min and discard supernatant.

3. Wash the pellet once with 500 µl TE (Section A, Reagent #3) and centrifuge at 14,000 RPM for 3 min.

4. Resuspend in 100 µl TE (Section A, Reagent 3).

5. Vortex approximately 10-30 sec to resuspend pellet.

6. Complete 3 freeze/thaw cycles, each 2 min in liquid nitrogen or a dry ice-ethanol bath followed by 2 min in a 98°C water bath.

7. Add 0.1 ± 0.02 g glass beads (Sigma #G-9139) to extract.

8. Agitate the suspension for 5 min with a vortex mixer or thermomixer, set at maximum speed. Then chill on ice for 5 min. A mixer attachment for holding multiple samples is useful at this step.

9. Centrifuge sample extract at 14,000 RPM (15,800 X *g*) for 3 min.

10. Transfer the supernatant to new sterile microcentrifuge tube. This extract can be stored frozen (-20°C) until ready for the PCR analysis, or if needed as reserve in case of template inhibition problems.

11. Combine 20 µl sample extract and 2 µl freshly prepared Non-fat Milk Solution (50 mg instant nonfat dried milk,]Janet Lee Brand or any food-grade equivalent(in 1 ml sterile deionized water. The entire 22 µl will be used as template in a 100 µl PCR amplification.

PCR Conditions:

1. Prepare reaction mix as indicated in Table 2:

Table 2: First PCR Reaction Components

Component	**5l**	**Final concentration**
sterile deionized water	43.5	
10X PCR Buffer II	10.0	50 mM KCl, 10 mM Tris-HCl, pH 8.3
dATP, dCTP, dGTP, dTTP 10 mM each dNTP mixed to give a final concentration of 2.5 mM	8.0	200 µM each dNTP
$MgCl_2$; 25 µM	8.0	2.0 mM
Primer CYCF1E (10 µM)	4.0	0.4 µM
Primer CYCR2B (10 µM)	4.0	0.4 µM
AmpliTaq polymerase, 5U/5l	0.5	2.5 Units
Template DNA	22.0	Sample extract prepared above

2. Mix tubes by gently tapping and add 50 µl (or 2 drops) sterile mineral oil if required for thermal cycler model.

3. All PCR analyses should include positive and negative controls for each experiment. Positive control DNA template can be prepared by the extraction of DNA from *Eimeria tenella* or *Cyclospora cayatenensis* oocysts using the method

described above. For the positive control reaction use extracted DNA equivalent to 25 to 50 oocysts as a template. In a separate reaction 20 µl of produce extract spiked with extracted DNA equivalent to 25 to 50 oocysts should be included as a control for detecting inhibition by produce extracts. If inhibition by 20 µl of berry extract is observed, a smaller volume (1 to 10µl) of the produce extract should be assayed in attempt to overcome the inhibition (appropriate controls should be included).

4. PCR cycling protocol is as shown in Table 3:

Table 3: First PCR thermal cycling parameters

Step	**Temperature/Duration**
Initial Inactivation/Denaturation: 1 cycle	94°C/3 min
Amplification: 45 cycles	
Denaturation	94°C/30 sec
Annealing	55°C/30 sec
Extension	72°C/90 sec
Final extension: 1 cycle	72°C/9 min
Hold (until next PCR round)	4° C

5. For the second round of PCR add 2 µl of the first PCR product to 48 µl of new reaction mix containing no template or test fraction. Refer to Table 4 for individual reaction components:

Table 4: Second PCR Reaction Components

Component	**µl**	**Final concentration**
sterile deionized water	30.75	
10X PCR Buffer II	5.0	50 mM KCl,10 mM Tris-HCl, pH 8.3
dATP, dCTP, dGTP, dTTP 10 mM each dNTP mixed to give a final concentration of 2.5 mM	4.0	200 micromolar each dNTP
$MgCl_2$; 25 mM	4.0	2.0 mM

Primer CYCF3E, 10 µM	2.0	0.4 µM
Primer CYCR4B, 10 µM	2.0	0.4 µM
AmpliTaq polymerase, 5U/5l	0.25	1.25 µM Units
Template DNA	2.00	From first PCR

6. The second PCR cycling conditions are identical to the first except that the annealing temperature is 60°C.

Gel Electrophoresis:

1. Mix 10 µl of the amplification product from the second round of PCR with 2 µl gel loading buffer (0.25% bromphenol blue, 0.25% xylene cyanole and 30% glycerol or equivalent). If mineral oil has been used, wipe the pipet tip before mixing reaction material with loading buffer.

2. Load the entire volume into a well of a 1.2% agarose gel prepared with Tris Borate EDTA (TBE) buffer and 0.5 µg/ml ethidium bromide. Alternatively, the gel (without ethidium bromide) can be post-stained for 15 min with ethidium bromide (1.0 µg/ml) in TBE. A 100 bp ladder Molecular Weight marker (BioMarker Low, BioVentures 101, Murfreesboro, TN or equivalent) is very useful for identifying the 308 bp DNA amplification product, especially from primer-dependent material visible at around 100 bp that is not *Cyclospora* DNA.

3. Electrophorese the gel at 8-V/cm for approximately 1 h.

4. PCR products are visualized by UV transillumination and photographed using Polaroid Type 667 film. The predicted size after F1E/R2B, is 651 bp; after F3E/R4B is 308 bp. Note: The amplified product after the first round may not be visible; therefore, only product from the second round of PCR should be electrophoresed.

E. Restriction Endonuclease Fragment Length Polymorphism (RFLP) Analysis of PCR Amplified Products:

1. A PCR product of 308 bp after the second PCR round in the nested PCR is a presumptive positive for *Cyclospora* or *Eimeria*. The band pattern resulting from digestion of the amplification product with the restriction enzyme *Mnl*I distinguishes *Cyclospora* from *Eimeria* amplification products.

   Combine 10 µl amplification product from the second round of PCR with one

unit *Mnl*I (Amersham Life Sciences Inc., Arlington Heights, IL) and 5 µl 10X Buffer M. Adjust the final reaction volume to 50 µl with sterile deionized water.

Prepare separate restriction digests for each presumptive positive PCR amplification product and the amplification products from control *Cyclospora cayetanensis* and *Eimeria tenella* strains. A digest including 1 µg Bacteriophage lambda DNA should also be prepared to demonstate complete digestion by the enzyme.

2. Incubate the restriction digests 1 h in a 37°C water bath.

3. Mix 10 µl of each restriction endonuclease digest with 2 µl of loading buffer (0.25% bromphenol blue, 0.25% xylene cyanole and 30% glycerol). Load the entire volume into a well on a 4% NuSieve 3:1 or GTG agarose (FMC, Rockland, ME) gel prepared with Tris Borate EDTA (TBE) buffer. Alternate lanes should contain a molecular size standard ladder (BioMarker Low, BioVentures 101, Murfreesboro, TN).

4. Electrophorese the gel at 5 volts/cm for 3 h or until the first dye front is approximately 1 cm from the end of the gel.

5. Post-stain the gel in 1X TBE containing 1 µg/ml ethidium bromide (Sigma Chemicals, St. Louis, MO) for 10 to 15 min and destain in deionized water for 1 to 5 min.

6. Place the gel on a UV transilluminator and photograph with Type 667 (1 second at f4.5) and/or Type 665 film (50-60 seconds at f4.5). Develop the negatives from Type 665 film as per manufacturer's instructions (Polaroid Corp., Cambridge, MA).

7. Acquire images by transmission densitometry from the Polaroid Type 665 film negatives with gel scanner (Sharp JX-325 with film scan unit or equivalent) as *.tif bitmap images (Adobe Photoshop v. 3.0 or equivalent). Gel analysis software (RFLPScan 3.0 (beta) Scanalytics Inc., Billerica MA or equivalent) can be used to analyze gels with user interactive algorithms for the band (peak) detection and relative peak area (% integrated optical density, OD) calculations. Band detection parameters of lane width of 39-29.66, band height threshold of 6-4, and smoothing operator pixel length of 2-50, allow for the automatic detection of all peaks. The molecular size standards are entered and properly associated with the standard peaks for the lanes containing the standards. The lanes are calibrated using the RFLPScan "de-smile" method with external lane standards and log piecewise linear regression. Band data may then be exported to a

spreadsheet program (EXCEL 5.0, Microsoft, Redmond, WA or equivalent).

Alternatively, the band position analysis may be performed by measuring the band migration distances from the bottom of the gel wells to the nearest 0.25 mm with a ruler and using the SeqAid II program ver. 3.81 (D.J. Roufa, Manhattan, KS or equivalent), or generating a calibration curve by plotting the logarithm of the number of base pairs of each standard band versus the migration distance. Table 5 lists the predicted fragment sizes for *Cyclospora* and *Eimeria* amplified products, observed fragment sizes should be within 5% of the predicted bp sizes.

Table 5: Predicted Restriction Endonuclease *Mnl*I Fragment Sizes(1).

	Predicted Fragment Sizes		
Organism	**Fragment 1**	**Fragment 2**	**Fragment 3**
***Cyclospora* spp.**	146	114	48
***Eimeria* spp.**	132	114	62

[1] The 18s rRNA gene sequences for 9 *Eimeria* spp. have been submitted to GenBank. This 3 band RFLP pattern is predicted for 7 of the *Eimeria* spp. based on the GenBAnk sequence data. Two of the *Eimeria* spp., *E. nieschulzi* and *E. bovis* are predicted to produce 2 RFLP patterns of 114 and 194 bp, and 115 and 195 bp, respectively. This RFLP pattern also would be distinct from the *Cyclospora* spp. RFLP pattern.

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1 FDA
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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Bacteriological Analytical Manual

Online

January 2001

Chapter 26

Detection and Quantitation of Hepatitis A Virus in Shellfish by the Polymerase Chain Reaction

Author

(Return to Table of Contents)

Hepatitis A virus (HAV), a major cause of infectious hepatitis in humans, is a positive strand RNA virus belonging to the hepatovirus group of the picornavirus family. Primary detection of HAV in clinical or biological samples is not routinely possible at present because wild-type HAV grows very poorly in cell culture. Except for virus preparations that have been adapted for rapid growth in cell culture, HAV does not produce a detectable cytopathic effect in infected cells. For these reasons, several laboratories have attempted to develop methods based on nucleic acid hybridization or the polymerase chain reaction (PCR) for detecting HAV. The reverse transcription-PCR (RT-PCR) procedure described here for detecting HAV in shellfish can be adapted for use in clinical samples, which have a much higher viral load and which contain less extraneous material that could interfere with RT-PCR. Also described here are the synthesis and use of a competitor template RNA for determination of the number of HAV genomic RNA molecules in a sample. We emphasize, however, that the method described here **does not** provide information about the infectious viral load in a sample, but simply the number

of RNA molecules having the HAV specific sequence.

A. **Equipment and materials**

1. DNA thermal cycler
2. Agarose gel electrophoresis set-up
3. Gel electrophoresis power supply
4. UV box
5. Liquid scintillation spectrometer
6. Computer for regression analyses of [^{32}P]nucleotide incorporation data
7. Water saturated phenol (from supplier)
8. 10 mM Tris-HCl (pH 8.00) - 1 mM EDTA (TE)
9. TE saturated phenol:chloroform:isoamyl alcohol (24:24:1) (PCIA)
10. Plasmid DNA purification kit (QIAGEN)
11. Eppendorf refrigerated microcentrifuge
12. Refrigerated high speed centrifuge
13. Water bath
14. Restriction enzymes *Bam*HI and *Hind*III (**see** ref. 1); also *Eco*RI.
15. SP6 RNA polymerase
16. Reverse transcriptase
17. Thermostable DNA polymerase
18. 3 M sodium acetate (pH 5.5)
19. RNAse-free DNAse
20. Vanadyl ribonucleoside complex
21. Forward primer 5'ATGCTATCAACATGGATTCATCTCCTGG3'
22. Reverse primer 5'CACTCATGATTCTACCTGCTTCTCTAATC3'
23. RNAse inhibitor, human placenta
24. Oligo $(dT)_{12\text{-}16}$
25. Unlabeled dNTPs
26. [^{32}P]dATP or dCTP
27. Plasmid pHAV6 (available from author)

B. **Synthesis of competitor template RNA**

The competitor template RNA is synthesized in vitro from the plasmid pHAV6 with SP6 RNA polymerase. The competitor RNA harbors a 63 base deletion in the region amplified by the PCR primer pair. This enables separation of the PCR product originating from wild-type viral RNA from the product generated from the competitor RNA in the same reaction tube. The design and construction of the plasmid pHAV6 was described in detail elsewhere (1).

For RNA synthesis, purify the plasmid by using a Qiagen plasmid kit and following the

manufacturer's instructions. Linearize the plasmid by using *Eco*RI; synthesize RNA and purify as previously described (1).

C. **Isolation of tissue RNA from hard shell clams**

Virtually any method of RNA isolation that provides good quality RNA will suffice. Isolate RNA by direct homogenization of fresh or frozen tissue in 10 volumes of 50 mM sodium acetate buffer (pH 5.5)-2 mM EDTA-1% SDS, and 10 volumes of water-saturated phenol in an Omni mixer at full speed for 1 min. Shake the mixture vigorously for 10 min and centrifuge (all centrifugations are at 10,000 x *g* for 10 min at 4C unless otherwise stated) to separate the phases. Re-extract the upper aqueous layer with an equal volume of water-saturated phenol as described above. Adjust the aqueous phase to 0.2 M in sodium acetate and precipitate total nucleic acids with 2.5 volumes of ethanol. Collect the bulky precipitate by centrifugation. Remove contaminating DNA, tRNA, and polysaccharides by three successive washes with ice cold 3 M sodium acetate (pH 5.5). Carry out these washes by thoroughly resuspending the pellet with a disposable plastic rod in 3 M sodium acetate, incubating on ice for 10-30 min, and centrifuging at 10,000 x *g* for 15-20 min. Dissolve the final pellet completely in water, and precipitate high molecular weight RNA from a mixture of 0.2 M sodium acetate and 2.5 volumes of ethanol. Remove any remaining DNA by digesting with RNase-free DNase in 10 mM Tris-HCl (pH 7.5)-10 mM magnesium chloride-100 mM sodium chloride, 5 mM vanadyl ribonucleoside complex-50 units/ml RNase-free DNase for 30 min at 37°C (this cleanup is not necessary when dealing with small amounts of tissue). Extract the RNA twice with PCIA (**see** A9) and then precipitate as described. Dissolve the final pellet in water and quantitate at an absorbance of A_{260}. The yield of RNA is approximately 1 mg/g of tissue with an A_{260}/A_{280} ratio of at least 1.8. When virus is added to tissue before RNA isolation, virus particles are added to 0.5 ml of a 10% homogenate of tissue in extraction buffer only. RNA is then isolated as described above by phenol extraction, except that all extractions are done in Eppendorf tubes. After adding an equal volume of phenol, vortex the tubes for 1 min and centrifuge to separate the phases. Repeat the phenol extraction once. Carry out the 3 M sodium acetate extractions with 0.5 ml of solution and omit the DNAse digestion (2).

D. **Reverse transcription (RT)**

Carry out RT of RNA in 20 µl reaction mixtures containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 10 mM $MgCl_2$, 1 mM concentrations of each of the four deoxyribonucleoside triphosphates (dNTPs), 10 mM dithiothreitol, 1 unit of human placental RNAse inhibitor per µl, 0.5 µg oligo$(dT)_{16}$, 15 units of avian myeloblastosis virus reverse transcriptase, and indicated amounts of seeded or unseeded tissue RNA and competitor template RNA. Incubate reactions at 22°C for 10 min followed by 50 min at

42°C, 5 min at 99°C, and 5 min at 4°C. Centrifuge the reaction tubes for 5 min at full speed in an Eppendorf centrifuge (2).

E. **PCR**

Perform PCR amplification in 50 µl volumes, which contain 5-10 µl cDNA pool from the RT reactions, 3 mM $mgCl_2$, 200 µM of each dNTPs, 0.5 µM of each amplification primer, and 1.5 units of *Taq* DNA polymerase. Denature the mixture initially for 3 min at 95°C, followed by 35-40 cycles of amplification, each consisting of 90 s at 94°C, 90 s at 63°C, and 120 s at 72°C. Perform a final extension at 72°C for 10 min; analyze 20 µl of PCR reaction by gel electrophoresis. When quantitation is desired, perform the PCR reaction in the presence of 5 µCi per reaction of [-^{32}P]dCTP in addition to unlabeled dCTP. Separate the products by gel electrophoresis, and excise and count bands corresponding to the wild-type and competitor template in a liquid scintillation counter (1).

F. **Quantitation of viral RNA molecules**

Figure 1 shows the results of an experiment to quantitate the number of viral RNA molecules in a crude virus preparation obtained from the Centers for Disease Control and Prevention, Atlanta, GA. To avoid losses during RNA isolation, crude virus preparation was diluted 500-fold in RNase-free water and heated at 95°C for 5 min to dissociate RNA-protein complexes and then chilled on ice. A 1 µl aliquot of heated virus was then mixed with several concentrations of competitor RNA and reverse transcribed. From each cDNA pool, 5 µl was then amplified by PCR as described above, except that ^{32}P dATP was used as the label. Products were separated in a 3% NuSieve-1% agarose gel. Bands corresponding to wild-type and competitor PCR products were cut out and counted. Incorporation into PCR product generated from competitor RNA was corrected for the loss of A and T residues caused by deletion of 63 base pairs. The ratio of incorporation in the two products was then plotted against the number of competitor RNA molecules added and a linear regression graph was obtained. The number of viral RNA molecules in the crude virus preparation is the same as the number of competitor RNA molecules added when the ratio of incorporation in the two PCR products is equal to one. Based on the results of the experiment, the number of viral RNA molecules in the virus preparation was estimated to be 2.5×10^8/ml.

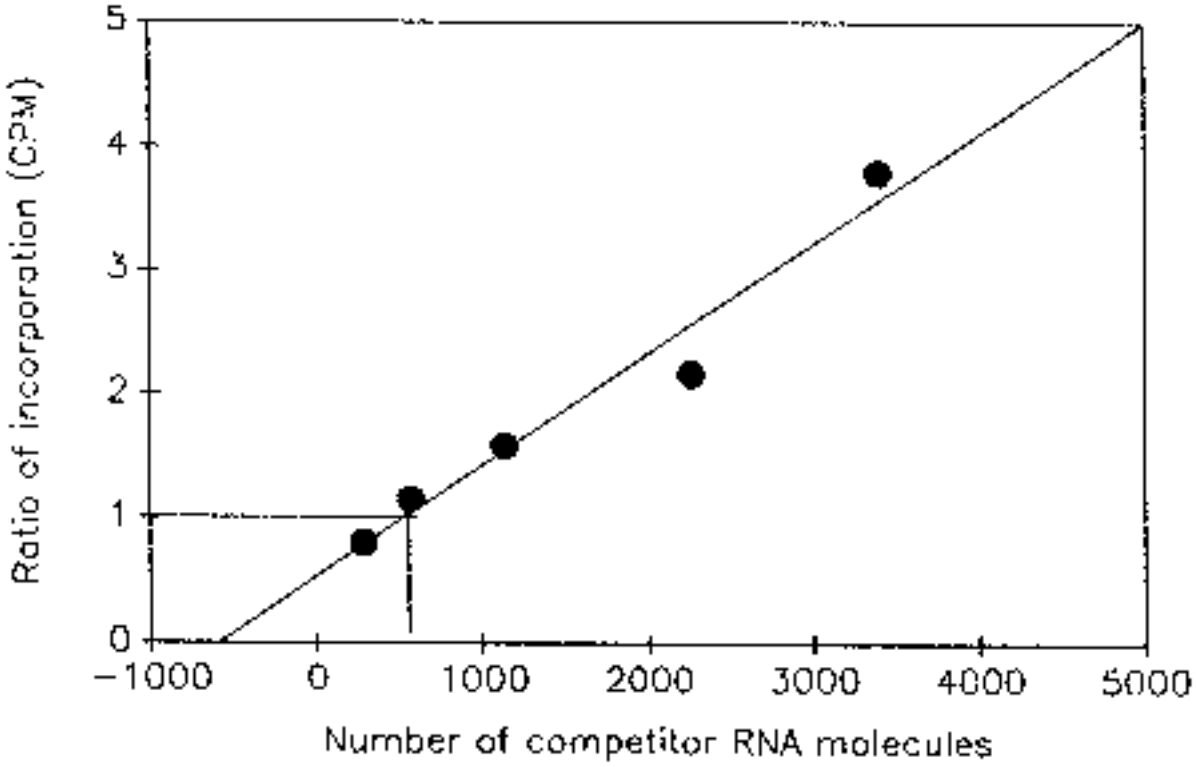

Figure 1. Quantitation of viral RNA molecules in a crude virus preparation by competitive PCR. A fixed concentration of heated virus was mixed with increasingly higher numbers of a competitor RNA molecule and reverse transcribed. A portion of the cDNA pool was then amplified as described in the text.

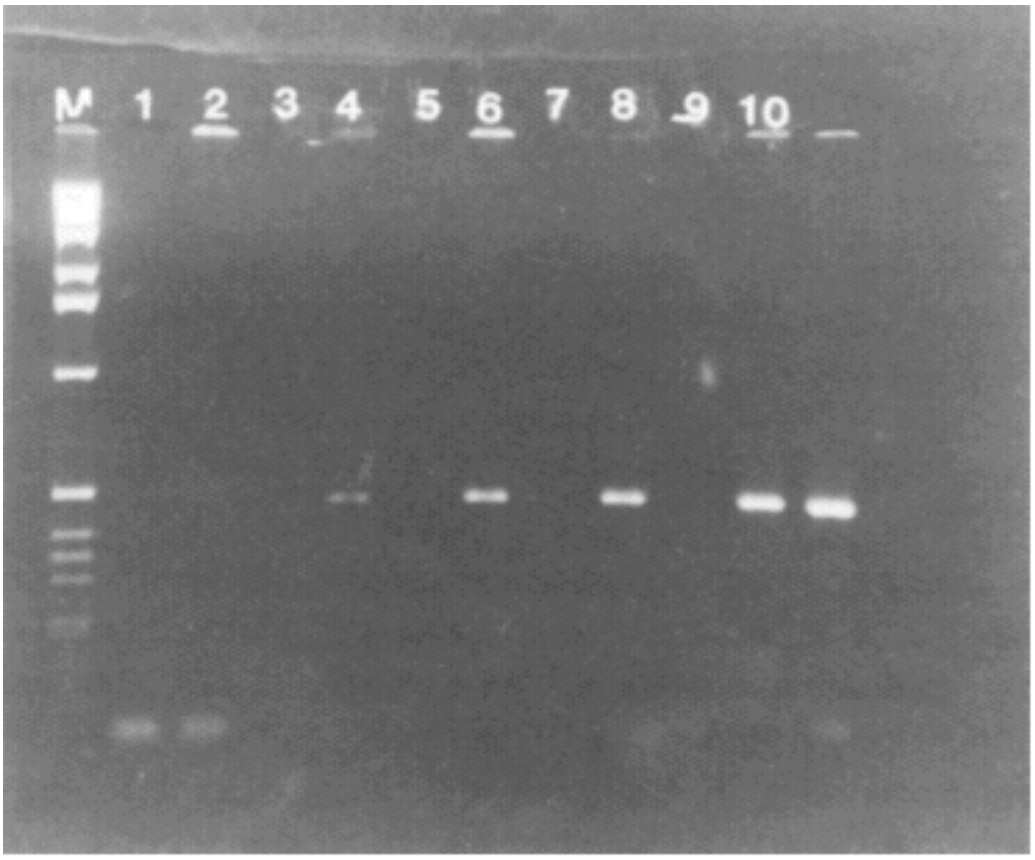

Figure 2. Detection of HAV sequence in RNA isolated from clam tissue seeded with wild-type HAV. RNA from seeded and unseeded tissue was isolated as described in the text. RNA representing 0-2500 particles of virus was then reverse transcribed and one-fifth of each cDNA pool was then amplified. Lanes 1 and 2, RNA isolated from unseeded tissue; lanes 3 and 4, RNA representing 50 particles of virus in the RT mixture; lanes 5 and 6, 250 particles of virus; lanes 7 and 8, 500 particles of virus; lanes 9 and 10, 2500 particles of virus; lane M, 1-kb DNA marker. Reverse transcriptase was omitted from the reaction mixtures in lanes 1, 3, 5, 7, and 9.

To detect HAV RNA in *Mercenaria mercenaria* seeded with wild-type HAV, RNA was isolated from 50 mg tissue seeded with 500 or 5000 particles of virus (based on the number of viral RNA molecules estimated from Fig. 1). As control, RNA was also isolated from tissue that had not been seeded with HAV. All RNA samples were then reverse transcribed and amplified as described above except that radioactive deoxynucleotide and competitor RNA were omitted. PCR products were analyzed by 1.6% agarose gel. As shown in Fig. 2, viral sequences were readily detected in samples seeded with virus before RNA isolation but were not detectable in the unseeded sample. No viral sequences were detected when reverse transcriptase was omitted from the reaction mixture. In subsequent studies, it was estimated that this method can detect 2000 virus particles per gram of shellfish tissue. The minimum infectious dose is

unknown, but presumably is less than this number. The lack of requirement of any special reagents such as specific antibodies should make this method generally applicable for the detection of HAV.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 13A

Staphylococcal Enterotoxins:

Micro-slide Double Diffusion and ELISA-based Methods

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(Return to Table of Contents)

Among the metabolites produced by *Staphylococcus aureus*, and other staphylo-coccal spp., enterotoxins (5,16,27) present the greatest foodborne risk to the health of consumers. Staphylococcal enterotoxins are basic proteins produced by certain *Staphylococcus* strains in a variety of environments, including food substrates. These structurally-related, toxicologically similar proteins are produced primarily by *Staphylococcus aureus*, although *S. intermedius* and *S. hyicus* also have been shown to be enterotoxigenic (1). Normally considered a veterinary pathogen (36,40), *S. intermedius* was isolated from butter blend and margarine in a food poisoning outbreak (15,29). A coagulase negative *S. epidermidis* was reported to have caused least one outbreak (17). These incidents support testing staphylococci other than *S. aureus* for enterotoxigenicity, if they are present in large numbers in a food suspected of causing a food poisoning outbreak.

When large numbers of enterotoxigenic staphylococci grow in foods, they may elaborate enough toxin to cause food poisoning after the foods are ingested. The most common symptoms of staphylococcal food poisoning, which usually begin 2-6 h after contaminated food is consumed, are nausea, vomiting, acute prostration, and abdominal cramps. To date, 8 enterotoxins (types A, B, C1, C2, C3, D, E, and H) have been identified as distinct serological entities. Current methods to detect enterotoxins use specific polyclonal or monoclonal antibodies (33,42,43).

The threshold amount of enterotoxin for causing illness in humans is not known. However, information from food poisoning outbreaks (16,25) and human challenge studies (24) indicates that individuals experiencing illness probably consumed at least 100 ng of enterotoxin A, the serotype most frequently involved in foodborne staphylococcal illness (20). The microslide gel double diffusion technique requires at least 30-60 ng of enterotoxin per gram of food. Chromatographic purification and concentration are used to achieve this toxin concentration so that the serological assay can be performed (4).

The microslide method is approved by AOAC International (4) and is the current standard for evaluating new methods. Other methods used for food extracts should be at least as sensitive as the microslide method, which requires concentrating extracts from 100 g food in as much as 600 ml to about 0.2 ml. Less sensitive methods are inadequate.

Techniques such as radioimmunoassay (RIA), agglutination, and enzyme-linked immunosorbent assay (ELISA), require less concentration of the food extracts ; thus, they save time and are more sensitive. Latex agglutination (16) appears promising as a serological tool for identifying staphylococcal enterotoxins. Several ELISA methods (26,28,30,32,37,38,39) have been proposed for the identification of enterotoxins in foods, but, except for a polyvalent ELISA (7,9), their specificity has not been studied extensively. Among ELISA methods, the "double antibody sandwich" ELISA is the method of choice, because reagents are commercially available in polyvalent and monovalent formats for both toxin screening and serotype specific identification(22). An automated enzyme-linked fluorescent immunoassay (ELFA) has been developed and is commercially available. This method has undergone specificity and sensitivity evaluations and has proven to be an effective serological system for the identification of staphylococcal enterotoxin in a wide variety of foods (14). Other methods, which have been used in the identification of the staphylococcal enterotoxins and may have application in foods, are the T-cell proliferation assay (35), and polyacrylamide gel

electrophoresis (PAGE) combined with Western blotting (2).

Examining staphylococci isolated from foods for enterotoxin production helps establish potential sources of enterotoxin in foods. Of the methods developed for laboratory testing of enterotoxin production, the semisolid agar procedure (19) is approved by AOAC International. It is simple to perform and requires minimal, routine laboratory equipment. Another simple approach is the use of pH 5.5 brain heart infusion (BHI) broth (14). The major problem with identifying enterotoxins in foods is that minute concentrations are sufficient to cause food poisoning. Pasteurization and thermal processing may render most toxin serologically unreactive. Consequently, false negatives may result, if detection methods lack sufficient sensitivity to detect active toxin (6).

This chapter presents a technique for the routine culturing of suspect staphylococci, procedures for the extraction of enterotoxin from foods and selected serological methods (Microslide gel double diffusion precipitation test, two manual ELISAs [Tecra™, Transia™], an automated qualitative "enzyme-linked fluorescent immunoassay" [ELFA™, Vidas™], and sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]-immunoblotting) for the identification of staphylococcal enterotoxin from isolates and from foods.

Recommended for routine analysis of foods for staphylococcal enterotoxin is the use, initially, of two different polyvalent ELISA kits. If results from different polyvalent ELISA methods yield conflicting results, retest using another method (e.g., another polyvalent ELISA method or the SDS-polyacrylamide gel electrophoresis-immunoblot assay for *S. aureus* enterotoxin A). Methods were developed to restore serological activity to heat-altered toxin in extracts of heat-processed foods (3,10,11,12,18,41,44). However, current toxin detection assays (described above) are sensitive enough to detect unaltered toxin that may persist after heat without such treatment (2).

These procedures are to be performed with extreme caution. Staphylococcal enterotoxins are highly toxic and procedures that may create aerosols should be performed in appropriate containment facilities, such as a biosafety hood.

Chromatographic Separation of Toxin from Foods for Micro-Slide Double Diffusion

A. Special equipment and materials

1. **Refrigerated cabinet or cold room**. The carboxymethyl cellulose (CMC) column extraction is performed at about 5°C, primarily because the column is allowed to run overnight. Storing food materials and extracts in a cold room or cabinet eliminates the need for a refrigerator.

2. **Waring blender or Omnimixer.** Grind foods into slurry for adequate extraction of enterotoxin. An Omnimixer (DuPont) is convenient for grinding food directly into stainless steel centrifuge tubes.

3. **pH meter**. The pH during extraction and the pH of buffers used in the extraction are important. Make adjustments within ± 0.1 pH unit.

4. **Refrigerated centrifuge**. Food extracts are centrifuged at relatively high speeds at 5°C in a refrigerated centrifuge, such as a Sorvall RC-2B, which can reach 20,000 rpm. The lower the centrifuge speed, the more difficult is the clarification of extracts.

5. **Carboxymethyl cellulose (CMC)**. The extract is partially purified by absorption onto CMC, Whatman CM 22, 0.6 meq/g (H. Reeve Angel, Inc., 9 Bridewell Place, Clifton, NJ). Soluble extractants are removed by this step.

6. **Centrifuge tubes**. Use 285 ml stainless steel centrifuge bottles (Sorvall No. 530).

7. **Magnetic stirrer**. A magnetic stirrer keeps test samples agitated during pH adjustments, dialysis, etc.

8. **Filter cloth**. At various stages in the procedures, food is filtered through several layers of coarse material such as cheesecloth placed in a funnel. Wetting cheesecloth before placing it in the funnel reduces adherence of food to cloth. The coarse material allows rapid flow with efficient removal of food particles, the chloroform layer, etc.

9. **Chromatographic tube (with stopcock or rubber tube attachment with finger clamp)**. Enterotoxin in food is

partially purified by using CMC, with elution in a chromatographic tube. For this purpose, a 19 mm id column, e.g., chromaflex, plain with stopcock, size 234 (Kontes Glass Co., Vineland, NJ) is recommended.

10. **Polyethylene glycol (PEG)**. Food extracts are concentrated with PEG (Carbowax 20,000; Union Carbide Corp., Chemical Division, 230 North Michigan Ave., Chicago, IL 60638).

11. **Lyophilizer**. The extract is finally concentrated by freeze-drying, which conveniently reduces the volume to 0.2 ml and completely recovers the extract.

12. **Dialysis tubing**. Cellulose casing of 1-1/8 inches flat width and an average pore diameter of 48 Angstrom units is used (12,000-14,000 mol wt exclusion).

13. **Separatory funnels**. Separatory funnels of various sizes are needed for $CHCl_3$ extractions and with the chromatographic column.

14. **Glass wool**. Glass wool makes ideal plugs for chromatographic columns.

15. **Chloroform**. Food extract is treated with $CHCl_3$ (several times in some instances) to remove lipids and other substances that interfere with concentration of extract to small volumes.

    Note: Chloroform is hazardous. Wear gloves, avoid contact with skin, and perform extraction in a chemical fume hood.

B. Reagents

1. 0.2 M $NaH_2PO_4 \cdot H_2O$
2. 0.2 M Na_2HPO_4
3. H_3PO_4 (0.005 M, 0.05 M)
4. Na_2HPO_4 (0.005 M, 0.05 M)
5. NaCl (crystal)
6. 1 N (or 0.1 N) NaOH
7. 1 N (or 0.1 N) HCl

C. Preparation of materials and reagents

1. **Polyethylene glycol (PEG)**. Prepare 30% (w/v) PEG 20,000 mol wt solution by adding 30 g PEG for each 70 ml distilled water. Cut dialysis tubing (1/8 inch flat width) long enough to accommodate food extract to be concentrated. Soak tubing in 2 changes of distilled water to remove glycerol coating. Tie one end of tubing with 2 knots close together. Fill tube with distilled water and test for leaks by squeezing filled sac while holding untied end tightly closed. Empty sac and place it in distilled water until use.

2. **Sodium phosphate buffer solutions**

    a. **Phosphate buffer, pH 5.7, 0.2 M (stock)**. Prepare solution by adding 0.2 M $NaH_2PO_4 \cdot H_2O$ (27.60 g in 1 liter water) to 0.2 M Na_2HPO_4 (28.39 g in 1 liter water) to final pH of 5.7.

    b. **0.005 M Phosphate buffer**. Dilute 0.2 M, pH 5.7 buffer (stock) with water (1 + 39). Adjust pH to 5.7 with 0.005 M H_3PO_4.

    c. **0.2 M Phosphate buffer, pH 6.4 (stock)**. Add 0.2 M Na_2HPO_4 to 0.2 M NaH_2PO_4 to pH 6.4.

    d. **0.05 M Sodium phosphate-NaCl buffer, pH 6.5**. Add NaCl (11.69 g/liter) to pH 6.4, 0.2 M solution (stock) to give 0.2 M NaCl, pH about 6.3. Dilute with water (1 + 3), and adjust to pH 6.5 with 0.05 M H_3PO_4 or 0.05 M Na_2HPO_4.

3. **Reservoir (separatory funnel)**. Attach about 60 cm latex tubing to stem of separatory funnel of appropriate size and attach other end of tube to glass tubing inserted through No. 3 rubber stopper to fit chromatographic column. Suspend separatory funnel from ring stand above chromatographic tube.

4. **Carboxymethy1 cellulose (CMC) column**. Suspend 1 g CMC in 100 ml 0.005 M sodium phosphate buffer, pH 5.7, in 250 ml beaker. Adjust pH of CMC suspension with 0.005 M H_3PO_4. Stir suspension intermittently for 15 min, recheck pH, and readjust to 5.7 if necessary. Pour suspension into 1.9 cm chromatographic tube, and let CMC particles settle. Withdraw liquid from column through stopcock to within 1 inch of surface of settled CMC. Place loosely packed plug of glass wool on top of CMC. Pass 0.005 M sodium phosphate buffer, pH 5.7, through column until washing is clear (150-200 ml). Check pH of last wash; if not 5.7, continue washing until pH is 5.7. Leave enough buffer in column to cover CMC and glass wool to prevent column from drying out.

D. Extraction and chromatographic separation of enterotoxin from food (**see** Fig. 1 - Scheme).

Note: This procedure and other procedures that may generate aerosols of pathogenic microorganisms should be performed in an approved biohazard hood.

Grind 100 g food in Waring blender at high speed for 3 min with 500 ml 0.2 M NaCl. Use Omnimixer for smaller quantities. Adjust pH to 7.5 with 1 N NaOH or HCl if food is highly buffered, and 0.1 N NaOH or HCl if food is weakly buffered (e.g., custards). Let slurry stand for 10 to 15 min, recheck pH, and readjust if necessary.

Transfer slurry to two 285 ml stainless steel centrifuge bottles. Centrifuge at 16,300 x *g* for 20 min at 5°C. Lower speeds with longer centrifuge time can be used, but clearing of some foods is not as effective. Separation of fatty materials is ineffective unless food is centrifuged at refrigeration temperature. Decant supernatant fluid into 800 ml beaker through cheesecloth or other suitable filtering material placed in a funnel. Re-extract residue with 125 ml of 0.2 M NaCl by blending for 3 min. Adjust pH to 7.5 if necessary. Centrifuge at 27,300 x *g* for 20 min at 5°C. Filter supernatant through cheesecloth, and pool filtrate with original extract.

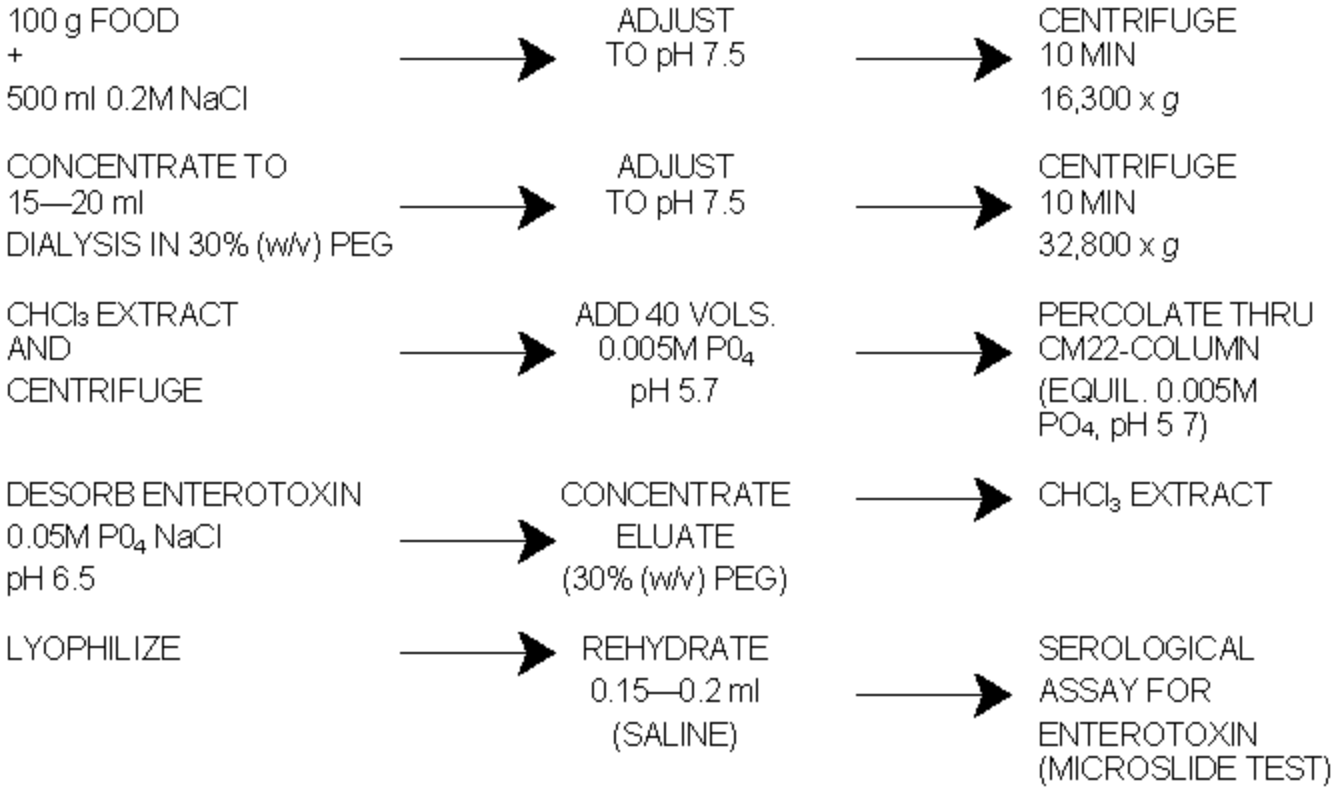

Fig. 1 Schematic diagram for the extraction and serological assay of enterotoxin in food.

Place pooled extracts in dialysis sac. Immerse sac in 30% (w/v) PEG at 5°C until volume is reduced to 15-20 ml or less (usually overnight). Remove sac from PEG and wash outside thoroughly with cold tap water to remove any PEG adhering to sac. Soak in distilled water for 1-2 min and in 0.2 M NaCl for a few min. Pour contents into small beaker.

Rinse inside of sac with 2-3 ml 0.2 M NaCl by running fingers up and down outside of sac to remove material adhering to sides of tubing. Repeat rinsing until rinse is clear. Keep volume as small as possible.

Adjust pH of extract to 7.5. Centrifuge at 32,800 x *g* for lO min. Decant supernatant fluid into graduated cylinder to measure volume. Add extract with < to = volume of $CHCl_3$ to separatory funnel. Shake vigorously 10 times through 90 degree arc. Centrifuge $CHCl_3$ extract mixture at 16,300 x *g* for 10 min at 5°C. Return fluid layers to separatory funnel. Draw off $CHCl_3$ layer from bottom of separatory funnel, and discard. Measure volume of water layer and dilute with 40 volumes of 0.005 M sodium phosphate buffer, pH 5.7. Adjust pH to 5.7 with 0.005 M H_3PO_4 or 0.005 M Na_2HPO_4. Place diluted solution in 2 liter separatory funnel.

Place stopper (attached to bottom of separatory funnel) loosely into top with liquid from separatory funnel. Tighten stopper in top of tube and open stopcock of separatory funnel. Let fluid percolate through CMC column at 5°C at 1-2 ml/min by adjusting flow rate with stopcock at bottom of column so that percolation can be completed overnight. If all liquid has not passed through column overnight, stop flow when liquid level reaches glass wool layer. If all liquid has passed through overnight, rehydrate column with 25 ml distilled water.

After percolation is complete, wash CMC column with 100 ml 0.005 M sodium phosphate buffer (1-2 ml/min); stop flow when liquid level reaches glass wool layer. Discard wash. Elute enterotoxin from CMC column with 200 ml 0.05 M sodium phosphate buffer, pH 6.5 (0.05 M phosphate-0.05 M NaCl buffer, pH 6.5), at flow rate of 1-2 ml/min at room temperature. Force last of liquid from CMC by applying air pressure to top of chromatographic tube.

Place eluate in dialysis sac. Place sac in 30% (w/v) PEG at 5°C and concentrate almost to dryness. Remove sac from PEG and wash. Soak sac in 0.2 M phosphate buffer, pH 7.4. Remove concentrated material from sac by rinsing 5 times with 2-3 ml 0.01 M sodium phosphate buffer, pH 7.4-7.5. Extract concentrated solution with $CHCl_3$. Repeat $CHCl_3$ extractions until precipitate is so lacy that it falls apart in $CHCl_3$ layer in cheesecloth.

Place extract in short dialysis sac (about 15 cm). Place sac in 30% (w/v) PEG, and let it remain until all liquid is removed from inside sac (usually overnight). Remove sac from PEG and wash outside with tap water. Place sac in distilled water for 1-2 min. Remove contents by rinsing inside of sac with 1 ml portions of distilled water. Keep volume below 5 ml. Place rinsings in test tube (18 x 100 mm) or other suitable container and freeze-dry. Dissolve freeze-dried test sample in as small an amount of saline as possible (0.1-0.15 ml). Check for enterotoxins by microslide method.

Microslide Gel Double Diffusion Test

A. Equipment and materials

1. Test tubes, 25 x 100 and 20 x 150 mm
2. Petri dishes, 15 x 100 and 20 x 150 mm, sterile
3. Bottles, prescription, 4 oz
4. Microscope slides, pre-cleaned glass, 3 x 1 inch (7.62 x 2.54 cm)
5. Pipets, sterile, 1, 5, and 10 ml, graduated
6. Centrifuge tubes, 50 ml
7. Sterile bent glass spreaders
8. Electrical tape, 0.25 mm thick, 10.1 mm wide, available from Scotch Branch, 3M Co., Electro-Products Divisions, St. Paul, MN 55011.
9. Templates, plastic (Fig. 2)
10. Silicone grease, high vacuum, available from Dow Corning Corp., Midland, MI 48640
11. Sponges, synthetic
12. Wooden applicator sticks
13. Glass tubing, 7 mm, for capillary pipets and de-bubblers
14. Pasteur pipets or disposable 30 or 40 1 pipets, available from Kensington Scientific Corp., 1165-67th St., Oakland,

CA 94601
15. Staining jars (Coplin or Wheaton)
16. Desk lamp
17. Incubator, 35 ± 1°C
18. Hot plate, electric
19. Sterilizer (Arnold), flowing steam
20. Blender and sterile blender jars
21. Centrifuge, high speed
22. Timer, interval

B. Media and reagents

1. Brain heart infusion (BHI) + 0.7% (w/v) agar (M23)
2. Agar, bacteriological grade, 0.2% (w/v)
3. Gel diffusion agar, 1.2% (w/v) (R28)
4. Baird-Parker medium (M17)
5. Nutrient agar, slants (M112)
6. Distilled water, sterile
7. Butterfield's phosphate-buffered dilution water (R11)
8. 0.2 M NaCl solution, sterile (R72)
9. Physiological saline solution, sterile (antisera diluent) (R63)
10. Thiazine Red R stain (R79)
11. Slide preserving solution (R69)
12. No. 1 McFarland standard (R42)
13. Antisera and reference enterotoxins (Toxin Technology Inc., 7165 Curtiss Ave., Sarasota, FL 34231)

C. Preparation of materials and media

1. **BHI with 0.7% (w/v) agar**. Adjust BHI broth to pH 5.3; add 7 g agar per liter broth [0.7 % (w/v)], and dissolve with minimal boiling. Dispense 25 ml portions into 25 x 200 mm test tubes and autoclave 10 min at 121°C. Just before use, aseptically pour sterile medium into standard petri dishes.

2. **No. 1 McFarland standard.** Prepare turbidity standard No. 1 of McFarland nephelometer scale (31). Mix 1 part 1% (w/v) $BaCl_2$ with 99 parts 1% (v/v) H_2SO_4 in distilled water.

3. **1.2% (w/v) Gel diffusion agar for gel diffusion slides.** Prepare fluid base for agar in distilled water as follows: NaCl 0.85% (w/v); sodium barbital 0.8% (w/v); merthiolate 1:10,000 (crystalline), available from Eli Lilly and Co., Terre Haute, IN. Adjust pH to 7.4. Prepare agar by adding 12 g per liter Noble special agar (Difco) [final concentration 1.2 %(w/v)]. Melt agar mixture in Arnold sterilizer (steamer) and filter while hot, in steamer, through 2 layers of grade filter paper (Whatman No. 1 or equivalent). Dispense small portions (15-25 ml) into 4 oz. prescription bottles. (Remelting more than twice may break down purified agar.)

4. **Thiazine Red R stain.** Prepare 0.1% (w/v) solution of Thiazine Red R stain in 1.0% (v/v) acetic acid.

5. **Preparation of slides.** Wrap double layer of electrician's plastic insulating tape around both sides of glass slide, leaving 2.0 cm space in center. Apply tape as follows: Start piece of tape (9.5-10 cm long) 0.5 cm from edge of undersurface of slide and wrap tightly around slide twice. Wipe area between tapes with cheesecloth soaked with 95% ethanol, and dry with dry cheesecloth. Coat upper surface area between tapes with 0.2% (w/v) agar in distilled water as follows: Melt 0.2% (w/v) bacteriological grade agar; maintain at 55°C or higher in screw-cap bottle. Hold slide over beaker placed on hot plate adjusted to 65-85°C and pour or brush 0.2% (w/v) agar over slide between 2 pieces of tape. Let excess agar drain into beaker. Return agar collected in beaker to original container for reuse. Wipe undersurface of slide. Place slide on tray and dry in dust-free atmosphere (e.g., incubator). **NOTE: If slide is not clean, agar will roll off without coating it uniformly.**

6. **Preparation of slide assembly.** Prepare plastic templates as described by Casman et al. (21) (**see** Fig. 2 for specifications). Spread thin film of silicone grease on side of template that will be placed next to agar, i.e., side with smaller holes. Place 0.4 ml 1.2% (w/v) gel diffusion agar, melted and cooled to 55-60°C, between tapes. Immediately

lay silicone-coated template on melted agar and edges of bordering tapes. Place one edge of template on one of the tapes and bring opposite edge to rest gently on the other tape. Soon after agar solidifies, place slide in prepared petri dish (C-7, below). Label slide with number, date, or other information.

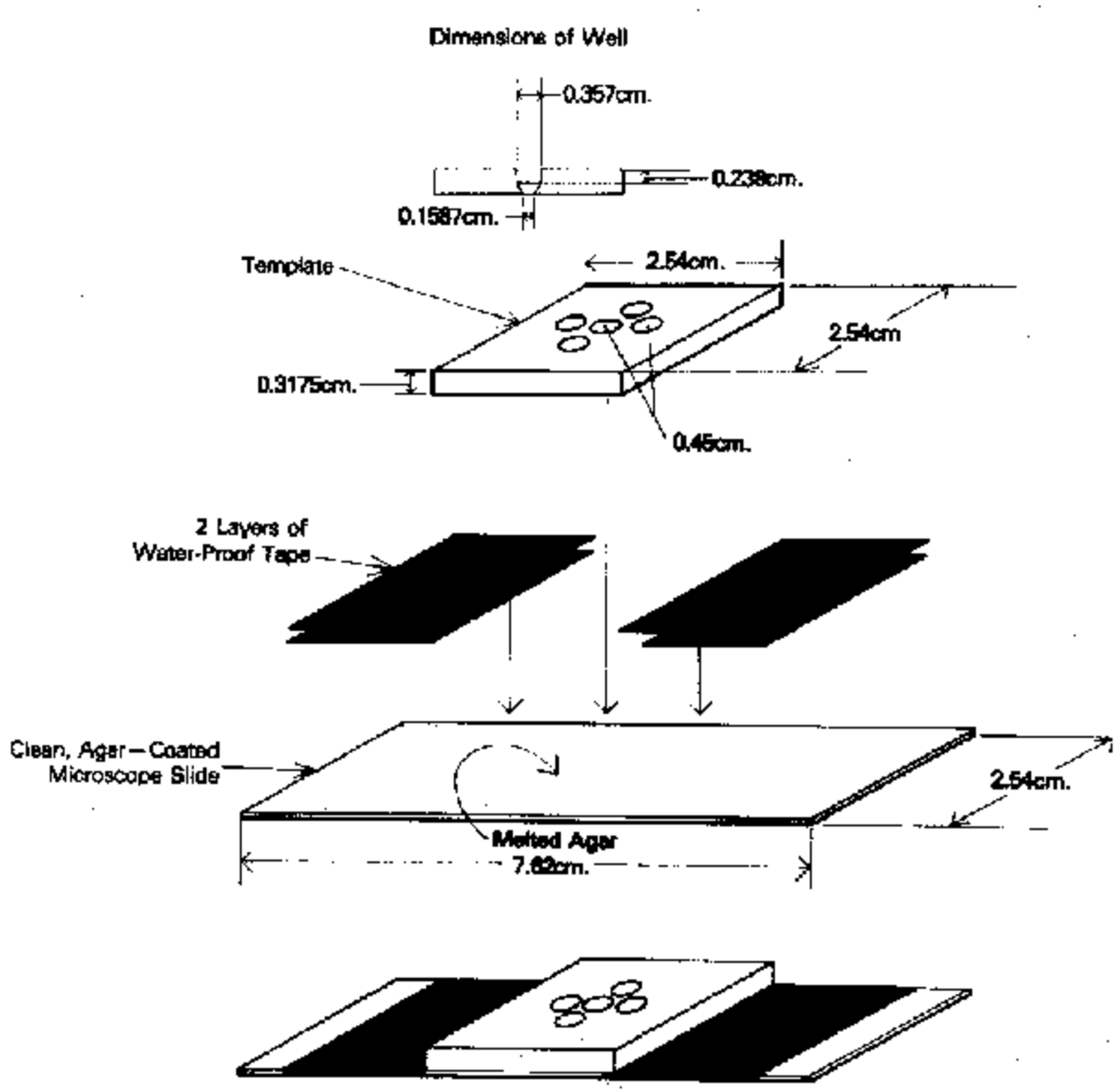

FIGURE 2
Microslide assembly with diagram for preparation and specifications for plastic template.

7. **Preparation of petri dishes for slide assemblies.** Maintain necessary high humidity by saturating 2 strips of synthetic sponge (about 1/2" inch wide x 1/2" inch deep x 2-1/2 inches long) with distilled water and placing them in each 20 x 150 mm petri dish. From 2 to 4 slide assemblies can be placed in each dish.

8. **Recovery of used slides and templates.** Clean slides without removing tape; rinse with tap water, brush to remove agar gel, and boil 15-20 min in detergent solution. Rinse about 5 min in hot running water, and boil in distilled water. Place slides on end, using test tube rack or equivalent, and place in incubator to dry. If slides cannot be uniformly coated with hot 0.2% (w/v) agar, they are not clean enough and must be washed again. Avoid exposing plastic templates to excessive heat or plastic solvents when cleaning. Place templates in pan, pour hot detergent solution over them, and let soak 10-15 min. Use soft nylon brush to remove residual silicone grease. Rinse sequentially with tap water, distilled water, and 95% ethanol. Spread templates on towel to dry.

9. **Directions for dissolving reagents used in slide gel.** Enterotoxins and their antisera are supplied as lyophilized preparations. Rehydrate antisera in physiological saline. Rehydrate reference enterotoxins in physiological saline containing 0.3% (w/v) proteose peptone, pH 7.0, or physiological saline containing 0.37% (w/v) BHI broth, pH 7.0.

Preparations should produce faint but distinct reference lines in slide gel diffusion test. Lines may be enhanced (E-3, below).

D. Procedure for enumeration and selection of staphylococcal colonies

To examine foods, use procedures described for detecting coagulase- positive staphylococci (**see** Chapter 12). Test isolates for enterotoxigenicity as described in E, below. To examine food in a suspected staphylococcal food poisoning outbreak, however, the following method is recommended:

1. **Enumeration of staphylococci/g.** Blend food with sterile 0.2 M NaCl solution for 3 min at high speed (20 g food in 80 ml 0.2 M NaCl, or 100 g in 400 ml, or whatever amount gives 1:5 dilution). Prepare decimal dilutions as follows: 10 = 1 part 1:5 dilution plus 1 part Butterfield's buffer; prepare dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. Place 0.1 ml portion of each dilution onto prepared Baird-Parker Agar and spread with sterile bent glass rod. Incubate plates inverted at 35°C for 48 ± 2 h. Count plates at dilution having 30-300 well-distributed colonies. Calculate staphylococci/g: total count x dilution factor of slurry x 10.

2. **Enumeration of enterotoxigenic staphylococci/g.** Note any variation in type or amount of pigment or other morphological characteristics produced by colonies. Count number of colonies in each group type, and record. Transfer two or more colonies from each type to nutrient agar slants or comparable medium. Test for enterotoxigenicity as described in E, below. Calculate enterotoxigenic staphylococci/g as follows: number of enterotoxigenic staphylococcal colonies x dilution factor of slurry x 10.

   NOTE: To determine presence of enterotoxin producers in food, add enough 0.2 M NaCl to slurry (1:5 dilution) to obtain 1:6 dilution, e.g., add additional 100 ml of 0.2 M NaCl to 1:5 dilution of slurry containing food and 400 ml of 0.2 M NaCl.

3. **Production of enterotoxin.** Of the methods described by Casman and Bennett (19) for production of enterotoxin, cultivation of staphylococci on semisolid BHI agar (pH 5.3) is simple and does not require special apparatus. Add loopful of growth from nutrient agar slants to 3-5 ml sterile distilled water or saline.

   Turbidity of suspension should be equivalent to No. 1 on McFarland nephelometer scale (approx. 3.00 x 10^8 organisms/ml). Using sterile 1.0 ml pipet, spread 4 drops of aqueous culture suspension over entire surface of BHI agar plate with sterile spreader and incubate at 35°C. Good surface growth is obtained after 48 h incubation, when pH of culture should have risen to 8.0 or higher. Transfer contents of petri dish to 50 ml centrifuge tube with wooden applicator stick or equivalent. Remove agar and organisms by high speed centrifugation (10 min at 32,800 x *g*). Examine supernatant for presence of enterotoxin by filling depots in slide gel diffusion assembly (**see** E, below).

E. **Slide gel diffusion test**. To prepare record sheet, draw hole pattern of template on record sheet, indicate contents of each well, and number each pattern to correspond with number on slide.

1. **Addition of reagents** (Fig. 3). Place suitable dilution of antienterotoxin (antiserum) in central well and place homologous reference enterotoxin in upper peripheral well (if diamond pattern is used); place test material in well adjacent to well containing reference enterotoxin(s). If bivalent system is used, place other reference toxin in lower well. Use reference toxins and antitoxins (antisera), previously balanced, in concentrations that give line of precipitation about halfway between their respective wells.

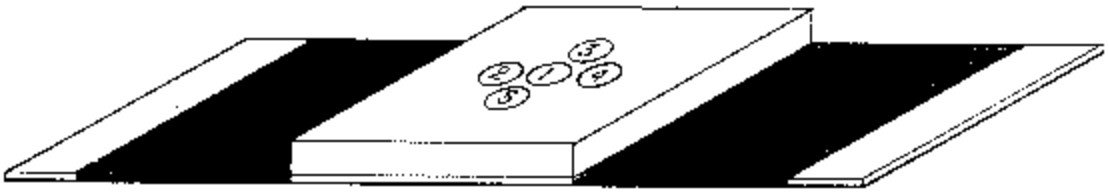

Figure 3. Arrangement of antiserum (antisera) and homologous reference enterotoxins for assay of test preparation(s) for presence of 2 serologically distinct enterotoxins (simultaneously (bivalent detections system) or for assay of dilutions of a test preparation system (monovalent detection system).

Adjust dilutions of reagents to give distinct but faint lines of precipitation for maximum sensitivity (**see** C-9, above). Prepare control slide with only reference toxin and antitoxin. Fill wells to convexity with reagents, using Pasteur pipet (prepared by drawing out glass tubing of about 7 mm outside diameter) or disposable 30 or 40 1 pipet. Remove bubbles from all wells by probing with fine glass rod. Make rods by pulling glass tubing very fine, as for capillary pipets; break into 2-1/2 inch lengths and melt ends in flame. It is best to fill wells and remove bubbles against a dark background. Insert rods into all wells to remove trapped air bubbles that may not be visible. Before examination , keep slides in covered petri dishes containing moist sponge strips at room temperature for 48-72 h or at 37°C for 24 h

2. **Reading the slide.** Remove template by sliding it to one side. If necessary, clean by dipping slide momentarily in water and wiping bottom; then stain as described below. To examine, hold slide over source of light and against dark background. Identify lines of precipitation through their coalescence with reference line of precipitation (Fig. 4). Excessive concentration of enterotoxin in test material will inhibit formation of reference line, and test material must then be diluted and retested. Figure 5, diagram A, shows typical precipitate line inhibition caused by enterotoxin excess by test preparation reactant arrangement in Fig. 3. Figure 6 shows typical line formation of diluted preparation. Occasionally, atypical precipitate patterns may be difficult for inexperienced analysts to interpret. One of the most common atypical reactions is formation of lines not related to toxin but caused by other antigens in test material. Examples of such patterns are shown in Fig. 7.

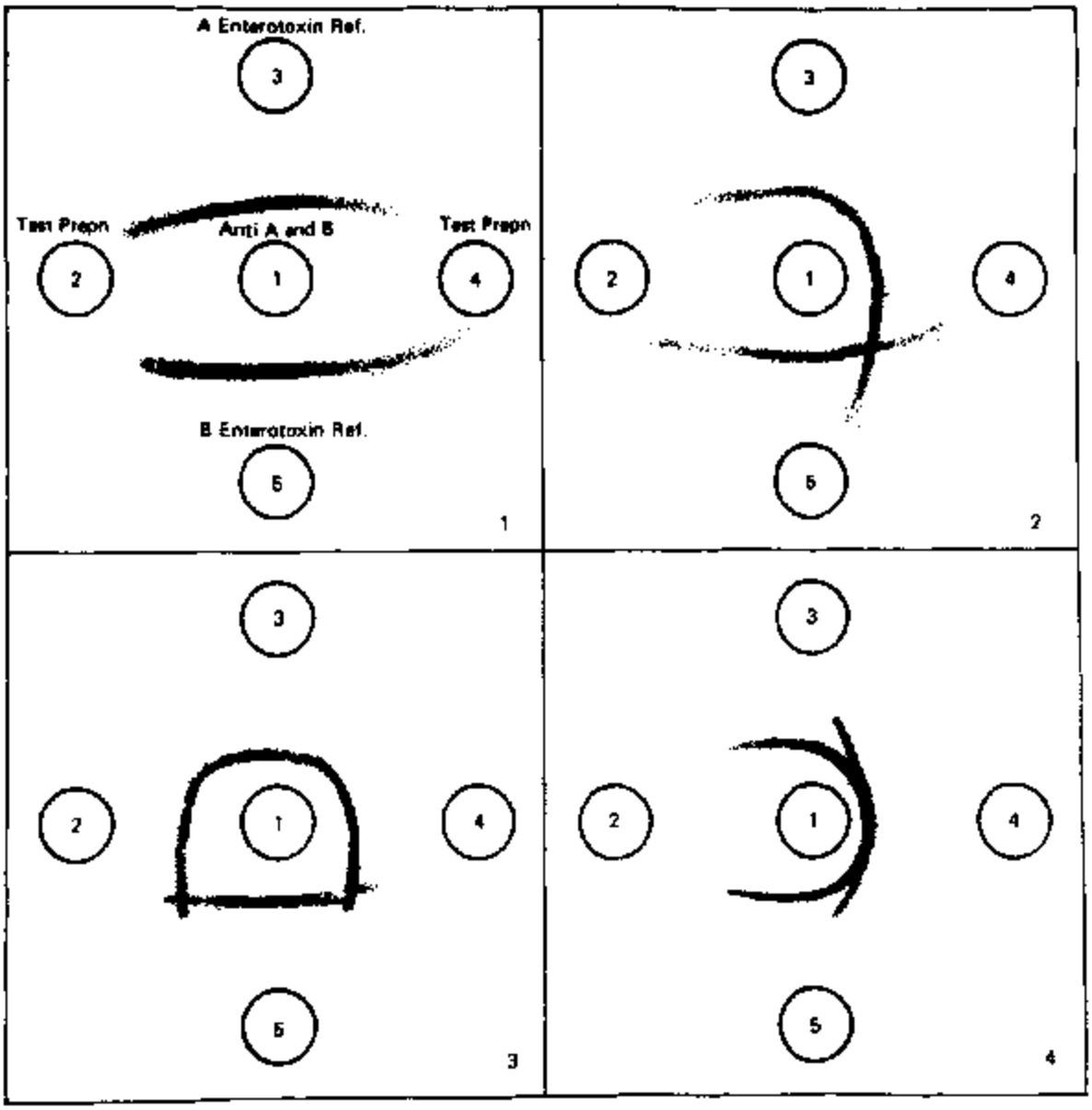

Fig. 4. Microslide gel diffusion test as bivalent detection system. Antisera to staphylococcal enterotoxins A and B are in well 1; known reference enterotoxins A and B are in wells 3 and 5, respectively, to produce reference lines of A and B; test preparations are in wells 2 and 4. Interpret 4 reactions as follows: (1) No line development between test preparations and antisera--absence of enterotoxins A and B; (2) coalescence of preparation line from

well 4 with enterotoxin A reference line (intersection of test preparation line with enterotoxin B reference line)-- absence of enterotoxins A and B in well 2, presence of enterotoxin A and absence of enterotoxin B in well 4; (3) presence of enterotoxin A and absence of enterotoxin B in both test preparations; and (4) absence of enterotoxins A and B in test preparation in well 2, presence of enterotoxins A and B in well 4.

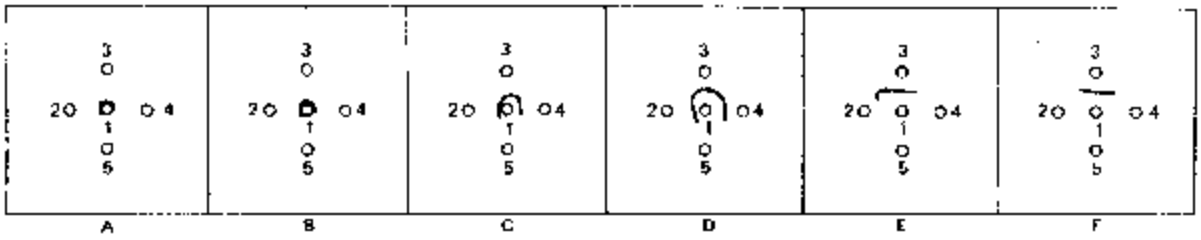

Fig. 5. Effect of amount of staphylococcal enterotoxin in test preparation on development of reference line of precipitation. Diagram A demonstrates inhibition (suppression) of reference line when 10 and 4 µg enterotoxin/ml, respectively, are used. Diagrams B-E show precipitate patterns when successively less enterotoxin (test preparation) is used. Diagram F shows typical formation of reference line of precipitation observed in slide test control system.

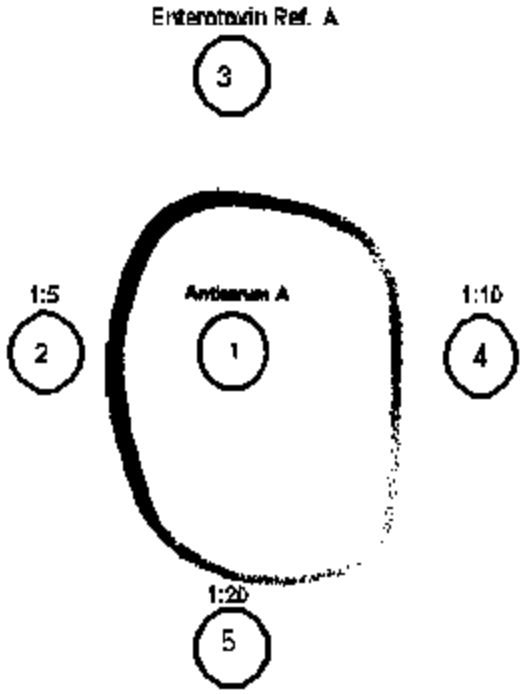

Fig. 6. Microslide gel double diffusion tests as monovalent detection system in which varying dilutions of test preparation are assayed for the presence of staphylococcal enterotoxin.

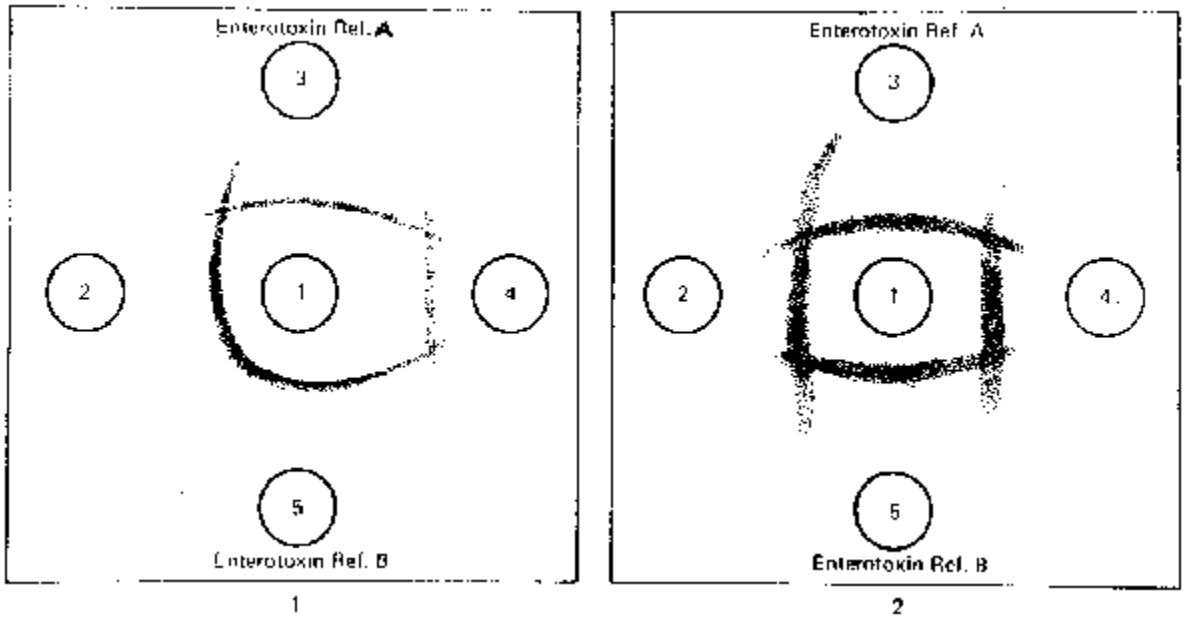

Fig. 7. *S. aureus* enterotoxin: Precipitate patterns in microslide gel diffusion test demonstrate nonspecific (atypical) lines of precipitation caused by other antigens reacting with antienterotoxin antibodies. In pattern 1, test preparation in well 4 produces atypical reaction indicated by nonspecific line of precipitation (lines of nonidentity with enterotoxin references A and B) which intersects with enterotoxin reference lines. In pattern 2, both test preparations (wells 2 and 4) are negative for enterotoxins A and B but, produce nonspecific lines of precipitation, which intersect enterotoxin A and B reference lines of precipitation.

3. **Staining of slides**. Enhance lines of precipitation by immersing slide 5-10 min in Thiazine Red R stain, and examine. Such enhancement is necessary when reagents have been adjusted to give lines of precipitation that are only faintly visible. Use staining procedure described by Crowle (23), modified slightly, when slide is to be preserved. Rinse away remaining reactant liquid by dipping slide momentarily in water and immersing it for 10 min in each of the following baths: 0.1% (w/v) Thiazine Red R in 1% (v/v) acetic acid; 1% (v/v) acetic acid; 1% (v/v) acetic acid; and 1% (v/v) acetic acid containing 1% (w/v) glycerol. Drain excess fluid and dry slide in 35°C incubator to store as permanent record. After prolonged storage, lines of precipitation may not be visible until slide is immersed in water.

Extraction of Enterotoxins from Foods for ELISA Assays

A. General precautions

- For raw or fermented foods and culture fluids from staphylococcal growth in laboratory media, check after extraction or collection of the culture fluid to determine if the test preparation contains peroxidase, which could interfere with the proper interpretation of results. To determine peroxidase presence, add 50 µl of sample to 50 µl of ELISA kit substrate reagent in an untreated microtiter plate (no antibody to staphylococcal enterotoxin) and let stand 10 min. If color changes to blue (or bluish-green), the sample contains intrinsic peroxidase, which must be inactivated. If sample remains colorless (or original color), analyze it for enterotoxin by ELISA. For inactivation of intrinsic peroxidase, prepare a 30% (w/v) solution of sodium azide and add 1 ml of this solution (30% w/v sodium azide) to 4 ml of test sample (final sodium azide concentration 6% (w/v)). Mix sample with azide solution, add extra sample additive, and let stand 1-2 min at room temperature. Retest sample for peroxidase presence (50 µl sodium azide-treated sample with 50 µl ELISA kit substrate reagent), as described above. If reaction is colorless (or original color), proceed with ELISA to identify enterotoxin in the peroxidase-inactivated sample. **CAUTION: Use appropriate safety waste containers for disposal of preparations containing sodium azide, a hazardous material.**

  When examining processed foods with obvious can defects which might result in the growth of organisms that produce peroxidase, test the extract for peroxidase production and inactivate as described above before testing for staphylococcal enterotoxin.

B. Procedures

Note: Raw food (e.g., vegetables), see, General Precautions, above. Follow directions under 5. Other Foods, below

1. **Milk and milk powder**. Reconstitute milk powder (25 g) by mixing with 125 ml 0.25 M Tris, pH 8.0. Treat reconstituted milk powder in same way as fluid milk. For milk samples (5.0 ml), ensure that pH is in range 7-8; then add 50 µlsample additive (in TECRA™ kit). For clearer extract, adjust pH to 4.0 with concentrated HCl. For milk samples (50 ml), ensure that pH is in range 7-8; then add 50 µl sample additive (in kit). Centrifuge sample for at least 10 min at 1000-3000 x *g*. Decant extract and pump about 5.0 ml through syringe containing wetted absorbent cotton into polypropylene tube. Readjust pH to 7.0-8.0 (use pH paper), add 50 µl additive (in kit), and mix thoroughly.

2. **Dehydrated food ingredients**. Add 125 ml 0.25 Tris, pH 8, to 25 g of food, and homogenize in blender for about 3 min at high speed. Centrifuge sample for about 10 min at 1000-3000 x *g* and collect extract. Remove plunger from plastic syringe containing prewetted absorbent cotton and carefully pump solution through, collecting eluate. Take 5 ml of eluate; adjust pH to 7.0-8.0; then add 50 µl of sample additive, and mix thoroughly.

3. **Cheeses**. Add 50 ml water to 25 g of cheese and homogenize for about 3 min at high speed in blender. Adjust pH to 4 (pH paper) with concentrated HCl. Centrifuge sample for about 10 min at 1000-3000 x *g*. Remove plunger of plastic syringe containing prewetted cotton, and place 5.0 ml of extract into syringe; insert plunger and carefully pump solution through, collecting eluate. Take 5 ml of eluate, and add NaOH to adjust pH to 7.0-8.0; add 50 µl of sample additive, and mix thoroughly.

4. **Other foods**. Prepare foods other than those described above as follows: Add 50 ml 0.25 M Tris, pH 8, to 25 g of food and homogenize for about 3 min at high speed in blender. Centrifuge sample for about 10 min in bench centrifuge at 1000-3000 x *g*. Remove plunger from plastic syringe containing prewetted absorbent cotton and place 5 ml of extract into syringe; insert plunger and carefully pump solution through, collecting eluate in polypropylene tube. Take 5 ml of eluate; adjust pH, if necessary, to 7.0-8.0; add 50 µl of sample additive, and mix thoroughly.

NOTE: Prepare food extracts immediately before testing.

C. Proceed to desired assay protocol.

1. Test 200 µl sample extract for TECRA kit
2. Test 500 µl for VIDAS or Transia kits

Visual ELISA: Polyvalent (Types A-E) Screening for Determining Enterotoxigenicity and Identifying Staphylococcal Enterotoxins in Foods

This visual immunoassay provides a rapid (4 h), sensitive (1.0 ng or greater per ml or g), specific screening test for the simultaneous identification of staphylococcal enterotoxins A-E. However, this kit cannot be used to distinguish among specific toxin serotypes. The ELISA is performed in a "sandwich" configuration. The kit is commercially available as TECRA™ (TECRA Diagnostics, 28 Barcoo St., NSW, P.0. Box 20, Roseville, 2069, Australia) and is distributed by International Bioproducts Inc., 14796 N.E. 95th St., Redmond, WA 98052. This method has been adopted "First Action" by AOAC International (13).

A. Special equipment and supplies

Materials supplied in kit:

1. Anti-SET antibody coated Removawells (48 or 96 wells)
2. Removawell holder for securing wells
3. Instruction booklet methods manual
4. Color comparator
5. Protocol sheet

Materials/equipment supplied by user:

1. Absorbent cotton

2. Pipets, 50-200 µl; 5-20 µl
3. Tips, plastic
4. Incubator, 35-37°C
5. Plastic film wrap or sealable plastic container
6. Omnimixer, Waring blender (or equivalent) for preparation of food extracts
7. pH paper (range 0-14)
8. Centrifuge and Centrifuge cups
9. Plastic squeeze bottle (500 ml)
10. Disposable plastic syringes (25 ml)
11. Microplate shaker (optional)
12. Microplate reader (optional, but dual wavelength is recommended)
13. Polypropylene tubes (12 x 75 mm)
14. Polyethylene glycol (PEG, 15,000 - 20,000 mol wt)
15. Dialysis tubing (12,000 - 14,000 mol wt exclusion)
16. Balance
17. Beakers (250 ml)

B. Reagents

Materials supplied in kit:

1. Wash concentrate
2. Sample additive
3. Positive control; negative control
4. Conjugate diluent; conjugate, lyophilized
5. Substrate diluent; substrate, lyophilized
6. Stop solution

Reagents supplied by user:

1. Tris buffer (0.25 M; 30.28 g TRIS/liter, pH 8.0)
2. Sodium hydroxide solution (1.0 N NaOH)
3. Hydrochloric acid
4. Deionized or distilled water
5. Sodium hypochlorite

C. Preparation of materials and reagents

1. BHI with 0.7% (w/v) agar,
2. **Syringe type filter (for foods)**. Prepare disposable plastic syringe (0.25 ml) by inserting plug of 0.5 cm thick absorbent cotton. Pump about 5.0 ml distilled water through to ensure tight packing. Do this just before filtering 5 ml of food extracts for treatment with additive provided in kit.
3. **Reconstitution of wash solution**. Dilute wash concentrate (as per kit directions) with distilled or deionized water in reagent bottle to 2 liters. Use this "wash solution" for washing wells and for diluting positive control when required.
4. **Reconstitution of conjugate**. Add conjugate diluent to conjugate and rehydrate at room temperature by gently mixing. This solution is referred to as "reconstituted conjugate."
5. **Reconstitution of substrate**. Add substrate diluent to substrate. Be sure contents have dissolved and are at room temperature before use.

D. General Precautions

- Note expiration date of kit. This is the last date on which product should be used. Prepare all reagents carefully and enter date of reconstitution on outside label of box. Use reconstituted kit within 56 days. Refrigerate all components (2-8°C) when not in use. **DO NOT FREEZE.**

- Components in SET visual immunoassay are intended for use as integral unit. Do not mix components of different batch numbers.

- Use a new pipet tip for each sample. Take care not to cross-contaminate wells. If plastic troughs are used to dispense conjugate and substrate, they must always be kept separate.
- Use positive and negative controls with each assay.
- Prepare trough containing 2% (v/v) sodium hypochlorite to be used for disposal of all samples that contain toxin.
- Store unused Removawell strips in pouch provided and re-seal with resealing strip after each use.

E. Laboratory production of toxin by suspect staphylococci (**see** Production of Enterotoxin, above).

F. Enterotoxin testing by polyvalent visual ELISA (Fig. 8)

Secure desired number of anti-SET antibody-coated Removawells in holder provided. Allow 1 well for each food sample, 1 well for negative control, and 1 well for positive control. Additional wells are required if optional positive (food) and negative controls are prepared. Fill each well with wash solution and let stand 10 min at room temperature (20-25°C). Empty wells by quickly inverting holder; remove residual liquid by firmly striking holder face-down on paper towel several times.

Transfer 200 µl aliquots of controls and samples (food extracts or culture fluids) into individual wells; record position of each sample on sample record sheet (original provided in kit). Gently tap holder containing test wells to ensure homogeneous distribution and contact of test material with walls of wells. Agitation of wells on microtiter plate shaker for 30 s is optional. To prevent evaporation, cover wells with plastic film or plate sealers (Dynex Technologies, Inc., 14340 Sullyfield Circle, Chantilly, VA 20151-1683) and incubate 2 h at 35-37°C. Wash well liberally with wash solution from squeeze bottles as follows: Press Removawells firmly into holder. Quickly invert holder, emptying contents into trough containing 2% (v/v) sodium hypochlorite. Remove residual liquid by firmly striking holder face-down on paper towel several times. Completely fill each well with wash solution. Repeat liberal washing 2-3 more times. Finally, empty wells.

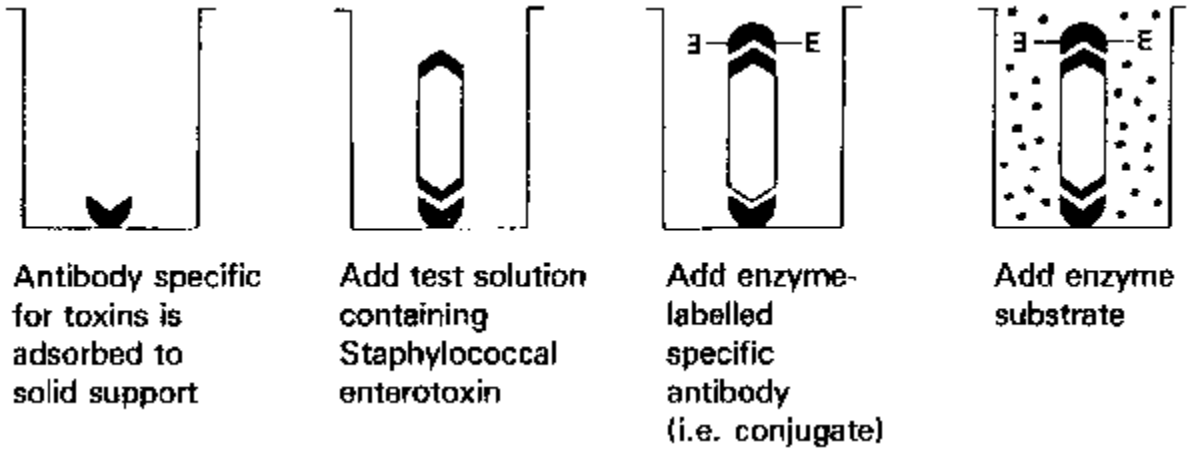

Figure 8. Typical double antibody "sandwich" ELISA scheme.

Add 200 µl reconstituted enzyme conjugate into each well. Cover tray and incubate 1 h at room temperature (20-25°C). Empty wells and wash them thoroughly 5 times, as above. Empty wells and remove residual liquid as described above.

Add 200 µl reconstituted substrate to each well. Leave at room temperature (20-25°C) for at least 30 min until positive control reaches absorbance greater than 1.0 or color darker than panel No. 4 on Color Comparator. Color development tends to concentrate around edge of wells. For accurate results, tap sides of plate gently to mix contents before reading. Add 20 µl of stop solution to each well. Tap sides of plate gently to mix contents. Assay is now complete. Determine results visually or with microtiter tray reader.

G. Interpretation of ELISA results

1. **Visual observation.** Place tray holding wells on white background; then compare individual test wells with Color

Comparator provided in kit. Positive toxin control (and positive food control, if used) should give strong green color, indicating that all reagents are functional. If negative control is significantly darker than "negative" panels on Color Comparator, washing step was probably inadequate and assay must be repeated.

Sample is considered positive when the following criteria are met:

(1) negative control is within negative range on Color Comparator, and

(2) sample has green (or blue) color greater than negative range on Color Comparator.

Sample is considered negative for enterotoxin when the following criteria are met:

(1) negative control is within negative range on Color Comparator, and

(2) sample is colorless or has color within negative range on Color Comparator.

2. **Absorbance measurement with microtiter tray reader.** Read absorbance (A) of samples at 414 ± 10 nm, using microtiter tray reader. Prepare dual wavelength reader blank against air, and set second "reference" wavelength at 490 ± 10 nm. Typical wavelength settings could be $A_{405-490}$ or $A_{414-492}$ for peroxidase-based systems such as the described ELISA. Prepare single wavelength instrument blank on well containing 200 µl of substrate (provided in the kit) or water. Absorbance of positive toxin control should be at least 1.0, indicating that all reagents are functional. If absorbance of negative control is greater than 0.200, washing of wells was probably inadequate and assay must be repeated. Refer to Troubleshooting Guide in kit.

   Sample is considered **positive** if absorbance is > 0.200.

   Sample is considered **negative** if absorbance is <= 0.200.

   Generally, culture fluids that contain toxin have absorbance readings significantly greater than 0.200. Some strains of *S. aureus* produce intrinsic peroxidase, which can be inactivated with sodium azide.

A. Recommended controls

1. **Positive toxin control.** Prepare by making 1:100 dilution of positive control solution (in kit) in wash solution (50 µl to 5 ml wash solution, as per kit directions) in a polypropylene tube. Run positive control whenever assay is performed to indicate that all reagents are functional and that assay has been conducted correctly. Discard unused diluted toxin control into sodium hypochlorite solution.

2. **Negative toxin control.** Use negative control solution provided in kit. No dilution of negative control solution is necessary. Use 200 µl of all controls.

3. **Positive food control (optional).** Add aliquot of positive control provided in kit to known enterotoxin-negative food product to serve as positive food control. Extract and assay sample under same conditions as suspect sample.

4. **Negative food control (optional).** Use same type of food as suspect food, but which is known to be toxin-free. Prepare negative food control in exactly the same manner as suspect food. This control will ensure that washing of wells was adequate and that no food components will interfere with test results. Extract and assay sample under same conditions as suspect sample.

B. Serotype toxin confirmation

If confirmation of serotype by the AOAC method is necessary, use $CHCl_3$ to extract the food extract as previously described, and proceed with remaining steps in procedure. Analyze chromatographed eluate with microslide gel double diffusion test. A faster and more sensitive option would be confirmation by SDS-PAGE-Western blotting described in this Chapter.

Automated multiparametric immunoanalyzer, Vidas™,
Vidas Staph (SET) for the identification of the staphylococcal enterotoxins

This kit can be purchased from biomerieux Vitek, Inc.,545 Anglum Dr., Hazelwood, Missouri 63042-2395.

A. Special equipment

Materials and reagents supplied in kit:

1. **30 SET Reagent Strips**

   The SET Reagent Strip (refer to the table below) is a polypropylene strip of 10 wells covered with a foil seal and paper label. The first well of the strip is for the sample. The last well of the strip, an optically clear cuvette, is for the fluorometric determination. The eight wells in the center of the strip contain the various reagents for the assay. (See description of reagent strip below).

DESCRIPTION OF THE STAPH ENTEROTOXIN REAGENT STRIP	
Wells	**Reagents**
1	Sample Well: 0.5 ml of food extract is placed into the well
2	Pre-Wash Solution (0.4 ml): TBS - Tween with 0.1% (w/v) sodium azide
3-4-5-7-8-9	Wash Solution (0.6 ml): TBS - Tween with 0.1% (w/v) sodium azide
6	Conjugate (0.4 ml): alkaline phosphatase labeled polyclonal antibodies with 0.1% (w/v) sodium azide
10	Cuvette with substrate (0.3 ml): 4-methyl-umbelliferyl phosphate with 0.1% (w/v) sodium azide
The name of the test, the lot number, and the expiration date of the kit are included on a bar code which is printed on the SET Reagent Strip. The test identification, lot number and calibration parameters are both clearly indicated in the kit's specification sheet and printed with a bar code.	

2. **30 SET SPRs**

   The interior of the SET SPR is coated at the time of manufacture with anti-enterotoxin antibodies.

3. **1 Bottle Standard (3 ml)**

   Purified staphylococcal enterotoxin B (5 ng/ml) with 0.1% (w/v) sodium azide and protein stabilizers.

   CAUTION: HANDLE WITH CARE!

4. **1 Bottle Positive Control (6 ml)**

   Purified staphylococcal enterotoxin B (5 ng/ml) with 0.1% (w/v) sodium azide and protein stabilizers. Control range indicated on the vial label. **CAUTION: HANDLE WITH CARE!**

5. **1 Bottle Negative Control (6 ml)**

   TRIS buffered saline (TBS) - Tween with 0.1% (w/v) sodium azide.

6. **1 Bottle Concentrated Extraction Buffer (55 ml)**

2.5 mol/1 TRIS - 1% (w/v) Tween with 1% (w/v) sodium azide.

MATERIALS REQUIRED BY USER BUT NOT PROVIDED IN KIT:

1. Pipette which will dispense a minimum of 0.5 ml.
2. Tips, Plastic, to deliver 500 µl.
3. Centrifugation/filtration tubes (bioMerieux Product Number: 30550) or plastic syringes (20 ml)- optional.
4. Omni mixer, Waring blender (or equivalent) for preparation of food extracts.
5. pH paper (range 0-14).
6. Centrifuge.
7. Centrifuge cups.
8. Polyethylene glycol (15,000-20,000 mol wt).
9. Sodium hydroxide solution (1.0 N Na0H).
10. Hydrochloric acid.
11. Sodium hypochlorite.
12. Dialysis tubing, flat width 32 mm or comparable.

B. General Precautions, recommendations and considerations.

1. **WARNINGS AND PRECAUTIONS**

   a. Routinely clean and decontaminate the VIDAS instrument. See the VIDAS Operator's Manual for the appropriate procedures.

   b. Reagents contain 0.1% (w/v) sodium azide which could react with lead or copper plumbing to form explosive metal azides. If liquid containing sodium azide is disposed of in the plumbing system, flush drains with large volumes of water to avoid build-up.

   c. The positive control and standard bottles contain purified staphylococcal enterotoxin. Handle with great care and use protective gloves. Consult physician immediately if ingested.

   d. Handle all kit components as potentially biohazardous material. Dispose of all used components and other contaminated material by acceptable procedures for potentially biohazardous material.

2. **STORAGE AND HANDLING**

   a. Store the VIDAS SET Kit at 2-8°C.

   b. Do not freeze reagents.

   c. Return unused components to 2-8°C.

   d. The indicator on the desiccant in the resealable SPR storage pouch should be blue. Do not use the remaining SPRs in the pouch if the indicator is pink. Completely reseal the pouch after removing SPR's; this will maintain their stability.

   e. When stored appropriately, all kit components are stable until the expiration date printed on the label. Do not use any kit components beyond the expiration date.

3. **LIMITATIONS OF THE ASSAY**

   a. Do not mix reagents or disposables of different lot numbers.

   b. Bring the reagents to room temperature before inserting them into the VIDAS.

   c. Mix the standard, controls and samples well before use to ensure reproducibility.

d. Improper sample processing or storage may yield incorrect results.

4. **SPECIFIC PERFORMANCE CHARACTERISTICS**

Staphylococcal enterotoxins A, B, C1, C2, C3, D, E are detected by the VIDAS SET Assay at the sensitivity of at least 1 ng/ml.

C. Procedures for Preparation of Controls and Extraction of Enterotoxins from Suspect Foods.

In addition to the food extraction procedures described here, a greater variety of food extraction procedures are described by the kit manufacturer. Prepare food extracts immediately before testing.

1. Recommended controls

a. **Positive toxin control.**

Dispense 500 µl of control reagent provided in the kit. Run positive control whenever assay is performed to indicate that all reagents are functional and that the assay has been conducted correctly.

b. **Negative toxin control.**

Use negative control solution provided in kit. No dilution of negative control solution is necessary. Add 500 µl of negative control reagent to test strip.

c. **Positive food control (optional).**

Add aliquot of positive control provided in kit to known enterotoxin-negative food product to serve as positive food control. Extract and assay sample under same conditions as suspect sample.

d. **Negative food control (optional).**

Use same type of food as suspect food, but which is known to be toxin-free. Prepare negative food control in exactly the same manner as suspect food. This control will ensure that washing of wells was adequate and that no food components will interfere with test results. Extract and assay sample under same conditions as suspect sample.

2. Extraction of toxin from foods.

a. see section on Extraction of Enterotoxins from Foods for ELISA Assays, above.

D. **ASSAY PROCEDURE, VIDAS**

Important: A standard must be run in duplicate for every lot of kits. The result is stored in the computer and automatically used for assay analysis. A standard may be run with each SET work list, or a stored standard result (stored in the computer) may be used. See the VIDAS Operator's Manual for complete instructions.

1. Remove the VIDAS Staph enterotoxin kit from the refrigerator and allow it to come to room temperature (approximately 30 minutes).

2. Remove necessary components from the kit and return all unused components to storage at 2-8°C.

3. In the space provided, label the SET Reagent Strips with the appropriate sample identification numbers.

4. Enter the appropriate assay information to create a work list. Type "SET" to enter the assay code, and enter the number of tests to be run. If a standard is being tested, type "S" ("S" then "1" on mini VIDAS) for the sample ID. The

standard may be run in any position of the work list. bioMerieux recommends running the standard in duplicate. See the VIDAS Operator's Manual for a complete explanation.

5. Pipette 0.5 ml of standard, control or sample into the center of the sample well of the SET Reagent Strip.

6. Load the SET Reagent Strips and the SET SPRs into the positions that correspond to the VIDAS section indicated by the work list. Check to make sure the color labels with the three letter assay code on the SPRs and the Reagent Strips match.

7. Dispose of all used SPRs and Reagent Strips in appropriate biohazard containers.

E. QUALITY CONTROL

A positive and negative control are provided to validate kit performance.

Test the positive and negative controls with each new lot or shipment to ensure that assay performance has remained unimpaired throughout shipping and storage. Test the controls as specified by your laboratory's regulatory guidelines. Controls are provided in ready-to-use form and must be thoroughly mixed and pipetted directly into the sample well of a reagent strip.

The expected positive control value will be: included in the range indicated on the vial label. If the results from testing the controls do not fall within this range, do not report sample results. NOTE: if the standard is out of range, the test value can be recalculated with another standard. See the VIDAS Operator's Manual for complete information.

F. INTERPRETATION OF RESULTS

Two instrument readings for fluorescence in the Reagent Strips's optical cuvette are taken for each specimen tested. The first reading is a background reading of the cuvette and substrate before the SPR is introduced into the substrate. The second reading is taken after the substrate has been exposed to the enzyme conjugate remaining on the interior of the SPR. The background reading is subtracted from the final reading to give a Relative Fluorescence Value (RFV) for the test result. A test value is generated for each sample by forming a ratio from the RFV of the sample to that of a standard. Test values from test samples and control samples are compared to a set of thresholds stored in the computer. The table below shows the thresholds and the interpreted results.

Thresholds and Interpretations

Test Value Threshold	Interpretation
$\leq$ 0.13	Negative
$\geq$0.13	Positive

A report is printed that records the type of test performed, the sample identification, the date and time, the lot number and expiration date of the reagent kit being used and each sample's RFV, test value and interpreted result.

Results with test values less than the low threshold indicate sample without detectable enterotoxin. Samples with test values greater than (or equal to) the high threshold are reported as positive.

Invalid results are reported when the background reading is above a pre-determined cut-off (indicating low-level substrate contamination). In this case, repeat the assay with the original sample.

An invalid result is also seen if there is no standard available for the lot number of the sample test strip. In this case, run a standard in duplicate in SET strips with the same lot number as the invalid sample test. The sample test result can then be recalculated using the new stored standard. See the VIDAS Operator's Manual for complete information.

G. Serotype toxin confirmation

If confirmation of serotype by the AOAC method is necessary, use $CHCl_3$ to extract the food extract as previously described, and proceed with remaining steps in procedure. Analyze chromatographed eluate with microslide gel double diffusion test. A more sensitive and faster option is the SDS-PAGE-Western blotting method described in this Chapter.

Transia™ Immunoenzymatic Test for the Identification of Staphylococcal Enterotoxin

This kit is produced by Transia-Diffchamb S.A. Lyon, France and is distributed by Idetek, Inc., Sunnyvale, CA. in the U.S.A.

A. **Special equipment**

Materials and reagents supplied in kit:

- **Bladed tubes** sensitized by a mixture of monoclonal antibodies specific for enterotoxin A, B, C, D and E, packed with a dehydrating agent into a plastic bag.
- **Reference ST 714B** contains 10 tubes.
- **Reference ST 724B** contains 20 tubes.
- **Reference ST 744B** contains 40 tubes.
- **Vial 1:** Negative control: ready to use.
- **Vial 2:** Positive control (concentrated 50X): mixture of staphylococcal enterotoxins A, B, C, D and E at 10 ng/ml. To be diluted to 1/50th before use.

  CAUTION: use gloves to handle.
- **Vial 3:** Washing buffer (concentrated 30X): to be diluted to 1/30th with distilled water.
- **Vial 4:** Conjugate: mixture of monoclonal and polyclonal anti-staphylococcal enterotoxin antibodies conjugated to peroxidase: ready to use solution.
- **Vial 5:** Substrate.
- **Vial 6:** Chromogen.
- **Vial 7:** Stopping solution: ready to use.

Equipment and reagents not provided in kit:

Equipment

1. Scales and weighing vessels.
2. homogenizer, mixer or stomacher.
3. Gloves.
4. Graduated pipettes.
5. Magnetic bars and magnetic stirrer.
6. Centrifuge tube.
7. Filter paper (Whatman or equivalent).
8. Laboratory centrifuge (1500 x *g* minimum).
9. Vortex mixer.
10. pH-meter or pH paper (range 0-14).

11. Clean glassware.
12. Test tube rack.
13. 100-1000 µl micropipettes and fitting tips.
14. Eppendorf type multipette, 5 and 2.5 ml tips for multipette.
15. Shaker (about 60 rpm).
16. 1 liter beaker.
17. Plastic squeeze bottle.
18. Absorbent paper.
19. Basin or another container: bleach- or soda-resistant.
20. Microcuvettes or unsensitized microtiter plastic wells.
21. Dialysis tubes (cut off threshold 12,000-14,000 Da)
22. Spectrophotometer for tubes or microcuvettes, with 450 nm filter (optional to microtiter plate reader).
23. Unsensitized (antibody free) removawell strips (plastic wells, Immulon 2® Removawell Strips cat. #011-010-6302, Dynatech Laboratories, Inc.) or equivalent.
24. Microtiter plate reader with 450 nm filter.

B. **Reagents**

1. Polyethylene glycol, mol wt 15,000-20,000
2. Decontamination solutions
3. TRIS buffer, 0.25 M, pH 8.0

- **To prepare the samples**:

1. Distilled water.
2. Extraction buffer: 0.25 M TRIS Buffer, pH 8.0.
3. for pH adjustment: NaOH 6N and HCl 6N.
4. For a possible concentration of the sample through dialysis: Polyethylene glycol (15,000-20,000 minimum) at 30% (w/v) in distilled water.

- **To decontaminate materials and reagents:**

- Chlorine bleach or 1N soda solution.

C. **Reagent preparation**

1. **Extraction buffer**:

   For preparation of 1 liter, add 30.28 g TRIS hydroxymethy - aminomethane to approx. 800 ml. of distilled H_20.Adjust to pH 8.0 and adjust volume to 1 liter.

2. **pH adjustment**:
   a. Sodium hydroxide solution (NaOH) 6N: dissolve 240 g in 1 liter of distilled water.
   b. Hydrochloric acid solution (HC1) 6N: dissolve 218,76 g in 1 liter of distilled water.

3. **Polyethylene glycol solution**:

   Dissolve 30 g of polyethylene glycol (PEG) in 100 ml of distilled water or use dry flakes of PEG

4. **Decontamination:**

   Bleach: dilute 50 ml of concentrated bleach in 950 ml of water.

   NaOH 1N: dissolve 40 g in 1 liter of distilled water.

D. **General Precautions and Recommendations.**

1. Store the kit at 2-8°C when not in use.
2. Read the instructions for use entirely before using the kit.
3. Enterotoxins represent a potential risk or food poisoning. The use of gloves is highly recommended when performing the test.
4. Decontaminate all materials and reagents that have been in contact with staphylococcal enterotoxins with bleach or NaOH.
5. Do not pipette reagents by mouth.
6. Strictly follow incubation times.
7. In case of contact of any reagent with eyes or skin, rinse immediately with plenty of water.
8. A safety data sheet is available on request to the manufacturer.

E. **Procedures for preparation of controls and the extraction of enterotoxins from suspect foods.**

In addition to the food extraction procedures described here, a greater variety of food extraction procedures are presented in manufacturer's directions.

NOTE: Prepare food extracts immediately before testing.

1. Recommended controls

    a. **Positive toxin control**.

    Positive control solution is prepared by adding 10 µl positive control to 500 µl of wash buffer in a polypropylene tube. Run positive control whenever assay is run to verify that all reagents are functional and that assay has been conducted correctly. Discard unused diluted toxin control into sodium hypochlorite solution.

    b. **Negative toxin control**.

    Use negative control solution provided in kit. No dilution of negative control solution is necessary. Use 500 µl of all controls.

    c. **Positive food control (optional)**.

    Add aliquot of positive control provided in kit to known enterotoxin-negative food product to serve as positive food control. Extract and assay sample under same conditions as suspect sample.

    d. **Negative food control (optional).**

    Use same type of food as suspect food, but which is known to be toxin-free. Prepare negative food control in exactly the same manner as suspect food. This control will ensure that washing of wells was adequate and that no food components will interfere with test results. Extract and assay sample under same conditions as suspect sample.

2. Extraction of toxin from foods (see Extraction of Enterotoxins from Foods for ELISA Assays, above.)

3. **Immunoenzymatic Test, Transia™**

Recommendation for Use

    a. Bring all the reagents and the samples to room temperature (18-25°C) one hour before use.

    b. Shake manually or vortex each vial before use.

    c. Return the reagents to 2-8°C storage after use.

d. Do not interchange individual reagents between kits of different batch numbers.

e. The washing step is very important: when washing, direct a strong stream to the bottom of the tubes.

f. Incubations of the immunoenzymatic test are to be done under agitation (about 600 rpm).

F. **Immunoenzymatic test**

See immunoenzymatic test flow chart (Figure 9)

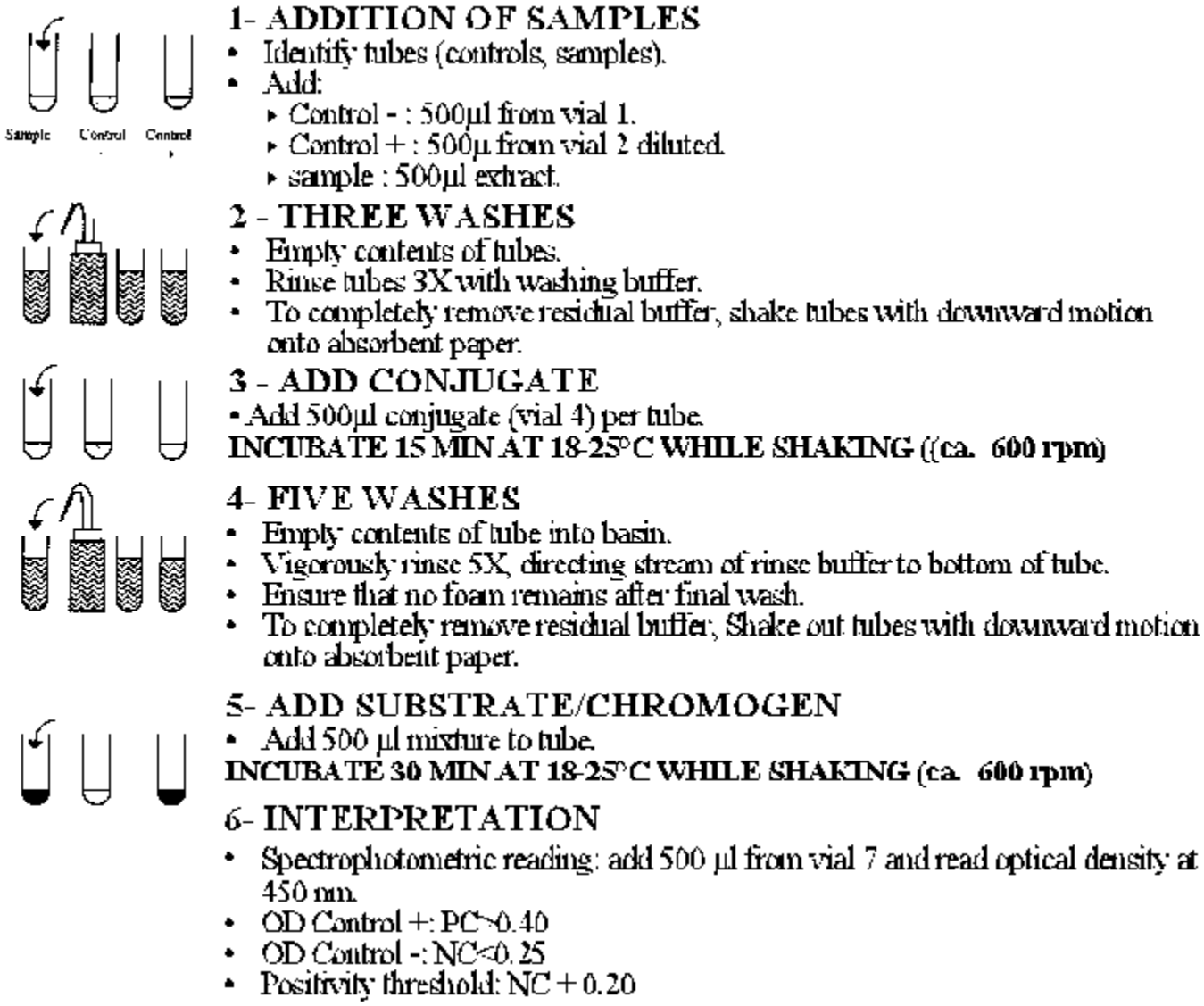

Figure 9. Scheme for Immunoenzymatic test.

a. Remove the number of tubes needed from the bag and put them into the test tube rack.

Allow:

1 tube for the negative control (**Vial 1**),
1 tube for the positive control (**Vial 2**),
1 tube per sample.

b. Return unused tubes to the plastic bag with dehydrating agent and close hermetically.

c. Identify the tubes with the reference of the sample to test and note their location on the work sheet (ENRCOM 180).

d. Prepare the washing buffer (**Vial 3**): Dilute the washing buffer in distilled water 30X, homogenize, and transfer to the plastic squeeze bottle.

e. Prepare the positive control: dilute 40 µl of the positive control 50X (**Vial 2**) in 2 ml of reconstituted washing buffer. Mix properly (the preparation of a larger volume than the required volume reduces the errors that may occur from pipetting of a too low positive control volume).

f. With a micropipette, add the controls and the samples: 500 µl per tube. Change tips each time.

g. Incubate for 15 min at room temperature (18-25°C) with shaking.

   NOTE: Increasing the first incubation period from 15 to 60 min improves the detection of enterotoxins.

h. Wash the tubes 3 times: empty the tubes by inverting them over a basin. Fill tubes rigorously with the washing buffer to the bottom of each tube and empty: repeat 3 times. Shake out the tubes with a downward motion on absorbent paper to completely remove any residual buffer.

i. Add 500 µl conjugate (**Vial 4**) in all the tubes.

j. Incubate for 15 min at room temperature (18-25°C) with shaking.

k. Wash the tubes 5 times as described above. Return the washing buffer to 2-8°C storage.

l. Prepare the substrate-chromogen mixture (**Vial 5 and Vial 6**) as follows: for n tubes, mix n x 300 µl of substrate with n x 300 µl of chromogen.

m. Add 500 µl of the substrate-chromogen mixture to all tubes.

   NB: Separate addition of the substrate and the chromogen can be done: distribute successively 250 µl of substrate (**Vial 5**) and then 250 µl of chromogen (**Vial 6**).

n. Incubate for 30 minutes at room temperature (18-25°C) while shaking.

o. If results are read with a spectrophotometer or microtiter plate reader, add with a multipipette 500 µl of the stopping solution (**Vial 7**) to all the tubes.

G. **Transia**™ test validation

The optical density of the positive control (PC) has to be higher than or equal to 0.40. The optical density of the negative control (NC) has to be lower than or equal to 0.25. The test can be validated if the optical densities of the controls meet the requirements defined above. If not, start the test again.

H. Interpretation of results.

Spectrophotometric reading

Read the optical densities at λ= 450 nm against an air blank. If you do not have a spectrophotometer for tubes, read the optical densities after having transferred the contents of the tubes into identified microcuvettes with 1 cm of optical pathway.

Microtiter plate reading

At FDA, a microtiter plate reader set at a wavelength of 450 nm is used to determine the optical density of each sample. This is accomplished by removing 200 µl of the test sample after addition of the stop solution and adding this volume (200 µl) to

plain (unsensitized) flat bottom polystyrene microwells (Removawell, Dynatech) designed to fit a Removawell strip Holder (Dynatech Laboratories, Inc.). Place holder containing the sample extracts in microtiter plate reader and determine their absorbances. Record the results from the microtiter plate reader printout on the worksheet.

- **Definitions:**

  Positivity threshold:

  Optical density of the negative control + 0.20.

  - **A sample is considered positive** if its optical density is higher than or equal to the positivity threshold.
  - **A sample is considered negative** if its optical density is lower than the positivity threshold: the tested sample does not contain enterotoxins or does not contain a detectable level by the used method.
  - **If the optical density of an extract is slightly lower** (between NC + 0.15 and positivity threshold) than the positivity threshold, results can be checked by concentrating the extract through dialysis against 30% (w/v) PEG(cut off threshold 12,000 to 14,000 Da) and by testing again: an increase in the optical density means the presence of enterotoxins in small quantity.

    NOTE: Some food samples containing peroxidase, protein A or endogenous substances may interfere with this method. False positives are rare and inconsistent.

I. Serotype toxin confirmation.

If confirmation of serotype by the AOAC method is necessary, use $CHCl_3$ to extract as previously described, and proceed with remaining steps in procedures described in BAM. Analyze chromatographed eluate with microslide gel double diffusion test. A faster, more sensitive option would be to confirm with the SDS-PAGE-Western blotting method described in this chapter.

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Center for **F**ood **S**afety & **A**pplied **N**utrition

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Chapter 13b

Electrophoretic and Immunoblot Analysis of Staphylococcal Enterotoxins in Food

Author

(Return to Table of Contents)

Immunoblotting can detect *Staphylococcus aureus* enterotoxin A in food. The method may also be adapted to other toxins in foods.

Staphylococcal enterotoxins (SE), a family of five major serological types of heat stable (1,6,9,11,18, 19,20, 21), emetic enterotoxins (SEA through SEE), are encoded by five genes, which share 50 to 85% homology at the predicted amino acid level (5,12). Enterotoxin A (SEA), a 27 kDa monomeric protein, is an extremely potent gastrointestinal toxin (2,7)and requires very sensitive methods to detect the low levels in foods (ng/g food) that can cause illness.

After antibodies to SEA were produced, immunological testing became the method of choice for SEA detection (4). Radioimmunoassay (13), microslide double diffusion and enzyme-linked immunosorbent assay (ELISA), have been used for testing food samples. ELISA is especially useful, because it is simple, sensitive (0.5 ng/ml), rapid, and available in commercial kits that

use distinct antibodies, either polyclonal or monoclonal.

Cross-reaction with unrelated antigens (15,16) or endogenous peroxides in particular foods that react with colorigenic reagents may not be distinguishable from positive results by some methods without extensive controls (17). In addition, heat-treated SEA (in heat processed foods) may give negative results, because heat-treated enterotoxin may aggregate, reducing its reactivity with antibodies. However, it may retain toxicity after heat treatment (1,3).

Methods for analysis of regulatory samples of foods must resolve or avoid "false positive" and "false negative" reactions. Before antibody is applied, the SDS-PAGE immunoblot method, described below, solubilizes and separates proteins, to discriminate cross reactions to heterologous proteins that may occur.

General Principle:

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a common method for protein separation (10,14). An electrical field is applied so that charged molecules migrate through a polyacrylamide matrix to the electrode bearing the opposite charge. The negatively-charged detergent, SDS, denatures and strongly binds proteins. Then, SDS-bound proteins migrate to the positive pole at rates inversely proportional to their molecular weights.

In general, two-part discontinuous gels are used (10). The sample is loaded onto the upper portion (stacking gel), which has a low acrylamide concentration, low pH, and low resolving ability. When a sample runs through the stacking gel, all proteins are concentrated into a narrow band. That narrow band then enters the lower portion (resolving gel) that separates proteins by size. The acrylamide concentration chosen for the resolving gel depends on the sizes of proteins to be separated. Smaller proteins are resolved at higher acrylamide concentrations and vice versa. SEs are 25-30 kDa; 12.5% acrylamide is useful for separating proteins in that range.

Immunoblotting (also known as "Western" blotting) is widely used for analyzing proteins separated by SDS-PAGE. The proteins are transferred from the gel to a membrane. Then, the membrane is probed with an antibody ("primary antibody") against the specific antigen. To detect the antibody-antigen complex, a secondary antibody is used. Usually, this is a polyclonal antibody (e.g. anti-mouse if the primary antibody is a mouse monoclonal) tagged with a biochemically detectable marker. Some common secondary antibody tags are fluorescent molecules (e.g. FITC, rhodamine), horseradish peroxidase, alkaline phosphatase, or biotin. Then, simple colorimetric reactions are carried out to reveal the location of the complex in a band on the membrane at a position corresponding to the molecular weight of the antigen.

Immunoblots for food testing

Immunoblots have two important advantages for food testing. First, even though heat and other treatments during food processing can cause proteins to aggregate, the aggregates are solubilized and unfolded in SDS gels. Other antibody-based methods of food analysis, such as ELISA, do not have an SDS solubilization step. Instead, the sample is applied directly to the antibody, because SDS in the sample would denature the detecting antibody. Second, cross-reacting antigens usually can be distinguished from the desired antigen on the basis of molecular weight in a Western blot. In ELISA, and other assays in which samples are evaluated without separation or purification, cross-reacting antigens increase the background.

A. Equipment and materials

1. Equipment: Electrophoretic apparatus: Vertical mini gel unit with 8 x 10 cm or 10 x 12 cm plates (Bio-Rad Mini-Protean II or Hoefer SE-260 or equivalent), 1.5 mm spacers, and 1.5 mm 10 well comb. (Wider combs and spacers for larger volumes may be custom-made).

2. Transfer unit, Mini electroblotting unit (Bio-Rad Mini-Trans-Blot or equivalent).

3. Power supply: Constant voltage of at least 200V and constant amperage of at least 400 mA (Bio-Rad PowerPac 300 or equivalent). A power supply with timer is recommended.

4. Microcentrifuge: A centrifuge that accomodates 1.5 ml (microcentrifuge) tubes and attains speeds of at least 10,000 rpm.

5. Homogenizer: An Omni H or other homogenizer for grinding small amounts of food.

6. Membrane - Nitrocellulose membranes (similar to Sigma N8142) the same size as gel.

7. Miscellaneous small equipment: Rotator or rocking platform for mixing samples, 100 x 15 mm square culture dishes (Falcon 1012 or equivalent), Pyrex baking dish (at least 10 inches square) and 1.5 ml plastic centrifuge (microfuge) tubes.

8. Scanner (recommended): A flatbed scanner with resolution of at least 600 DPI (similar to Hewlett Packard 4C).

B. **Reagents**

1. 30% Acrylamide/bis-acrylamide (0.8% bis) pre-mixed solution (Sigma A-3699 or equivalent)

   WARNING! ACRYLAMIDE IS NEUROTOXIC. ALWAYS WEAR GLOVES AND OTHER APPROPRIATE PROTECTION WHEN USING.

2. BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium), alkaline phosphatase substrate solution (Sigma B-6404 or equivalent).

3. Protein molecular weight color markers (6-200 kDa)(Sigma C-3437 or equivalent)

4. Purified Staphylococcal enterotoxin A (similar to Sigma S9399 or equivalent

5. Molecular biology grade reagents:
   a. Tris base
   b. SDS
   c. EDTA
   d. Tween 20 (Polyoxyethylenesorbitan Monolaurate)
   e. NaCl
   f. 6N HCl
   g. Glycine
   h. Ammonium Persulfate
   i. TEMED
   j. methanol
   k. non-fat dry milk (similar to Carnation)
   l. ß-mercaptoethanol
   m. bromphenol blue

6. **Antibodies:**
   a. Mouse monoclonal anti-SEA (IGEN Mab 3A #506-022-01)
   b. Rabbit polyclonal anti-SEA (Sigma S-7656 or equivalent)
   c. Goat anti-rabbit alkaline phosphatase conjugate (Sigma A2556 or equivalent)
   d. Goat anti-mouse alkaline phosphatase conjugate (Sigma A4937 or equivalent)

C. **Preparation of materials**

1. **1 M Tris, pH 7**. Dissolve 121.1 g Tris base in 750 ml H_2O. Add 6N HCl to pH 7.0 (approximately 160 ml.). Add H_2O to final volume of 1 liters.

2. **1M Tris, pH 8** Dissolve 121.1 g Tris base in 750 ml H_2O. Add 6N HCl to pH 8.0 (approximately 90 ml.). Add H_2O to final volume of 1 liters.

3. **20% (W/V) SDS** (wear a mask when weighing SDS)

4. **Gel Buffer A (3M Tris, pH 8.8)** Dissolve 181.6 g Tris base in 250 ml H_2O. Add 40 ml 6N HCl. Add H_2O to final volume of 500 ml.

5. **Gel Buffer D (0.25M Tris, pH 6.8)** Dissolve 15.1 g Tris base in 50 ml H_2O. Add 18 ml 6N HCl. Add H_2O to final volume of 100 ml.

6. **10X Running buffer (250mM Tris; 1.92M glycine; 1.0 % SDS)** Dissolve 121 g Tris base and 576 g glycine in 4 l H_2O. Stir until completely dissolved. Add 40 g SDS (wear a mask when weighing SDS).

7. **10X Western blot transfer buffer (250mM Tris; 1.92M glycine**) Add 121 g Tris base and 576 g glycine to 4 l H_2O; stir until completely dissolved.

8. **1X Western blot transfer buffer**. Mix 400 ml 10X Western blot transfer buffer with 2800 ml H_2O. Then add 800 ml methanol.

9. **Tris-Tween blocking buffer (10 mM Tris, pH8; 500 mM NaCl; 0.5% Tween-20)** Mix 116 g NaCl with 3 L H_2O. Add 40 ml 1.0 M Tris pH 8 and stir until dissolved. Add 20 ml Tween-20 and stir gently. Add water to 4 liters.

10. **Loading buffer (250mM Tris, pH 7; 4% SDS; 20% glycerol; 10% -mercaptoethanol; 0.05% bromphenol blue:** Mix 12.5 ml 1.0 M Tris pH 7 with 10 ml glycerol and 5 ml -mercaptoethanol. Add H_2O to 40 ml. Add 10 ml 20% SDS and 25 mg bromphenol blue.

11. **10% (w/v) APS (Ammonium persulfate)** - prepare fresh weekly; cover in aluminum foil.

D. **Casting gels**

Commercially Prepared gels are available through suppliers (depending on apparatus).

1. **Assemble the casting tray** - The gel is cast between two glass plates. Follow the

manufacturer's instructions and assemble the plates for casting two gels, using the 1.5 mm spacers. Always cast two gels; the second can be stored, used as a duplicate or as a backup for the first.

2. **Prepare lower gel** - Always wear gloves when handling acrylamide solutions Prepare the resolving (lower) gel (Table 1) in a 50 ml tube. Add acrylamide last. The amount of APS and TEMED is higher than normally recommended, so the solution polymerizes rapidly. If you find it hard to work fast enough, half the amount of APS and TEMED.

Table 1. **Lower (resolving) gel (for two 0.75mm gels)**

% Gel	**12.5%**	**15%**	**16%**	**17%**
Buffer A	2.8 ml	2.8 ml	2.8 ml	2.8 ml
H_2O	1.5 ml	0.83 ml	0.58 ml	0.33 ml
20% SDS	50 µl	50 µl	50 µl	50 µl
10% APS	30 µl	30 µl	30 µl	30 µl
TEMED	30 µl	30 µl	30 µl	30 µl
Acrylamide solution	3.1 ml	3.75 ml	4 ml	4.25 ml
Total	7.5 ml			

3. **Pour lower gel** - Quickly pour the gel solution between the glass plates to 2/3 of the height. Immediately overlay (**GENTLY)** with 300 µl H_2O or isobutanol. Note, however, that isobutanol may react with the plastic of the apparatus

4. **Prepare gel to add stacking gel** - The gel will polymerize within 15 minutes, forming a clearly visible interface between the water and the gel. The gel can now be used immediately or covered with Saran Wrap and stored (at 4°C) for later use. Right before using the gel, pour off the water (or isobutanol).

5. **Prepare and pour stacking (upper) gel** - Prepare the stacking gel solution (table 2), pour to top of plates and insert 1.5 mm comb. After the gel polymerizes, **GENTLY** remove the comb and wash the wells with H_2O. Drain the wells carefully.

Table 2. **Stacking gel (for two 0.75mm gels)**

% Gel	4.5%
Buffer D	0.625 ml
H_2O	1.5 ml
20% SDS	30 µl
10% APS	20 µl
TEMED	20 µl
Acrylamide solution	0.375 ml
Total	2.57 ml

6. **Checking the gels** - assemble the electrode unit (follow the manufacturer's instructions) and fill the upper buffer with H_2O to test for leaks.

E. **Sample Preparation**

1. Mushroom samples - Homogenize at least 1 g mushrooms (follow sampling procedure) with the homogenizer. Add an equal amount (w/v) of the can brine and homogenize again. Transfer 300 µl (approximately) to a 1.5 ml tube. Volume measurements may be inaccurate because these samples are very viscous. It may help to cut the micropipette tip to a larger aperture, or add approx.300 mg to a preweighed tube. Add an equal volume of Loading Buffer. Heat at 90°C for 2 min and centrifuge for 1 min.

2. **Purified SEA (positive control)** - Make a 1 µg/ml stock solution in H_2O. For 1 ng add 1 µl to 19 µl H_2O, and add an equal volume of Loading Buffer. Heat at 90°C for 2 min and centrifuge for 1 min.

F. **Running the gel**

1. **Loading** - Load gels with 40 µl (sample size depends on combs and spacer use; wider combs and spacers for larger volumes are available through the FDA upon request) of the sample per well. Whenever possible, skip one lane between samples, to minimize cross-contamination. Be sure to apply the positive control several lanes from any test samples. Place 5 µl (25 µg protein) of prestained (color) MW marker in a nearby well to monitor the progress of the run, the effectiveness of transfer and the size of the bands.

2. **Running the gel** - Pour the running buffer into the outer chamber and then **GENTLY** add buffer to the upper chamber, using a pipette so as not to disturb

the samples. When attaching the leads, **VERIFY** correct electrical polarity. Wrong electrical orientation is the most common mistake in SDS-PAGE. Run at 150 V for 1.5 h (one gel) or 100 V for 2.2 h (two gels), or until the bromphenol blue reaches the bottom of the resolving gel. When running two gels, make sure not to overheat the apparatus (lower the voltage if necessary).

G. **Immunoblotting**

1. Stop the gel, dissemble the electrophoresis unit, and remove the top glass plate of the gel.

2. **Assembling the transfer apparatus - Use gloves when handling nitrocellulose filters.** Assemble the transfer unit according to manufacturer's instructions, in a baking dish filled with cold transfer buffer to avoid air bubbles.

3. **Transferring proteins** - Add ice to the unit's cooling reservoir to keep it cool while running. Connect the electric lead and **VERIFY** correct electrical polarity. Electroblot at 400 mA for one and half hour (you may need to change the ice). **[Consult your manual].**

4. **Dissemble the unit** - Confirm successful transfer of colored MW markers. Discard gel and put membrane into a baking dish.

5. **Block the membrane** - Incubate in Tris-Tween Blocking Buffer for 20 min with gentle shaking in a square culture dish; use at least 20 ml solution.

6. **Primary antibody** - Add anti-SEA in Tris-Tween Blocking Buffer. If using the monoclonal anti-SEA from IGEN, dilute 1:300. Use at least 10 ml of solution (20 ml for two membranes). For SIGMA anti SEA dilute 1:1000. Shake gently for 2 hours.

7. **Washing** - Wash for 20 minutes in 20 ml or more of Tris-Tween Blocking Buffer with gentle shaking.

8. **Secondary antibody** - Incubate 1-2 hours with the secondary antibody. If a mouse monoclonal was used as the primary antibody, use an anti-mouse alkaline phosphatase conjugate diluted 1:1000 in 10 ml Tris-Tween Blocking Buffer.

9. **Washing and color development** - Wash vigorously three times for 20 min with at least 20 ml Tris-Tween Blocking Buffer. Add 10 ml BCIP/ NBT color reagent for detection. Watch for signal development and for background to determine the

correct incubation time empirically for each sample and membrane (approximately 10 min)**.**

H. **Densitometry (recommended)**

1. Dry the membrane and then scan the blot to quantitate the signal. Set the scan mode to 256 gray scale black and white photograph scanning. The approximate size of the file (in TIFF format) of a mini gel blot is 1.4 Mb. While contrast and brightness can be adjusted to improve the data for presentation, this will affect the quantitation of the image. Quantitation should always be performed with the raw data compared to a standard curve of known amounts of toxin. While there is no established method for immunoblot quantitation, the bands can be quantitated using NIH Image software (public domain software for MacIntosh).

I. **Data presentation. Test samples should be recorded using the form suggested here. This form contains all information necessary for Western blot data analysis.**

EXAMPLES

A. **Example 1- Western immunoblotting of food contaminated with SEA**

Western blotting was tested for the ability to detect SEA in foods that are commonly associated with food poisoning. Each sample was homogenized, spiked with purified SEA (2 ng/40 µl), and applied directly to the gel.

SEA was detectable in each sample, regardless of which food was present (Fig. 1). Undiluted milk samples distorted SEA mobility (data not shown), but ten-fold diluted milk samples ran correctly. Heterologous antigens cross-reacted in several samples, because polyclonal anti-SEA antibodies reacted with components from the food matrix. For example, the antibodies recognized a 66 KDa protein in milk, whether or not SEA is present in milk (Lanes 2 and 3, Fig. 1). This unrelated band did not affect the assay for SEA, because SEA is determined by the intensity of the 27 kDa band, detected only in the "spiked" sample.

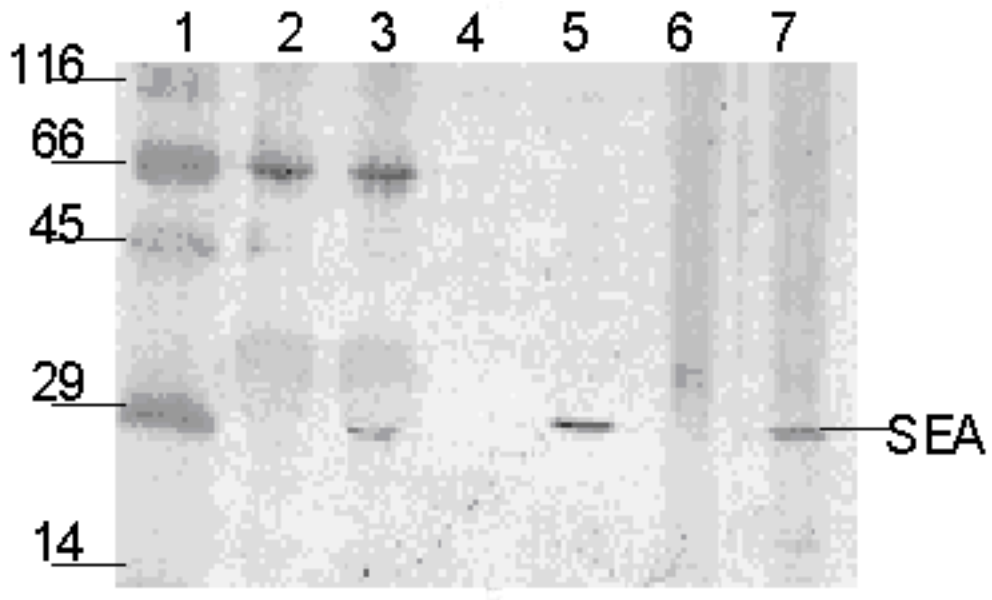

Figure 1. Western immunoblots of foods contaminated with SEA. Food samples were homogenized and spiked with purified SEA. The sample (40 µl) was then applied directly to the gel and assayed by Western blot. Milk, potato salad and meat product with or without SEA were tested. Lane 1 -- Protein Standards; Lane 2 -- milk; Lane 3 -- milk+SEA; Lane 4 -- potato salad; Lane 5 -- potato salad+SEA; Lane 6 -- meat; Lane 7 - meat+SEA.

B. **Example 2 - Detection of SEA in heat-treated mushrooms by Western blotting**

Canned foods are problematic for ELISA because ELISA often fails to detect heat-treated SEA. Canned mushrooms were used to see if Western blots can detect heat-treated SEA in food.

The contents of a can of mushrooms (113 g in 500 ml flask) were inoculated with an overnight culture (10^6 cells /ml) of *S. aureus* (ATCC13565), then cells were grown for 6 h at 37C with shaking. Samples were taken hourly to measure bacterial growth and SEA production. Each sample was autoclaved at 121°C for 20 min to simulate canning and then assayed by Western blot. As shown in Figure 2. SEA was detected in contaminated mushrooms at 130 min (lane 3), at mid-log phase. Although there are additional cross-reacting bands, they have different molecular weights from SEA, and do not affect the analysis. There is no 27 kDa band in the uninfected control (lane 1).

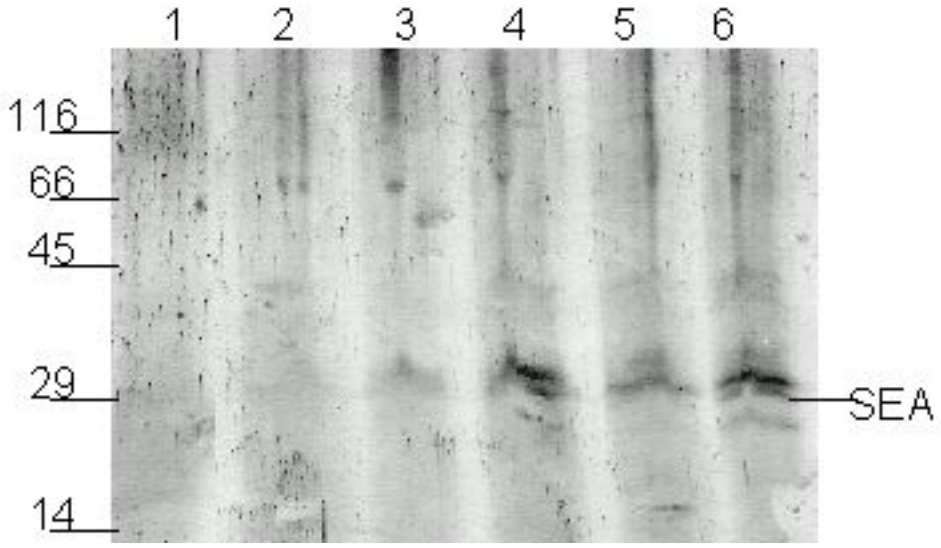

Figure 2. Detection of SEA produce by S. aureus (ATCC No. 13565) grown on mushrooms. Lane 1 -- sample after 0 min; Lane 2 -- 60 min; Lane 3 -- 130 min ; Lane 4 -- 180 min; Lane 5 -- 240 min; Lane 6 -- 300 min.

Problems and troubleshooting:

Problem	**Cause**
Slow or no polymerization of the gel	APS is old, OR APS, TEMED or acrylamide were left out
No tracking dye observed	Wrong polarity
Smile effect or gel overheats	High voltage leads to excessive heat.
Sample floats in the well or diffuses out of well	Wrong concentration of glycerol in the loading dye
No transfer	Wrong polarity or problem with transfer buffer.
Transfer OK but no signal in the positive control, membrane turns purple	problem with antibodies.
Transfer OK but no signal in the positive control, membrane is colorless	problem with detection reagents
High background	problems with blocking. increase time and/or add 0.5%-1% non-fat dry milk to the Blocking Buffer
Diffuse /distorted marker bands	Too little SDS
Distorted SEA band in samples	Too much protein in sample (overloading). Dilute sample or use chromatography or immunoprecipitation to remove major proteins

Limitations of Western blotting:

Western blotting has some limitations, which are important to recognize when applying the method to food analysis.

1. First, inactive and active SE are nearly indistinguishable by Western blotting (or any other antibody-based method).

2. Second, only small sample volumes (30-50 µl) can be loaded onto a gel (Wider combs and spacers for larger volumes are available through the FDA upon request)., which may limit the sensitivity of the method. Preparative methodology (tube gels and preparative electrophoresis), which is under development, may overcome this limitation. The present technology with small samples is nevertheless extremely sensitive. When compared directly with ELISA using contaminated mushroom samples, Western blotting was as sensitive as ELISA with native samples and much more sensitive with heat-denatured samples.

3. A third limitation of Western blots is that cross-reactive bands potentially could co-migrate with the antigen. Cross-reactivity is an inherent problem with all immunological methodology, because antibodies recognize small regions of proteins and similar epitopes may occur in other proteins. This is a major concern in ELISA and other methods in which the proteins are not separated. It is a smaller concern with Western blotting because the proteins are separated, but false positives are still a potential problem.

   There are several ways to minimize this problem. One is to increase the specificity of the reaction by using monoclonal antibodies. Alternatively, several independently isolated antibodies and control samples of uncontaminated similar food can be used to determine whether the bands represent toxin or unrelated antigens.

4. Finally, it is important to note that Western blots have a linear response over a broad range of toxin concentrations. However, at very low levels, the signal is not linear.

FDA ELECTROPHORETIC ANALYSIS REPORT			
FDA lab:	name:	record:	date:
Sample:			
Sample preparation:			

Gel: % acrylamide	spacer mm	sample vol. ml	apparatus:	
Running volts	time: min	remarks:		
Transfer mA	time: min	remarks:		
Blocking:	time: min	volume: ml	remarks:	
Antibody 1:	time: min	volume: ml	supplier:	dilution:
Antibody 2:	time: min	volume: ml	supplier:	dilution:
Wash buffer:	vol : ml	wash#1: min	wash#2: min	wash#3: min
Developing:	vol: ml	time min	Background:	

Densitometry:	resolution: dpm	setting:
Image analysis:	software:	setting:

Remarks:

Gel #1:			
lane #1	signal	lane#2	signal
lane #3	signal	lane#4	signal
lane #5	signal	lane#6	signal
lane #7	signal	lane#8	signal
lane #9	signal	lane#10	signal
Gel #2:			
lane #1	signal	lane#2	signal
lane #3	signal	lane#4	signal
lane #5	signal	lane#6	signal
lane #7	signal	lane#8	signal
lane #9	signal	lane#10	signal

Remarks:

Gel#1	Gel#2

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Top

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Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual *Online*

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Chapter 15

Bacillus cereus Diarrheal Enterotoxin

Author

(Return to Table of Contents)

Bacillus cereus is an aerobic sporeformer that is commonly found in soil, on vegetables, and in many raw and processed foods. Consumption of foods that contain large numbers of *B. cereus* (10^6 or more/g) may result in food poisoning, especially when foods are prepared and held for several hours without adequate refrigeration before serving. Cooked meat and vegetables, boiled or fried rice, vanilla sauce, custards, soups, and raw vegetable sprouts have been incriminated in past outbreaks (1). Two types of illness are attributed to the consumption of foods contaminated with *B. cereus*. The first and better known is characterized by abdominal pain and diarrhea; it has an incubation period of 4-16 h and symptoms that last for 12-24 h (4,5). The second, which is characterized by an acute attack of nausea and vomiting that occurs within 1-5 h after a meal; diarrhea is not a common feature in this type of illness.

Although certain physiological and cultural characteristics are necessary for identifying *B. cereus* (4), its enterotoxigenicity indicates whether a suspect strain may be a public health hazard. Evidence shows that diarrheal toxin is a distinct serological entity; in vitro methods that use specific antibodies have been developed to detect the toxin in culture fluids. The evidence for the emetic toxin, however, is still incomplete. This chapter presents a method for the routine culturing of suspect *Bacillus* spp., using a semisolid agar medium and a serological procedure (the microslide gel double diffusion test) to identify the enterotoxin.

A. Equipment and materials

1. Test tubes, 25 x 100 and 20 x 150 mm
2. Petri dishes, 15 x 100 and 20 x 150 mm, sterile
3. Bottles, prescription, 4 oz
4. Microscope slides, glass, pre-cleaned, 3 x 1 inch (7.62 x 2.54 cm)
5. Pipets, sterile, 1, 5, and 10 ml, graduated
6. Centrifuge tubes, 50 ml
7. Sterile bent glass spreaders
8. Electrical tape, 0.25 mm thick, 19.1 mm wide, available from Scotch Brand, 3M Co., Electro-Products Division, St. Paul, MN 55011.
9. Templates, plastic (Fig. 1)
10. Silicone grease, high vacuum, available from Dow Corning Corp., Midland, MI 48640
11. Sponges, synthetic
12. Wooden applicator sticks
13. Glass tubing, 7 mm, for capillary pipets and de-bubblers

14. Pasteur pipets or disposable 30 or 40 µl pipets, available from Kensington Scientific Corp., 1165-67th St., Oakland, CA 94601, if capillary pipets are not available.
15. Staining jars (Coplin or Wheaton)
16. Desk lamp
17. Incubator, 35 ± 1°C
18. Hot plate, electric
19. Sterilizer (Arnold), flowing steam
20. Blender and sterile blender jars (**see Chapter 1)**
21. Centrifuge, high speed
22. Timer, interval

B. Media and reagents

1. Brain heart infusion (BHI) broth (M24)
2. Glucose, dextrose anhydrous
3. Gel diffusion agar, 1.2% (R28)
4. Nutrient agar slants (M112)
5. Distilled water, sterile
6. Phosphate-buffered dilution water (Butterfield's buffer) (R11)
7. Normal (physiological) saline, sterile (antisera diluent) (R63)
8. Thiazine Red R stain (R79)
9. Slide preserving solution for stained slides, 1% acetic acid and 1% glycerol (R69)
10. No. 1 McFarland standard (R42)
11. Antisera and reference enterotoxins

C. Preparation of materials and media

1. **BHIG, 0.1%**. Adjust BHI broth containing 0.1% glucose to pH 7.4 and dissolve by stirring. Distribute medium in 30 ml portions in 125 ml flasks and autoclave at 121°C for 10 min.

2. **No. 1 McFarland standard**. Prepare turbidity standard No. 1 of McFarland nephelometer scale (5). Mix 1 part 1% $BaCl_2$ with 99 parts 1% H_2SO_4 in distilled water.

3. 1.2% Gel diffusion agar for gel diffusion slides. Prepare fluid base for agar in distilled water as follows: NaCl 0.85%; sodium barbital 0.8%; merthiolate 1:10,000 (crystalline), available from Eli Lilly and Co., Terre Haute, IN. Adjust pH to 7.4. Prepare agar by adding 1.2% Noble special agar (Difco). Melt agar mixture in Arnold sterilizer (steamer) and filter while hot, in steamer, through 2 layers of filter paper; dispense in small portions (15-25 ml) in 4 oz prescription bottles. (Remelting more than twice may break down purified agar.)

4. **Thiazine Red R stain**. Prepare 0.1% solution of Thiazine Red R stain in 1.0% acetic acid.

5. **Preparation of slides**. Wrap double layer of electrician's plastic insulating tape around both sides of glass slide, leaving 2.0 cm space in center. Apply tape as follows: Start a piece of tape 9.5-10 cm long about 0.5 cm from edge of undersurface of slide and wrap tightly around slide twice. Wipe area between tapes with cheesecloth soaked with 95% ethanol, and dry with dry cheesecloth. Coat upper surface area between tapes with 0.2% agar in distilled water as follows: Melt 0.2% bacteriological grade agar, and maintain at 55°C or higher in screw-cap bottle. Hold slide over beaker placed on hot plate adjusted to 65-85°C and pour or brush 0.2% agar over slide between 2 pieces of tape. Let excess agar drain into beaker. Return agar collected in beaker to original container for reuse. Wipe undersurface of slide. Place slide on tray and dry in dust-free atmosphere (e.g., incubator). **NOTE**: If slides are not clean, agar will roll off slide without coating it uniformly.

6. **Preparation of slide assembly**. Prepare plastic templates as described by Casman et al. (2) (Fig. 1). Spread thin film of silicone grease on side of template that will be placed next to agar, i.e., the side with the smaller holes. Place about 0.4 ml melted and cooled (55-60°C) 1.2% diffusion agar between tapes. Immediately lay

silicone-coated template on melted agar and edges of bordering tapes. Place one edge of template on one of the tapes and bring opposite edge to rest gently on the other tape. Place slide in prepared petri dish (**see** C-7, below) soon after agar solidifies and label slide with number, date, or other information.

7. **Preparation of petri dishes for slide assemblies**. Maintain necessary high humidity by saturating 2 strips of synthetic sponge (about 1/2 inch wide x 1/2 inch deep x 2-1/2 inches long) with distilled water and placing them in each 20 x 150 mm petri dish. From 2 to 4 slide assemblies can be placed in each dish.

8. **Recovery of used slides and templates**. Clean slides without removing tape; rinse with tap water, brush to remove agar gel, boil in detergent solution for 15-20 min, rinse about 5 min in hot running water, and boil in distilled water. Place slides on end, using test tube rack or equivalent, and place in incubator to dry. If slides cannot be uniformly coated with hot 0.2% agar, they are not clean enough and must be washed again. Avoid exposure to excessive heat or plastic solvents when cleaning plastic templates. Place templates in a pan and pour hot detergent solution over them; let them soak 10-15 min. Use soft nylon brush to remove residual silicone grease. Rinse sequentially with tap water, distilled water, and 95% ethanol. Spread templates on towel to dry.

9. **Directions for dissolving reagents used in slide gel**. The reagents are supplied as lyophilized preparations of enterotoxins and their antisera. Rehydrate antisera in physiological saline. Rehydrate reference enterotoxins in physiological saline containing 0.3% proteose peptone, pH 7.0, or physiological saline containing 0.37% dehydrated BHI broth, pH 7.0. These preparations should produce faint but distinct reference lines in the slide gel diffusion test. The lines may be enhanced (**see** E-3, below).

D. **Procedure for enumeration and selection of B. cereus colonies**. For examining food products, use procedures described for detecting B. *cereus* (**see** Chapter 14). Test isolates for enterotoxigenicity as described in E, below.

Production of enterotoxin. Of the methods described for the production of enterotoxin, cultivation of B. *cereus* in BHIG (0.1% glucose, pH 7.4) is simple and requires no special apparatus other than a shaker. Add loopful of growth from nutrient agar slants to 3-5 ml sterile distilled water or saline. Inoculate BHIG with 0.5 ml of this aqueous suspension, which should contain about 300 million organisms/ml. Turbidity of suspension should be equivalent to No. 1 on McFarland nephelometer scale. Deliver suspension with sterile 1.0 ml pipet. Shake flasks at 3 ± 2°C at 84-125 cycles/ml for 12 h. Good surface growth is obtained after 12 h of incubation. Transfer contents of flasks to 50 ml centrifuge tube. Remove organisms by high speed centrifugation (10 min at 32,800 x **g**). Examine supernatant for presence of enterotoxin by filling depots in slide gel diffusion assembly, as directed in E, below.

E. Slide gel diffusion test. To prepare record sheet, draw hole pattern of template on record sheet, indicate contents of each well, and give each pattern on record sheet a number to correspond with number on slide.

1. **Addition of reagents** (Fig. 2). Place suitable dilution of anti-enterotoxin (antiserum) in central well and place homologous reference enterotoxin in upper peripheral well (if diamond pattern is used); place material(s) under examination in well adjacent to well containing reference enterotoxin(s). Use reference toxins and antitoxins (antiserum), previously balanced, in concentrations that give line of precipitation about halfway between their respective wells. Adjust dilutions of reagents to give distinct but faint lines of precipitation for maximum sensitivity. (**See** C-9 for directions for dissolving reagents.) Prepare control slide with only reference toxin and antitoxin.

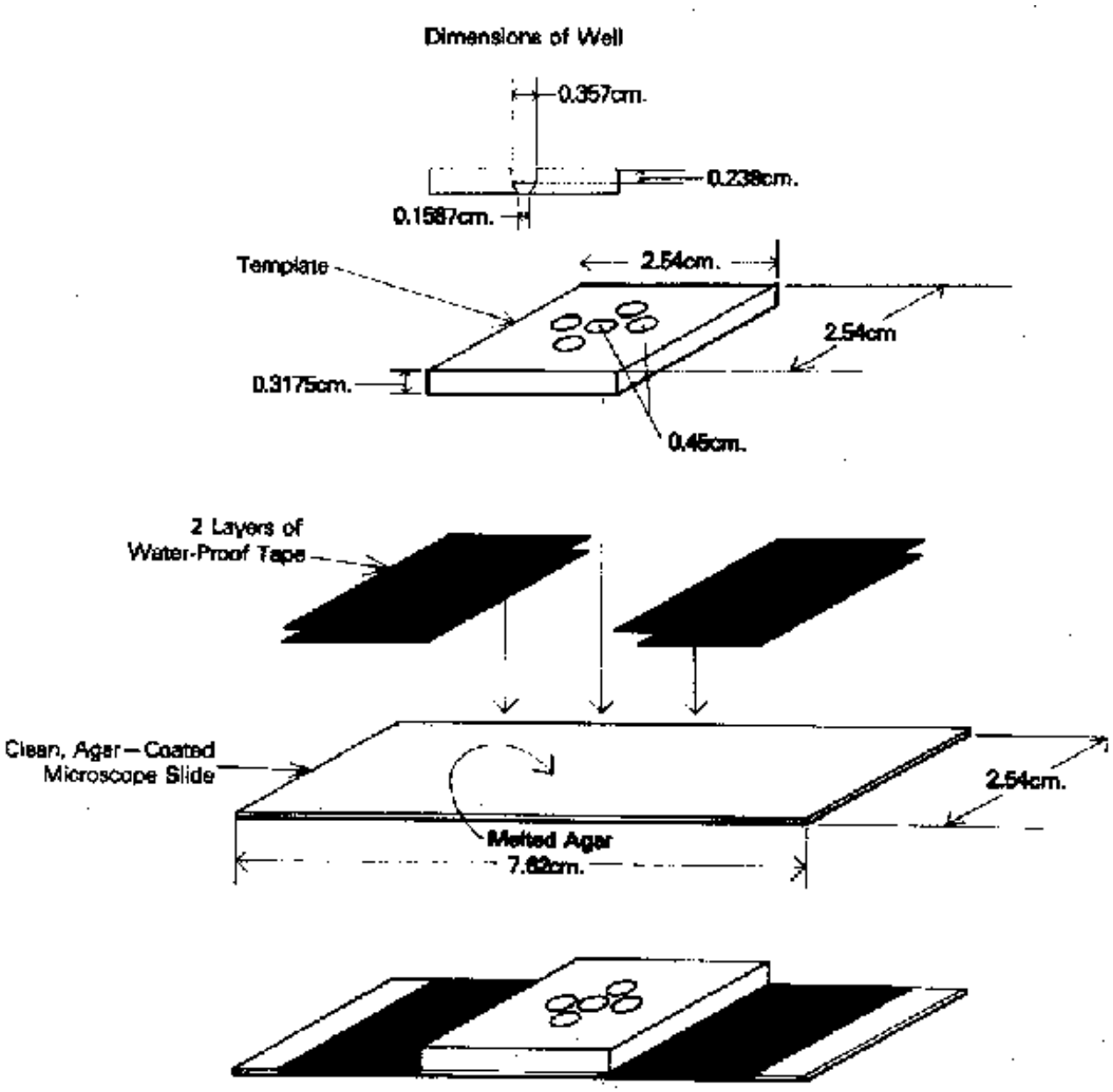

Fig. 1. Microslide assembly with diagram for preparation and specifications for plastic template.

Fill wells to convexity with reagents, using Pasteur pipet (prepared by drawing out glass tubing of about 7 mm od) or disposable 30 or 40 µl pipet. Remove bubbles from all wells by probing with fine glass rod. Make rods by pulling glass tubing very fine, as in making capillary pipets, breaking it into about 2-1/2 inch lengths, and melting ends in flame. It is best to fill wells and remove bubbles against a dark background. Insert rods into all wells to remove trapped air bubbles that may not be visible. Let slides remain at room temperature in covered petri dishes containing moist sponge strips for 48-72 h before examination or for 24 h at 37°C.

2. **Reading the slide**. Remove template by sliding it to one side. If necessary, clean slide by dipping momentarily in water and wiping bottom of slide; then stain as described below. Examine slide by holding over source of light and against dark background. Identify lines of precipitation through their coalescence with reference line of precipitation (Fig. 3). If concentration of enterotoxin in test material is excessive, formation of reference line will be inhibited; test material must then be diluted and retested. Figure 4, diagram A, shows typical precipitate line inhibition caused by enterotoxin excess in test preparation reactant arrangement in Fig. 2. Figure 5 shows typical line formation. Figure 6 shows a diluted preparation. Occasionally, atypical precipitate patterns that form may be difficult for inexperienced analysts to interpret. One of the most common atypical reactions is formation of lines not related to toxin but caused by other antigens in test material (Fig. 7).

3. **Staining of slides**. Enhance lines of precipitation by immersing slide in Thiazine Red R strain for 5-10 min, and then examine. Such enhancement is necessary when reagents have been adjusted to give lines of precipitation that are only faintly visible. Use staining procedure described by Crowle (3), modified slightly, when slide is to be preserved. Rinse away any reactant liquid remaining on slide by dipping slide momentarily in water and immersing it for 10 min in each of the following baths: 0.1% Thiazine Red R in 1% acetic acid; 1% acetic acid; and 1% acetic acid containing 1% glycerol. Drain excess fluid from slide and dry in 35°C incubator for storage as permanent record. After prolonged storage, lines of precipitation may not be visible until slide is immersed in water.

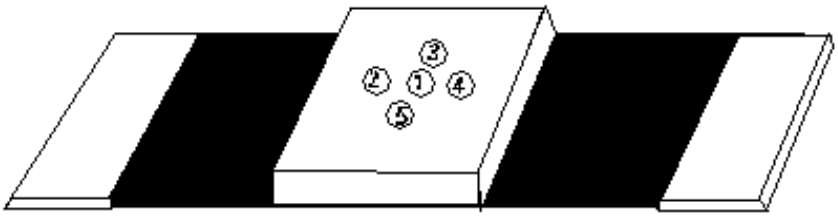

Fig. 2. Reagent arrangement for serologic identification of *B. cereus* diarrheal antigen: 1) antiserum to *B. cereus* antigen; 2) test preparation; 3) *B. cereus* toxin reference; 4) and 5) test preparations.

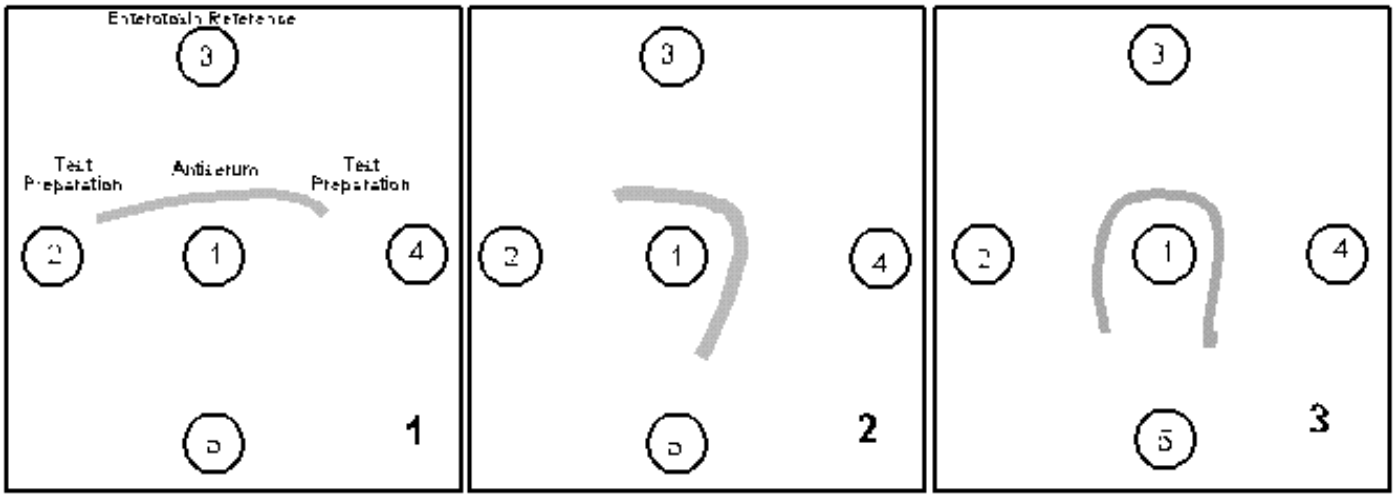

Fig. 3. Microslide gel diffusion test as toxin detection system; Antiserum to *B. cereus* diarrheagenic antigen is in well 1; known reference enterotoxin is in well 3 to produce reference lines; test preparations are in wells 2 and 4. Interpret reactions as follows: 1) No line development between test preparations and antisera -- absence of *B. cereus* toxin; 2) coalescence of test preparation line from well 4 with enterotoxin reference line -- presence of enterotoxin in well 4; 3) coalescence of test preparation line from wells 2 and 4 with enterotoxin reference -- presence of enterotoxin in wells 2 and 4.

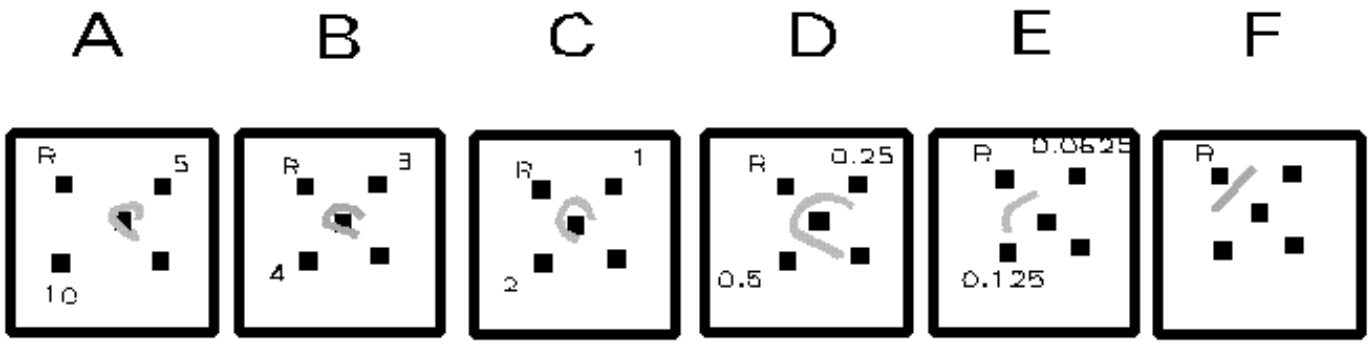

Fig. 4. Effect of amount of *B. cereus* enterotoxin in test preparation on development of reference line of precipitation. A, inhibition (suppression) of reference line when 10 and 5 µg enterotoxin/ml, respectively, are used. B-E, precipitate patterns when successively less enterotoxin (test preparation) is used. F, typical formation of reference line of precipitation in slide test control system.

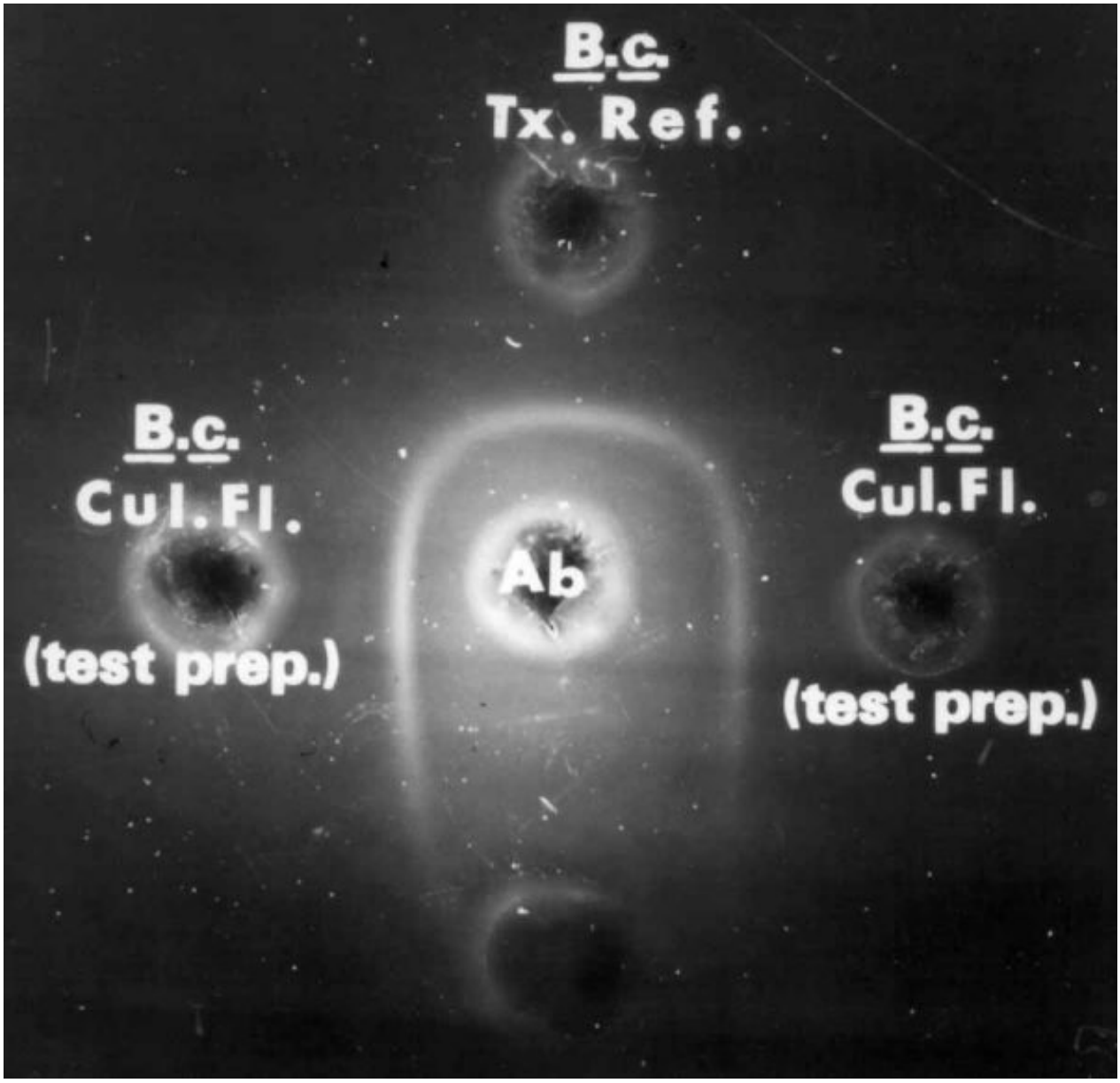

Fig. 5. Diarrheal antigen-antibody line of precipitation with microslide test. Interpretation of reaction: *B. cereus* culture fluids (right, left, and adjacent to reference toxin) contain diarrheagenic component, indicated by lines of

precipitation coalescing with reference line.

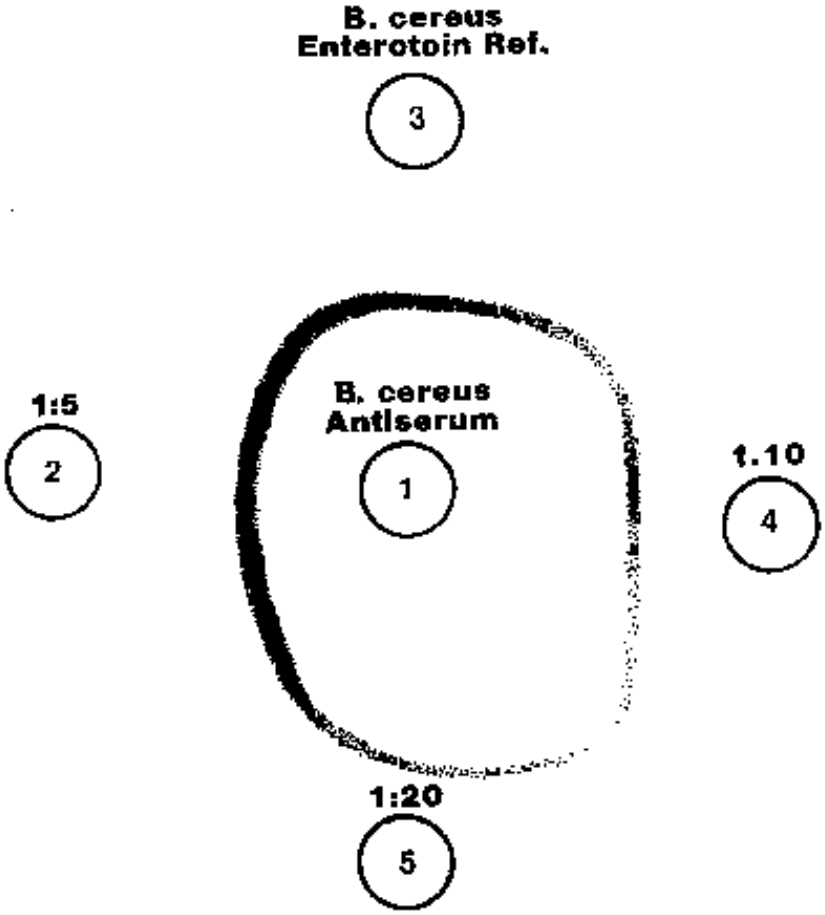

Fig. 6. Typical lines of precipitation of diluted *B. cereus* culture fluids, using microslide test.

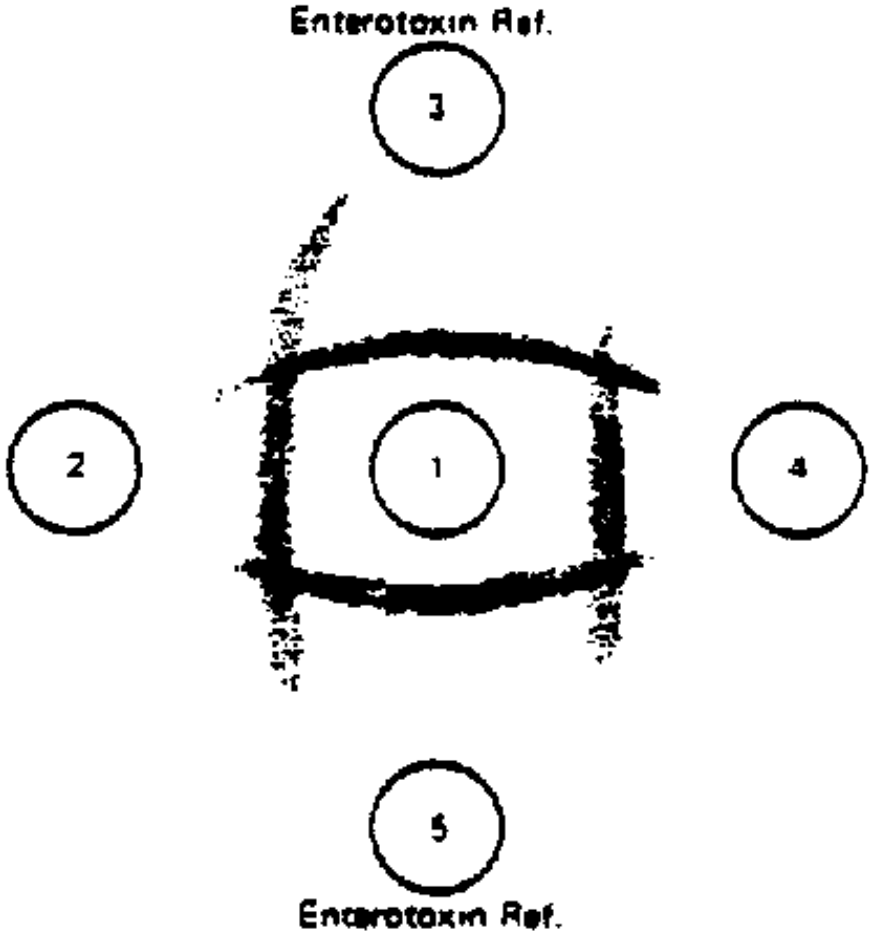

Fig. 7. *B. cereus* enterotoxin: Precipitate patterns in microslide gel diffusion test demonstrate nonspecific (atypical) lines of precipitation caused by other antigens reacting with nonenterotoxin antibodies. Test preparations (wells 2 and 4) are negative for enterotoxin but produce nonspecific lines of precipitation which intersect enterotoxin reference lines of precipitation.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 24

Identification of Foodborne Bacterial Pathogens by Gene Probes

Authors

(Return to Table of Contents)

DNA Hybridization

The identification of bacteria by DNA probe hybridization methods is based on the presence or absence of particular genes. This is in contrast to most biochemical and immunological tests that are based on the detection of gene products such as antigens or chemical end products of a metabolic pathway.

The physical basis for gene probe tests stems from the structure of DNA molecules themselves. Usually, DNA is composed of two strands of nucleotide polymers wound around each other to form a double helix. These long nucleotide chains are held together by hydrogen bonds between specific pairs of nucleotides. Adenine (A) in one strand binds to thymine (T) in the complementary strand. Similarly, guanine (G) in one strand forms a hydrogen bond with cytosine (C) in the opposite strand. For a discussion of the structure of DNA and nucleic acid hybridization, **see** Watson et al. (107). An overview (49) of DNA hybridization technology gives a more detailed explanation of hybridization theory, sample preparation, labeling, and formats.

The hydrogen bonds holding the strands together can usually be broken by raising the pH above 12 or the temperature above 95°C. Single-stranded molecules result and the DNA is considered denatured. When the pH or temperature is lowered, the hydrogen bonds are reestablished between the AT and GC pairs, reforming double-stranded DNA. The source of the DNA strands is inconsequential as long as the strands are complementary. If the strands of the double helix are from different sources, the molecules are called hybrids and the process is termed hybridization.

A gene probe is composed of nucleic acid molecules, most often double-stranded DNA. It consists of either an entire gene or a fragment of a gene with a known function. Alternatively, short pieces of single-stranded DNA can be synthesized, based on the nucleotide sequence of the known gene. These are commonly referred to as oligonucleotides. Both natural and synthetic oligonucleotides are used to detect complementary DNA or RNA targets in samples. Double-stranded DNA probes must be denatured before the hybridization reaction; oligonucleotide and RNA probes, which are single-stranded, do not need to be denatured. Target nucleic acids are denatured by high temperature or high pH, and then the labeled gene probe is added. If the target nucleic acid in the sample contains the same nucleotide sequence as that of the gene probe, the probe will form hydrogen bonds with the target. Thus the labeled probe becomes specifically associated with the target (Fig. 1). The unreacted, labeled probe is removed by washing the solid support, and the presence of probe-target complexes is signalled by the bound label.

In addition to DNA, probes and/or their targets can be made of RNA. A number of commercially available gene probe kits use synthetic DNA probes specific for ribosomal RNA targets. DNA:RNA and RNA:RNA hybrids are somewhat more thermally stable than DNA:DNA duplexes, but RNA molecules are quite labile at alkaline pH.

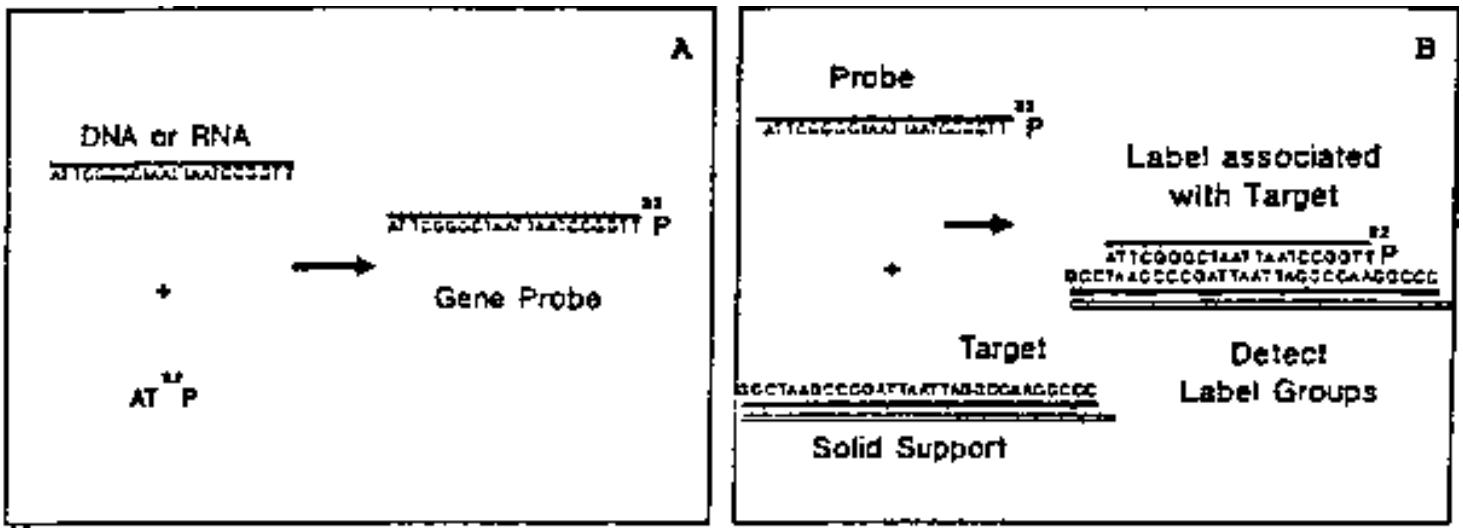

Fig. 1. A) Oligonucleotide of known sequence (derived from gene of known function) is end-labeled with radioactivity, using AT^{32}P and bacteriophage T4 polynucleotide kinase. B) DNA probe is allowed to incubate with DNA extracted from a sample. If target DNA (immobilized on a solid support) contains sequences complementary to those of the probe, the probe (and its radioactive label) will bind with the sample DNA. For purposes of illustration, this schematic shows the two strands of DNA parallel to each other. In reality, they are wrapped around one another to form a double helix.

Colony Hybridization

DNA hybridization tests may be performed in many ways. One format, the colony hybridization assay (29,59), will be described here. Generally, an aliquot of a homogenized food is spread-plated on an appropriate agar. After incubation, the colonial pattern is transferred to a solid support (usually a membrane or paper filter) by pressing the support onto the agar surface. Next, the cells are lysed in situ by a combination of high pH and temperature (0.5 M NaOH and/or steam or microwave irradiation), which also denatures and affixes the DNA to the support. The solid support with the attached target DNA is incubated with a ^{32}P- or enzyme-labeled probe. The labeled probe DNA that fails to reform the double helix is removed by washing the probe-target complexes on the support at an appropriate temperature and salt

concentration.

Great care must be taken to ensure that the washing temperature is correct; this parameter is usually determined empirically. If the temperature of the washing solution is too high, all the hydrogen bonds between the probe and target may be broken, producing a false-negative result. If the washing temperature is too low, strands of DNA will not match up accurately, and noncomplementary strands may be formed, leading to a false-positive outcome. If the temperature allows only accurately rejoined strands to remain together, the conditions are termed "high stringency." If the temperature is too low, so that mismatched strands exist, the stringency is low. For a review of hybridization using solid supports, **see** Meinkoth and Wahl (62).

The radioactive probe DNA that is bound to the target on the support is often detected by autoradiography. An X-ray film is placed over the support. Radioactive decays expose the film, so that when it is developed, black spots appear where cells are harboring the same gene as the probe (Fig. 2). If an enzyme-labeled probe is used, a chromogenic substrate is added. Where the probe-associated enzyme is present, a colored spot will develop. Each spot represents a bacterial colony that has arisen from a single cell. The number of cells harboring the target gene in the original sample can be calculated by multiplying the number of spots by the dilution factor.

Table 1. Some gene probes used to detect pathogenic bacteria in foods

Bacteria	**Target[a]**	**Reference**
Campylobacter jejuni	r-RNA	86
Escherichia coli	Heat-stable toxin (ST)	74
		103
		35
	Heat-labile toxin (LT)	104
		112
	Shiga-like toxins	41
	Invasive genes	54
	O157:H7	20
Listeria species	r-RNA	50
L. monocytogenes	Listeriolysin O	11
	Major secreted polypeptide (*msp*)	10, 24
Salmonella species	r-RNA	7
Shigella species	Invasive genes	54
Staphylococcus aureus	Enterotoxin B	78
Vibrio cholerae	Cholera enterotoxin	57

V. parahaemolyticus	Thermostable direct hemolysin (*tdh*)	75
V. vulnificus	Cytotoxin-hemolysin	112
Yersinia enterocolitica	Cytotoxicity/Sereny	65
	Invasive gene (*ail*)	68
Y. pseudotuberculosis	Invasive gene	19

[a] **See** text under description of individual probes to identify targets.

Target Selection

The first step in developing a gene probe assay is to decide what information is needed. If a particular taxonomic group is to be identified, the probe must be directed toward a gene or region of a gene that is conserved throughout a particular species or genus. On the other hand, one may want to know if a microorganism carrying a particular gene is present. Probes to specific determinants of virulence are useful in assessing a risk to public health posed by bacterial contamination.

Table 1 lists probes that have been used or are of potential use for detecting bacterial pathogens in foods. In the section, "Probes and Their Targets," the development of each probe is described briefly along with what is known about the probe target and its significance. The first probes designed to detect all members of a taxonomic group were constructed by screening randomly cloned DNA fragments. As data on the evolution of ribosomal RNA nucleotide sequences accumulate, probes are being directed toward these targets. Conserved regions can be used to identify large taxons, whereas the variable regions may be unique for a particular genus or species. Furthermore, as a cell contains more than 1000 copies of ribosomal RNA, test sensitivity is increased, because fewer cells are required to produce a positive signal.

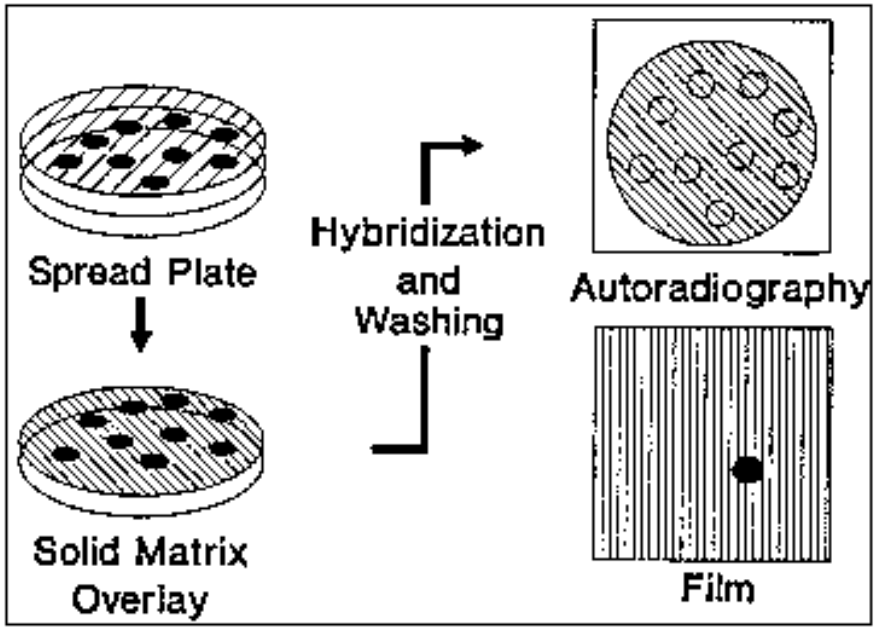

Fig. 2. Aliquot of homogenized sample is spread-plated on appropriate medium (cross-hatched area) and

incubated until colonies are formed. Colonies are transferred by gentle contact to solid support such as a filter (hatched area). Colony cells are lysed in situ by high pH and/or steam or microwave irradiation, which immobilizes single-stranded target DNA. Filters are then incubated with a labeled gene probe. (In this figure, a radioactive label was used.) Unbound probe is removed by washing the filter at a temperature that allows well-matched double strands to remain joined; poorly matched strands are separated. If DNA from a colony contains the same genetic information as the probe, that area of the filter will become radioactive. Radioactivity is observed as a dark spot on an X-ray film. Count the spots to calculate the number of cells containing specific gene present in the original sample.

Probe Specificity

The relatively short length of synthetic oligonucleotide probes means that they are specific for particular regions of DNA. There is only about 1 chance in 15,000 that a sequence length of 18 bases would appear more than once in the *E. coli* genome. With a 22-base probe, the chance drops to about 1 in 4 million. To avoid mismatches that reduce specificity, filter washings are conducted at high stringency so that a single base-pair difference between target and probe could not result in hybridization and produce a negative result. Such changes occur as the result of rare mutations. The use of two nonoverlapping probes would significantly reduce the probability of false negatives.

Construction of Probes

Recombinant DNA techniques have made gene probes possible. Probe tests require preparations of relatively pure, specific segments of DNA. The first probes were obtained by inserting these regions into plasmids and transforming the plasmids into the appropriate host cells to increase the amount of probe DNA. Plasmids were purified, and in some cases the inserted fragments were isolated. These cloned, natural DNA probes served quite well, although a considerable amount of effort was required for their production and purification. Through the development of DNA sequencing and automated oligonucleotide synthesis, short (18-30 bases) DNA probes were produced in the laboratory by chemical means. The ready availability of probes considerably expanded their use and application.

Probe Labeling

For probes consisting of cloned DNA fragments, the nick translation method (89) for labeling DNA with radioactivity is very popular. Cloned DNA can also be labeled by a random priming technique (18). Several kits to perform these reactions are commercially available; however, these techniques are unsatisfactory for labeling short oligonucleotides. Oligonucleotide probes are usually labeled on the 5' end with ^{32}P, using bacteriophage T4 polynucleotide kinase and gamma-AT ^{32}P (88). Although radioactive gene probes seem to have the greatest sensitivity in colony hybridization procedures, they are a potential biohazard, and disposal of radioactive waste can be expensive.

Many schemes are being examined for the nonradioactive labeling of gene probes. Some of these techniques have been incorporated into commercial tests designed to signal the presence or absence of a particular gene. For example, alkaline phosphatase has been conjugated to synthetic oligonucleotides without affecting the kinetics or specificity of the hybridization reaction (40).

The Polymerase Chain Reaction

At present it is not practical to use gene probes to detect bacteria directly in foods. Current methods require about 10^5-10^6 copies of the target sequence to yield a clear, positive result. To make this number of copies, cells are allowed to replicate in liquid media or to form colonies on agar plates. The growth period is usually overnight, adding 16-24 hours to the length of the test.

It is now possible to amplify specific DNA segments enzymatically to a million-fold in 1-3 hours. This process is called the polymerase chain reaction (PCR) (92). The reaction has been automated by using a thermostable enzyme and a programmable heating block (93). Because of the rapid amplification of target DNA, 1-day probe tests may be developed in the near future. A review of PCR has been published (16). PCR has been used to detect enteroinvasive *E. coli* and *Shigella* spp. (54), *V. vulnificus* (37) Hepatitis A virus (**see** Chapter 26), and *V. cholerae* (**see** Chapter 28) in foods.

Description of Probes and Their Development

The design and construction of gene probes requires careful scientific experimentation and a series of complex decisions. A first step is to determine if the gene probe is to be targeted to a particular pathogenic strain or to an entire taxonomic group. A target must be chosen so that all of the microorganisms to be detected contain such a gene. For probes designed to detect all members of a genus or species, ribosomal RNA has been a popular target because it contains both conserved and variable regions. If a pathogenic strain is sought, a probe is usually targeted to a virulence factor gene responsible for causing disease. A considerable amount of research is needed to identify the genes involved and the role they play in pathogenesis.

Probes and Their Targets

Campylobacter jejuni: Ribosomal RNA

A probe that is specific for *C. jejuni* ribosomal RNA genes has been developed (86,87) and is available commercially. A pool of randomly selected and tested chromosomal fragments is also specific for *C. jejuni*, but the target has not been reported (83).

Escherichia coli: Heat-labile enterotoxin genes

The heat-labile enterotoxins (LT) of *E. coli* are a closely related group of proteins; they are distinguished from heat-stable enterotoxins (ST) by being immunogenic and are inactivated by heating at 60°C for 10 Min (31). The toxins stimulate adenylate cyclase (30) and can be detected by tissue culture assays of Chinese hamster ovary cells (30) or mouse Y-l adrenal cells (13). Using these tests, So et al. (102) localized and cloned the structural gene for LT; Dallas et al. (8) recloned a smaller fragment into plasmid pEWD299. Although there are several different genes for LT, as evidenced by their nucleotide sequences (56,73,103,110,111), they all share a significant amount of genetic similarity. The region of the LT genes chosen as a gene probe target is identical in each of these genes, so that all strains with the genetic potential to produce LTs should be detected.

The LT probe, eltA11, is a 20 base synthetic oligonucleotide that encodes amino acids 45-51 of the A subunit of the *E. coli* LT (111).

E. coli: Heat-stable enterotoxin genes

The heat-stable enterotoxin (ST) of *E. coli* is distinguished from LT (above) by heat stability and lack of immunogenicity. It can be detected by the suckling mouse bioassay (12) and acts by stimulating guanylate cyclase (22). There are at least two different types: ST I (also known as STa and STP) and ST II (also known as STb and STH). The latter toxin is not active in the infant mouse assay. These genes have been cloned and the nucleotide sequences of the region encoding STa and STb have been determined (74,82,101).

The STP probe is a 22 base synthetic oligonucleotide for the toxin type strain first isolated from pigs. It targets the region of the gene that encodes amino acids 4-12 of the toxin protein.

The STH probe is targeted to the ST elaborated by a strain of *E. coli* isolated from a human. The probe is also 22 bases long and targets the region of the STH that encodes amino acids 19-26 of the toxin.

Both of these probes have been tested for their specificity, and data are available on their ability to detect a few ST-producing cells against a high level of ST-negative microorganisms (35). The reliability of the colony hybridization technique with oligonucleotide probes was tested by collaborative study, using pure cultures of strains harboring the STH or STP genes (36).

Enteroinvasive *Escherichia coli* (EIEC) and *Shigella*: Invasive gene

Some strains of *E. coli* invade colonic epithelial cells, multiply intracellularly, and spread intercellularly, causing a dysenteric enteritis similar to that caused by *Shigella* (15). However, an important difference is that the infectious dose for *Shigella* may be as low as 1-10 organisms, whereas 10^8 EIEC cells are necessary to cause disease. A number of genetic determinants that encode virulence factors of EIEC and *Shigella* spp. are located on a large [220 kilobase (kb) pair] invasion plasmid (96). Loss of this virulence plasmid renders the bacterium avirulent (97). A 17 kb *Eco*RI fragment was used as a hybridization probe to detect invasive *Shigella* species and EIEC (4).

Small and Falkow (100) demonstrated that a 2.5 kb pair *Hind*III fragment of the large plasmid is required for invasion of human epithelial cells. Plasmid DNA involved in the invasion of HeLa cells by *S. flexneri* has also been cloned (61). These regions of the plasmid have been sequenced and are genetically similar (54). A probe from this region of the plasmid is specific for tissue culture cell-invasive EIEC and *Shigella*. Such probes also identify strains that are invasive in the guinea pig eye assay (98). Of 41 probe-positive isolates tested by the guinea pig method, 2 were negative, indicating that a few strains may be invasive in tissue culture assays but not in tests that require a greater number of pathogenic determinants (108). A synthetic probe of 18 bases has been constructed. Its target is within a gene that encodes for a virulence factor.

Enterohemorrhagic *E. coli* (EHEC): Shiga-like toxin (SLT) genes

Human illnesses ranging from simple diarrhea to hemorrhagic colitis and hemorrhagic uremic syndrome have been associated with strains of *E. coli* that produce moderate to high levels of Shiga-like toxins (SLTs). Strains of *E. coli* serotype O157:H7 are the most significant pathogens associated with hemorrhagic colitis; strains of serotype O26:H11 are also classified as EHEC. More than 50 other serotypes

of *E. coli* that produce SLTs have been identified, but the correlation of these serotypes with disease is uncertain. Two related but distinct cytotoxins, SLT I and SLT II, have been characterized. Individual strains produce one or both cytotoxins. For example, *E. coli* O157:H7 produces SLT I, SLT II, or both, whereas O26:H11 produces only SLT I. The DNA sequences of SLT I and SLT II structural genes have been published, and analysis shows that SLT I has 99% homology with the Shiga toxin gene of *S. dysenteriae* type 1, but SLT II has only 60% homology (41). Two synthetic oligodeoxyribonucleotide probes were prepared from sequence data from the A-subunit regions of the SLT I and SLT II genes (nucleotides 473-490 and 472-490, respectively). HC agar (M62) (**see** ref. 105) was the selective medium chosen to screen isolates and foods for *E. coli* strains that carry the SLT gene because the growth of *E. coli* O157:H7 is less inhibited on HC agar than on other selective media. HC agar contains NaCl and a lower concentration of bile salts No. 3. Its plating efficiency of a strain of *E. coli* O157:H7 at 37 and 43°C for 17 h was similar to that of plate count agar. Plating efficiencies of other *E. coli* serotypes that carry the SLT gene have not been determined. The modified enrichment procedure of Doyle and Schoeni (14) is included in the method for detection of low level contamination of foods. For additional information about enterohemorrhagic strains of *E. coli*, see the review by Karmali (46).

Enterohemorrhagic *E. coli* (EHEC): O157:H7 serotype-specific probe

The fluorogenic MUG assay for *E. coli* is based on the activity of the -glucuronidase (GUD) enzyme, which is encoded by the *uidA* gene in *E. coli*. Although isolates of serotype O157:H7 are negative with the MUG assay, genetic studies have shown that this EHEC serogroup also contains *uidA* gene sequences for the GUD enzyme (21). Sequencing analysis has determined that the *uidA* gene of O157:H7 serotype contains several base mutations; therefore, it is not identical to the *uidA* gene of MUG assay (+) *E. coli*. Although the base mutations in the *uidA* allele of O157:H7 do not appear to be responsible for the absence of the MUG phenotype, one of the base changes was found to be conserved among the O157:H7 serogroup. An oligonucleotide probe, PF-27, directed to this base alteration was developed and determined to be specific only for EHEC isolates of serotype O157:H7. Other SLT-producing EHEC and other pathogenic *E coli* or enteric bacteria failed to hybridize with PF-27 (20).

***Listeria monocytogenes*: Invasion-associated protein (iap) and hemolysin (*hly*) genes**

Of the seven *Listeria* species that have been isolated from a variety of foods, including dairy, vegetable, meat, and poultry products, only *L. monocytogenes* has been implicated in human disease. Genetic and physiological studies have incriminated an extracellular hemolysin as one of the virulence factors in *L. monocytogenes* (5,26,47). This hemolysin (also called listeriolysin O or alpha-listeriolysin) has been cloned and sequenced (11,55,64). Several oligonucleotides (including AD13) were constructed by using the sequence of the listeriolysin O gene (64) and can specifically identify *L. monocytogenes* in foods by colony hybridization (11,70).

A 5.3 kb DNA fragment encoding a 60 kilodalton (Kdal) protein (*msp*) associated with hemolytic activity has been cloned (24). Kuhn and Goebel (53) reported the cloning and sequencing of a gene (*iap*) whose product (a 60 Kdal protein) may be involved in the uptake of *L. monocytogenes* by nonprofessional phagocytes. Sequence analysis revealed that the *msp* and *iap* share extensive homology, which indicates that *msp* and *iap* may be the same gene (51). An internal region of this gene was sequenced (Datta, unpublished results) and a synthetic probe, AD07, was used to identify and enumerate *L. monocytogenes* in a number of foods (9,10,34). Thus, either AD07 (for the *iap* gene) or AD13 (for the *hly* gene) can be used to detect and enumerate *L. monocytogenes* in foods. To avoid false-negative results because of "silent"

mutations in the gene (nucleotide changes that affect DNA probe binding but do not change the gene function), both probes should be used in combination (designated AD713).

Salmonella species:

Originally, several restriction endonuclease fragments selected randomly from the *Salmonella* chromosome were used as probes to identify members of the genus (23). Although these molecules served as specific probes, the role played by the target genes was never reported. More recently, probes were developed for regions of the bacterial ribosomal gene that are unique for salmonellae. These probes were used to develop a commercial kit that also uses a nonisotopic labeling and detection system (7).

Staphylococcus aureus: *ent*B probe

Six groups (A, B, C_1, C_2, D, and E) of related enterotoxins associated with pathogenicity are elaborated by some strains of *S. aureus* and can cause symptoms of staphylococcal food poisoning if ingested (1). The genes for enterotoxins A, B, C_1, and E (*entA*, *entB*, *ent*C_1, and *entE*) have been cloned and sequenced (2,3,6,43,85).

Three synthetic oligonucleotide probes were synthesized according to the sequence of the *entB* gene and used to test 210 strains of *S. aureus* (78). One probe was specific for *entB*; the others hybridized with strains producing enterotoxin C. The former probe was used to detect EntB-producing *S. aureus* in artificially contaminated crabmeat (Trucksess and Williams, manuscript in preparation). Although the nucleotide sequences of enterotoxin genes for groups A, C_1, and E are known, synthetic probes have not been reported.

Vibrio cholerae: Cholera toxin

The classical cholera enterotoxin (CT) is a major virulence factor in pathogenic strains of *V. cholerae*. The mechanism of action and immunological reactivity is quite similar to the LT of *E. coli*. Genes encoding this multisubunit protein were cloned and sequenced (57,58,63). Non-O1 *V. cholerae* enterotoxin genes are apparently similar to classical CT (33). Two sequences from the A subunit structural gene for production of the classical enterotoxin are used as probes to detect the CT gene: *ctxA*11 (bases 702-721) and *ctxA*12 (bases 718-735).

Vibrio parahaemolyticus: Thermostable direct hemolysin

An important foodborne pathogen often associated with seafood, *V. parahaemolyticus* can produce a thermostable direct hemolysin (TDH) (95), also referred to as the Kanagawa phenomenon-associated hemolysin (69). This phenotype is commonly associated with strains isolated from humans with gastroenteritis but rarely found in environmental isolates (44). It is not yet known if this hemolysin is a virulence factor, but epidemiological evidence suggests that it is. The gene for the hemolysin has been cloned and sequenced (45,75,106). The specificity of both the cloned probes (76) and a synthetic oligonucleotide probe, tdh3 (77) has been established. The tdh3 probe is 18 bases long and its target encodes amino acids 122-128 of the *tdh* gene.

Vibrio vulnificus: Cytotoxin/hemolysin

V. vulnificus has been implicated as a cause of human infections and septicemia. The primary source of infection appears to be raw or undercooked seafood, especially raw oysters (71). This lactose-positive vibrio produces a cytotoxin/hemolysin which was implicated as a virulence factor (28), and the gene has been cloned (109). A 3.2 kb DNA fragment carrying the structural gene for this protein is a specific probe for *V. vulnificus* (72) and has been sequenced (112). One synthetic probe (VV6) exhibited 100% specific for 166 laboratory and environmental strains of *V. vulnificus* (FDA Contract No. 223-84-2031, Task XIII).

Yersinia pseudotuberculosis: Invasive gene (INV-3)

A chromosomal gene of *Y. pseudotuberculosis*, *inv*, which plays an integral part in *Yersinia* pathogenicity, has been cloned and sequenced (38,39). Oligonucleotide probe INV-3, based on published *inv* sequence (39) is 21 nucleotide bases long and targeted to a region 200 base pairs away from the 5' terminus of the *inv* gene of *Y. pseudotuberculosis* (19). Tests of INV-3 using Southern and colony hybridizations were compared with HeLa cell invasion studies and shown to be specific only for invasive *Y. pseudotuberculosis* isolates. Although there are homologous sequences between the *inv* genes of *Y. enterocolitica* and *Y. pseudotuberculosis*, this homology is not detectable by INV-3.

Yersinia enterocolitica: Chromosomal invasion gene (PF-13)

The genes responsible for mammalian cell invasion are also carried on the chromosome in *Y. enterocolitica* (66). Unlike *Y. pseudotuberculosis*, however, *Y. enterocolitica* has two loci that encode the invasion phenotype. The *inv* locus, homologous to the *inv* gene of *Y. pseudotuberculosis*, allows high level invasion of several tissue culture cell lines, whereas the *ail* gene shows more host specificity (66). Analysis of *Yersinia* serotypes and species using cloned probes from *inv* and *ail* showed that all disease-causing isolates are tissue culture-invasive; all these isolates reacted with the AIL gene probe (67). The INV probe reacted with both tissue culture-invasive and noninvasive isolates; however, recent evidence suggests that the *inv* in these noninvasive strains may not be expressed. The oligonucleotide probe PF13 is targeted specifically to a region 60 base pairs away from the 3' terminus of the *ail* gene of *Y. enterocolitica*. The probe is 18 nucleotides in length. A comparison of colony and Southern hybridization studies of 150 yersiniae and non-yersiniae isolates and HeLa cell invasion studies showed that PF13 hybridized only with invasive *Y. enterocolitica* isolates (19).

Yersinia enterocolitica: Plasmid gene (SP-12)

All pathogenic *Yersinia* species carry a 42-48 Mdal plasmid (pYV), which encodes for many of the virulence-associated phenotypes (84). These include Ca^{2+}-dependent growth, mouse lethality, cytotoxicity, Sereny reaction, production of V and W antigens, serum resistance, and production of outer membrane proteins (YOPs). The pYV plasmid of *Y. enterocolitica* was subcloned and the region encoding for HEp-2 cell cytotoxicity and Sereny reaction was identified and sequenced (91). A 24 base oligonucleotide probe, SP12, targeted to this region was shown to be specific for the virulence plasmid. The use of SP12 for detecting pathogenic *Y. enterocolitica* isolates in artificially inoculated foods was also evaluated (65).

For additional information on specific probes, contact the authors as follows:

At FDA Seafood Research Center, 22201 23rd Dr., S.E., Bothell, WA 98021:

- Walter E. Hill - *C.jejuni*; ribosomal DNA; *E. coli* LT and ST enterotoxin genes; *S. aureus entB*; *Vibrio* spp. probes.

At FDA, 200 C Street, S.W., Washington, DC 20204:

- Atin R. Datta - *L. monocytogenes iap* and *hly* genes
- Peter Feng - *Yersinia* spp. probes and O157:H7 serotype-specific probe
- Keith Lampel - *E. coli* (EIEC) and *Shigella* invasive gene probes; *Salmonella* probes
- William L. Payne - *E. coli* (EHEC) and Shiga-like toxin genes

Methods

In the future it may be possible to carry out DNA probe tests with one standardized condition. Unfortunately, the inherent differences in the length and sequence composition of oligonucleotide probes and the variable susceptibility of microorganisms to lysing conditions require the use of several buffers and hybridization and washing temperatures. The types of microorganisms to be tested dictate the media and growth conditions. To minimize any confusion about similar but slightly different conditions to be used with various gene probes, each procedure is listed separately in this chapter. Although this results in much repetition, each protocol is complete and can be followed step by step. However, it is recommended that all the method sections be read, since some concepts and techniques discussed in the context of a particular probe may be applied in the use of others. In addition, for reference, a procedure for the end-labeling of oligonucleotides is presented.

Four different techniques can be used for colony hybridization tests:

1. Direct plating of samples for enumeration.
2. Direct plating of cultures after enrichment to determine presence/absence.
3. Spotting of individual colonies or pure cultures for an additional hybridization assay to confirm a positive result from colony hybridization with a mixed culture.
4. Returning to a "master" replica plate to make pure cultures of positive colonies for further study and analysis.

The first three techniques differ as to when solid media are inoculated. In the first, aliquots of the homogenized sample are plated immediately after blending. In the second, plates are inoculated after aliquots of the homogenized sample have been incubated under selective conditions. Samples from the first and second techniques are plated onto selective agar media whenever such appropriate media are available. For the third technique, individual positive colonies are re-streaked and an additional colony hybridization test is conducted to ensure that the initial positive or negative results can be repeated. With the last technique, a pure culture can be obtained without selective enrichment, and additional microbiological tests requiring a pure culture can then be performed.

Control cultures

Strains that are positive or negative for the various probe tests must be properly stored and periodically tested for the appropriate phenotypic characteristics. A test methodology other than a gene probe must be used to independently verify the genotype of the control microorganisms. Control cultures must also be

stored appropriately to minimize the possibility of genetic change. Usually, freezing liquid cultures at -70°C in 10-25% glycerol will suffice, except for *Vibrio* species, which are particularly sensitive to cold. Appropriate control strains have been listed, but other strains can be used if they have been properly characterized.

Preparation of controls

The importance of running controls cannot be overemphasized. Perhaps the most rigorous (and time-consuming) procedure for preparing controls is the inoculation of a food sample with a known number of positive (or negative) control cells. The food sample should have been previously tested by conventional microbiological techniques and shown to be free of the pathogen currently being sought. To prepare controls, streak out positive and negative cultures (or spot them in an array if pure cultures are being tested) onto the same medium as that used for food samples. Process the controls and the sample unknowns in an identical fashion at the same time. Such controls will signal if the cell growth, lysis, and hybridization steps have been successful. Although not serving as filter preparation controls, filter test strips will control for hybridization conditions. They can be prepared in advance by spotting control cultures into a repeated array. After cell lysis, cut the support into strips and add a strip to the hybridization mixture to control for conditions.

A. Materials and equipment

1. Whatman 541 filter paper (82-88 mm diameter)
2. Absorbent paper for pads, such as Whatman 3 or 3MM
3. Plastic-backed absorbent paper, such as Kaydry, Benchkote, or Labmat
4. X-ray film, such as Kodak XAR-2 or equivalent and appropriate developer and fixer

B. Media and reagents

1. Butterfield's buffer (R11)
2. Normal physiological saline (R63)
3. MacConkey agar (M91)
4. 50X Denhardt's solution (R17)
5. Sonicated calf thymus or salmon-sperm DNA (R75)
6. Hybridization mixture (6X SSC) (R35)
7. HC agar (M62)
8. Trypticase soy broth (modified) (M156)
9. Novobiocin solution (R50)
10. 20X SSC, pH 7.0 (R77)
11. SSC (6X, 3X, 2X) (R77)
12. 0.5 M EDTA, pH 8.0 (R20)
13. 10 N Sodium hydroxide (R74)
14. LPM agar (M81)
15. Trypticase soy agar with yeast extract (TSAYE) (M153)
16. Trypticase soy broth with yeast extract (TSBYE) (M157)
17. 10X Kinase buffer (R37)
18. Scintillation fluid (R68)
19. 4 M Ammonium acetate (R1)

20. 0.25 M Ammonium acetate (R2)

PROCEDURES

Enterotoxigenic *Escherichia coli*: Heat-Stable Enterotoxin (Human), Heat-Stable Enterotoxin (Porcine), and Heat-Labile Enterotoxin

Growth

1. Aseptically add 25 g of sample to 225 ml Butterfield's buffer and blend according to BAM procedures.
2. Spread-plate 0.1 ml directly from blender onto each of 2 MacConkey agar plates.
3. Make tenfold dilution from blender in Butterfield's buffer and spread 0.1 ml on each of 2 MacConkey plates. (Additional dilutions may be plated, depending on level of microbial contaminants suspected and health hazard concern with the specific pathogen.)
4. Incubate plates for 18-24 h at 35-37°C.

Filter preparation--**NOTE**: If pure cultures are to be sought by picking colonies from original master plate after "positive" areas are found, use sterile Whatman 541 filters. Wrap filters in aluminum foil and autoclave on liquid cycle.

1. Mark filters (with pencil) and agar plates so that they may be oriented correctly with respect to each other.
2. Carefully apply filter over surface of colonies. Remove any air pockets, using gentle pressure on glass spreading rod.
3. Lyse cells by transferring filter (colony side up) to petri dish containing absorbent paper that has been thoroughly wetted (but not soaked) with normal saline. Very carefully remove any air pockets. Store agar plates at 4°C if confirmation is necessary.
4. Microwave for 30 s at 600-700 W.
5. Repeat step 3, using filters wetted with 0.5 M NaOH in 1.5 M NaCl (final concentrations). If possible, transfer filters horizontally to minimize DNA flow across filter. Let sit 10 min.
6. Neutralize NaOH by transferring filters to absorbent filter wetted with 1.0 M Tris-HCl (pH 7) in 2.0 M NaCl (final concentrations). Let filters sit 5-10 min.
7. Filters may be used immediately or air-dried and stored in vacuum desiccator at room temperature for several months.

Hybridization

1. Prepare 50 ml of fresh hybridization mixture (6X SSC, 5X Denhardt's solution, 10 MM EDTA, pH 8.0) in plastic tube.
2. Boil 1.0 ml sonicated calf-thymus DNA for 5 min and add to hybridization mixture.
3. Dispense 5-10 Ml into petri dish to thoroughly wet colony hybridization filter as prepared above.
4. Calculate volume required to contain 10^6 cpm of radioactive probe, allowing for half-life of 14.2 days. (**NOTE**: Do not use probes labeled more than 15 days previously.) Add this amount to each filter and mix briefly.
5. Incubate overnight at 37°C. This temperature is not critical but should be between 35 and 45°C for

LT and ST probes.

(**NOTE**: Several filters may be processed in the same petri dish. **See** Kaysner et al. (48) for protocol.)

Washing

1. Remove spent radioactive hybridization solution, preferably by using disposable pipet. Dispose of radioactive waste properly.
2. Transfer radioactive filter to fresh petri dish and add about 10 Ml 6X SSC containing 0.1% SDS that has been prewarmed to 50°C.
3. Incubate 20 min at 50°C with occasional gentle agitation. Drain.
4. Remove used wash liquid and repeat steps 2 and 3, but without transferring to new petri dish.
5. Rinse filters briefly in 2X SSC at room temperature.
6. Air-dry filters on absorbent paper.

Autoradiography

1. Carefully tape radioactive filters, colony side up, to paper support and cover with plastic sheet. Qualitatively estimate amount of radioactivity bound to filter with a Geiger counter. This will help estimate exposure time (**see** below).
2. In darkroom with appropriate safelight, place X-ray film on top of plastic and place in film cassette with intensifying screens.
3. If Geiger counter reads a significant level (more than 5 cps), a 4-h film exposure may be sufficient. If less radioactivity is present, consider exposing film at -70°C overnight.
4. Let cassette warm somewhat before opening it in the darkroom. Use correct safelight.
5. Develop film according to manufacturer's instructions. Development may be stopped after 30 s to 4 min, when spots appear on film. Dry the film.

Controls

These control strains must have been confirmed for enterotoxin production by an appropriate non-probe assay, such as the suckling mouse test for ST or the mouse Y1 adrenal cell test for LT. Because the genes for these toxins are usually found on plasmids, control strains should be stored frozen at -70°C in 10% glycerol.

The following strains are suitable for use as controls:

Strain	**Reaction**	**Reference**
C600 (pEWD299)	LT+	8
H10407	STH+, STP+, LT+	110
ATCC 25922	-	
HB101 (pBR313)	-	

C600 (pBR322) -

Interpretation of results

1. Compare intensity of control spots with filters from sample.
2. Record number of positive colonies from each dilution.
3. Calculate concentration of ETEC in food sample.

Confirmation

1. Place film under master plate used to make that filter and locate positive colonies.
2. Pick colonies that correspond to darkened areas of film and perform tests for identification of *E. coli* on cultures made from those colonies.

Invasive *Shigella* Species and *Escherichia coli*

Growth--enumeration method

1. Aseptically add 25 g sample to 225 ml Shigella broth without novobiocin and blend according to BAM procedures.
2. Spread-plate 0.1 ml directly from blender onto each of 2 MacConkey agar plates.
3. Make tenfold dilution from blender in Butterfield's buffer and spread 0.1 ml on each of 2 MacConkey plates. (Additional dilutions may be plated, depending on level of microbial contaminants suspected and health hazard concern with specific pathogen.)
4. Incubate plates for 18-24 h at 35-37°C.

Growth--presence/absence method

1. Aseptically add 25 g sample to 225 ml Shigella broth without novobiocin contained in sterile 500 ml Erlenmeyer flask.
2. Shake contents at 37°C for 24 h.
3. Withdraw 0.1 ml aliquot at 0, 4, and 24 h; dilute in diluent buffer, and spread 0.1 ml of diluted cultures onto MacConkey agar plates. If total aerobic plate count is desired, also plate onto trypticase soy agar (TSA) plates. Incubate at 37°C overnight. **NOTE**: For 0-h aliquot, shake flask for 2 min before dilution.

Filter preparation--**see** NOTE under PROCEDURES, above.

1. Mark filters (with pencil) and agar plates so that they may be oriented correctly with respect to each other.
2. Carefully apply filter over surface of colonies. Remove any air pockets, using gentle pressure on glass spreading rod.
3. Lyse cells by transferring filter (colony side up) to plastic-backed paper such as Kaydry, Benchkote, or Labmat (nonabsorbent side up) wetted with 2.0 ml 0.5 N NaOH for 7 min. Very carefully remove any air pockets by repositioning filter. Store agar plates at 4°C if confirmation is necessary.

4. If possible, transfer filters horizontally to minimize DNA flow across filter to a pad wetted with 2.0 ml 1.0 M Tris-HCl, pH 7.4, for 2 min.
5. Repeat step 4.
6. Place filters onto pads wetted with 2.0 ml 1.0 M Tris-HCl in 1.5 M NaCl (final concentrations) for 2 min.
7. Filters may be used immediately or air-dried and stored in vacuum desiccator at room temperature for several months.

To prepare filters using a microwave, use filter preparation method for *E. coli* SLT.

Hybridization

1. Prepare 50 ml of fresh hybridization mixture (6X SSC, 5X Denhardt's solution, 10 MM EDTA, pH 8.0) in a plastic tube.
2. Boil 1.0 ml sonicated calf-thymus DNA for 5 min and add to hybridization mixture.
3. Dispense 5-10 Ml into petri dish to thoroughly wet a colony hybridization filter as prepared above.
4. Calculate volume required to contain 10^6 cpm of radioactive probe, allowing for half-life of 14.2 days. (**NOTE**: Do not use probes labeled more than 15 days previously.) Add this amount to each filter and mix briefly.
5. Incubate overnight at 37°C. (This temperature is not critical but should be between 35 and 45°C for the invasive probe.)

NOTE: Several filters may be processed in the same petri dish. **See** Kaysner et al. (48) for protocol.

Washing

1. Remove spent radioactive hybridization solution, preferably by using disposable pipet, and dispose of radioactive waste properly.
2. Transfer radioactive filter to fresh petri dish and add about 10 Ml of 6X SSC containing 0.1% SDS that has been prewarmed to 54°C.
3. Incubate 1 h at 54°C with occasional gentle agitation. Drain.
4. Remove used wash liquid and repeat steps 2 and 3, but without transferring to new petri dish.
5. Rinse filters briefly in 2X SSC at room temperature.
6. Air-dry filters on absorbent paper.

Autoradiography--**see** procedure under Enterotoxigenic *E. coli*, above.

Controls

The following strains have been used successfully as controls for the *inv* gene probe:

Strain	**Reaction**	**Reference**
S. flexneri M90T	Positive	97

S. flexneri M90T-55	Negative	97
E. coli (EIEC) M41 63	Positive	32
HB101 (pBR313)	Negative	32

Interpretation of results

1. Compare intensity of control spots with filters from sample.
2. Record number of positive colonies from each dilution.
3. Calculate concentration of organisms with invasion gene in food sample.

Isolation of suspected colonies

Match spots present on autoradiogram to area on plate from which filter was made. Pick colony and establish pure culture. Follow procedure described in Chapter 6 to isolate and identify any *Shigella* spp.

NOTE: Compositing procedures may be applied to this method (**see** Chapter 1, on sample handling).

The most difficult problem is deciding what dilutions are necessary for plating. Because the bacterial flora in each food varies, a fixed number for the dilutions would be inadequate. For foods with a low microbial flora, dilutions of 10^2, 10^3, and 10^4 are adequate, whereas foods with a high bacterial background, e.g., alfalfa sprouts, require higher dilutions (10^3-10^6). Duplicate plates should be made for each dilution.

Confirm any positive cultures by using the Congo Red assay (94).

Escherichia coli **Shiga-Like Toxin (SLT)**

Growth--standard procedure

1. Blend, according to BAM procedures, 25 g of sample in 225 ml Butterfield's buffer.
2. Spread-plate 0.1 ml of sample on each of 4 HC agar plates.
3. Incubate plates inverted for 24 h at 43°C (humidity incubator); include a plate with positive and negative strains as controls.

Growth--low levels expected

If very low levels of EHEC are suspected, prepare filters from enriched food samples, although this will make it impossible to obtain quantitative results.

1. Blend 25 g sample in 225 ml modified trypticase soy broth (mTSB).
2. Incubate with shaking (about 100 rpm) for 18-24 h at 37°C.
3. Dilute enrichment cultures in Butterfield's phosphate buffer and spread 0.1 ml of each dilution on duplicate HC agar plates.
4. Invert and incubate at 43°C for 24 h.

5. Select 2 plates of the same dilution with approximately 10^3 colonies for preparing filters.

Growth--control strains and pure cultures for screening

NOTE: Store isolates in 10% glycerol at -70°C if possible.

1. Inoculate 5 ml brain heart infusion (BHI) broth or TSB with 0.6% yeast extract (TSBYE) and incubate overnight at 37°C with shaking.
2. Dilute overnight cultures and either spread-plate 0.1 ml containing 10^2-10^3 colonies or streak onto HC agar; alternatively, spot cultures in orderly asymmetric pattern with a transfer device such as a Replaclone (LAO Enterprises, Gaithersburg, MD) or sterile toothpicks.
3. Incubate overnight at 43°C.

Filter preparation--**see** NOTE under PROCEDURES, above.

1. Mark filters (with pencil) and agar plates so that they may be oriented correctly with respect to each other.
2. Carefully apply filter over surface of colonies. Remove any air pockets, using gentle pressure on glass spreading rod. Let sit 5 min.
3. Lyse cells by transferring filter (colony side up) to glass petri dish containing absorbent paper (such as Whatman No. 3) that has been thoroughly wetted (but not soaked) with 4-5 ml 0.5 N NaOH in 1.5 M NaCl (final concentrations). Very carefully remove any air pockets. Let stand for 5 min. Store agar plates at 4°C if confirmation is necessary.
4. Microwave for 30 s at 30% maximum power.
5. Transfer filter to glass petri dish containing absorbent paper wetted with 4-5 ml 1.0 M Tris-HCl (pH 7.0) in 2.0 M NaCl (final concentrations) for 5 min.
6. Filters may be used immediately or air-dried and stored in a vacuum desiccator at room temperature for several months.

Hybridization

1. Prepare 50 ml of fresh hybridization mixture (6X SSC, 5X Denhardt's solution, 10 MM EDTA, pH 8.0) in plastic tube.
2. Boil 1.0 ml sonicated calf-thymus DNA for 5 min and add to hybridization mixture.
3. Dispense 8-10 Ml into petri dish to thoroughly wet colony hybridization filter as prepared above. Let filter set at room temperature for 15 min.
4. Calculate volume required to contain 10^6 cpm of radioactive probe, allowing for half-life of 14.2 days. (**NOTE**: Do not use probes labeled more than 15 days previously.) Add this amount to each filter and mix briefly.
5. Incubate overnight with gentle shaking at 37°C.

NOTE: Several filters may be processed in the same petri dish; however, this method has not been tested with this probe. **See** Kaysner et al. (48) for protocol.

Washing

1. Remove spent radioactive hybridization solution, preferably by using disposable pipet. Dispose of radioactive material properly.
2. Transfer radioactive filter to fresh 20 mm petri dish and rinse for 10 s in 30 ml 3X SSC prewarmed to 56°C. Drain.
3. Add 40 ml 3X SSC prewarmed to 56°C and incubate 1 h at 56°C with gentle agitation. Drain.
4. Repeat step 3.
5. Rinse filters briefly in 2X SSC at room temperature.
6. Air-dry filters on absorbent paper. Store under vacuum at room temperature if not used immediately.

Autoradiography--see procedure given under Enterotoxigenic *E. coli*, above.

Controls

Store control strains (and other pure cultures to be tested) preferably at -70°C in 10% glycerol or on BHI slants at room temperature. The following strains have been tested for the presence of the SLT gene by cytotoxin-neutralization assays and by colony hybridization with gene probes.

Strain	**Reaction**	**Reference**
C600	Negative	104
O157:H7 (933)	SLT I and SLT II	90
C600 (933J)	SLT I	81, 104
O26:H11 (H30)	SLT I	52, 80

Interpretation of results

1. Compare intensity of control spots with filters from sample.
2. Record number of positive colonies from each dilution.
3. Calculate concentration of cells with SLT gene in food sample only for sample where enrichment was not used.

Confirmation

Pick probe-positive colonies from master plates (which were stored at 4°C) and confirm as *E. coli*. Serotype these isolates (17,60) and test for production of SLT by cytotoxicity assay (27) as modified by O'Brien (80).

Perform toxin neutralization assay according to O'Brien and LaVeck (79).

EHEC O157:H7 - Specific Probe (PF-27)

Growth and filter preparation--**see** procedures under *E. coli* Shiga-like toxin (SLT).

Hybridization and washing--**see** procedures under *Yersinia pseudotuberculosis* INV-3, but use 60°C as the optimal washing temperature.

Autoradiography--**see** procedure under Enterotoxigenic *E. coli*, above.

Controls--Available from Peter Feng, Division of Microbiological Studies (HFS-516), FDA, 200 C Street, S.W., Washington, DC 20204.

Strain	**Reaction**
EC260 (O157:H7)	Positive
EC258 (MUG [+] *E. coli*)	Negative
EC36 (MUG [-] *E. coli*)	Negative

Listeria monocytogenes: Combination of Invasion-Associated Protein (*iap*) and Hemolysin (*hly*) Gene Probes - AD713

Growth--enumeration method

1. Homogenize 25 g sample in 225 ml Listeria enrichment broth (**see** Chapter 10) and make dilutions in the same medium.
2. Plate 0.1 ml of each dilution onto LPM agar on duplicate plates and incubate 48 h at 37°C.
3. Count total number of colonies and pick some "blue" (presumptive *Listeria*) colonies for future characterization.

Growth--pure cultures

1. Grow pure cultures in TSBYE at 37°C for 24 h.
2. Spot cultures in regular array on TSAYE plates, using sterile needle or loop; to facilitate production of multiple filters, transfer 200 1 of liquid culture to wells of sterile microtiter plate. Then, using sterile replicating device, such as Replica Plater (Sigma:R2383) transfer cultures onto duplicate TSAYE plates.
3. Incubate 24 h at 37°C.

Filter preparation

1. For best results, select LPM plate with not more than 300 colonies.
2. Place properly marked Whatman 541 filters onto LPM plates (enumeration method) or TSAYE plates (for pure cultures) and press gently with stirring rod.
3. Let sit 5 min. Carefully lift filter and place into plastic petri dish on paper wetted with saline (about 3 ml), colony side up. Place cover on dish.
4. Microwave for 30 s on high (about 700 W).
5. Transfer filter paper to absorbent paper wetted with 0.5 N NaOH in 1.5 M NaCl (final

concentrations) for 5 min.
6. Neutralize 5 min on paper wetted with 1.0 M Tris (pH 7) in 2.0 M NaCl (final concentrations).
7. Air-dry and store under vacuum if not used immediately.

Hybridization

1. Prepare 50 ml of fresh hybridization mixture (6X SSC, 5X Denhardt's solution, 10 MM EDTA, pH 8.0) in plastic tube.
2. Boil 1.0 ml sonicated calf-thymus DNA for 5 min and add to hybridization mixture.
3. Dispense 10-15 ml into petri dish to thoroughly wet a colony hybridization filter as prepared above.
4. Calculate volume required to contain 2-5 x 10^6 cpm of end-labeled radioactive AD713 probe, allowing for half-life of 14.2 days. (**NOTE**: Do not use probes labeled more than 15 days previously.) Add this amount to each filter and mix briefly.
5. Incubate overnight at 37°C with gentle shaking.

Washing

1. Remove spent radioactive hybridization solution, preferably by using disposable pipet. Dispose of radioactive material properly.
2. Transfer radioactive filter to fresh petri dish and add about 10 Ml 3X SSC that has been prewarmed to 50°C.
3. Incubate 1 h at 50°C with occasional gentle agitation. Drain.
4. Remove used wash liquid and repeat steps 2 and 3, but without transferring to new petri dish.
5. Rinse filters briefly in 2X SSC at room temperature.
6. Air-dry filters on absorbent paper.

Autoradiography--**see** procedure given under Enterotoxigenic *E. coli*, above.

Controls: Use filters inoculated with *L. monocytogenes* and *L. innocua*.

Strain	**Reaction**	**Reference**
L. monocytogenes Scott A	Positive	25
L. innocua ATCC 33090	Negative	25

Interpretation of results

1. Compare intensity of control spots with filters from sample.
2. Record number of positive colonies (dark spots) from each dilution.
3. Calculate concentration of *L. monocytogenes* in sample by using number of dark spots on filters prepared by enumeration method.

Confirm positive cultures with BAM procedures recommended for *Listeria*.

*Staphylococcus aureus ent*B

Growth

1. Aseptically add 25 g of sample to 225 ml Butterfield's buffer and blend according to BAM procedures.
2. Spread-plate 0.1 ml directly from blender onto each of 2 Baird-Parker agar plates.
3. Make tenfold dilution from blender in Butterfield's buffer and spread 0.1 ml onto each of 2 Baird-Parker plates. (Additional dilutions may be plated, depending on level of microbial contaminants suspected and health hazard concern with the specific pathogen.)
4. Incubate plates 18-24 h at 35-37°C.

Filter preparation--**see** NOTE under PROCEDURES, above.

With this probe, nylon supports, such as Nylon 66 (Micron Separations Inc., Westborough, MA 01581), generate less nonspecific background than do Whatman 541 filters.

1. Mark filters (with pencil) and agar plates so that they may be oriented correctly with respect to each other.
2. Carefully apply filter over surface of colonies. Remove any air pockets, using gentle pressure on glass spreading rod.
3. Lyse cells by transferring filter (colony side up) to petri dish containing absorbent paper that has been thoroughly wetted (but not soaked) with 0.5 M NaOH in 1.5 M NaCl (final concentrations). Very carefully remove any air pockets. Store agar plates at 4°C if confirmation is necessary.
4. Microwave for 30 s at 600-700 W.
5. As in step 3, transfer filters to pads wetted with 0.5 M NaOH in 1.5 M NaCl (final concentrations). If possible, transfer filters horizontally to minimize DNA flow across the filter. Let sit 10 min.
6. Neutralize NaOH by transferring filters to absorbent filter wetted with 1.0 M Tris-HCl (pH 7) in 2.0 M NaCl (final concentrations). Let filters sit 5-10 min.
7. Filters may be used immediately or air-dried and stored in vacuum desiccator at room temperature for several months.

Hybridization

1. Prepare 50 ml of fresh hybridization mixture (6X SSC, 10X Denhardt's solution, 10 MM EDTA, pH 8.0) in plastic tube.
2. Boil 1.0 ml sonicated calf-thymus DNA for 5 min and add to hybridization mixture.
3. Dispense 5-10 Ml into petri dish to thoroughly wet a colony hybridization filter as prepared above.
4. Calculate volume required to contain 10 cpm of radioactive probe, allowing for half-life of 14.2 days. (**NOTE**: Do not use probes labeled more than 15 days previously.) Add this amount to each filter and mix briefly.
5. Incubate overnight at 60°C.

Washing

1. Remove spent radioactive hybridization solution, preferably by using disposable pipet. Dispose of

radioactive material properly.
2. Transfer radioactive filter to fresh petri dish and add about 10 Ml 6X SSC containing 0.1% SDS that has been prewarmed to 65°C.
3. Incubate 20 min at 65°C with occasional gentle agitation. Drain.
4. Remove used wash liquid and repeat steps 2 and 3, but without transferring to new petri dish.
5. Rinse filters briefly in 2X SSC at room temperature.
6. Air-dry filters on absorbent paper.

Autoradiography--**see** procedure given under Enterotoxigenic *E. coli*, above.

Strain	**Reaction**
SA 49 (243)	Positive
SA 54 (D87)	Negative

Interpretation of results

1. Compare intensity of control spots with filters from sample.
2. Record number of positive colonies from each dilution.
3. Calculate concentration of cells with gene for enterotoxin B.

Confirm toxin production by using a rapid method, as listed in Appendix 1.

***Vibrio cholerae ctxA*11**

Growth--pure cultures

1. Transfer individual isolates onto TSA with 2% NaCl.
2. Incubate about 18 h at 37°C.

Filter preparation--**see** NOTE under PROCEDURES, above.

1. Mark filters (with pencil) and agar plates so that they may be oriented correctly with respect to each other.
2. Carefully apply filter over surface of colonies. Remove any air pockets by using gentle pressure on glass spreading rod.
3. Lyse cells by transferring filter (colony side up) to petri dish containing absorbent paper that has been thoroughly wetted (but not soaked) with 3 ml normal saline. Very carefully remove any air pockets. Store agar plates at room temperature if confirmation is necessary.
4. Microwave for 30 s at 600-700 W.
5. Repeat step 3, using filters wetted with 0.5 M NaOH in 1.5 M NaCl (final concentrations). If possible, transfer filters horizontally to minimize DNA flow across filter. Let sit 10 min.
6. Neutralize NaOH by transferring filters to absorbent filter wetted with 1.0 M Tris-HCl (pH 7) in 2.0 M NaCl (final concentrations). Let filters sit 5-10 min.
7. Filters may be used immediately or air-dried and stored in vacuum desiccator at room temperature

for several months.

Hybridization

1. Prepare 50 ml of fresh hybridization mixture (6X SSC, 5X Denhardt's solution, 10 MM EDTA, pH 8.0) in plastic tube.
2. Boil 1.0 ml sonicated calf-thymus DNA for 5 min and add to hybridization mixture.
3. Dispense 8-10 Ml into petri dish to thoroughly wet a colony hybridization filter as prepared above.
4. Calculate volume required to contain 10^6 cpm of radioactive probe, allowing for half-life of 14.2 days. (**NOTE**: Do not use probes labeled more than 15 days previously.) Add this amount to each filter and mix briefly.
5. Incubate overnight at 37°C. This temperature is not critical but should be between 35 and 45°C.

NOTE: Several filters may be processed in the same petri dish. **See** Kaysner et al. (48) for protocol.

Washing

1. Remove spent radioactive hybridization solution, preferably by using disposable pipet. Dispose of radioactive waste properly.
2. Transfer radioactive filter to fresh petri dish and add about 10 Ml 6X SSC, containing 0.1% SDS that has been prewarmed to 45°C.
3. Incubate 20 min at 45°C with occasional gentle agitation. Drain.
4. Remove used wash liquid and repeat steps 2 and 3, but without transferring to a new petri dish.
5. Rinse filters briefly in 2X SSC at room temperature.
6. Air-dry filter on absorbent paper.

Autoradiography--**see** procedure given under Enterotoxigenic *E. coli*, above.

Controls

Strain	**Reaction**	**Reference**
ATCC 14033	Positive	99
E. coli H10407	Negative (high stringency)	99
E. coli H10407	Positive (low stringency)	99

Interpretation of results: Compare intensity of control spots with sample spots. Confirm positive cultures with BAM procedures recommended for *V. cholerae*.

***Vibrio parahaemolyticus* tdh3**

The colony hybridization protocol is identical to that of *V. cholerae*, including the 45°C washing temperature. Confirm by performing those tests required to identify *V. parahaemolyticus*.

Strain	Reaction	Reference
WP1	Positive	75
S162-71	Negative	75
ATCC 17802	Positive	75

Vibrio vulnificus VV6

Plating medium is modified CPC agar (M98). The colony hybridization protocol is identical to that for *V. cholerae*, except that washing temperature is 60°C. Confirm by performing those tests required to identify *V. vulnificus*.

Strain	Reaction
ATCC 27562	Positive
ATCC 14033	Negative

Yersinia pseudotuberculosis INV-3

Growth--contaminated foods

1. Follow general instructions for sampling and blending detailed in Chapter 8.
2. Spread-plate 0.1 ml from appropriate dilutions on MacConkey agar and incubate.

NOTE: If samples are suspected to be heavily contaminated with other microflora, this level may be reduced by brief treatment with alkali, as demonstrated by Jagow and Hill (42).

Growth--pure culture testing for invasiveness and pathogenicity

1. Spot cultures onto TSA plates in regular array, using sterile needle or toothpick.
2. Incubate overnight at 25°C.

Filter preparation--**see** NOTE under PROCEDURES, above.

1. Mark filters (with pencil) and agar plates so that they may be oriented correctly with respect to each other.
2. Carefully apply filter over surface of the colonies. Remove any air pockets by using gentle pressure on glass spreading rod.
3. Lyse cells by transferring filter (colony side up) to petri dish containing absorbent paper that has been thoroughly wetted (but not soaked) with normal saline. Very carefully remove any air pockets. Store agar plates at 4°C if confirmation is necessary.
4. Microwave for 30 s at 30% maximum power.

5. Repeat step 3, using filters wetted with 0.5 M NaOH in 1.5 M NaCl (final concentrations) to denature the DNA. If possible, transfer filters horizontally to minimize DNA flow across filter. Let sit 5 min.
6. Neutralize NaOH by transferring filters to absorbent filter wetted with 1.0 M Tris-HCl (pH 7) in 2.0 M NaCl (final concentrations). Let filters sit 5 min.
7. Filters may be used immediately or air-dried and stored in vacuum desiccator at room temperature for several months.

Hybridization

1. Prepare 50 ml of fresh hybridization mixture (6X SSC, 10X Denhardt's solution, 10 MM EDTA pH 8.0) in plastic tube.
2. Boil 1.0 ml sonicated calf-thymus or salmon-sperm DNA (5 mg/ml) for 5 min and add to hybridization mixture.
3. Dispense 5-10 Ml into petri dish to thoroughly wet a colony hybridization filter as prepared above.
4. Prehybridize filters for 3-4 h at 45°C.
5. Calculate volume required to contain 10^6 cpm of radioactive probe, allowing for half-life of 14.2 days. (**NOTE**: Do not use probes labeled more than 15 days previously.) Add this amount to each filter and mix briefly.
6. Incubate overnight at 37°C.

NOTE: Several filters may be processed in the same petri dish. **See** Kaysner et al. (48) for protocol.

Washing

1. Remove spent radioactive hybridization solution, preferably by using disposable pipet. Dispose of radioactive waste properly.
2. Transfer radioactive filter to fresh petri dish and add about 10 Ml 6X SSC containing 0.1% SDS that has been prewarmed to 58°C.
3. Incubate 30 min at 58 ± 2°C with occasional gentle agitation. Drain.
4. Remove used wash liquid and repeat steps 2 and 3, but without transferring to new petri dish.
5. Rinse filters briefly in 2X SSC at room temperature.
6. Air-dry filters on absorbent paper.
7. Optimal washing temperature is 58°C but may vary 1 or 2°C.

Autoradiography--**see** procedure given under Enterotoxigenic *E. coli*, above.

Controls

Strain	**Reaction**
Yp188 isolate 82-599	Positive
Ye133 (8081)	Negative

Interpretation of results

1. Compare intensity of control spots with filters from sample.
2. Record number of positive colonies from each dilution.
3. Calculate concentration of cells with invasive gene.

Yersinia enterocolitica Chromosomal Probe PF-13

The colony hybridization protocol is identical to that described for INV-3, except that the optimal wash temperature for this probe is 47°C.

Controls

Strain	Reaction
Ye133 (8081)	Positive
Yp188	Negative

Yersinia enterocolitica Plasmid Probe SP-12

The colony hybridization protocol is identical to that described for INV-3,except that the optimal wash temperature for this probe is 57°C.

Controls

Strain	Reaction
Ye133 (8081)	Positive
Yf225 (*Y. fredericksenii*)	Negative

End-Labeling of Oligonucleotides

1. Rehydrate lyophilized preparation of synthetic oligonucleotides with 0.25-1.0 ml water to yield stock solution with A_{260} between 1 and 10 units (50-400 g/ml).
2. Dilute preparation and read at A_{260}. A reading of 1 corresponds to about 33 g/ml. The molecular weight (MW) of a nucleotide is about 330 daltons.
3. Calculate concentration of stock solution and prepare 10 M working solution. For example, the MW of a 22 base oligonucleotide is about 7260; thus, a 10 M solution is 72.6 g/ml (10 pmoles/l).
4. Mix 5 l of working solution with 2.5 l kinase buffer, 15 l water, 1.5 l gamma ^{32}P ATP (3000-7000 Ci/mmol) and 1 l (20 units/l) T4 kinase in plastic 500 l conical microcentrifuge tube on ice.
5. Mix well and incubate at 37°C for 1 h.
6. Add 2 l 0.5 M EDTA to stop reaction.
7. Add 1.6 l 4.0 ammonium acetate solution to bring ammonium acetate concentration to 0.25 M.
8. Equilibrate NACS PREPAC column with 0.25 M ammonium acetate and load reaction mixture onto

column.

9. Wash column, using gravity or gentle pressure, with a minimum of 4 ml 0.25 M ammonium acetate to remove free ATP.
10. Elute bound DNA with 200 l aliquots of 4 M ammonium acetate, but do not force through column.
11. Collect 3 fractions in 500 l conical plastic centrifuge tubes.
12. Determine amount of radioactivity in each fraction by spotting 2 l onto paper and count by any method suitable for detecting beta decay. Most of the radioactivity is eluted in fractions 1 and 2. Use fraction 1, but if more counts are needed, fractions 1 and 2 can be pooled. Usually 1-2 x 10^8 cpm is obtained if ATP of a specific activity of 3000-7000 Ci/mmol is used.
13. Store at -20°C. For best results use probe within 15 days of labeling.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Bacteriological Analytical Manual

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Chapter 20A

Inhibitory Substances in Milk

Author

(Return to Table of Contents)

Two methods for detecting substances that inhibit microbes in milk are the cylinder plate method, using *Micrococcus luteus* as the test organism, and a paper disk method, using *Bacillus stearothermophilus*. The cylinder plate method, which is the official method for quantitative detection of -lactam residues (3), is described in Chapter 16, Dairy Products, in *Official Methods of Analysis* (2). The analyst must also refer to Chapter 42, Drugs in Feeds (*Official Methods*), for preparation of media and certain other details. The entire method is presented here for convenience.

A description of the cylinder plate method for detecting penicillin in dry powdered milk is given by Kramer et al. (6). The same basic procedure can be applied to the assay of penicillin in fluid milk. Normal whole milk is used as a control diluent in place of the dry powdered milk plus buffer control diluent listed under the procedure for Preparation of Standard Curve. The normal whole milk that is to serve as the control diluent should be tested before use to ensure that it exhibits no antibacterial activity against the test organism. No sample preparation is necessary; milk samples submitted for examination are tested just as they are received. In all cases, the milk samples should be fresh. If a milk sample is suspected to contain penicillin at a level greater than 0.2 units/ml, it should be diluted with the control diluent to an estimated 0.05

unit/ml concentration before being tested. All other parts of the procedure remain the same. The disk method, which is the official method for qualitative detection of inhibitory substances in milk (1), is a modification of the method approved by the International Dairy Federation for the qualitative detection of penicillin in milk (4).

Micrococcus luteus Cylinder Plate Method

A. Equipment and materials

1. Stainless steel cylinders, 8 + 0.1 mm od, 6 + 0.1 mm id, 10 + 0.1 mm long. Available from S & L Metal Products Corp., 58-29 57th Drive, Maspeth, NY 11378.

2. Shaw cylinder dispenser. Available from E.C. Condit, P.0. Box 75, Middle Haddam, CT 06456, or Arthur E. Farmer, P.O. Box 1785, Trenton, NJ 08618.

3. Petri dishes, 20 x 100 mm, with porcelain covers, glazed on outside, or cover lids with filter pads able to absorb water of syneresis. Comparable plastic petri dishes may be used if desired. Plastic covers may be used if they are raised slightly to let water escape.

B. Media and reagents

1. Antibiotic Medium No. l (Ml4). Penassay seed agar (Difco) and seed agar (BBL) are satisfactory.

2. Antibiotic Medium No. 4 (M15). Yeast beef agar (Difco and BBL) are satisfactory.

3. Phosphate buffer, 1% (pH 6.0 + 0.1). Dissolve 8.0 g monobasic potassium phosphate and 2.0 g dibasic potassium phosphate in distilled water and dilute to 1 liter with distilled water.

4. Penicillinase (-lactamase) (R55). Available from Difco Laboratories, Box 1058A, Detroit, MI 48232; BBL, Div. of BioQuest, P.O. Box 243, Cockeysville, MD 21030; ICN Nutritional Biochemicals, 26201 Miles Road, Cleveland, OH 44128; Schwarz/Mann, Orangeburg, NY 10962; Calbiochem, 10933 N. Torrey Pines Road, La Jolla, CA 92037.

5. Penicillin G working standard. Authentic penicillin G reference standard may be obtained from U.S.P. Reference Standards, 12601 Twinbrook Parkway, Rockville, MD 20852. Follow label directions for preparation and storage. Prepare stock solutions by carefully weighing, in an atmosphere of 50% humidity or less, a small amount of standard ana diluting weighed powder in appropriate diluent to obtain solution of convenient concentration.

6. Physiological saline solution, 0.85% (sterile) (R63)

C. Preparation of *Micrococcus luteus*

Cultures of *Micrococcus luteus* (ATCC 9341) may be obtained from American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. Maintain as stock culture on agar slants of Antibiotic Medium No. 1 and transfer to fresh slant about once every 2 weeks. Prepare suspension as follows: Streak agar slant heavily with test organism and incubate 18-24 h at 32-35°C. Wash growth from slant with 1-2 ml sterile physiological saline and transfer to dry surface of Roux bottle containing 300 ml Antibiotic Medium No. 1. Spread suspension evenly over entire surface with aid of sterile glass beads. Incubate 18-24 h at 32-35°C. Wash growth from agar surface with 50 ml saline. Before actual assay, prepare trial plates to determine optimum amount of bulk suspension to be added to seed agar to obtain best zones of inhibition. Generally this will range only from 0.1 to 0.5 ml inoculum per 100 ml Antibiotic Medium No. 4. Store this stock suspension in refrigerator no longer than 2 weeks.

D. Preparation of plates

Add 10 ml Antibiotic Medium No. 1 to each petri dish. Distribute medium evenly and let harden on flat, level surface. Melt Antibiotic medium No. 4, cool to 48°C, and add optimum amount of culture per 100 ml, as determined above. Mix thoroughly. Add 4.0 ml of this inoculated agar to each plate. Distribute agar evenly by tilting plates from side to side in circular motion. Let harden. Use plates the same day they are prepared.

E. Preparation of standard curve

Prepare stock solution of penicillin G by dissolving accurately weighed portion of penicillin standard in enough phosphate buffer (B-3, above) to obtain solution containing 1000 units/ml. This stock solution may be used for 2 days. Prepare control diluent from phosphate buffer and antibiotic-free dry powdered milk at concentration of 3 ml buffer per 1.0 g milk. Further dilute penicillin G stock solution in control diluent to obtain concentrations of 0.00625, 0.0125, 0.025, 0.05, 0.1, and 0.2 unit/ml. Reference concentration is 0.05 unit/ml. Place 6 cylinders, equally spaced, on inoculated agar surface of prepared petri plates (D, above). Fill 3 cylinders with 0.05 unit/ml standard

solution and 3 cylinders with one other concentration of standard, alternating so that each 0.05 unit/ml cylinder is followed by cylinder of the other concentration. Use 3 plates for each point in curve, for total of 15 plates. The 3 plates containing lowest concentration of standard (0.00625 unit/ml) are intended to produce negative results; other 12 plates are used to construct standard response line. This gives 45 determinations for 0.05 unit/ml and 9 determinations for each of other points on the line.

Replace covers and incubate plates 16-18 h at 30°C. After incubation, invert plates to remove cylinders. Measure diameter of each zone of inhibition as accurately as possible (at least to nearest 0.5 mm). Average readings of 0.05 unit/ml concentration and of point tested on each set of 3 plates. Also average all 45 readings of 0.05 unit/ml concentration to obtain correction point for curve.

Correct average value for each point to value it would be if average of 0.05 unit/ml readings for that set of 3 plates were same as correction point. Example: If average of 45 readings of 0.05 unit/ml concentration is 20 mm, and average of nine 0.05 unit/ml cylinders on set of three 0.025 unit/ml plates is 19.8 mm, correction is + 0.2 mm. If average reading for nine 0.025 unit/ml cylinders on these 3 plates is 17.0 mm, corrected value is 17.2 mm. Plot corrected values, including average of 0.05 unit/ml concentration, on 2-cycle, semilog graph paper, placing concentration in units/ml on logarithmic scale and diameter of zone of inhibition on arithmetic scale. Construct best straight line through these points, either by inspection cr by using following equations:

$$L = \frac{(3a + 2b + c - e)}{5}$$

$$H = \frac{(3e + 2d + c - a)}{5}$$

where L and H = calculated zone diameters for lowest and highest (0.0125 and 0.2 unit/ml) concentrations of standard response line; c = average zone diameter of 45 zone diameters for reference concentration; and a, b, d, e = corrected average zone diameters for each of other concentrations used for standard response line.

F. Preparation of sample

Accurately weigh 10 g dry powdered milk sample and add 30 ml phosphate buffer (B-3, above). Mix thoroughly. If concentration of penicillin G greater than 0.2 unit/ml in this mixture is expected, dilute aliquot of reconstituted powdered milk with control diluent

(E, above) to estimate 0.05 unit/ml. To identify activity as penicillin, take portion of sample, add penicillinase concentrate (B-4, above) at rate of 0.5 ml/10 ml sample, and incubate 30 min at 37°C. On 3 plates, fill 2 cylinders with unit/ml reference standard. 2 cylinders with untreated sample, and 2 cylinders with penicillinase-treated sample. Incubate plates and follow same procedure as in E, above. Zone of inhibition with untreated sample and no zone with penicillinase-treated sample is positive test for penicillin G.

G. Calculation of potency

Average the zone readings of standard and zone readings of sample on 3 plates. If average zone size of sample is larger than average for standard, add difference between them to zone size of reference standard on curve. If average sample value is lower than standard value, subtract difference between them from zone size of reference standard on curve. From curve, read concentration corresponding to this adjusted sample zone size. Multiply concentration in units/ml by dilution factor of 4X to obtain final concentration of penicillin G in units/g. If sample powder has been additionally diluted, appropriate dilution factor must be taken into account in calculation of final potency.

H. Controls

In using these assay procedures, the analyst must be certain that any antibiotic activity detected derives from the sample and not from environmental conditions (including the analyst), the equipment, or reagents used. Good laboratory practices require that proper controls be maintained throughout the analytical process. These should always include controls to indicate the degree of precision and accuracy of the determinations to be reported. The lowest standard concentration (0.00625 unit/ml) is intended to be a control that may produce negative results. This lowest concentration represents control diluent to which the drug has been added at a level that is normally below the limit of detectability. On occasion, the lowest concentration (0.00625 unit/ml) will produce a measurable zone of inhibition. The next highest concentration (0.0125 unit/ml) should always produce positive results. The sensitivity of this assay is normally 0.01 unit/ml. The control diluent should always produce negative results.

Bacillus stearothermophilus **Disk Assay - Qualitative Method II**

A. Equipment and materials

1. PM indicator agar (M6)
2. Trypticase (tryptic) soy broth without dextrose (M154)

3. Trypticase (tryptic) soy agar (M152)
4. Petri dishes (see A-3, above)
5. Paper disks, 12.7 mm (Schleicher & Schuell)
6. Penicillinase (ß-lactamase) (R55)
7. Control disks. Prepare fresh daily from positive control milk containing 0.008 unit/ml penicillin.
8. 90 µl pipettor with disposable tips
9. Vernier calipers
10. Fine tip forceps

B. Penicillin G working standard

Accurately weigh, in an atmosphere of less than 50% relative humidity, about 30 mg U.S.P. sodium penicillin G reference standard (U.S.P.). Dissolve in enough phosphate buffer to obtain stock concentration of 100-1000 units/ml. Store in dark for <2 days at 0-4.4°C.

C. Preparation of *Bacillus stearothermophilus*

Cultures of *B. stearothermophilus* var. *calidolactis* (ATCC 10149) (5) may be obtained from American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. Maintain on trypticase soy agar slants and transfer weekly. Prepare spore suspension as follows: Inoculate three 300 ml Erlenmeyer flasks, each containing 150 ml trypticase soy broth without dextrose. Incubate flasks at 64 ± 2°C. Periodically make spore stains to determine extent of sporulation. When sufficient sporulation has occurred (about 80%, usually in 72 h), centrifuge cells 15 min at 5000 rpm. Decant supernatant, resuspend cells in physiological saline, and recentrifuge; repeat. After discarding supernatant from final wash, suspend cells in 30 ml physiological saline and store at 0-4.4°C. Spore suspension will remain viable 6-8 months. Commercially prepared spore suspension is satisfactory. Check viability periodically by preparation of trial test plates.

D. Preparation of standard milk solution

Prepare standard milk solution by diluting penicillin G stock solution in inhibitor-free milk to concentration of 0.008 unit/ml. Store at 0-4.4°C for not more than 2 days, or distribute in small amounts and freeze in non-frost-free freezer. Store frozen no longer than 6 months. Difco PM positive controls and Penicillin Assay, Inc., penicillin standards are satisfactory.

E. Preparation of plates

Inoculate PM indicator agar, cooled to 64°C, with previously prepared spore suspension of B. stearothermophilus (C, above). Adjust inoculum level to provide 1 x 10^6 spores/ml. Pipet 6 ml inoculated agar into each petri dish and let harden on level surface. Use plates fresh or store in sealed plastic sacks at 0-4.4°C and use within 5 days of preparation.

F. Assay - Screening

Use 90 µl pipettor. With tip securely fastened and pipettor positioned vertically, depress plunger completely to first stop. Insert tip 1 cm below surface of well-mixed sample (if necessary, tilt sample to avoid foam). Release plunger slowly to fill tip properly. (If plunger is released too quickly, amount of milk taken up will not be uniform. If tip is not appropriately filled after releasing plunger, repeat above procedure.) With clean, dry forceps, remove and place a blank disk (within identified section) on agar surface, pressing disk gently with forceps to ensure good contact. Immediately deliver sample to disk. Holding pipettor vertically with tip approximately 1 cm above center of disk, depress plunger in slow, continuous motion to first stop. With plunger completely depressed (go to the second stop, if one exists) touch off tip once to center of disk. Observe that tip is empty before discarding. (Repeat above for all samples.) Prepare control disk, containing 0.008 unit penicillin/ml, as for above samples.

Alternatively, with clean, dry forceps, touch paper disk to surface of well-mixed milk. (Agitate raw samples by shaking 25 times in 7 s through arc of 1 ft or completely invert retail containers 25 times. Let bubbles break up before sampling. Sample must be taken within 3 min of agitation.) Let milk be absorbed by capillary action. Drain excess milk by touching edge of disk once to inside surface of sterile petri dish lid. Immediately place disk on agar surface, pressing gently to ensure good contact. (Repeat above for all samples.) Place control disk containing 0.008 unit penicillin/ml on agar surface as above (if multiple plates, vary location of control on each plate, center or edge). Identify each disk or section on which it is placed. (Place a maximum of 7 disks/plate, 6 edge and 1 center.)

Before inverting plate(s) check to see that disks are completely and uniformly filled by observing through bottom of petri dish at light source. Also determine that no milk is visible beyond edge of disks. Invert plates and incubate at 64 ± 2°C until well-defined zones of inhibition (16-20 mm) are obtained with 0.008 unit/ml control (2.5-3.5 h). Examine plate(s) for clear zone(s) of inhibition surrounding disks. (Measure zone(s) with Vernier calipers.) Clear zones of 14 mm indicate presence of inhibitory substances. Zones of <14 mm are read as negative. Zones of 16 mm must be confirmed for the presence of inhibitor.

G. Assay - Confirmatory

Heat test sample to 82°C for >2 min and cool promptly to room temperature. Use 90 µl pipettor procedure or, alternatively, with clean, dry forceps, touch paper disk to surface of well-mixed milk and let milk be absorbed by capillary action. Add 0.05 ml penicillinase to 5 ml sample and fill disk. Drain excess milk by touching disk to inside surface of sterile petri dish. Immediately place each disk on agar surface, pressing gently to ensure good contact. Place control disk containing 0.008 unit/ml on plate, or use 90 µl pipettor (F, above). Invert plate and incubate at 64 ± 2°C until well-defined zones of inhibition (16-20 mm) are obtained with the 0.008 unit/ml control. Examine plate for clear zone of inhibition (>16 mm) surrounding disk, indicating presence of inhibitory substance.

H. Interpretation--Assay of test milk in screening and confirmatory test may produce the following results:

- No zone around disk containing untreated milk in screening test is a negative test for inhibitory substances.
- Zone around disk containing untreated milk but no zone around disk containing penicillinase-treated milk in the confirmatory test is a positive test for -lactam residue.
- Clear zone of equal size around both disks in confirmatory test indicates presence of inhibitors other than -lactam residues.
- Clear zone of 4 mm around penicillinase-treated milk smaller than that around untreated milk disk in confirmatory test indicates presence of -lactam residues as well as another inhibitor(s).

The penicillin-positive control solution at 0.008 unit/ml should produce clear, well-defined zones of inhibition (16-20 mm). If no zone of inhibition is produced by penicillin-positive control, test sensitivity is not adequate and test should be repeated.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

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Chapter 20B

Rapid HPLC Determination of Sulfamethazine in Milk

Authors

(Return to Table of Contents)

A simple, relatively rapid HPLC procedure has been developed for determining sulfamethazine in raw bovine milk in the low parts per billion (ppb) range. A separatory funnel is used to extract sulfamethazine from milk with chloroform, the chloroform is evaporated, and the fatty residue is dissolved in hexane. The sulfamethazine is then partitioned into an aqueous potassium phosphate solution which is injected directly onto the chromatograph.

A. Equipment and materials

1. Liquid chromatograph. Series 4 or 410 pump equipped with Model LC-95 UV/Vis detector (Perkin-Elmer Corp., Instrument Div., Norwalk, CT 06056), or equivalent
2. Column heater and controller capable of maintaining 35 ± 0.2°C (Fiatron, Oconomowoc, WI 53066), or equivalent
3. Column, 250 x 4.6 mm, packed with LC-18-DB (Supelco, Bellefonte, PA)
4. Guard column, 2 cm long, LC-18-DB (Supelco)

5. Precolumn filter, 3 mm diameter frit, 0.5 m porosity (Supelco)
6. Rotary evaporator (Buchi Laboratory Techniques Ltd, Flawil, Switzerland), or equivalent
7. Vortex mixer (Genie Scientific, Fountain Valley, CA), or equivalent
8. Freezer capable of holding temperature at -50 to -80°C
9. Polypropylene tubes, 50 ml, with screw caps (Fisher Scientific, Pittsburgh, PA)
10. Micro weighing funnel (Radnoti Glass Technology, Inc., Monrovia, CA), or equivalent
11. Volumetric pipets, 1, 2, and 5 ml; two 10 and 20 ml class A, or equivalent
12. Volumetric flasks, six 100 ml, class A
13. Laboratory refrigerator
14. Eppendorf pipettors, 10-100 µl and 100-1000 µL, or equivalent
15. Eppendorf maxipettor, positive displacement, 1-10 ml, or equivalent
16. HPLC solvent filtering apparatus, 1, 2, or 4 L capacity
17. Nylon-66 HPLC solvent filter, 0.4 m porosity
18. Volumetric flask, 2 L, class A
19. Graduated cylinders, 50 ml, 1 L
20. Intermediate containers, at least 3 glass bottles, 4 L, with Teflon-lined caps. **NOTE**: Plastic storage vessels are not satisfactory for LC solvents.
21. Hewlett-Packard model llC calculator or equivalent
22. Glass repeater pipettors, two 5 ml (Fisher Scientific)
23. Rings and clamps or funnel stand and appropriate laboratory hardware

For each replicate to be analyzed, the following are needed:

24. Separatory funnel, 125 ml, with ground glass stopper and Teflon stopcock
25. Short stem funnel, 75 mm diameter
26. Fluted filter paper, 12.5 cm (Schleicher & Schuell)
27. Pear-shaped flask, 100 ml, with 24/40 standard taper neck with stoppers (Kontes Glass Co., Vineland, NJ)
28. Pasteur pipet
29. Glass autosampler vial or glass test tube

B. Reagents

1. Sulfamethazine standard (Sigma Chemical Co., St. Louis, MO)
2. Potassium dihydrogen phosphate, HPLC grade
3. Methanol, HPLC grade
4. Purified water, distilled, deionized, HPLC grade
5. Chloroform, B&J or equivalent (Burdick & Jackson, Muskegon, MI 49442)
6. Hexane, HPLC grade

7. Solutions (water is considered to be distilled, deionized water)

    1. **Potassium dihydrogen phosphate** (PDP), 0.1 M solution. Dissolve 27.2 g PDP in water, dilute to 2 L, mix, and filter through 0.4 m porosity nylon-66 filter. Store PDP solution at room temperature in intermediate container(s), properly labeled with expiration date 3 months after date of preparation. In this text, PDP solution means filtered PDP solution.

    2. **Mobile phase**. Dilute 600 ml methanol, filtered through nylon-66 filter, to 2 L with PDP solution, and mix. Store mobile phase at room temperature in intermediate container(s), properly labeled with expiration date 3 months after date of preparation of PDP solution.

    3. **Flush solution**. Dilute 1200 ml methanol to 2 L with water, mix, and filter through nylon-66 filter. Store flush solution at room temperature in intermediate container(s), properly labeled with expiration date 3 months after date of preparation.

    4. **Standard solutions**. All standard solutions have expiration date 3 months after date of preparation of master solution. Store all standard solutions below 10°C. **NOTE**: After washing, rinse all glassware with 1 N HC1 as precaution against sulfamethazine cross-contamination; then thoroughly rinse with water, and finally rinse with methanol. Remove stopcock from separatory funnel for rinses.

        a. **Master solution**. Weigh 100 mg sulfamethazine standard at room temperature in glass weighing boat and transfer to 100 ml volumetric flask. Dissolve in methanol, dilute to volume with methanol, and mix.

        b. **Sulfamethazine solution, 10,000 ng/ml**. Measure 1 ml master solution with 1 ml volumetric pipet into 100 ml volumetric flask, dilute to volume with water, and mix.

        c. **Fortification solution -- sulfamethazine solution 1000 ng/ml**. Transfer 10 ml of the 10,000 ng/ml sulfamethazine solution to 100 ml volumetric flask with 10 ml volumetric pipet, dilute to volume with water, and mix.

Table 1. Recovery of sulfamethazine added to milk at 0, 5, 10, and 20 ppb and incurred in milk[(a)]

5 ppb		10 ppb		20 ppb		Incurred
Rec. ppb	**Rec. %**	**Rec. ppb**	**Rec. %**	**Rec. ppb**	**Rec. %**	**Found ppb**
3.99	79.8	7.51	75.1	14.89	74.5	4.46
4.23	84.6	7.22	72.2	15.07	75.4	4.64
4.40	88.0	7.98	79.8	15.19	76.0	4.29
3.47	69.4	7.51	75.1	14.07	70.4	4.35
4.05	81.0	7.92	79.2	13.84	69.2	4.52
Average	80.6		76.3		73.1	4.45
SD	7.0		3.2		3.1	0.14
CV, %	8.7		4.2		4.2	3.1

[a] Sulfamethazine was not detected in the 5 control samples. Taken from Weber & Smedley, J.Assoc. Off. Anal. Chem. 72:445-447 (1989).

d. **Solutions for standard curve** (prepare as follows):

20 ppb standard: Dilute 20 ml fortification solution to 100 ml with water, using 20 ml volumetric pipet and 100 ml volumetric flask. (**NOTE**: This is a 200 ng/ml solution, which is equivalent to a 20 ppb standard.)

10 ppb standard: Dilute 10 ml fortification solution to 100 ml with water, using 10 ml volumetric pipet and 100 ml volumetric flask.

NOTE: Residues from 10 ml of milk are extracted into a final 1 ml of PDP, resulting in a tenfold concentration of residues. Therefore, a standard or final extract with a concentration of 100 ng/ml is equivalent to 10 ppb of that analyte in milk. One hundred µl of both sample and standards are injected onto the LC system.

5 ppb standard: Dilute 5 ml fortification solution to 100 ml with water,

using 5 ml volumetric pipet and 100 ml volumetric flask.

D. Sample storage

Store fresh raw milk in refrigerator at <10°C. However, if milk will not be used within 2-3 days, subdivide into polypropylene plastic tubes and store at -80°C. If a freezer of this nature is not available, store samples frozen at as low a temperature as is available. Thaw frozen milk slowly in slightly warm tap water on the day sample is to be analyzed. Mix milk gently before sampling. Some samples have been successfully analyzed 1-2 years after freezing. However, degradation of milk was noted when it was stored at -15°C for only a few months.

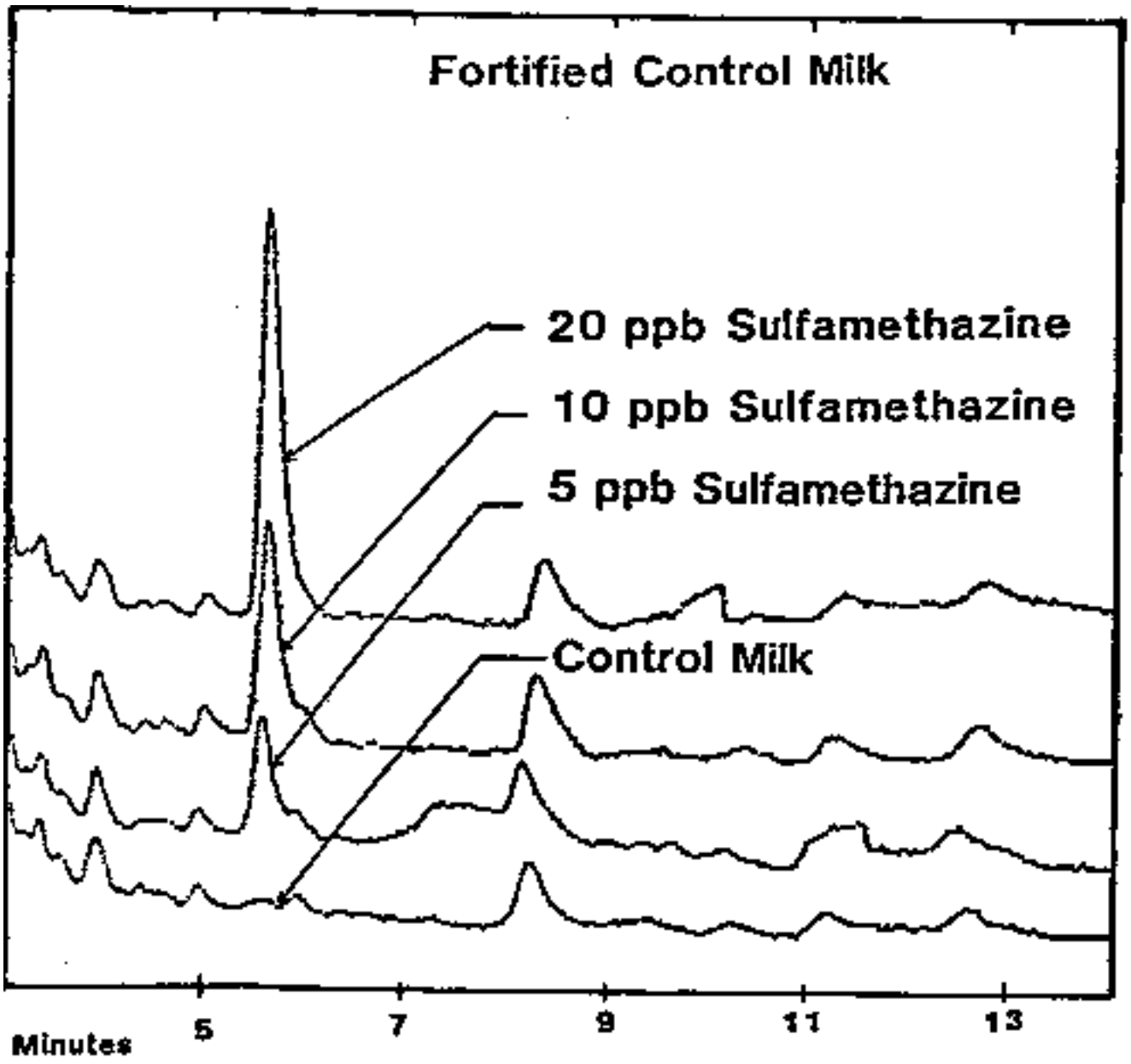

Figure 1. Chromatograms of control milk and fortified control milk. No data were collected during first 3 min after injection to conserve electronic data storage space.

E. Analytical method

1. Extraction of sulfamethazine from milk

In a hood, set 75 mm short stem funnel with fluted filter paper on rack or stand. Using a 5 ml repeater pipettor, wash filter paper with 5 ml chloroform and discard chloroform wash. Place 100 ml pear-shaped flask under funnel as receiver. Using 10 ml maxipettor, add 10 ml milk to 125 ml separatory funnel. For recovery studies, fortify samples at this point. (**See** Table 1 for expected recoveries.) Add 50, 100, or 200 µl of fortification solution to the 10 ml of milk in the separatory funnel to obtain 5, 10, or 20 ppb fortified samples. Using a graduated cylinder, add 50 ml chloroform to 125 ml separatory funnel, and stopper. Shake mixture of milk and chloroform vigorously for 1 min; excess pressure is then carefully vented through stopper. Shake again for 1 min, vent, and let phases separate for 1 min. Repeat shaking for 1 min, vent, and shake for 1 min more. Vent and let phases separate for at least 5 min.

VENTING IS A CRITICAL POINT. Venting separatory funnel through stopper is important. **NOTE**: Venting through stopcock often clogs stopcock with milk solids, so that chloroform cannot be conveniently drawn off. Venting through stopper resolves the problem.

Draw off and filter chloroform through fluted filter paper into 100 ml pear-shaped flask. Using 5-ml repeater pipettor, rinse filter paper twice with 5 ml portions of chloroform, and collect washings in same pear-shaped flask.

2. Prepare sample for injection on HPLC

Evaporate chloroform solution in pear-shaped flask just to dryness on rotary evaporator at 32 ± 2°C. Using another 5 ml repeater pipettor, add 5 ml hexane to flask, stopper, and dissolve residue by agitating vigorously on vortex mixer for 1 min. Using the 100-1000 µL Eppendorf pipettor, immediately add 1 ml PDP solution to hexane in pear-shaped flask. Agitate vigorously on vortex mixer for about 1 min, 3 or 4 times over a minimum of 15 min.

CONTACT TIME IS A CRITICAL POINT. Contact time is as important as vigor of agitation. **NOTE**: Recovery improves up to about 15 min. Longer times,

up to 1 h, can be safely used, but will not improve recovery.

Transfer aqueous layer from bottom of flask to autoinjector vial with Pasteur pipet, taking care not to transfer any hexane to autoinjector vial. If autoinjector is not available, transfer PDP solution to glass test tube. The sample is ready for injection.

3. Chromatography (**see** Fig. 1)

Inject standards at beginning and end of every sample set. Construct standard curve by using peak heights of sulfamethazine standards; calculate sample concentration as described in calculation section.

a. Chromatographic conditions

Heat column to 35 ± 0.2°C. Set mobile phase flow rate to 1.5 ml/min, with UV detector set at 265 nm wavelength. **NOTE**: Use premixed mobile phase and pump isocratically because a small change in the methanol to PDP solution ratio results in a significant change in retention time of sulfamethazine. Let system pump under these conditions for at least 45 min before injecting standards. Set run time at 15 min with equilibrium time of 1 min between runs. **NOTE:** A late eluting peak in some milk samples may appear in the subsequent run, but does not coelute with the sulfamethazine peak. If this causes a problem with the analysis, increase the run by a few minutes as necessary. Inject 100 µl of standard or sample solution. Use flush solution to rinse autoinjector. Set sensitivity and/or recorder ranges to give 75-90% full deflection for 20 ppb standard. Pump flush solution through column for minimum of 45 min as part of system shut-down at end of day.

b. Chromatographic suitability test

Standard curve. Inject duplicate 100 µl aliquots of 5, 10, and 20 ppb sulfamethazine standards. Correlation coefficient of standard curve prepared by using peak heights should be 0.98 or greater. If value is less, repeat injection of standards.

Calculations. Calculate linear regression equation, using least square fit for sulfamethazine standard solutions (concentrations vs peak heights) to obtain values for:

$$Y = mX + b;$$

then solve for concentrations of unknown, X, from equation of line where Y = peak height at sulfamethazine retention time of samples, and b and m are y-intercept and slope, respectively.

F. Additional comments

- If fresh milk is to be stored more than 2 or 3 days, subdivide sample in 50 ml polypropylene tubes and store between -50 and -80°C.
- We have run more than 1000 injections of samples and standards combined without having to replace the LC-18-DB column.
- Prepared samples, in sealed injection vials, have been stored in the autoinjector (at room temperature) for up to 24 h before being injected. No changes in retention time or peak heights of standard solutions were observed.

This method has been successfully validated by an AOAC collaborative study (4). The original single residue method has been modified to simultaneously determine 10 sulfonamide drugs at 10 ppb and above in raw bovine milk (2). Eight of these drugs were selected to be validated in another AOAC collaborative study (1).

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 21A

Examination of Canned Foods

Authors

(Return to Table of Contents)

The incidence of spoilage in canned foods is low, but when it occurs it must be investigated properly. Swollen cans often indicate a spoiled product. During spoilage, cans may progress from normal to flipper, to springer, to soft swell, to hard swell. However, spoilage is not the only cause of abnormal cans. Overfilling, buckling, denting, or closing while cool may also be responsible. Microbial spoilage and hydrogen, produced by the interaction of acids in the food product with the metals of the can, are the principal causes of swelling. High summer temperatures and high altitudes may also increase the degree of swelling. Some microorganisms that grow in canned foods, however, do not produce gas and therefore cause no abnormal appearance of the can; nevertheless, they cause spoilage of the product.

Spoilage is usually caused by growth of microorganisms following leakage or underprocessing. Leakage occurs from can defects, punctures, or rough handling. Contaminated cooling water sometimes leaks to the interior through pinholes or poor seams and introduces bacteria that cause spoilage. A viable mixed microflora of bacterial rods and cocci is indicative of leakage, which may usually be confirmed by can examination. Underprocessing may be caused by undercooking; retort operations that are faulty because of inaccurate or improperly functioning thermometers, gauges, or controls; excessive contamination of the product for which normally adequate processes are insufficient; changes in formulation or handling of the product that result in a more viscous product or tighter packing in the container, with consequent lengthening of the heat penetration time; or, sometimes, accidental bypassing of the retort operation altogether. When the can contains a spoiled

product and no viable microorganisms, spoilage may have occurred before processing or the microorganisms causing the spoilage may have died during storage.

Underprocessed and leaking cans are of major concern and both pose potential health hazards. However, before a decision can be made regarding the potential health hazard of a low-acid canned food, certain basic information is necessary. Naturally, if *Clostridium botulinum* (spores, toxin, or both) is found, the hazard is obvious. Intact cans that contain only mesophilic, Gram-positive, sporeforming rods should be considered underprocessed, unless proved otherwise. It must be determined that the can is intact (commercially acceptable seams and no microleaks) and that other factors that may lead to underprocessing, such as drained weight and product formulation, have been evaluated.

The preferred type of tool for can content examination is a bacteriological can opener consisting of a puncturing device at the end of a metal rod mounted with a sliding triangular blade that is held in place by a set screw. The advantage over other types of openers is that it does no damage to the double seam and therefore will not interfere with subsequent seam examination of the can.

Table 1. Useful descriptive terms for canned food analysis.

Exterior can condition	**Internal can condition**		
leaker	normal		
dented	peeling		
rusted	slight, moderate or severe etching		
buckled	slight, moderate or severe blackening		
paneled	slight, moderate or severe rusting		
bulge	mechanical damage		
Micro-leak test	**Product odor**	**Product liquor**	
packer seam	putrid	cloudy	
side panel	acidic	clear	
side seam	butyric	foreign	
cut code	metallic	frothy	
pinhole	sour		
	cheesy		
	fermented		
	musty		
	sweet		
	fecal		
	sulfur		
	off-odor		

Solid product	Liquid product	Pigment	Consistency
digested	cloudy	darkened	slimy
softened	clear	light	fluid
curdled	foreign	changed	viscous
uncooked	frothy		ropy
overcooked			

Flat - a can with both ends concave; it remains in this condition even when the can is brought down sharply on its end on a solid, flat surface.
Flipper - a can that normally appears flat; when brought down sharply on its end on a flat surface, one end flips out. When pressure is applied to this end, it flips in again and the can appears flat.
Springer - a can with one end permanently bulged. When sufficient pressure is applied to this end, it will flip in, but the other end will flip out.
Soft swell - a can bulged at both ends, but not so tightly that the ends cannot be pushed in somewhat with thumb pressure.
Hard swell - a can bulged at both ends, and so tightly that no indentation can be made with thumb pressure. A hard swell will generally "buckle" before the can bursts. Bursting usually occurs at the double seam over the side seam lap, or in the middle of the side seam.

The number of cans examined bacteriologically should be large enough to give reliable results. When the cause of spoilage is clear-cut, culturing 4-6 cans may be adequate, but in some cases it may be necessary to culture 10-50 cans before the cause of spoilage can be determined. On special occasions these procedures may not yield all the required information, and additional tests must be devised to collect the necessary data. Unspoiled cans may be examined bacteriologically to determine the presence of viable but dormant organisms. The procedure is the same as that used for spoiled foods except that the number of cans examined and the quantity of material subcultured must be increased.

A. Equipment and materials

1. Incubators, thermostatically controlled at 30, 35, and 55°C
2. pH meter, potentiometer
3. Microscope, slides, and coverslips
4. Can opener, bacteriological can opener, and can punch, all sterile
5. Petri dishes, sterile
6. Test tubes, sterile
7. Serological pipets, cotton-plugged, sterile
8. Nontapered pipets, cotton-plugged (8 mm tubing), sterile
9. Soap, water, brush, and towels, sterile and nonsterile
10. Indelible ink marking pen
11. Diamond point pen for marking cans

12. Examination pans (Pyrex or enamel baking pans)

B. Media and reagents

1. Bromcresol purple (BCP) dextrose broth (M27)
2. Chopped liver broth (M38) or cooked meat medium (CMM) (M42)
3. Malt extract broth (M94)
4. Liver-veal agar (without egg yolk) (LVA) (M83)
5. Acid broth (M4)
6. Nutrient agar (NA) (M112)
7. Methylene blue stain (R45), crystal violet (R16), or Gram stain (R32)
8. Sabouraud's dextrose agar (SAB) (M133)
9. 4% Iodine in 70% ethanol (R18)

C. Can preparation

Remove labels. With marking pen, transfer subnumbers to side of can to aid in correlating findings with code. Mark labels so that they may be replaced in their original position on the can to help locate defects indicated by stains on label. Separate all cans by code numbers and record size of container, code, product, condition, evidence of leakage, pinholes or rusting, dents, buckling or other abnormality, and all identifying marks on label. Classify each can according to the descriptive terms in Table 1. Before observing cans for classification, make sure cans are at room temperature.

D. Examination of can and contents

Classification of cans. **NOTE**: Cans must be at room temperature for classification.

1. **Sampling can contents**

    a. **Swollen cans**. Immediately analyze springers, swells, and a representative number (at least 6, if available) of flat and flipper cans. Retain examples of each, if available, when reserve portion must be held. Place remaining flat and flipper cans (excluding those held in reserve) in incubator at 35°C. Examine at frequent intervals for 14 days. When abnormal can or one becoming increasingly swollen is found, make note of it. When can becomes a hard swell or when swelling no longer progresses, culture sampled contents, examine for preformed toxin of *C. botulinum* if microscopic examination shows typical *C. botulinum* organisms or Gram-positive rods, and perform remaining steps of canned food examination.

    b. **Flat and flipper cans**. Place cans (excluding those held in reserve) in incubator at 35°C. Observe cans for progressive swelling at frequent intervals for 14 days. When swelling occurs, follow directions in l-a, above. After 14 days remove flat and flipper cans from incubator and test at least 6, if available. (It is not

necessary to analyze all normal cans.) Do not incubate cans at temperatures above 35°C. After incubation, bring cans back to room temperature before classifying them.

2. **Opening the can**. Open can in an environment that is as aseptic as possible. Use of vertical laminar flow hood is recommended.

    a. **Hard swells, soft swells, and springers**. Chill hard swells in refrigerator before opening. Scrub entire uncoded end and adjacent sides of can using abrasive cleanser, cold water, and a brush, steel wool, or abrasive pad. Rinse and dry with clean sterile towel. Sanitize can end to be opened with 4% iodine in 70% ethanol for 30 min and wipe off with sterile towel. **DO NOT FLAME**. Badly swollen cans may spray out a portion of the contents, which may be toxic. Take some precaution to guard against this hazard, e.g., cover can with sterile towel or invert sterile funnel over can. Sterilize can opener by flaming until it is almost red, or use separate presterilized can openers, one for each can. At the time a swollen can is punctured, test for headspace gas, using a qualitative test or the gas-liquid chromatography method described below. For a qualitative test, hold mouth of sterile test tube at puncture site to capture some escaping gas, or use can-puncturing press to capture some escaping gas in a syringe. Flip mouth of tube to flame of Bunsen burner. A slight explosion indicates presence of hydrogen. Immediately turn tube upright and pour in a small amount of lime water. A white precipitate indicates presence of CO_2. Make opening in sterilized end of can large enough to permit removal of sample.

    b. **Flipper and flat cans**. Scrub entire uncoded end and adjacent sides of can using abrasive cleanser, warm water, and a brush, steel wool, or abrasive pad. Rinse and dry with clean sterile towel. Gently shake cans to mix contents before sanitizing. Flood end of can with iodine-ethanol solution and let stand at least 15 min. Wipe off iodine mixture with clean sterile towel. Ensure sterility of can end by flaming with burner in a hood until iodine-ethanol solution is burned off, end of can becomes discolored from flame, and heat causes metal to expand. Be careful not to inhale iodine fumes while burning off can end. Sterilize can opener by flaming until it is almost red, or use separate presterilized can openers for each can. Make opening in sterilized end of can large enough to permit removal of sample.

3. **Removal of material for testing**. Remove large enough portions from center of can to inoculate required culture media. Use sterile pipets, either regular or wide-mouthed. Transfer solid pieces with sterile spatulas or other sterile devices. Always use safety devices for pipetting. After removal of inocula, aseptically transfer at least 30 ml or, if less is available, all remaining contents of cans to sterile closed containers, and refrigerate at about 4°C. Use this material for repeat examination if needed and for possible toxicity tests. This is the reserve sample. Unless circumstances dictate

otherwise, analyze normal cans submitted with sample organoleptically and physically (**see** 5-b, below), including pH determination and seam teardown and evaluation. Simply and completely describe product appearance, consistency, and odor on worksheet. If analyst is not familiar with decomposition odors of canned food, another analyst, preferably one familiar with decomposition odors, should confirm this organoleptic evaluation. In describing the product in the can, include such things as low liquid level (state how low), evidence of compaction, if apparent, and any other characteristics that do not appear normal. Describe internal and external condition of can, including evidence of leakage, etching, corrosion, etc.

4. **Physical examination**. Perform net weight determinations on a representative number of cans examined (normal and abnormal). Determine drained weight, vacuum, and headspace on a representative number of normal-appearing and abnormal cans (1). Examine metal container integrity of a representative number of normal cans and all abnormal cans that are not too badly buckled for this purpose (**see** Chapter 22). **CAUTION**: Always use care when handling the product, even apparently normal cans, because botulinal toxin may be present.

5. **Cultural examination of low-acid food (pH greater than 4.6)**. If there is any question as to product pH range, determine pH of a representative number of normal cans before proceeding. From each container, inoculate 4 tubes of chopped liver broth or cooked meat medium previously heated to 100°C (boiling) and rapidly cooled to room temperature; also inoculate 4 tubes of bromcresol purple dextrose broth. Inoculate each tube with 1-2 ml of product liquid or product-water mixture, or 1-2 g of solid material. Incubate as in Table 2.

Table 2. Incubation times for various media for examination of low acid foods (pH > 4.6).

Medium	No. of tubes	Temp. (°C)	Time of incubation (h)
Chopped liver (cooked meat)	2	35	96-120
Chopped liver (cooked meat)	2	55	24-72
Bromcresol purple dextrose broth	2	55	24-48
Bromcresol purple dextrose broth	2	35	96-120

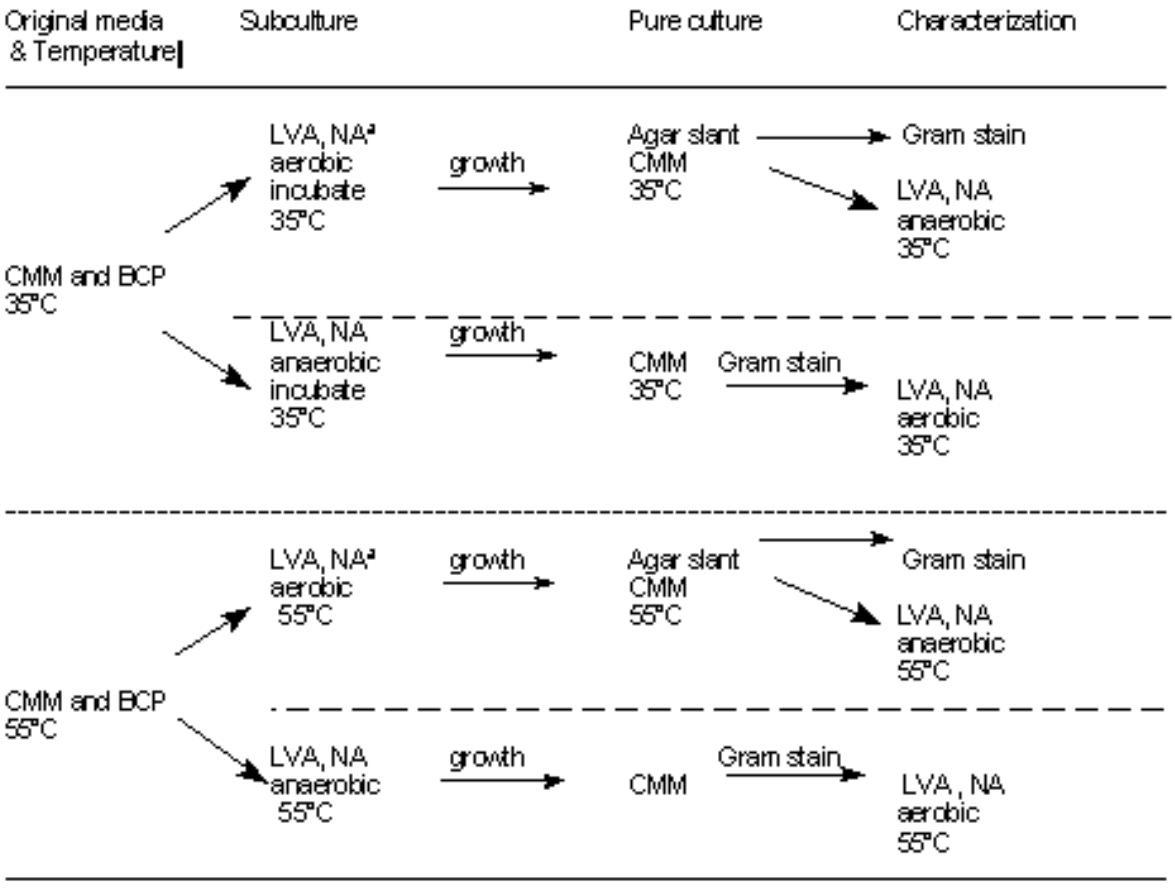
After culturing and removing reserve sample, test material from cans (other than those classified as flat) for preformed toxins of *C. botulinum* when appropriate, as described in Chapter 17.

a. **Microscopic examination**. Prepare direct smears from contents of each can after culturing. Dry, fix, and stain with methylene blue, crystal violet, or Gram

stain. If product is oily, add xylene to a warm, fixed film, using a dropper; rinse and stain. If product washes off slide during preparation, examine contents as wet mount or hanging drop, or prepare suspension of test material in drop of chopped liver broth before drying. Check liver broth before use to be sure no bacteria are present to contribute to the smear. Examine under microscope; record types of bacteria seen and estimate total number per field.

b. **Physical and organoleptic examination of can contents**. After removing reserve sample from can, determine pH of remainder, using pH meter. **DO NOT USE pH PAPER**. Pour contents of cans into examination pans. Examine for odor, color, consistency, texture, and overall quality. **DO NOT TASTE THE PRODUCT**. Examine can lining for blackening, detinning, and pitting.

Table 3.Schematic diagram of culture procedure for low-acid canned foods

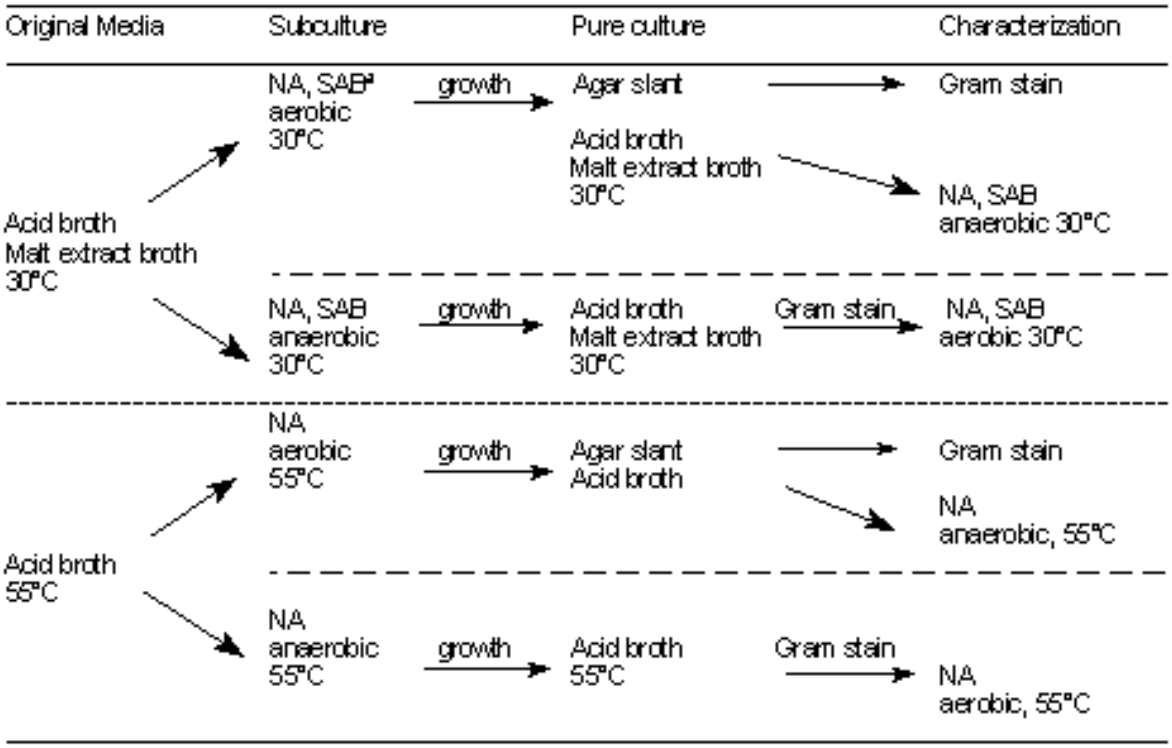


[a] LVA, liver-veal agar; NA, nutrient agar; CMM, cooked meat medium; BCP, bromcresol purple dextrose broth.

Table 4. Incubation of acid broth and malt extract broth used for acid foods (pH 4.6)

Medium	No. of tubes	Temp. (°C)	Time of incubation (h)
Acid broth	2	55	48
Acid broth	2	30	96
Malt extract broth	2	30	96

Table 5. Pure culture scheme for acid foods (pH 4.6).



[a] NA, nutrient agar; SAB, Sabouraud's dextrose agar.

E. Cultural findings in cooked meat medium (CMM) and bromcresol purple dextrose broth (BCP)

Check incubated medium for growth at frequent intervals up to maximum time of incubation (Table 2). If there is no growth in either medium, report and discard. At time growth is noted

streak 2 plates of liver-veal agar (without egg yolk) or nutrient agar from each positive tube. Incubate one plate aerobically and one anaerobically, as in schematic diagram (Table 3). Reincubate CMM at 35°C for maximum of 5 days for use in future toxin studies. Pick representatives of all morphologically different types of colonies into CMM and incubate for appropriate time, i.e., when growth is sufficient for subculture. Dispel oxygen from CMM broths to be used for anaerobes but not from those to be used for aerobes. After obtaining pure isolates, store cultures to maintain viability.

1. **If mixed microflora is found only in BCP**, report morphological types. If rods are included among mixed microflora in CMM, test CMM for toxin, as described in Chapter 17. If Gram-positive or Gram-variable rods typical of either *Bacillus* or *Clostridium* organisms are found in the absence of other morphological types, search to determine whether spores are present. In some cases, old vegetative cells may appear to be Gram-negative and should be treated as if they are Gram-positive. Test culture for toxin according to Chapter 17.

Table 6. Classification of food products according to acidity

Low acid--pH greater than 4.6	**Acid pH 4.6 and below**
Meats	Tomatoes
Seafoods	Pears
Milk	Pineapple
Meat and vegetable Mixtures and "specialties"	Other fruit
Spaghetti Soups	Sauerkraut
Vegetables	Pickles
Asparagus Beets Pumpkin Green beans Corn Lima beans	Berries Citrus Rhubarb

Table 7. Spoilage microorganisms that cause high and low acidity in various vegetables and fruits

Spoilage type	**pH groups**	**Examples**

Thermophilic		
Flat-sour	≥5.3	Corn, peas
Thermophilic[a]	≥4.8	Spinach, corn
Sulfide spoilage[a]	≥5.3	Corn, peas
Mesophilic		
Putrefactive anaerobes[a]	≥4.8	Corn, asparagus
Butyric anaerobes	≥4.0	Tomatoes, peas
Aciduric flat-sour[a]	≥4.2	Tomato juice
Lactobacilli	4.5-3.7	Fruits
Yeasts	≤3.7	Fruits
Molds	≤3.7	Fruits

[a] The responsible organisms are bacterial sporeformers.

Table 8. Spoilage manifestations in low-acid products

Group of organisms	Classification	Manifestations
Flat-sour	Can flat	Possible loss of vacuum on storage
	Product	Appearance not usually altered; pH markedly lowered, sour; may have slightly abnormal odor; sometimes cloudy liquor
Thermophilic anaerobe	Can swells	May burst
	Product	Fermented, sour, cheesy or butyric odor
Sulfide spoilage	Can flat	H_2S gas absorbed by product
	Product	Usually blackened; rotten egg odor
Putrefactive anaerobe	Can swells	May burst
	Product	May be partially digested; pH slightly above normal; typical putrid odor
Aerobic sporeformers	Can flat or swollen	Usually no swelling, except in cured meats when nitrate and sugar present; coagulated evaporated milk, black beets

Table 9. Spoilage manifestations in acid products

Type of organism	Classification	Manifestation
Bacillus thermoacidurans (flat, sour tomato juice)	Can flat	Little change in vacuum
	Product	Slight pH change; off-odor
Butyric anaerobes (tomatoes and tomato juice)	Can swells	May burst
	Product	Fermented, butyric odor
Nonsporeformers (mostly lactic types)	Can swells	Usually burst, but swelling may be arrested
	Product	Acid odor

Table 10. Laboratory diagnosis of bacterial spoilage

	Underprocessed	Leakage
Can	Flat or swelled; seams generally normal	Swelled; may show normal defects[a]
Product appearance	Sloppy or fermented	Frothy fermentation; viscous
odor	Normal, sour or putrid, but generally consistent from can to can	Sour, fecal; generally varying from can to can
pH	Usually fairly constant	Wide variation
Microscopic and cultural	Cultures show sporeforming rods only	Mixed cultures, generally rods and cocci; only at usual temperatures
	Growth at 35 and/or 55°C. May be characteristic on special growth media, e.g., acid agar for tomato juice. If product misses retort completely, rods, cocci,yeast or molds, or any combination of these may be present.	

History	Spoilage usually confined to certain portions of pack	Spoilage scattered
	In acid products, diagnosis may be less clearly defined; similar organisms may be involved in understerilization and leakage.	

[a] Leakage may be due not to can defects but to other factors, such as contamination of cooling water or rough handling, e.g., can unscramblers, rough conveyor system.

Table 11. pH range of a few selected commercially canned foods

Food	pH range	Food	pH range
Apples, juice	3.3 - 3.5	Jam, fruit	3.5 - 4.0
Apples, whole	3.4 - 3.5	Jellies, fruit	3.0 - 3.5
Asparagus, green	5.0 - 5.8	Lemon juice	2.2 - 2.6
Beans		Lemons	2.2 - 2.4
Baked	4.8 - 5.5	Lime juice	2.2 - 2.4
Green	4.9 - 5.5	Loganberries	2.7 - 3.5
Lima	5.4 - 6.3	Mackerel	5.9 - 6.2
Soy	6.0 - 6.6	**Milk**	
Beans with pork	5.1 - 5.8	Cow, whole	6.4 - 6.8
Beef, corned, hash	5.5 - 6.0	Evaporated	5.9 - 6.3
Beets, whole	4.9 - 5.8	Molasses	5.0 - 5.4
Blackberries	3.0 - 4.2	Mushroom	6.0 - 6.5
Blueberries	3.2 - 3.6	Olives, ripe	5.9 - 7.3
Boysenberries	3.0 - 3.3	Orange juice	3.0 - 4.0
Bread		Oysters	6.3 - 6.7
White	5.0 - 6.0	Peaches	3.4 - 4.2
Date and nut	5.1 - 5.6	Pears (Bartlett)	3.8 - 4.6
Broccoli	5.2 - 6.0	Peas	5.6 - 6.5
Carrot juice	5.2 - 5.8	**Pickles**	
Carrots, chopped	5.3 - 5.6	Dill	2.6 - 3.8

Cheese		Sour	3.0 - 3.5
Parmesan	5.2 - 5.3	Sweet	2.5 - 3.0
Roquefort	4.7 - 4.8	Pimento	4.3 - 4.9
Cherry juice	3.4 - 3.6	**Pineapple**	
Chicken	6.2 - 6.4	Crushed	3.2 - 4.0
Chicken with noodles	6.2 - 6.7	Juice	3.4 - 3.7
Chop suey	5.4 - 5.6	Sliced	3.5 - 4.1
Cider	2.9 - 3.3	Plums	2.8 - 3.0
Clams	5.9 - 7.1	Potato salad	3.9 - 4.6
Cod fish	6.0 - 6.1	**Potatoes**	
Corn		Mashed	5.1
Cream style	5.9 - 6.5	White, whole	5.4 - 5.9
On-the-cob	6.1 - 6.8	Prune juice	3.7 - 4.3
Whole grain		Pumpkin	5.2 - 5.5
Brine-packed		Raspberries	2.9 - 3.7
Vacuum-packed	6.0 - 6.4	Rhubarb	2.9 - 3.3
Crab apples, spiced	3.3 - 3.7	Salmon	6.1 - 6.5
Cranberry		Sardines	5.7 - 6.6
Juice	2.5 - 2.7	Sauerkraut	3.1 - 3.7
Sauce	2.3	Juice	3.3 - 3.4
Currant juice	3.0	Shrimp	6.8 - 7.0
Dates	6.2 - 6.4	**Soups**	
Duck	6.0 - 6.1	Bean	5.7 - 5.8
Figs	4.9 - 5.0	Beef broth	6.0 - 6.2
Frankfurters	6.2 - 6.2	Chicken noodle	5.5 - 6.5
Fruit cocktail	3.6 - 4.0	Clam chowder	5.6 - 5.9
Gooseberries	2.8 - 3.1	Duck	5.0 - 5.7
Grapefruit		Mushroom	6.3 - 6.7
Juice	2.9 - 3.4	Noodle	5.6 - 5.8
Pulp	3.4	Oyster	6.5 - 6.9
Sections	3.0 - 3.5	Pea	5.7 - 6.2
Grapes	3.5 - 4.5	Spinach	4.8 - 5.8
Ham, spiced	6.0 - 6.3	Squash	5.0 - 5.8

Hominy, lye	6.9 - 7.9	Tomato	4.2 - 5.2
Huckleberries	2.8 - 2.9	Turtle	5.2 - 5.3
		Vegetable	4.7 - 5.6
Strawberries	3.0 - 3.9	**Miscellaneous products**	
Sweet potatoes	5.3 - 5.6	Beers	4.0 - 5.0
Tomato juice	3.9 - 4.4	Ginger ale	2.0 - 4.0
Tomatoes	4.1 - 4.4	**Human**	
Tuna	5.9 - 6.1	Blood plasma	7.3 - 7.5
Turnip greens	5.4 - 5.6	Duodenal contents	4.8 - 8.2
Vegetable juice	3.9 - 4.3	Feces	4.6 - 8.4
Vegetables, mixed	5.4 - 5.6	Gastric contents	1.0 - 3.0
Vinegar	2.4 - 3.4	Milk	6.6 - 7.6
Youngberries	3.0 - 3.7	Saliva	6.0 - 7.6
		Spinal fluid	7.3 - 7.5
		Urine	4.8 - 8.4
		Magnesia, milk of	10.0 -10.5
		Water	
		Distilled, CO_2	6.8 - 7.0
		Mineral	6.2 - 9.4
		Sea	8.0 - 8.4
		Wine	2.3 - 3.8

2. **If no toxin is present**, send pure cultures for evaluation of heat resistance to Cincinnati District Office, FDA, 1141 Central Parkway, Cincinnati, OH 45202, if cultures meet the following criteria:

    - Cultures come from intact cans that are free of leaks and have commercially acceptable seams. (Can seams of both ends of can must be measured; visual examination alone is not sufficient.)

    - Two or more tubes are positive and contain similar morphological types.

3. **Examination of acid foods (pH 4.6 and below) by cultivation**. From each can, inoculate 4 tubes of acid broth and 2 tubes of malt extract broth with 1-2 ml or 1-2 g of

product, using the same procedures as for low-acid foods, and incubate as in Table 4. Record presence or absence of growth in each tube, and from those that show evidence of growth, make smears and stain. Report types of organisms seen. Pure cultures may be isolated as shown in Table 5.

F. Interpretation of results (**see** Tables 6-11)

1. The presence of only sporeforming bacteria, which grow at 35°C, in cans with satisfactory seams and no microleaks indicates underprocessing if their heat resistance is equal to or less than that of C. *botulinum*. Spoilage by thermophilic anaerobes such as *C. thermobutylicum* may be indicated by gas in cooked meat at 55°C and a cheesy odor. Spoilage by *C. botulinum*, *C. sporogenes*, or *C. perfringens* may be indicated in cooked meat at 35°C by gas and a putrid odor; rods, spores, and clostridial forms may be seen on microscopic examination. Always test supernatants of such cultures for botulinal toxin even if no toxin was found in the product itself, since viable botulinal spores in canned foods indicate a potential public health hazard, requiring recall of all cans bearing the same code. Spoilage by mesophilic organisms such as *Bacillus thermoacidurans* or *B. coagulans* and/or thermophilic organisms such as *B. stearothermophilus*, which are flat-sour types, may be indicated by acid production in BCP tubes at 35 and/or 55°C in high-acid or low-acid canned foods. No definitive conclusions may be drawn from inspection of cultures in broth if the food produced an initial turbidity on inoculation. Presence or absence of growth in this case must be determined by subculturing.

2. Spoilage in acid products is usually caused by nonsporeforming lactobacilli and yeasts. Cans of spoiled tomatoes and tomato juice remain flat but the products have an off-odor, with or without lowered pH, due to aerobic, mesophilic, and thermophilic sporeformers. Spoilage of this type is an exception to the general rule that products below pH 4.6 are immune to spoilage by sporeformers. Many canned foods contain thermophiles which do not grow under normal storage conditions, but which grow and cause spoilage when the product is subjected to elevated temperatures (50-55°C). *B. thermoacidurans* and *B. stearothermophilus* are thermophiles responsible for flat-sour decomposition in acid and low-acid foods, respectively. Incubation at 55°C will not cause a change in the appearance of the can, but the product has an off-odor with or without a lowered pH. Spoilage encountered in products such as tomatoes, pears, figs, and pineapples is occasionally caused by *C. pasteurianum*, a sporeforming anaerobe which produces gas and a butyric acid odor. *C. thermosaccolyticum* is a thermophilic anaerobe which causes swelling of the can and a cheesy odor of the product. Cans which bypass the retort without heat processing usually are contaminated with nonsporeformers as well as sporeformers, a spoilage characteristic similar to that resulting from leakage.

3. A mixed microflora of viable bacterial rods and cocci usually indicates leakage. Can examination may not substantiate the bacteriological findings, but leakage at some time in the past must be presumed. Alternatively, the cans may have missed the retort

altogether, in which case a high rate of swells would also be expected.

4. A mixed microflora in the product, as shown by direct smear, in which there are large numbers of bacteria visible but no growth in the cultures, may indicate precanning spoilage. This results from bacterial growth in the product before canning. The product may be abnormal in pH, odor, and appearance.

5. If no evidence of microbial growth can be found in swelled cans, the swelling may be due to development of hydrogen by chemical action of contents on container interiors. The proportion of hydrogen varies with the length and condition of storage. Thermophilic anaerobes produce gas, and since cells disintegrate rapidly after growth, it is possible to confuse thermophilic spoilage with hydrogen swells. Chemical breakdown of the product may result in evolution of carbon dioxide. This is particularly true of concentrated products containing sugar and some acid, such as tomato paste, molasses, mincemeats, and highly sugared fruits. The reaction is accelerated at elevated temperatures.

6. Any organisms isolated from normal cans that have obvious vacuum and normal product but no organisms in the direct smear should be suspected as being a laboratory contaminant. To confirm, aseptically inoculate growing organism into another normal can, solder the hole closed, and incubate 14 days at 35°C. If any swelling of container or product changes occur, the organism was probably not in the original sample. If can remains flat, open it aseptically and subculture as previously described. If a culture of the same organism is recovered and the product is normal, consider the product commercially sterile since the organism does not grow under normal conditions of storage and distribution.

Headspace Gas Determination by Gas-Liquid Chromatography

Nitrogen, the principal gas normally present in canned foods during storage, is associated with lesser quantities of carbon dioxide and hydrogen. Oxygen included in the container at the time of closure is initially dissipated by container corrosion and/or product oxidation. Departure from this normal pattern can serve as an important indication of changes within the container, since the composition of headspace gases may distinguish whether bacterial spoilage, container corrosion, or product deterioration is the cause of swollen cans (2). Use of the gas chromatograph for analyzing headspace gases of abnormal canned foods has eliminated the possibility of false-negative tests for different gases. It has also allowed the analyst to determine the percentage of each gas present, no matter what the mixture is. By knowing these percentages, the analyst can be alerted to possible can deterioration problems or bacterial spoilage. A rapid gas-liquid chromatographic procedure is presented here for the determination of carbon dioxide, hydrogen, oxygen, nitrogen, and hydrogen sulfide from the headspace of abnormal canned foods.

The analysis of 2352 abnormal canned foods, composed of 288 different products by a gas-liquid chromatography showed viable microorganisms in 256 cans (3). Analysis of this data showed that greater than 10 percent carbon dioxide in the headspace gas was indicative of microbial growth. Although greater than 10 percent carbon dioxide is found in a container, long periods of storage at normal temperatures can result in autosterilization and absence of viable microorganisms. Carbon dioxide my be produced in sufficient quantities to swell the container. Storage at elevated temperatures accelerates this action. Hydrogen can be produced in cans when the food contents react chemically with the metal of the seam (3).

A. Equipment and materials

1. Fisher Model 1200 Gas Partitioner, with dual thermal conductivity cells and dual in-line columns. Column No. 1 is 6-1/2 ft x 1/8 inch, aluminum packed, with 80-100 mesh Columpak^{TM} PQ. Column No. 2 is 11 ft x 3/16 inch, aluminum packed, with 60-80 mesh molecular sieve 13X (Fig. 1).

   NOTE: Other gas chromatograph instruments equipped with the appropriate columns, carrier gas, detector and recorder or integrator may also be suitable for this analysis.

   Operating conditions: column temperature, 75°C; attenuation, 64/256; carrier gas, argon, with in-let pressure of 40 psig; flow rate, 26 ml/min through gas partitioner and 5 ml/min through flush line; bridge current, 125 mA; column mode, 1 & 2; temperature mode, column; injector temperature, off.

   NOTE: **Installation of flush system**. Injection of gas samples through either sample out port or septum injection port may lead to damaged filaments in detector and excessive accumulation of moisture on columns due to bypassing the sample drying tube. To avoid this, make all injections in the sample in port. To avoid cross-contamination, install a flush line off the main argon line (Fig. 2), and flush sample loop between injections.

2. Strip chart recorder, with full scale deflection and speed set at 1 cm/min, 1 mv

3. Can puncturing press (Fig. 3)

4. Sterile stainless steel gas piercers (Fig. 4)

5. Miniature inert valve, with 3-way stopcock and female luer on left side (Popper & Sons, Inc., 300 Denton Ave., New Hyde Park, NY 11040), or equivalent (Fig. 5)

6. Plastic disposable 10-50 ml syringes, with restraining attachment for maximum volume control (Fig. 6). Syringes may be reused.

7. Gas chromatograph and caps, for capping syringes (Alltech Associates, Inc., 202

Campus Drive, Arlington Heights, IL 60004), or equivalent (Fig. 6)

8. Beaker, 1 liter, glass or metal

9. Plastic gas tubing, 3 ft x 1/8 inch id, for exhaust tubing

10. Soap solution, for detecting gas leaks ("SNOOP" Nuclear Products Co., 15635 Saranac Road, Cleveland, OH 44110), or equivalent

11. Small pinch clamp, to weigh down exhaust tubing in beaker

12. Nupro Valve, flow-regulating valve for flush line, 1/8 inch, Angle Pattern Brass (Alltech), or equivalent (Fig. 2)

13. Silicone rubber tubing, seamless, red, autoclavable, 1/8 inch bore x 3/16 inch wall thickness (Arthur H. Thomas Co., Vine St. at 3rd, Philadelphia, PA), or equivalent

B. Calibration of gas chromatograph

Calibration gases of known proportions are commercially available. Construct calibration curves from analysis of pure gases and at least 2-3 different percentage mixtures of gases. Plot linear graph of various known concentrations of each gas as peak height (mm) vs percent gas (Fig. 7).

C. Preparation of materials

Prepare gas collection apparatus as illustrated in Figs. 8 and 9. Adjust height of gas collection apparatus to height of can to be examined. Attach male terminal of miniature valve to female Luer-Lok terminal mounted on top of brass block on can-puncturing press. Attach one end of gas exhaust tubing to female terminal of miniature valve. Attach small pinch clamp to other end of gas exhaust tubing and place in beaker partially filled with water. Attach disposable syringe to other female Luer-Lok terminal on miniature valve. Turn 2-way plug so that gas entering from piercer will flow toward disposable syringe. Place sterile gas piercer in position on male terminal mounted on bottom of brass block on can-puncturing press.

D. Collection of headspace gas

Place can under gas press (cans to be cultured should first be cleaned and sterilized). Lower handle until gas piercer punctures can and seals. Hold in position until adequate volume of gas has been collected (minimum of 5 ml); then turn 2-way plug to release excess gas through exhaust tubing. Release handle, remove syringe, and cap immediately. Identify syringe appropriately.

E. Injection of gas into gas chromatograph

Turn on gas chromatograph and recorder. Let stabilize for about 2 h. Make sure flush line is attached and gas sampling valve is open to allow flushing of sample loop. Turn on chart drive on recorder. Remove flush line, uncap, and immediately attach syringe to Sample-In Injection Port. Inject 5-10 ml of gas and immediately close gas sampling valve. Remove syringe and cap. Reattach flush line onto Sample-In Port and open gas sample valve to allow flushing of system before next injection. Observe chromatogram and switch attenuation from 64 to 256 after carbon dioxide peak has been recorded and returned back to base line. This allows hydrogen peak to be retained on scale. After hydrogen peak returns to base line, switch attenuation back to 64. After instrument has separated gases (about 6 min), determine retention time and peak height for each gas recovered from unknown sample and percent determined from standard graph by comparing retention times and peak heights with known gases, usually associated with headspace gases from abnormal canned food products. Mount chromatogram on mounting paper and identify properly as in Fig. 10. For each sample examined, inject control gases for each type of headspace gas recovered.

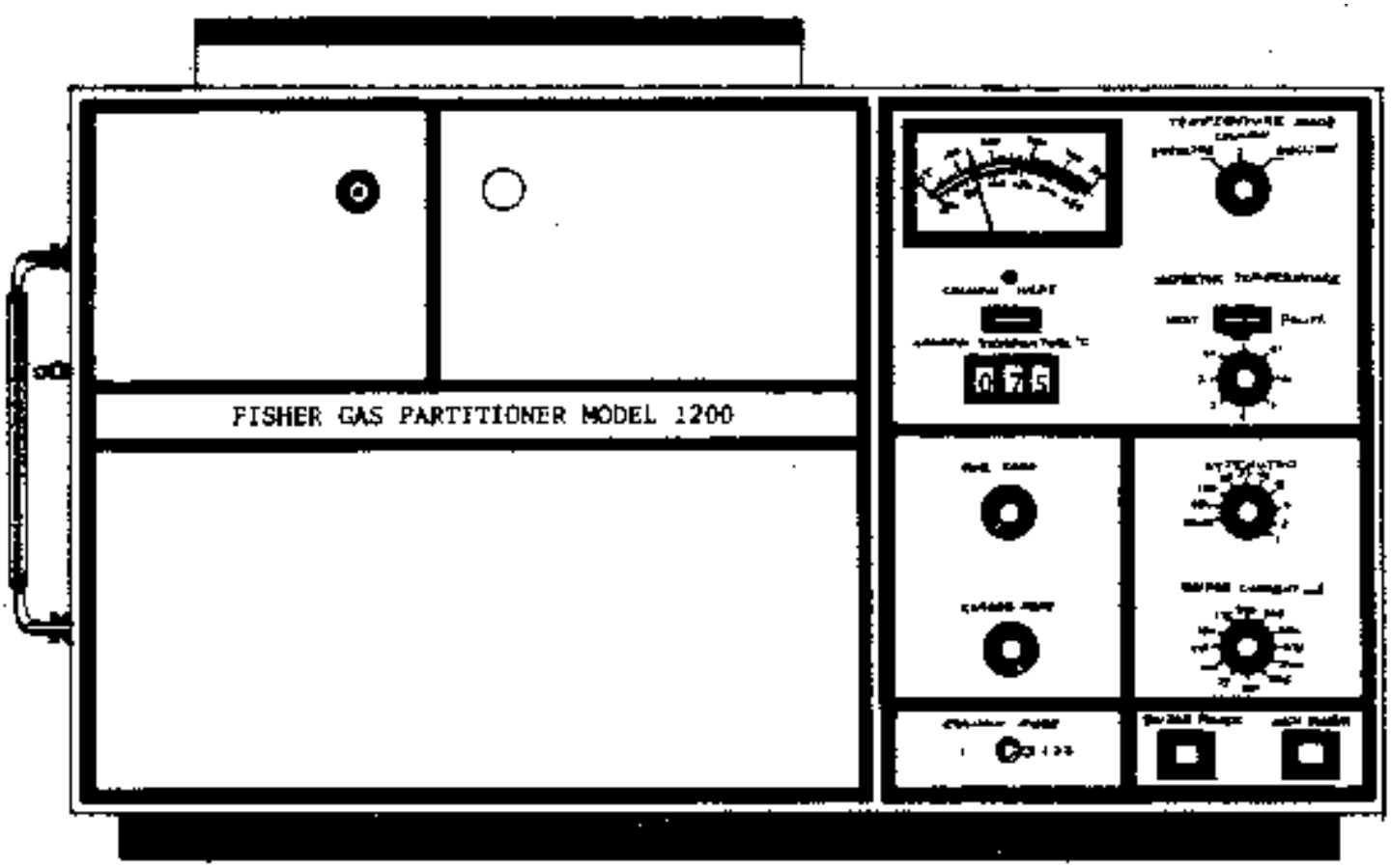


Figure 1. Fisher Model 1200 gas partitioner.

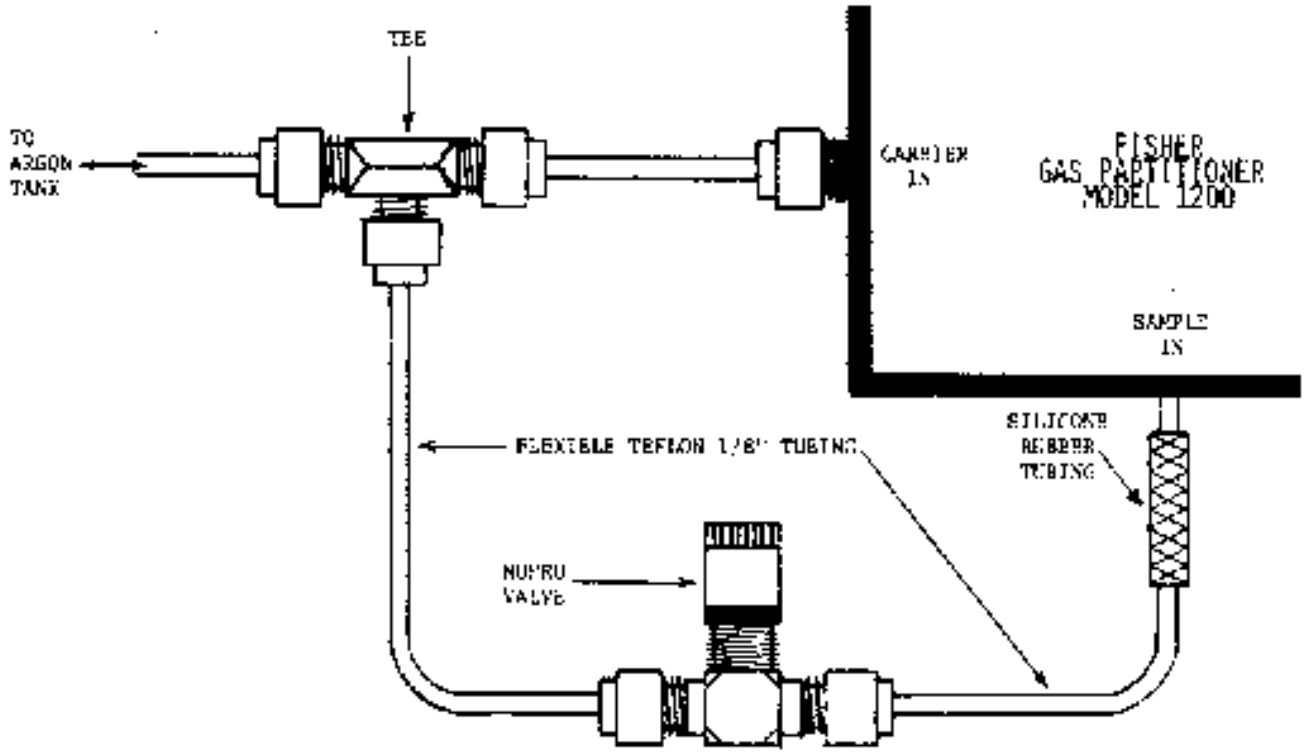


Figure 2. Flush System.

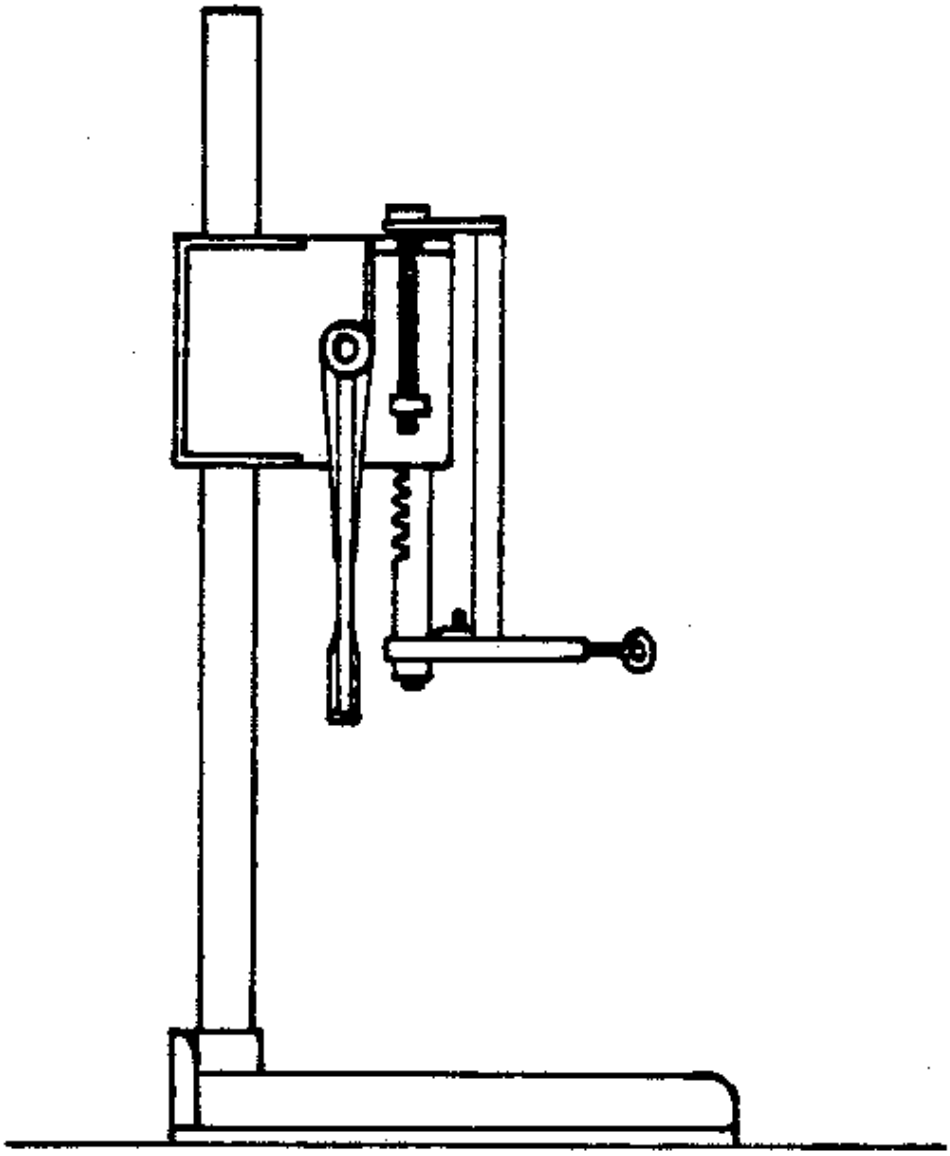


Figure 3. Can puncturing press.

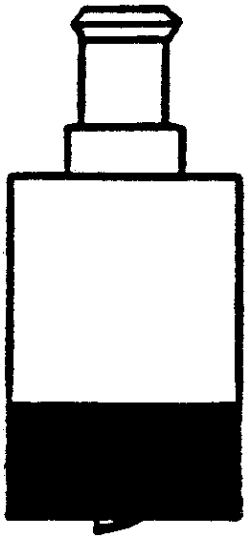


Figure 4. Stainless steel gas piercer.

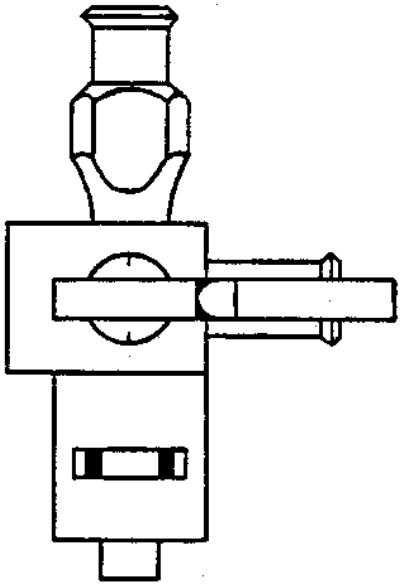


Figure 5. Miniature inert valve.

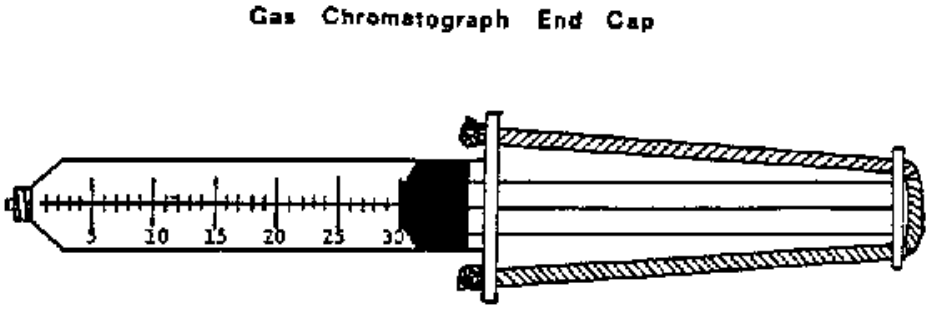


Figure 6. Plastic disposable syringe with restraining attachment.

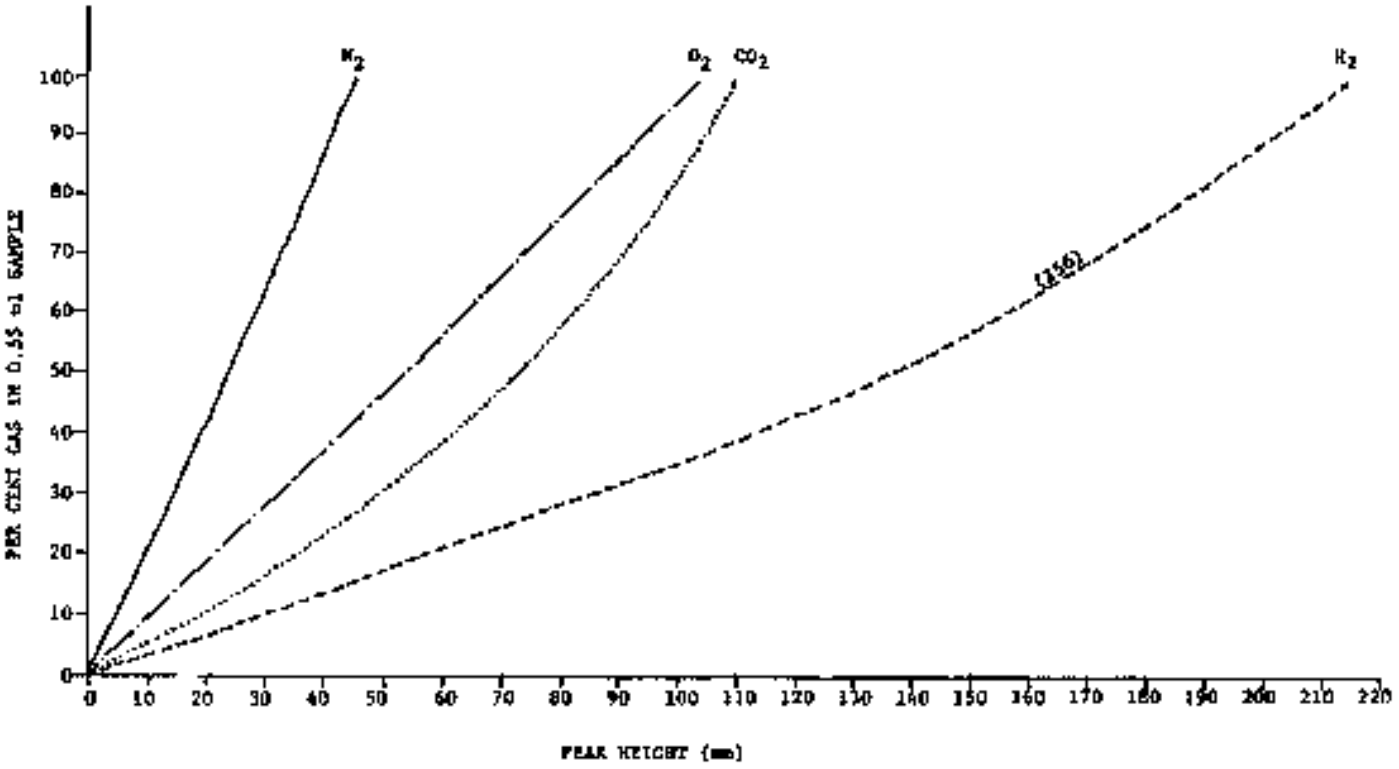


Figure 7. Calibration graph for gas chromatography of headspace gas, using pure and unknown mixtures.

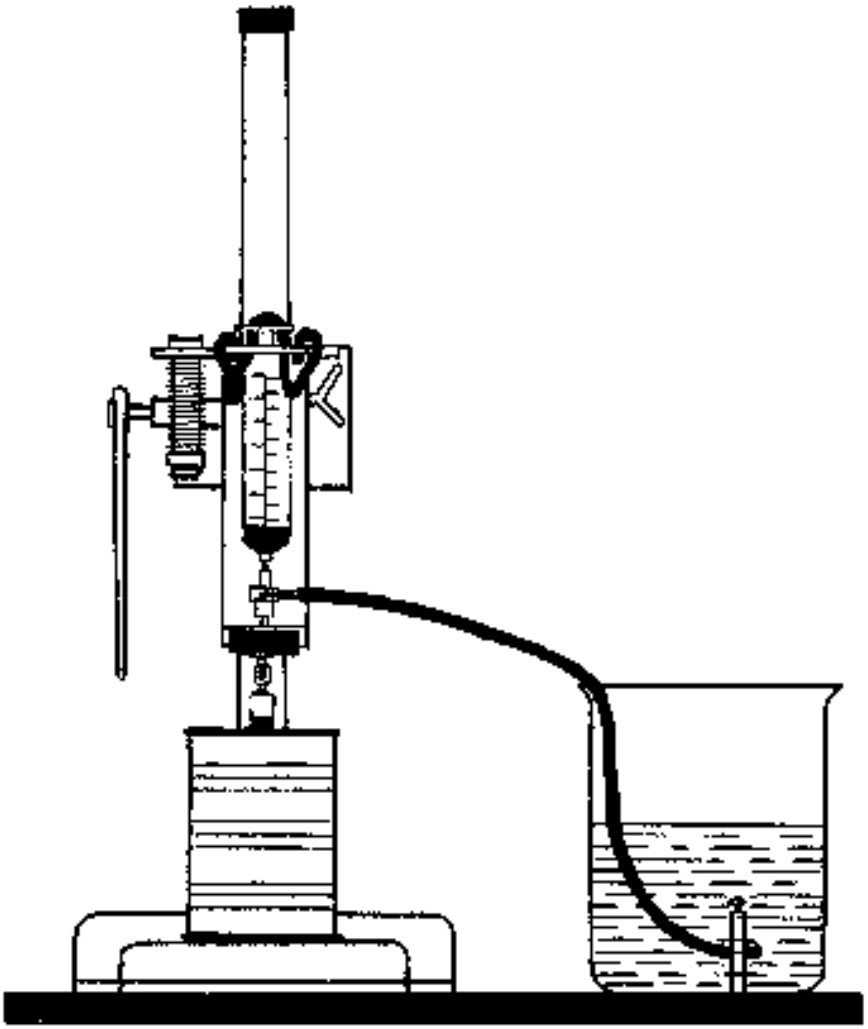


Figure 8. Gas collection apparatus.

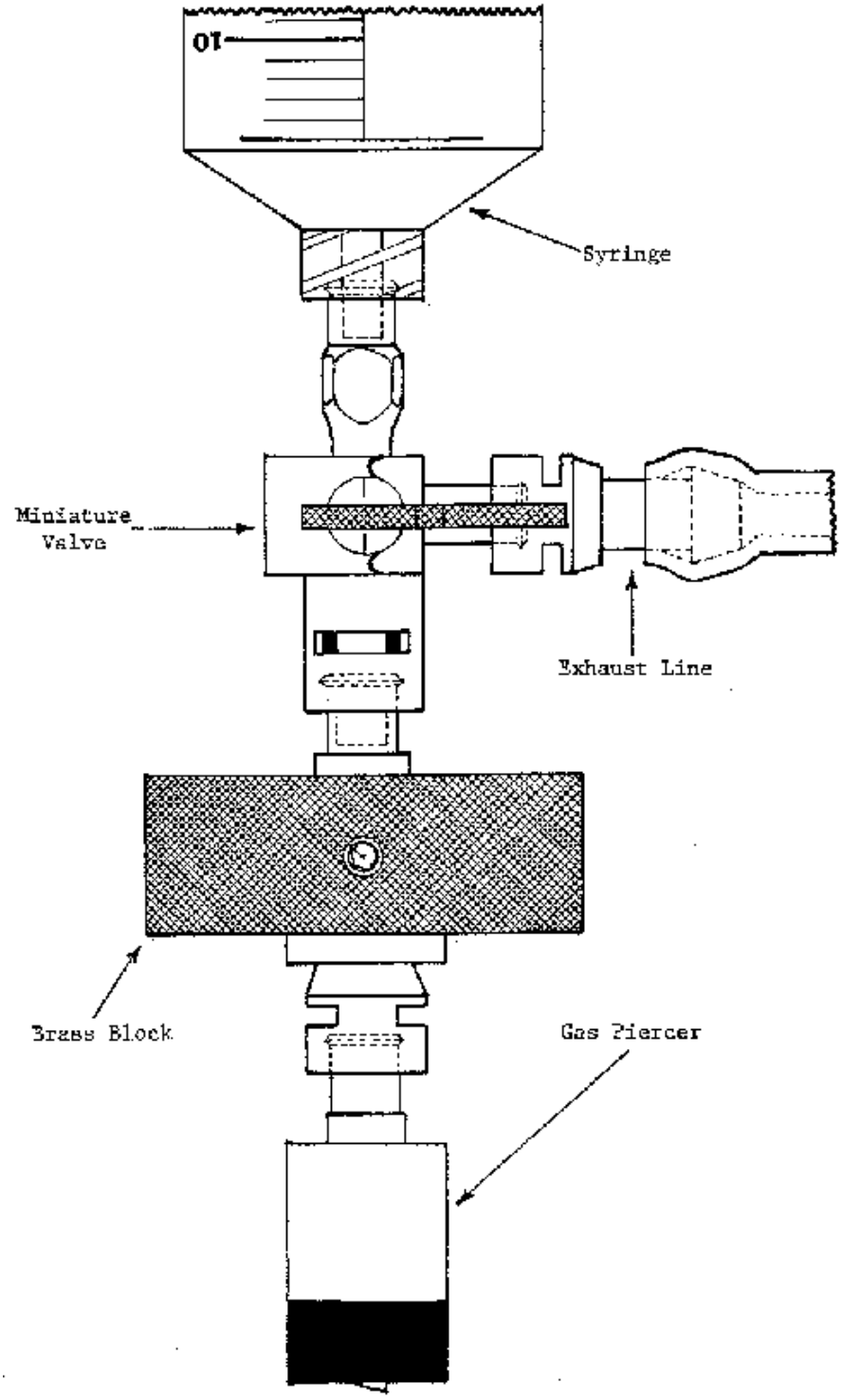


Figure 9. Gas collection apparatus (detail).

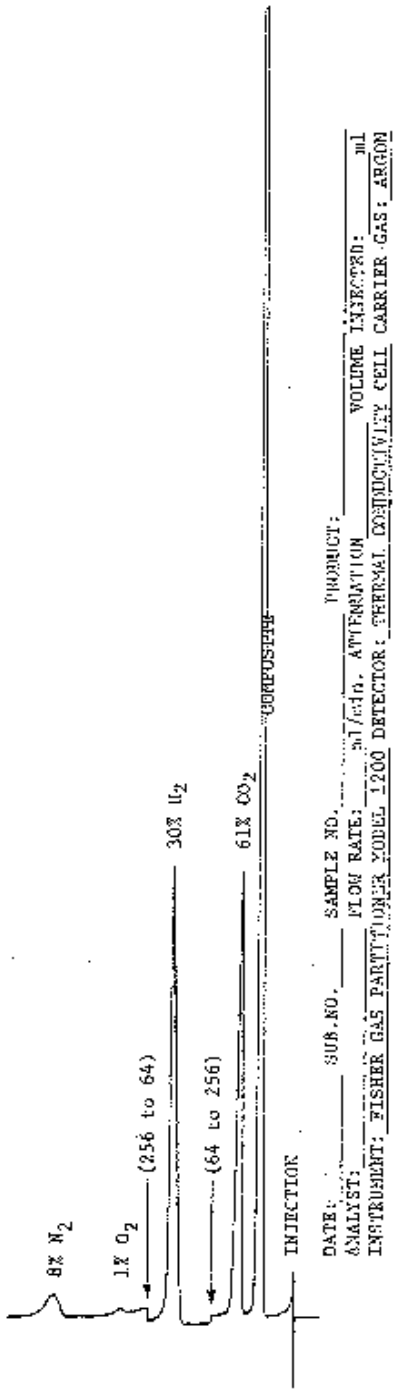


Figure 10. Gas chromatograph of headspace gas.

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Authors: Warren L. Landry, Albert H. Schwab, and Gayle A. Lancette

Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

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U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual *Online*

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Chapter 21B

Modification of Headspace Gas Analysis Methodology, Using the SP4270 Integrator

Authors

(Return to Table of Contents)

This section may be used as an alternative method by laboratories that have the necessary equipment and expertise.

The use of the SP4270 Integrator in place of the strip chart recorder will free the analyst from time-consuming calculations and identification procedures because the analyst need not monitor the instrument constantly to change the attenuation and stop the procedure between injections. With the integrator, the analyst can simply enter a few basic instructions and a little pertinent information before injections and the integrator will print a report with the analyst's name or initials, sample number, sub number, chromatogram, and the gases detected with their percentage.

A. Equipment

1. Fisher gas partitioner Model 1200, with dual thermal conductivity cells and dual in-line columns. Column No. 1 is 6-1/2 ft x 1/8 inch, aluminum packed, with 80-100 mesh Columpak^{TM} PQ. Column No. 2 is 11 ft x 3/16 inch aluminum packed with 60-80 mesh molecular sieve 13X (Fig. 1).

   Note: Other gas chromatograph instruments equipped with the appropriate columns, carrier gas, detector and recorder or integrator may also be suitable for this analysis.

   Operating conditions

   Column temperature = 75°C
   Attenuation = 64
   Carrier gas = Argon, with in-let pressure of 60 psig
   Flow rate = 41 ml/min through gas partitioner 5 ml/min through flush line
   Bridge Current = 125 mA
   Column mode = 1 & 2
   Temperature mode = Column
   Injector temperature = Off

2. Standby power system, Model SPS 200-117, SAFT America Inc., Electronic Systems Division, 2414 W. 14th St., Tempe, AZ 85281 (Fig. 2).

3. Spectra-physics integrator, Model SP4270, Spectra-Physics, Autolab Division, 3333 N. First St., San Jose, CA 95134 (Fig. 3).

B. Integrator programming

After turning system on and entering date and time, enter dialog portion of system and program all information as listed below.

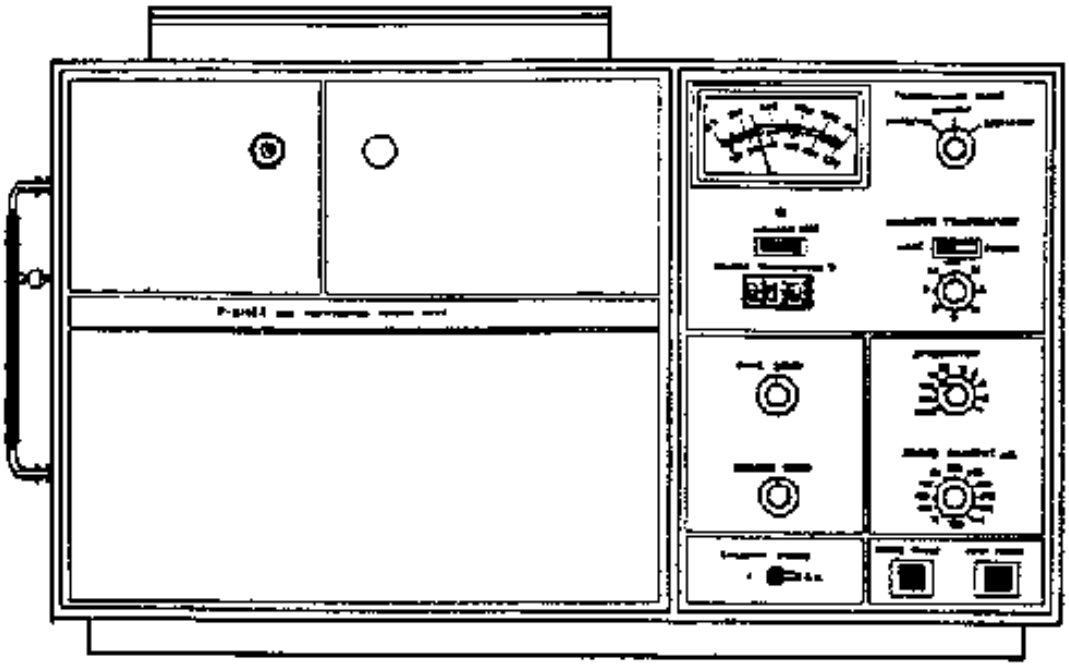


Figure 1. Fisher gas partitioner Model 1200

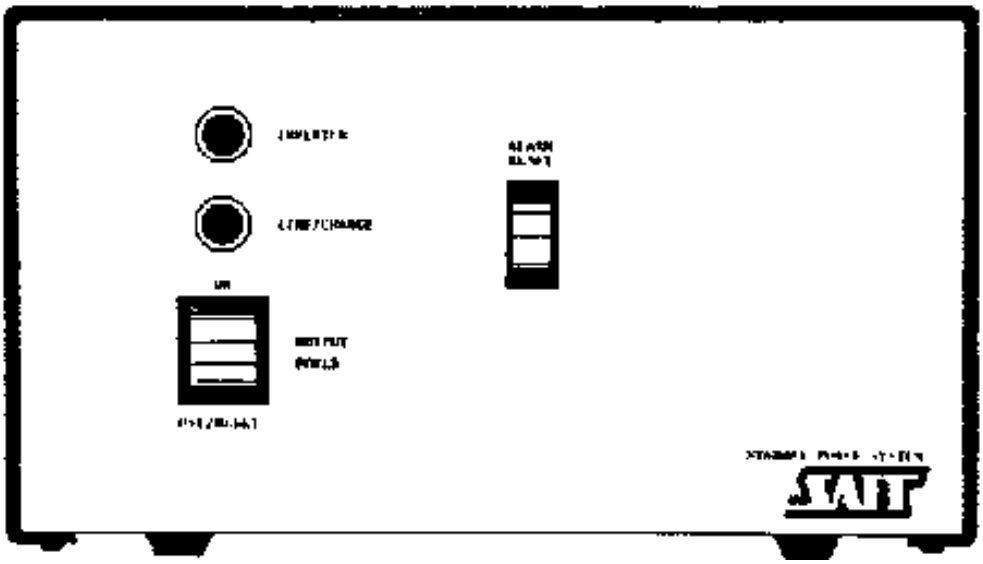


Figure 2. Standby power system, Model SPS 200-117

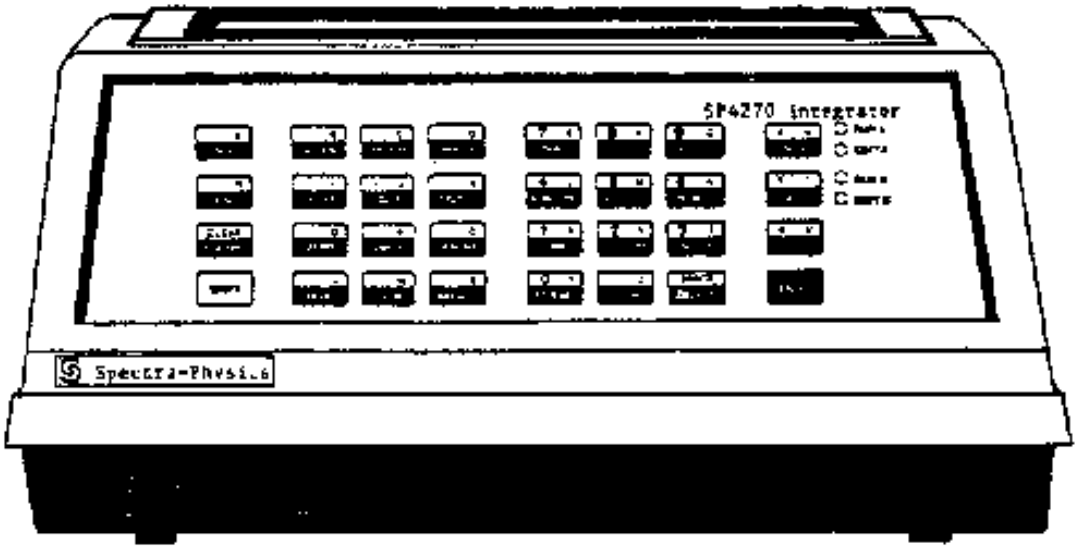


Figure 3. Spectra-physics integrator, Model SP4270

```
  READY
DATE * 05/06/85
TIME * 9:25                              (ENTER DIALOG)
FI= 1.      FE= 1.      MN= 0.
PRESS 'ENTER' TO SKIP ENTRY
FILE NAME=* CALC                         (ENTER NAME OF YOUR PROGRAM)
TIME        FUNCTION   VALUE
TT= .01     TF=* II    TV= 1
TT= .7      TF=* II    TV= 0
TT= 2.5     TF=* ER    TV= 1
TT=

METHOD NUMBER:MN= 5
IF NV=0 THEN NO CALIB
IF NV=1 THEN NORMAL CALIB
IF NV>1 THEN MULTI-LEVEL CALIB
NUMBER OF LEVELS:NV= 14
INJECTIONS/LEVEL:INJ/LEV= 1
TOTAL CALIB INJECTIONS,RC= 14.
LINEAR(1) OR NONLINEAR(2):LS= 2
COMPONENT TABLE...
RET TIME        NAME
RT= .55         CN=" COMP
RT= .98         CN=" CO2
RT= 1.90        CN=" H2
RT= 2.45        CN=" O2
RT= 2.92        CN=" H2
RT=
RRT REF PEAK:RP(I)=                      (PRESS ENTER)
MULTILEVEL CALIB VALUES:
RETENTION TIMES:
  RT( 1)=    0.55 ( 2)=    0.9  ( 3)=    1.9  ( 4)=    2.45 ( 5)=    2.9
2
CONCENTRATIONS:
  VA( 1)= .001  VA( 2)= 100   VA( 3)= .01   VA( 4)= .01   VA( 5)= .01
  VB( 1)= .001  VB( 2)= .01   VB( 3)= 100   VB( 4)= .01   VB( 5)= .01
  VC( 1)= .001  VC( 2)= .01   VC( 3)= .01   VC( 4)= 100   VC( 5)= .01
  VD( 1)= .001  VD( 2)= .01   VD( 3)= .01   VD( 4)= .01   VD( 5)= 100
  VE( 1)= .001  VE( 2)= 10    VE( 3)= 90    VE( 4)= .01   VE( 5)= .01
  VF( 1)= .001  VF( 2)= 25    VF( 3)= 75    VF( 4)= .01   VF( 5)= .01
  VG( 1)= .001  VG( 2)= 49.1  VG( 3)= 50.9  VG( 4)= .01   VG( 5)= .01
  VH( 1)= .001  VH( 2)= 75.3  VH( 3)= 24.7  VH( 4)= .01   VH( 5)= .01
  VI( 1)= .001  VI( 2)= 90.17 VI( 3)= 9.83  VI( 4)= .01   VI( 5)= .01
  VJ( 1)= .001  VJ( 2)= .01   VJ( 3)= .01   VJ( 4)= 10    VJ( 5)= 90
  VK( 1)= .001  VK( 2)= .01   VK( 3)= .01   VK( 4)= 25    VK( 5)= 75
  VL( 1)= .001  VL( 2)= .01   VL( 3)= .01   VL( 4)= 50    VL( 5)= 50
```

```
VG( 1)= .001  VG( 2)= 49.1  VG( 3)= 50.9  VG( 4)= .01   VG( 5)= .01
VH( 1)= .001  VH( 2)= 76.2  VH( 3)= 24.7  VH( 4)= .01   VH( 5)= .01
VI( 1)= .001  VI( 2)= 90.17 VI( 3)= 9.83  VI( 4)= .01   VI( 5)= .01
VJ( 1)= .001  VJ( 2)= .01   VJ( 3)= .01   VJ( 4)= 10    VJ( 5)= 90
VK( 1)= .001  VK( 2)= .01   VK( 3)= .01   VK( 4)= 25    VK( 5)= 75
VL( 1)= .001  VL( 2)= .01   VL( 3)= .01   VL( 4)= 50    VL( 5)= 50
VM( 1)= .001  VM( 2)= .01   VM( 3)= .01   VM( 4)= 75    VM( 5)= 25
VN( 1)= .001  VN( 2)= .01   VN( 3)= .01   VN( 4)= 90.13 VN( 5)= 9.87
SAMPLE TABLE...
ANALYST:AN=* WLL                    (ENTER ANALYST INITIALS)
INJECTIONS/SAMPLE:RA= 1
SAMPLES BETWEEN CALIB:CI= 0
CONC UNITS:CU=* %
SAM IDX   NAME          SAM AMT      SCALE FACTOR
S1=                                 (PRESS ENTER)
END OF DIALOG
TF=* PM TV= 1
```

C. Procedure for calibration injections

With gas partitioner on and integrator connected in the same fashion as strip chart recorder had been previously, make calibration injections with concentrations listed, where:

VA(l)/VN(1) = percentage composite
VA(2)/VN(2) = percentage CO_2
VA(3)/VNt 3) = percentage H_2
VA(4)/VN(4) = percentage 0_2
VA(5)/VN(5) = percentage N_2

- Detach flush line from Sample-In Port.
- Attach calibration gas onto Sample-In Port and inject 10 ml.
- Immediately push in plunger to inject gas and push INJ A button on integrator.
- Remove calibration gas and attach flush line.
- Pull out plunger to flush sample loop.
- After integrator has computed calibration (approximately 4 min) repeat these steps until all 14 calibrations have been performed.

At the end of these calibration injections the integrator will compute and print the coefficients of least squares fit to a quadratic equation. The calibration procedure of the instrument has now been completed and is ready for use.

By following the instructions below the analyst can enter his initials, the control gases to be used in conjunction with the sample, the sample No. (omitting the year prefix), and the subsample No. This will program the integrator to print the information so that the final printout is identified and ready for submission with the sample report.

Shown below is an example of the final printout ready for submission with the sample report.

Analyst Input	Computer Output	Analyst Input	Analyst Input	Analyst Input
Sample	Sample Table			
	Analyst: AN="	WLL		ENTER
	INJECTIONS/SAMPLE: RA=	1		ENTER
	SAMPLES BETWEEN CALIB:CI=	0		ENTER
	CONC. UNITS:CU="	%		ENTER
	SAM IDX			
	SI=	1		ENTER
	SN="		CO(SHIFT)2	ENTER
	SA=			ENTER
	XF=			ENTER
	SI=	2		ENTER
	SN="	SHIFT	(w/o 92) SAMPLE "-SUB"	ENTER
	SA=			ENTER
	XF=			ENTER
	SI=			ENTER
	END OF DIALOG			

D. Discussion | Accessibility/Help

Past experience has shown that the system, although fairly linear, is not linear enough. When the integrator is programmed to use a nonlinear program, it uses quadratic equations to determine nonlinear curves.

A series of standard concentrations was used to achieve a low, middle, and high concentration of each particular gas for greater accuracy. Fewer calibrations could possibly be carried out, but a loss of accuracy might result.

The analyst should be aware that the SP4270 uses a 3-function keypad. The lower section of each key initiates a system function, while the upper portion represents numeric/punctuation characters (upper left) and alpha characters (upper right) (**see** Figs. 3 and 4).

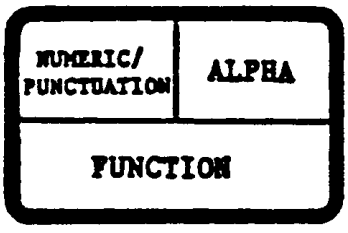


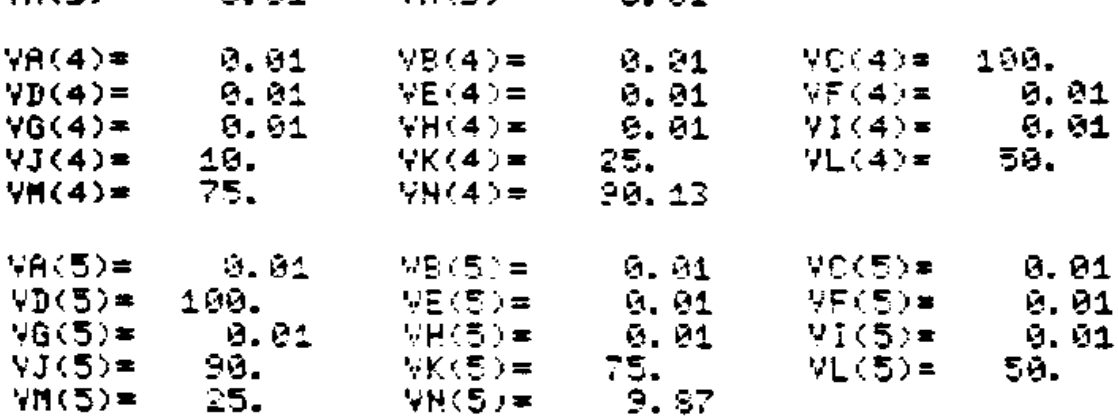
Figure 4. Three Function Key.

The SHIFT key is used to shift the keypad from one key section to the other. The EDIT lights -- located on the right-hand side of the keypad -- indicate which key section is currently being accessed for which channel.

This is important for entering information such as CO_2, which is a mixture of numeric and alpha characters, or the sample number, where the integrator is programmed to accept alpha characters but the numeric characters must be entered.

E. Operating conditions for integrator

```
LEVEL = 1007
MN=  5.        REM   FE=  1.        CH= "A"   PS=  1.

NM    = "CALC "

PW    = 6.           PT    = 12.          CA    = 0.
RN    = 20.          IX    = 1.           OD    = 1.
PH    = 0.           TB    = 0.           CZ    = 5.
LS    = 2.           NV    = 14.          SI    = 1.
SZ    = 2.           RC    = 14.          CI    = 0.
RA    = 1.           SP    = 1.

TT(1)=     0.01      TF(1)="II"       TV(1)=     1.
TT(2)=     0.7       TF(2)="II"       TV(2)=     0.
TT(3)=     3.5       TF(3)="ER"       TV(3)=     1.

RT(1)=     0.55      CN(1)= "COMP "

RT(2)=     0.93      CN(2)= "CO2  "

RT(3)=     1.8       CN(3)= "H2   "

RT(4)=     2.4       CN(4)= "O2   "

RT(5)=     2.91      CN(5)= "N2   "

VA(1)=     0.001     VB(1)=     0.001     VC(1)=     0.001
VD(1)=     0.001     VE(1)=     0.001     VF(1)=     0.001
VG(1)=     0.001     VH(1)=     0.001     VI(1)=     0.001
VJ(1)=     0.001     VK(1)=     0.001     VL(1)=     0.001
VM(1)=     0.001     VN(1)=     0.001

VA(2)=   100.        VB(2)=     0.01      VC(2)=     0.01
VD(2)=     0.01      VE(2)=    10.        VF(2)=    25.
VG(2)=    49.1       VH(2)=    75.3       VI(2)=    90.17
VJ(2)=     0.01      VK(2)=     0.01      VL(2)=     0.01
VM(2)=     0.01      VN(2)=     0.01

VA(3)=     0.01      VB(3)=   100.        VC(3)=     0.01
VD(3)=     0.01      VE(3)=    90.        VF(3)=    75.
VG(3)=    50.9       VH(3)=    24.7       VI(3)=     9.83
VJ(3)=     0.01      VK(3)=     0.01      VL(3)=     0.01
VM(3)=     0.01      VN(3)=     0.01

VA(4)=     0.01      VB(4)=     0.01      VC(4)=   100.
VD(4)=     0.01      VE(4)=     0.01      VF(4)=     0.01
VG(4)=     0.01      VH(4)=     0.01      VI(4)=     0.01
```



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*Authors: Warren L. Landry and Margarito J. Uribe

Top

Home | B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 22A

Examination of Containers for Integrity

I. Examination of Metal Containers for Integrity

Authors

(Return to Table of Contents)

The quality of a food container is determined by its ability to protect the product it contains from chemical deterioration or microbiological spoilage. Good double and side seams are essential to a good hermetic seal, particularly at the cross-over (juncture); however, the performance of a can is also affected by many other factors. Spoilage within the can may be caused by leakage, underprocessing, or elevated storage temperatures. Leaker spoilage occurs mainly from seam defects and mechanical damage. Improper pressure control during retorting and cooling operations may stress the seam, resulting in poor seam integrity and subsequent leaker spoilage. However, the rate of container defects that might result in food spoilage is usually low.

The suitability of the particular food to be preserved also affects the performance of the container. Hydrogen swells and sulfide stains caused by chemical corrosion sometimes occur. In addition, prolonged storage of cans at elevated temperatures promotes corrosion and may result in perforation. Improper retorting operations, such as rapid pressure changes, may cause can deformation and damage seam integrity. Postprocess contamination by nonchlorinated

cooling water or excessive buildup of bacteria in can-handling equipment may also cause spoilage, and abusive handling of containers may result in leaker spoilage.

Although the incidence of spoilage in canned foods is low, it is necessary to know how to proceed with the investigation of the integrity of the can when spoilage does occur. This chapter presents methods for seam examination and leakage detection.

Container examinations associated with food spoilage are usually accompanied by pH determination of the product, gas analysis of can headspace, and microbiological testing of the product (**see** Chapter 21). Analytical results that depart from normal patterns may indicate changes within the container and help to pinpoint the cause of spoilage.

The double seam (Fig. 1) consists of 5 thicknesses of plate (7 thicknesses at the juncture of end and side seam for 3-piece soldered cans, and 6.3-6.4 thicknesses at the juncture for welded cans) interlocked or folded and pressed firmly together, plus a thin layer of sealing compound. It is formed in 2 rolling operations. The side seam is bonded by welding or with solder or adhesive cement. Side seams of soldered cans consist of 4 thicknesses of metal body plate, except at the laps or cross-over areas, which have 2 thicknesses of metal. Side seams of welded and cemented cans have 2, or parts of 2, thicknesses of metal body plate (1.3-1.4 times the metal plate thickness for welded cans). [**NOTE**: the use of lead solder to close the seams of food cans is rapidly being prohibited in many nations.] Welded 3-piece cans permit reduction of side seam thickness and double seam thickness at the cross-over. Drawn cans eliminate the side seam and bottom end seam, resulting in fewer areas that affect can integrity. Can ends (Fig. 2) are punched from sheets; edges of the ends are curled, and a sealing compound is applied and dried in the lining channel (curl and flat areas) of the can end. Once the lined can end is double seamed onto a can body, the sealing compound in this compressive seal fills the voids (spaces) between the folds of metal in the properly made double seam to form an abuse-resistant hermetic seal.

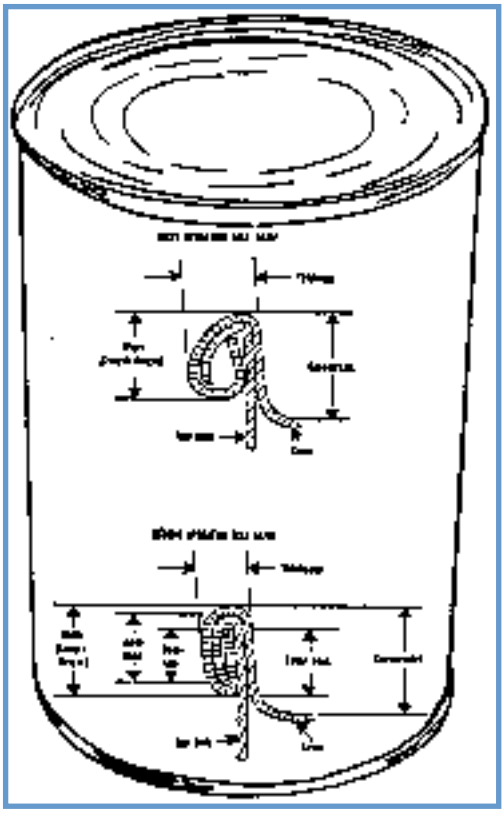

Figure 1. Double seam.

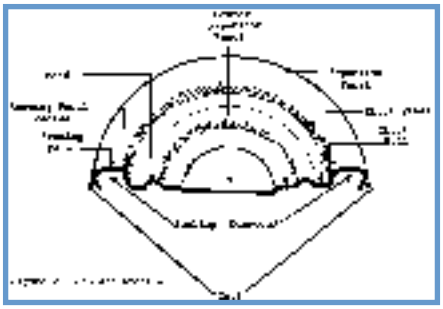

Figure 2. Can end profile.

A. Sampling and sample size

Sample size required for product analysis and can examination depends on the type of spoilage and complexity of the problem. When the cause of spoilage is clear, 4-6 cans may be enough. In more complex cases, it may be necessary to examine 50 or more cans. An adequate number of normal (flat) cans should be taken from the same case or lot for examination.

B. Preliminary examination

Remove labels, assign subnumbers, if necessary, and separate code numbers. Use same

coding or subnumber system for both product and container examinations. Before removing any product sample, perform complete external can examination, observing such defects as evidence of leakage, pinholes or rusting, dents, buckling, and general exterior conditions.

Classify each can as (a) flat, (b) flipper, (c) springer, (d) soft swell, or (e) hard swell according to criteria in Chapter 21, Table 1. Leakage tests and external double seam dimensions may not be valid when cans are buckled. However, these cans should be examined and then torn down and re-examined for seam defects that may have existed before buckling. If possible, set aside reserve cans representing the classifications noted. Refrigerate reserve cans to prevent bursting.

Examine cans classified as springers and soft and hard swells immediately. **Do not incubate**. Remove sample from uncoded end of can in a manner that will not disturb double seam, e.g., with bacteriological can opener (Fig. 3). If can end has been punctured as a result of gas sampling, bacteriological can opener may be used if puncture is in center of end. If puncture is not in center, remove end with a pair of metal cutters.

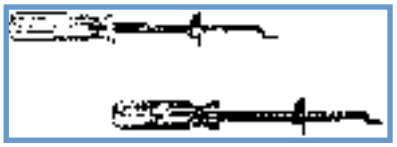

Figure 3. Bacteriological can opener.

C. Can examination

Note condition of cans (exterior and interior) and quality of seams. Observe and feel for gross abnormalities, mechanical defects, perforations, rust spots, and dents. Perform pressure and/or vacuum tests to detect invisible microleaks either in double seams or side seams. Measure seam dimensions and perform teardown examination. Note condition of double seam formation and construction (by micrometer, seam scope, or seam projector). Chemical, instrumental, metallographic, and other techniques may be required.

1. Visual examination

   Use hand as well as eye. A magnifying glass with proper illumination is helpful. Run thumb and forefinger around seam on inside (chuck wall) and outside of seam to locate any roughness, uneveness, or sharpness. Examine by sight and touch for the following defects that may result in can leakage (for definition of

terms, **see** glossary at end of this chapter):

- sharp seam
- code cut
- cutovers or cut-throughs
- false seam (although some false seams may not be detected by external examination)
- dents
- deadheads (incomplete seam)
- excessive droop
- jumpover
- excessive scuffing in chuck wall area
- knocked-down flange
- cable cuts on double seam
- excess solder

2. Microleak detection

The microleak tests are not listed in their order of sensitivity, nor is it necessary to use them all. Each has its advantages and disadvantages, depending on the particular set of conditions. In some instances a test may be chosen as a personal preference. They are all presented to provide the analyst with all the procedures and available options. Make all external measurements of can double seams before any microleak testing. **See** section on double seam measurements.

Figure 4. Vacuum leak test apparatus (NFDA).

a. National Food Processors Association (NFPA, formerly National Canners Association) Vacuum Leak Test (19) (Fig. 4).

This test applies a vacuum to the can, which more closely duplicates the condition in the can when it contains product and is sealed. Proponents of this method feel that using a vacuum to detect leakage in a can designed to hold a vacuum is more effective than using pressure. The use of a vacuum may remove food particles from leakage paths in can seams; pressure may force particles more deeply into leakage paths.

1. Materials

   Bacteriological can opener (Fig. 3) (Wilkens-Anderson Co., 4515 W. Division St., Chicago, IL 60651) or equivalent
   Plexiglas plate
   Plastic tubing
   Rubber gasket, to fit container being examined
   Vacuum source with gauge
   Nonfoaming wetting agent, e.g., Triton X-100 (R86)
   Outside light source, such as high-intensity lamp

2. Procedure

   Remove uncoded end of can with bacteriological can opener adjusted to cut out can end, leaving 1/4 inch border around outer edge. Empty and wash container with water and suitable detergent to remove food particles lodged in seam area. (Ultrasonic cleaner may be used to remove small food particles lodged in seam areas.) Add wetting agent plus water to depth of about 1 inch. Place Plexiglas plate with tubing attached and wetted rubber gasket on open end of container. Increase vacuum until gauge indicates vacuum of 15-25 inches. Swirl water in container to dissipate small bubbles produced by application of vacuum. Tilt container slowly to immerse all seam surfaces, letting light source focus through Plexiglas into can for better observation. Rotate tilted can so that all surfaces are observed and covered with water. Depending on size of hole, path of leakage, pressure differential, and surface tension of the test water, bubbles will appear smaller or larger and with lesser or greater frequency. Release vacuum by first closing main vacuum petcock and then opening intake petcock.

Figure 5. Mead Jar Test apparatus.

b. Mead jar test (1) (Fig. 5)

This nondestructive examination determines leakage paths of finished cans that have both ends double seamed. It is used primarily for vacuum- or nonvacuum-packed dried or semidried products. Deaerated water is preferred because, as vacuum is pulled on the jar, dissolved air in the water comes out of solution as bubbles, preventing a clear view of the container and any leakage bubbles. This test would rarely be used for items thermally processed in metal cans.

1. Materials

Mead jar (battery jar), glass or plastic
Mesh protector, to fit around Mead jar
Metal top with rubber gasket on bottom side and air inlet, vacuum inlet, and vacuum gauge on top side
Vacuum source
Rubber tubing
Deaerated water (prepare by applying 25-30 inches of vacuum on water for 8 h or overnight)
Vaseline or stopcock grease
Device or weight to hold can under water

2. Procedure

Place enough deaerated water into Mead jar to completely submerge test container. Put container in water and, if necessary, place device or weight on it to hold it under water. Place mesh protector around jar, and put Vaseline on rubber gasket on underside of top piece to act as sealant between gasket and lip of Mead jar. Place lid on top of jar and rotate it to improve seal. Attach rubber hose to vacuum line. Turn off air inlet stopcock and open vacuum line stopcock. Turn on vacuum and record vacuum reading on gauge if leakage or air bubbles emanate from container. Note location of leakage point. Turn up to full vacuum if no leakage is noted. Turn off vacuum and open air inlet valve to release vacuum. Take off lid, remove container, and mark leakage point.

CAUTION: Do not use defective or cracked Mead jars because of danger of collapsing them. Always put wire mesh protector in place before turning on vacuum. For a container to be classified as a leaker, a continuous stream of bubbles from a single point is necessary. Unless leakage is obvious, the can should be observed under test conditions for at least 30 s. Generally, a few bubbles will be seen when the vacuum is first applied because of air entrapment in the double seams. These should not be confused with leakage point bubbles.

Figure 6. Air pressure test apparatus.

c. Air pressure test (1) (Fig. 6)

For detection of container leakage caused by minute body pinholes and perforations, and/or defective side seams, air pressure testing is the most convenient and conclusive. It is also helpful for locating double seam leaks. During pressure testing, double seams may be distorted, producing false leakers or sealing off minute leakage paths. For this reason, the air pressure testing method should be used in conjunction with the fluorescein test or penetrant dye test to trace the actual leakage path through double seams.

1. Materials

   Single pocket (or multiple pocket) foot tester with attached air and water source
   Manually operated air pressure control valve and pressure gauge installed on air line
   Two gate valves installed in air line, one to pressurize and the other to exhaust the can during testing

2. Procedure

Preparation of samples. Open filled cans at one end with bacteriological can opener so that double seam remains intact. Manufacturer's end is usually opened, but if enough samples are available, half the cans may be opened at packer's end. After removing contents, wash cans thoroughly. Wash containers of fatty or oily products with warm detergent and water, or boil them in detergent and water; use ultrasonic cleaner to remove all fat or oil trapped in the double seam. Dry cans at least 8 h at 100-120°F before pressure testing.

Pressure test. Fill foot tester with warm water (100-120°F) to about 2 inches from top. Set air pressure control valve at desired pressure level (20 lb for most sanitary cans). Seat open end of test can against rubber base plate, side seam up, and lower pressure bar against can with just enough pressure to hold can in place. Secure pressure bar in this position with 2 nuts so that adjustment is not necessary for testing additional cans of same size. Close exhaust gate valve and completely immerse can in tank by stepping on foot pedal. With can completely immersed, open pressurized gate valve, letting air flow into can. Hold can in this position long enough to detect any leakage points.

Leakage may be shown by steady stream of large bubbles or continued intermittent escape of very tiny bubbles. If leakage is present, close pressurized gate valve and exhaust can by opening exhaust gate valve. Release foot pressure and lift can out of water. Rotate can 180 against rubber base plate until new area of can is exposed, and repeat pressure testing operation. Mark location of any leaks noted during test for use as reference when cans are examined further. Do not exceed 30 psi because can may burst beyond this level.

After pressure testing, use fluorescein test to obtain additional information on presence of any leakage paths, or strip can seams for further examination if point of leakage is conclusively located.

3. General comments

During pressure testing operation, pay particular attention to cross-over areas and double seams on both ends of can. Also observe side seam area closely and scan bodies for pinholes or perforations. Use warm water during test. During drying, small leakage paths will be

opened; these may be reclosed by contraction of the can during testing in cold water. (**NOTE**: The air pressure test must not be rushed. Very small leaks may take several seconds to show up, and then the only evidence may be the intermittent flow of very tiny bubbles.

d. Fluorescein dye test (1)

Fluorescein dye testing has been used for many years to detect minute double seam, lap, and side seam leakage paths in all types of containers. The fluorescein test is especially useful for examining sanitary-style containers that are normally packed with some initial vacuum. Fluorescein dye can often be used to detect minute leakage paths on suspected cans that do not leak under the air pressure test. Fluorescein testing of most types of containers under vacuum simulates actual packed condition, i.e., with ends pulled inward.

1. Materials

Vacuum line, with bleed-valve attachment for regulating amount of vacuum to be used
Rubber-faced plate, for attaching containers to vacuum line
Ultraviolet (UV) light (Black Light Lamp, Fig. 7)

Figure 7. Ultraviolet light for fluorescein dye test.

Fluorescein dye solution. Mix 100 ml triethylene glycol, 300 ml water, 15 g glycerine, 3 g wetting agent, such as Triton X-100 (R86), and 3 g sodium fluorescein, technical grade. (Zyglo dye solution ZL-4B is available from Magnaflux Corporation, Chicago, IL.)

2. Procedure

Preparation of sample. Open filled cans at one end with bacteriological can opener so that double seam remains intact. Manufacturer's end is usually opened, but if enough samples are available, half the containers may be opened at packer's end. After removing contents, wash and dry containers thoroughly. Wash containers of fatty or oily products with hot detergent and water, or boil them in detergent and water to remove all fat or oil trapped in double seams. Use ultrasonic bath to remove small food particles lodged in seam areas.

Vacuum application (Fig. 8). Place opened end of emptied and dried container against vertically mounted rubber-faced plate connected to vacuum line. As a general rule, 15-20 inches of vacuum is used for most pressure-processed sanitary-style containers. For other container styles, maximum allowable vacuum to be used depends on panel resistance of bodies.

After container is mounted in place and vacuum drawn, apply fluorescein solution with a small brush or eyedropper to outside double seam and side seam areas. **OR** place can in dye bath to cover seam and/or score areas to be examined. Run test for 30 min to 2 h, depending on style of container being examined. Longer test period may be required to detect microleaks. If in doubt as to time needed to demonstrate leakage, remove containers every 30 min and check inside with UV light for presence of fluorescein. Since fluorescein solution runs off the mounted container, apply fresh solution at 15 min intervals.

Container examination. At end of vacuum test period, thoroughly remove fluorescein remaining on outside of containers with water; then wipe dry. Take special care not to splash any fluorescein into opened end of container. After cleaning excess solution from outside, strip the double seam and examine it under UV light to detect any fluorescein on the inside. Do not allow wet solution trapped in outside of double seam to creep inside container, thus giving false-positive results. Also, keep tools clean of fluorescein to prevent contamination during stripping. Examine containers immediately after vacuum testing, since dried fluorescein solution does not fluoresce (Fig. 8).

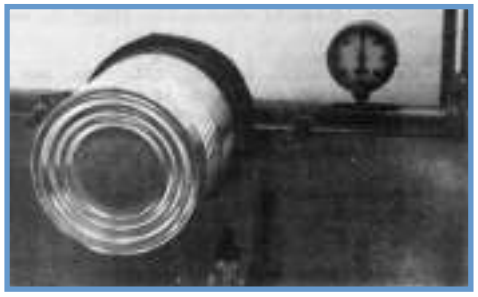

Figure 8. Application of vacuum for fluorescein dye test.

3. Double seam measurement (1,2)

   NOTE: Sample report forms for double seam examination (Chart 1) and for the complete container integrity examination (Chart 2) are included at the end of this chapter.

   a. Measure seam width (length, height), thickness, and countersink on both can ends before can is opened, if possible. Seam width and thickness may be measured after opening if bacteriological can opener is used. However, take care to prevent distortion of area around seaming panel. Countersink cannot be measured after end is removed. Perform teardown examination and record results for seam integrity evaluation. Use seam scope, projector technique, or micrometer measurement (or all 3 systems) for verification, to determine adequacy of double seam formation.

   b. Teardown and cross-section strip examinations

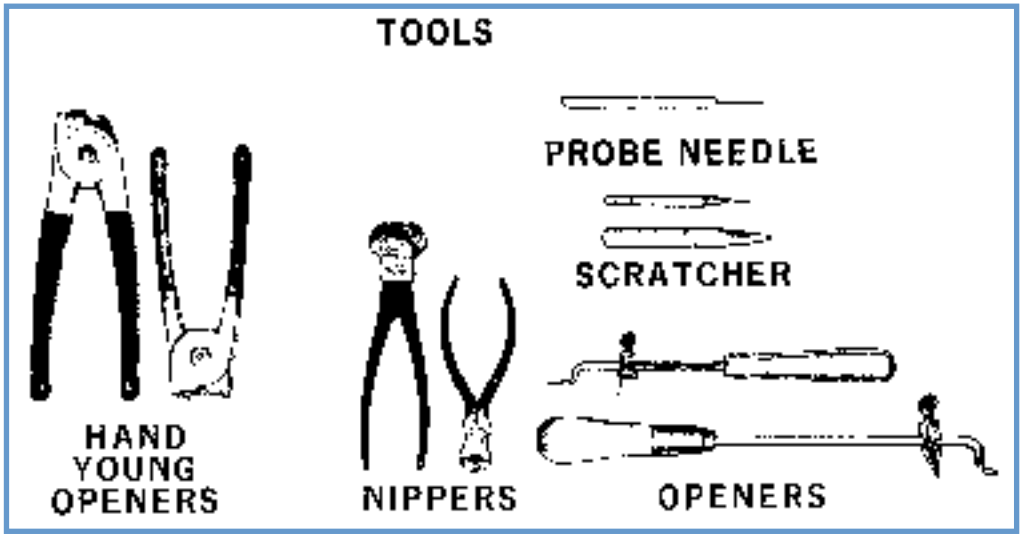

Figure 9. Materials for teardown and cross-section strip examination.

1. Materials (stripping tools, Fig. 9)

2. Procedure. **NOTE**: The seam projector examination is easier to perform before the cover hook is removed and after the seam has been cut. If uncoded end is still on can, remove with bacteriological can opener. Empty contents from can. Wash, dry, and test can for leakage, as previously described. After leakage test, cut 2 strips about 3/8 inch wide through double seam and into body of can about 1-1/4 inches, leaving bottom of strips attached to can. One strip should be at least 1/2 inch counterclockwise from juncture; the other should be 180 from lap or side seam. This will leave an adequate length of cover hook for thorough examination. Remove remaining end metal, beginning at cutout strip closest to side seam, moving clockwise until entire cover hook is removed (Figs. 10 and 11). If rocker-like motion does not work, pull out and away (Fig. 11). Take care to prevent any injury or cut. Partially remove coded end with bacteriological can opener, leaving it attached about 90 counterclockwise from side seam so that the can may be identified after stripping. Then cut strips into coded end of can and tear it down, using procedures previously described.

Figure 10. Removal of cover hook.

Figure 11. Removal of cover hook (alternative procedure).

Strip off cover hook to point where remaining end metal has not been removed (about 1/2 inch from second metal strip). Measure body and cover hooks. Grade cover hook for tightness by examining and evaluating wrinkles. Examine cross-over area of 3-piece soldered cans for wrinkles and for crawled laps (body hook of one side of cross-over drops down or crawls below bottom of other side) in soldered cans.

Observe double seams for plate fractures. Inspect interior of body wall in double seam area for well-defined continuous impression around circumference of can. This is referred to as pressure ridge and is one indication of seam tightness, although pressure ridge may or may not be present in a good seam. Tape cover hooks and coded end to can so that they may be identified as belonging to the can in question. If cover hooks are still attached, bend them inward inside the can body to prevent cut injury.

3. General comment

As mentioned previously, measure countersinks while ends of can are still intact. On swelled cans these measurement are seldom meaningful because of possible distention caused by swelling. However, measurements that indicate deep countersinks are useful because they represent the condition as is.

4. Side seam examination

NOTE: Food products are packed in 2 basic types of cans: the 2- and 3-piece cans. The 2-piece can has a drawn body, no side seam, and only one double seamed end, thus the term 2-piece. Side seam and lap examination are not

applicable to these cans. Three-piece can bodies are made from a flat plate, which is rounded and seamed to form a cylinder. These cans have 2 double seam ends, thus the term 3-piece. The side seam is sealed by solder, adhesive, or welding, and is in addition to the double seam, which is susceptible to leakage.

a. Soldered side seam examination

1. Materials (stripping tools and side seam breaker)

2. Procedure

   Break open and observe both laps. Note solder voids and channels, and discolored and stained solder. Identify hot and cold breaks in solder. Observe flange area of the lap closely for defects. Examine side seam construction, pulling apart solder bond with side seam breaker (Fig. 12) as follows. Place can over body horse of appropriate can diameter and pull down on breaker arm to expand can and open solder bond. Observe side seam for solder voids, channels, and discolored areas.

   Figure 12. Side seam breaker.

   Examine laps for heavy solder, evidenced by excessive solder adjacent to lap inside and outside can, or on body hook, causing severely crawled lap or cutover. Break open lap to see if excessive solder is still present in lap.

3. General comments

   The portion of the cover hook that intersects the side seam is called the juncture area and is about 3/8 inch wide. In an ideally made 3-piece can, length of cover hook is not reduced at the juncture, even though cover hook is depressed at this point. In most commercial 3-piece cans, length of the cover hook is reduced at the side seam in

varying degrees. The shortening of the cover hook at the side seam is caused by the 2 additional thicknesses of body flange at the side seam. These additional layers of metal prevent the cover hook from being tucked up under the body hook as it is under the body hook away from the side seam.

A good juncture with sufficiently long cover and body hooks is essential. Without a good juncture, the overlap at the side seam may be critically short, making the can less abuse-resistant. As with the tightness rating, which is based on the wrinkle-free metal of the hook length, the juncture rating is based on the amount of droop-free metal for the existing hook length away from the juncture. Because the cover hook is frequently shorter at the juncture in the form of a droop, the juncture is rated on a percentage scale, starting at 100% (ideal) and decreasing in 25% increments. If the cover hook in the juncture is even with the rest of the cover hook, it is rated 100%. If the cover hook reduction at the juncture is about 1/4 the length of the cover hook, the juncture is rated 75%. If the cover hook at the juncture is 1/2 the length of the rest of the cover hook, the juncture is rated 50%. Similarly, if the cover hook length at the juncture is only 1/4 the length of the rest of the cover hook, it is rated 25%.

A severe condition of crawled laps results in an area of little overlap at the cross-over, creating a possible leakage point. This condition is usually, but not always, accompanied by an external droop at the cross-over. Cover hooks made with double cold reduced (2 CR) ends may look wrinkled, but these wrinkles may be reverse wrinkles, which do not indicate a loose seam. Compound wrinkles do not give wavy cut edges to the cover hook and are not used to establish tightness ratings. Discolored or stained solder may indicate previous leakage through the lap. It is not necessary to have a solderless channel for leakage to occur at the lap. This can happen as a result of either a hot break or a cold break in the solder. Both of these conditions are conducive to container leakage. A hot break is identified by solder that appears relatively smooth; it occurs in the soldering operation during manufacture when the lap opens up before the solder solidifies completely. A cold break gives the solder a round, mottled appearance; if leakage has occurred at this point, the area shows a dark discoloration. Cold breaks may occur at almost any time after the solder has hardened. They are difficult for manufacturing plants to detect because they

usually occur after manufacturing. Cold breaks are usually caused by weak soldering bonds or poor manufacturing techniques.

In general, if pressure testing shows leakage at the cross-over, if the fluorescein test indicates a path through the flange area, and if no other double seam or lap defects are found at the cross-over, the most likely cause of leakage is a solder break at the flange area of the lap. It is also possible to have no solder at this area. This defect is easier to detect visually than the solder break. The leakage path may be confirmed by a fluorescein path or by stained or darkened solder. A condition known as an "island" is often observed in the side seam. An island is an isolated area in the side seam fold that is void of solder, but without a connecting solderless path, or break, leading to the outside of the can. This condition is not necessarily associated with leakage, but does indicate a weak side seam.

b. Welded side seam examination

1. Materials (stripping tools and side seam breaker)

2. Procedure

Examine the flange area for flange cracks, the weld for blow holes or weld splashes, and the lap area for fishtail. **NOTE**: A fishtail is a piece of metal extending beyond the flange at the lap area that might cause double seam difficulty. Any of the above defects in the weld may result in leakage. However, this should be supported by a valid leak test.

c. Micrometer measuring system [21 CFR 113.60(a)(1)]

1. Materials

Use a micrometer especially made for measuring double seams and reading to nearest 0.001 inch. Be sure to adjust micrometer properly. When micrometer is set at zero position, zero graduation on movable barrel should match exactly with index line on stationary member. If zero adjustment is more than 1/2 space from index line at this setting, adjust it.

2. Procedure

Make seam measurements on round cans, at minimum of 3 points about 120 apart, around circumference of can, beginning about an inch to one side of cross-over (or at least 1/2 inch away from cross-over).

Obtain the 5 required measurements: seam thickness; seam width (length, height); body hook length; cover hook length; and tightness (observation for wrinkle).

The 2 optional measurements are countersink depth and overlap, calculated by the following formula:

$$\text{Overlap} = CH + BH + T - W$$

where CH is cover hook length, BH is body hook length, T is cover plate thickness, and W is seam width (height, length). Grade tightness (wrinkle) (2) of double seam by examining cover hook wrinkle according to percentages illustrated in Figs. 13 and 14. Drawing shows cover hook with 0-100% tightness, with wrinkle number shown below it. Tightness can also be indicated by flatness of cover hook; that is, cover hook should not appear round. Make this observation on cover hook removed from seam that has been sectioned with seam saw (Fig. 15). This method is good verification for wrinkle method but should not be a substitute for it. Tightness may be expressed in terms of percentage of cover hook not included in wrinkle or by rating number equivalent to distance up cover hook. Both procedures are listed below. Percentage method is preferred.

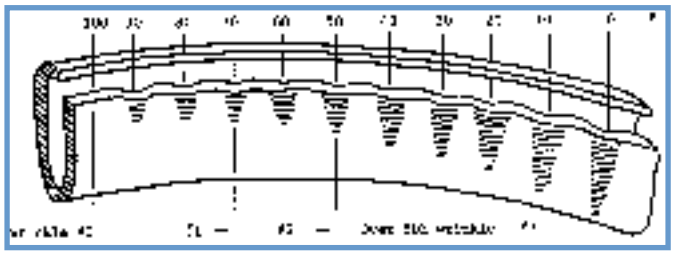

Figure 13. Tightness (wrinkle) rating in percent.

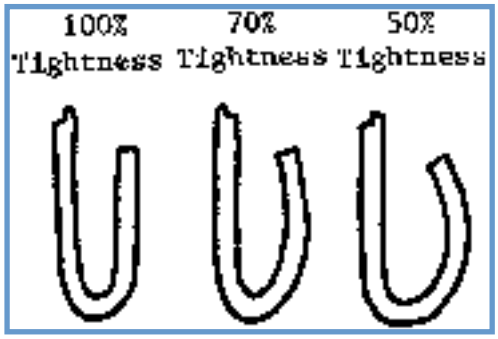

Figure 14. Evaluation of tightness by flatness of the cover hook.

Figure 15. Waco Saw for sectioning, to left, and seam projector for examining cross sections of double seams, to right in photograph.

Tightness, expressed in terms of wrinkles: No. 0 - Smooth, no wrinkle No. 1 - Wrinkle up to 1/3 distance from edge

No. 2 - Wrinkle up to 1/2 distance from edge No. 3 - Wrinkle up to more than 1/2 distance from edge

Tightness ratings expressed in % of cover hook not included in the wrinkle (preferred method):

100	-	Equivalent to No. 0
90	-	Equivalent to between Nos. 0 and 1
70	-	Equivalent to No. 1
50	-	Equivalent to No. 2
<50	-	Equivalent to No. 3

Rate the juncture as previously described. Measure free space to determine seam condition of 2-piece oblong and 2-piece oval cans, as follows:

$$FS = ST - (2BPT + 3CPT)$$

where ST is seam thickness, BPT is body plate thickness, and CPT is cover plate thickness. **NOTE**: Specifications are not yet well established.

d. Seam projector measuring system [21 CFR 113.60(a)(1)]

As an alternative to the use of the micrometer, or as a verification, cross-sections of double seams may be examined visually with a seam projector. A section of the double seam is cut in the form of a metal strip that remains attached to the can body and that is then placed in the projector. From the image projected on the screen, the seam width, hook lengths, and overlap dimensions may be measured with a specially calibrated caliper. General seam formation and, in some instances, seam tightness may be observed. The seam projector method facilitates examination of the critical overlap area at the cross-over; this is especially valuable for examining 3-piece soldered No. 10 cans, which are particularly vulnerable to leakage at this point.

1. Materials

Seam projector (Wilkens-Anderson) (Fig. 15)
Waco saw (Wilkens-Anderson) (Fig. 15)
Micrometer

2. Procedure

Obtain the 4 required measurements: body hook length, overlap, seam thickness, and tightness (observation for wrinkle). The 3 optional measurements are width (length, height), cover hook length, and countersink depth.

Measure each double seam characteristic at 2 different locations on each double seam, excluding the cross-over. Cut cross-sections through double seams with Waco seam saw. Polish cross-section

surface with fine emery cloth to ensure bright surface that will project clear image on screen. Place polished section in clamp on side of projector, look into shadow box, and observe image. Bring calipers in instrument into position. Note any looseness, tightness, or other malformations. With calibrated calipers, carefully measure and record width, cover hook, body hook, and overlap on image. Repeat this procedure in all 4 different locations along double seam. To properly evaluate seam for degree of looseness, strip cover hook from can, and visually grade for wrinkle formation. Observe absence or discontinuities of sealing compound after cover hook is removed from double seam. Enter observations on Chart 2 (shown at end of this chapter). Sealing compound should form complete 360 circle around edge of lid.

Overlap percentage is a measure of both how well end hook and body hook overlap and how well hooks match each other in length. It is also a ratio of the existing distance between body hook and cover hook compared to the distance the hooks would lap for the given seam. Overlap percentage is measured directly when seam projector is used with nomograph placed on viewing screen (Fig. 16). Calculate percentage from seam length, body hook, cover hook, and body and end plate thicknesses.

$$\text{Overlap, \%} = 100 \times (BH + CH + EPT - W)/[W - (2EPT + BPT)]$$

where BH is body hook length, CH is cover hook length, EPT is end plate thickness, W is width (seam height, length), and BPT is body plate thickness. Use minimum value found for each measurement (maximum value for W), to approximate lowest possible overlap percentage. Overlap may also be measured in thousandth inches or millimeters with calibrated calipers, as above.

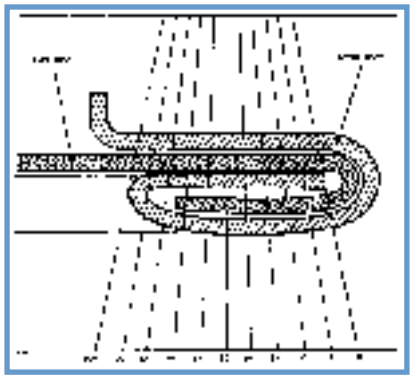

Figure 16. Nomograph for use with seam projector.

Open calipers as wide as possible and place nomograph card on screen. Position nomograph card so that image appears on it and reference lines of nomograph are parallel to hook images. Adjust position of nomograph to place zero line on side of body hook radius of image; then move it forward or backward until the 100 line is on inside of end hook radius. Now, move nomograph, keeping reference lines parallel to hooks and allowing no forward or backward motion, until zero line is at end of end hook; read nomograph at end of body hook. This value is percent overlap. Rate the juncture as previously described.

D. Special tests on rectangular and oval cans (William R. Cole, Division of Hazard Analysis Critical Control Point (HACCP) Programs, FDA)

Sardines are packed predominantly in a shallow rectangular (or oblong) 405x301x014 2-piece (drawn) aluminum can, with a scored, pull-tab type lid (known as "quarter-pound" can; Fig. 17). A larger volume of fish is packed into an oval 607x403x108 2-piece (drawn) aluminum can (known as "pound oval"; Fig. 18). A 405 tin-plated steel can and a 405 drawn aluminum can, both without the scored pull-tab top, are also used. The longer western sardine (pilchard) is packed in oval and round cans in 1/4 and l-lb units. Examination of imported sardines for container integrity is much like that for round sanitary cans. This section covers areas that are evaluated differently and specific problems inherent in these cans.

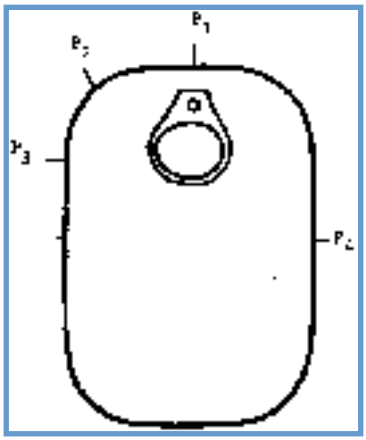

Figure 17. Measurement point template. Double seam examination of a "quarter-pound" aluminum sardine can.
P_1 -- Locus for seam cut and/or micrometer measuring point
P_2 -- Cover hook (and body wall) section number, pertinent to examination for wrinkle and pressure ridge

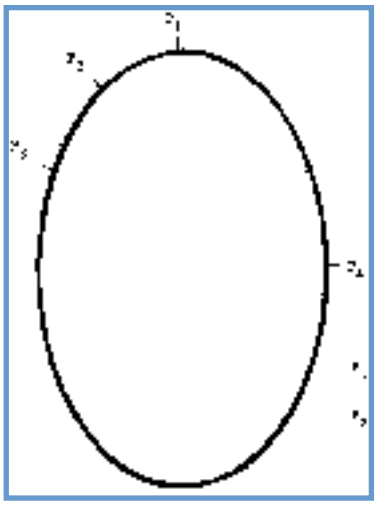

Figure 18.

Measurement point template. Double seam examination of a "one-pound oval" aluminum sardine can.
P_1 -- Locus for seam cut and/or micrometer measuring point
P_2 -- Cover hook (and body wall) section number, pertinent to examination for wrinkle and pressure ridge

A major integrity problem with these cans is the formation of the so-called cover hook "droop," usually at or near one of the 4 corners of the can, with resulting short overlaps at the droop area. An additional factor is the degree of wrinkle at the corners. If this wrinkle is used to rate cover hook tightness, it often indicates a tightness rating below specifications established by most domestic can manufacturers. However, the remainder of the cover hook away from the corners will, at the same time, appear to have an acceptable tightness rating. The following procedures have been developed for the examination of the 405 and 607 drawn aluminum sardine cans.

1. Visual examination

   Visual examination of cans is applicable for 405 and 607 drawn aluminum sardine cans. Be alert to such conditions as minute leakage of packing medium around scored area of lid, as well as base or attachment point of pull tab, on the 405 can. The primary gross closure defect associated with 405 containers has been cover hook droop, usually at or near one of the 4 corners. One cause of

droop is product overhanging the can flange before seaming. Visually examine the area surrounding the droop for evidence of product trapped in the seam.

2. Microleak detection

National Food Processors Association (NFPA) Vacuum Leak Test (19). Can examination (microleak detection, NFPA vacuum leak test), above, is also applicable for 405 and 607 sardine cans.

Fluorescein dye test. Use bacteriological cutter to remove portion of no-lid end. Check lids, score, and rivet area for evidence of leakage.

3. Double seam measurements

    a. General comments

    405x301x014 (quarter-pound) oblong can. The location for micrometer and/or seam projector measurement (i.e., standard for the round sanitary can) may be sufficient to adequately interpret the integrity of the 405 drawn aluminum sardine can. Figure 17 illustrates the template that was constructed as a guide for double seam examination of the 405 can. Four points, P_1 to P_4, were selected as locations for seam (projector and/or micrometer) measurements. The P_2 location is stripped and examined for wrinkle and presence of pressure ridge. Length, thickness, body hook, and cover hook are generally measured at P_3 location, and the data are recorded. With respect to examination for wrinkle and pressure ridge, firms that made seam scope measurements would either strip sections of the cover hook from between the measurement points or completely strip another can pulled along with the first.

    607x403x108 (l-lb) oval can. Figure 18 illustrates template constructed as industry guide for double seam examination of the 607 can. Four points, P_1 to P_4, were selected as locations for seam micrometer and/or seam projector measurements. As with Fig. 17, P_2 represents cover hook and body wall sections that are to be stripped and examined for tightness and presence of pressure ridge.

    b. Data sheet for double seam examination of aluminum sardine cans (607x403x108 and 405x301x014)

    As specific points on both the 405 and 607 containers were indicated as

potential problem areas, with respect to droop, overlap, and tightness, the 5 recommended points for measurement may be entered under the last column of Chart 1 to allow for easier identification of these points. However, the selection of the P_1 location on both cans was designed to provide a general reference point (the 301 side closest to the pull-tab on the 405 can; the point on the 607 can closest to the embossed code) similar to the side seam on a 3-piece container. P_3 and its 3 corresponding points along the straight section of the double seam on the 405 can generally reflect the measurement points used by industry. P_2 and 3 other corner points and adjacent areas have indicated potential weakness with respect to droop, low overlap, and low tightness. Therefore, the data sheet shown in Chart 2 allows immediate comparison of data obtained on 2 sets of points. If the visual and microleak examinations indicate one particular weak area of the seam, double seam teardown could be performed in that particular area.

With a bacteriological can opener (Fig. 3), remove circular disk, about 1 to 1-1/2 inches in diameter, from center of bottom panel of container. Then, with metal cutters or tin snips, remove most of remainder of bottom, leaving about 1/2 inch border around outer edge of container. Empty and wash container with detergent and warm water; use a brush if necessary. Next, boil container in detergent and water to remove as much as possible of any product that may be trapped in the seam. Or place container in ultrasonic washer with detergent and wash at about 100-120°F for at least 2 h. In either case, dry the container in an oven at about 100-120°F for at least 2 h.

Add distilled water plus wetting agent to a depth that just covers entire lid area. Place Plexiglas with tubing attached and wetted rubber gasket on open bottom of container. Begin with initial vacuum of 5 inches, increasing gradually to maximum of 20-22 inches. Swirl water in container to dissipate small bubbles produced by application of vacuum. Tilt container slowly to immerse all seam surfaces and scored area of the 405 container, letting light source focus through Plexiglas into can.

E. Other methods under development

1. **Laser holographic method**. This nondestructive optical technique uses an electronic processing system for leak detection of hermetically sealed containers. Wagner et al. (21) studied the method and used it successfully to determine seal integrity of implanted cardiac pacemakers.

Hermetically sealed food cans are tested in a chamber (Fig. 19) under either vacuum or pressure, by applying a predetermined amount of stress to cans. The can surface is viewed for fringes as the can ends deform in response to the applied stress. A hologram showing the image of fringes is recorded by a reflected laser beam of a subject illuminated by a portion of the laser beam. A hologram of cans within the test chamber is recorded on video tape and may also be exposed and developed in place with a liquid gate film holder. The pattern of fringes that occurs on the can surface indicates the relative size of the leak. To locate the leak, the stress applied to cans in the testing chamber is slowly varied, the seam area is photographically enlarged, and fringe control techniques are applied. Figure 20 is a Polaroid picture taken from a TV video monitor. The can on the left, which does not show fringes in the hologram, is the leaker.

Figure 19. Test chamber.

Figure 20. Hologram showing the image of fringes.

Equipment for leak detection includes:

Helium-neon laser, 5 milliwatts or 5 watts argon laser
Test chamber
Recording and display system
Optical components

2. **Helium leak test** (U.S. Rhea and J.E. Gilchrist, FDA). (NOTE: This test is AOAC Final Action method 984.36, *Official Methods of Analysis*, 15th ed., 1990)

Flat cans are exposed to helium pressure in an enclosed tank for a specified

period of time. Cans previously exposed to pressurized helium are observed for evidence of leakage: a swollen can is an indication of a leaker; a paneled can or a can with vacuum is usually not a leaker. Headspace gas is analyzed by gas chromatography to confirm the presence of helium. Cans with large amounts of internal air (e.g., dried or semidried products) are crushed by helium pressure and should not be tested by this procedure.

A swollen can is first depressurized, and, if necessary, a sample of the contents is removed for microbiological analysis. After resealing, the can is tested for a leak. Swollen cans may be sampled for headspace gas analysis by gas chromatography. The helium leak test detects holes as small as 1 m. Results are reported as percent helium in the headspace gas.

a. Equipment and materials

Figure 21. Gas chromatograph for use in helium leak test.

Gas chromatograph (Fig. 21) capable of separating and measuring nitrogen, oxygen, hydrogen, helium, and carbon dioxide (Chapter 21)
Strip chart recorder or other readout system
Puncturing press (Chapter 21)
Helium exposure tank tested to 100 psi (American Society of Mechanical Engineers paint tank, 10 gal) equipped with inlet and outlet micro control valves
Pressurized helium tank with 2-stage regulator
Pressure regulator
Combination vacuum-pressure gauge for use on puncturing press Timer and solenoid to automate release of helium from exposure tank
Helium gas standards
Cyanoacrylate glue
Can opener, bacteriological (Fig. 3)
Rubber disks, 2-3/8 x 1/8 inch

b. Procedure

Calibration of gas chromatograph. For gas chromatographs equipped with side port loop (0.5 ml), inject 5.0 ml calibrated helium standards (suggested range 5, 15, 25, 50, and 75% helium). For instruments not equipped with side port loop, inject appropriate volume of standards. Use same volume for analysis of headspace gas samples. Plot percent helium vs helium peak height at attenuation used. Depending on quality of instrument, plot should approximate a linear or continuous curve.

Figure 22. Helium exposure tank.

Helium exposure tank (Fig. 22). Control the rate of introduction of helium into exposure tank and time that cans are exposed to helium pressure at 45 psi. Timer, solenoid, and microvalves with Vernier scales can facilitate procedure. Connect helium source to exposure tank. Turn timer on to close outlet solenoid valve. Adjust inlet and outlet microvalves to settings of 0.25 and 0.5, respectively, on Vernier scale. At these settings, it should take about 20 min to reach 45 psi in tank. Make minor adjustments if necessary. Adjust timer to expose cans to helium pressure at 45 psi for 30 min, in addition to time necessary to reach 45 psi.

Figure 23. Sealing an open can.

Sealing an open can (Fig. 23). A swollen can must be depressurized and resealed before exposure to helium. Release pressure by puncturing and, if necessary, cut hole 1.5 inches in diameter to remove sample for

microbiological analysis as described in Chapter 21. Depress any high edges and roughen area around hole with emery cloth. Pool glue (cyanoacrylate) around hole and press rubber disk 2-3/8 inches in diameter (1/8 inch thick) into glue. Take care to remove any air bubbles. Place weight (400 ml beaker filled with water) on disk for at least 1 h to obtain effective seal before exposure to helium.

Collection and analysis of headspace gas. After exposure to helium, make visual observation of cans (Chapter 21, Table 1). Can piercing assembly is shown in Fig. 24. Before piercing can, close gauge valve and pull plunger on syringe to remove air from silicone tubing. Close syringe valve and expel air from syringe. Puncture can and open gauge valve to read vacuum or pressure. Turn gauge valve and syringe valve to release gas into syringe. If gas sample is >5.0 ml, withdraw only 5.0 ml and inject into side port of gas chromatograph. If gas sample is <5.0 ml, force collected gas back into can. Close syringe valve to retain gas in tubing and can. Use syringe to add 40 ml room air to can, and pump syringe twice to mix gas. Let syringe equilibrate to atmospheric pressure and record syringe volume. From this dilute gas, obtain sample for gas chromatograph. Divide percent helium measured by dilution factor to determine correct percent helium in headspace gas. Use the following formula:

Figure 24. Can piercer and gas collection apparatus.

Dilution factor = (equilibrated syringe volume - 40 ml air + headspace volume)/(equilibrated syringe volume + headspace volume)

Example: (43 - 40 + 9)/(43 + 9) = 12/52 = 0.23 dilution factor % helium in can = % helium measured/dilution factor

Example: 5% helium/0.23 = 22% helium in can

Measure headspace volume by piercing a control can that still has vacuum. Measure both amount of vacuum (inches Hg) and volume of air pulled in from syringe.

Headspace volume = measured volume from syringe x 30 inches of mercury/measured vacuum in can in inches of mercury

Example: If 6 ml air was pulled into can and vacuum was 20 inches of mercury:

Headspace volume = 6 ml x 30 inches Hg/20 inches Hg = 9 ml

For performing additional work on the can, the collected gas may be stored in a capped syringe for a few hours without appreciable change in its composition.

Interpretation of results. Report can as leaker if, after exposure to pressurized helium, can internal pressure is 8 psi or helium is 1%. Report can as nonleaker if, after exposure to pressurized helium, can internal vacuum is 5 inches, or helium is <1%.

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*Authors: Rong C. Lin, Paul H. King, and Melvin R. Johnston

Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

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Chapter 22B

Examination of Containers for Integrity

II. Examination of Glass Containers for Integrity

Authors

(Return to Table of Contents)

Almost all low-acid foods packaged in glass containers are sealed with vacuum-type closures. Currently 4 types of vacuum closures are widely used on low-acid food products: LT (lug-type twist) cap, PT (press-on twist-off) cap, pyr-off (side seal) cap, and CT (continuous thread) screw cap (Fig. 25). Packers' tests and examinations to ensure a reliable hermetic seal of containers are required by 21 CFR 113.60 (a) (2) and (3).

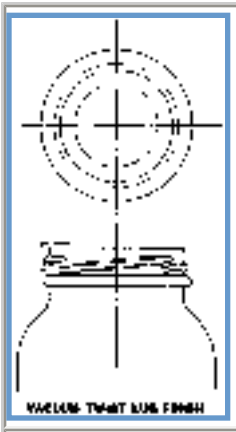
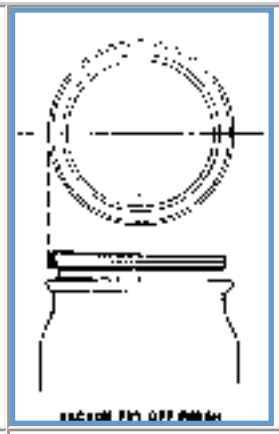
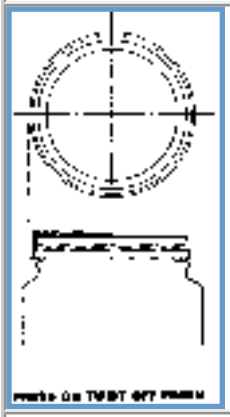
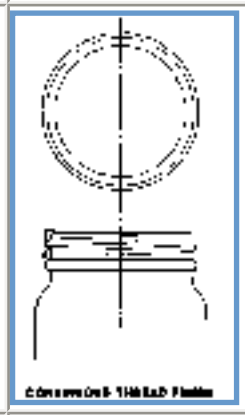
	
Vacuum Twist Lug Finish	Vacuum Pry Off Finish
	
Press On Twist Off Finish	Continuous Thread Finish

Figure 25. Types of vacuum closures and glass finishes.

A. Visual examination for closure and glass defects (for definition of terms, **see** the glossary section of this chapter)

cap tilt
crushed lug
chipped glass finish
cut-through
cocked cap
stripped cap
cracked glass finish

B. Seal integrity examination

1. **Vacuum**. Use standard open-closed type of vacuum gauge or USG No. 12118 gauge with both vacuum and pressure scales (Fig. 26). Wet rubber gasket on piercing device with water. Shake off excess water. Puncture closure, using

piercing needle attached to vacuum gauge. Read and record vacuum in inches (0-30 inches), or pressure (0-15 psi).

Figure 26. Vacuum gauge for seal integrity examination.

2. **Removal torque (cam-off) for PT or LT type closures (Fig. 27)**. Properly secure jar on torque meter. Ease closures off in smooth, continuous motion rather than rapid, jerking motion. Use one hand to twist cap counterclockwise to open cap from sealed jar while avoiding any downward pressure on cap. Record maximum torque in inch-pounds required to open cap.

Figure 27. Torque meter.

3. **Security values (lug tension) on lug-type twist cap (Fig. 28)**. Make vertical line on cap and corresponding line on container wall with marking pen. Turn closure counterclockwise just until vacuum is broken. Reapply closure to container just until gasket compound touches glass finish and closure lug touches glass thread (or until closure is at 2 inch-pound reapplication torque to achieve uniformity for application). Measure and record, in 1/16 inch increments, distance in front of vertical lines that were made before opening. Security is considered positive if line on cap is to right of line on container, and negative if line on cap is to left of

line on container.

Figure 28. Security measurement for lug type twist closure.

4. **Pull-up (lug position) for lug-type twist cap (Fig. 29)**. Mark vertical neck ring seam on glass finish. Measure distance from this vertical line, in 1/16 inch increments, to leading edge of cap lug position nearest it. Record lug position measurements made on right side of vertical neck ring seam (as analyst looks at package) as positive (+) and those to left side of parting line as negative (-).

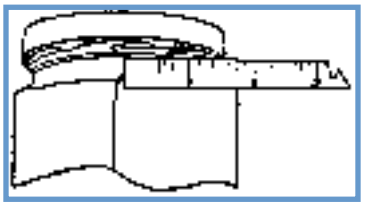


Figure 29. Pull-up lug position of LTD measurement.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 22C

Examination of Containers for Integrity

III. Examination of Flexible and Semirigid Food Containers for Integrity

George W. Arndt, Jr. (NFPA)

Author

(Return to Table of Contents)

Flexible and semirigid food packages are composed mainly or in part of plastic materials. Closure is achieved by heat sealing or double seaming. The 4 main groups of packages that cause similar integrity concerns and that are examined by common methods are paperboard packages, flexible pouches, plastic cups and trays with flexible lids, and plastic cans with double-seamed metal ends.

The purpose of a hermetic closure is to provide a barrier to microorganisms and to prevent oxygen from degrading the food. Closure integrity is significant because sealing surfaces may contain food particles and moisture that contribute to heat-seal and double-seam defects. Critical control must be exercised in this operation. Visual examination will reveal most defects. For many flexible packages, seal strength may be ascertained by squeezing.

A. Package examination

Note condition of package (exterior and interior) and quality of seals or seams; observe and feel for gross abnormalities, mechanical defects, perforations, malformations, crushing, flex cracks, delamination, and swelling. Measure dimensions as recommended by manufacturer of closing equipment or packaging material. Perform teardown procedure as described. Note condition of package and closure. If there is evidence that a package may lose or has lost its hermetic seal, or that microbial growth has occurred in the package contents, further investigation is required.

1. Visual examination

Use hand as well as eye. A magnifying glass with proper illumination is helpful. Rub thumb and forefinger around seal area, feeling for folds and ridges. Rub fingers over flat surfaces to feel for delamination, roughness, or unevenness. By sight and touch, determine presence of defects. Mark location of defects with indelible ink. **See** Fig. 30 for visual inspection criteria for closure seal.

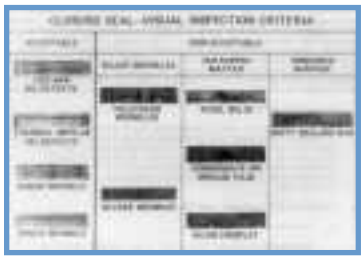

Figure 30. Visual inspection criteria for closure seal.
(**Courtesy of Brik Pak, Inc**.)

2. Examination of packages (**see** Tables 1 and 2)

Table 1. Test methods for plastic packages containing food (5)

	Package type			
Test methods	**Paper board**	**Flexible pouch**	**Plastic, heat-sealed lid**	**Plastic, double-seam metal end**
Air leak testing	O	O	O	O
Biotesting	O	O	O	O

Burst testing	O	R	R	O
Chemical etching	O	O	O	NA
Compression, squeeze testing	R	O	O	O
Distribution (abuse) test	O	O	O	O
Dye penetration	R	O	R	O
Electester	O	NA	NA	NA
Electrolytic	R	O	R	NA
Gas leak detection	O	O	O	O
Incubation	R	R	R	R
Light	NA	O	O	O
Machine vision	O	O	O	O
Proximity tester	O	O	O	R
Seam scope projection	NA	NA	NA	R
Sound	R	NA	R	R
Tensile (peel) testing	NA	R	R	NA
Vacuum testing	NA	O	R	O
Visual inspection	R	R	R	R

Abbreviations: R, test method is recommended by NFPA Bulletin 41-L, Flexible Package Integrity Bulletin; O, other commercially accepted test method applications; NA, test method is inappropriate for this style package.

Table 2. List of visible package defects provided by National Food Processors Association (5)

	Package type[(a)]			
Defect	**Paper board**	**Flexible pouch**	**Plastic, heat-sealed lid**	**Plastic, double-seam metal end**
Abrasion	+	+	+	+
Blister	-	+	-	-
Burnt seal	-	-	+	-
Channel leak(er)	-	+	+	-

Clouded seal	-	+	-	-
Compressed seal	-	+	-	-
Contaminated seal	-	+	+	-
Convolution	-	+	-	-
Corner dent	+	-	-	-
Corner leaker	+	-	-	-
Crooked seal	-	+	-	-
Crushed	+	-	+	+
Defective seal	-	-	+	-
Deformed	+	-	-	-
Deformed seal	+	-	-	-
Delamination	+	+	+	+
Embossing	-	+	-	-
Flexcracks	-	+	+	+
Foreign matter (inclusion)	-	-	+	+
Fracture	-	+	+	+
Gels	-	-	+	+
Hotfold	-	+	-	-
Incomplete seal	-	-	+	-
Label foldover	-	-	+	-
Leaker	-	+	-	-
Loose flaps	+	-	-	-
Malformed	-	-	+	+
Misaligned seal	-	-	+	+
Nonbonding	-	+	-	-
Notch leaker	-	+	-	-
Puncture	+	+	+	+
Seal creep	-	+	-	-
Seal leaker	+	-	-	-
Seal width variation	-	-	+	-
Shrinkage wrinkle	-	-	+	-

Stringy seal	-	+	-	-
Swell (swollen package)	+	+	+	+
Uneven impression	-	-	+	-
Uneven seal junction	-	+	-	-
Waffling	-	+	-	-
Weak seal	+	-	-	-
Wrinkle	-	+	+	-
[a] +, Definition is applicable to that package type; -, definition is not applicable.				

a. Paperboard packages (20)

Teardown procedures. Unfold all flaps (except gable top packages); check integrity and tightness of transverse (top and bottom) and side (vertical or longitudinal) seals by firmly squeezing package. If package has longitudinal sealing (LS) strip, pull off overlapping paper layer at side (longitudinal) seal. Check air gap of longitudinal sealing strip application (about 1 mm). Squeeze package and check that there are no leaks or holes in the LS strip.

Next, on side opposite side seal, puncture container with sharp scissors and empty contents. Saving side seal portion, cut near fold at each end of package and down length of package to remove a large rectangular body portion. Observe this large rectangular body portion for holes, scratches, or tears anywhere on the surface. Pay close attention to corners of package, particularly directly under end seals and near the straw hole or pull tab, if present. Now cut remaining package in half through the center of the side seam. Wash both halves of remaining package and dry them with a paper towel. Mark to identify the package.

Evaluation procedures for seal quality differ between package designs, constructions, and sealing methods. Obtain specific procedures for a given package from the manufacturer. For example, seal evaluation may consist of starting at one end of the seal, and very slowly and carefully pulling the seal apart. In some packages the seal is good if the polymer stretches the entire length of the seal (that is, stretching of polymer film continues to a point beyond which paper and laminates have separated). In other packages, fiber tear can be seen the entire length of the seal (that is, raw paperboard is visible on both sides of the separated seal areas). This is known as 100% fiber tear and indicates a good seal. Test all 3 seals of

each package half. Problems to look for are absence of (or narrow) fiber tear, lack of polymer stretch, "cold spots" (no polymer bond in seal area), and "tacking" (polymer melt but no stretch or fiber tear). For longitudinal sealing strip-type packages, additional tests (such as centering examination, heat mark examination, and appearance of aluminum foil examination when stripped) should be made according to manufacturer's directions.

Electrolytic and dye testing. These tests differ according to each system manufacturer's filed procedure. Contact the individual manufacturer, obtain recommendations, and follow them.

b. Flexible pouches (20)

1. **Teardown procedures**. Check tightness of both head and side seals by squeezing each package from each fill tube or sealing lane. Important points are corners and crossing of head and side seals. This is a rapid determination of obvious defects. Each seal must be accurately torn apart and evaluated for correct integrity. Carefully inspect edges of each head and side seal for evidence of product in seal areas. No product should be visible.

   Observe width of each seal area. Width must comply with machine-type specifications: for example, 1/16 inch minimum on all head and side seals for fill tube or sealing lane machines. Look for presence of smooth seal junction along inside edge of seal. Open each package to check side seals and head seals. Visually inspect for such defects as misaligned seal, flex cracking, nonbonding, and seal creep. If applicable, tear the seals by doing a seal tensile strength test or a burst test. Then observe appearance of tear at each seal. Seals should tear evenly so that foil and part of laminated layer from one side of package tears off, adhering to seal on other side of package. The seal should appear rough and marbleized. The seal is also adequate if the foil is laid bare across entire length of seal. Retain records of test results as required.

2. Other test procedures

   Squeeze test. Apply manual kneading action that forces product against interior seal surface. The sealing surface must be smooth, parallel, and free of wrinkles. Examine all seal areas for evidence of product leakage or delamination. Packages that exhibit

delamination of the outer ply on seal area but not at product edge should be tested further by manually flexing the suspect area 10 times and examining all seal areas for leakage or reduction in the width of the seal area to less than 1/16 inch.

Seal tensile strength. Results to be expressed in pounds per linear inch, average of sample (that is, 3 adjacent specimens cut from that seal) should not be less than specified for the material and application.

Burst strength test. With internal pressure resistance as the measurement to check all seals, apply uniform pressure, under designated test conditions, to a level of not less than specified for a material or application, for 30 s. Then evaluate seals to ensure that proper closure seal is still in effect.

c. Plastic package with heat-sealed lid (20)

Container integrity testing. Peel test procedures of form fill and seal containers. Squeeze container side walls of entire set from a mold. Squeeze each cup to cause 1/8 inch bulge of lid area. Lid should not separate from package when package is squeezed. Observe sealing area for fold-over wrinkles in sealant layer of lidstock. From a first set of containers, visually observe embossed ring in sealed area for completeness. (Embossed ring should be at least 90% complete if present.) Remove a second set of containers (1 cup per mold) and gently peel back each lid at approximately a 45 angle. Observe the peeled area for a generally frosty appearance on both the lid and cup sealed surfaces. Observe entire package for holes, scratches, even flange widths, smooth inside surfaces, and any deformities caused by dirty mold or sealing die.

Leak test procedures (optional). These tests differ according to each system manufacturer's filed procedure. Contact the individual manufacturer, obtain recommendations, and follow them.

Electrolytic test. Plastic packages generally do not conduct a flow of low-voltage electricity unless a hole is present. Use a volt meter or amp meter to determine the presence of a closed circuit. If a voltage flow can be measured, use a dye solution to identify the presence of a hole.

Dye penetration test. Use a dye to locate leaks in packages or to demonstrate that no leaks exist.

Air pressure or vacuum test. Apply pressure or vacuum to a closed package to test for holes and to observe any loss of pressure or vacuum. Underwater vacuum testing may reveal a steady stream of small bubbles emitting from a hole in a package.

d. Plastic cans with double-seamed metal ends (20)

Procedures for examining metal cans with double seams are described in Chapter 21 and in 21 CFR, Part 113. Use these methods to examine plastic cans with double-seamed metal ends. Make the following changes to 21 CFR 113.60 (a,1,i,a and b).

B. Micrometer measurement system

Metal cans. Required: cover hook, body hook, width (length, height), tightness (observation for wrinkle), and thickness. Optional: overlap (by calculation) and countersink.

Plastic cans with double-seamed metal ends. Required in addition to seam scope examination: thickness and tightness. Compare seam thickness to that calculated from individual thicknesses of plastic flange and neck and metal end, excluding compound. Optional: cover hook, countersink, and width (length, height).

Seam scope of projector

Metal cans. Required: body hook, overlap, tightness (observation for wrinkle), and thickness by micrometer. Optional: width (length, height), cover hook, and countersink.

Plastic cans with double-seamed metal ends. Required: overlap, body hook, countersink, width (length, height). Optional: cover hook.

Visual examination for plastic cans with double-seamed metal ends. Required: tightness. Note compression of pressure ridge or flange during overlap measurement. Remove entire cover and examine pressure ridge for continuity. Under 21 CFR 113.60 (a,1,i,c) add the following: pressure ridge for plastic cans with double-seamed ends; impression around complete inside periphery of can body in double seam area.

C. Microleak detection

Microleak testing methods are not listed in order of sensitivity, nor is it necessary to use

them all. Each test has advantages and disadvantages, depending on the package, equipment, and set of conditions. Optional methods are appropriate when additional information will clarify the nature of various package defects. Some test methods are not appropriate for some package materials, closures, or package styles. Refer to the manufacturer of the package or closure system for recommended test methods or see Table 1. Common methods are presented to provide the analyst with procedures and options. Visible defects of the 4 flexible package groups are summarized in Fig. 30.

Measure packages before testing for microleaks. Mark visually detected defects to aid location during or after microleak testing (non-water soluble markers are recommended). Record all results, methods used, and environmental conditions (temperature, relative humidity) and retain these records. Conduct all tests in the standard laboratory atmosphere of 23 + 2°C and 50 + 5% relative humidity. When this is not possible, report temperature and relative humidity along with test results (14).

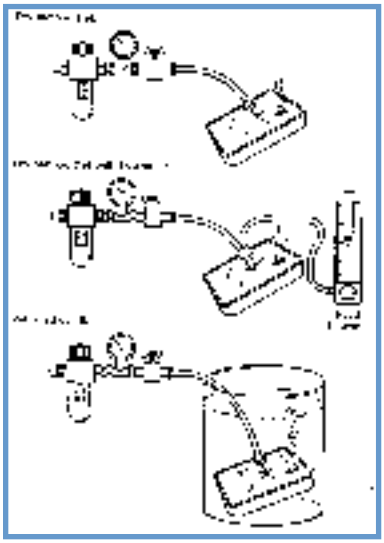

Figure 31. Air leak testing of packages.

1. Airleak testing (5) (Fig. 31)

    a. Dry method

        1. Materials

            Compressed air with regulator
            Needle, valve, hoses
            Pressure gauge or flow meter

        2. Procedure

Puncture container wall with needle. Inject air while increasing at 1 psi/s until a standard pressure is reached. Standard pressure used for testing should be less than the normal unrestrained burst pressure for the package. Observe pressure gauge for loss of internal pressure over a 60 s period. If a flow meter is used, observe for airflow, which indicates presence of openings in the test package. Dye testing may be used to locate air leaks that are not visible with the dry method. Inject air to create internal pressure within the package without causing it to burst. Observe all surfaces and seals for air leaks. Observe flow meter for indication of air loss from the package.

b. Wet method

1. Materials

Compressed air with regulator
Needle, valve, hoses
Water
Transparent container to observe bubbles

2. Procedure

Inject air to create internal pressure within the package without causing it to burst. Immerse package in water and inspect visually for a stream of bubbles emitting from a common source.

c. Results

Positive. - A steady stream of bubbles comes from the package at one or more locations.

Negative. - No bubbles are emitted from the package.

False positive. - Bubbles are emitted from point at which needle entered package; or bubbles cling to surface of the package after package is submerged in water.

False negative. - Food particles block holes through which air might escape from defective package; or air pressure used is insufficient to force air through minute holes in package.

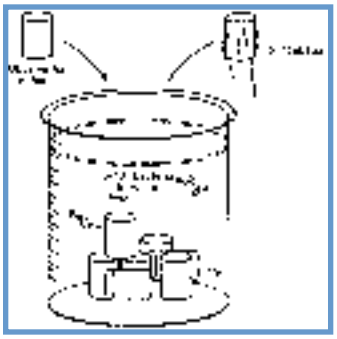

Figure 32. Biotesting of packages.

2. Biotesting (5,21) (Fig. 32)

   The objective of biotesting is to detect the presence of holes in hermetic packages by placing them in an agitated solution of fermentation bacteria in water for an extended period of time.

   Obtain representative packages and submerged them in an agitated solution of active bacteria. The bacterial concentration should be $>10^7/cm^3$. The temperature of the solution that surrounds the packages should be maintained at a temperature that permits rapid growth of the bacteria within any packages they may enter. However, growth of the bacteria in the liquid surrounding the submerged packages is not desirable. The bacteria must cause fermentation of the product within the package if they penetrate and must not be pathogenic. Packages should be flexed during immersion to expose cracks and holes to incursion. The solution that surrounds the packages should be maintained at a temperature that permits rapid growth of bacteria within defective packages. After biotesting, packages are incubated for 3 weeks at 95-100F. This test should be used only to evaluate new package designs or to validate packaging systems. It should not be used as routine quality control procedure. Other methods are cheaper, simpler, and just as reliable.

   a. Materials

      Water bath with temperature control and agitation solution of *Enterobacter aerogenes* for foods, pH >5.0. Solution of *Lactobacillus cellobiosis* for foods, pH <5.0
      Sample packages
      Apparatus to flex packages
      Incubator

b. Procedure

Obtain representative samples. Mix active bacteria in water at about 1.0 x 10^7/ml. Immerse samples in mixture. Agitate water bath and flex sample for 30 min. Remove packages and rinse with chlorinated water. Incubate samples for 2 weeks at 95-100F. Observe packages for swelling for 3 weeks. Open each package by cutting in half across the middle, leaving a hinge and observe contents for spoilage. Thoroughly wash insides of both halves from each spoiled package. Subject each half to a dye test to locate leaks.

c. Results. Report location of leaks.

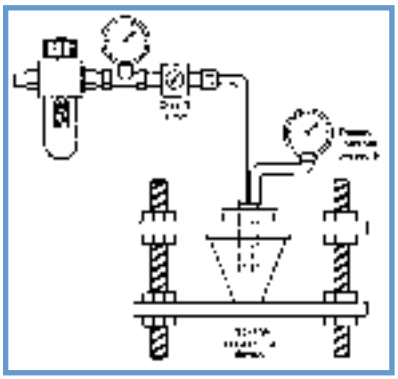

Figure 33. Burst tester.

Figure 34. Pouch air burst tester.

3. Burst testing (5) (Figs. 33 and 34)

The objective of burst testing is to provide a means for determining the ability of a hermetically sealed package to withstand internal pressure (psig). The entire package is subjected to uniform stress and failure generally reveals the weakest

point. Both restrained and unrestrained burst testing may be used. Restraint limits expansion by minimizing the angle of the package seal, which becomes greater as a package is inflated. With restraint, packages with strong seals fail at greater internal pressure than do packages with weak seals. Thus, use of a restraining device during burst testing permits noticeable separation between packages having strong or weak seals.

Fused seals are stronger than the walls of a flexible package. Burst failure generally occurs adjacent to fused seals. Peelable seals are weaker than the walls of a flexible package, and less pressure is needed to induce pressure failure. Lower pressure and a longer time increment are required to burst test peelable seals.

Dynamic burst testing involves a steady increase of internal pressure until failure occurs. Static burst testing involves a steady increase in internal pressure to a pressure less than failure, followed by a 30-s hold. Both methods are used for packages with fused seals. Peelable seals are burst-tested by inflating at a steady rate to a point less than failure pressure and held for 30 s, followed by a 0.5 psig pressure increase and another 30-s hold. Pressure and time indexing is continued with observation of the seal area for seal separation (peeling) until failure occurs.

a. Materials

   Compressed air or water
   Regulation valve
   Needle with gasket and pressure tubing
   Solenoid with timer(s)
   Pressure indicator(s), digital or gauge with sweep hand
   Restraining device (optional)

b. Procedure

   Use empty sealed package, or cut and remove contents of a filled package. Place package in restraining fixture (if used). Pierce package with gasketted needle(s) and inject air or water. Inflate at 1 psig/s.

   Dynamic method. Continue inflation at 1 psig/s until failure occurs. Record internal pressure at failure.

   Static method. Inflate at 1 psig/s to specified internal pressure, and hold at specified pressure for 30 s. Record as pass or fail.

Indexed method. Inflate to 5 psig and hold for 30 s, inflate additional 0.5 psig and hold for 30 s. Continue increase and hold sequence until failure occurs. Observe peelable seal separation. Report internal pressure at failure.

c. Results

Positive. Pressure failure occurs below specified level of performance, indicating a hole in the package.

Negative. No pressure failure occurs below specified level of performance.

False positive. A leak is present at point where air or water is injected into package and pressure cannot be maintained.

False negative. A small leak occurs, but is not sufficient to reduce pressure noticeably.

4. Chemical etching (5)

Multilaminate and composite packaging materials may be etched to remove overlying layers, revealing the hermetic seal of packages that have polyolefin heat seals. This allows comparison of visually detected package defects on the external surface before etching and within the seal area after all external layers have been removed.

Composite paperboard packages. The outer layers of a package are removed by tearing, abrasion, and chemical action to expose the sealant layer intact. By photographing or photocopying the package before etching, the etched seal can be compared with the photograph to determine the significance of visually discernible defects.

a. Materials

Water bath and heater with thermostat
Three l-L Pyrex glass beakers
Running tap water
Graduated cylinder
Automatic stirring device (heated is preferred)
Drying oven equilibrated to 65°C (150F)

Paper towels
Rubber gloves, protective goggles, apron, tongs
Fume hood with chemical-resistant surface

Chemicals for etching of paperboard aseptic packages:
hydrochloric acid (HCl) solution, 3.7 N
acidified solution of copper chloride ($CuC1_2$)
saturated solution of bisodium carbonate (Na_2CO_3) in water

e. Preparation of solutions. **CAUTION**: Always pour acid into water; never pour water into acid.

Pour 0.5 L of concentrated HCl into 1 L of cold distilled water. Pour slowly, as heat will be produced when acid and water mix. Stir until mixed completely. Cover to prevent evaporation. Solution will be 3.7 N HCl.

Pour 0.5 L of concentrated HCl into 1.5 L of cold distilled water. Add 10 g of $CuC1_2$. Stir until completely mixed. Cover beaker and let warm to room temperature before using.

Pour enough Na_2CO_3 into a container to make a saturated solution at room temperature. Some undissolved Na_2CO_3 should remain on bottom of beaker after stirring.

f. Procedure

Cut transversal seal from package approximately 1 inch from end. Identify multiple samples by notching cut edge with scissors. Manually strip paper from sample to be etched. Place sample in hot HCl solution (65°C) for 5 min. Remove sample with tongs and immerse it in Na_2CO_3 solution to neutralize the acid. Remove sample from the Na_2CO_3 solution with tongs and rinse it in running tap water. Pull off polyethylene layer that lies between paperboard layer and aluminum foil.

Using a glass stirring rod to manipulate the sample, drop it into the $CuC1_2$ solution so that it is completely immersed. Observe closely while stirring to ensure that the heat of the reaction does not damage the polyethylene sealant layer as the foil is dissolved. Remove from solution.

Dip sample in Na_2CO_3 solution to neutralize it, and then rinse it with

water. Press sample gently between soft absorbent paper towels and place in oven at 65°C (150F) until dry. Apply alcohol-based dye solution to inner and outer seal edges. (**See** fluorescein dye solution formula, described above).

Observe pattern of ink dispersion and check for leaks and channels within fused seal area. Use overhead projector to enlarge seal samples and provide a more accurate visual inspection.

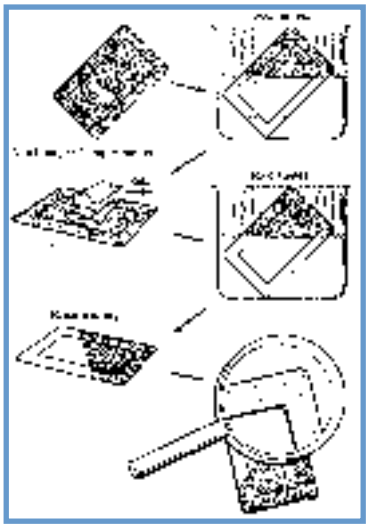

Figure 35. Chemical etching of package seal.

Retortable pouches (Fig. 35)

1. Materials

   Two l-L Pyrex beakers
   Running tap water
   Paper towels
   Rubber gloves, apron
   Protective goggles, tongs
   Fume hood with chemical-resistant surface
   Chemicals for etching retortable pouches
   6 N HCl solution, commercial grade
   Tetrahydrofurant (THF), commercial grade, stabilized

2. Procedure

   Cut off end of pouch and remove contents. Wash inside of pouch. Dry the pouch. Cut all but suspected area away from area of interest, leaving about

1 inch adjacent to seal. Soak sample in tetrahydrofurant (THF) to remove outer polyester layer by softening adhesive and/or inks. Do this in a fume hood; wear protective gloves resistant to THF. (If separation cannot be obtained, proceed to next step.) Remove most of the ink and adhesive from aluminum foil with THF and paper towels. Soak remaining structure in 6 N HCl in a fume hood to remove aluminum foil by etching. Rinse sealant layers with water and dry with paper towels.

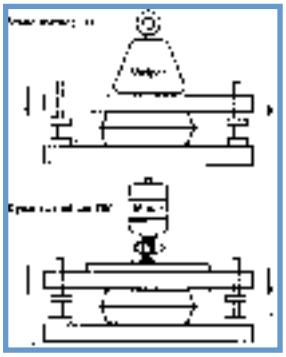

Figure 36. Compression testing of packages.

5. Compression test (5) (Fig. 36)

Place a filled and sealed food package on flat surface and apply pressure while observing for leaks.

a. Materials

Flat surface or conveyor belt
Sealed package
Heavy flat object or mechanical press
Timer

b. Procedure

Static method. Place sealed package on flat surface and lay a flat-surfaced weight on it. Observe effect of weight on integrity of package seals over time. A similar test may be performed by applying a constant weight to a package moving on a conveyor belt. The speed of the moving belt determines the time of compression.

Dynamic method. Use a press to continually increase the force applied to a package at a constant rate. Observe the maximum force required to cause

failure of the package.

Squeeze test. Apply a manual kneading action that forces product against the interior seal surface area. Examine all seal areas for evidence of product leakage or delamination. Packages that exhibit delamination of the outer ply on the seal area but not at product edge should be tested further by again manually flexing the suspect area 10 times and examining all seal areas for leakage or short-width.

c. Results

Positive. Holes form in package or its seals or seams, with measurable movement of top plate or deflection on a force gauge.

Negative. No loss of hermetic integrity, and no measurable movement of top plate or deflection on a force gauge.

False positive. Underfilled or weak packages deflect in a manner that simulates failure without loss of hermetic integrity.

False negative. Holes form in package but food product closes off the holes, permitting pressure to increase within package.

6. Distribution (abuse) test (5)

Packages are subjected to vibration, compression, and impact at levels typical of the distribution system for which they are designed. After the test, which is a conditioning regimen, the packages are examined. Defects are quantified and described in relation to package failures observed in normal distribution. Fragility is eliminated by design changes in the package system. Whenever possible all samples should be incubated for 2 weeks at 100F before abuse-testing (Fig. 37).

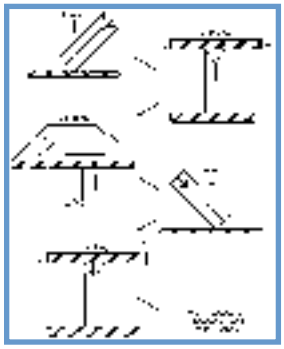

Figure 37. Distribution abuse testing.

a. Materials

Packages to be tested
Drop tester
Vibration table
Compression tester
Standard laboratory conditions 23 + 2°C, 50 + 5% relative humidity
Incubator at 100F to contain all test packages

b. Procedure. **See** ASTM D-4169 Standard Practice for Performance Testing of Shipping Containers and Systems (15).

Select distribution cycle 6 for flexible packages in shipping cases transported by motor freight. Before testing, incubate all packages for 14 days at 100F and inspect visually for defects.

Perform the following 10 steps (**see** Section 9 of ASTM D-4169) (15).

1. Define shipping unit - Shipping unit to be tested is a typical pallet load.

2. Establish assurance level - Assurance level II will be used, based on value and volume of shipment.

3. Determine acceptance criteria at assurance level II: **Criterion 1** - no product damage; **criterion 2** - all packages in good condition.

4. Select distribution cycle (DC) - DC-6 will be used for pallet shipments.

5. Write test plan (values for X must be determined before conducting the test). Select representative samples for test. Condition samples to 23 + 1°C, 50 + 2% relative humidity, in accordance with Practice D 4332 (14).

6. Perform tests in accordance with test plan in step 5, as directed in the referenced ASTM standards and in the special instructions for each shipment.

7. Evaluate results - Examine products and packages to determine if acceptance criteria have been met.

8. Document test results (16) - Write a report to cover all steps in detail.

9. Report fully all the steps taken. At a minimum, the report should include all the criteria in step 10.

10. Description of product and shipping unit DC and test plan
    Assurance levels and rationale
    Number of samples tested
    Conditioning used
    Acceptance criteria
    Variation from recommended procedures
    Condition of specimens after test

    After testing, examine all failed (positive) packages to determine location and cause of damage. Incubate all containers that do not fail (negative) during testing for 14 days at 100F and inspect visually for defects before destructive testing by other methods listed in this chapter.

c. Results

Positive. A package loses hermetic integrity during any one phase of the testing protocol or during the incubation period that follows.

Type test	**ASTM Method**	**Level**
Handling (12)	D-1083	One impact on 2 opposite base edges from X inches
Stacking (10)	D-642	Compression to X lb (individual container) *
Vibration (13)	D-999 Method C	Search 3-100 Hz at 0.5 g peak. Dwell 10 min at 0.5 g peak
Handling (11)	D-959	One impact on 2 opposite base edges from X inches

(12)	D-1083	
(17)	D-997	
(18)	D-775	
Stacking (10)	D-642	Compression to X lb (individual container) *
*Alternative full pallet load compression test, X lb per bottom tier container.		

Negative. A package retains hermetic integrity through the test, and contents do not show evidence of microbial growth after incubation.

False positive. A package appears to be defective, yet confirmational testing by incubation or dye penetration reveals that no loss of the hermetic barrier occurred during the abuse test.

False negative. A package appears to pass testing but later exhibits failure when incubated.

7. Dye testing (5)

Dye or ink is applied to inside surface of a cleaned package at the seal or suspected location of failure and observed to determine whether it can pass through to the outside (Figs. 24, 38, 39).

Figure 24. Can piercer and gas collection apparatus.

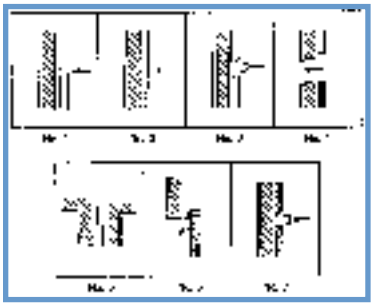

Figure 38. Dye testing.

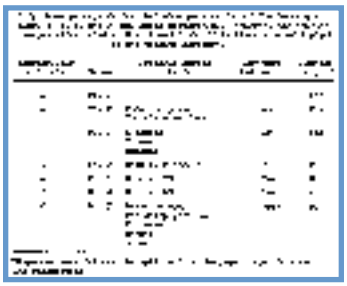

Figure 39. Dye testing results.
(**Courtesy of Brik Pak, Inc**.)

a. Materials

Disposable plastic gloves
Dye solution: 1 L of isopropanol (solvent) and 5 g of rhodamine (powder) mixed (or other appropriate dye solution)
Sink
Scissors or knife
Oven to dry sample packages
Paper towels
Magnifying glass or low-power microscope

b. Procedure

Open and empty a package; wash, and dry by wiping or by oven drying (180F, 15 min). Apply low surface-tension solution containing dye along the closure or on side of package at suspected location of hole. The solution moves by capillary action through the hole and appears on opposite side of package wall. After dye is completely dry, cut package with scissors and examine the hole closely.

Cut open cans, tubs, or bowls through bottom (leaving seal areas or double

seams untouched) and remove product. Cut pouches and paperboard containers along equator, leaving a hinge (so that both ends can be tested), and remove product. Wash package with water containing mild detergent, rinse thoroughly with tap water, and wipe dry. Holding package upside down and at slight angle, place 1 drop of dye solution at inside edge of seal surface. Rotate to allow dye to wet entire inside seal circumference.

CAUTION: A number of dyes are known or suspected to cause cancer. Rhodamine B is a possible carcinogen. Wear disposable plastic gloves and avoid skin contact with dyes.

Let dye solution dry completely. Very slowly peel the seal completely and observe the frosty, white, sealed surfaces for evidence of dye. In some packages the innermost laminates must be carefully observed for stretching as the seal is peeled.

c. Results

Positive. Dye penetrates hole in package, indicating loss of hermetic barrier.

Negative. Dye does not pass through the package (wall or seal).

False positive. Solution dissolves packaging material, creating hole in package, or dye is accidentally splattered on outside of package, indicating hole or leakage where none exists.

False negative (for paperboard only). Solution penetrates holes in hermetic barrier layers but fails to reach outside of package where it would be visible.

8. Electester (5)

The objective is to determine changes in viscosity of liquid foods after incubation of filled packages (Fig. 40).

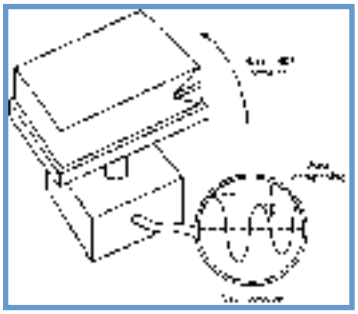

Figure 40. Electester

Microbial fermentation can cause changes in the viscosity of still liquids. If all factors are constant, shock waves will dampen at different rates in liquids with different viscosities. Incubation of shelf-stable liquid foods and nondestructive testing of each package may identify containers that have been subjected to microbial activity.

a. Materials

   Packages filled with still, liquid food, incubated
   Electesting device
   Fixture to restrain test packages

b. Procedure

   Remove representative samples from production line and incubate at 95°F for 4 days. Place packages containing still liquids in restraining device with largest flat surface of package facing downward. Rotate package 90 horizontally and back to its original position very rapidly; do this only one time. The motion creates a shock wave. Fixture holding the package is precisely balanced to minimize outside interference and minimize dampening as shock wave moves back and forth within package. Motion is sensed and displayed on an oscilloscope with alarms alerting operator to vibrations that dampen more quickly or more slowly than normal for a specific liquid food product. Examine contents with a microscope and determine pH to confirm spoilage if there is any doubt.

c. Results

   Positive. Wave dampens more quickly or slowly than normal, indicating change in product viscosity.

Negative. Rate of wave dampening is within range established by testing "normal" liquid product that did not display microbial spoilage during incubation.

False positive. Range of acceptance is too narrow, and normal product is incorrectly identified as spoiled.

False negative. Range of acceptance is too broad, and spoiled product is incorrectly identified as normal.

9. Electroconductivity (5)

The objective is to detect holes in hermetic packages by sensing the flow of electrical current. Plastics are generally poor conductors of electricity. Consequently, plastic food packages without holes will form an effective barrier to mild electrical current; therefore, this method may be used to detect minute breaks in plastic food packages. A detectable flow of low-voltage electrical current generally indicates that the hermetic barrier has been lost.

a. Materials

1% NaCl in water (brine solution)
Scissors
Battery 9V, three 12-inch lengths of wire, 9V light bulb, or a conductivity meter (VOM). Remove insulation from each end of wires. One wire from positive pole goes to light bulb that has a wire as probe. The second wire from the negative pole is the other probe (Fig. 41).
Plastic bowl large enough to submerge package

Figure 41. An electrolytic cell for leak examination.

b. Procedure

Obtain sample food package and cut off one end with scissors. Aseptic

paperboard packages and flexible pouches may be cut on all but one edge along package equator and folded 180 on uncut side to form 2 equal halves. Wash samples to remove all food contents and any dried plugs that may occlude holes. Oven drying at 180F is recommended but not required before immersion. Wipe the cut edges with a paper towel if necessary, as wet edges may result in false-positive test results. Place samples in bowl containing brine solution and partially fill sample with brine so that it stands upright and is almost completely submerged. Place conductivity meter or light bulb with one probe inside the package and the other outside the package. Submerge both probes into their respective brine solutions. Test the other half of package similarly for current flow.

c. Results

Positive. Current flow indicates break in hermetic barrier.

Negative. No current flow indicates hermetic barrier exists.

False positive. Aluminum foil conducts electricity. A pinhole or partial break through inner layers of a package may expose the foil layer, resulting in false-positive test result. Dye testing will confirm presence or absence of holes. Moisture may form a bridge over cut edge of a package, creating a false positive.**False negative**. Dried product may occlude minute holes in a package. If plugs do not rehydrate quickly, they will not conduct electricity when packages are immersed.

10. Gas detection (5)

The objective is to detect microleaks in hermetically sealed packages with sensors tuned to detect only gas leaking from within package. The package must be a barrier to the test gas so that the rate of gas permeation through the package wall will not raise the normal background concentration in atmosphere of testing area. Gas concentrations may be detected by impact to a sensor. The sensor may be a heated element in which electrical resistance varies in relation to gas molecules removing heat as they impact. Examples of test gases suitable for package include oxygen, nitrogen, hydrogen, carbon dioxide, and helium.

a. Procedure for detecting helium leak

Gas obtained from storage tanks or air fractioning may be used to displace headspace gases within food packages before closure. Concentration of gas within package must be greater than the concentration of that gas in

the atmosphere where packages are tested. There are three modes for detection: ASTM E493, inside-out tracer mode (6); ASTM E498, tracer probe testing mode (7); and ASTM E499, detector probe testing mode (8). Slight compression of a package may assist the movement of gas molecules through microleaks.

b. Results

Positive. Detection of gas concentrations greater than the normal atmospheric concentration indicates break in hermetic barrier of sample package. Confirm with dye testing to locate hole in sample package.

Negative. No detection of test gas concentration greater than the normal atmospheric concentration indicates hermetically sealed container.

False positive. Detection of gas concentrations in excess of the normal background level may result from increase in test gas concentration in the testing area. Test background concentration before and after testing sample. Packages with high permeability may lose gas.

False negative. Internal gas concentration may be reduced through absorption by the product, reaction with a component inside the package, or permeability if over an extended storage period.

11. Incubation (5)

The objective is to determine whether a package has lost hermetic barrier by holding containers at an ideal temperature for sufficient time to ensure microbial growth. Hermetic integrity is the condition that bars entry of microorganisms into a package. Growth of microorganisms indicates either insufficient processing or loss of hermetic barrier. Growth may be observed as gas formation, change in pH, growth of viable organisms, or changes in the appearance of food.

a. Materials

Insulated box or room to serve as an incubator
Heater with thermostat
Storage racks
Recording thermometer
Temperature recording charts
Knife, scissors

pH meter
Inoculating loop and flame
Sterile culture dishes and tubes, and culture media

b. Procedure

Obtain representative sample packages containing processed product. Inspect all samples visually for defects. Place packages in incubator for recommended period of time at recommended temperature.

Products stored in incubator at 95°F (35°C)
FDA products - 14 days
USDA products - 10 days
Products stored in warehouse
85-95°F (29-35°C) 30 days
70-85°F (21-29°C) 60 days
60-70F (16-21°C) 90 days

Visually inspect packages for evidence of spoilage. Open and inspect all (or some) packages for visible signs of microbial growth, aroma, and change in pH. Never taste incubated product if spoilage may have occurred. Aseptically obtain product samples to culture microbiologically and confirm cause of spoilage. Conduct appropriate integrity test on package to identify presence or absence of microleaks. Dispose of product safely. Autoclave any product or packages showing spoilage before disposal.

c. Results

Positive. Spoilage has occurred and is evident as swelling, putrefactive odor, change in product pH from normal, or change in appearance.

Negative. Spoilage has not occurred.

False positive. Chemical reaction or enzymatic activities alter product characteristics without microbial activity.

False negative. Should not occur because this would be commercial sterility.

12. Light (5)

a. **Infrared light**. The objective is to observe differences in the absorbance and transmittance of heat energy (infrared light) in a package or seal. Infrared light may be absorbed, transmitted, and emitted by a package or a seal. Differences between these parameters provide a means for visual interpretation when sensed automatically and enhanced for visibility.

    1. Materials

       Infrared light or oven (180F)
       Visual infrared light detector

    2. Procedure: Expose samples to infrared radiation before examining.

b. **Laser light**. The objective is to measure small changes in the relative position of similar surfaces on separate packages as they are subjected to changes in external pressure. Flexible packages possessing some headspace gas may be flexed by altering the external pressure in a closed chamber. Packages are held by fixtures so that a split laser beam may be directed to the same position on both packages. The reflected beams are recombined with mirrors and prisms. Laser light has a well-defined wave length that does not change by reflection. However, if packages move differently when flexed, one beam segment will travel a greater distance than the other. When beam segments are recombined, differences in position of reflecting surfaces will cause the recombined laser beam to be out of phase. This condition can be sensed and used to segregate packages that do not flex in the normal manner from those that do.

    1. Materials: Laser set up with chamber and means to read the differences.

    2. Procedure: Flex packages in chamber by applying and releasing vacuum. Observe any difference in the 2 packages and determine by controls which package leaks.

c. **Polarized light**. The objective is to observe differences in the transmittance of visible light through translucent and transparent heat seals (Fig. 42). Polarized light filters are composed of minute parallel lines on a glass surface or plastic film. When 2 polarized filters are rotated to be 90 different, no light will pass through. At 0, both sets of lines are parallel and a light bulb set in line with the 2 polarized filters will be visible.

Figure 42. Visual inspection of transparent seals with polarized light.

During heat sealing of transparent and translucent plastic materials, energy is added, providing free movement of polymer chains. Close packing and increased hydrogen bonding occurs, resulting in alignment of carbon chains and increased crystalline structure. Differences between random, oriented, and crystalline configuration affect both light absorption and transmission in these materials. A seal sample placed between 2 polarizing filters is first illuminated by polarized light. To enhance color changes resulting from differences in crystalline structure, rotate the other filter to block most of the transmitted light. Inspect visually to determine degree of crystalinity within fused seals. Uniform crystalinity, seen as uniform color tone along the inner edge of the primary seal, is one indicator of fusion. Areas that are not fused appear as a different color. Colors differ with materials and thickness.

1. Materials

   Light bulb, white, 40, 75, 100 W
   Polarized camera filters, 2 each
   Frame to hold filters and permit free rotation of both filters in line with light bulb and sample

2. Procedure

   Obtain a clean transparent seal sample. Turn on light. Place seal sample between polarized filters. Rotate one filter to obtain maximum difference in color between fused seal and nonseal area. Examine fused seal area for uniformity.

d. **Visible light**. The objective is to detect holes in packages by sensing transmitted and reflected visible light. Package is placed over low-wattage light bulb in darkened room to enhance visual inspection. Aluminum foil will block all light transmission except where holes and flexcracks in foil are present. Close inspection is required to determine whether other lamina overlay holes in foil layer. Dye testing is required to establish presence or

absence of minute holes. Chemical etching may be used to remove materials external to polyolefin seals. Magnification of etched seals with backlighting aids inspection.

1. Materials

   Light bulb
   Scissors
   Sink with running water
   Paper towels
   Darkened room
   Indelible marking pen
   Dye (optional)

2. Procedure

   Remove contents, wash, and dry container. Inspect package for light leaks. Mark location of light leaks with a marking pen; draw a circle around the defect location. Closely examine defects for presence of holes through all layers. Use dye test to verify presence or absence of holes.

3. Results

   Positive. A hole through all layers is detected in a package.

   Negative. No light leaks are detected.

   False positive. A hole in the foil layers permits light to pass, but no holes exist in overlying layers and hermetic barrier is maintained.

   False negative. A hole through all layers is not aligned so that light can be transmitted.

13. Machine vision (5)

The objective is to detect holes in hermetic packages by computer evaluation of images with previously defined patterns of acceptance. This system is designed to eliminate visual inspection of packages. Packages are positioned before a camera to present a consistent pattern. The video image obtained is digitized. Both grayscale and color density may be evaluated. The computer compares coded

patterns with acceptable patterns stored in memory. Some systems evaluate one image at a time. Others use parallel computers to evaluate different segments of the video image in less time. Patterns that do not match the acceptance criteria are rejected and the package is automatically rejected from the production line.

a. Materials

Video imaging system
Computer with stored images for acceptance criteria
Strobe light (optional)
Packages

b. Results

Positive. Image does not match acceptance criteria.

Negative. Image matches acceptance criteria.

False positive. Image was not presented to camera correctly and does not match acceptance criteria.

False negative. Acceptance criteria include defects.

14. Proximity devices (5)

The objective is to detect holes by measuring changes in the shape of hermetically sealed packages as a function of time. The position of a package containing metal may be established by the strength of a magnetic field, detected with a galvanometer. By comparing 2 readings as a function of time, a determination can be made as to whether the shape of a package has changed.

a. Materials

Proximity detection system
Computer with stored acceptance criteria
Packages

b. Procedure

Compare multiple packages to a standard value. Fix limits of acceptance or alter automatically by computing a running average and standard

deviation. Packages displaying stronger or weaker disturbances to a magnetic field sensed by a galvanometer may fall outside of the limits of acceptance. Mark these packages for removal from packaging line.

Read magnetic fields of single packages at one location and, after a period of time, make a second reading at a downstream location. If shape of container changes, mark package for removal from packaging line. Confirm with dye testing to locate holes in packages.

c. Results

Positive. Disturbance of magnetic field exceeding limits of acceptance.

Negative. Disturbance of magnetic field within limits of acceptance.

False positive. External disturbance of magnetic field or imprecise positioning of package resulted in values that exceeded limits of acceptance.

False negative. Distortion of package sufficient to cause disturbance of magnetic field outside normal range of acceptance.

15. Seam scope projection (5)

The objective is to measure critical dimensions in the closure profile of plastic packages. Packages are cut in cross-section to reveal all components in their proper thickness and relative position. The cut edge is magnified with a projector to aid measurement and visual inspection.

a. Materials

Knife, saw, or scissors
Microprojector
Micrometer, calipers, ruler, or measurescope

b. Procedure

Cut directly across seal or closure with knife, saw, or scissors and remove section containing adjacent material. Magnify cross section. Compare observed dimensions with criteria for acceptance or rejection provided by manufacturer of package or closure machine. Accept or reject sample.

c. Results

Positive. Dimensions of sample exceed limits of criteria for acceptance.

Negative. Dimensions within limits of criteria for acceptance.

False positive. Magnification with incorrect scale or measuring error results in rejection of acceptable sample.

False negative. Measuring error results in acceptance of defective sample.

16. Sound (5)

Ultrasonic. The objective is to passively sense air moving through small orifices in packages possessing internal vacuum or pressure by monitoring the presence or absence of high-frequency sound waves.

a. Materials

Microphone
Audiofilters
Oscilloscope with alarm system
Packages

b. Procedure

Place packages in a chamber to eliminate external disturbances and subject to changes in external pressure. Air movement through small holes in package wall generates ultrasonic sound waves. A microphone senses the vibration. Audiofilters eliminate all frequencies except those of interest.

c. Results

Positive. Package exhibits ultrasonic whistling sound, indicating a leak is present, permitting air to enter or exit package.

Negative. No sound is emitted by package within range of frequencies monitored.

False positive. Background noise occurred within range monitored.

False negative. Hole does not emit a noise within the range monitored, or hole was occluded by moisture or food.

Echo. The objective is to actively sense the frequency of echoes in hermetically sealed containers. When a package possessing a vacuum is tapped, the tightness of the package creates a sound that is audibly different from that of the same package without a vacuum. Two changes can be monitored: frequency and amplitude. Changes in frequency (vibrations per second) are recognized as differences in tone (pitch). Changes in amplitude are recognized as 2 relative difference in volume. Loss of hermetic integrity will result in microbial growth within the contents of a food package during incubation. Changes in sound accompany changes in viscosity. Consequently, this method may be used as a nondestructive test for a number of product/package combinations.

a. Materials

Control sample
Samples to be evaluated
Tapping device (electronic device or unsharpened pencil to be used like a drumstick)
Incubator

b. Procedure

Obtain sample packages, either newly packed or incubated, and a control package (known to be properly sealed) containing the same product as sample packages. Tap the section of the package covering that is taut. Listen to the echo for differences between packages. Commercial devices are available that electronically monitor the echos, allowing for a less subjective determination.

c. Results

Positive. Package displays audibly different sound, indicating loss of hermetic integrity.

Negative. Package resonates at same frequency as control package.

False positive. Differences in vacuum level or fill volume create different sounds in test packages.

False negative. Audible difference between control package and test package cannot be differentiated.

17. Tensile strength (5)

The objective is to measure the tensile strength required to cause separation of peelable or fused seals. A section of a seal is obtained by cutting a 1/2 or 1 inch strip perpendicular to the seal edge. The strip is then clamped by opposing grippers and pulled at constant speed and defined angle until failure is obtained. The peak force required to fully separate the 2 halves is recorded as the strength of the seal.

a. Materials

Sample packages
Sample cutting apparatus
Scissors (sample dimensions are critical to precision)
Tensile strength testing device

b. Procedure. **See** ASTM D-882 - Standard test methods for tensile properties of thin plastic sheeting (9).

Remove representative sample from production line. Cut open sample and remove contents. Do not disturb seal to be tested. Cut a segment of the seal to produce a test strip. Test strip must be cut perpendicular to the seal to be tested. Secure both ends of test strip in separate clamps. With screwdriver, move one screw clamp away from the other, creating a 180° separation of the seal. Observe force required to fully separate seal. Fixtures are required to hold samples at angles different from 180°.

c. Results

Positive. Sample separates at peak tensile strength less than established standard.

Negative. Sample separates uniformly at peak tensile strength greater than or equal to established standard.

False positive. Sample separates at peak tensile strength less than established standard because of equipment miscalibration or greater separation speed of jaws.

False negative. Sample separates at tensile strength greater than or equal to established standard. However, a different portion of the same sample failed at a tensile strength less than the standard.

18. Vacuum testing (5)

The objective is to cause the movement of air out of a sealed container through leaks by using external vacuum within a testing chamber. Closed packages are placed inside a sealed testing chamber and vacuum is created to cause movement of air through leaks in the packages. Deflection of the package may be measured as a function of time to determine whether leakage has occurred. If vacuum chamber contains water, bubbles from holes in packages may be observed.

a. Materials

Bell jar (glass or plastic) with tight-fitting lid
Water to cover package within bell jar
Weighted fixture to keep package below water level during test
Vacuum pump
Vacuum gauge
Valve
Grease for tight gasketing of lid on chamber

b. Procedure

Obtain representative sample from production line. Place one sample inside vacuum chamber. Evacuate chamber. Observe package swelling and any movement of air (bubbles) or product through holes that may be present or may have developed. When vacuum is released, observe packages to determine if original shape is retained or if atmospheric pressure causes sample to appear slightly crushed.

c. Results

Positive. Leak in test package causes air or product to escape through holes in container. Container ruptures or lid separates because of weak closure. When vacuum is released, package appears distorted or crushed by atmospheric pressure.

Negative. Package distorts under vacuum but no loss of product or air is observed. When vacuum is released, package assumes its original

configuration.

False positive. Air clinging to surface of package or within paper laminates is mistaken for bubbles emitting from a defect.

False negative. Food particles prevent movement of air out of a hole in container while under vacuum.

19. Visual inspection (5)

The objective is to visually observe defects in food packages. Representative samples are obtained from production line. External surfaces are examined for holes, abrasions, delamination, and correct design. Critical dimensions are measured and observations recorded.

a. Materials

Strong light without glare, for visual inspection of packages
Measuring devices, such as ruler, calipers, micrometer
Scissors or knife

b. Procedure

Refer to examination procedures for paperboard packages, flexible pouches, plastic packages with heat-sealed lids, and plastic cans with double-seamed metal ends.

c. Results

Positive. Visually detected defect.

Negative. No visually detected defects.

False positive. Visual identification of defect not actually present.

False negative. Defect is present, but not visually detected.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 22D

Examination of Containers for Integrity

Glossary and References

Authors

(Return to Table of Contents)

Glossary

BASE PLATE PRESSURE. Force of the base plate that holds the can body and end against the chuck during the double seaming operation. In general, it has the following effect on the seam formation: low pressure, short body hook; high pressure, long body hook.

BODY - The principal part of a container, usually the largest part in one piece comprising the sides. The body may be cylindrical, rectangular, or another shape.

BODY HOOK - The flange of the can body that is turned down in the formation of the double seam.

BOTTOM SEAM - Double seam of the can end put on by the can manufacturer, also known as factory end seam.

CABLE CUTS - Cuts or grooves worn into can ends and bodies by cables of the runway conveyor system.

CAN, SANITARY - Full open-top 2-piece drawn can and 3-piece can with double seamed bottom. Cover or top end is attached with a double seam by the packer after filling. Ends are compound-lined. Also known as packer's can or open-top can.

CANNER'S END - **See** packer's end.

CAP TILT - Cap should be essentially level with transfer bead or shoulder.

CHIPPED GLASS FINISH - Defect in which a piece of glass has broken away (chipped) from the finish surface.

CHUCK - Part of a closing machine that fits inside the end countersink and acts as an anvil to support the cover and body against the pressure of the seaming rolls.

CHUCK WALL - Part of the can end that comes in contact with the seaming chuck (Fig. 2).

COCKED CAP - Cap not level because cap lug is not properly seated under glass lug.

CODE CUT - Fracture in the metal of a can end caused by improper code embossing.

COLD WELD - Weld appears narrower and lighter than normal and may be scalloped. Fails the pull test, possibly exhibiting a zipper or sawtooth type of failure.

CONTAMINATION IN WELD AREA - Any visible burn at one or more points along side seam.

COMPOUND - Sealing material consisting of a water or solvent dispersion or solution of rubber and placed in the curl of the can end. The compound aids in producing a hermetic seal by filling spaces or voids in the double seam

COUNTERSINK DEPTH - Measurement from top edge of double seam to end panel adjacent to chuck wall.

COVER - **See** packer's end.

COVER HOOK - The part of the double seam formed from the curl of the can end. Wrinkling and other visual defects can be observed by stripping off the cover hook.

CRACKED GLASS FINISH - Actual break in the glass over the sealing surface of the finish. Also known as split finish.

CRAWLED LAPS - Occurs when two layers of metal are bent and the outer layer looks shorter because it has a greater radius to traverse than the inner layer, which has a smaller radius, perhaps being bent almost double. Also known as creep.

CROSS-OVER - The portion of a double seam at the juncture with the side seam of the body.

CROSS-SECTION - A section cut through the double seam for the purpose of evaluating the seam.

CRUSHED LUG - Lug on cap forced over glass lug, causing the cap lug not to seat under glass lug.

CURL - Extreme edge of the cover that is turned inward after the end is formed. In metal can double seaming, the curl forms the cover hook of the double seam. For the closure for glass containers, the curl is the rolled portion of metal at the bottom of the closure skirt (may be inward or outward).

CUTOVER - A break in the metal at top of inside portion of double seam caused by a portion of the cover being forced over the top of the seaming chuck. This condition usually occurs at the cross-over. Also known as a cut through by some can manufacturers. These manufacturers refer to a cutover as the same condition without the break.

CUT THROUGH - Gasket damage caused by excessive vertical pressure.

DEADHEAD - An incomplete double seam resulting from the seaming chuck spinning in the end's countersink during the double seaming operation. Also known as a spinner, skidder, or slip.

DELAMINATION - Any separation of plies (laminate materials) that results in questionable pouch integrity.

DOUBLE SEAM - Closure formed by interlocking and compressing the curl of the end and the flange of the can body. It is commonly produced in 2 operations. The first operation roll preforms the metal to produce the 5 thicknesses or folds; the second presses and flattens them together to produce double seam tightness.

DROOP - Smooth projection of the double seam outside and below the bottom of the normal seam. Usually occurs at the side seam lap area.

FACTORY END - **See** manufacturer's end.

FALSE SEAM - Double seam where a portion of the cover hook and body hook are not interlocked, i.e., no hooking of body and cover hooks.

FINISH - That part of the glass container for holding the cap or closures.

FLANGE - Outward flared edge of the can body cylinder that becomes the body hook in the double seaming operation. For weld cans, any flange crack at or immediately adjacent to the weld is a major defect.

FLEXIBLE CONTAINER - A container, the shape or contour of which, when filled and sealed, is affected by the enclosed product.

HEAVY LAP - A lap containing excess solder. Also called a thick lap.

HOOK, BODY - **See** body hook.

HOOK, COVER - **See** cover hook.

IMPROPER POUCH SEAL - A defect (e.g., entrapped food, grease, moisture, voids, or fold-over wrinkles) in that area of the closure seal that extends 1/8 inch vertically from edge of seal on food product side and along full length of seal.

IRREGULAR WELD WIDTH - Any obvious irregularity in weld width along length of side seam.

JUMPED SEAM - **See** jumpover.

JUMPOVER - Double seam that is not rolled tight enough adjacent to the cross-over; caused by jumping of the seaming rolls at the lap.

JUNCTURE - The junction of the body side seam and the end double seam, or that point where the 2 seams come together. Also known as the cross-over.

KNOCKED-DOWN FLANGE - Common term for a false seam where the bottom of the flange is visible below the double seam. A portion of the body flange is bent back against the body without being engaged with the cover hook.

LAP - The section at the end of the side seam consisting of 2 layers of metal bonded together.

As the term implies, the 2 portions of the side seam are lapped together to allow for the double seam, rather than hooked, as in the center of the side seam.

LID - **See** packer's end.

LIP - Projection where the cover hook metal protrudes below the double seam in one or more "V" shapes. Also known as a vee.

LUG CAP - Closure with raised internal impressions that intermesh with identical threads on the finish of the glass container. It is a closure with horizontal protrusions that seat under angled threads on the glass container finish.

MANUFACTURER'S END - End of the can that is attached by the can manufacturer.

NOTCH - Small cut-out section in the lap designed to facilitate the formation or the body hook at cross-over.

OPEN LAP - A lap that is not properly soldered or has failed by separating or opening because of various strains in the solder.

OVERLAP - Distance the cover hook laps over the body hook. Any observable loss of overlap along the side seam is a critical defect.

PACKER'S END - End of the can attached and coded by the food packer. Also known as the canner's end.

PLATE - General term for tinplate, aluminum, and the steel sheets from which cans are made. It is usually tin plate, which is black plate with tin applied to it.

PRESSURE RIDGE - Impression (chuck impression) around the inside of the can body directly opposite the double seam.

PULL-UP - Term applied to distance measured from the leading edge of the closure lug to the vertical neck ring seam.

SAWTOOTH - Partial separation of the weld side seam overlap at one or more points along the seam. If observed after performing the pull test, it is considered a critical defect.

SEAM NARROWING - A steadily visible narrowing of the weld at either end of the weld side seam is a critical defect.

SEAM THICKNESS - Maximum dimension of double seam measured across or perpendicular to layers of seam.

SEAM WIDTH (LENGTH OR HEIGHT) - Maximum dimension of double seam measured parallel to folds of seam.

SECURITY - Residual clamping force remaining in the closure application when gasket has properly seated after processing and cooling.

SEMIRIGID CONTAINER - A container, the shape or contour of which, when filled and sealed, is not affected by the enclosed product under normal atmospheric temperature and pressure, but which may be deformed by external mechanical pressure of less than 10 psi (0.7 kg/cm^2) (i.e., normal firm finger pressure).

SIDE SEAM - The seam joining the 2 edges of the body blank to form a can body.

SKIDDER - Can with incompletely finished double seam because the can slipped in the seaming chuck. In this defect, part of the seam will be incompletely rolled out. The term has the same meaning as deadhead when referring to seamers that revolve the can. Also known as a spinner.

SOFT CRAB - Colloquial term used to describe a breakdown in the packer's can resulting in a hole between end and body.

SPINNER - **See** deadhead and skidder.

STRIPPED CAP - Lug closure applied with too much torque, which causes lugs to pass over glass lugs. May have vacuum but has no security value.

TIGHTNESS - Degree to which the double seam is compressed by the second operation roll. Tightness is determined primarily by the degree of freedom from wrinkles in the cover hook. Tightness rating is a percentage that ranges from 100 to 0, depending on the depth of the wrinkle: 100% indicates no wrinkle and 0% indicates a wrinkle extending completely down the face of the cover hook. A well-defined continuous impression around the circumference of the can in the double seam area indicates a tight seam. This impression is known as a pressure ridge.

TOP SEAM - Top of packer's end seam.

UNEVEN HOOK - Body or cover hook that is not uniform in length.

WELD CRACK - Class I corrosion products plus any observable seam crack, and any cracks that extend 25% or more across the width of the weld at any point along the weld seam are considered critical defects.

WELD PROTRUSION - Protrusion of the weld in excess of 1/16 inch beyond the leading or trailing edge of the can body.

WRINKLE (COVER HOOK) - A waviness occurring in the cover hook from which the degree of double seam tightness is determined.

ZIPPER - Gross separation of the side seam overlap along all or any part of the side seam. If observed during pull test, it is a critical defect.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Bacteriological Analytical Manual

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Chapter 23

Microbiological Methods for Cosmetics

Authors

(Return to Table of Contents)

The ability of microorganisms to grow and reproduce in cosmetic products has been known for many years. Microorganisms may cause spoilage or chemical changes in cosmetic products and injury to the user (4,5,10,14-16,20,21). Methods for isolation of microorganisms from cosmetic products are direct colony counts and enrichment culturing. Products that are not soluble in water are initially treated to render them miscible before isolation procedures are conducted. Dilution and plating media that partially inactivate preservative systems commonly found in cosmetic products are used. The isolated microorganisms are identified by routine microbiological methods or by commercial identification kits. The scheme for these analyses is summarized in Fig. 1.

A. Equipment and materials

1. Pipets, sterile, 1, 5, and 10 ml, graduated
2. Gauze pads, sterile, 4 x 4 inch
3. Sterile instruments: forceps, scissors, scalpel and blades, spatulas, and microspatulas
4. Test tubes, screw-cap, 13 x 100, 16 x 125, and 20 x 150 mm

5. Dilution bottles, screw-cap
6. Balance, sensitivity of 0.01 g
7. Petri dishes, sterile, plastic, 15 x 100 mm
8. Bent glass rods, sterile
9. Incubators, 30 ± 2°C and 35 ± 2°C
10. Anaerobic atmosphere generating envelopes, indicator strips, and jars (BBL or Oxoid), or anaerobic incubator, 35 ± 2°C, or anaerobic glove box, 35 ± 2°C.
11. Candle jars or CO_2 incubator, 35 ± 2°C.
12. Laminar flow hood with HEPA filter, if available
13. Vitek or equivalent automated computerized identification system

B. Media for enumeration and identification of Gram-positive bacteria and fungi

1. Anaerobe agar (M11)
2. Bile esculin agar (M18)
3. Brain heart infusion (BHI) agar and broth (M24)
4. Malt extract agar (MEA) (M93)
5. Potato dextrose agar (PDA) (M127)
6. Mannitol salt agar (M97)
7. Modified letheen agar (MLA) (M78) and broth (MLB) (M79)
8. Oxidative-fermentative (OF) test medium (M117)
9. Sabouraud's dextrose broth (M133)
10. Blood agar base (M20a)
11. Starch agar (M143)
12. Trypticase (tryptic) soy agar (TSA) (M152) and broth (TSB) (M154)
13. Baird-Parker (BP) agar (M17)
14. Catalase test (R12)
15. Vogel-Johnson (VJ) agar (optional) (M176)
16. Commercial bacterial identification kit (API or equivalent)

Fig. 1: Scheme for Enumeration, Isolation, and Identification of Cosmetic Microbes

- Sample preparation.
- Dilute prepared samples in MLB.
- Spread duplicate 0.l ml samples on

(a)	(b)	(c)	(d)

MLA 48h, 30°C	PDA (or MEA) with chlortetracycline 7 days, 30°C	BP (or VJ) agar 48h, 35°C (optional)	Anaerobic agar MLA 2-4 days, 35°C

- Enrich MLB dilutions for 7 days, at 30°C. Purify growth only if no colonies on MLA.
- Count colonies and subculture different colony types on MLA and MacConkey agar (and BP or VJ agars if used in c, above). For fungal isolates, **see** text.
- Determine Gram reaction, cell shape, and catalase production of purified isolates.
- Proceed with identification of bacterial isolates as described in text, or use identification kits.

Fig. l. Abbreviations: MLB, modified letheen broth; MLA, modified letheen agar; PDA, potato dextrose agar; MEA, malt extract agar; BP, Baird-Parker; VJ, Vogel-Johnson.

C. Media for identification of *Enterobacteriaceae*

1. Andrade's carbohydrate broth and indicator (M13) for testing metabolism of rhamnose, mannitol, sorbitol, arabinose
2. Lysine iron agar (M89)
3. Malonate broth (M92) or phenylalanine malonate broth (Difco)
4. Motility-indole ornithine medium (M99)
5. MR-VP broth (M104)
6. Simmons citrate agar (M138)
7. Triple sugar iron (TSI) agar (M149)
8. Christensen's urea agar (M40)
9. MacConkey agar (M91)
10. Lysine decarboxylase medium for Gram-negative nonfermentative bacteria (M88)
11. Phenylalanine deaminase agar (M123) (**see also C-3, above)**
12. API 20E, Roche Enterotube, or other equivalent identification kits

Incubate all biochemical tests using media in B and C, above, at 35-37°C for 18-24 h, except malonate broth (48 h) and MR-VP broth (48 h or longer).

D. Media and reagents for identification of Gram-negative nonfermentative (NF) bacilli

1. Acetamide medium (M2)

2. Clark's flagellar stain (R14)
3. Esculin agar, modified (CDC) (M53)
4. Nutrient gelatin (CDC) (M115)
5. Indole medium (M64) and indole medium (CDC) (M65)
6. King's B medium (M69)
7. Lysine decarboxylase (LDC) medium for Gram-negative NF bacteria (M88)
8. Motility nitrate medium (M101)
9. Nitrate broth, enriched (CDC) (M109)
10. King's OF basal medium (M70) for testing metabolism of sucrose, lactose, fructose, esculin, xylose, glucose (dextrose), mannitol, salicin, sorbitol, and maltose
11. Oxidase test strips
12. Christensen's urea agar (M40)
13. Decarboxylase basal medium (for arginine decarboxylase) (M44)
14. Yeast extract (YE) agar (M181)
15. Pseudomonas agars F (M128 and P (M129) (Difco)
16. Cetrimide agar (Pseudosel™, BBL; Difco), or equivalent (M37)
17. Glycerol, sterile (Difco), or equivalent
18. API, NFT, or other equivalent commercial identification system
19. Koser's citrate broth (M72)

E. Other media and reagents

1. Aqueous solution of 70% ethanol and 1% HCl (v/v) **or** 4% iodine in 70% ethanol solution or 2% glutaraldehyde solution
2. Tween 80 (Polysorb 80)
3. Ethanol, 95% (v/v)
4. Lyophilized rabbit coagulase plasma with EDTA
5. 3% (v/v) Aqueous solution of hydrogen peroxide
6. Gram stain (R32) and endospore stain (R32a)
7. Cooked meat medium (M42)

F. Handling of cosmetic samples for microbiological analysis

Analyze samples as soon as possible after their arrival. If necessary, store samples at room temperature. Do not incubate, refrigerate, or freeze samples before or after analysis. Inspect samples carefully before opening and note any irregularities of sample container. Disinfect surface of sample container with aqueous mixture of 70% ethanol (v/v) and 1% HCl (v/v) or other disinfectant (**see** E-l) before opening and removing contents. Use laminar flow hood if possible. Dry surface with sterile gauze before opening. Use representative portion of contents for microbial analysis, e.g., 1 g (ml) portion of sample.

For products weighing less than 1 g (ml), analyze entire contents. If only one sample unit is available and multiple analyses are requested (i.e., microbial, toxicological, and chemical), take subsample for microbiological examination before those for other analyses. In this situation, amount of subsample used for microbial analysis will depend on other analyses to be performed. For example, if total sample content is 5 ml, use 1 or 2 ml portion for microbial analyses.

G. Preliminary sample preparation

Amounts of sample and diluent given here can be adjusted according to amount of sample available. If sample has many subsamples, amount of test material can be increased and workload streamlined by compositing. Analysts should use their best judgment as to when and how much material to composite.

1. **Liquids**. Decimally dilute 1 ml liquid directly into 9 ml modified letheen broth (MLB) in 20 x 150 mm screw-cap test tube for the 10^{-1} dilution.

2. **Solids and powders**. Aseptically remove and weigh 1 g sample into 20 x 150 mm screw-cap test tube containing 1 ml sterile Tween 80. Disperse product in Tween 80 with sterile spatula. Add 8 ml sterile MLB and mix thoroughly. This will be the 10^{-1} dilution.

3. **Cream and oil-based products**. Aseptically remove and weigh 1 g sample into 20 x 150 mm screw-cap tube containing 1 ml sterile Tween 80 plus five to seven 5-mm glass beads (or ten to fifteen 3-mm glass beads). Mix total contents with Vortex mixer. Adjust total volume to 10 ml with sterile MLB (8 ml) for the 10^{-1} dilution.

4. **Aerosols of powders, soaps, liquids, and other materials**. Decontaminate nozzle of spray can as much as possible by swabbing with gauze pad moistened with 70% (v/v) aqueous ethanol. Expel some product to flush out nozzle; then spray appropriate amount into tared dilution bottle, e.g., 1 g of product into 9 ml sterile MLB. Thoroughly mix product and broth, and reweigh. This will be a 10^{-1} dilution if exactly 1 g of sample was obtained.

5. **Anhydrous materials**. Treat as in G-2 or G-3, as appropriate.

H. Microbiological evaluations

Not all analyses described below will necessarily be performed; however, the aerobic plate count, enrichment culture, and the fungal count should be performed routinely.

1. **Aerobic plate count (APC)**. Use spread plate technique to facilitate recognition of different colony types and, if necessary, for differential count. Also use Baird-Parker (BP) or Vogel-Johnson (VJ) agar if *Staphylococcus* spp. are suspected. Prepare and label duplicate sets of petri dishes containing modified letheen agar (MLA) and BP agar for samples of 10^{-1} to 10^{-6} dilutions. Add either 5 or 10 ml of prepared cosmetic preparation (**see** G, above) to 45 or 90 ml, respectively, of MLB, for 10^{-2} dilution.

   Dilute samples decimally in MLB (**NOTE**: save dilutions for enrichment step) to obtain complete dilution series from 10^{-1} to 10^{-6}. (Begin with 10^{-2} if all the 10^{-1} dilution is used up.) Thoroughly mix dilutions and pipet 0.1 ml of each dilution onto surfaces of solid media in prelabeled petri dishes. Spread inoculum over entire surface with bent glass rod that was first sterilized by dipping in 95% ethanol and quickly flamed to remove the ethanol. Let medium absorb inoculum before inverting and incubating plates for 48 h at 30 ± 2°C (35°C for BP plates). Use new spreader for each dilution (at low dilutions) because some product residue may carry over and adversely affect the flame-sterilization procedure. For effective inoculum absorption, be sure agar surface is dried (30 min at 35°C) when agar is freshly made.

   Count all colonies in plates containing 25-250 colonies, and record results per dilution counted. Average the colony counts obtained, and multiply the average by 10 and then by the appropriate dilution factor (10^{-1} - 10^{-6}). Report results as APC/g (ml) sample. If plates do not contain 25-250 colonies, record dilution counted and note number of colonies found.

   For BP plates, count well-distributed colonies that are convex, shiny black, either with or without a clear zone surrounding the colony. (**NOTE**: Coagulase-positive colonies produce clearing, but coagulase-negative colonies may or may not produce clearing. If coagulase-negative colonies clear, their irregularity reportedly distinguishes them from coagulase-positive colonies.) Select plates having more than 250 colonies when those at a greater dilution do not contain colonial types described above. Plates from minimal dilutions having fewer than 25 colonies may also be used if necessary. From each BP plate demonstrating growth, pick one or more typical colonies to confirm their coagulase reaction. Transfer colonies to agar slants of any suitable maintenance medium, e.g., trypticase (tryptic) soy agar (TSA), brain heart infusion (BHI) agar. Incubate slants until growth is evident.

Calculate number of *Staphylococcus aureus* organisms present by first determining the fraction of colonies tested that are coagulase positive. Multiply this fraction by average number of *Staphylococcus* colonies counted on the BP plates. Multiply the number obtained by the appropriate dilution factor and report as number of *S. aureus*/g (ml) sample.

If no colonies are obtained on MLA or BP media, observe already prepared MLB dilutions while enriching them at 30 ± 2°C for 7 days. Examine enrichments daily for growth. After 7 days of incubation, or when growth is suspected, subculture all enrichments onto both MLA and MacConkey agar plates. Incubate plates 48 h at 30 ± 2°C.

2. **Fungi, yeast, and mold plate count**. Transfer 0.1 ml portions of dilution series (H-l, above) to appropriately labeled duplicate plates of either malt extract agar (MEA) or potato dextrose agar (PDA), both containing 40 ppm chlortetracycline. Spread inoculum over surface of medium with sterile glass spreader rod. After inoculum is absorbed by medium, invert plates, incubate at 30 ± 2°C, and observe daily for 7 days. Average the counts obtained on duplicate plates, multiply by 10 to allow for the volume plated (0.1 ml), multiply by the dilution factor, and report as yeast or mold count/g (ml) sample. For fungal enrichments (optional), dilute prepared sample decimally in Sabouraud's dextrose broth and incubate as described above for MLB dilutions. If growth occurs, streak on Sabouraud's dextrose agar, MEA, or PDA. The latter two agars should both contain 40 ppm chlortetracycline.
3. **Anaerobic plate count (use only for talcs and powders)**. The main purpose of this procedure is to detect the tetanus bacillus (*Clostridium tetani*), which can occur in these products. Perform as described above for APC, using MLA agar, pre-reduced anaerobe agar, and 5% defibrinated sheep blood agar for plating. Incubate blood agar plates in 5-10% carbon dioxide atmosphere (candle jar or CO_2 incubator), and anaerobe agar plates in anaerobic jars. Incubate both for 48 h before counting. Reincubate for 2 more days if no colonies appear at 48 h. Pre-reduce anaerobic agar plates before inoculation by placing them in an anaerobic atmosphere overnight (12-16 h). Incubate anaerobe agar plates in anaerobic atmosphere (anaerobic jar, incubator, or glove box) for 2 days at 35 ± 2°C; incubate MLA plates aerobically for 2 days at 35 ± 2°C as aerobic control. Strict anaerobes will grow only in the anaerobic jars. It is recommended that a small amount (0.1 ml) of inoculum be used to minimize spreading of growth caused by wetness, and that inoculated plates be placed in an anaerobic atmosphere within minutes after inoculation to minimize exposure to oxygen. Suspected anaerobic organismsmust be subcultured aerobically (under CO_2) and anaerobically to establish their true oxygen relationship. Check for terminally located spores in cooked meat broth incubated at 35°C for 2 days. Use of a differential spore stain to detect spores is mandatory. Other methods may detect nonspore artifacts,

which could lead to wasted identification efforts. If an obligate anaerobic sporeformer is isolated, consult A.D. Hitchins, FDA, Washington, DC 20204, for information about how to proceed.

4. **Screening test for total numbers of microorganisms present in used and unused cosmetics**. Solid media, incubation temperatures and times described in H 1-3 can be applied, as appropriate, to samples prepared as shown in G 1-5 to screen cosmetics for total counts before performing complete microbiological evaluations as described in H 1-3 above. If sample contains <10 cfu per ml or g product, a screening test using 1 ml of the 10^{-1} dilution in the pour plate technique should yield negative growth results. If sample contains <100 cfu per ml or g product, a screening test using 0.1 ml of the 10^{-1} dilution in the spread plate technique should yield a negative growth outcome.

Identification of Microbes

Molds and yeasts should be purified and yeasts identified as far as possible using kits, e.g., Vitek yeast card and API yeast assimilation strip. If necessary, send fungal isolates to Valerie H. Tournas, FDA, Washington, DC 20204, for speciation. For bacteria, examine all plates and streak morpho-logically dissimilar colonial types onto MacConkey and MLA media. Prepare Gram stain of all morphologically dissimilar colonial types obtained in pure culture. With methods given here, isolates may be identified to genus level in general; tests for speciation are listed when necessary. Test results should be evaluated using *Bergey's Manual* (12) or Madden's methods (14). Commercial identification kits, e.g., API, Roche, Vitek, Hewlett-Packard (**see** Appendix 1), are strongly recommended.

A. Identification methods

1. **Gram-positive rods**. Report aerobic Gram-positive rods as either sporeforming or nonsporeforming. To enhance sporulation, inoculate starch agar plate with isolate and incubate 48 h at room temperature. Prepare either Gram stain or endospore stain from isolated colony and note position of endospore within vegetative cell (central, terminal, or subterminal), shape of endospore (round or ellipsoidal), and morphology of sporulating cell's sporangium (swollen or not swollen). Test all aerobic sporeforming rods for motility by either of two methods:

    a. **Cultivation method**. Stab-inoculate tube of motility test or motility-indole-ornithine medium. Incubate aerobically 18-24 h at room temperature. Growth from line of stab (indicated by turbidity of medium around stab) constitutes a positive test.

b. **Microscopic examination**. Inoculate isolated colony into suitable broth. Incubate aerobically 18-24 h at room temperature. Place one drop of broth culture on clean microscope slide and cover with coverslip. Motility is indicated by individual bacterial cells moving in random directions. Observe at either 400X or under oil immersion.

Further characterization of Gram-positive rods is generally unnecessary. Consult refs. 7 and 12 if further characterization of these organisms is required.

2. **Gram-positive cocci**. Streak MLA plate from APC media (MLA or BP), incubate 18-24 h at 35 ± 2°C, and test resultant growth for catalase activity and coagulase production (if catalase-positive).

   a. **Catalase test**. Add a drop of 3% H_2O_2 either to isolated colony or to clean microscope slide and place platinum loop carrying some isolate into the drop. Reaction is positive if oxygen gas evolves rapidly (bubble formation). (Nichrome wire loops may give false-positive reactions.) When H_2O_2 is placed directly on a colony the bacteria will be killed. Positive control (*Staphylococcus* or an enteric bacterium) and negative control (*Streptococcus*) should be run concurrently to ensure the quality of the H_2O_2 solution.
   b. **Coagulase test**. Inoculate small amount of growth from maintenance slant into 13 x 100 mm tube containing 0.2 ml BHI broth. Incubate 18-24 h at 35 ± 2°C; then add 0.5 ml reconstituted lyophilized rabbit coagulase plasma (with EDTA) and mix thoroughly. Incubate at 35 ± 2°C for 6 h and examine for clotting. Weakly coagulase-producing strains may require overnight incubation for clot formation to be evident. Include known coagulase-positive and known coagulase-negative organism with every set of samples. Consider all strains that yield positive coagulase reaction as *S. aureus*.

If no catalase is produced, inoculate bile esculin agar slant, a tube of TSB containing 6.5% NaCl, and a 5% sheep blood agar plate. Incubate 18-24 h at 35 ± 2°C. If organism blackens bile esculin medium and will grow in presence of 6.5% NaCl, report it as "Group D enterococcus" (*Enterococcus* spp.). If it blackens bile esculin medium, but will not grow in presence of 6.5% NaCl, report it as "Group D *Streptococcus*, not enterococcus." If it does not blacken bile esculin medium, report it as either alpha, beta, or gamma hemolytic *Streptococcus*. If 5% sheep blood agar is not available, report it as "*Streptococcus*, not Group D." Perform additional speciation of streptococci if required, using procedures outlined in ref.

7 or serological kits commercially available for this purpose, e.g., Phadebact (Pharmacia Diagnostics, Piscataway, NJ).

If catalase is produced, inoculate the following media with freshly isolated colony: mannitol salt agar, duplicate tubes of oxidative-fermentative (OF) medium with dextrose (overlay 1 tube with sterile vaspar or mineral oil; leave 1 tube loosely capped with no overlay), and enriched agar slant for use in coagulase test.

Report organism as *S. aureus* if it is coagulase-positive and/or will ferment mannitol; *S. epidermidis* if it is fermentative as well as oxidative on OF dextrose, is coagulase-negative, and will not ferment mannitol; or *Micrococcus* species if it is oxidative only on OF dextrose.

3. **Gram-negative rods**. Inoculate TSI agar slant, MacConkey agar plate, cetrimide agar, and MLA plate with all Gram-negative rods. Incubate 18-24 h at 35 ± 2°C. TSI slant/butt reactions of A/A or K/A (A = acidic; K = alkaline) + H_2S indicate an *Enterobacteriaceae* isolate. K/K, K/NC (NC = no change) or NC/NC reactions indicate nonfermentative (NF) Gram-negative bacilli. If TSI reactions are masked by hydrogen sulfide production, inoculate lactose and glucose carbohydrate broths and incubate 18-24 h at 35 ± 2°C. For an *Enterobacteriaceae* isolate, perf the following tests and use refs. 3, 6, 11-13 to interpret results. The API 20E or equivalent commercial kit may be used to identify to species level. Media necessary for tests are listed in C, above.

   If an organism grows on cetrimide agar or is identified as an NF Gram-negative bacillus, determine fluorescent and nonfluorescent pigment production, aerobic production of acid from either glucose, sucrose, xylose, or mannitol, and the production of nitrogen gas from an inorganic nitrogen source; carry out other necessary tests using media listed in D, above. Acetamide utilization, growth at 42°C, and gelatin liquefaction are important tests for distinguishing the three *Pseudomonas* species, *P. aeruginosa*, *P. fluorescens*, and *P. putida*. To interpret results of these tests, use refs. 4, 11, 12, 19 or the API nonfermentative kit and data base. Confirm putative *P. aeruginosa* isolates by the method outlined below.

4. Method for identification of *Pseudomonas aeruginosa*

   Identification of *P. aeruginosa* is of particular concern because this organism survives in eye-area cosmetics (21) and has been implicated in eye infections (20). It is opportunistically pathogenic to humans (10) and highly resistant to antibacterial agents such as quaternary ammonium compounds, penicillin, and many broad-spectrum antibiotics.

a. Presumptive identification

TSI agar slants. Transfer well-isolated typical colonies from cetrimide agar plates to TSI agar slants. Streak surface and stab butt. Incubate at 35°C for 24 ± 2 h. All slants having growth and an alkaline (red) slant and alkaline (red) butt should be considered as presumptive positive for *Pseudomonas* spp. and tested for oxidase and other biochemical reactions. Some pseudomonads may produce slight hydrogen sulfide in TSI, but this can be confused with soluble pigments produced by some species.

Oxidase Test

Oxidase test strips (for *Pseudomonas* spp.)	
Tetramethyl-**p**-phenylenediamine-dihydrochloride	1.0 g
Ascorbic acid	0.1 g
Distilled water	100 ml

Cut filter paper (Whatman No. 40) into small strips of about 10 x 40 mm. Shade in reagent. Drain. Spread strips on paper towels on a tray. Shade with paper towels, because light degrades the reagent; dry in 35°C incubator. (Reagent also degrades at higher temperature.) When dry, store in brown bottle at room temperature. Strips must be protected from light and moisture; they should be white. The strips are stable indefinitely.

Use platinum loop to smear mass of cells on portion of strip. (Nichrome wire gives false-positive reactions.) Read at 10 s, **no longer**. Positive is indicated by a deep purple color; negative is indicated by the absence of color or when a purple color appears after 10 s. *Pseudomonas* spp. are oxidase-positive.

b. **Biochemical tests**

From each positive presumptive TSI agar slant inoculate duplicate YE agar slants, Koser's citrate broth, malonate broth, decarboxylase basal medium containing arginine, motility nitrate agar, nutrient gelatin (CDC), Pseudomonas agar F, and Pseudomonas agar P.

YE agar slants. Inoculate duplicate YE agar slants. Incubate one slant at 35°C for 24 ± 2 h and one slant at 42°C for 24 ± 2 h. (**NOTE**: Eq agar slants to 42°C before inoculation, since other species of *Pseudomonas* may grow slightly at 42°C on nonpreincubated media, but will not grow on prewarmed slants.) Few *Pseudomonas* spp. other than *P. aeruginosa* will grow at 42°C. *P. aeruginosa* produces a fishy odor of trimethylamine on YE agar. Approximately 4% of all cultures routinely encountered fail to produce pigment. Because they are often confused with *Alcaligenes* spp., *Achromobacter* spp., or other species, the nonpigmented isolates should be tested further before they are discarded.

Koser's citrate broth. Inoculate broth and incubate at 35°C for 24 and 48 h. Citrate utilization is indicated by marked turbidity.

Malonate broth. Inoculate malonate broth. Incubate at 35°C for 24 ± 2 h. A positive test for utilization of malonate as a sole source of carbon is indicated by an indicator change from green to blue (alkaline).

Nitrate motility agar. To inoculate nitrate motility agar, streak surface and stab butt. Incubate at 35°C for 24 ± 2 h. Add a few drops each of sulfanilic acid and -naphthylamine. A resulting deep pink or red color indicates reduction of nitrate to nitrite. A negative color along with the presence of gas bubbles or cracks in the medium is considered a positive reaction and indicates the reduction of nitrate to nitrite to free nitrogen. **NOTE**: Always test a control tube incubated under the same conditions.

Arginine decarboxylase broth. Inoculate decarboxylase basal medium containing arginine. Screw down caps tightly to prevent aeration. Incubate at 35°C for 24 ± 2 h. Examine for growth. A positive reaction for arginine decarboxylation is indicated by no change in color of purple medium. A negative reaction is indicated by an indicator change to yellow (acid).

Gelatin liquefaction. Inoculate nutrient gelatin tubes and stab butt. Incubate at 25°C (room temperature) for at least 72 h. Chill before examining for liquefaction. Incubate negative tubes 1 week. Negative tubes are normally kept up to 6 weeks before discarding but clearly this is not practical. However, *P. aeruginosa* usually liquefies gelatin rapidly.

Pseudomonas agar F and Pseudomonas agar P. Inoculate poured plates of agar F and agar P by streaking. Incubate at 25°C for at least 3 days. Examine agar F with black light (longwave UV). Fluorescent water-

soluble pigments (pyoverdines) will diffuse into agar adjacent to streaks containing *Pseudomonas* spp.

Break up agar P with glass rod in approximately equal amount of distilled water and shake vigorously until water has removed as much pigment as possible. Decant into separator. In a chemical hood, add 5-10 ml chloroform to water in separator and shake (venting occasionally to prevent internal pressure). The blue pyocyanine will migrate to chloroform. Draw off chloroform layer into test tube. Add about 3 ml distilled water. Add 1 drop 1 N H_2SO_4. Pyocyanine becomes red and migrates to water. Discard solvent in special chloroform-waste bottle.

Flagella stain. If culture meets all other requirements for *P. aeruginosa*, including pigments, flagella stain is not needed. Use Clark methods or follow any other suitable method (7), e.g., Leifson or Bailey methods. A rapid wet mount method, using Ryu stain (8) has been reported. *P. aeruginosa* has a single polar flagellum. The other fluorescent pseudomonads have several flagella.

B. Biochemical results. Examine data in order as shown. **Do not skip**.

TSI		
Acid butt, alkaline slant, + gas	-	Not *P. aeruginosa*
Acid butt, acid slant, + gas	-	Not *P. aeruginosa*
Alkaline slant and butt, + H_2S	-	Possibly *P. aeruginosa*
YE agar		
No growth at 42°C	-	Not *P. aeruginosa*
Growth at 42°C	-	Probably *P. aeruginosa*
Arginine decarboxylase		
Negative	-	Probably not *P. aeruginosa*

Positive	-	Probably *P. aeruginosa*
Koser's citrate		
No growth	-	Probably not *P. aeruginosa*
Growth	--	Possibly *P. aeruginosa*
Malonate utilization		
Negative	--	Probably not *P. aeruginosa*
Positive	-	Possibly *P. aeruginosa*
Nitrate reduction		
Negative, no gas	-	Probably not *P. aeruginosa*
Positive	-	Possibly *P. aeruginosa*
Motility		
Negative	-	Probably not *P. aeruginosa*
Positive	-	Possibly *P. aeruginosa*
Flagellation		
Polar flagella	-	Possibly *P. aeruginosa*
Any other flagellation	-	Not *P. aeruginosa*
Pseudomonas agar F		
No fluorescent pigment	-	Not *P. aeruginosa*
Fluorescent water-soluble pigment (pyoverdine)	-	*P. aeruginosa*
Pseudomonas agar P		
No pigment	-	Not *P. aeruginosa*

If pigment is present, confirm as procyanine	-	*P. aeruginosa*

C. Interpretation

Cosmetic products are not expected to be aseptic; however, they must be completely free of high-virulence microbial pathogens, and the total number of aerobic microorganisms per gram must be low. Since there are no widely acceptable standards for numbers, temporary guidelines are used instead. For eye-area products, counts should not be greater than 500 colony forming units (CFU)/g; for non-eye-area products, counts should not be greater than 1000 CFU/g. The presence of pathogens would be particularly important in evaluating as unacceptable a cosmetic with a marginally acceptable count, e.g., 400 CFU/g for an eye-area product. Pathogens or opportunistic pathogens whose incidence would be of particular concern, especially in eye-area cosmetic products, include *S. aureus*, *Streptococcus pyogenes*, *P. aeruginosa* and other species, and *Klebsiella pneumoniae*. Some microbes normally regarded as nonpathogenic may be opportunistically pathogenic, e.g., in wounds.

D. **Cosmetic preservative efficacy**. The above guidelines for interpretation of results apply to cosmetic products before the time of use. Cosmetics contain antimicrobial preservatives and thus are expected to withstand a certain amount of abuse by users. Formerly, there were no validated tests for cosmetic preservative efficacy (9), although the test for pharmaceutical preservative efficacy in the U.S. Pharmacopeia (2) or the cosmetic test in the technical guidelines of the Cosmetic, Toiletry, and Fragrance Association (CTFA) (1) were used. Recently, the CTFA test has been AOAC validated (2b) for use with liquid cosmetics. A test for solid cosmetic preservative efficacy has been proposed (18). Cosmetics in reusable test kits, such as those in retail stores, can be microbiologically evaluated semiquantitatively by a sterile swab test (17).

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Chapter 27
Screening Method for Phosphatase (Residual) in Cheese

Author

(Return to Table of Contents)

Over the past ten years outbreaks of foodborne diseases have been caused by the consumption of dairy products made with raw or improperly pasteurized milks. Milk is pasteurized by heating at 62.8°C for 30 minutes or 71.7°C for 15 seconds. These temperatures kill all nonsporeforming pathogens and inactivate the native alkaline phosphatase (ALP) enzyme found in milk. The thermal resistance of ALP [EC 3.1.3.1] ortho-phosphoric monoester phosphohydrolase] is greater than that of nonsporeforming pathogenic microorganisms; therefore, liquid milk and milk products that show a negative result when tested for phosphatases are considered properly pasteurized and safe (4). Soft cheeses produced by fermentation (e.g., blue, Swiss, Camembert) show a positive result when analyzed for ALP, because ALP is produced by the microorganisms used during fermentation of the cheeses (2,3).

The method given below is an expansion of phosphatase (residual) in milk to cheese (1). The buffer has been changed because it was found that carbonate ions are inhibitory to bovine ALPs (5). Unlike the previous AOAC (16th ed.) method (sec. 946.03), the analysis is done in a single test tube and the reagents are the same for all of the cheeses tested.

This method is a screening method. A sample which exceeds the levels in Table 1 must be reanalyzed using AOAC method 946.03 (16th ed.) which is equivalent to AOAC method 16.275-16.277 (13th ed.), cited in 21 CFR 133.5.

A. Equipment and materials

1. Pipets or digital pipetter, able to dispense volumes ranging from 100 µl to 1 ml (Eppendorf or equivalent)
2. Pipetter tips
3. Centrifuge capable of holding 10-15 ml tubes and spinning them at least at RCF 2400 x *g*
4. Ice bath
5. Test tubes, 10-15 ml
6. Two water baths. The first is a boiling water bath used for pasteurization of controls; the second is maintained at 40°C and is used to incubate the test mixture.
7. Spectrophotometer capable of measuring at 650 nm.

B. Reagents

1. **AMP buffer.** Dissolve 10.0 g 2-amino-2-methyl-1-propanol in water. Adjust pH to 10.1 with 6 M HCl, add 10 ml Tergitol type 4, and dilute to 1 L with distilled water.
2. **Buffer substrate.** Dissolve 0.5 g phenol-free crystalline disodium phenyl phosphate in AMP buffer solution and dilute to 500 ml with AMP buffer. Prepare fresh daily.
3. **n-Butyl alcohol**, n-BuOH, b.p. 116-118°C
4. **Catalyst.** Dissolve 200 mg $CuSO_4.5H_2O$ in distilled water and dilute to 100 ml.
5. **CQC solution.** Dissolve 40 mg crystalline 2,6-dichloro-quinonechloroimide in 10 ml MeOH and transfer to dark bottle. Or prepare solution by dissolving 1 Indo-Phax tablet (containing catalyst) (available from Applied Research Institute, 141 Lewis St., Perth Amboy, NJ 08861) in 5 mL MeOH. Store in refrigerator. Discard after 1 week or when brown.
6. **CQC-catalyst solution.** Mix equal volumes of CQC solution and catalyst together. **Prepare fresh daily.**
7. **6 M HCL solution.** To 50 ml water add 50 ml concentrated hydrochloric acid. **ADD ACID TO WATER.**
8. **Phenol standard solutions.**

    a. **Stock solution**--Accurately weigh 1.000 g pure phenol, transfer to 1 L volumetric flask, dilute to volume with 0.1 N HCl, and mix (1 ml = 1 mg

phenol). Solution is stable several months in the refrigerator.

b. **Working solution**--Dilute 100 µl solution (a) to 100 ml with AMP buffer, and mix (1 ml = 1 µg phenol). **Prepare fresh daily.**
c. **Color standard solutions**--Dilute 0.0, 0.25, 0.5, 1.0, 2.5, and 5.0 ml of working solution (b) to 5.0 ml with AMP buffer in a series of test tubes. Add 0.5 ml water to each tube.

9. **Tergitol type 4 (also called Niaproof type 4).** This is an anionic surfactant, 7-ethyl-2 methyl-4 undecanol hydrogen sulfate, sodium salt (Sigma No. 4). **No substitutions**.
10. **Water**. Distilled or deionized.

C. Sampling of cheeses

Hard cheese. Take sample from interior with **clean** spatula or knife.

Soft and semisoft ripened cheese. Harden cheese by chilling in freezer. Avoid contaminating sample with microbial phosphatases from contamination that may be present on the surface. Sample by either of the following methods:

- Cut portion from end of loaf or side of cheese, extending in 5 cm (2 inches) if possible, to a point somewhat beyond center in case of small cheese. Cut slit 6-12 mm (1/4-1/2 inch) deep at least half way around portion and midway between top and bottom. Break portion into 2 parts, pulling apart so that break occurs on line with slit and taking care not to contaminate freshly exposed broken surface. Remove sample from freshly exposed surface at or near center of cheese.

- Remove surface of area to be sampled (e.g., end and adjacent sides) with clean knife or spatula to depth of 6 mm (1/4 inch). Clean instruments and hands with hot water and phenol-free soap, and wipe dry. Remove freshly exposed surface from same or greater depth, and repeat cleaning. Take sample from center of freshly exposed area, preferably at or near center of cheese if cheese is small.

Processed cheese and cheese spreads. Take sample from beneath surface with clean knife or spatula.

D. Screening test for phosphatase (residual) in cheese

Collect and weigh two 0.5 g samples into test tubes using the directions in section C. Add 0.5 ml water (one tube is the test portion; the second tube is for the boiled control or blank). Mash with a glass rod. Heat controls in a boiling water bath for 2 min, and

rapidly cool in ice bath.

To test portion and boiled controls add 5 ml buffer substrate and mix by vortex or inversion. Immediately incubate test portions, boiled controls, and color standard solutions in water bath 15 min at 40 ± 1°C (allow 1 min warm-up time for total of 16 min). Mix samples once during incubation.

Remove from water bath and add 0.2 ml of CQC catalyst solution or 0.1 ml of Indo-Phax solution. Mix, and immediately place back in 40°C water bath for 5 min. Remove from bath and cool in ice-water bath 5 min.

Add 3 ml butanol and extract by inverting the parafilm covered tubes 6 full turns. Chill in ice water bath 5 min. Centrifuge 5 min at 2400 x *g*.

Remove butanol (upper) layer into cuvettes with Pasteur pipet. Read absorbance of butanol extracts with spectrophotometer at 650 nm. Standard curve of µg phenol against absorbance should be a straight line. Subtract the µg phenol/g cheese of the boiled blank from the corresponding sample to get the µg phenol/g cheese for the sample. If less than zero, record as zero.

E. Interpretation of results

Table 1 lists different types of cheese, maximum amounts of phenol equivalents/g of cheese, and appropriate CFR reference. Any sample exceeding the levels listed in Table 1 is violative. If a particular cheese is not listed in Table 1, then it shall be considered violative if it exceeds 12 µg phenol equivalents/g cheese.

Table 1. List of cheeses[(a)] from CFR 21

Cheese	µg phenol/g cheese	CFR
Brick	20	133.108(a)(2)
Cheddar	12	133.113(a)(2)
Colby	12	133.118(c)(2)
Cook cheese, Koch Kaese	12	133.127(a)(2)
Washed curd and soaked curd	12	133.136(a)(2)
Edam	12	133.138(a)(2)

Gouda	12	133.144(a)(2)
Gruyere	12	133.149(a)(2)
Hard	12	133.150(c)(2)
Limburger	16	133.152(a)(2)
Monterey & Monterey Jack	12	133.153(a)(2)
Mozzarella & Scamorza	12	133.155(a)(2)
Low moisture Mozzarella & Scamorza	12	133.156(a)(2)
Muenster	12	133.16 (a)(2)
Pasteurized process	12	133.169(a)(2)
Process cheese food	12	133.173(a)(2)
Pasteurized Neufchatel, cheese spread with other foods	12	133.178(a)(2)
Provolone	12	133.181(a)(2)
Samsoe	12	133.185(a)(2)
Semisoft	20	133.187(c)(2)
Semisoft part-skim	20	133.188(c)(2)
Spiced	12	133.190(a)(2)
Swiss & Emmentaler	12	133.193(a)(2)

[a] **NOTE**: Different cheeses have different levels. The value per gram of cheese is obtained by multiplying the value listed in the CFR by 4.

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Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy | Accessibility/Help

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

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Appendix 1

Rapid Methods for Detecting Foodborne Pathogens

Author

(Return to Table of Contents)

Introduction

Authors Note: This section differs from others in this manual in that it lists methods that are not necessarily used by FDA. In addition, the detailed protocols for these methods are not presented, and the user is referred to the instructions that accompany the test kits. One reason for this departure is the incremental rate of change and innovation in rapid testing technology. The best of these new techniques should be evaluated individually by user labs for their particular needs, and also collaboratively for possible adoption as official methods by the AOAC International (1).

The following text and tables list many of the commercially available rapid methods; they are classified by the principles underlying the procedure used. The assay principles and some of the detailed procedures are discussed in other chapters of this manual and/or in the literature cited in the tables. The AOAC status of rapid tests is indicated for those methods that have been validated or evaluated by AOAC (1) and have been adopted as AOAC Official methods.

However, these methods continue to be modified or adapted, so that published information may not be the most current. Rapid methods are generally used as screening techniques, with negative results accepted as is, but positive results requiring **confirmation** by the appropriate official method, which, in many instances, is cultural. In many other instances, the rapid method has not been validated; therefore, the listing of a method or kit in this chapter in no way constitutes FDA recommendation or approval.

Rapid Methods

The rapid detection of pathogens and other microbial contaminants in food is critical for ensuring the safety of consumers. Traditional methods to detect foodborne bacteria often rely on time-consuming growth in culture media, followed by isolation, biochemical identification, and sometimes serology. Recent advances in technology make detection and identification faster, more convenient, more sensitive, and more specific than conventional assays -- at least in theory. These new methods are often referred to as "rapid methods", a subjective term used loosely to describe a vast array of tests that includes miniaturized biochemical kits, antibody- and DNA-based tests, and assays that are modifications of conventional tests to speed up analysis (8, 15, 16, 24, 36). Some of these assays have also been automated to reduce hands-on manipulations. With few exceptions, almost all assays used to detect specific pathogens in foods require some growth in an enrichment medium before analysis.

Experts who were surveyed in 1981 (19) about future developments in methods used for food microbiology, accurately predicted the widespread use of miniaturized biochemical kits for the identification of pure cultures of bacteria isolated from food. Most consist of a disposable device containing 15 - 30 media or substrates specifically designed to identify a bacterial group or species. With the exception of a few kits where results can be read in 4 hrs, most require 18-24 hrs incubation. In general, miniaturized biochemical tests are very similar in format and performance, showing 90-99% accuracy in comparison to conventional methods (5, 16, 21). However, kits that have been in use longer may have a more extensive identification database than newer tests. Most miniaturized kits are designed for enteric bacteria, but kits for the identification of non-*Enterobacteriaceae* are also available, including for *Campylobacter*, *Listeria*, anaerobes, non-fermenting Gram-negative bacteria and for Gram-positive bacteria (Table 1).

Advances in instrumentation have enabled automation of the miniaturized biochemical identification tests. These instruments can incubate the reactions and automatically monitor biochemical changes to generate a phenotypic profile, which is then compared with the provided database stored in the computer to provide an identification (8, 23, 35). Other automated systems identify bacteria based on compositional or metabolic properties, such as fatty acid profiles, carbon oxidation profiles (28) or other traits (Table 1).

Not forecast in that 1981 survey were the potential applications of immunological and genetic techniques in food microbiology (19). During the 1980s, major advances in basic research were transferred rapidly to applied areas, as "biotechnology" companies emerged and sought markets in the diagnostic field (11). DNA and antibody-based assays for numerous microbes or their toxins are now available commercially (12).

There are many DNA-based assay formats, but only probes, PCR and bacteriophage have been developed commercially for detecting foodborne pathogens. Probe assays generally target ribosomal RNA (rRNA), taking advantage of the fact that the higher copy number of bacterial rRNA provides a naturally amplified target and affords greater assay sensitivity (6, 14, 25, 37) (Table 2).

The basic principle of DNA hybridization is also being utilized in other technologies, such as the polymerase chain reaction (PCR) assay, where short fragments of DNA (probes) or primers are hybridized to a specific sequence or template, which is then enzymatically amplified by *Taq* polymerase using a thermocycler (2, 22). Theoretically, PCR can amplify a single copy of DNA by a million fold in less than 2 hrs; hence its potential to eliminate, or greatly reduce the need for cultural enrichment. However, the presence of inhibitors in foods and in many culture media can prevent primer binding and diminish amplification efficiency (26, 34), so that the extreme sensitivity achievable by PCR with pure cultures is often reduced when testing foods. Therefore, some cultural enrichment is still required prior to analysis (Table 2).

The highly specific interaction of phage with its bacterial host has also been used to develop assays for foodborne pathogens (38). One example is an assay for *Salmonella*, in which a specific bacteriophage was engineered to carry a detectable marker (ice nucleation gene). In the presence of *Salmonella*, the phage confers the marker to the host, which then expresses the phenotype to allow detection (Table 2).

The highly specific binding of antibody to antigen, especially monoclonal antibody, plus the simplicity and versatility of this reaction, has facilitated the design of a variety of antibody assays and formats, and they comprise the largest group of rapid methods being used in food testing (3, 10, 12, 33). There are 5 basic formats of antibody assays (12), the simplest of which is latex agglutination (LA), in which antibody-coated colored latex beads or colloidal gold particles are used for quick serological identification or typing of pure culture isolates of bacteria from foods (7, 12). A modification of LA, known as reverse passive latex agglutination (RPLA), tests for soluble antigens and is used mostly in testing for toxins in food extracts or for toxin production by pure cultures (12) (Table 3).

In the immunodiffusion test format, an enrichment sample is placed in a gel matrix with the antibody; if the specific antigen is present, a visible line of precipitation is formed (30).

The enzyme-linked immunosorbent assay (ELISA) is the most prevalent antibody assay format used for pathogen detection in foods (3, 33). Usually designed as a "sandwich" assay, an antibody bound to a solid matrix is used to capture the antigen from enrichment cultures and a second antibody conjugated to an enzyme is used for detection. The walls of wells in microtiter plates are the most commonly used solid support; but ELISAs have also been designed using dipsticks, paddles, membranes, pipet tips or other solid matrices (12) (Table 3).

Antibodies coupled to magnetic particles or beads are also used in immunomagnetic separation (IMS) technology to capture pathogens from pre-enrichment media (31). IMS is analogous to selective enrichment, but instead of using antibiotics or harsh reagents that can cause stress-injury, an antibody is used to capture the antigen, which is a much milder alternative. Captured antigens can be plated or further tested using other assays.

Immunoprecipitation or immunochromatography, still another antibody assay format, is based on the technology developed for home pregnancy tests. It is also a "sandwich" procedure but, instead of enzyme conjugates, the detection antibody is coupled to colored latex beads or to colloidal gold. Using only a 0.1 ml aliquot, the enrichment sample is wicked across a series of chambers to obtain results (9). These assays are extremely simple, require no washing or manipulation and are completed within 10 minutes after cultural enrichment (Table 3).

The last mentioned "category" of rapid methods includes a large variety of assays, ranging from specialized media to simple modifications of conventional assays, which result in saving labor, time, and materials. Some, for instance, use disposable cardboards containing dehydrated media, which eliminates the need for agar plates, constituting savings in storage, incubation and disposal procedures (4, 5). Others incorporate specialized chromogenic and fluorogenic substrates in media to rapidly detect trait enzymatic activity (13, 17, 20, 27, 29). There are also tests that measure bacterial adenosine triphosphate (ATP), which (although not identifying specific species), can be used to rapidly enumerate the presence of total bacteria (Table 4).

Applications and Limitations of Rapid Methods

Almost all rapid methods are designed to detect a single target, which makes them ideal for use in quality control programs to quickly screen large numbers of food samples for the presence of a particular pathogen or toxin. A positive result by a rapid method however, is only regarded as presumptive and must be confirmed by standard methods (11). Although confirmation may extend analysis by several days, this may not be an imposing limitation, as negative results are most often encountered in food analysis.

Most rapid methods can be done in a few minutes to a few hours, so they are more rapid than traditional methods. But, in food analysis, rapid methods still lack sufficient sensitivity and

specificity for direct testing; hence, foods still need to be culture-enriched before analysis (12). Although enrichment is a limitation in terms of assay speed, it provides essential benefits, such as diluting the effects of inhibitors, allowing the differentiation of viable from non-viable cells and allowing for repair of cell stress or injury that may have resulted during food processing.

Evaluations of rapid methods show that some perform better in some foods than others. This can be attributed mostly to interference by food components, some of which can be especially troublesome for the technologies used in rapid methods. For example, an ingredient can inhibit DNA hybridization or *Taq* polymerase, but has no effect on antigen-antibody interactions and the converse situation may also occur (12). Since method efficiencies may be food dependent, it is advisable to perform comparative studies to ensure that a particular assay will be effective in the analysis of that food type.

The specificity of DNA based assays is dictated by short probes; hence, a positive result, for instance with a probe or primers specific for a toxin gene, only indicates that bacteria with those gene sequences are present and that they have the potential to be toxigenic. But, it does not indicate that the gene is actually expressed and that the toxin is made. Likewise, in clostridial and staphylococcal intoxication, DNA probes and PCR can detect only the presence of cells, but are of limited use in detecting the presence of preformed toxins (12).

Currently, there are at least 30 assays each for testing for *E. coli* O157:H7 and for *Salmonella*. Such a large number of options can be confusing and overwhelming to the user, but, more importantly, has limited the effective evaluation of these methods. As a result, only few methods have been officially validated for use in food testing (1,11).

Conclusions

As a rapid method is used more frequently, its benefits and at the same time, its limitations also become more apparent. This section only briefly described some of the rapid method formats and selected problems encountered when using these assays in food analysis. However, because of the complex designs and formats of these tests, coupled with the difficulties of testing foods, users must exercise caution when selecting rapid methods and to also evaluate these tests thoroughly, as some may be more suitable than others for distinct testing situations or for assaying certain types of food. Lastly, technology continues to advance at a great pace and next generation assays, such as biosensors (18) and DNA chips (32) already are being developed that potentially have the capability for near real-time and on-line monitoring of multiple pathogens in foods.

NOTE: The listings provided in Tables 1-4 are intended for general reference only and do not indicate endorsement or approval by FDA for use in food analysis.

Table 1. Partial list of miniaturized biochemical kits and automated systems for identifying foodborne bacteria* (5, 8, 15, 16, 21, 35, 36).

System	Format	Manufacturer	Organisms
API[b]	biochemical	bioMerieux	*Enterobacteriaceae, Listeria, Staphylococcus, Campylobacter*, Non-fermenters, anaerobes
Cobas IDA	biochemical	Hoffmann LaRoche	*Enterobacteriaceae*
Micro-ID[b]	biochemical	REMEL	*Enterobacteriaceae, Listeria*
EnterotubeII	biochemical	Roche	*Enterobacteriaceae*
Spectrum 10	biochemical	Austin Biological	*Enterobacteriaceae*
RapID	biochemical	Innovative Diag.	*Enterobacteriaceae*
BBL Crystal	biochemical	Becton Dickinson	*Enterobacteriaceae, Vibrionaceae*, Non-fermenters, anaerobes
Minitek	biochemical	Becton Dickinson	*Enterobacteriaceae*
Microbact	biochemical	Microgen	*Enterobacteriaceae*, Gram negatives, Non-fermenters, *Listeria*
Vitek[b]	biochemical[a]	bioMerieux	*Enterobacteriaceae*, Gram negatives, Gram positives
Microlog	C oxidation[a]	Biolog	*Enterobacteriaceae*, Gram negatives, Gram positives
MIS[b]	Fatty acid[a]	Microbial-ID	*Enterobacteriaceae, Listeria, Bacillus, Staphylococcus, Campylobacter*

Walk/Away	biochemical[a]	MicroScan	*Enterobacteriaceae, Listeria, Bacillus, Staphylococcus, Campylobacter*
Replianalyzer	biochemical[a]	Oxoid	*Enterobacteriaceae, Listeria, Bacillus, Staphylococcus, Campylobacter*
Riboprinter	nucleic acid[a]	Qualicon	*Salmonella, Staphylococcus, Listeria, Escherichia coli*
Cobas Micro-ID	biochemical[a]	Becton Dickinson	*Enterobacteriaceae*, Gram negatives, Non-fermenters
Malthus[b]	conductance[a]	Malthus	*Salmonella, Listeria, Campylobacter, E. coli, Pseudomonas*, coliforms
Bactometer	impedance[a]	bioMerieux	*Salmonella*

* Table modified from: Feng, P., App.I., FDA Bacteriological Analytical Manual, 8A ed.
[a] Automated systems
[b] Selected systems adopted AOAC Official First or Final Action.

NOTE: This table is intended for general reference only and lists known available methods. Presence on this list does not indicate verification, endorsement, or approval by FDA for use in food analysis.

Table 2. Partial list of commercially-available, nucleic acid-based assays used in the detection of foodborne bacterial pathogens* (2, 5, 8, 12, 14, 22, 25, 36, 37).

Organism	Trade Name	Format	Manufacturer
Clostridium botulinum	Probelia	PCR	BioControl
Campylobacter	AccuProbe	probe	GEN-PROBE
	GENE-TRAK	probe	GENE-TRAK
Escherichia coli	GENE-TRAK	probe	GENE-TRAK
E. coli O157:H7	BAX	PCR[a]	Qualicon
	Probelia	PCR	BioControl

Listeria	GENE-TRAK[c]	probe	GENE-TRAK
	AccuProbe	probe	GEN-PROBE
	BAX	PCR	Qualicon
	Probelia	PCR	BioControl
Salmonella	GENE-TRAK[c]	probe	GENE-TRAK
	BAX	PCR	Qualicon
	BIND[b]	phage	BioControl
	Probelia	PCR	BioControl
Staphylococcus aureus	AccuProbe	probe	GEN-PROBE
	GENE-TRAK	probe	GENE-TRAK
Yersinia enterocolitica	GENE-TRAK	probe	GENE-TRAK

* Table modified from: Feng, P., App.I, FDA Bacteriological Analytical Manual, 8A ed.

[a] Polymerase chain reaction

[b] Bacterial Ice Nucleation Diagnostics

[c] Adopted AOAC Official First or Final Action

NOTE: This table is intended for general reference only and lists known available methods. Presence on this list does not indicate verification, endorsement, or approval by FDA for use in food analysis.

Table 3. Partial list of commercially-available, antibody-based assays for the detection of foodborne pathogens and toxins* (3, 5, 8, 12, 33, 36).

Organism/toxin	Trade Name	Assay Format[a]	Manufacturer
Bacillus cereus diarrhoeal toxin	TECRA	ELISA	TECRA
	BCET	RPLA	Unipath
Campylobacter	Campyslide	LA	Becton Dickinson
	Meritec-campy	LA	Meridian
	MicroScreen	LA	Mercia

	VIDAS	ELFA[b]	bioMerieux
	EiaFOSS	ELISA[b]	Foss
	TECRA	ELISA	TECRA
Clostridium botulinum toxin	ELCA	ELISA	Elcatech
C. perfringens enterotoxin	PET	RPLA	Unipath
Escherichia coli			
EHEC**[c] O157:H7	RIM	LA	REMEL
	E. coli O157	LA	Unipath
	Prolex	LA	PRO-LAB
	Ecolex O157	LA	Orion Diagnostica
	Wellcolex O157	LA	Murex
	E. coli O157	LA	TechLab
	O157&H7	sera	Difco
	PetrifilmHEC	Ab-blot	3M
	EZ COLI	Tube-EIA	Difco
	Dynabeads	Ab-beads	Dynal
	EHEC-TEK	ELISA	Organon-Teknika
	Assurance[e]	ELISA	BioControl
	HECO157	ELISA	3M Canada
	TECRA	ELISA	TECRA
	E. coli O157	ELISA	LMD Lab
	Premier O157	ELISA	Meridian
	E. coli O157:H7	ELISA	Binax
	E. coli Rapitest	ELISA	Microgen
	Transia card	ELISA	Transia
	E. coli O157	EIA/capture	TECRA
	VIP[e]	Ab-ppt	BioControl

	Reveal	Ab-ppt	Neogen
	Quix Rapid O157	Ab-ppt	Universal HealthWatch
	ImmunoCardSTAT	Ab-ppt	Meridian
	VIDAS	ELFA[b]	bioMerieux
	EiaFOSS	ELISA[b]	Foss
Shiga toxin (Stx)	VEROTEST	ELISA	MicroCarb
	Premier EHEC	ELISA	Meridian
	Verotox-F	RPLA	Denka Seiken
ETEC [c]			
Labile toxin (LT)	VET-RPLA	RPLA	Oxoid
Stabile toxin (ST)	E. coli ST	ELISA	Oxoid
Listeria	Microscreen	LA	Microgen
	Listeria Latex	LA	Microgen
	Listeria-TEK[e]	ELISA	Organon Teknika
	TECRA[e]	ELISA	TECRA
	Assurance[e]	ELISA	BioControl
	Transia Listeria	ELISA	Transia
	Pathalert	ELISA	Merck
	Listertest	Ab-beads	VICAM
	Dynabeads	Ab-beads	Dynal
	VIP[e]	Ab-ppt	BioControl
	Clearview	Ab-ppt	Unipath
	RAPIDTEST	Ab-ppt	Unipath
	VIDAS[e]	ELFA[b]	bioMerieux
	EiaFOSS	ELISA[b]	Foss
	UNIQUE	Capture-EIA	TECRA
Salmonella	Bactigen	LA	Wampole Labs

	Spectate	LA	Rhone-Poulenc
	Microscreen	LA	Mercia
	Wellcolex	LA	Laboratoire Wellcome
	Serobact	LA	REMEL
	RAPIDTEST	LA	Unipath
	Dynabeads	Ab-beads	Dynal
	Screen	Ab-beads	VICAM
	CHECKPOINT	Ab-blot	KPL
	1-2 Test[e]	diffusion	BioControl
	SalmonellaTEK[e]	ELISA	Organon Teknika
	TECRA[e]	ELISA	TECRA
	EQUATE	ELISA	Binax
	BacTrace	ELISA	KPL
	LOCATE	ELISA	Rhone-Poulenc
	Assurance[e]	ELISA	BioControl
	Salmonella	ELISA	GEM Biomedical
	Transia	ELISA	Transia
	Bioline	ELISA	Bioline
	VIDAS[e]	ELFA[b]	bioMerieux
	OPUS	ELISA[b]	TECRA
	PATH-STIK	Ab-ppt	LUMAC
	Reveal	Ab-ppt	Neogen
	Clearview	Ab-ppt	Unipath
	UNIQUE[e]	Capture-EIA	TECRA
Shigella	Bactigen	LA	Wampole Labs
	Wellcolex		Laboratoire Wellcome
Staphylococcus aureus	Staphyloslide	LA	Becton Dickinson
	AureusTest[e]	LA	Trisum

	Staph Latex	LA	Difco
	S. aureus VIA	ELISA	TECRA
enterotoxin	SET-EIA	ELISA	Toxin Technology
	SET-RPLA	RPLA	Unipath
	TECRA[e]	ELISA	TECRA
	Transia SE	ELISA	Transia
	RIDASCREEN	ELISA	R-Biopharm
	VIDAS	ELFA[b]	bioMerieux
	OPUS	ELISA[b]	TECRA
Vibrio cholera	choleraSMART	Ab-ppt	New Horizon
	bengalSMART	Ab-ppt	New Horizon
	choleraScreen	Agglutination	New Horizon
	bengalScreen	Agglutination	New Horizon
enterotoxin	VET-RPLA[d]	RPLA	Unipath

* Table modified from: Feng, P., App.I, FDA Bacteriological Analytical Manual, 8A ed.

[a] Abbreviations: ELISA, enzyme linked immunosorbent assay; ELFA, enzyme linked fluorescent assay; RPLA, reverse passive latex agglutination; LA, latex agglutination; Ab-ppt, immunoprecipitation.

[b] Automated ELISA

[c] EHEC - Enterohemorrhagic *E. coli*; ETEC - enterotoxigenic *E. coli*

[d] Also detects *E. coli* LT enterotoxin

[e] Adopted AOAC Official First or Final Action

**** CAUTION: unless the assays claim that they are specific for the O157:H7 serotype, most of these tests detect only the O157 antigen; hence will also react with O157 strains that are not of H7 serotype. These O157, non-H7 strains, generally do not produce Shiga toxins and are regarded as not pathogenic for humans. Furthermore, some antibodies to O157 can also cross react with *Citrobacter, E. hermanii* and other enteric organisms.**

NOTE: This table is intended for general reference only and lists known available methods. Presence on this list does not indicate verification, endorsement, or approval by FDA for use in food analysis.

Table 4. Partial list of other commercially available rapid methods and specialty substrate media for detection of foodborne bacteria* (4, 8, 13, 20, 27, 36).

Organism	Trade Name	Format[a]	Assay Manufacturer
Bacteria	Redigel[b]	Media	RCR Scientific
	Isogrid[b]	HGMF	QA Labs
	Enliten	ATP	Promega
	Profile-1	ATP	New Horizon
	Biotrace	ATP	Biotrace
	Lightning	ATP	Idexx
	Petrifilm[b]	media-film	3M
	Sim Plate	media	Idexx
Coliform/ *E. coli*	Isogrid[b]	HGMF/MUG	QA Labs
	Petrifilm[b]	media-film	3M
	SimPlate	media	Idexx
	Redigel	Media	RCR Scientific
	ColiQuik[c]	MUG/ONPG	Hach
	ColiBlue[c]	media	Hach
	Colilert[b,c]	MUG/ONPG	Idexx
	LST-MUG[b]	MPN media	Difco & GIBCO
	ColiComplete[b]	MUG-Xgal	BioControl
	Colitrak	MPN-MUG	BioControl
	ColiGel & E*Colite[c]	MUG-Xgal	Charm Sciences
	CHROMagar	Medium	CHROMagar
E. coli	MUG disc	MUG	REMEL
	CHROMagar	Medium	CHROMagar

EHEC[d]	Rainbow Agar	Medium	Biolog
	BCMO157:H7	Medium	Biosynth
	Fluorocult O157:H7	Medium	Merck
Listeria monocytogenes	BCM	Medium	Biosynth
Salmonella	Isogrid[b]	HGMF	QA Labs
	OSRT	Medium/ motility	Unipath (Oxoid)
	Rambach	Medium	CHROMagar
	MUCAP	C8esterase	Biolife
	XLT-4	Medium	Difco
	MSRV[b]	Medium	
Yersinia	Crystal violet	Dye binding	Polysciences

* Table modified from: Feng, P., App.I, FDA Bacteriological Analytical Manual, 8A ed.

[a] Abbreviations: APC, aerobic plate count; HGMF, hydrophobic grid membrane filter; ATP, adenosine triphosphate; MUG, 4-methylumbelliferyl-ß-D-glucuronide; ONPG, O-nitrophenyl ß-D-galactoside; MPN, most probable number.

[b] Adopted AOAC Official First or Final Action.

[c] Application for water analysis

[d] EHEC - enterohemorrhagic *Escherichia coli*

NOTE: This table is intended for general reference only and lists known available methods. Presence on this list does not indicate verification, endorsement, or approval by FDA for use in food analysis.

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Author: Peter Feng

Top

B A M | B A M Media | B A M Reagents | Bad Bug Book

Foods Home | FDA Home | Search/Subject Index | Disclaimers & Privacy Policy

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

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Appendix 2
Most Probable Number from Serial Dilutions

Author

(Return to Table of Contents)

Background

The most probable number (MPN) is particularly useful for low concentrations of organisms (<100/g), especially in milk and water, and for those foods whose particulate matter may interfere with accurate colony counts. The following background observations are adapted and extended from the article on MPN by James T. Peeler and Foster D. McClure in the *Bacteriological Analytical Manual* (BAM), 7th edition.

Only viable organisms are enumerated by the MPN determination. If, in the microbiologist's experience, the bacteria in the prepared sample in question can be found attached in chains that

are not separated by the preparation and dilution, the MPN should be judged as an estimate of growth units (GUs) or colony-forming units (CFUs) instead of individual bacteria. For simplicity, however, this appendix will speak of these GUs or CFUs as individual bacteria.

The following assumptions are necessary to support the MPN method. The sample is prepared in such a way that the bacteria are distributed randomly within it. The bacteria are separate, not clustered together, and they do not repel each other. The growth medium and conditions of incubation have been chosen so that every inoculum that contains even one viable organism will produce detectable growth.

The essence of the MPN method is the dilution of a sample to such a degree that inocula will sometimes but not always contain viable organisms. The "outcome", i.e., the numbers of inocula producing growth at each dilution, will imply an estimate of the original, undiluted concentration of bacteria in the sample. In order to obtain estimates over a broad range of possible concentrations, microbiologists use serial dilutions, incubating several tubes (or plates, etc.) at each dilution.

The first accurate estimation of the number of viable bacteria by the MPN method was published by McCrady (1915). Halvorson and Ziegler (1933), Eisenhart and Wilson (1943), and Cochran (1950) published articles on the statistical foundations of the MPN. Woodward (1957) recommended that MPN tables should omit those combinations of positive tubes (high for low concentrations and low for high concentrations) that are so improbable that they raise concerns about laboratory error or contamination. De Man (1983) published a confidence interval method that was modified to make the tables for this appendix.

Confidence Intervals

The 95 percent confidence intervals in the tables have the following meaning.

> Before the tubes are inoculated, the chance is at least 95 percent that the confidence interval associated with the eventual result will enclose the actual concentration.

It is possible to construct many different sets of intervals that satisfy this criterion. This manual uses a modification of the method of de Man (1983). De Man calculated his confidence limits iteratively from the smallest concentrations upward. Because this manual estimates concentrations of pathogens, the intervals have been shifted slightly upward by iterating from the largest concentrations downward.

Improbable Outcomes

When excluding improbable outcomes, de Man's (1983) preferred degree of improbability was adopted. The included combinations of positive tubes are those that would be among the 99.985 percent most likely to result if their own MPNs were the actual bacterial concentrations. Therefore the entire set of results on any 10 different samples will be found in these tables at least 99 percent of the time.

Precision, Bias, and Extreme Results

The MPNs and confidence limits have been expressed to 2 significant digits. For example, the entry "400" has been rounded from a number between 395 and 405.

Numerous articles have noted a bias toward over-estimation of microbial concentrations by the MPN. Garthright (1993) has shown, however, that there is no appreciable bias when the concentrations are expressed as logarithms, the customary units used for regressions and for combining results. Therefore, these MPNs have not been adjusted for bias.

Prior to the first revision, the 8th edition tables showed the MPN for the (0,0,0) outcomes as less than the MPN of the (1,0,0) outcome. This made good numerical sense, but made for unacceptable complexity in trying to write acceptance standards for raw materials in terms of the BAM. This revision returns to the prior practice of recording the MPN for the (0,0,0) outcomes as less than the MPN for the (0,0,1) outcome, so that standards can once again be written in a simple manner in terms of all-negative outcomes.

Since no particular density is indicated for an outcome of (0,0,0), a density must be assigned arbitrarily (and stated explicitly in the report) in order to calculate statistics. For the logarithm of the density, log[0.5*MPN(0,0,1)] is a reasonable choice. For statistics using the (non-logarithmic) density itself, calculate once with a density of 0.0 and once with a density of 0.5*MPN(0,0,1). Either report both statistics or report one statistic accompanied by a comment on the difference between that statistic and the other one.

Selecting Three Dilutions for Table Reference

An MPN can be computed for any numbers of tubes at any numbers of dilutions. MPN values based on 3 decimal dilutions, however, are very close approximations to those based on 4 or more dilutions. When more than three dilutions are used in a decimal series of dilutions, refer to the 3 dilution table according to the following two cases, illustrated by the table of examples below (with 5 tubes at each dilution).

Case 1. One or more dilutions show all tubes positive. Select the highest dilution that gives positive results in all tubes (even if a lower dilution gives negative results) and the next two higher dilutions (ex. a and b); if positive results occur in higher unselected dilutions, shift each

selection to the next higher dilution (ex. c). If there are still positive results in higher unselected dilutions, add those higher-dilution positive results to the results for the highest selected dilution (ex. d). If there were not enough higher dilutions tested to select three dilutions, then select the next lower dilutions (ex. e).

Case 2. No dilutions show all tubes positive. Select the 3 lowest dilutions (ex. f). If there are positive results in higher unselected dilutions, add those higher-dilution positive results to the results for the highest selected dilution (ex. g).

Example	1.0 g	0.1 g	0.01 g	0.001 g	0.0001 g	Combination of Positives	MPN/g
a	5	5	1	0	0	5-1-0	33
b	4	5	1	0	0	5-1-0	33
c	5	4	4	1	0	4-4-1	40
d	5	4	4	0	1	4-4-1	40
e	5	5	5	5	2	5-5-2	5400
f	0	0	1	0	0	0-0-1	0.20
g	4	4	1	1	0	4-4-2	4.7

Other compendia of methods require that no excluded lower dilutions may have any negative tubes. This manual differs when the highest dilution that makes all tubes positive follows a lower dilution that has one or more negative tubes. Example b above would be read according to other compendia as (4, 5, 1, 0, 0) with MPN 4.8/g. The BAM reading, 33/g, is 7 times larger. The BAM selection method is based on FDA experience that for some organisms in some food matrices such outcomes as (2, 5, 1, 0, 0) and (0, 3, 1, 0, 0) occur too often to be random occurrences. In these cases, it appears that some factor (a competing organism or adverse set of compounds) is present at the lowest dilutions in such concentrations that it can reduce the detection of the target microbes.

Until further research clarifies this situation, analysts should continue to exclude dilutions lower than the highest dilution with all tubes positive. The findings should, however, report the extent to which such lower, partially-negative dilutions have been excluded. Analysts working with materials with known limited complexity in research settings will want to use their professional judgement to read outcomes such as (4, 5, 1, 0, 0) as (4, 5, 1, 0, 0). They may also read outcomes such as (3, 5, 1, 0, 0) as too improbable to record, because they are not included in the tables.

Inconclusive Tubes

In special cases where tubes or plates cannot be judged either positive or negative (e.g., plates overgrown by competing microflora at low dilutions), these tubes or plates should be excluded from the results. The entire dilutions at or below those in which exclusion occurs may be excluded. If it is not desired to exclude the remaining tubes at or below the dilution of the excluded tubes, the results will now have an unequal number of tubes at several dilutions. These MPNs can be solved by computer algorithms or estimated by Thomas's Rule below. Their confidence limits can be solved by Haldane's method, described below Thomas's rule.

Conversion of Table Units

The tables below apply directly to inocula 0.1, 0.01, and 0.001 g. When different inocula are selected for table reference, multiply the MPN/g and confidence limits by whatever multiplier is required to make the inocula match the table inocula. For example, if the inocula were 0.01, 0.001, and 0.0001 for 3 tubes each, multiplying by 10 would make these inocula match the table inocula. If the positive results from this 3 tube series were (3, 1, 0), one would multiply the Table 1 MPN/g estimate, 43/g, by 10 to arrive at 430/g.

Approximations for an Unusual Series of Dilutions

The MPNs for a series of dilutions not addressed by any tables (e.g., resulting from accidental loss of some tubes) may be computed by iteration or may be estimated as follows. First, select the lowest dilution that doesn't have all positive results. Second, select the highest dilution with at least one positive result. Finally, select all the dilutions between them. Use only the selected dilutions in the following formula of Thomas (1942):

$$\mathbf{MPN/g = P/[(N*T)^{(1/2)}]}$$

where $(\ )^{1/2}$ means square root,

P is the number of positive results,

T is the total grams of sample in the selected dilutions, and

N is the grams of sample in the selected negative tubes.

The following examples will illustrate the application of Thomas's formula. We assume that the dilutions are 1.0, 0.1, 0.01, 0.001, and 0.0001 g.

Example (1). Dilution results are (5/5, 10/10, 4/10, 2/10, 0/5). We use only(--,--, 4/10, 2/10,--); so T= 10*0.01 + 10*0.001 = 0.11. There are 6 negative tubes at 0.01 and 8 negative tubes at 0.001, so N = 6*0.01 + 8*0.001 = 0.068. There are 6 positive tubes, so

$$\mathbf{MPN/g=6/(0.068 * 0.11)^{(1/2)}=6/0.086=70/g}$$

Example (2). Dilution results are (5/5, 10/10, 10/10, 0/10, 0/5). We use only(--,--, 10/10, 0/10,--), so by Thomas's formula,

$$\mathbf{MPN/g=10/(0.01 \times 0.11)^{(1/2)}=10/.0332=300/g}$$

These two approximated MPNs compare well with the MPNs for (10, 4, 2) and (10,10,0) (i.e., 70/g and 240/g, respectively).

Example (2) above is a special case for which an exact solution for the two selected dilutions can be calculated directly, as follows. When all the results at the highest dilutions are negative, all the results at the remaining dilutions are positive, and when V is an individual inoculum at the highest dilution with all positive tubes, then

$$\mathbf{MPN/g=(1/V)[2.303 \log_{10} (T/N)]}$$

where T and N are defined as for Thomas's formula. For the second example above, the third dilution is the highest with positive portions, so V = 0.01. The MPN for the third and fourth dilution would be exactly

$$\mathbf{MPN/g=(1/0.01) \times [2.303 \log_{10} (0.11/0.01)]=240/g}$$

UPDATED

Approximate confidence limits for any dilution test outcome can be calculated by first estimating the standard error of $\log_{10}$(MPN) by the method of Haldane. We describe the method for 3 dilutions, but it can be shortened to 2 or extended to any number.

Let z1, z2, z3 denote the inoculation amounts at the largest to the smallest amounts (e.g., z1 = 0.1 g, z2 = 0.01 g, z3 = 0.001 g in these tables).

Let p1, p2, p3 denote the numbers of positive tubes at the corresponding dilutions. For legibility, we denote y^x by "y**x" and "y times x" by "y*x". As usual, we denote the base of the natural logarithm by "e".

Now we compute

$$\mathbf{T1 = e^{**}(-mpn*z1),\ T2 = e^{**}(-mpn*z2),\ etc.}$$

Then we compute

$$\mathbf{B = [p1*z1*z1*T1/((T1 - 1)^{**}2)] + ... + [p3*z3*z3*T3/((T3 - 1)^{**}2)].}$$

Finally, we compute

$$\mathbf{Standard\ Error\ of\ Log_{10}(mpn) = 1/(2.303*mpn*(B^{**}0.5))}$$

Now the 95 percent confidence intervals, for example, are found at

$$\mathbf{Log_{10}(mpn) \pm 1.96*(Standard\ Error).}$$

MPN for a Single Dilution

The MPN for a single dilution is very simple to compute. Let "t" denote the number of tubes that will be inoculated, let "n" denote the number of negative tubes that result, and let "z" denote the grams of inoculate planted in each individual tube.

Then

$$\mathbf{MPN/g = (1/z)*2.303*log_{10}(t/n).}$$

By Haldane's approximation, the standard error is found as above with the following simpler formula for B:

$$\mathbf{B = z*z*n*t/(t-n).}$$

Special requirements and tables included

Requests for special computations and different designs will be honored as resources permit. Designs may be requested with more or less than 3 dilutions, uneven numbers of tubes, different confidence levels, etc. (Telephone or write the Division of Mathematics, FDA/CFSAN, 200 C St., SW, Washington, DC 20204.) The most-published designs, three 10-

fold dilutions with 3, 5, 8, or 10 tubes at each dilution, are presented here.

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In response to requests, an 8 tube MPN tube has been developed and can be accessed by activating the hyperlink for Table 4 below.

Tables

Table 1. For 3 tubes each at 0.1, 0.01, and 0.001 g inocula, the MPNs per gram and 95 percent confidence intervals.

Pos. tubes			MPN/g	Conf. lim.		Pos. tubes			MPN/g	Conf. lim.	
0.10	0.01	0.001		Low	High	0.10	0.01	0.001		Low	High
0	0	0	<3.0	--	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1100	420	--

Table 2. For 5 tubes each at 0.1, 0.01, and 0.001 g inocula, the MPNs and 95 percent confidence intervals.

Pos. Tubes			MPN/g	Conf. lim.		Pos. tubes			MPN/g	Conf.lim.	
0.1	0.01	0.001		Low	High	0.1	0.01	0.001		Low	High
0	0	0	<1.8	--	6.8	4	0	2	21	6.8	40
0	0	1	1.8	0.09	6.8	4	0	3	25	9.8	70
0	1	0	1.8	0.09	6.9	4	1	0	17	6	40
0	1	1	3.6	0.7	10	4	1	1	21	6.8	42
0	2	0	3.7	0.7	10	4	1	2	26	9.8	70
0	2	1	5.5	1.8	15	4	1	3	31	10	70
0	3	0	5.6	1.8	15	4	2	0	22	6.8	50
1	0	0	2	0.1	10	4	2	1	26	9.8	70
1	0	1	4	0.7	10	4	2	2	32	10	70
1	0	2	6	1.8	15	4	2	3	38	14	100
1	1	0	4	0.7	12	4	3	0	27	9.9	70
1	1	1	6.1	1.8	15	4	3	1	33	10	70
1	1	2	8.1	3.4	22	4	3	2	39	14	100
1	2	0	6.1	1.8	15	4	4	0	34	14	100
1	2	1	8.2	3.4	22	4	4	1	40	14	100
1	3	0	8.3	3.4	22	4	4	2	47	15	120
1	3	1	10	3.5	22	4	5	0	41	14	100
1	4	0	11	3.5	22	4	5	1	48	15	120
2	0	0	4.5	0.79	15	5	0	0	23	6.8	70
2	0	1	6.8	1.8	15	5	0	1	31	10	70
2	0	2	9.1	3.4	22	5	0	2	43	14	100
2	1	0	6.8	1.8	17	5	0	3	58	22	150
2	1	1	9.2	3.4	22	5	1	0	33	10	100
2	1	2	12	4.1	26	5	1	1	46	14	120
2	2	0	9.3	3.4	22	5	1	2	63	22	150
2	2	1	12	4.1	26	5	1	3	84	34	220
2	2	2	14	5.9	36	5	2	0	49	15	150
2	3	0	12	4.1	26	5	2	1	70	22	170
2	3	1	14	5.9	36	5	2	2	94	34	230
2	4	0	15	5.9	36	5	2	3	120	36	250

3	0	0	7.8	2.1	22	5	2	4	150	58	400
3	0	1	11	3.5	23	5	3	0	79	22	220
3	0	2	13	5.6	35	5	3	1	110	34	250
3	1	0	11	3.5	26	5	3	2	140	52	400
3	1	1	14	5.6	36	5	3	3	180	70	400
3	1	2	17	6	36	5	3	4	210	70	400
3	2	0	14	5.7	36	5	4	0	130	36	400
3	2	1	17	6.8	40	5	4	1	170	58	400
3	2	2	20	6.8	40	5	4	2	220	70	440
3	3	0	17	6.8	40	5	4	3	280	100	710
3	3	1	21	6.8	40	5	4	4	350	100	710
3	3	2	24	9.8	70	5	4	5	430	150	1,100
3	4	0	21	6.8	40	5	5	0	240	70	710
3	4	1	24	9.8	70	5	5	1	350	100	1100
3	5	0	25	9.8	70	5	5	2	540	150	1700
4	0	0	13	4.1	35	5	5	3	920	220	2600
4	0	1	17	5.9	36	5	5	4	1600	400	4600
						5	5	5	>1600	700	--

Table 3. For 10 tubes at each of 0.1, 0.01, and 0.001 g inocula, the MPNs and 95 percent confidence intervals.

Pos. tubes			MPN/g	Conf. lim.		Pos. tubes			MPN/g	Conf. lim.	
0.1	0.01	0.001		Low	High	0.1	0.01	0.001		Low	High
0	0	0	<.90	--	3.1	8	2	0	17	7.7	34
0	0	1	0.9	0.04	3.1	8	2	1	19	9	34
0	0	2	1.8	0.33	5.1	8	2	2	21	10	39
0	1	0	0.9	0.04	3.6	8	2	3	23	11	44
0	1	1	1.8	0.33	5.1	8	3	0	19	9	34

0	2	0	1.8	0.33	5.1	8	3	1	21	10	39
0	2	1	2.7	0.8	7.2	8	3	2	24	11	44
0	3	0	2.7	0.8	7.2	8	3	3	26	12	50
1	0	0	0.94	0.05	5.1	8	4	0	22	10	39
1	0	1	1.9	0.33	5.1	8	4	1	24	11	44
1	0	2	2.8	0.8	7.2	8	4	2	26	12	50
1	1	0	1.9	0.33	5.7	8	4	3	29	14	58
1	1	1	2.9	0.8	7.2	8	5	0	24	11	44
1	1	2	3.8	1.4	9	8	5	1	27	12	50
1	2	0	2.9	0.8	7.2	8	5	2	29	14	58
1	2	1	3.8	1.4	9	8	5	3	32	15	62
1	3	0	3.8	1.4	9	8	6	0	27	12	50
1	3	1	4.8	2.1	11	8	6	1	30	14	58
1	4	0	4.8	2.1	11	8	6	2	33	15	62
2	0	0	2	0.37	7.2	8	7	0	30	14	58
2	0	1	3	0.81	7.3	8	7	1	33	17	73
2	0	2	4	1.4	9	8	7	2	36	17	74
2	1	0	3	0.82	7.8	8	8	0	34	17	73
2	1	1	4	1.4	9	8	8	1	37	17	74
2	1	2	5	2.1	11	9	0	0	17	7.5	31
2	2	0	4	1.4	9.1	9	0	1	19	9	34
2	2	1	5	2.1	11	9	0	2	22	10	39
2	2	2	6.1	3	14	9	0	3	24	11	44
2	3	0	5.1	2.1	11	9	1	0	19	9	39
2	3	1	6.1	3	14	9	1	1	22	10	40
2	4	0	6.1	3	14	9	1	2	25	11	44
2	4	1	7.2	3.1	15	9	1	3	28	14	58
2	5	0	7.2	3.1	15	9	1	4	31	14	58
3	0	0	3.2	0.9	9	9	2	0	22	10	44
3	0	1	4.2	1.4	9.1	9	2	1	25	11	46
3	0	2	5.3	2.1	11	9	2	2	28	14	58

3	1	0	4.2	1.4	10	9	2	3	32	14	58
3	1	1	5.3	2.1	11	9	2	4	35	17	73
3	1	2	6.4	3	14	9	3	0	25	12	50
3	2	0	5.3	2.1	12	9	3	1	29	14	58
3	2	1	6.4	3	14	9	3	2	32	15	62
3	2	2	7.5	3.1	15	9	3	3	36	17	74
3	3	0	6.5	3	14	9	3	4	40	20	91
3	3	1	7.6	3.1	15	9	4	0	29	14	58
3	3	2	8.7	3.6	17	9	4	1	33	15	62
3	4	0	7.6	3.1	15	9	4	2	37	17	74
3	4	1	8.7	3.6	17	9	4	3	41	20	91
3	5	0	8.8	3.6	17	9	4	4	45	20	91
4	0	0	4.5	1.6	11	9	5	0	33	17	73
4	0	1	5.6	2.2	12	9	5	1	37	17	74
4	0	2	6.8	3	14	9	5	2	42	20	91
4	1	0	5.6	2.2	12	9	5	3	46	20	91
4	1	1	6.8	3	14	9	5	4	51	25	120
4	1	2	8	3.6	17	9	6	0	38	17	74
4	2	0	6.8	3	15	9	6	1	43	20	91
4	2	1	8	3.6	17	9	6	2	47	21	100
4	2	2	9.2	3.7	17	9	6	3	53	25	120
4	3	0	8.1	3.6	17	9	7	0	44	20	91
4	3	1	9.3	4.5	18	9	7	1	49	21	100
4	3	2	10	5	20	9	7	2	54	25	120
4	4	0	9.3	4.5	18	9	7	3	60	26	120
4	4	1	11	5	20	9	8	0	50	25	120
4	5	0	11	5	20	9	8	1	55	25	120
4	5	1	12	5.6	22	9	8	2	61	26	120
4	6	0	12	5.6	22	9	8	3	68	30	140
5	0	0	6	2.5	14	9	9	0	57	25	120
5	0	1	7.2	3.1	15	9	9	1	63	30	140

5	0	2	8.5	3.6	17	9	9	2	70	30	140
5	0	3	9.8	4.5	18	10	0	0	23	11	44
5	1	0	7.3	3.1	15	10	0	1	27	12	50
5	1	1	8.5	3.6	17	10	0	2	31	14	58
5	1	2	9.8	4.5	18	10	0	3	37	17	73
5	1	3	11	5	21	10	1	0	27	12	57
5	2	0	8.6	3.6	17	10	1	1	32	14	61
5	2	1	9.9	4.5	18	10	1	2	38	17	74
5	2	2	11	5	21	10	1	3	44	20	91
5	3	0	10	4.5	18	10	1	4	52	25	120
5	3	1	11	5	21	10	2	0	33	15	73
5	3	2	13	5.6	23	10	2	1	39	17	79
5	4	0	11	5	21	10	2	2	46	20	91
5	4	1	13	5.6	23	10	2	3	54	25	120
5	4	2	14	7	26	10	2	4	63	30	140
5	5	0	13	6.3	25	10	3	0	40	17	91
5	5	1	14	7	26	10	3	1	47	20	100
5	6	0	14	7	26	10	3	2	56	25	120
6	0	0	7.8	3.1	17	10	3	3	66	30	140
6	0	1	9.2	3.6	17	10	3	4	77	34	150
6	0	2	11	5	20	10	3	5	89	39	180
6	0	3	12	5.6	22	10	4	0	49	21	120
6	1	0	9.2	3.7	18	10	4	1	59	25	120
6	1	1	11	5	21	10	4	2	70	30	150
6	1	2	12	5.6	22	10	4	3	82	38	180
6	1	3	14	7	26	10	4	4	94	44	180
6	2	0	11	5	21	10	4	5	110	50	210
6	2	1	12	5.6	22	10	5	0	62	26	140
6	2	2	14	7	26	10	5	1	74	30	150
6	2	3	15	7.4	30	10	5	2	87	38	180
6	3	0	12	5.6	23	10	5	3	100	44	180

6	3	1	14	7	26	10	5	4	110	50	210
6	3	2	15	7.4	30	10	5	5	130	57	220
6	4	0	14	7	26	10	5	6	140	70	280
6	4	1	15	7.4	30	10	6	0	79	34	180
6	4	2	17	9	34	10	6	1	94	39	180
6	5	0	16	7.4	30	10	6	2	110	50	210
6	5	1	17	9	34	10	6	3	120	57	220
6	5	2	19	9	34	10	6	4	140	70	280
6	6	0	17	9	34	10	6	5	160	74	280
6	6	1	19	9	34	10	6	6	180	91	350
6	7	0	19	9	34	10	7	0	100	44	210
7	0	0	10	4.5	20	10	7	1	120	50	220
7	0	1	12	5	21	10	7	2	140	61	280
7	0	2	13	6.3	25	10	7	3	150	73	280
7	0	3	15	7.2	28	10	7	4	170	91	350
7	1	0	12	5	22	10	7	5	190	91	350
7	1	1	13	6.3	25	10	7	6	220	100	380
7	1	2	15	7.2	28	10	7	7	240	110	480
7	1	3	17	7.7	31	10	8	0	130	60	250
7	2	0	13	6.4	26	10	8	1	150	70	280
7	2	1	15	7.2	28	10	8	2	170	80	350
7	2	2	17	7.7	31	10	8	3	200	90	350
7	2	3	19	9	34	10	8	4	220	100	380
7	3	0	15	7.2	30	10	8	5	250	120	480
7	3	1	17	9	34	10	8	6	280	120	480
7	3	2	19	9	34	10	8	7	310	150	620
7	3	3	21	10	39	10	8	8	350	150	620
7	4	0	17	9	34	10	9	0	170	74	310
7	4	1	19	9	34	10	9	1	200	91	380
7	4	2	21	10	39	10	9	2	230	100	480
7	4	3	23	11	44	10	9	3	260	120	480

7	5	0	19	9	34	10	9	4	300	140	620
7	5	1	21	10	39	10	9	5	350	150	630
7	5	2	23	11	44	10	9	6	400	180	820
7	6	0	21	10	39	10	9	7	460	210	970
7	6	1	23	11	44	10	9	8	530	210	970
7	6	2	25	12	46	10	9	9	610	280	1300
7	7	0	23	11	44	10	10	0	240	110	480
7	7	1	26	12	50	10	10	1	290	120	620
8	0	0	13	5.6	25	10	10	2	350	150	820
8	0	1	15	7	26	10	10	3	430	180	970
8	0	2	17	7.5	30	10	10	4	540	210	1300
8	0	3	19	9	34	10	10	5	700	280	1500
8	1	0	15	7.1	28	10	10	6	920	350	1900
8	1	1	17	7.7	31	10	10	7	1200	480	2400
8	1	2	19	9	34	10	10	8	1600	620	3400
8	1	3	21	10	39	10	10	9	2300	810	5300
						10	10	10	>2300	1300	--

Table 4: 8 tube MPN table

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Author: Wallace E. Garthright

Top

Hypertext updated by rim/rxb/kwg 2001-MAR-12

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Bacteriological Analytical Manual

Online

January 2001

M1

A-1 Medium

Tryptone	20 g
Lactose	5 g
NaCl	5 g
*Triton X-100 (Rohm & Haas)	1 ml
Salicin	0.5 g
Distilled water	1 liter

Dissolve ingredients in 1 liter distilled water. Adjust pH to 6.9 ± 0.1. Dispense 10 ml portions of single strength broth into 18 x 150 mm tubes containing inverted fermentation vials. For double strength broth, use 22 x 175 mm tubes containing inverted fermentation vials. Medium may be cloudy before sterilization. Autoclave 10 min at 121°C.

Store in dark up to 7 days.

(Commercially available A-1 medium is unacceptable.)

Storage dark, up to 7 days

*Triton X-100 may be purchased from Fisher Scientific Company, Fairlawn, NJ 07410.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

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BacteriologicalAnalyticalManual

Online

January 2001

M29a

Abeyta-Hunt Bark Agar

(Return to Table of Contents)

Heart infusion agar (Difco)	40 g
Yeast extract	2 g
Distilled water	950 ml

Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Cool and add sodium cefoperazone (**6.4 ml** if using broth preparation or **4 ml** of the agar preparation[below]), 4 ml rifampicin, 4 ml amphotericin B, and 50 ml lysed horse blood.

After pouring plates, dry plates overnight on bench.If plates must be used the same day, place them in 42°C incubator for several hours. Do not dry in a hood with lids open. Even very brief surface drying will inhibit campylobacter growth.

1. **Sodium cefoperazone**. Prepare as described for broth for final concentration of **32 mg/liter**, adding 6.4 ml to agar. OR dissolve 0.8 g in 100 ml water in a 100 ml volumetric flask, filter and add 4 ml to agar.

2. **Rifampicin**. Dissolve 0.25 g **slowly** into 60-80 ml alcohol in a 100 volumetric, swirling repeatedly. When powder is dissolved completely, bring to the line with distilled water. Store up to 1 year at -20°C. Final concentration is 10 mg/liter.

3. UPDATED **Amphotericin B**, **solubilized** (Sigma Cat. No. A9528). Dissolve 0.05 g in water in a 100 ml volumetric flask and bring to the line. Filter sterilize and store at -20°C for 1 year. Final concentration is 2 mg/liter. Add 4 ml per liter.
4. **FBP**. Dissolve 6.25 g Sodium pyruvate in 10-20 ml distilled water. Pour into a 100 ml volumetric. Add 6.25 g Ferrous sulfate and 6.25 g Sodium metabisulfite. Bring to the line with distilled water and filter sterilize. Use 4 ml/liter agar.

   Note: FBP is light sensitive and absorbs oxygen rapidly. Prepare only the amount needed. 10-25 ml amounts can be filtered with a 0.22 µm syringe filter. Freeze unused portions in 5 ml amounts at -70°C as soon as possible after preparation. It can be stored at -70°C for 3 mos or -20°C for 1 mo.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998. Updated: 29-DEC-2000

Hypertext updated by rim/cjm 2001-OCT-24

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Bacteriological Analytical Manual

Online

January 2001

M2

Acetamide Medium

(Return to Table of Contents)

Stock basal medium

KH_2PO_4	0.5 M	14 ml
K_2HPO_4	0.5 M	6 ml
Agar		0.5 g
Distilled water		400 ml

Heat with agitation to dissolve agar. Add 1 ml PR-CV (500X concentrate).

Stock acetamide, 1%

Acetamide	1 g
Distilled water	100 ml

Store over $CHCl_3$ in screw-cap container. Stable indefinitely at room temperature.

PR-CV (500X concentrate)

Phenol red	2 g
Crystal violet	0.2 g
Distilled water	200 ml

Add 5 N NaOH until ingredients are dissolved.

Final medium

Add 0.8 ml basal medium to 13 x 100 mm tube. Add 0.2 ml acetamide solution. Steam (100°C) 10 min. Cool.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/ear 2001-NOV-26

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M3

Acetate Agar

(Return to Table of Contents)

Sodium acetate	2 g
NaCl	5 g
$MgSO_4$ (anhydrous)	0.2 g
Ammonium phosphate	1 g
K_2HPO_4	1 g
Bromthymol blue	0.08 g
Agar	20 g
Distilled water	1 liter

Add all ingredients except $MgSO_4$ to 1 liter distilled water. Heat to boiling with stirring. Add $MgSO_4$ and adjust pH. Dispense 8 ml portions into 16 x 150 mm tubes. Autoclave 15 min at 121°C. Incline tubes to obtain 5 cm slant. Final pH, 6.7.

NOTE:This medium contains a slightly smaller concentration of acetate than the formula recommended in Ewing (1986).

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Hypertext updated by rim/ear 2001-NOV-26

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M4

Acid Broth

(Return to Table of Contents)

Proteose peptone	5 g
Yeast extract	5 g
Dextrose	5 g
K_2HPO_4	4 g
Distilled water	l liter

Dissolve ingredients and dispense 12-15 ml portions into 20 x 150 mm tubes. Autoclave 15 min at 121°C. Final pH, 5.0.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-20

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Bacteriological Analytical Manual

Online

January 2001

M5

AE Sporulation Medium, Modified

(for *Clostridium perfringens*)

(AE base is available commercially)

(Return to Table of Contents)

Polypeptone	10.0 g
Yeast extract	10.0 g
Na_2HPO_4	4.36 g
KH_2PO_4	0.25 g
Ammonium acetate	1.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Distilled water	1 liter

Dissolve ingredients and adjust to pH 7.5 ± 0.1, using 2 M sodium carbonate. Dispense 15 ml into 20 x 150-mm screw-cap tubes and sterilize by autoclaving for 15 min at 121°C.

After sterilization, add 0.6 ml of sterilized 10% raffinose and 0.2 ml each of filter-sterilized 0.66 M sodium carbonate and 0.32% cobalt chloride ($CoCl_2 \cdot 6H_2O$) dropwise to each tube.

Check pH of one or two tubes; it should be 7.8 ± 0.1. Just before use, steam medium for 10 min; after cooling, add 0.2 ml of filter-sterilized 1.5% sodium ascorbate (prepared daily) to each

tube.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M6

Agar Medium P

(Return to Table of Contents)

Beef extract	3 g	NaCl	0.5 g
Peptone	5 g	K_2HPO_4	0.25 g
Tryptone	1.7 g	Polysorbate 80	1 g
Soytone	0.3 g	Bromcresol purple	0.06 g
Dextrose	5.25 g	Agar	15 g
Distilled water			l liter

Heat with agitation to dissolve agar. Autoclave 15 min at 121°C. Final pH, 7.8 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M7

AKI Medium

(Return to Table of Contents)

Peptone	15 g
Yeast extract	4 g
NaCl	5 g
Distilled water	970 ml
$NaHCO_3$, 10% aqueous, filter-sterilized	30 ml

On day of use, dissolve peptone, yeast extract, and NaCl in distilled water. Autoclave 15 min at 121°C. Cool. Add 30 ml freshly prepared, filter-sterilized $NaHCO_3$, and mix. Dispense aseptically into screw-capped tubes (use 15 ml for 16 x 125 mm tubes). Final pH, 7.4 ± 0.2.

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Hypertext updated by rim 2000-MAR-23

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Bacteriological Analytical Manual

Online

January 2001

M8

Alkaline Peptone Agar

(Return to Table of Contents)

Peptone	10 g
NaCl	20 g
Agar	15 g
Distilled water	1 liter

Boil to dissolve ingredients. Adjust pH so that value after sterilization is 8.5 ± 0.2. Autoclave 15 min at 121°C. Solidify agar in tubes as slants.

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M9

Alkaline Peptone Salt Broth (APS)

(Return to Table of Contents)

Peptone	10 g
NaCl	30 g
Distilled water	1 liter

Dissolve ingredients. Adjust pH so that value after sterilization is 8.5 ± 0.2. Dispense 10 ml into tubes. Autoclave 10 min at 121°C.

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Bacteriological Analytical Manual

Online

January 2001

M10

Alkaline Peptone Water

(Return to Table of Contents)

Peptone	10 g
NaCl	10 g
Distilled water	l liter

Adjust pH so that value after sterilization is 8.5 ± 0.2. Dispense into screw-cap tubes. Autoclave 10 min at 121°C.

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Bacteriological Analytical Manual

Online

January 2001

M11

Anaerobe Agar

(Return to Table of Contents)

Base

Trypticase (tryptic) soy agar	40 g
Agar	5 g
Yeast extract	5 g
L-Cysteine (dissolved in 5 ml 1 N NaOH)	0.4 g
Distilled water	l liter

Heat with agitation to dissolve agar. Adjust pH to 7.5 ± 0.2. Autoclave 15 min at 121°C. Cool to 50°C.

Hemin solution

Suspend 1 g hemin in 100 ml distilled water. Autoclave 15 min at 121°C. Refrigerate at 4°C.

Vitamin K_1 solution

Dissolve 1 g vitamin K_1 (Sigma Chemical Co., St. Louis, MO) in 100 ml 95% ethanol. Solution may require 2-3 days with intermittent shaking to dissolve. Refrigerate at 4°C.

Final medium

To 1 liter base add 0.5 ml hemin solution and 1 ml Vitamin K_1 solution. Mix and pour 20 ml portions into 15 x 100 mm petri dishes. Medium must be reduced before inoculation by 24 h anaerobic incubation in anaerobic glove box or GasPak jar.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

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Bacteriological Analytical Manual

Online

January 2001

M12

Anaerobic Egg Yolk Agar

(Return to Table of Contents)

Agar base

Yeast extract	5 g
Tryptone	5 g
Proteose peptone	20 g
NaCl	5 g
Agar	20 g
Distilled water	1 liter

Autoclave 15 min at 121°C. Adjust pH to 7.0 ± 0.2.

2 Fresh Eggs

Treatment of eggs

Wash 2 fresh eggs with stiff brush and drain. Soak eggs in 70% ethanol for 1 h. Crack eggs aseptically. Retain yolks. Drain contents of yolk sacs into sterile stoppered graduate and discard sacs. Add yolk to equal volume of sterile 0.85% saline. Invert graduate several times to mix. Egg yolk emulsion (50%) is available commercially.

Preparation of medium

To 1 liter melted medium (48-50°C) add 80 ml yolk-saline mixture (available from Difco as Bacto Egg Yolk Enrichment 50%), and mix. Pour plates immediately. After solidification dry 2-3 days at ambient temperature or at 35°C for 24 h. Check plates for contamination before use. After drying, plates may be stored for a short period in refrigerator.

Storage	4°C

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Bacteriological Analytical Manual

Online

January 2001

M13

Andrade's Carbohydrate Broth and Indicator

(Return to Table of Contents)

Base

Beef extract	3 g
Peptone or gelysate	10 g
NaCl	10 g
Distilled water	1 liter

Adjust pH to 7.2 ± 0.2. Autoclave 15 min at 121°C.

Andrade's indicator

Acid fuchsin	0.2 g
Distilled water	10 ml
1 N NaOH	16 ml

Allow to decolorize before use. Add 1-2 ml NaOH if necessary. Add 10 ml indicator to 1 liter base.

Carbohydrate stock solution.

Prepare dextrose, lactose, sucrose, and mannitol in 10% solutions. Prepare dulcitol, salicin, and other carbohydrates in 5% solutions. Sterilize by filtration through 0.20 µm membrane. Dilute sugar solutions 1:10 in base with Andrade's indicator to give recommended concentration. Mix gently.

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Bacteriological Analytical Manual

Online

January 2001

M14

Antibiotic Medium No. 1

(Agar Medium A)

(Return to Table of Contents)

Gelatone or gelysate	6 g
Tryptone or trypticase	4 g
Yeast extract	3 g
Beef extract	1.5 g
Dextrose	1 g
Agar	15 g
Distilled water	1 liter

Autoclave 15 min at 121°C. Final pH, 6.5-6.6. Commercially available in dehydrated form as Difco Penassay Seed Agar or BBL Seed Agar.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M15

Antibiotic Medium No. 4

(Agar Medium B)

(Return to Table of Contents)

Tryptone or trypticase	6 g
Yeast extract	3 g
Beef extract	1.5 g
Dextrose	1 g
Agar	15 g
Distilled water	1 liter

Autoclave 15 min at 121°C. Final pH, 6.5-6.6. Commercially available in dehydrated form as Difco Yeast Beef Agar or BBL Yeast Beef Agar.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-20

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M16

Arginine-Glucose Slant

(AGS)

(Return to Table of Contents)

Peptone	5 g
Yeast extract	3 g
Tryptone	10 g
NaCl	20 g
Glucose	1 g
L-Arginine (hydrochloride)	5 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.3 g
Bromocresol purple	0.02 g
Agar	13.5 g

Suspend ingredients in distilled water and boil to dissolve. Dispense into tubes (for 13 x 100 mm tubes use 5 ml). Autoclave 10-12 min at 121°C. After sterilization, solidify as slants. Final pH, 6.8-7.0.

Distilled water	1 liter

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M17

Baird-Parker Medium,

pH 7.0

(Return to Table of Contents)

Basal medium

Tryptone	10 g
Beef extract	5 g
Yeast extract	1 g
Sodium pyruvate	10 g
Glycine	12 g
Lithium chloride.$6H_2O$	5 g
Agar	20 g

Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2. If desired for immediate use, maintain melted medium at 48-50°C before adding enrichment. Otherwise, store solidified medium at 4 ± 1°C up to 1 month. Melt medium before use.

Enrichment

Bacto EY tellurite enrichment.

Complete medium

Aseptically add 5 ml prewarmed (45-50°C) Bacto EY tellurite enrichment to 95 ml melted base. Mix well (avoiding bubbles) and pour 15-18 ml portions into sterile 15 x 100 mm petri dishes. The medium must be densely opaque. Dry plates before use. Store prepared plates at 20-25°C for up to 5 days. See *Official Methods of Analysis* of AOAC International, 15th Edition (1990), p. 429, for more information.

Storage **20-25°C** **5 days**

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998. Revised by rim 2000-03-28

Hypertext updated by rim 2000-MAR-28

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M18

Bile Esculin Agar

(Return to Table of Contents)

Beef extract	3 g
Peptone	5 g
Esculin	1 g
Oxgall	40 g
Ferric citrate	0.5 g
Agar	15 g
Distilled water	1 liter

Heat with agitation to dissolve. Dispense into tubes, autoclave 15 min at 121°C, and slant until solidified. Final pH, 6.6 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-5

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M19

Bismuth Sulfite Agar

(Wilson and Blair)

(Return to Table of Contents)

Polypeptone (or peptone)	10 g
Beef extract	5 g
Dextrose	5 g
Na_2HPO_4 (anhydrous)	4 g
$FeSO_4$ (anhydrous)	0.3 g
Bismuth sulfite (indicator)	8 g
Brilliant green	0.025 g
Agar	20 g
Distilled water	1 liter

Mix thoroughly and heat with agitation. Boil about 1 min to obtain uniform suspension. (Precipitate will not dissolve.) Cool to 45-50°C. Suspend precipitate by gentle agitation, and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Let plates dry about 2 h with lids partially removed; then close plates. Final pH, 7.7 ± 0.2

DO NOT AUTOCLAVE. Prepare plates on day before streaking and store in dark. Selectivity decreases in 48 h.

CAUTION	DO NOT AUTOCLAVE
PREPARATION	Day before use.
STORAGE	In Dark
Loss of Selectivity after 48 h	

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

BacteriologicalAnalyticalManual

Online

January 2001

M20

Blood Agar

(Return to Table of Contents)

Tryptone	15 g
Phytone or soytone	5 g
NaCl	5 g
Agar	15 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Autoclave 15 min at 121°C. Cool to 50°C. Add 5 ml defibrinated sheep red blood cells to 100 ml melted agar. Mix and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Final pH of base, 7.3 ± 0.2.

Tryptic soy agar, tryptic soy agar blood base, or trypticase soy agar [soybean-casein digest agar (M152)] may be used as the basal medium. Commercially available sheep blood agar plates are satisfactory. Blood agar base is available from Difco, BBL, and Oxoid.

For *Vibrio hollisae*, add NaCl to a final concentration of 1%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-6

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M20a

Blood Agar Base

(commercially available blood agar base may be used)

(Return to Table of Contents)

Infusion from beef heart	500 g
Tryptose	10 g
NaCl	5 g
Agar	15 g
Sheep blood (sterile, defibrinated)	50 ml
Distilled water	1 liter

Suspend ingredients except blood to dissolve. Autoclave 15 min at 121°C. Cool to 45-50°C. Add 50 ml blood (room temperature), and mix. Dispense into sterile petri dishes. Final pH, 6.8 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/ear 2000-NOV-26

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M21

Blood Agar Base (Infusion Agar)

(Return to Table of Contents)

Heart muscle, infusion from	375 g
Thiotone	10 g
NaCl	5 g
Agar	15 g
Distilled water	1 liter

Heat gently to dissolve. Autoclave 20 min at 121°C. Final pH, 7.3 ± 0.2. Commercially available dehydrated heart infusion agars may be used.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-8

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M22

Blood Agar Base #2

(Difco)

(Return to Table of Contents)

Proteose peptone	15 g
Liver digest	2.5 g
Yeast extract	5 g
NaCl	5 g
Agar	12 g
Distilled Water	1 liter

Autoclave at 121°C for 15 min. For blood agar, reduce water to 950 ml. Add 50 ml defibrinated (whole or lysed) horse blood and FBP (4 ml to agar + blood) after autoclaving and cooling to 48°C. Final pH, 7.4 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-12

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M23

Brain Heart Infusion (BHI) Agar (0.7%)

(for staphylococcal enterotoxin)

(Return to Table of Contents)

Prepare a suitable quantity of brain heart infusion broth (M24). Adjust pH to 5.3 with 1 N HCl. Add agar to give 0.7% concentration. Dissolve by minimal boiling. Dispense 25 ml portions into 25 x 200 mm test tubes. Autoclave 10 min at 121°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-6

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M24

Brain Heart Infusion (BHI) Broth and Agar (Equivalent media are available from Difco and BBL)

(Return to Table of Contents)

Medium 1

Calf brain, infusion from	200 g
Beef heart, infusion from	250 g
Proteose peptone (Difco) or polypeptone (Bioquest)	10 g
NaCl	5 g
Na_2HPO_4*	2.5 g
Dextrose	2.0 g

Dissolve ingredients of **Medium 1** in distilled water with gentle heat.

Distilled water	1 liter

*Difco does not specify waters of hydration.

Medium 2

Brain heart-infusion	6.0 g
Peptic digest of animal tissue	6.0 g
NaCl	5.0 g
Dextrose	3.0 g
Pancreatic digest of gelatin	14.5 g
Na_2HPO_4 +	2.5 g
Distilled water	1 liter

Suspend ingredients of **Medium 2** in distilled water and boil for 1 min to completely dissolve.

+BBL does not specify waters of hydration.

For both Medium 1 and Medium 2, dispense broth into bottles or tubes for storage. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Commercially available BHI is acceptable.

To prepare brain heart infusion agar, add 15 g agar to 1 liter BHI broth. Heat to dissolve agar before dispensing into bottles or flasks. Autoclave 15 min at 121°C.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-20

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M25

Brilliant Green Lactose Bile Broth

(Return to Table of Contents)

Peptone	10 g
Lactose	10 g
Oxgall	20 g
Brilliant green	0.0133 g
Distilled water	1 liter

Dissolve peptone and lactose in 500 ml distilled water. Add 20 g dehydrated oxgall dissolved in 200 ml distilled water. The pH of this solution should be 7.0-7.5. Mix and add water to make 975 ml. Adjust pH to 7.4. Add 13.3 ml 0.1% aqueous brilliant green in distilled water. Add distilled water to make 1 liter.

Dispense into fermentation tubes, making certain that fluid level covers inverted vials.

Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.1.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-20

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M26

Bromcresol Purple Broth

(Return to Table of Contents)

Base

Peptone	10 g
Beef extract	3 g
NaCl	5 g
Bromcresol purple	0.04 g
Distilled water	1 liter

Dispense 2.5 ml portions of base solution into 13 x 100 mm test tubes containing inverted 6 x 50 mm fermentation tubes. Autoclave 10 min at 121°C. Final pH, 7.0 ± 0.2.

Sterilize stock solutions of carbohydrates (50% w/v) separately by autoclaving or, preferably, by filtration (0.2 µm pore size). Add 0.278 ± 0.002 ml stock carbohydrate solution to 2.5 ml basal medium to give 5% w/v final carbohydrate concentration.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M27

Bromcresol Purple Dextrose Broth (BCP)

(Return to Table of Contents)

Dextrose	10 g
Beef extract	3 g
Peptone	5 g
Bromcresol purple (1.6% in ethanol)	2 ml
Distilled water	1 liter

Dissolve ingredients in distilled water. Dispense 12-15 ml into tubes. Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-6

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M28a

Campylobacter enrichment broth

(Bolton formula)

(Return to Table of Contents)

(Oxoid AM-7526 (manufactured by Med-Ox Chemicals Ltd.) or Malthus Diagnostics Lab)

Enrichment Broth Base

Meat Peptone	10 g
Lactalbumin Hydrolysate	5 g
Yeast Extract	5 g
NaCl	5 g
Haemin	0.01 g
Sodium Pyruvate	0.5 g
Alpha-Ketoglutamic Acid	1 g

Final pH, 7.4 ± 0.2.

THOROUGHLY MIX 27.61 g in 1 liter water. Let soak 10 min. Swirl again and autoclave 15 min at 121°C (in screw-capped bottles if possible). Before use, add 2 rehydrated vials of Campylobacter enrichment broth (Bolton formula) supplement (Oxoid NDX131 or Malthus Diagnostics X-131). If supplement is not available add 4 ml each of antibiotic concentrates (formulas below, solutions made separately).

Sodium Metabisulphite	0.5 g
Sodium Carbonate	0.6 g
Total Dry Incredients	27.61 g
Distilled Water	1000 ml

Store powdered media in a tightly fastened container in a cool, dry area to reduce oxygen infusion and peroxide formation, which can inhibit recovery of microaerophiles. Use prepared broth within 1 month of preparation (preferably less than 2 weeks).

Item	Storage conditions	Shelf life
Complete medium	cool, dry	2 weeks - 1 month
Sodium cefoperazone	4°C	5 days
	-20°C	14 days
	-70°C	5 months
Trimethoprim lactate	4°C	1 year
vancomycin	4°C	2 months
Cycloheximide*	4°C	1 year
*Note: Substitute solubilized amphotericin B (Sigma Cat. No. A9528) for cycloheximide if cycloheximide not available. See instructions below.		

Campylobacter Enrichment Broth Supplements (Prepare each solution separately)

1. **Sodium cefoperazone**. Weigh 0.5 g into 100 ml distilled water in volumetric flask and dissolve. Only prepare amount required and filter-sterilize, 0.22 5m, using a syringe filter if 25 ml. Store solution 5 days at 4°C, 14 days at -20°C, and 5 months at -70°C. Freeze in sterile plastic tubes or bottles. Powder may be purchased from Sigma or obtained free from Pfizer by writing Roering Division, Pfizer, 235 E. 42nd St., New York, NY 10017. Request 2-4 g for in vitro use. Final concentration is 20 mg/liter.

2. **Trimethoprim**

i. **Trimethoprim lactate** (Sigma Cat. No. T0667). Dissolve 0.66 g in 100 ml distilled water, and filter. May be stored 1 year at 4°C. Add 4 ml/liter to yield final concentration of 20 mg/liter Trimethoprim.

or

ii. **Trimethoprim** (Sigma Cat. No. T7883)[a lower cost alternative]. To prepare Trimethoprim (TMP)-HCl: add 0.5 g TMP to 30ml 0.05N HCl at 50°C (stir until dissolved using hot plate with magnetic stirrer). Adjust volume to 100ml with distilled water. Add 4 ml/liter to yield final concentration of 20 mg/liter Trimethoprim.

3. **Vancomycin** (Sigma). Dissolve 0.5 g in 100 ml distilled water and filter. Store up to 2 months at 4°C. Because of short shelf life, prepare smaller amounts. Add 4 ml/liter for final concentration of 20 mg/liter
4. UPDATED **Cycloheximide** Dissolve 1.25 g in 20-30 ml ethanol in a 100 ml volumetric flask and bring to line with water. Filter-sterilize. Store at 4°C up to 1 year. Add 4 ml for final a concentration of 50 mg/liter. Use amphotericin B as described below if cycloheximide is not available.

   a. UPDATED **Amphotericin B**, **solubilized** (Sigma Cat. No. A9528). Dissolve 0.05 g in water in a 100 ml volumetric flask and bring to the line. Filter sterilize and store at -20°C for 1 year. Final concentration is 2 mg/liter. Add 4 ml per liter.

Note: For information on cycloheximide availability problems, see Advisory for BAM Users on Reported Supply Problems for Cycloheximide

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Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998. Updated and revised: 29-DEC-2000.

Hypertext updated by rim/kwg/cjm 2001-OCT-24

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M31

Cary-Blair Transport Medium

(Return to Table of Contents)

Sodium thioglycollate	1.5 g
Na_2HPO_4	1.1 g
NaCl	5 g
Agar	5 g
$CaCl_2$ (1% solution)	9 ml
Distilled water	991 ml

Heat with agitation to dissolve dry ingredients. Cool to 50°C and add $CaCl_2$.Adjust pH to 8.4. Dispense 7 ml portions into 9 ml screw-cap tubes and immediately steam exactly 15 min. Cool, and tighten caps.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-20

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M32

Casamino Acids-Yeast Extract (CYE) Broth

(Return to Table of Contents)

Casamino acids	30 g
Yeast extract	4 g
K_2HPO_4	0.5 g
Distilled water	1 liter

Dissolve ingredients. Adjust pH to 7.4 ± 0.2. Dispense 10 ml into 50 ml flasks. Autoclave 15 min at 121°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 1999-DEC-6

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M34

Casamino Acids-Yeast Extract-Salts Broth

(CA-YE)

(Gorbach)

(Return to Table of Contents)

Casamino acids	20 g
Yeast extract	6 g
NaCl	2.5 g
K_2HPO_4	8.71 g
Trace salts solution (below), optional	1 ml
Distilled water	1 liter

Adjust pH so that value after autoclaving is 8.5 ± 0.2. Autoclave 15 min at 121°C.

Trace salts solution (optional)

$MgSO_4$	50 g
$MnCl_2$	5 g
$FeCl_2$	5 g
Distilled water	1 liter

Suspend ingredients. Add enough 0.1 N H_2SO_4 to dissolve. Sterilize by filtration through 0.45 µm membrane. Add 1 ml to 1 liter base. Dispense 10 ml portions into 50 ml flasks.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 1999-DEC-6

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M35

Cefsulodin-Irgasan Novobiocin (CIN) Agar or *Yersinia* Selective Agar (YSA)

(Return to Table of Contents)

A. Basal medium

Special peptone	20 g
Yeast extract	2 g
Mannitol	20 g
Pyruvic acid (Na salt)	2 g
NaCl	1 g
$MgSO_4.7H_2O$ (10 mg/ml)	1 ml

Agar	12 g
Distilled water	756 ml

B. ## Irgasan (Ciba-Geigy) solution

Irgasan 0.40% in 95% ethanol	1 ml

May be stored at -20°C up to 4 weeks.

C. ## Desoxycholate solution

Sodium desoxycholate	0.5 g
Distilled water	200 ml

Bring to boil with stirring; cool to 50-55°C.

D. Sodium hydroxide, [5 N] 1 ml
E. Neutral red, [3 mg/ml] 10 ml
F. Crystal violet, [0.1 mg/ml] 10 ml
G. Cefsulodin (Abbott Labs), [1.5 mg/ml] 10 ml
H. Novobiocin, [0.25 mg/ml] 10 ml
I. Strontium chloride, [10%; filter-sterilized] 10 ml

May be stored at -70°C. Thaw to room temperature just before use.

Preparation: Add ingredients for solution A (basal medium) to water and bring to boil with stirring. Cool to about 80°C (10 min in 50°C water bath). Add solution B (Irgasan) and mix well. Cool to 50-55°C. Add solution C (desoxycholate); solution should remain clear. Add solutions D through H. Slowly add solution I with stirring. Adjust pH to 7.4 with 5 N NaOH. Dispense 15-20 ml into each petri dish. Commercially prepared dehydrated Yersinia selective agar (Difco) with supplements may be substituted. Follow manufacturer's instructions for preparation.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-20

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M36

Cell Growth Medium

(Return to Table of Contents)

Mix the following sterile solutions aseptically:

Minimal essential medium with Hanks' salts (MEMH)	1 liter
L-15 medium (Leibovitz) (containing 100 units penicillin G, 100 5g streptomycin, and 50 5g gentamicin per ml)	1 liter

Mix on magnetic stirrer, filter through 0.20 µm membrane, and dispense into sterile 2 liter Erlenmeyer flask. Final pH, 7.5. Cap and store at 5°C.

Just before use add:

Fetal bovine serum	200 ml

$NaHCO_3$ (7.5%)	50 ml

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M187

Cellulase Solution

(Return to Table of Contents)

Dissolve 1 g cellulase in 99 ml sterile distilled water. Filter sterilize through a 0.45 µm filter. Cellulase solution may be stored at 2-5°C for 2 weeks.

Adapted from Chapter 5, Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.
created 2000-MAR-01

Hypertext updated by rim 2000-MAR-07

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M37

Cetrimide Agar

(Return to Table of Contents)

Pancreatic digest of gelatin	20.0 g
Magnesium chloride	1.4 g
Potassium sulfate	10.0 g
Agar	13.6 g
Cetrimide (cetyl trimethyl ammonium bromide)	0.3 g

Suspend 45.3 g of ingredients or commercial powder (Pseudosel™, BBL; Becton-Dickinson) in 1 liter of purified water. Add 10 ml glycerol. Mix well. Heat to boiling, agitating frequently; maintain boiling for 1 min to dissolve powder.

Autoclave at 118°C for 15 min.

Final pH, 7.2 ± 0.2. Bacto cetrimide agar base plus glycerol (Difco) is a similar medium.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M38

Chopped Liver Broth

(Return to Table of Contents)

Fresh beef liver	500 g
Peptone	10 g
K_2HPO_4	1 g
Soluble starch	1 g
Distilled water	1 liter

Grind liver into the water. Heat to boiling and simmer 1 h. Cool, adjust pH to 7.0, and boil 10 min. Filter through cheesecloth and press out excess liquid. Add other ingredients and adjust pH to 7.0. Add water to make 1 liter. Filter through coarse filter paper.

Store broth and meat separately in freezer. To 18 x 150 or 20 x 150 mm test tubes, add chopped liver to depth of 1.2-2.5 cm and 10-12 ml of broth. Autoclave 15 min at 121°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M39

Christensen Citrate Agar

(Return to Table of Contents)

Sodium citrate	3 g
Glucose	0.2 g
Yeast extract	0.5 g
Cysteine monohydrochloride	0.1 g
Ferric ammonium citrate	0.4 g
KH_2PO_4	1 g
NaCl	5 g
Sodium thiosulfate	0.08 g
Phenol red	0.012 g
Agar	15 g

Suspend ingredients, mix thoroughly and heat with occasional agitation. Boil about 1 min to dissolve ingredients. Fill 16 x 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave for 15 min at 121°C. Final pH, 6.9 ± 0.2. Before media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt. The Difco formulation does not include ferric ammonium citrate and sodium thiosulfate.

Distilled water	1 liter

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 1999-DEC-6

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M40

Christensen's Urea Agar

(Return to Table of Contents)

Base

Peptone	1 g
NaCl	5 g
Dextrose	1 g
KH_2PO_4	2 g
Phenol red (6 ml of 1:500 solution)	0.012 g
Agar	15 g
Distilled water	900 ml

Dissolve all ingredients except urea in 900 ml water (basal medium).

For halophilic *Vibrio* spp., add extra 15 g NaCl (final NaCl concentration, 2%).

Autoclave 15 min at 121°C. Cool to 50-55°C.

Urea concentrate

Urea	20 g
Distilled water	100 ml

Dissolve urea in 100 ml water.

Filter-sterilize; add aseptically to cooled basal medium. Mix. Final pH, 6.8 ± 0.1. Dispense to sterile tubes or petri dishes. Slant tubes for 2 cm butt and 3 cm slant.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M41

Congo Red BHI Agarose (CRBHO) Medium*

(Return to Table of Contents)

Brain heart infusion (M20	37 g
$MgCl_2$	1 g
Agarose	12 g
Congo Red dye (375 mg/100 ml of distilled water)	20 ml

Dissolve, autoclave at 121°C for 15 min and pour 20 ml per plate.

*From S. Bhaduri, USDA, Philadelphia, PA. ASM Abstracts, 1989, No. P26.

U.S. Patent application ser. no. 07/493,662.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M42

Cooked Meat Medium

(Return to Table of Contents)

Beef heart	454 g
Proteose peptone	20 g
Dextrose	2 g
NaCl	5 g
Distilled water	1 liter

Follow directions as in M38, or suspend 12.5 g commercial dehydrated cooked medium in 100 ml cold distilled water. Mix and let stand 15 min to wet particles thoroughly. Or distribute 1.25 g into 20 x 150 mm test tubes, add 10 ml cold distilled water, and mix thoroughly to wet all particles. Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.2. Steam the sterilized medium and cool, without agitation, just before use.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-7

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M43

Cooked Meat Medium (Modified)

(Return to Table of Contents)

(a) Cooked meat medium

(commercially available in dehydrated form)

Beef heart	454 g
Proteose peptone	20 g
Dextrose	2 g
NaCl	5 g

(b) Diluent

(not available commercially)

Tryptone	10 g
Sodium thioglycollate	1 g
Soluble starch	1 g
Dextrose	2 g
Neutral red (1% aqueous)	5 ml
Distilled water	1 liter

Adjust pH to 6.8 ± 0.2. Add 1 g dehydrated (a) and 15 ml (b) to 20 x 150 mm tubes. Let meat particles rehydrate. Autoclave 15 min at 121°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M44

Decarboxylase Basal Medium

(Arginine, Lysine, Ornithine)

(Return to Table of Contents)

Base

Peptone or gelysate	5 g
Yeast extract	3 g
Glucose	1 g
Bromcresol purple	0.02 g
Distilled water	1 liter

Adjust pH so that value after sterilization is 6.5 ± 0.2. Dispense 5 ml portions into 16 x 125 mm screw-cap tubes. Autoclave loosely capped tubes 10 min at 121°C. Screw the caps on tightly for storage and after inoculation. For control, use unsupplemented base.

For	to 1 liter base, add:
Arginine Broth	5 g L-arginine
Lysine Broth (Falkow)	5 g L-lysine
Ornithine Broth	5 g L-ornithine

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/ear 2000-NOV-26

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M184

Dichloran 18% glycerol (DG18) agar

(Return to Table of Contents)

Glucose	10.0 g
Peptone	5.0 g
Potassium phosphate monobasic	1.0 g
Magnesium sulfate heptahydrate	0.5 g
Dichloran (0.2% in ethanol, w/v)	1.0 ml
Agar	15.0 g
Chloramphenicol	0.1 g
Distilled water	800 ml

Mix above items and steam to dissolve agar, then bring volume to 1000 ml with distilled water. Add 220 g glycerol and sterilize by autoclaving at 121°C for 15 min. The final pH should be 5.6 and the final a_w, 0.955.

This medium is used as a general purpose mold enumeration medium and is preferred when the a_w of the analyzed food is 0.95 or lower. The low water activity of this medium reduces interference by bacteria and fast-growing fungi. When both yeasts and molds must be enumerated, DRBC agar should be used.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-31

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M183

Dichloran rose bengal chloramphenicol (DRBC) agar

(Return to Table of Contents)

Glucose	10.0 g
Bacteriological peptone	5.0 g
Potassium phosphate, monobasic	1.0 g
Magnesium sulfate heptahydrate	0.5 g
Rose bengal (5% soln., w/v)	0.5 ml
Dichloran (2,6-dichloro-4-nitroaniline) solution (0.2%(w/v) in ethanol)	1.0 ml
Chloramphenicol	0.1 g
Distilled water	1 liter

Final pH should be 5.6. Mix ingredients, heat to dissolve agar and sterilize by autoclaving at 121°C for 15 min. Temper to 45 ± 1°C in a water bath and pour plates.

Agar	15.0 g

Notes:

1. DRBC agar is especially useful for analyzing sample containing "spreader" molds (e.g. Mucor, Rhizopus, etc.), since the added dichloran and rose bengal effectively slow down the growth of fast-growing fungi, thus readily allowing detection of other yeast and mold propagules, which have lower growth rates.
2. Media containing rose bengal are light-sensitive;
   relatively short exposure to light will result in the formation of inhibitory compounds. Keep these media in a dark, cool place until used. DRBC agar should be used for spread plates only.

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Hypertext updated by rim 2000-MAR-31

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M45

Duncan-Strong (DS) Sporulation Medium, Modified

(for *C. perfringens*)

(Return to Table of Contents)

Proteose peptone	15 g
Yeast extract	4 g
Sodium thioglycollate	1 g
$Na_2HPO_4 \cdot 7H_2O$	10 g
Raffinose	4 g
Distilled water	1 liter

Dissolve ingredients and sterilize by autoclaving for 15 min at 121°C. Adjust to pH 7.8 ± 0.1, using filter-sterilized 0.66 M sodium carbonate.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-7

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M46

Eagle's Minimal Essential Medium (MEME)

(with Earle's salts and nonessential amino acids)

(Return to Table of Contents)

L-Alanine	8.9 mg	L-Valine	46.0 mg
L-Arginine HCl	126.0 mg	L-Tyrosine (disodium salt)	52.1 mg
L-Asparagine-H_2O	150.0 mg	D-Calcium pantothenate	1.0 mg
L-Aspartic acid	13.3 mg	Choline chloride	1.0 mg
L-Cystine.2HCl	31.29 mg	Folic acid	1.0 mg
L-Glutamic acid	14.7 mg	Isoinositol	2.0 mg
L-Glutamine	292.0 mg	Nicotinamide	1.0 mg
L-Glycine	7.5 mg	Pyridoxal HCl	1.0 mg

Dissolve ingredients in

L-Histidine $HCl.H_2O$	42.0 mg	Riboflavin	0.1 mg
L-Isoleucine	52.0 mg	Thiamine HCl	1.0 mg
L-Leucine	52.0 mg	Glucose	1000.0 mg
L-Lysine HCl	72.5 mg	$CaCl_2.2H_2O$	265.0 mg
L-Methionine	15.0 mg	KCl	400.0 mg
D-Phenylalanine	32.0 mg	$MgSO_4.7H_2O$	200.0 mg
L-Proline	11.5 mg	NaCl	6800.0 mg
L-Serine	10.5 mg	$NaHCO_3$	2200.0 mg
L-Threonine	48.0 mg	$NaH_2PO_4.H_2O$	140.0 mg
L-Tryptophan	10.0 mg	Phenol red	10.0 mg
Distilled water			1 liter

distilled water. Sterilize by filtration. Final pH, 7.2 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-FEB-9

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M47

Earle's Balanced Salts

(Phenol Red-Free)

(Return to Table of Contents)

NaCl	6.8 g
KCl	400 mg
$CaCl_2 72H_2O$	265 mg
$MgSO_4 77H_2O$	200 mg
$NaH_2PO_4 7H_2O$	140 mg
Glucose	1.0 g
$NaHCO_3$	2.2 g
Distilled water	1 liter

Dissolve ingredients in the water. Sterilize by filtration. Final pH, 7.2 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-7

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M48

EB Motility Medium

(Return to Table of Contents)

Beef extract	3 g
Peptone or gelysate	10 g
NaCl	5 g
Agar	4 g
Distilled water	1 liter

Heat with agitation and boil 1-2 min to dissolve agar. Dispense 8 ml portions into 16 x 150 mm screw-cap tubes. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-7

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M49
EC Broth

(Return to Table of Contents)

Trypticase or tryptose	20 g
Bile salts No. 3	1.5 g
Lactose	5 g
K_2HPO_4	4 g
KH_2PO_4	1.5 g
NaCl	5 g
Distilled water	1 liter

Distribute 8 ml portions to 16 x 150 mm test tubes containing inverted 10 x 75 mm fermentation tubes. Autoclave 15 min at 121°C. Final pH, 6.9 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-7

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M50

EC-MUG Medium

(Return to Table of Contents)

Prepare as for EC broth (M49), but add 50 mg 4-methylumbelliferyl-β-D-glucuronide (MUG) per liter before autoclaving (15 min, 121°C). EC-MUG medium is available commercially.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Home | B A M | B A M Media | Bad Bug Book

Hypertext updated by rim 2000-MAR-27

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M51

Egg Yolk Emulsion, 50%

(Return to Table of Contents)

Wash fresh eggs with a stiff brush and drain. Soak eggs 1 h in 70% ethanol. Drain ethanol. Crack eggs aseptically and discard whites. Remove egg yolks with sterile syringe or wide-mouth pipet. Place yolks in sterile container and mix aseptically with equal volume of sterile 0.85% saline. Store at 4°C until use. Egg yolk emulsion (50%) is available commercially.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-31

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Bacteriological **A**nalytical **M**anual

Online

January 2001

M52

Buffered Enrichment Broth,

(for *Listeria*)
pH 7.3 ± 0.1

(Return to Table of Contents)

TSBYE supplemented with:

Monopotassium phosphate (anhydrous)	1.35 g/liter
Disodium phosphate	9.6 g/liter
Acriflavin HCl	10 mg/liter
Nalidixic acid (sodium salt)	40 mg/liter
Cycloheximide	50 mg/liter
Pyruvic acid (Sodium salt) (Sigma), 10% (w/v) aqueous solution	11.1 ml/liter

Sterilize enrichment broth without the 3 selective agents by autoclaving at 121°C for 15 min. Then add 2.5 ml 10% (w/v) filter-sterilized sodium pyruvate. Add the 3 selective ingredients aseptically to 225 ml enrichment broth plus the 25 g food sample after 4 h incubation at 30°C. Prepare acriflavin and nalidixic supplements as 0.5% (w/v) stock solutions in distilled water. Prepare cycloheximide supplement as 1.0% (w/v) stock solution in 40% (v/v) solution of ethanol in water.

Filter-sterilize the three selective ingredients. Add stock solutions: 0.455 ml acriflavin, 1.8 ml sodium nalidixate, and 1.15 ml cycloheximide to 225 ml enrichment broth plus the 25 g food sample.

Note: For information on cycloheximide availability problems, see Advisory for BAM Users on Reported Supply Problems for Cycloheximide.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.
(Revised SEP-26-2000)

Hypertext updated by rim/cjm/kwg 2001-FEB-09

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M53
Esculin Agar,
Modified (CDC)

(Return to Table of Contents)

Infusion agar	40 g
Esculin	1 g
Ferric citrate	0.5 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Cool to 55°C. Adjust pH to 7.0 ± 0.2. Dispense 4 ml portions to 13 x 100 mm tubes. Autoclave 15 min at 121°C. Slant tubes.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-FEB-9

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Bacteriological **A**nalytical **M**anual

Online

January 2001

M30b

Freezing medium

for *Campylobacter*

(Return to Table of Contents)

Bolton broth base	9.5 ml
Fetal bovine serum	1 ml (filtered, 0.22 µm pore size)
Glycerol (10%)	1 ml

Mix well before use.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M54

Gelatin Agar (GA)

(Return to Table of Contents)

Peptone	4 g
Yeast extract	1 g
Gelatin	15 g
Agar	15 g
Distilled water	1 liter

Suspend ingredients with constant stirring to prevent scorching gelatin, and boil to dissolve gelatin and agar. Adjust to pH 7.2 ± 0.2. Autoclave 15 min at 121°C. Cool to 45-50°C. Pour plates.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-12

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M55

Gelatin Salt Agar (GS)

(Return to Table of Contents)

Prepare gelatin agar (M54), but add 30 g NaCl per liter. Suspend ingredients and boil to dissolve gelatin and agar. Adjust to pH 7.2 ± 0.2. Autoclave 15 min at 121°C. Cool to 45-50°C. Pour plates. If necessary, to inhibit spreading by *Vibrio* spp. such as *V. alginolyticus*, use 25-30 g agar/liter.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-12

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M57

Gentamicin Sulfate Solution

(Return to Table of Contents)

Gentamicin sulfate	500,000 µg
Distilled water	100 ml

Sterilize by filtration through 0.20 µm membrane. Store at -20°C.

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Home | B A M | B A M Media | Bad Bug Book

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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M58
Ham's F-10 Medium

(commercial preparation is preferred)

(Return to Table of Contents)

L-Alanine	8.91 mg	Sodium pyruvate	110 mg
L-Arginine Hcl	211.00 mg	Thymidine	0.727 mg
L-Asparagine-H_2O	15.00 mg	Biotin	0.024 mg
L-Aspartic acid	13.30 mg	Choline chloride	0.698 mg
L-Cysteine·2HCl	35.12 mg	Folic acid	1.320 mg
L-Glutamine	146.20 mg	Isoinositol	0.541 mg

Dissolve ingredients in water. Sterilize by filtration.

L-Glutamic acid	14.70 mg	Niacinamide	0.615 mg
Glycine	7.51 mg	D-Calcium pantothenate	0.715 mg
L-Histidine HCl·H_2O	21.00 mg	Pyridoxine HCl	0.206 mg
L-Isoleucine	2.60 mg	Riboflavin	0.376 mg
L-Leucine	13.10 mg	Thiamine HCl	1.010 mg
L-Lysine HCl	29.30 mg	Vitamin B_{12}	1.360 mg
L-Methionine	4.48 mg	$CaCl_2 \cdot 2H_2O$	44.10 mg
D-Phenylalanine	4.96 mg	$CuSO_4 \cdot 5H_2O$	0.0025 mg
L-Proline	11.50 mg	$FeSO_4 \cdot 7H_2O$	0.83 mg
L-Serine	10.50 mg	KCl	285.00 mg
L-Threonine	3.57 mg	KH_2PO_4	83.00 mg
L-Tryptophan	0.60 mg	$MgSO_4 \cdot 7H_2O$	152.80 mg
L-Tyrosine	1.81 mg	NaCl	7400.00 mg
L-Valine	3.50 mg	$NaHCO_3$	1200.00 mg
Glucose	1100.0 mg	$NaH_2PO_4 \cdot H_2O$	290.0 mg
Hypoxanthine	4.08 mg	$ZnSO_4 \cdot 7H_2O$	0.028 mg

Final pH, 7.0 ± 0.2. Check sterility before use.

Lipoic acid	0.2 mg	Phenol red	1.2 mg
Distilled water			1 liter

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M59

Heart infusion agar (HIA)

(Difco)

(Return to Table of Contents)

Beef heart infusion (Difco)	500 g
Tryptose	10 g
NaCl	5 g
Agar	15 g
Distilled water	1 liter

Or

Blood Agar Base No. 2 (Difco)	1 liter
Proteose peptone	15 g

Liver digest	2.5 g
Yeast extract	5 g
NaCl	5 g
Agar	12 g

Autoclave either base at 121°C for 15 min. If preparing blood agar, reduce water to 950 ml. Add 50 ml defibrinated (whole or lysed) horse blood and FBP (4 ml to agar + blood) after autoclaving and cooling to 48°C. Final pH, 7.4 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-22

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M60

Heart Infusion (HI) Broth and Agar (HIA)

(for *Vibrio*)

(Return to Table of Contents)

Beef heart, infusion from 500 g	1 liter
Tryptose	10 g
NaCl	5 g

Dissolve ingredients and dispense into tubes.

For halophilic *Vibrio* spp. add an extra 15 g NaCl per liter.

Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2.

For heart infusion agar, add 15 g agar/liter and boil to dissolve before dispensing and sterilizing.

Commercially available from Difco.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-12

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M61

Hektoen Enteric (HE) Agar

(Return to Table of Contents)

Caution!!	
Storage	1 day only

Peptone	12 g	NaCl	5 g
Yeast extract	3 g	Sodium thiosulfate	5 g
Bile salts No. 3	9 g	Ferric ammonium citrate	1.5 g
Lactose	12 g	Bromthymol blue	0.065 g
Sucrose	12 g	Acid fuchsin	0.1 g
Salicin	2 g	Agar	14.0 g
Distilled water			1 liter

Heat to boiling with frequent agitation to dissolve. Boil no longer than 1 min. Do not overheat. Cool in water bath. Pour 20 ml portions into sterile 15 x 100 mm petri dishes. Let dry 2 h with lids partially removed. Final pH, 7.5 ± 0.2. Do not store more than 1 day.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M62

HC Agar

(for "Hemorrhagic Colitis" *E. coli* strains)

(Return to Table of Contents)

Tryptone	20.0 g
Bile salts No. 3	1.12 g
NaCl	5.0 g
Sorbitol	20.0 g
MUG reagent	0.1 g
Bromcresol purple	0.015 g
Agar	15.0 g
Distilled water (deionized)	1 liter

Dissolve ingredients in distilled water by heating with stirring. Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.2.

NOTE: The MUG reagent is not essential for the enzyme-labeled monoclonal antibody procedure.

For DNA probe: If colonies are not to be isolated by growth and metabolic characteristics, MUG reagent may be omitted. Plates may be kept 3-4 weeks if wrapped and stored at 4°C. Colonies do not attach well on filters if plates are too dry.

If HC agar is to be utilized fully, see Chapter 24, ref. 105.

MUG reagent may be purchased from HACH Company, P.O. Box 389, Loveland, CO.

Storage:	3-4 wk at 4°C if plates wrapped.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

R63

Hugh-Leifson Glucose Broth (HLGB)

(Return to Table of Contents)

Peptone	2 g
Yeast extract	0.5 g
NaCl	30 g
Dextrose	10 g
Bromcresol purple	0.015 g
Agar	3 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Adjust pH to 7.4 ± 0.2. Autoclave 15 min at 121°C.

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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M64

Indole Medium

(Return to Table of Contents)

L-Tryptophan	1 g
NaCl	1 g
K_2HPO_4	3.13 g
KH_2PO_4	0.27 g
Distilled water	200 ml

Dissolve ingredients. Dispense 1 ml portions to 13 x 100 mm screw-cap tubes. Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-19

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M65

Indole Medium (CDC)

(Return to Table of Contents)

Tryptone or trypticase	20 g
Distilled water	1 liter

Adjust pH to 7.3 ± 0.2. Dispense 4 ml portions to 13 x 100 mm tubes. Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Home | B A M | B A M Media | Bad Bug Book

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U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M66

Indole Nitrite Medium

(Tryptic Nitrate)

(Return to Table of Contents)

Trypticase	20 g
Na_2HPO_4	2 g
Dextrose	1 g
KNO_3	1 g
Agar	1 g
Distilled water	1 liter

Heat with agitation to dissolve ingredients. Mix and dispense 11 ml portions into 16 x 150 mm tubes. Autoclave 15 min at 118°C. Final pH, 7.2 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by ear/rim 2000-MAR-27

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M67

Irgasan-Ticarcillin-Chlorate (ITC) Broth

(Return to Table of Contents)

Tryptone	10 g
Yeast extract	1 g
$MgCl_2 \cdot 6H_2O$	60 g
NaCl	5 g
Potassium chlorate	1 g
Malachite green, 0.2%	5 ml
Distilled water	1 liter

Adjust to pH 7.6 ± 0.2 and autoclave at 121°C for 15 min.

Add the following:

Ticarcillin	(1 mg/ml)	1 ml
Irgasan DP300	(1 mg/ml)	1 ml

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M68

Iron Milk Medium (Modified)

(Return to Table of Contents)

Fresh whole milk	1 liter
Ferrous sulfate.$7H_2O$	1 g
Distilled water	50 ml

Dissolve ferrous sulfate in 50 ml distilled water. Add slowly to 1 liter milk and mix with magnetic stirrer. Dispense 11 ml medium into 16 x 150 mm culture tubes. **Autoclave 12 min at 118°C.**

Prepare fresh medium before use.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-27

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M69

King's B Medium

(Return to Table of Contents)

Proteose peptone No. 3	20 g
Glycerol, C.P.	10 ml
K_2HPO_4	1.5 g
$MgSO_4$	1.5 g
Agar	15 g
Distilled water	1 liter

Add all ingredients except $MgSO_4$. Heat with agitation to dissolve agar. Adjust pH to 7.2 ± 0.2. Add $MgSO_4$ slowly and mix. Dispense 4 ml portions to 13 x 100 tubes. Autoclave 15 min at 121°C.

Incline tubes to give half butt and half slant.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-24

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M70

King's O/F Basal Medium

(Return to Table of Contents)

Base

Trypticase (or casitone)	2 g
Phenol red, 1.5% solution	2 ml
Agar	3 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Adjust pH to 7.3 ± 0.2. Dispense 100 ml portions to flasks. Autoclave 15 min at 121°C. Cool to 50°C.

Carbohydrates, 10%.

Dissolve 10 g quantities of carbohydrates in 100 ml distilled water. Sterilize by filtration

through 0.22 µm membrane. Add 10 ml concentrate to 90 ml melted base and mix. Aseptically dispense 3 ml portions to sterile 13 x 100 mm tubes.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-FEB-10

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M71

Kligler Iron Agar

(Return to Table of Contents)

Polypeptone peptone	20 g
Lactose	20 g
Dextrose	1 g
NaCl	5 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.5 g
Agar	15 g
Phenol red	0.025 g
Distilled water	1 liter

Heat with agitation to dissolve. Dispense into 13 x 100 mm screw-cap tubes and autoclave 15 min at 121°C. Cool and slant to form deep butts. Final pH, 7.4 ± 0.2.

Commercially available from Difco, BBL, and Oxoid.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-27

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M72

Koser's Citrate Broth

(Return to Table of Contents)

$NaNH_4HPO_4 \cdot 4H_2O$	1.5 g
KH_2PO_4 (monobasic)	1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Sodium citrate$\cdot 2H_2O$	3 g
Distilled water	1 liter

Dispense into screw-cap tubes as desired. Autoclave 15 min at 121°C. Final pH, 6.7 ± 0.2. This formulation is listed in *Official Methods of Analysis* of AOAC International, and *Standard Methods for the Examination of Wastewater* of the APHA. It differs from the composition of commercially available dehydrated medium. The latter is satisfactory.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-27

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M73

L-15 Medium (Modified) Leibovitz

(Return to Table of Contents)

D-Galactose	90 mg	L-Tyrosine	300 mg
Phenol red, Na	10 mg	DL-Valine	200 mg
Sodium pyruvate	550 mg	D-Calcium pantothenate	1 mg
DL--Alanine	450 mg	Choline chloride	1 mg
L-Arginine (free base)	500 mg	Folic acid	1 mg
L-Asparagine·H_2O	250 mg	i-Inositol	2 mg
L-Cysteine (free base)	120 mg	Nicotinamide	1 mg
L-Glutamine	300 mg	Pyridoxine HCl	1 mg
Glycine	200 mg	Riboflavin-5-phosphate, Na	0.1 mg
L-Histidine (free base)	250 mg	Thiamin monophosphate·$2H_2O$	1.0 mg

Filter through 0.20 µm membrane and dispense into 2 liter Erlenmeyer flask. Final pH,

L-Isoleucine	250 mg	$CaCl_2$ (anhydrous)	140 mg
L-Leucine HC1	125 mg	KC1	400 mg
DL-Methionine	150 mg	KH_2PO_4	60 mg
L-Phenylalanine	250 mg	$MgCl_2$ (anhydrous)	93.68 mg
L-Serine	200 mg	NaCl	8000 mg
DL-Threonine	600 mg	Na_2HPO_4 (anhydrous)	190 mg
L-Tryptophan	20 mg	Distilled water	1 liter

7.5. Cap and store at 5°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M74

Lactose Broth

(Return to Table of Contents)

Beef extract	3 g
Peptone	5 g
Lactose	5 g
Distilled water	1 liter

Dispense 225 ml portions into 500 ml Erlenmeyer flasks. After autoclaving 15 min at 121°C and just before use, aseptically adjust volume to 225 ml. Final pH, 6.9 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-21

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M75

Lactose-Gelatin Medium

(for *Clostridium perfringens*)

(Return to Table of Contents)

Tryptose	15 g
Yeast extract	10 g
Lactose	10 g
Phenol red (1% solution in 95% ethanol)	5.0 ml
Gelatin	120 g
Distilled water	1 liter

Heat to dissolve tryptose, yeast extract, and lactose in 400 ml water. Suspend gelatin in 600 ml water and heat at 50-60°C with agitation to dissolve. Mix 2 solutions. Adjust pH to 7.5 ± 0.2. Add phenol red and mix. Dispense 10 ml portions into 16 x 150 mm screw-cap tubes. Autoclave 10 min at 121°C. If not used within 8 h, deaerate by heating at 50-70°C for 2-3 h before use.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M76

Lauryl Tryptose (LST) Broth

(Return to Table of Contents)

Tryptose or trypticase	20 g
Lactose	5 g
K_2HPO_4	2.75 g
KH_2PO_4	2.75 g
NaCl	5 g
Sodium lauryl sulfate	0.1 g
Distilled water	1 liter

Dispense 10 ml portions into 20 x 150 mm tubes containing inverted 10 x 75 mm fermentation tubes. Autoclave 15 min at 121°C. Final pH, 6.8 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-28

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M77

Lauryl Tryptose MUG (LST-MUG) Broth

(Return to Table of Contents)

Tryptose or trypticase	20 g
Lactose	5 g
K_2HPO_4	2.75 g
KH_2PO_4	2.75 g
NaCl	5 g
Sodium lauryl sulfate	0.1 g
4-Methylumbelliferyl-β-D-glucuronide (MUG)*	50 mg
Distilled water	1 liter

Prepare lauryl tryptose broth and add MUG. Dissolve with gentle heat if necessary. Dispense 10 ml portions into 20 x 150 mm test tubes containing inverted 10 x 75 mm fermentation tubes. Autoclave 15 min at 121°C. Final pH, 6.8 ± 0.2.

*MUG may be obtained from several sources, e.g., HACH Co., Loveland, CO.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-FEB-11

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M78

Letheen Agar (Modified)

(Return to Table of Contents)

Letheen agar (Difco or BBL)	32 g
Trypticase peptone	5 g
Thiotone peptone	10 g
Yeast extract	2 g
NaCl	5 g
Sodium bisulfite	0.1 g
Agar	5 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Autoclave 15 min at 121°C. Aseptically dispense 20 ml into 15 x 100 mm petri dishes. Final pH, 7.2 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-28

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

August 2001

M79

Letheen Broth (Modified)

(Return to Table of Contents)

Letheen broth	25.7 g
Trypticase peptone	5 g
Thiotone peptone	10 g
Yeast extract	2 g
Sodium bisulfite	0.1 g
Distilled water	1 liter

Dispense 90 ml into screw-cap bottle. Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.
Revisions: 2001-AUG-20 Formulation corrected, letheen broth amount corrected from 26.7 to 25.7.

Hypertext updated by rim/cjm 2001-OCT-24

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M80

Levine's Eosin-Methylene Blue (L-EMB) Agar

(Return to Table of Contents)

Peptone	10 g
Lactose	10 g
K_2HPO_4	2 g
Agar	15 g
Eosin Y	0.4 g
Methylene blue	0.065 g
Distilled water	1 liter

Boil to dissolve peptone, phosphate, and agar in 1 liter of water. Add water to make original volume. Dispense in 100 or 200 ml portions and autoclave 15 min at not over 121°C. Final pH, 7.1 ± 0.2.

Before use, melt, and to each 100 ml portion add:

a. 5 ml sterile 20% lactose solution;

b. 2 ml aqueous 2% eosin Y solution; and
c. 4.3 ml 0.15% aqueous methylene blue solution.

When using complete dehydrated product, boil to dissolve all ingredients in 1 liter water. Dispense in 100 or 200 ml portions and autoclave 15 min at 121°C. Final pH, 7.1 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M81

Lithium Chloride-Phenylethanol-Moxalactam (LPM) Medium

(Return to Table of Contents)

Phenylethanol agar (Difco)	35.5 g
Glycine anhydride (**NOTE**: not glycine)	10 g
Lithium chloride	5 g
Moxalactam stock solution, 1% in phosphate buffer, pH 6.0	2 ml
Distilled water	1 liter

Sterilize medium (without moxalactam) at 121°C for 15 min. Cool to 48-50°C and add filter-sterilized moxalactam solution.

Moxalactam stock solution

Consists of 1 g moxalactam salt (ammonium or sodium) in 100 ml 0.1 M potassium phosphate

buffer, pH 6.0. Store filter-sterilized stock solution frozen in 2 ml aliquots.

Moxalactam (Eli Lilly Co.) is retailed by Sigma Chemical Co. The LPM medium is most effective in the Henry illumination system when poured thin, i.e., 12-15 ml per standard petri dish. To avoid drying of thin agar, refrigerate and use plates rapidly. LPM basal medium is commercially available as a powder.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-24

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M82

LPM Plus Esculin and Ferric Iron

(Return to Table of Contents)

Esculin	1.0 g
Ferric ammonium citrate	0.5 g

Add these components to those for LPM (M81).

Sterilize medium, temper, and add filter-sterilized moxalactam, as described for LPM medium.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M83

Liver-Veal Agar

(Return to Table of Contents)

Liver, infusion from	50 g	Starch, soluble	10 g
Veal, infusion from	500 g	Casein, isoelectric	2 g
Proteose peptone	20 g	NaCl	5 g
Neopeptone	1.3 g	Sodium nitrate	2 g
Tryptone	1.3 g	Gelatin	20 g
Dextrose	5 g	Agar	15 g
Distilled water			1 liter

Heat with agitation to dissolve. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-24

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M84

Liver-Veal-Egg Yolk Agar

(Return to Table of Contents)

Fresh eggs, yolks only	2 or 3
Liver veal agar	48.5 g
Distilled water	500 ml

Heat with agitation to dissolve. Autoclave 15 min at 121°C. Cool to 50°C.

Egg yolk emulsion

To each 500 ml of melted liver veal agar, add 40 ml egg yolk-saline suspension (**see** M51 for instructions). Mix thoroughly and pour into sterile 15 x 100 mm petri dishes. Dry plates at room temperature for 2 days or at 35°C for 24 h. Check plates for sterility and store sterile plates in refrigerator. In certain instances the medium may be used without addition of egg yolk emulsion.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M85

Long-term Preservation Medium

(Return to Table of Contents)

Yeast extract, 0.3%	3 g
Peptone	10 g
NaCl	30 g
Agar	3 g
Distilled water	1 liter

Heat to dissolve ingredients. Dispense 4 ml portions to 13 x 100 mm screw-cap tubes. Autoclave 15 min at 121°C. Cool and tighten caps for storage. No pH adjustment is necessary.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-24

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M86

Lysine Arginine Iron Agar (LAIA)

(Return to Table of Contents)

Peptone	5 g
Yeast extract	3 g
Glucose	1 g
L-Lysine	10 g
L-Arginine	10 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.04 g
Bromcresol purple	0.02 g
Agar	15 g

Adjust pH to 6.8. Heat to boiling and dispense 5 ml into each 13 x 100 mm screw-cap culture tube. Autoclave at 121°C for 12 min. Cool tubes in slanted position. (This medium may also be prepared by supplementing Difco lysine iron agar (LIA) with 10 g L-arginine per liter.)

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-24

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M87

Lysine Decarboxylase Broth

(Falkow)

(for *Salmonella*)

(Return to Table of Contents)

Gelysate or peptone	5 g
Yeast extract	3 g
Glucose	1 g
L-Lysine	5 g
Bromcresol purple	0.02 g
Distilled water	1 liter

Heat until dissolved. Dispense 5 ml portions into 16 x 125 mm screw-cap tubes. Autoclave loosely capped tubes 15 min at 121°C. Screw the caps on tightly for storage and after inoculation. Final pH, 6.8 ± 0.2.

For halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-24

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M88

Lysine Decarboxylase (LDC) Medium

(for Gram-negative nonfermentative bacteria)

(Return to Table of Contents)

L-Lysine HCl	0.5 g
Dextrose	0.5 g
KH_2PO_4	0.5 g
Distilled water	100 ml

Dissolve ingredients. Adjust pH to 4.6 ± 0.2. Autoclave 15 min at 121°C. Aseptically dispense 1 ml portions to sterile 13 x 100 mm tubes.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-24

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M89

Lysine Iron Agar

(Edwards and Fife)

(Return to Table of Contents)

Gelysate or peptone	5 g
Yeast extract	3 g
Glucose	1 g
L-Lysine hydrochloride	10 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate (anhydrous)	0.04 g
Bromcresol purple	0.02 g
Agar	15 g
Distilled water	1 liter

Heat to dissolve ingredients. Dispense 4 ml portions into 13 x 100 mm screw-cap tubes. Autoclave 12 min at 121°C. Let solidify in slanted position to form 4 cm butts and 2.5 cm slants. Final pH, 6.7 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-24

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M90

Lysozyme Broth

(Return to Table of Contents)

Base

Prepare nutrient broth as recommended. Dispense 99 ml portions to 170 ml bottles. Autoclave 15 min at 121°C. Cool to room temperature before use.

Lysozyme solution

Dissolve 0.1 g lysozyme in 65 ml sterile 0.0l N HCl. Heat to boiling for 20 min. Dilute to 100 ml with sterile 0.0l N HCl. Alternatively, dissolve 0.1 g lysozyme in 100 ml distilled water. Sterilize by filtration through 0.45 µm membrane. Test for sterility before use. Add 1 ml lysozyme solution to 99 ml nutrient broth. Mix and dispense 2.5 ml portions to sterile 13 x 100 mm tubes.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M91

MacConkey Agar

(Return to Table of Contents)

Proteose peptone or polypeptone	3 g
Peptone or gelysate	17 g
Lactose	10 g
Bile salts No. 3 or bile salts mixture	1.5 g
NaCl	5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Distilled water	1 liter

Suspend ingredients and heat with agitation to dissolve. Boil 1-2 min. Autoclave 15 min at 121°C, cool to 45-50°C, and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Dry at room temperature with lids closed.

DO NOT USE WET PLATES. Final pH, 7.1 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M92

Malonate Broth

(Return to Table of Contents)

Yeast extract	1 g
$(NH_4)_2SO_4$	2 g
K_2HPO_4	0.6 g
KH_2PO_4	0.4 g
NaCl	2 g
Sodium malonate	3 g
Glucose	0.25 g
Bromthymol blue	0.025 g
Distilled water	1 liter

Dissolve by heating, if necessary. Dispense 3 ml portions into 13 x 100 mm test tubes. Autoclave 15 min at 121°C. Final pH, 6.7 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M185

Malt Agar (MA)

(Return to Table of Contents)

Malt extract, powdered	20.0 g
Agar	20.0 g
Distilled water	1.0 liter

Mix ingredients, steam to dissolve agar and sterilize for 15 min at 121°C. Temper medium to 45°C and pour plates under aseptic conditions.

To prepare slants dispense 5-6 ml of steamed medium (before autoclaving) into each of several 16 x 125 mm screw-cap tubes, loosely cap tubes and sterilize as above. After autoclaving lay tubes in a slanting position and let them cool.

This medium is recommended as a general maintenance medium.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-31

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M93

Malt Extract Agar

(Cosmetics-General Microbiology)

(Return to Table of Contents)

Malt extract	30 g
Agar	20 g
Distilled water	1 liter

Boil to dissolve ingredients. Avoid overheating, which causes softening of agar and darkening of medium color. Autoclave 15 min at 121°C. Dispense 20-25 ml into sterile 15 x 100 mm petri dishes. Final pH, 5.5 ± 0.2.

For cosmetics use

Cool medium to 47-50°C after autoclaving. Dispense 4 ml stock filter-sterilized chlortetracycline HCl solution (1 g/100 ml) per liter of medium to yield final concentration of 40 ppm chlorotetracycline HCl. Mix thoroughly and dispense 20 ml portions into 15 x 100 mm petri dishes.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M182

Malt Extract Agar for Yeasts and Molds (MEAYM)

(Return to Table of Contents)

Malt extract, powdered	20.0 g
Glucose	20.0 g
Peptone	1.0 g
Agar	20.0 g
Distilled water	1 liter

Final pH 5.4.

Mix ingredients, heat to dissolve agar and sterilize at 121°C for 15 min. Temper media to 45°C and pour plates under aseptic conditions. Dehydrated MA is commercially available, but since several MA formulas exist, check for the correct composition.

This medium is recommended for identification of *Aspergillus* and *Penicillium*.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-31

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M94

Malt Extract Broth

(Difco)

(Return to Table of Contents)

Malt extract base	6.0 g
Maltose, technical	1.8 g
Dextrose	6.0 g
Yeast extract	1.2 g
Water	1 liter

Final pH, 4.7 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M95

Mannitol-Egg Yolk-Polymyxin (MYP) Agar

(Return to Table of Contents)

Base

Beef extract	1 g
Peptone	10 g
Mannitol	10 g
NaCl	10 g
Phenol red (1% solution in 95% ethanol)	2.5 ml
Agar	15 g

Heat with agitation to dissolve agar. Adjust pH so that value after sterilization is 7.2 ± 0.2. Dispense 225 ml portions to 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°C. Cool to 50°C. MYP agar is commercially available from Difco.

Distilled water	900 ml

Polymyxin B solution, 0.1%

Dissolve 500,000 units polymyxin B sulfate in 50 ml distilled water. Filter-sterilize and store in the dark at 4°C until needed.

Egg yolk emulsion, 50%

(see M51). Also available from commercial suppliers.

Final medium

To 225 ml melted base add 2.5 ml polymyxin B solution and 12.5 ml egg yolk emulsion. Mix and dispense 18 ml portions to sterile 15 x 100 mm petri dishes. Dry plates at room temperature for 24 h before use.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M96

Mannitol Maltose Agar

(Return to Table of Contents)

Phytone	5 g
Polypeptone	5 g
Beef Extract	5 g
D-Mannitol	10 g
Maltose	10 g
NaCl	20 g
Agar	13 g
1000X Dye stock solution*	1 ml
Distilled water	1 liter

Suspend ingredients and boil to dissolve. Adjust to pH 7.8 ± 0.2 Autoclave 15 min at 121°C.

*1000X Dye stock solution

See formulation under modified cellobiose-polymyxin B-colistin (mCPC) agar (M98).

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm/kwg 2001-MAR-28

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M97

Mannitol Salt Agar

(Return to Table of Contents)

Beef extract	1 g
Polypeptone	10 g
NaCl	75 g
Mannitol	10 g
Phenol red	0.025 g
Agar	15 g
Distilled water	1 liter

Heat with agitation to dissolve agar and boil 1 min. Dispense 20 ml portions into 15 x 100 mm petri dishes. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M30a

Modified Campylobacter Blood-Free Selective Agar Base (CCDA)

(Return to Table of Contents)

CCDA agar base (OXOID)	45.5 g
Yeast extract	2 g
Distilled water	1 liter

Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Cool and add of sodium cefoperazone (**6.4 ml** of strength used for broth or **4 ml** of the solution for A-H agar), 4 ml rifampicin, and 4 ml amphotericin B. See A-H directions for precautions when drying plates.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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Bacteriological**A**nalytical**M**anual

Online

January 2001

M98

Modified Cellobiose-Polymyxin B-Colistin (mCPC) Agar

(Return to Table of Contents)

Solution 1

Peptone	10 g
Beef extract	5 g
NaCl	20 g
1000X Dye stock solution*	1 ml
Agar	15 g
Distilled water	900 ml

Adjust to pH 7.6. Boil to dissolve agar. Cool to 48-55°C.

*1000X Dye stock solution

Bromthymol blue	4.0 g
Cresol red	4.0 g
Ethanol, 95%	100 ml

For consistent medium color, use dye solution rather than repeatedly weighing out dry dyes. Dissolve dyes in ethanol for 4% (w/v) stock solution. Using 1 ml of this solution per liter of mCPC agar gives 40 mg bromthymol blue and 40 mg cresol red per liter.

Solution 2

Cellobiose	10 g
Colistin	400,000 units
Polymyxin B	100,000 units
Distilled water	100 ml

Dissolve cellobiose in distilled water by heating gently. Cool. Add antibiotics.

Add Solution 2 to cooled Solution 1, mix, and dispense into petri dishes. Final color, dark green to green-brown.

NOTE: This medium, like TCBS, is very inhibitory and does not require autoclaving. Medium may be stored 2 weeks at refrigeration temperatures.

Storage: limit: 2 wks Temperature: 4°C

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

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Bacteriological Analytical Manual

Online

January 2001

M99

Motility-Indole-Ornithine (MIO) Medium

(Return to Table of Contents)

Yeast extract	3 g
Peptone	10 g
Tryptone	10 g
L-Ornithine HCl	5 g
Dextrose	1 g
Agar	2 g
Bromcresol purple	0.02 g
Distilled water	1 liter

Dispense 4 ml portions into 13 x 100 mm tubes. Autoclave 15 min at 121°C. Final pH, 6.5 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/cjm 2000-APR-26

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Bacteriological Analytical Manual

Online

January 2001

M100

Motility Medium (for *B. cereus*)

(Return to Table of Contents)

Trypticase	10 g
Yeast extract	2.5 g
Dextrose	5 g
Na_2HPO_4	2.5 g
Agar	3 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Dispense 100 ml portions to 170 ml bottles. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Cool to 50°C. Aseptically dispense 2 ml portions to sterile 13 x 100 mm tubes. Store at room temperature 2 days before use.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M101

Motility Nitrate Medium

(for Cosmetics)
(for Gram-negative nonfermentative bacteria)

(Return to Table of Contents)

Tryptose	10 g
Heart infusion agar (Difco)	8 g
Potassium (or sodium) nitrate	1 g
Glucose	0.5 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Dispense 4 ml portions into 13 x 100 mm screw-cap tubes. Autoclave 15 min at 121°C. Available as prepared medium from BBL and Scott.

NOTE: Make sure nitrates are nitrite-free.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-31

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Bacteriological Analytical Manual

Online

January 2001

M102

Motility-Nitrate Medium, Buffered

(for *C. perfringens*)

(Return to Table of Contents)

Beef extract	3 g
Peptone (Difco)	5 g
KNO_3	1 g
Na_2HPO_3	2.5 g
Agar	3 g
Galactose	5 g
Glycerin (reagent grade)	5 ml
Distilled water	1 liter

Dissolve all ingredients except agar. Adjust pH to 7.3 ± 0.1. Add agar and heat to dissolve. Dispense 11 ml portions into 16 x 150 mm tubes. Autoclave 15 min at 121°C. If not used within 4 h, heat 10 min in boiling water or flowing steam. Chill in cold water.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-28

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M103

Motility Test Medium (Semisolid)

(Return to Table of Contents)

Beef extract	3 g
Peptone or gelysate	10 g
*NaCl	5 g
Agar	4 g
Distilled water	1 liter

General Instructions: Heat with agitation and boil 1-2 min to dissolve agar. For use with *Salmonella*, see instructions listed below. Dispense 8 ml portions into 16 x 150 screw-cap tubes. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2.

*For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

For *Listeria*: Keep individual tubes tightly screw capped and sealed with parafilm. Store in refrigerator for up to two weeks.

For *Salmonella*: Dispense 20 ml portions into 20 x 150 mm screw-cap tubes, replacing caps loosely. Autoclave 15 min at 121°C. Cool to 45°C after autoclaving. Tighten caps, and

refrigerate at 5-8°C. To use, remelt in boiling water or flowing steam, and cool to 45°C. Aseptically dispense 20 ml portions into sterile 15 x 100 mm petri plates. Cover plates and let solidify. Use same day as prepared. Final pH, 7.4 ± 0.2.

CAUTION:	**For *Salmonella:* USE SAME DAY AS PREPARED**

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.
(Revised SEP-26-2000)

Hypertext updated by cjm/rim/kwg 2001-FEB-08

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Bacteriological Analytical Manual

Online

January 2001

M104

MR-VP Broth

(Return to Table of Contents)

Medium 1

Buffered peptone-water powder (Difco or BBL)	7 g
Glucose	5 g
K_2HP0_4	5 g
Distilled water	1 liter

Medium 2

Pancreatic digest of casein	3.5 g
Peptic digest of animal tissue	3.5 g

Dextrose	5.0 g
Potassium phosphate	5.0 g
Distilled water	1 liter

Dissolve ingredients in water with gentle heat if necessary. Dispense 10 ml into 16 x 150 mm test tubes and autoclave 15 min at 118-121°C. Final pH, 6.9 ± 0.2.

Medium 3

Peptone	5.0 g
Glucose	5.0 g
Phosphate buffer	5.0 g
Distilled water	1 liter

Dissolve ingredients in water. Dispense 10 ml into 16 x 150 mm test tubes and autoclave 15 min at 121°C. Final pH, 7.5 ± 0.2.

For *Salmonella*: Dispense 10 ml into 16 x 150 mm test tubes and autoclave 12-15 min at 121°C.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-28

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M105

Mucate Broth

(Return to Table of Contents)

Peptone	10 g
Mucic acid	10 g
Bromthymol blue	0.024 g
Distilled water	1 liter

Dissolve peptone. Dissolve mucic acid by slowly adding 5 N NaOH and stirring. Dispense 5 ml portions into 13 x 100 mm screw-cap tubes. Autoclave 10 min at 121°C. Final pH, 7.4 ± 0.1.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M106

Mucate Control Broth

(Return to Table of Contents)

Peptone	10 g
Bromthymol blue	0.024 g
Distilled water	1 liter

Dissolve ingredients. Dispense 5 ml portions into 13 x 100 mm screw-cap tubes. Autoclave 10 min at 121°C. Final pH, 7.4 ± 0.1.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M107

Mueller-Hinton Agar

(Return to Table of Contents)

Beef, infusion from	300 g
Acidicase Peptone (BBL) or Casamino Acids (Difco)	17.5 g
Starch	1.5 g
Agar	17 g
Distilled water	1 liter

Heat to boiling for 1 min. Autoclave 15 min at 116°C. Final pH, 7.3 ± 0.2.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M108

Nitrate Broth

(Return to Table of Contents)

Beef extract	3 g
Peptone	5 g
KNO_3 (nitrite-free)	1 g
Distilled water	1 liter

Dissolve ingredients. Dispense 5 ml portions into 16 x 125 mm tubes. Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M109

Nitrate Broth, Enriched (CDC)

(Return to Table of Contents)

Infusion broth	25 g
KNO_3 (nitrite-free)	2 g
Distilled water	1 liter

Dispense 4 ml portions into 13 x 100 mm tubes with inverted Durham tubes. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M110

Nitrate Reduction Medium and Reagents

(Return to Table of Contents)

Culture medium

Prepare nitrate broth (M108) from nutrient broth (M114) containing 1.0 g/liter potassium nitrate.

Reagent A. Dissolve 0.5 g α-naphthylamine (a carcinogen) in 100 ml 5 N acetic acid by gently heating. Prepare 5 N acetic acid by adding distilled water to 28.7 ml glacial acetic acid (17.4 N) to give final volume of 100 ml.

Reagent B. Dissolve 0.8 g sulfanilic acid in 100 ml 5 N acetic acid by gently heating.

Reagent C. Dissolve 1 g α-naphthol in 200 ml acetic acid.

Zinc powder.

Cadmium reagent. Place zinc rods in 20% solution of cadmium sulfate for several hours. Draw off precipitated cadmium and add to 1 N HCl.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-29

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M111

Nonfat Dry Milk (Reconstituted)

(Return to Table of Contents)

Nonfat dry milk	100 g
Distilled water	1 liter

For *Salmonella*:

Suspend 100 g dehydrated nonfat dry milk in 1 liter distilled water. Swirl until dissolved. Autoclave 15 min at 121°C.

For monkey kidney cell culture:

Dispense 500 ml into 1 liter Erlenmeyer flasks.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M112

Nutrient Agar

(Return to Table of Contents)

Beef extract	3 g
Peptone	5 g
Agar	15 g
Distilled water	1 liter

Heat to boiling to dissolve ingredients. Dispense into tubes or flasks. Autoclave 15 min at 121°C. Final pH, 6.8 ± 0.2. If used as base for blood agar, add 8 g NaCl to prevent hemolysis of blood cells.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M113

Nutrient Agar

(for *Bacillus cereus*)

(Return to Table of Contents)

For slants, prepare nutrient agar and dispense 6.5 ml portions into 16 x 125 mm screw-cap tubes. Autoclave 15 min at 121°C. Slant tubes until medium solidifies. For plates, dispense 100-500 ml portions into bottles or flasks and autoclave. Cool to 50°C and dispense 18-20 ml into sterile 15 x 100 mm petri dishes. Dry plates for 24-48 h at room temperature before use.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M114

Nutrient Broth

(Return to Table of Contents)

Beef extract	3 g
Peptone	5 g
Distilled water	1 liter

Heat to dissolve. Dispense 10 m1 portions into tubes or 225 ml portions into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 6.8 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M115

Nutrient Gelatin (CDC)

(for Gram-negative nonfermentative bacteria)

(Return to Table of Contents)

Infusion broth	25 g
Gelatin	120 g
Distilled water	1 liter

Heat with agitation to dissolve. Cool to 55°C and adjust pH to 7.4 ± 0.2. Dispense 4 ml portions into 13 x 100 mm screw-cap tubes. Autoclave 15 min at 121°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M116

OF Glucose Medium, Semisolid

(Return to Table of Contents)

Tryptone (trypticase)	2 g
NaCl	5 g
Dipotassium phosphate	0.3 g
Bromthymol blue dye	0.03 g
Agar	2 g
Glucose	10 g
Distilled water	1 liter

Boil ingredients to dissolve.

For halophilic *Vibrio* spp., add 15 g NaCl (2% NaCl, final concentration) to medium.

Dispense 5 ml into 13 x 100 mm tubes and autoclave 15 min at 121°C. Final pH, 6.8 ± 0.2.

*To use OF medium with sugars other than glucose, prepare medium without glucose in 900 ml water, sterilize as above, and cool to 45-50°C. Add 100 ml of 10% solution of filter-sterilized sugar to basal medium. Aseptically dispense 5 ml into sterile 13 x 100 mm tubes.

For *Campylobacter*, prepare half the tubes with glucose and half without.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M118

Oxford Medium

(Return to Table of Contents)

Columbia blood agar base	39.0 g
Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Lithium chloride	15.0 g
Cycloheximide	0.4 g
Colistin sulfate	0.02 g
Acriflavin	0.005 g
Cefotetan	0.002 g
Fosfomycin	0.010 g
Distilled water	1 liter

Add the 55.5 g of the first 4 components (basal medium) to 1 liter distilled water. Bring gently to boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 min. Cool to 50°C and aseptically add supplement, mix, and pour into sterile petri dishes. To prepare supplement, dissolve cycloheximide, colistin sulfate, acriflavin, cefotetan, and fosfomycin in 10 ml of 1:1 mixture of ethanol and distilled water. Filter-sterilize supplement before use. Oxford basal medium and supplement mixture are available commercially.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M117

Oxidative-Fermentative (OF) Test Medium

(Return to Table of Contents)

Base

Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Bromthymol blue	0.03 g
Agar	2.5 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Dispense 3 ml portions into 13 x 100 mm tubes. Autoclave 15 min at 121°C. Cool to 50°C; pH, 7.1.

Carbohydrate stock solution

Dissolve 10 g carbohydrate in 90 ml distilled water. Sterilize by filtration through 0.22 µm membrane. Add 0.3 ml stock solution to 2.7 ml base in tube. Mix gently and cool at room temperature.

Inoculate tubes in duplicate. Layer one tube with sterile mineral oil. Incubate 48 h at 35°C.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M118a

PALCAM Listeria Selective Agar

(Return to Table of Contents)

Basal medium

Peptone	23 g
Starch	1 g
NaCl	5 g
Columbia agar	13 g
Mannitol	10 g
Ferric ammonium citrate	0.5 g
Esculin (aesculin)	0.8 g
Dextrose (glucose)	0.5 g

Lithium chloride	15.0 g
Phenol red	0.08 g
Distilled water	1000 ml

Selective agents

Polymyxin B sulfate	10 mg
Acriflavin	5 mg
Ceftazidine	20 mg
Distilled water	2 ml

To make 500 ml of medium, weigh 34.4 g basal medium powder (all ingredients except the three selective agents) and suspend in 500 ml distilled water. Sterilize by autoclaving at 121°C for 15 min. Dissolve the selective agent supplement mixture in sterile distilled water at 17.5 mg/ml and filter sterilize. Add **1 ml** selective agent supplement solution to 500 ml sterile basal medium that has been cooled to 50°C. Mix gently and pour plates. Final pH, 7.2 ± 0.1.

*__NOTE__: The basal medium powder and lyophilized supplement mixture are available commercially; follow the manufacturer's instructions for preparing the medium.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/kwg 2000-DEC-15

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M56a

Papain

(Return to Table of Contents)

M56 - Gelatinase has been replaced by M56a

Papain	5 g
Distilled water	95 ml

Add papain to sterile, distilled water and swirl to dissolved completely. Dispense 100 ml portion into bottles.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M119

Penicillin-Streptomycin Solution

(antibiotic concentrate)

(Return to Table of Contents)

Penicillin G	500,000 IU
Streptomycin	500,000 5g
Distilled water	100 ml

Dissolve antibiotics in water and sterilize by filtration. Store at 5°C.

For *V. cholerae*, use commercially available penicillin G-streptomycin sulfate solution. Add 5 ml solution per 500 ml medium. Store at -20°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M120

Peptone Sorbitol Bile Broth

(Return to Table of Contents)

Na_2HPO_4	8.23 g
$NaH_2PO_4 \cdot H_2O$	1.2 g
Bile salts No. 3	1.5 g
NaCl	5 g
Sorbitol	10 g
Peptone	5 g
Distilled water	1 liter

Dispense 100 ml into Wheaton bottles. Autoclave 15 min at 121°C. Final pH, 7.6 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M121

Phenol Red Carbohydrate Broth

(Return to Table of Contents)

Trypticase or proteone peptone No. 3	10 g
NaCl	5 g
Beef extract (optional)	1 g
Phenol red (7.2 ml of 0.25% phenol red solution)	0.018 g
Distilled water	1 liter
Carbohydrate*	

*Dissolve either 5 g dulcitol, 10 g lactose, or 10 g sucrose (as specified in the *Salmonella* test) in this basal broth.

Dispense 2.5 ml portions into 13 x 100 mm test tubes containing inverted 6 x 50 mm fermentation tubes. Autoclave 10 min at 118°C. Final pH, 7.4 ± 0.2.

Alternatively, dissolve ingredients, omitting carbohydrate, in 800 ml distilled water with heat and occasional agitation. Dispense 2.0 ml portions into 13 x 100 mm test tubes containing inverted fermentation tubes. Autoclave 15 min at 118°C and let cool. Dissolve carbohydrate in 200 ml distilled water and sterilize by passing solution through bacteria-retaining filter. Aseptically add 0.5 ml sterile filtrate to each tube of sterilized broth after cooling to less than

45°C. Shake gently to mix. Final pH, 7.4 ± 0.2.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M122

Phenol Red Glucose Broth

(Return to Table of Contents)

Proteose peptone No. 3	10 g
NaCl	5 g
Beef extract (optional)	1 g
Dextrose	5 g
Phenol red (7.2 ml of 0.25% solution)	0.018 g
Distilled water	1 liter

Dispense 2.5 ml portions into 13 x 100 mm tubes. Autoclave 10 min at 118°C. Final pH, 7.4 ± 0.2.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M123

Phenylalanine Deaminase Agar

(Return to Table of Contents)

Yeast extract	3 g
L-Phenylalanine or	1 g
DL-Phenylalanine	2 g
Na_2HPO_4	1 g
NaCl	5 g
Agar	12 g
Distilled water	1 liter

Heat gently to dissolve agar. Tube and autoclave 10 min at 121°C. Incline tubes to obtain long slant. Final pH, 7.3 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M124

Plate Count Agar

(Standard Methods)

(Return to Table of Contents)

Tryptone	5 g
Yeast extract	2.5 g
Dextrose	1 g
Agar	15 g
Distilled water	1 liter

Heat to dissolve ingredients. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2.

For viable yeasts and molds, dispense 20-25 ml portions into sterile 15 x 100 mm petri dishes.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M125

PMP Broth

(Return to Table of Contents)

Na_2HPO_4	7.9 g
NaH_2PO_4	1.1 g
Peptone	2.5 g
D-Mannitol	2.5 g
Distilled water	1 liter

Adjust pH to 7.6. Autoclave at 121°C for 15 min.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-29

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M126

*Potassium Cyanide (KCN) Broth

(Return to Table of Contents)

*Potassium cyanide (KCN)	0.5 g
Proteose peptone No. 3 or polypeptone	3 g
NaCl	5 g
KH_2PO_4	0.225 g
Na_2HPO_4	5.64 g
Distilled water	1 liter

Dissolve above ingredients **except potassium cyanide** and autoclave 15 min at 121°C. Cool and refrigerate at 5-8°C. Final pH, 7.6 ± 0.2.

Prepare KCN stock solution by dissolving 0.5 g KCN in 100 ml sterile distilled water cooled to 5-8°C.

Using bulb pipetter, add 15 ml cold KCN stock solution to 1 liter cold, sterile base.

DO NOT PIPET BY MOUTH!!!
Handle with gloves!!

Mix and aseptically dispense 1.0-1.5 ml portions to 13 x 100 mm sterile tubes. Using aseptic technique, stopper tubes with No. 2 corks impregnated with paraffin. Prepare corks by boiling in paraffin about 5 min. Place corks in tubes so that paraffin does not flow into broth but forms a seal between rim of tubes and cork. Store tubes at 5-8°C **no longer than 2 weeks before use**.

Caution:	***POISON!! DO NOT PIPET BY MOUTH! Use protective equipment/gloves when handling!**

STORAGE CONDITIONS	**5-8° Retain no longer than two weeks before use.**

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M127

Potato Dextrose Agar

(Return to Table of Contents)

Potato infusion	200 g
Dextrose	20 g
Agar	20 g
Distilled water	1 liter

To prepare potato infusion, boil 200 g sliced, unpeeled potatoes in 1 liter distilled water for 30 min. Filter through cheesecloth, saving effluent, which is potato infusion (or use commercial dehydrated form). Mix in other ingredients and boil to dissolve. Autoclave 15 min at 121°C. Dispense 20-25 ml portions into sterile 15 x 100 mm petri dishes. Final pH, 5.6 ± 0.2.

Medium should not be re-melted more than once. Medium powder is available commercially but may require supplementing with extra agar to a final concentration of 20 g/liter. To BBL or Difco dehydrated medium, add 5 g of agar.

For potato dextrose salt agar, prepare potato dextrose agar, as above, and add 75 g NaCl per liter.

For cosmetics, cool medium to 47-50°C after autoclaving. Add 40 ppm (final concentration) chlortetracycline. Mix thoroughly and dispense 20 ml portions into 15 x 100 mm petri dishes. Dispense 4 ml of stock filter-sterilized chlortetracycline HCl (1 g/100 ml) per liter of medium.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M128

Pseudomonas Agar F

(for fluorescein production)

(Return to Table of Contents)

Use Difco product, if available.

Proteose peptone No. 3 (Difco)	20.0 g
Tryptone (Difco)	10.0 g
Dipotassium phosphate	1.5 g
Magnesium sulfate	0.73 g
Glycerol (Difco)	10.0 g
Agar	15.0 g
Distilled water	1 liter

Add medium powder and glycerol to water; mix. Heat to boiling to dissolve ingredients. Autoclave at 121°C for 15 min. Final pH, 7.0.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M129

Pseudomonas Agar P

(for pyocyanine production)

(Return to Table of Contents)

Peptone (Difco)	20.0 g
Potassium sulfate	10.0 g
Magnesium chloride	1.4 g
Glycerol (Difco)	10.0 g
Agar	15.0 g
Distilled or deionized water	1 liter

Prepare as described for Pseudomonas Agar F (M128).

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Bacteriological Analytical Manual

Online

January 2001

M130

Purple Carbohydrate Broth

(Return to Table of Contents)

Proteose peptone No. 3	10 g
Beef extract	1 g
NaCl	5 g
Bromcresol purple	0.02 g
Distilled water	1 liter

Prepare as for phenol red carbohydrate broth (M121).

Final pH, 6.8 ± 0.2.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M130a

Purple Carbohydrate Fermentation Broth Base

(Return to Table of Contents)

Purple broth base (Becton-Dickinson)	15 g
Distilled water	900 ml

Add purple broth base to distilled water. Dispense 9 ml into 16 x 125 mm tubes containing Durham tubes. Autoclave at 121°C for 15 min. Prepare carbohydrates, except esculin, as sterile 5% solutions.

Filter-sterilize or autoclave, as appropriate. Add 1 ml carbohydrate solution to 9 ml broth base to yield 0.5% carbohydrate in broth.

Add esculin directly into base broth to make a 0.5% solution and autoclave 15 min at 115°C. At room temperature, a 5% solution of esculin is a gel that cannot be pipetted.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M131
Pyrazinamidase Agar

(Return to Table of Contents)

Tryptic soy agar (M152)	30 g
Yeast extract	3 g
Pyrazine-carboxamide	1 g
0.2 M Tris-maleate, pH 6.0	1 liter

Heat to boiling; dispense 5 ml in 16 x 125 mm tubes. Autoclave at 121°C for 15 min. Cool slanted.

*See Chapter 8, *Yersinia*, ref. 26.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Home | B A M | B A M Media | Bad Bug Book

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U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M132

Rappaport-Vassiliadis Medium

(Return to Table of Contents)

Broth base

Tryptone	5 g
NaCl	8 g
KH_2PO_4	1.6 g
Distilled water	1 liter

Magnesium chloride solution

$MgCl_2 \cdot 6H_2O$	400 g
Distilled water	1 liter

Malachite green oxalate solution

Malachite green oxalate	0.4 g
Distilled water	100 ml

To prepare the complete medium, combine 1000 ml broth base, 100 ml magnesium chloride solution, and 10 ml malachite green oxalate solution (total volume of complete medium is 1110 ml). Broth base must be prepared on same day that components are combined to make complete medium. Magnesium chloride solution may be stored in dark bottle at room temperature up to 1 year. To prepare solution, dissolve entire contents of $MgCl_2 \cdot 6H_2O$ from newly opened container according to formula, because this salt is very hygroscopic. Malachite green oxalate solution may be stored in dark bottle at room temperature up to 6 months. Merck analytically pure malachite green oxalate is recommended because other brands may not be equally effective. Dispense 10 ml volumes of complete medium into 16 x 150 mm test tubes. Autoclave 15 min at 115°C. Final pH, 5.5 ± 0.2. Store in refrigerator and **use within 1 month**.

This medium must be made from its individual ingredients. Use of commercially available dehydrated media is not recommended. Users of this medium should be aware that there are formulations and incubation temperatures for this medium other than those recommended in this manual.

Storage Conditions:	Refrigerator [4-8°C]
Shelf Life:	1 month

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by ear/rim 2000-MAR-29

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M133

Sabouraud's Dextrose Broth and Agar

(Return to Table of Contents)

Polypeptone or neopeptone	10 g
Dextrose	40 g
Distilled water	1 liter

Dissolve completely and dispense 40 ml portions into screw-cap bottles. Final pH, 5.8. Autoclave 15 min at 118-121°C. Do not exceed 121°C.

For Sabouraud's dextrose agar, prepare broth as above and add 15-20 g agar, depending on gel strength desired. Final pH, 5.6 ± 0.2. Dispense into tubes for slants and bottles or flasks for pouring plates. Autoclave 15 min at 118-121 °C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M134

Selenite Cystine Broth

(Return to Table of Contents)

Medium 1 (modification of Leifson's formulation for selenite broth)

Tryptone or polypeptone	5 g
Lactose	4 g
Sodium acid selenite ($NaHSeO_3$)	4 g
Na_2HPO_4	10 g
L-Cystine	0.01 g
Distilled water	1 liter

Heat to boiling to dissolve. Dispense 10 ml portions into sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. **DO NOT AUTOCLAVE.** Final pH, 7.0 ± 0.2. The medium is not sterile. Use same day as prepared.

Medium 2 (North-Bartram modification)

Polypeptone	5 g
Lactose	4 g
Sodium acid selenite ($NaHSeO_3$)	4 g
Na_2HPO_4	5.5 g
KH_2PO_4	4.5 g
L-Cystine	0.01 g
Distilled water	1 liter

Heat with agitation to dissolve. Dispense 10 ml portions into sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. **DO NOT AUTOCLAVE.** Use same day as prepared.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

March 2001

M30d

Semisolid Medium, modified,

for Biochemical Identification of *Campylobacter*

(Return to Table of Contents)

Base Medium

Campylobacter Enrichment Broth (Bolton)(Oxoid AM-7526)	27.6 g
Agar	1.8 g
Distilled water	1000 ml
Neutral red solution (see below)	10 ml

Biochemicals (see below)

Neutral red solution, 0.2% Dissolve 0.2 g neutral red in 10 ml EtOH in a 100 ml volumetric and bring to line with d. water.

Mix base medium ingredients, then boil. Divide into four 250 ml portions. Add 2.5 ml neutral red to **3** of the 4 portions. Add glycine, NaCl and cysteine-HCl to the 3 portions containing neutral red. Add potassium nitrate in the portion without neutral red. Adjust the pH of each portion to 7.4 ± 0.2. Dispense 10 ml per 16 X 125 mm screw-cap tube. Autoclave 121°C, 15 min.

a. **Potassium nitrate** (for Final Concentration of 1% (w/v)). Add **2.5 g to 250 ml** (10 g/liter) semi-solid mixture **without** neutral red.

b. **Glycine** (for Final Concentration of 1% (w/v)). Add **2.5 g to 250 ml** (10 g/liter) semi-solid mixture with neutral red.

c. **NaCl** (for Final Concentration of 3.5% (w/v)). Add **7.5 g to 250 ml** (30 g/liter) semi-solid mixture with neutral red.

d. **Cysteine-HCl** (for Final Concentration of 0.02% (w/v)). Add **0.05 g to 250 ml** (0.2 g/liter) semi-solid mixture with neutral red.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.
Modified on 2001-MAR-08

Hypertext updated by rim/kwg/cjm 2001-OCT-24

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M30c

Semi-Solid Medium, modified,

for Culture Storage

(Return to Table of Contents)

Campylobacter Enrichment Broth (Bolton) (Oxoid AM-7526)	27.6 g
Agar	1.8 g
Sodium Citrate	0.1 g
Distilled water	1000 ml

Mix ingredients, pH to 7.4 ± 0.2, boil and dispense 10 ml per 16 X 125 screw-cap tube. Autoclave 121°C, 15 min. Keep tubes tightly capped during storage.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-JAN-6

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M135

Sheep Blood Agar

(Return to Table of Contents)

Blood agar base (Oxoid No. 2)	95 ml
Sterile sheep blood, defibrinated	5 ml

Rehydrate and sterilize base as recommended by manufacturer. Agar and blood should both be at 45-46°C before blood is added and plates are poured.

Commercial pre-poured sheep blood agar plates may be used.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Home | B A M | B A M Media | Bad Bug Book

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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M136

Shigella Broth

(Return to Table of Contents)

Base

Tryptone	20 g
K_2HPO_4	2 g
KH_2PO_4	2 g
NaCl	5 g
Glucose	1 g
Tween 80	1.5 ml
Distilled water	1 liter

Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2.

Novobiocin solution

Weigh 50 mg novobiocin into 1 liter distilled water.

Sterilize by filtration through 0.45 µm membrane. Add 2.5 ml concentrate to 225 ml base.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M137
SIM Motility Medium

(Return to Table of Contents)

Pancreatic digest of casein (casitone)	20.0 g
Peptic digest of animal tissue (beef extract)	6.1 g
Ferrous ammonium sulfate	0.2 g
Sodium thiosulfate	0.2 g
Agar	3.5 g

Rehydrate and add 6 ml medium per 16 x 125 mm screw-cap tube. Sterilize according to manufacturer's instructions. Final pH, 7.3 ± 0.2. Medium is available from BBL.

DO NOT USE Difco SIM.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M138

Simmons Citrate Agar

(Return to Table of Contents)

Sodium citrate*	2 g
NaCl	5 g
K_2HPO_4	1 g
$NH_4H_2PO_4$	1 g
$MgSO_4$	0.2 g
Bromthymol blue	0.08 g
Agar	15 g
Distilled water	1 liter

Heat gently with occasional agitation. Boil 1-2 min until agar dissolves. Fill 13 x 100 or 16 x 150 mm screw-cap tubes 1/3 full. Autoclave 15 min at 121°C. Before medium solidifies, incline tubes to obtain 4-5 cm slants and 2-3 cm butts. Final pH, 6.8 ± 0.2.

*Difco does not specify waters of hydration.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M139

Sorbitol-MacConkey Agar

(Return to Table of Contents)

Peptone or gelysate	17.0 g
Protease peptone No. 3 or polypeptone	3.0 g
Sorbitol	10.0 g
Bile salts, purified	1.5 g
NaCl	5.0 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Distilled water	1 liter

Dissolve ingredients in distilled water by heating with stirring. Autoclave 15 min at 121°C. Final pH, 7.1 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M140

Sporulation Broth

(for *Clostridium perfringens*)

(Return to Table of Contents)

Polypeptone	15 g
Yeast extract	3 g
Starch, soluble	3 g
$MgSO_4$ (anhydrous)	0.1 g
Sodium thioglycollate	1 g
Na_2HPO_4	11 g
Distilled water	1 liter

Adjust pH to 7.8 ± 0.1. Dispense 15 ml portions into 20 x 150 mm screw-cap tubes. Autoclave 15 min at 121°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M141

Spray's Fermentation Medium

(for *Clostridium perfringens*)

(Return to Table of Contents)

Tryptone	10 g
Neopeptone	10 g
Agar	2 g
Sodium thioglycollate	0.25 g

Dissolve all ingredients except agar and adjust pH to 7.4 ± 0.2. Add agar and heat with agitation to dissolve. Dispense 9 ml portions into 16 x 125 mm tubes. Autoclave 15 min at 121 °C. Before use, heat in boiling water or flowing steam for 10 min. Add 1 ml of sterile 10% carbohydrate solution to 9 ml base.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M142

Staphylococcus Agar No. 110

(Return to Table of Contents)

Gelatin	30 g
Trypticase peptone	10 g
Yeast extract	2.5 g
Lactose	2 g
Mannitol	10 g
NaCl	75 g
K_2HPO_4	5 g
Agar	15 g
Distilled water	1 liter

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M143

Starch Agar

(Return to Table of Contents)

Nutrient agar	23 g
Potato starch	10 g
Distilled water	1 liter

Heat to dissolve agar in 500 ml water. Dissolve starch in 250 ml water. Combine and dilute to 1 liter. Autoclave 15 min at 121°C.

Note: add 3 g agar to Difco's dehydrated starch agar.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M144

T_1N_1 Medium

(Return to Table of Contents)

Trypticase	10 g
NaCl	10 g
Agar (if solid medium is preferred)	20 g
Distilled water	1 liter

Heat if necessary to dissolve ingredients. Dispense into 16 x 125 mm screw-cap tubes (if tubed medium is required). Autoclave 15 min at 121°C. Slant tubes until cool or let medium cool to 50 °C and pour into 15 x 100 mm petri dishes. Final pH, 7.1 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M145

Tetrathionate (TT) Broth

(Return to Table of Contents)

Tetrathionate broth base

Polypeptone	5 g
Bile salts	1 g
Calcium carbonate	10 g
Sodium thiosulfate.$5H_2O$	30 g
Distilled water	1 liter

Suspend ingredients in 1 liter distilled water, mix, and heat to boiling. **DO NOT AUTOCLAVE**. (Precipitate will not dissolve completely.) Cool to less than 45°C. Store at 5-8°C. Final pH, 8.4 ± 0.2.

Iodine-Potassium Iodide (I_2-KI) solution

Potassium iodide	5 g
Iodine, resublimed	6 g
Distilled water, sterile	20 ml

Dissolve potassium iodide in 5 ml sterile distilled water. Add iodine and stir to dissolve. Dilute to 20 ml.

Brilliant green solution

Brilliant green dye, sterile	0.1 g
Distilled water, sterile	100 m1

On day of use, add 20 ml I_2-KI solution and 10 ml brilliant green solution to 1 liter base. Resuspend precipitate by gentle agitation and aseptically dispense 10 ml portions into 20 x 150 or 16 x 150 mm sterile test tubes. Do not heat medium after addition of I_2-KI and dye solutions.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M146

Thioglycollate Medium (Fluid) (FTG)

(Return to Table of Contents)

L-Cystine	0.5 g
Agar (granulated)	0.75 g
NaCl	2.5 g
Dextrose	5 g
Yeast extract	5 g
Tryptone	15 g
Sodium thioglycollate or thioglycollic acid	0.5 g
Resazurin, sodium solution (1:1000), fresh	1 ml
Distilled water	1 liter

Mix L-cystine, NaCl, dextrose, yeast extract, and tryptone with 1 liter water. Heat in Arnold steamer or water bath until ingredients are dissolved. Dissolve sodium thioglycollate or thioglycollic acid in solution and adjust pH so that value after sterilization is 7.1 ± 0.2. Add sodium resazurin solution, mix, and autoclave 20 min at 121°C. If commercial media are used, dispense 10 ml portions to 16 x 150 mm tubes and autoclave 15 min at 121°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-29

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M147

Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar

(Return to Table of Contents)

Yeast extract	5 g	Oxgall	5 g
Peptone	10 g	NaCl	10 g
Sucrose	20 g	Ferric citrate	1 g
Sodium thiosulfate75H_2O	10 g	Bromthymol blue	O.04 g
Sodium citrate72H_2O	10 g	Thymol blue	0.04 g
Sodium cholate	3 g	Agar	15 g
Distilled water			1 liter

Prepare in flask at least 3 times larger than required volume of medium. Add ingredients to warm distilled water and heat to dissolve. Bring just to boil, and immediately remove from heat. **DO NOT AUTOCLAVE!** Cool to 50°C and pour into petri dishes. Dry the plates overnight or

at 37-45°C before use.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M148

Toluidine Blue-DNA Agar

(Return to Table of Contents)

Deoxyribonucleic acid (DNA)	0.3 g
Agar	10 g
$CaCl_2$ (anhydrous)	1.1 mg
NaCl	10 g
Toluidine blue O	0.083 g
Tris (hydroxymethyl) aminomethane	6.1 g
Distilled water	1 liter

Dissolve Tris (hydroxymethyl) aminomethane in 1 liter distilled water. Adjust pH to 9.0. Add the remaining ingredients except toluidine blue O and heat to boiling to dissolve. Dissolve toluidine blue O in medium. Dispense to rubber-stoppered flasks. Sterilization is not necessary if used immediately. The sterile medium is **stable at room temperature for 4 months** and is satisfactory after several melting cycles.

Storage	room temperature
Shelf life	4 months

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M149

Triple Sugar Iron Agar (TSI)

(Return to Table of Contents)

Medium 1		Medium 2	
Polypeptone	20 g	Beef extract	3 g
NaCl	5 g	Yeast extract	3 g
Lactose	10 g	Peptone	15 g
Sucrose	10 g	Proteose peptone	5 g
Glucose	1 g	Glucose	1 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.2 g	Lactose	10 g
$Na_2S_2O_3$	0.2 g	Sucrose	10 g
Phenol red	0.025 g	$FeSO_4$	0.2 g

These two media are interchangeable for general use.

Suspend ingredients of **Medium 1** in distilled water, mix thoroughly, and heat with occasional agitation. Boil about 1 min to dissolve ingredients. Fill 16 x 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave **Medium 1** for 15 min at 118°C.

Prepare **Medium 2** in the same manner as **Medium 1**, except autoclave 15 min at 121°C.

Agar	13 g	NaCl	5 g
Distilled water	1 liter	$Na_2S_2O_3$	0.3 g
		Phenol red	0.024 g
		Agar	12 g
		Distilled water	1 liter

Before the media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt. Final pH, 7.3 ± 0.2 for **Medium 1** and 7.4 ± 0.2 for **Medium 2**.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M150

Trypticase Novobiocin (TN) Broth

(Return to Table of Contents)

Trypticase soy broth	30 g
Bile salts No. 3	1.5 g
Dipotassium phosphate	1.5 g
Novobiocin	20 mg
Distilled water	1 liter

Dissolve all ingredients except novobiocin by heating and stirring; autoclave at 121°C for 15 min. Prepare stock solution of novobiocin by adding 20 mg monosodium novobiocin per ml of distilled water. Filter-sterilize.

Make fresh stock each time of use, or store frozen at -10°C in the dark (compound is light-sensitive) for not more than 1 month (half-life is several months at 4°C). Add 1 ml stock solution per liter of medium.

Make up fresh or

Storage:	Frozen [-10°C] dark conditions up to 1 month

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-FEB-01

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M151

Trypticase-Peptone-Glucose-Yeast Extract Broth

(TPGY)

(Return to Table of Contents)

Trypticase	50 g
Peptone	5 g
Yeast extract	20 g
Dextrose	4 g
Sodium thioglycollate	1 g
Distilled water	l liter

Dissolve solid ingredients and dispense 15 ml in 20 x 150 mm tubes. Autoclave tubes 10 min at 121°C. Final pH, 7.0 ± 0.2. Refrigerate at 5°C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-02

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M151a

Trypticase-Peptone-Glucose-Yeast Extract Broth with Trypsin

(TPGYT)

(Return to Table of Contents)

Base

Trypticase	50 g
Peptone	5 g
Yeast extract	20 g
Dextrose	4 g
Sodium thioglycollate	1 g
Distilled water	1 liter

Dissolve solid ingredients of base and dispense 15 ml in 20 x 150 mm tubes or 100 ml in 170 ml prescription bottles. Autoclave tubes 10 min at 121°C and bottles 15 min at 121°C. Final pH, 7.0 ± 0.2. Refrigerate at 5°C. Add trypsin immediately before use.

Trypsin solution

trypsin (1:250)	1.5 g
Distilled water	100 ml

Stir trypsin in water to suspend. Let particles settle and filter-sterilize supernatant through 0.45 µm membrane.

Before use, steam or boil base for 10 min to expel dissolved oxygen. Add 1 ml trypsin to each 15 ml of broth or 6.7 ml trypsin to 100 ml of broth.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-02

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M152

Trypticase (Tryptic) Soy Agar

(Return to Table of Contents)

Trypticase peptone	15 g
Phytone peptone	5 g
NaCl	5 g
Agar	15 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Boil 1 min. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-APR-20

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M153

Trypticase Soy Agar with 0.6% Yeast Extract

(TSAYE)

(Return to Table of Contents)

Trypticase soy agar	40 g
Yeast extract	6 g
Distilled water	1 liter

Weigh ingredients, add water, mix, and autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2. After autoclaving, swirl to disperse molten agar.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-02

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Bacteriological Analytical Manual

Online

January 2001

M154

Trypticase (Tryptic) Soy Broth

(Return to Table of Contents)

Trypticase peptone	17 g
Phytone peptone	3 g
NaCl	5 g
K_2HPO_4	2.5 g
Glucose	2.5 g
Distilled water	1 liter

Heat with gentle agitation to dissolve. Dispense 225 ml into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

For trypticase soy broth without dextrose, prepare as above, but omit 2.5 g dextrose.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2002

M156

Trypticase Soy Broth Modified

(mTSB)

(Return to Table of Contents)

Trypticase soy broth	30.0 g
Bile salts No. 3	1.5 g
Dipotassium phosphate	1.5 g
Novobiocin solution (R50)	0.2 ml
Deionized water	1 liter

Autoclave mTSB without novobiocin and let cool to room temperature. Final pH, 7.4 +/- 0.2. For chapter 24, gene probe method, add novobiocin just before adding food.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.
Revisions: January 28, 2002 Required pH specified.

Hypertext updated by rim/kwg 2002-FEB-04

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M186

Trypicase (Tryptic) Soy Broth with ferrous sulfate

(Return to Table of Contents)

Trypticase peptone	17 g
Phytone peptone	3 g
NaCl	5 g
K_2HPO_4	2.5 g
Glucose	2.5 g
Ferrous sulfate	35 mg
Distilled water	1.0 liter

Heat with gentle agitation to dissolve. Dispense 225 ml into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

Hypertext Source: adapted from Chapter 5, Bacteriological Analytical Manual, 8th Edition, Revision A, 1998. Created 2000-MAR-01

Hypertext updated by rim 2000-MAR-31

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M155

Trypticase (Tryptic) Soy Broth (TSB) with Glycerol

(Return to Table of Contents)

Trypticase peptone (tryptone)	17 g
Phytone peptone (soytone)	3 g
NaCl	15 g
K_2HPO_4	2.5 g
Glycerol	240 ml
Distilled water	760 ml

Suspend ingredients in distilled water and heat to dissolve. Dispense into bottles or vials. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-30

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M154a

Trypticase (Tryptic) Soy Broth with 10% NaCl and 1% Sodium Pyruvate

(Return to Table of Contents)

Trypticase or tryptose (pancreatic digest of casein)	17 g
Phytone (papaic digest of soya meal)	3 g
NaCl	100 g
K_2HPO_4	2.5 g
Dextrose	2.5 g
Sodium pyruvate	10 g
Distilled water	1 liter

Dehydrated trypticase or tryptic soy broth is satisfactory with 95 g NaCl and 10 g sodium pyruvate added per liter. Adjust to pH 7.3. Heat gently if necessary. Dispense 10 ml into 16 x 150 mm tubes. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2. Store up to 1 month at 4 ± 1°C.

Storage	
Temperature	4 ± 1° C
Duration	1 month

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M157

Trypticase Soy Broth with 0.6% Yeast Extract

(TSBYE)

(Return to Table of Contents)

Trypticase soy broth	30 g
Yeast extract	6 g
Distilled water	1 liter

Weigh ingredients and dissolve in water. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-17

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M158

Trypticase Soy-Polymyxin Broth

(Return to Table of Contents)

Prepare trypticase soy broth (M154) and dispense 15 ml portions into 20 x 150 mm tubes. Autoclave 15 min at 121°C.

Polymyxin B solution, 0.15%

Dissolve 500,000 units polymyxin B sulfate in 33.3 ml distilled water. Filter-sterilize and store in the dark at 4°C until needed. Before use, add 0.1 ml sterile 0.15% polymyxin B solution to 15 ml tryptic soy broth, and mix thoroughly.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-30

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Bacteriological **A**nalytical **M**anual

Online

January 2001

M159

Trypticase Soy-Sheep Blood Agar

(Return to Table of Contents)

Prepare Trypticase Soy Agar (M152). Sterilize as recommended and cool to 50°C. Add 5 ml defibrinated sheep blood to 100 ml agar. Mix and dispense 20 ml portions to 15 x 100 mm petri dishes. (Commercial trypticase soy-blood agar plates are satisfactory.)

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim/ear 2001-NOV-26

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M160

Trypticase Soy-Tryptose Broth

(Return to Table of Contents)

Trypticase soy broth (commercial, dehydrated)	15 g
Tryptose broth (commercial, dehydrated)	13.5 g
Yeast extract	3 g
Distilled water	1 liter

Dissolve ingredients in 1 liter water. Heat gently to dissolve. Dispense 5 ml portions into 16 x 150 mm test tubes. Autoclave 15 min at 1210C. Final pH, 7.2 1 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-30

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M164

Tryptone (Tryptophane) Broth, 1%

(Return to Table of Contents)

Tryptone or trypticase	10 g
Distilled water	1 liter

Dissolve and dispense 5 ml portions into 16 x 125 or 16 x 150 mm test tubes. Autoclave 15 min at 121°C. Final pH, 6.9 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-3

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M161

Tryptone Broth and Tryptone Salt Broths

(T_1N_0, T_1N_1, T_1N_3, T_1N_6, T_1N_8, T_1N_{10})

(Return to Table of Contents)

Trypticase or tryptone	10 g
NaCl	0, 10, 30, 60, 80, or 100 g
Distilled water	1 liter

Dissolve ingredients in distilled water.

For T_1N_0, add no NaCl.
For T_1N_1, use 10 g NaCl [1% (w/v) NaCl].
For T_1N_3, use 30 g NaCl/liter [3% (w/v) NaCl], etc.

Dispense into 16 x 125 mm screw-cap tubes. Tighten caps to maintain correct salt concentration in tube. Autoclave 15 min at 121°C. Final pH, 7.2 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-APR-20

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M162

Tryptone Phosphate (TP) Broth

(for enteropathogenic *Escherichia coli*)

(Return to Table of Contents)

Tryptone	20 g
K_2HPO_4	2 g
KH_2PO_4	2 g
NaCl	5 g
Polysorbate 80 (Tween 80)	1.5 ml
Distilled water	1 liter

Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-03

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M163

Tryptone Salt Agar

(T_1N_1 Agar and T_1N_2 Agar)

(Return to Table of Contents)

Trypticase or tryptone	10 g
NaCl	10 g
Agar	20 g
Distilled water	1 liter

Suspend ingredients and boil to dissolve agar. For slants, dispense into tubes. Autoclave 15 min at 121°C. Solidify tubes as slants. Cool medium for plates to 45-50°C and pour into sterile petri dishes.

For T_1N_2 agar, use 20 g NaCl rather than the 10 g specified for T_1N_1 agar.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-03

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M165

Tryptone Yeast Extract Agar

(Return to Table of Contents)

Tryptone	10 g
Yeast extract	1 g
*Carbohydrate	10 g
Bromcresol purple	0.04 g
Agar	2 g
Distilled water	1 liter

Dissolve agar with heat and gentle agitation. Adjust pH to 7.0 ± 0.2. Fill 16 x 125 mm tubes 2/3 full. Autoclave 20 min at 115°C. Before use, steam medium 10-15 min. Solidify by placing tubes in ice water.

*Glucose and mannitol are the carbohydrates used for identification of *Staphylococcus aureus*.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-03

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M166

Tryptose Blood Agar Base

(Return to Table of Contents)

Tryptose	10 g
Beef extract	3 g
NaCl	5 g
Agar	15 g
Distilled water	1 liter

Suspend ingredients in distilled water, mix thoroughly, and heat with occasional agitation. Boil about 1 min. Fill 16 x 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave 15 min at 121°C. Before media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-03

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M167

Tryptose Broth and Agar (for serology)

(Return to Table of Contents)

Difco Bacto tryptose	20 g
NaCl	5 g
Dextrose	1 g
Agar	15 g
Distilled water	1 liter

Weigh ingredients, add water, and mix. Autoclave 15 min at 121°C. Swirl to disperse molten agar. Final pH, 7.2 ± 0.2.

For broth, omit agar from formulation.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M168

Tryptose Phosphate Broth (TPB)

(for cell culture)

(Return to Table of Contents)

Tryptose	20 g
Dextrose	2 g
NaCl	5 g
Na_2HPO_4	2.5 g
Distilled water	1 liter

Sterilize by filtration through 0.20 µm membrane. Available from Flow Laboratories, Inc., McLean, VA 22102.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-03

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M169

Tryptose-Sulfite-Cycloserine (TSC) Agar

(Return to Table of Contents)

Tryptose	15 g
Yeast extract	5 g
Soytone	5 g
Ferric ammonium citrate (NF Brown Pearls)	1 g
Sodium metabisulfite	1 g
Agar	20 g
Distilled water	900 ml

Heat with agitation to dissolve. Adjust pH to 7.6 ± 0.2. Dispense 250 ml portions to 500 ml flasks. Autoclave 15 min at 121°C. Maintain medium at 50°C before use. Dehydrated SFP agar base (Difco) is satisfactory for base.

D-cycloserine solution

Dissolve 1 g D-cycloserine (white crystalline powder) in 200 ml of distilled water. Sterilize by filtration and store at 4°C until use. D-cycloserine powder is available from Sigma Chemical Co., St. Louis, MO.

Final medium

For pour plates, add 20 ml of D-cycloserine solution to 250 ml base. To prepare prepoured plates containing egg yolk, also add 20 ml of 50% egg yolk emulsion (M51). Mix well and dispense 18 ml into 15 x 100 mm petri dishes. Cover plates with a towel and let dry overnight at room temperature before use.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-03

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M170

Tyrosine Agar

(Return to Table of Contents)

Base

Prepare Nutrient Agar (M112). Dispense 100 ml portions into 170 ml bottles. Autoclave 15 min at 121°C. Cool to 48°C.

Tyrosine suspension

Suspend 0.5 g L-tyrosine in 10 ml distilled water in 20 x 150 mm culture tube. Mix thoroughly with Vortex mixer. Autoclave 15 min at 121°C.

Final medium

Combine 100 ml base with sterile tyrosine suspension. Mix thoroughly by gently inverting bottle 2 or 3 times. Aseptically dispense 3.5 ml into 13 x 100 mm tubes with frequent mixing. Slant tubes and cool rapidly to prevent separation of tyrosine.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-03

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M188

Universal Preenrichment Broth

(Return to Table of Contents)

Tryptone	5 g
Proteose peptone	5 g
KH_2PO_4	15 g
Na_2HPO_4	7 g
NaCl	5 g
Dextrose	0.5 g
$MgSO_4$	0.25 g
Ferric ammonium citrate	0.1 g
Sodium pyruvate	0.2 g
Distilled water	1.0 liter

Heat with gentle agitation to dissolve. Dispense 225 ml into 500 ml Erlenmeyer flasks. Autoclave 15 min at 121°C. Final pH, 6.3 ± 0.2.

Creation date: 2000-MAR-01

Hypertext updated by rim 2000-MAR-07

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Bacteriological **A**nalytical **M**anual

Online

January 2001

M171
Urea Broth

(Return to Table of Contents)

Urea	20 g
Yeast extract	0.1 g
Na_2HPO_4	9.5 g
K_2HPO_4	9.1 g
Phenol red	0.01 g
Distilled water	1 liter

Dissolve ingredients in distilled water. **DO NOT HEAT.** Sterilize by filtration through 0.45 µm membrane. Aseptically dispense 1.5-3.0 ml portions to 13 x 100 mm sterile test tubes. Final pH, 6.8 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-06

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M172

Urea Broth (Rapid)

(Return to Table of Contents)

Urea	20 g
Yeast extract	0.1 g
KH_2PO_4	0.091 g
Na_2HPO_4	0.095 g
Phenol red	0.01 g
Distilled water	1 liter

Prepare as for urea broth (M171).

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-6

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M173

Veal Infusion Agar and Broth

(Return to Table of Contents)

Veal, infusion from	500 g
Proteose peptone No. 3	10 g
NaCl	5 g
Agar	15 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Dispense 7 ml portions into 16 x 150 mm tubes. Autoclave 15 min at 121°C. Incline tubes to obtain 6 cm slant. Final pH, 7.3 ± 0.2.

Veal infusion broth

Prepare as above, but omit the 15 g agar. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-31

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M174

Violet Red Bile Agar (VRBA)

(Return to Table of Contents)

Yeast extract	3.0 g
Peptone or gelysate	7.0 g
NaCl	5.0 g
Bile salts or bile salts No. 3	1.5 g
Lactose	10.0 g
Neutral red	0.03 g
Crystal violet	0.002 g
Agar	15.0 g
Distilled water	1.0 liter

Suspend ingredients in distilled water and let stand for a few min. Mix thoroughly and adjust to pH 7.4 ± 0.2. Heat with agitation and boil for 2 min. Do not sterilize. Before use, cool to 45°C and use as a plating medium. After solidification, add a cover layer above the agar of approximately 3.0 to 4.0 ml to prevent surface growth and spreading of colonies.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-06

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Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological Analytical Manual

Online

January 2001

M175

Violet Red Bile-MUG Agar (VRBA-MUG)

(Return to Table of Contents)

Add 0.1 g 4-methylumbelliferyl-β-D-glucuronide (MUG) to the ingredients for 1 liter of VRBA (M174), and continue as for preparation of VRBA.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-06

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M176

Vogel-Johnson (VJ) Agar

(Return to Table of Contents)

Trypticase	10 g
Yeast extract	5 g
Mannitol	10 g
K_2HPO_4	5 g
Lithium chloride	5 g
Glycine	10 g
Phenol red	0.025 g
Agar	15 g
Distilled water	1 liter

Heat with agitation to dissolve agar. Autoclave 15 min at 121°C. Cool to 50°C. Add 20 ml Chapman tellurite solution, 1%, commercially available from Difco Laboratories, Detroit, MI. Mix and pour into plates. Final pH, 7.2 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-06

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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M177

Voges-Proskauer Medium (Modified)

(Return to Table of Contents)

Proteose peptone	7 g
NaCl	5 g
Dextrose	5 g
Distilled water	1 liter

Dissolve ingredients in water and adjust pH if necessary. Dispense 5 ml portions into 20 x 150 mm tubes. Autoclave 10 min at 121°C. Final pH, 6.5 ± 0.2.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-06

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M178

Wagatsuma Agar

(Return to Table of Contents)

Yeast extract	3 g
Peptone	10 g
NaCl	70 g
K_2HPO_4	5 g
Mannitol	10 g
Crystal violet	0.001 g
Agar	15 g
Distilled water	1 liter
Human or rabbit red blood cells, fresh (24 h), with anticoagulant	50 ml

Mix fresh (within 24 h of drawing) human or rabbit blood with same or larger volume of physiological saline. Centrifuge cells at about 4000 x *g* at 4°C for 15 min. Pour off saline and wash 2 more times. After 3rd wash, pour off saline and resuspend cells to original volume with saline.

Suspend ingredients, except blood, in distilled water and boil to dissolve agar. Adjust to pH 8.0 ± 0.2. Steam 30 min. **DO NOT AUTOCLAVE**. Cool to 45-50°C. Add 50 ml of washed red blood cells to the cooled medium. Mix and pour into sterile petri dishes. Dry plates thoroughly and use promptly. Medium can be made in smaller volumes (requiring less blood) when few plates are needed.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-06

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M179

Xylose Lysine Desoxycholate (XLD) Agar

(Return to Table of Contents)

Yeast extract	3 g	Ferric ammonium citrate	0.8 g
L-lysine	5 g	Sodium thiosulfate	6.8 g
Xylose	3.75 g	NaCl	5 g
Lactose	7.5 g	Agar	15 g
Sucrose	7.5 g	Phenol red	0.08 g
Sodium desoxycholate	2.5 g	Distilled water	1 liter

Heat with agitation just until medium boils. Do not overheat. Pour into plates when medium has cooled to 50°C. Let dry about 2 h with covers partially removed. Then close plates. Final pH, 7.4 ± 0.2. **Do not store more than 1 day.**

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-06

U.S. Food & Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

Bacteriological **A**nalytical **M**anual

Online

January 2001

M180

Y-1 Adrenal Cell Growth Medium

Escherichia coli, V. cholerae

(Return to Table of Contents)

	Escherichia coli	*Vibrio cholerae*
Ham's F-10 medium.	90 ml	500 ml
Fetal bovine serum	10 ml	75 ml
Penicillin-streptomycin solution (M119)	1 ml	5 ml

These ingredients are commercially available. Filter-sterilize. Store at 4-50C.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-06

U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual

Online

January 2001

M181

Yeast Extract (YE) Agar

(Return to Table of Contents)

Proteose peptone	10 g
Yeast extract	3.0 g
NaCl	5.0 g
Agar	15.0 g
Distilled water	1 liter

Adjust pH to 7.2-7.4. Autoclave at 121°C for 15 min.

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998.

Hypertext updated by rim 2000-MAR-07

Bad Bug Book

U.S. Food & Drug Administration

Center for Food Safety & Applied Nutrition

Foodborne Pathogenic Microorganisms and Natural Toxins Handbook

The *"Bad Bug Book"*

This handbook provides basic facts regarding foodborne pathogenic microorganisms and natural toxins. It brings together in one place information from the Food & Drug Administration, the Centers for Disease Control & Prevention, the USDA Food Safety Inspection Service, and the National Institutes of Health.

Some technical terms have been linked to the National Library of Medicine's Entrez glossary. Recent articles from Morbidity and Mortality Weekly Reports have been added to selected chapters to update the handbook with information on later outbreaks or incidents of foodborne disease. At the end of selected chapters on pathogenic microorganisms, hypertext links are included to relevant Entrez abstracts and GenBank genetic loci. A more complete description of the handbook may be found in the Preface.

PATHOGENIC BACTERIA

- Miscellaneous enterics
- *Streptococcus*

ENTEROVIRULENT ESCHERICHIA COLI GROUP (EEC Group)

- *Escherichia coli* - enterotoxigenic (ETEC)
- *Escherichia coli* - enteropathogenic (EPEC)
- *Escherichia coli* O157:H7 enterohemorrhagic (EHEC)
- *Escherichia coli* - enteroinvasive (EIEC)

PARASITIC PROTOZOA and WORMS

- *Giardia lamblia*
- *Entamoeba histolytica*
- *Cryptosporidium parvum*
- *Cyclospora cayetanensis*
- *Anisakis* sp. and related worms
- *Diphyllobothrium* spp.
- *Nanophyetus* spp.
- *Eustrongylides* sp.
- *Acanthamoeba* and other free-living amoebae
- *Ascaris lumbricoides* and *Trichuris trichiura*

VIRUSES

- Hepatitis A virus
- Hepatitis E virus
- Rotavirus
- Norwalk virus group
- Other viral agents

NATURAL TOXINS

- Ciguatera poisoning

- Shellfish toxins (PSP, DSP, NSP, ASP)
- Scombroid poisoning
- Tetrodotoxin (Pufferfish)
- Mushroom toxins
- Aflatoxins
- Pyrrolizidine alkaloids
- Phytohaemagglutinin (Red kidney bean poisoning)
- Grayanotoxin (Honey intoxication)

OTHER PATHOGENIC AGENTS

- Prions

APPENDICES

- Infective dose
- Epidemiology summary table
- Factors affecting microbial growth in foods
- Foodborne Disease Outbreaks, United States 1988-1992
- Additional Foodborne Disease Outbreak Articles and Databases.

Hypertext last updated by las/ear 2001-JUN-20

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Recent News

- What's New
- Press Announcements and Fact Sheets
- Food Safety and Terrorism

Moving

CFSAN has finished Moving

Overview

- Overview and History of FDA and the Center for Food Safety and Applied Nutrition
- CFSAN 1996-2002 Activities
  - CFSAN 2002 Program Priorities
  - CFSAN 2001 Program Priorities Report Card
- Other regulatory agencies with responsibilities for foods

Program Areas

- Acidified and Low Acid Canned Foods
- Biotechnology
- Color Additives
- Cosmetics
- Dietary Supplements
- Food Additives and Premarket Approval
- Foodborne Illness
  - Bad Bug Book
  - Microbiological Methods & BAM
- Food Labeling and Nutrition
- HACCP Hazard Analysis and Critical Control Point
- Imports and Exports
- Inspections, Compliance, Enforcements and Recalls
- Pesticides and Chemical Contaminants
  - Total Diet Study
- Seafood and Regulatory Fish Encyclopedia (Fish Images)

- Selected Topics: BSE / Food Allergens / Subject Index

National Food Safety Programs

- National Food Safety Programs
  Produce and Import Initiative / Eggs / Fruit Juice / Sprouts / *Listeria* / Research / Risk Assessment
  - Food Safety Progress Report FY '00

- www.FoodSafety.gov
  Gateway to Government Food Safety Information

Special Interest Areas

- Consumer Advice
- Federal/State Food Programs
  Milk, Retail, & Shellfish; including 2001 Food Code
- Industry Assistance
  - Starting a Food Business
- International / Codex / Other Languages
- Kids, Teens & Educators
- Selected Health Topics
- Seniors

- Women's Health

Interacting with the Center

- Comment on Proposed Regulations
- E-Mail and Phone Numbers
- Public Meetings/Advisory Committees
- Report Problems to FDA
    - Report Websites selling illegal foods/drugs/supplements
- Request Information
- Submit Petitions
- Toll Free Food Information Line 1-888-SAFEFOOD

Obtain FDA Documents

- Code of Federal Regulations
- FDA Dockets
- Federal Register Documents
- Food & Cosmetic Guidance Documents
- Freedom of Information Request
- Frequently Asked Questions
- Laws Enforced by FDA
  Includes Food, Drug and Cosmetic Act
- Other Language Documents
- Selected Vacancy Announcements
- Videos on the Web

Other Sources of Food Information

- Joint Institute for Food Safety and Applied Nutrition (JIFSAN), College Park, Maryland
- National Center for Food Safety and Technology Illinois
- National Center for Toxicological Research
- www.FoodSafety.gov: Gateway to Government Food Safety Information
- Selected Non-FDA Sources of Food and Nutrition or Chemical or Biological information
- Center Resources Page

- Your First Click to the U.S. Government FIRSTGOV

U.S. Department of Health and Human Services

Food and Drug Administration

2002 FDA Science Forum Presentations

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A-Z Index

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Products FDA Regulates

Food
Foodborne Illness, Nutrition, Dietary Supplements...

Drugs
Prescription, Over-the-Counter, Generic...

Medical Devices
Pacemakers, Contact Lenses, Hearing Aids...

Biologics
Vaccines, Blood Products...

Animal Feed and Drugs
Livestock, Pets...

Cosmetics
Safety, Labeling...

Radiation-Emitting Products
Cell Phones, Lasers, Microwaves...

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FDA NEWS

FDA Warns About Potentially Dangerous OB/GYN Devices. Certain obstetric and gynecological surgical devices manufactured by A&A and labeled as sterile may not have undergone sterilization, creating a potential hazard of serious injury or death. FDA is urging the company to conduct a recall of the products and will take measures to ensure the products are removed as soon as possible. Full story.

More FDA News
Press Releases, Meetings, Speeches

Safety Alerts

Risk of Severe Liver Injury Associated with Kava Dietary Supplements

Other Recalls and Alerts

Product Approvals

New Test Screens Blood Donors for Both HIV and Hepatitis C

Other Recent Approvals

Let Us Hear From You

Report a Problem with a Product Regulated by FDA

Tell Us Your Views on FDA's Proposed Regulations

How to Petition FDA

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Code of Federal Regulations

Federal Register

Dockets

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• Regulatory Research
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Hot Topics

- Bioterrorism
- Buying Medicines Online
- BSE *(Mad Cow Disease)*
- Antibiotic Resistance
- LASIK Surgery
- PPA

More Hot Topics...

Jobs@FDA

FDA Activities

- Advisory Committees
- Clinical Trials/Human Subject Protection
- FDA Modernization
- Field Operations
- Freedom of Information
- Imports
- International
- MedWatch: Safety Information/ Adverse Event Reporting
- Science @ FDA
- User Fees

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FDA/Office of Public Affairs
Web page created by zwr. Last updated by tg Mar. 28, 2002.

Search and Subject Index

1. Search all FDA web sites - updated daily

   Search

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2. Search only the Center for Food Safety and Applied Nutrition web site. (Excludes food and cosmetic documents in FDA documents and all inspectional, compliance and import references in FDA's Office of Regulatory Affairs.) (Updated weekly)

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   Search Clear

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   - ( ) **All** of the words entered
   - ( ) **Exact word phrase** entered

3. **Subject Index:**

4. Search Federal Government Food Safety web sites (FDA, FSIS, CDC & EPA)

5. Search the CFSAN
   - Dietary Supplement Adverse Event Monitoring System
   - Seafood List

6. Employee locators

7. Frequently Asked Questions

8. FIRSTGOV

Hypertext updated by cjm/dms/ear 2001-DEC-13

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