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

Bacteriological Analytical Manual *Online*

January 2001

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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 Alan.Evans@cfsan.fda.gov.

[Introduction](#)

GEORGE J. JACKSON
ROBERT I. MERKER
and RUTH BANDLER
BAM Project Coordinators

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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.

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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.

- 2.** Microscopic Examination
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- 4.** *Escherichia coli*
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- 4A.** *Escherichia coli*
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- 1.** Rapid Methods
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- M28a.** *Campylobacter* enrichment broth (Bolton formula)
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- 103.** Motility 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 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

Medium (Semisolid)
September 2000
156. Trypticase Soy
Broth Modified
January 2002

**Advisories for
BAM Users**

Cycloheximide
Supply Update
January 2001

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

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Chapter 1

Food Sampling and Preparation of Sample Homogenate

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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-	Fresh, frozen, and canned fruits and juices, concentrates, and nectars; dried fruits for

22	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., *Bacillus cereus*, 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 ([R11](#)), 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-1, 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-1, 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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U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

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Chapter 4

Enumeration of *Escherichia coli* and the Coliform Bacteria

Authors

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Escherichia coli, originally known as *Bacterium coli* commune, was identified in 1885 by the German pediatrician, Theodor Escherich ([14](#), [29](#)). *E. coli* is widely distributed in the intestine of humans and warm-blooded animals and is the predominant facultative anaerobe in the bowel and part of the essential intestinal flora that maintains the physiology of the healthy host ([9](#), [29](#)). *E. coli* is a member of the family *Enterobacteriaceae* ([15](#)), which includes many genera, including known pathogens such as *Salmonella*, *Shigella*, and *Yersinia*. Although most strains of *E. coli* are not regarded as pathogens, they can be opportunistic pathogens that cause infections in immunocompromised hosts. There are also pathogenic strains of *E. coli* that when ingested, causes gastrointestinal illness in healthy humans (see Chap. 4A).

In 1892, Sharding proposed the use of *E. coli* as an indicator of fecal contamination. This was based on the premise that *E. coli* is abundant in human and animal feces and not usually found in other niches. Furthermore, since *E. coli* could be easily detected by its ability to ferment glucose (later changed to lactose), it was easier to isolate than known gastrointestinal pathogens. Hence, the presence of *E. coli* in food or water became accepted as indicative of recent fecal contamination and the possible presence of frank pathogens.

Although the concept of using *E. coli* as an indirect indicator of health risk was sound, it was complicated in practice, due to the presence of other enteric bacteria like *Citrobacter*, *Klebsiella* and *Enterobacter* that can also ferment lactose and are similar to *E. coli* in phenotypic characteristics, so that they are not easily distinguished. As a result, the term "coliform" was coined to describe this group of enteric bacteria. Coliform is not a taxonomic classification but rather a working definition used to describe a group of Gram-negative, facultative anaerobic rod-shaped bacteria that ferments lactose to produce acid and gas within 48 h at 35°C. In 1914, the U.S. Public Health Service adopted the enumeration of coliforms as a more convenient standard of sanitary significance.

Although coliforms were easy to detect, their association with fecal contamination was questionable because some coliforms are found naturally in environmental samples (6). This led to the introduction of the fecal coliforms as an indicator of contamination. Fecal coliform, first defined based on the works of Eijkman (12) is a subset of total coliforms that grows and ferments lactose at elevated incubation temperature, hence also referred to as thermotolerant coliforms. Fecal coliform analyses are done at 45.5°C for food testing, except for water, shellfish and shellfish harvest water analyses, which use 44.5°C (1, 3, 30). The fecal coliform group consists mostly of *E. coli* but some other enterics such as *Klebsiella* can also ferment lactose at these temperatures and therefore, be considered as fecal coliforms. The inclusion of *Klebsiella* spp in the working definition of fecal coliforms diminished the correlation of this group with fecal contamination. As a result, *E. coli* has reemerged as an indicator, partly facilitated by the introduction of newer methods that can rapidly identify *E. coli*.

Currently, all 3 groups are used as indicators but in different applications. Detection of coliforms is used as an indicator of sanitary quality of water or as a general indicator of sanitary condition in the food-processing environment. Fecal coliforms remain the standard indicator of choice for shellfish and shellfish harvest waters; and *E. coli* is used to indicate recent fecal contamination or unsanitary processing. Almost all the methods used to detect *E. coli*, total coliforms or fecal coliforms are enumeration methods that are based on lactose fermentation (4). The Most Probable Number (MPN) method is a statistical, multi-step assay consisting of presumptive, confirmed and completed phases. In the assay, serial dilutions of a sample are inoculated into broth media. Analysts score the number of gas positive (fermentation of lactose) tubes, from which the other 2 phases of the assay are performed and then uses the combinations of positive results to consult a statistical tables (Appendix 2), to estimate the number of organisms present. Typically only the first 2 phases are performed in coliform and fecal coliform analysis, while all 3 phases are done for *E. coli*. The 3-tube MPN test is used for testing most foods. The 5-tube MPN is used for water, shellfish and shellfish harvest water testing and there is also a 10-tube MPN method that is used to test bottled water or samples that are not expected to be highly contaminated (3).

There is also a solid medium plating method for coliforms that uses Violet Red Bile Agar, which contains neutral red pH indicator, so that lactose fermentation results in formation of pink colonies. There are also membrane filtration tests for coliform and fecal coliform that measure aldehyde formation due to fermentation of lactose. This chapter also includes variations of above tests that use fluorogenic substrates to detect *E. coli* (18), special tests for shellfish analysis, a brief consideration of bottled water testing and a method for testing large volumes of citrus juices for presence of *E. coli* in conjunction with the Juice HACCP rule.

I. Conventional Method for coliforms, fecal coliforms and *E. coli*

A. Equipment and materials

1. Covered water bath, with circulating system to maintain temperature of $45.5 \pm 0.2^{\circ}\text{C}$. Water level should be above the medium in immersed tubes.
2. Immersion-type thermometer, $1\text{--}55^{\circ}\text{C}$, about 55 cm long, with 0.1°C subdivisions, certified by National Institute of Standards and Technology (NIST), or equivalent
3. Incubator, $35 \pm 1.0^{\circ}\text{C}$
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 polyethylene screw caps equipped with Teflon liners. Commercially prepared dilution bottles containing sterile Butterfield's phosphate buffer can also be used.
9. Quebec colony counter, or equivalent, with magnifying lens
10. Longwave UV light [~ 365 nm], not to exceed 6 W.
11. pH meter

B. [Media](#) and [Reagents](#)

Brilliant green lactose bile (BGLB) broth, 2% ([M25](#))

Lauryl tryptose (LST) broth ([M76](#))

EC broth ([M49](#))

Levine's eosin-methylene blue (L-EMB) agar ([M80](#))

Tryptone (tryptophane) broth ([M164](#))

MR-VP broth ([M104](#))

Koser's citrate broth ([M72](#))

Plate count agar (PCA) (standard methods) ([M124](#))

Butterfield's phosphate-buffered water ([R11](#)) or equivalent diluent (except for shellfish)

Kovacs' reagent ([R38](#))

Voges-Proskauer (VP) reagents ([R89](#))

Gram stain reagents ([R32](#))

Methyl red indicator ([R44](#))

Violet red bile agar (VRBA) ([M174](#))

VRBA-MUG agar ([M175](#))

EC-MUG medium ([M50](#))

Lauryl tryptose MUG (LST-MUG) broth ([M77](#))

Peptone Diluent, 0.1% ([R56](#))

C. MPN - Presumptive test for coliforms, fecal coliforms and *E. coli*

Weigh 50 g food into sterile high-speed blender jar. (see Chapter 1 and current FDA compliance programs for instructions on sample size and compositing) Frozen samples can be softened by storing it for ≤ 18 h at 2-5°C, but do not thaw. Add 450 mL of Butterfield's phosphate-buffered water and blend for 2 min. If <50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades.

Prepare decimal dilutions with sterile Butterfield's phosphate diluent. Number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm arc or vortex mix 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 at least 3 consecutive dilutions. Hold pipet at angle so that its lower edge rests against the tube. Let pipet drain 2-3 s. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media.

NOTE: Use 5-tube MPN for analysis of shellfish and shellfish harvest waters.

Incubate LST tubes at 35°C. Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 2 h. Perform confirmed test on all presumptive positive (gas) tubes.

D. MPN - Confirmed test for coliforms

From each gassing LST tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. Incubate BGLB tubes at 35°C and examine for gas production at 48 ± 2 h. Calculate most probable number (MPN) (see Appendix 2) of coliforms based on proportion of **confirmed** gassing LST tubes for 3 consecutive dilutions.

E. MPN - Confirmed test for fecal coliforms and *E. coli*

From each gassing LST tube from the Presumptive test, transfer a loopful of each suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). Incubate EC tubes 24 ± 2 h at 45.5 °C and examine for gas production. If negative, reincubate and examine again at 48 ± 2

h. Use results of this test to calculate fecal coliform MPN. To continue with *E. coli* analysis, proceed to Section F below. The EC broth MPN method may be used for seawater and shellfish since it conforms to recommended procedures (1). (Caution: see Note below).

NOTE: Fecal coliform analyses are done at $45.5 \pm 0.2^\circ\text{C}$ for all foods, except for water testing and in shellfish and shellfish harvest water analysis, which uses an incubation temperature of $44.5 \pm 0.2^\circ\text{C}$.

F. MPN - Completed test for *E. coli*.

To perform the Completed test for *E. coli*, gently agitate each gassing EC tube and streak for isolation, a loopful to a L-EMB agar plate and incubate for 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 up to 5 suspicious colonies from each L-EMB plate to PCA slants incubate for 18-24 h at 35°C and use for further testing.

NOTE: Identification of any 1 of the 5 colonies as *E. coli* is sufficient to regard that EC tube as positive; hence, not all 5 isolates may need to be tested.

Perform Gram stain. All cultures appearing as Gram-negative, short rods should be tested for the IMViC reactions below and also re-inoculated back into LST to confirm gas production.

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.

Voges-Proskauer (VP)-reactive compounds. Inoculate tube of MR-VP broth and incubate 48 ± 2 h at 35°C . Transfer 1 mL to 13 x 100 mm tube. Add 0.6 mL α -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.

Methyl red-reactive compounds. After VP test, incubate MR-VP tube additional 48 ± 2 h at 35°C . Add 5 drops of methyl red solution to each tube. Distinct red color is positive test. Yellow is negative reaction.

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

Gas from lactose. Inoculate a tube of LST and incubate 48 ± 2 h at 35°C . Gas production (displacement of medium from inner vial) or effervescence after gentle agitation is positive reaction.

Interpretation: All cultures that (a) ferment lactose with gas production within 48 h at 35°C , (b) appear as Gram-negative nonsporeforming rods and (c) give IMViC patterns of +++ (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*.

NOTE: Alternatively, instead of performing the IMViC test, use API20E or the automated VITEK biochemical assay to identify the organism as *E. coli*. Use growth from the PCA slants and perform these assays as described by the manufacturer.

G. Solid medium method - Coliforms

Prepare and autoclave violet red bile agar (VRBA) according to manufacturer's instructions. Cool to 48°C before use. Prepare, homogenize, and decimal dilute sample as described in section I. C above so that isolated colonies will be obtained when plated. Transfer two 1 mL aliquots of each dilution to petri dishes, and use either of the following two pour plating methods, depending on whether injured or stressed cells are suspected to be present (1).

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 a 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.

Invert solidified plates and incubate 18-24 h at 35°C. Incubate dairy products at 32°C (2). Examine plates under magnifying lens and with illumination. 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. To confirm that the colonies are coliforms, pick at least 10 representative colonies and transfer each to a tube of BGLB broth. Incubate tubes at 35°C. Examine at 24 and 48 h for gas production.

NOTE: If gas-positive BGLB tube shows a pellicle, perform Gram stain to ensure that gas production was not due to Gram-positive, lactose-fermenting bacilli.

Determine the number of coliforms per gram by multiplying the number of suspect colonies by percent confirmed in BGLB by dilution factor.

Alternatively, *E. coli* colonies can be distinguished among the coliform colonies on VRBA by adding 100 µg of 4-methyl-umbelliferyl-β-D-glucuronide (MUG) per mL in the VRBA overlay. After incubation, observe for bluish fluorescence around colonies under longwave UV light. (see LST-MUG section II for theory and applicability.)

H. Membrane Filtration (MF) Method - coliforms: see Section III. Bottled Water.

NOTE: Food homogenates will easily clog filters, hence MF are most suitable for analysis of water samples; however, MF may be used in the analysis of liquid foods that do not contain high levels of particulate matter.

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

The LST-MUG assay is based on the enzymatic activity of β-glucuronidase (GUD), which cleaves the substrate 4-methylumbelliferyl β-D-glucuronide (MUG), to release 4-methylumbelliferone (MU). When exposed to longwave (365 nm) UV light, MU exhibits a bluish fluorescence that is easily visualized in the medium or around the colonies. Over 95% of *E. coli* produces GUD, including anaerogenic (non-gas-producing) strains. One exception is enterohemorrhagic *E. coli* (EHEC) of serotype O157:H7, which is consistently GUD negative (11, 17). The lack of GUD phenotype in O157:H7 is often used to differentiate this serotype from other *E. coli*, although GUD positive variants of O157:H7 do exist (24, 26). The production of GUD by other members of the family *Enterobacteriaceae* is rare, except for some shigellae (44-58%) and salmonellae (20-29%) (18, 27). However, the inadvertent detection of these pathogens by GUD-

based assays is not considered a drawback from a public health perspective. Expression of GUD activity is affected by catabolite repression (8) so on occasion, some *E. coli* are GUD-negative, even though they carry the *uidA* gene (*gusA*) that encodes for the enzyme (19). In most analyses however, about 96% of *E. coli* isolates tested are GUD-positive without the need for enzyme induction (27).

MUG can be incorporated into almost any medium for use in detecting *E. coli*. But some media such as EMB, which contain fluorescent components, are not suitable, as they will mask the fluorescence of MU. When MUG is incorporated into LST medium, coliforms can be enumerated on the basis of gas production from lactose and *E. coli* are presumptively identified by fluorescence in the medium under longwave UV light, thus it is capable of providing a presumptive identification of *E. coli* within 24 h (18, 28). The LST-MUG method described below has been adopted as Official Final Action by the AOAC for testing for *E. coli* in chilled or frozen foods, exclusive of shellfish (28). For information on MUG assay contact Dr. Peter Feng, FDA, CFSAN, College Park, MD, 20740; 301-436-1650; pfeng@cfsan.fda.gov.

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 adequate and safe. When using a more powerful UV source, such as a 15-watt fluorescent lamp, wear protective glasses or goggles. Also, prior to use in MUG assays, examine all glass tubes for auto fluorescence. Cerium oxide, which is sometimes added to glass as a quality control measure, will fluoresce under UV light and interfere with the MUG test (25). The use of positive and negative control strains for MUG reaction is essential.

1. **Equipment and material:** see section I.A above and in addition,
New, disposable borosilicate glass tubes (100 x 16 mm)
New, disposable borosilicate glass Durham vials (50 x 9 mm) for gas collection.
Longwave UV lamp, 6-watt or equivalent
2. **Media and reagents:** see section I.B above
3. **Presumptive LST-MUG test for *E. coli*.**

Prepare food samples and perform the MPN Presumptive test as described in section I.C. above, except use LST-MUG tubes instead of LST. Be sure to inoculate one tube of LST-MUG with a known GUD-positive *E. coli* isolate as positive control (ATCC 25922). In addition, inoculate another tube with a culture of *Enterobacter aerogenes* (ATCC 13048) as negative control, to facilitate differentiation of sample tubes that show only growth from those showing both growth and fluorescence. Incubate tubes for 24 to 48 ± 2 h at 35°C. Examine each tube for growth (turbidity, gas) then examine tubes in the dark under longwave UV lamp (365 nm). A bluish fluorescence is a positive presumptive test for *E. coli*. Studies by Moberg et al. (28) 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 (28). Perform a confirmed test on all presumptive positive tubes by streaking a loopful of suspension from each fluorescing tube to L-EMB agar and incubate 24 ± 2 h at 35°C. Follow protocols outlined in I. F, above, for Completed test for *E. coli*. Calculate MPN of *E. coli* based on combination of confirmed fluorescing tubes in 3 successive dilutions.

III. Examination of Bottled Water

Consumption of bottled water is increasing rapidly worldwide. In the U.S. alone, over 3.6 billion gallons of

bottled water were consumed in 1998 (International Bottled Water Association, Alexandria, VA). Unlike potable water, which is regulated by the U.S. EPA, bottled water is legally classified as food in the U.S. and regulated by the FDA (Federal Register. 1995. 21 CFR Part 103 et al. beverages: bottled water; final rule. 60(218) 57076-57130). FDA defines bottled water as "water that is intended for human consumption and that is sealed in bottles or other containers with no added ingredients except that it may contain safe and suitable antimicrobial agents" and, within limitations, some added fluoride. Bottled water may be used as a beverage by itself or as an ingredient in other beverages. These regulations do not apply to soft drinks or similar beverages. In addition to "bottled water" or "drinking water", in 21 CFR Part 103 FDA also defines various types of bottled water that meet certain criteria. These identities include "artesian or artesian well water", "ground water", mineral water", "purified or demineralized water", "sparkling bottled water", "spring water" and "well water". Additionally "sterile water" is defined as water that meets the requirements under the "Sterility Test" in the United States Pharmacopeia.

Coliform organisms are not necessarily pathogens and are rarely found in bottled water, however, they serve as an indicator of insanitation or possible contamination. Surveys have shown that coliforms are useful indicators of bottled water quality, but some countries also monitor additional microbial populations as indicators of bottle water quality (10, 33). Under the current bottled water quality standard, FDA has established a microbiological quality requirement that is based on coliform detection levels. These levels may be obtained by membrane filtration (MF) or by 10-tube MPN analysis of ten 10-mL analytical units. For information on bottled water methods contact Dr. Peter Feng, FDA, CFSAN, College Park, MD, 20740; 301-436-1650; pfeng@cfsan.fda.gov.

1. Equipment and Materials.

- a. Incubator at $35^{\circ} \pm 0.5^{\circ}\text{C}$.
- b. Membrane filtration units (filter base and funnels): glass, plastic, or stainless steel; wrapped in foil or paper and sterilized.
- c. Ultraviolet sterilization chamber for sterilizing filter base and funnels (optional).
- d. Filter manifold or vacuum flask to hold filter funnels.
- e. Vacuum source (line vacuum, electric vacuum pump or water aspirator).
- f. Membrane filters; sterile, white, gridded, 47 mm diameter, $0.45\ \mu\text{m}$ pore size (or equivalent, as specified by the manufacturer) for enumeration of bacteria.
- g. Petri dishes, sterile, plastic, 50 x 12 mm, with tight fitting lids.
- h. Forceps designed to transfer membranes without damage.

2. Culture media.

- a. Lauryl sulfate tryptose (LST) broth ([M-76](#)).
- b. Brilliant green lactose bile broth (BGLB) ([M-25](#)).
- c. M-Endo medium or LES Endo Agar (for formulation see Standard Method for Examination of

Water and Wastewater, 1998, 20th ed, p. 9-58 (3).

3. Ten tube MPN coliform test - Presumptive and Confirmed procedures.

For routine examination of bottled water, take 100 mL of sample and inoculate 10 tubes of 2X LST (10 mL of medium) with 10 mL of undiluted sample each. Incubate tubes at 35°C. Examine tubes at 24 ± 2 h for growth and gas formation as evidenced by displacement of medium in fermentation vial or effervescence when tubes are gently agitated. If negative at 24 h, reincubate tubes for an additional 24 h and examine again for gas. Perform a confirmed test on all presumptive positive (gassing) tubes as follows: gently agitate each positive LST tube and, using a 3.0 - 3.5 mm sterile loop, transfer one or more loopfuls of suspension to a tube of BGLB broth. Sterile wooden applicator sticks may also be used for transfer by inserting it at least 2.5 cm into the broth culture. Incubate BGLB tubes for 48 ± 2 h at 35°C. Examine for gas production and record. Calculate MPN using 10 tube MPN Table (9221.III), p. 9-52, Standard Methods for the Examination of Water and Wastewater (3).

4. Membrane filter method for coliforms.

Filter 100 mL of test sample and transfer the filter to M-Endo medium or LES Endo Agar and incubate at 35 °C for 22-24 h. Count colonies that are pink to dark red with a green metallic surface sheen. The sheen may vary from pinpoint to complete coverage of the colony. Use of a low power, dissecting-type microscope to examine filters is recommended.

Confirmation - If there are 5 to 10 sheen colonies on the filter, confirm all by inoculating growth from each sheen colony into tubes of LST and incubate at 35 °C for 48 h. If the number of sheen colonies exceeds 10, randomly select and confirm 10 colonies that are representative of all sheen colonies. Any gas positive LST tubes should be sub cultured to BGLB and incubated at 35°C for 48 hr. Gas production in BGLB within 48 h is a confirmed coliform test. Report results as number of coliform colonies per 100 mL.

NOTE: Standard Method, 1998, 20th ed, p. 9-60 (3), allows for simultaneous inoculation of LST and BGLB during verification. However, BGLB is somewhat inhibitory so the method described above, where samples are sub cultured from LST into BGLB is regarded as a more sensitive verification assay and therefore, recommended.

IV. Examination of Shellfish and Shellfish Meats

The official FDA procedure for bacteriological analysis of domestic and imported bivalve molluscan shellfish is fully and properly described in the APHA's *Recommended Procedures the Examination of Sea Water and Shellfish*, 4th ed. 1970 (1). The methods, including the conventional 5-tube MPN for coliform, fecal coliform and standard total plate count for bacteria (see Chapter 3), are described below for examining shell stock, fresh-shucked meats, fresh-shucked frozen shellfish, and shellfish frozen on the half shell. These procedures do 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. Also, many methods that are used for testing for environmental water or for shellfish harvest waters will not be covered here. Environmental water analyses are done by the U.S. EPA (3) and the quality of shellfish harvest waters are mainly the responsibilities of each State's Shellfish Control Authorities (20).

1. Sample Preparation

Using 10-12 shellfish, obtain 200 g of shellfish liquor and meat. Blend 2 min, with 200 mL sterile

phosphate buffered dilution water or 0.5% peptone water to yield a 1:2 dilution of sample. Analysis of the ground sample must begin within 2 min after blending. Make serial dilutions in 0.5% sterile peptone water or sterile phosphate buffered dilution water.

2. MPN - Presumptive and Confirmed Test for Coliform

Use Lactose Broth (M74) or Lauryl Tryptose Broth (M76), at single strength in 10 ml volumes. Inoculate as follows:

- a. To each of 5 tubes, add 2 mL of the blended homogenate (equivalent to 1 g of shellfish).
- b. To each of 5 tubes, add 2 mL of 1:10 dilution of homogenate (0.1 g shellfish).
- c. To each of 5 tubes, add 2 mL of 1:100 dilution of homogenate (0.01 g shellfish).
- d. To each of 5 tubes, add 2 mL of 1:1000 dilution of homogenate (0.001 g shellfish).

Further dilutions may be necessary to avoid indeterminate results. Incubate tubes at 35°C then follow instructions in section 1.C and perform Confirmed test as in 1.D above, under "Conventional Method for Coliforms, fecal coliforms and *E. coli*". Calculate MPN as described in section 1.D above, except that shellfish analysis specifies that the coliform density be expressed as MPN per 100 g of sample rather than per g.

3. MPN - Presumptive and Confirmed Test for Fecal Coliforms in Shellfish

Perform presumptive test as described in section 2 above. To confirm positive tubes, transfer one loopful from gas positive LST tubes to EC broth and incubate in a covered circulating waterbath at 44.5±0.2°C for 24 ± 2 hr. Gas production in EC is a positive confirmed test for fecal coliforms. Calculate the MPN for fecal coliforms as described above for coliform.

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

The MUG assay for β -glucuronidase (GUD) described above for detecting *E. coli* in chilled and frozen food can also be used for testing for *E. coli* in shellfish meats; but with slight modifications. This is due to the fact that foods, such as shellfish meats contain natural GUD activity (32). As a result, oyster homogenate inoculated directly into LST-MUG tubes in the Presumptive phase of the MPN test can cause false positive fluorescence reactions. Hence, in the analysis of *E. coli* in shellfish meats, the MUG reagent is added to the EC medium and used in the confirmatory phase of the assay. The EC-MUG tubes, incubated at 44.5°C, can be used in the confirmatory phase of a conventional 5-tube MPN assay to determine fecal coliform levels in shellfish meats, then by examining tubes for fluorescence under longwave UV, an *E. coli* MPN can also be readily obtained (32).

See section 1.A and 1.B above for materials and reagents required. Use commercially prepared dehydrated EC-MUG, or prepare medium by adding MUG to EC broth (0.05 g/L) (M50). Several sources of MUG compound are suitable: Marcor Development Corp., Hackensack, NJ; Biosynth International, Skokie, IL; Sigma Chemical Co., St. Louis, MO and Hach Chemical, Loveland, CO. Dispense 5 mL into new disposable borosilicate glass tubes (100 x 16 mm) containing, new disposable borosilicate glass Durham vials (50 x 9 mm) for gas collection. Sterilize EC-MUG broth tubes at

121°C for 15 min; store up to 1 week at room temperature or up to a month under refrigeration.

Perform the 5-tube MPN Presumptive and Confirmed Test for Fecal Coliforms in Shellfish as described above in Section 3, except use EC-MUG tubes instead of EC for the confirmed test. Determination of fluorescence in EC-MUG broth requires the use of 3 control tubes, one inoculated with *E. coli* as positive control; one with *K. pneumoniae* as negative control; and an uninoculated tube as EC-MUG medium batch control. Inoculate the positive and negative controls at the time when Confirmed test is being performed and incubate all tubes at $44.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ for 24 h.

Read fluorescence as described above under LST-MUG assay. Note that some (<10%) *E. coli* are anaerogenic (gas-negative), but should be MUG-positive. Include all fluorescence positive tubes in the *E. coli* MPN calculations. Determine *E. coli* MPN from tables using combination of fluorescence positive tubes at each dilution.

V. Analysis for *E. coli* in citrus juices

Analysis for *E. coli* has been implemented to identify potentially contaminated juices or for verifying the effectiveness of HACCP during processing of unpasteurized juices (21 CFR Part 120, Vol. 66, No. 13, January 19, 2001). The standard method commonly used for testing for *E. coli* is the MPN however, it does not seem adequate for juice testing because of the acidity (pH 3.6 to 4.3) of juices, which can interfere with the test, plus it only allows for testing 3.33 mL of sample. Unlike most *E. coli* methods, which are enumeration assays, the following method is a simple Presence/Absence test that can examine 10-mL volume of juices (34, 35). This assay, designated as modified ColiComplete (CC) Method, is a modification of AOAC Official Method 992.30, which uses MUG for detection of *E. coli* (see Section on LST-MUG Method for details).

1. Equipment and materials

- Covered water bath, with circulating system to maintain temperature of $44.5 \pm 0.2^{\circ}\text{C}$. Water level should be above the medium in immersed tubes.
- Incubator, $35 \pm 0.5^{\circ}\text{C}$
- Longwave UV light [~ 365 nm], not to exceed 6 W.

2. Media and reagents:

Universal Preenrichment Broth (UPEB) (M188) or can be purchased from Difco Laboratories, Detroit, MI

EC-MUG medium ([M50](#))

ColiComplete (CC) discs - Biocontrol, Bellview, WA

3. Sample preparation, enrichment and analysis

Perform assay in duplicate. Aseptically, inoculate 10-mL portion of juice into 90 mL of UPEB and incubate at 35°C for 24 h. After enrichment, mix and transfer 1-mL from each UPEB enrichment broth

into 9 mL of EC broth containing a CC disc. Incubate EC/CC broth tubes at 44.5°C in a circulating water bath for 24 h. Include a tube inoculated with a MUG (+) *E. coli* strain as positive control and another with *K. pneumoniae* as negative control. Examine tubes in the dark and under long wave UV light. The presence of blue fluorescence in either tube is indicative that *E. coli* is present in the sample. Note: The CC discs also contain X-gal, which when cleaved by β -galactosidase will yield blue color on or around the disc. This reaction is analogous to measuring acid/gas production from fermentation of lactose hence, the presence of blue color is indicative of coliforms.

VI. Other Methods for Enumerating Coliforms and *E. coli*

For information and reference purposes there is a Table in Appendix I, which contains a partial listing of commercially-available assays, many of which uses fluorogenic reagents like MUG or other chromogenic substrates for presumptive detection and identification of coliform and *E. coli* in foods. Many of these tests, such as the Petrifilm dry rehydratable film, the hydrophobic grid membrane filter/MUG (HGFM/MUG) method (13), ColiComplete disc (16), etc, have been evaluated by collaborative studies and adopted as official first or final action by the AOAC. There are also many modifications of the membrane filtration assays that have been developed for testing for coliform, fecal coliform and *E. coli* and some of these may be useful in testing foods such as milk and beverages, but they are used mostly for water, environmental waters, and shellfish harvest waters analysis (5, 7, 20, 22, 23, 31).

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Chapter 4A

Diarrheagenic *Escherichia coli*

Authors

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Escherichia coli is one of the predominant species of facultative anaerobes in the human gut and usually harmless to the host; however, a group of pathogenic *E. coli* has emerged that causes diarrheal disease in humans. Referred to as Diarrheagenic *E. coli* ([20](#)) or commonly as pathogenic *E. coli*, these groups are classified based on their unique virulence factors and can only be identified by these traits. Hence, analysis for pathogenic *E. coli* often requires that the isolates be first identified as *E. coli* before testing for virulence markers. The pathogenic groups includes enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and perhaps others that are not yet well characterized ([15](#), [20](#)). Of these, only the first 4 groups have been implicated in food or water borne illness. Some properties and symptoms of these 4 subgroups are discussed below and summarized in Table 1.

ETEC is recognized as the causative agent of travelers' diarrhea and illness is characterized by watery diarrhea with little or no fever. ETEC infections occurs commonly in under-developed countries but, in the U.S., it has been implicated in sporadic waterborne outbreaks as well as due to the consumption of soft cheeses, Mexican-style foods and raw vegetables. Pathogenesis of ETEC is due to the production of any of several enterotoxins. ETEC may produce a heat-labile enterotoxin (LT) that is very similar in size (86 kDa), sequence, antigenicity, and function to the cholera toxin (CT). ETEC may also produce a heat stable toxin (ST) that is of low molecular size (4 kDa) and resistant to boiling for 30 min. There are several variants of ST, of which ST1a or STp is found in *E. coli* isolated from both humans and animals, while ST1b or ST_h is predominant in human isolates only. The infective dose of ETEC for adults has been estimated to be at least 10^8 cells; but the young, the elderly and the infirm may be susceptible to lower levels. Because of its high infectious dose, analysis for ETEC is usually not performed unless high levels of *E. coli* have been found in a food. Also, if ETEC is detected, levels should also be enumerated to assess the potential hazard of the contaminated food. Production of LT can be detected by Y-1 adrenal cell assays ([20](#)) or serologically by commercial reverse passive latex agglutination assay and ELISA (see Appendix 1). The production of ST can also be detected by ELISA or by infant mouse assay ([26](#)). Both LT and ST genes have also been sequenced and PCR ([28](#), [30](#)) and gene probe assays (see chapter 24) are available. Analysis of colonies on plating media using gene probe/colony hybridization also allows enumeration of ETEC in foods.

EIEC closely resemble *Shigella* and causes an invasive, dysenteric form of diarrhea in humans (6). Like *Shigella*, there are no known animal reservoirs; hence the primary source for EIEC appears to be infected humans. Although the infective dose of *Shigella* is low and in the range of 10 to few hundred cells, volunteer feeding studies showed that at least 10⁶ EIEC organisms are required to cause illness in healthy adults. Unlike typical *E. coli*, EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose, so they are anaerogenic. Pathogenicity of EIEC is primarily due its ability to invade and destroy colonic tissue. The invasion phenotype, encoded by a high molecular weight plasmid, can be detected by invasion assays using HeLa or Hep-2 tissue culture cells (6, 18) or by PCR and probes specific for invasion genes (see chapter 24).

EPEC causes a profuse watery diarrheal disease and it is a leading cause of infantile diarrhea in developing countries. EPEC outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Through volunteer feeding studies the infectious dose of EPEC in healthy adults has been estimated to be 10⁶ organisms. Pathogenesis of EPEC involves intimin protein (encoded by *eae* gene) that causes attachment and effacing lesions (11); but it also involves a plasmid-encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells (27). Production of EAF can be demonstrated in Hep-2 cells and the presence of *eae* gene can be tested by PCR assays (20).

EHEC are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome (HUS). EHEC are typified by the production of verotoxin or Shiga toxins (Stx). Although Stx1 and Stx2 are most often implicated in human illness, several variants of Stx2 exist. There are many serotypes of Stx-producing *E. coli* (STEC), but only those that have been clinically associated with HC are designated as EHEC. Of these, O157:H7 is the prototypic EHEC and most often implicated in illness worldwide (2, 10, 14, 20). The infectious dose for O157:H7 is estimated to be 10 - 100 cells; but no information is available for other EHEC serotypes. EHEC infections are mostly food or water borne and have implicated undercooked ground beef (2, 10), raw milk (23), cold sandwiches (14), water (25), unpasteurized apple juice (1) and sprouts and vegetables (3, 13). EHEC O157:H7 are phenotypically distinct from *E. coli* in that they exhibit slow or no fermentation of sorbitol and do not have glucuronidase activity (see chapter 4. LST-MUG for details); hence, these traits are often used to isolate this pathogen from foods. The production of Stx1 and Stx2 can be tested by cytotoxicity assays on vero or HeLa tissue culture cells or by commercially available ELISA or RPLA kits (see Appendix 1). Gene probes (see chapter 24) and PCR assays specific for *stx1* and *stx2* and other trait EHEC markers are also available (9, 12) (and see below).

Table 1. Some properties and symptoms associated with pathogenic *E. coli* subgroups.

	ETEC	EPEC	EHEC	EIEC
Toxin	LT/ST ^a	-	Shiga or Vero toxin (Stx or VT)	-
Invasive	-	-	-	+
Intimin	-	+	+	-
Enterohemolysin	-	-	+	-
Stool	Watery	Watery, Bloody	Watery, very bloody	Mucoid, bloody
Fever	Low	+	-	+

Fecal leukocytes	-	-	-	+
Intestine involved	Small	Small	Colon	Colon, lower small
Serology	various	O26, O111 & others	O157:H7, O26, O111 & others	various
I _D ^b	High	High	Low	High
^a LT, labile toxin; ST, stable toxin. ^b I _D , infective dose.				

Isolation and Identification of Pathogenic *Escherichia coli* - Except EHEC of serotype O157:H7

Since pathogenic *E. coli* are identified based on its unique virulence properties, the analytical procedure for these pathogens in foods generally requires the isolation and identification of the organisms as *E. coli* before testing for the specific virulence traits. Following is a general procedure for enrichment and isolation of pathogenic *E. coli* from food ([17](#)).

A. Equipment and materials

1. Balance, ≥ 2 kg with 0.1 g sensitivity
2. Blender, Waring or equivalent model with low speed operation at 8000 rpm, with 1 liter glass or metal jar
3. Incubators, $35 \pm 0.5^\circ\text{C}$ and $44 \pm 1^\circ\text{C}$
4. Petri dishes 20 x 150 mm
5. Pipets, Pasteur
6. 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 broth ([M26](#)) supplemented individually with 0.5% (w/v) of each: glucose, adonitol, cellobiose, sorbitol, arabinose, mannitol, and lactose
9. Urea broth ([M171](#))
10. Lysine decarboxylase broth, Falkow ([M87](#)).
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- β -D-galactopyranoside) disks ([R53](#))
3. Phosphate buffered saline solution, (sterile) (PBS) ([R60](#)), or Butterfield's Phosphate-buffered dilution water (BPBW) ([R11](#)).
4. Kovac's 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. Enumeration.

Refrigerate samples promptly after receipt. Do not freeze except to hold frozen products until just prior to analysis. Analyze samples as soon as possible. If enumeration is required, prepare a homogenate of 25 g in 225 mL of PBS or BPBW. Perform decimal dilutions (in PBS or BPBW) from the homogenate and direct plate onto MacConkey agar to yield isolated colonies. After incubating the plates for 20 h at 35°C, perform colony lifts and hybridization with specific gene probes for virulence genes (see chapter 24). This enumeration procedure is most effective if *E. coli* constitutes at least 10% of the microbial growth on isolation agars and it is present at a level of >1,000 cells/g.

E. Enrichment for Pathogenic *E. coli*

The approach recommended here permits qualitative determination of the presence of pathogenic *E. coli*. Aseptically weigh 25 g of sample into 225 ml of BHI broth (dilution factor of 1:10). If necessary, sample size may deviate from 25 g depending on availability of the sample, as long as the diluent is adjusted proportionally. Blend or stomach briefly. Incubate the homogenate for 10 min at room temperature with periodic shaking then allow the sample to settle by gravity for 10 min. Decant medium carefully into a sterile container and incubate for 3 h at 35°C to resuscitate injured cells. Transfer contents to 225 mL double strength TP broth in a sterile container and incubate 20 h at 44.0 ± 0.2°C. After incubation, streak to L-EMB and MacConkey agars. Incubate these agars for 20 h at 35°C.

F. Selection.

Typical lactose-fermenting colonies on L-EMB agar appear dark centered and flat, with or without metallic sheen. Typical colonies on MacConkey agar appear brick red. Lactose non-fermenting biotypes on both agars produce colorless or slightly pink colonies.

NOTE: EIEC do not ferment lactose and there may also be atypical non-lactose fermenting strains in the other pathogenic *E. coli* groups; therefore, as many as 20 colonies (10 typical and 10 atypical) should be picked for further characterization.

G. Conventional Biochemical Screening and identification (7, 22)

Use the procedures described in Chapter 4 for biochemical and morphological identification of *E. coli*. However, because many enteric bacteria can also grow in the TP enrichment broth, plus anaerogenic, non-motile and slow or lactose non-fermenting strains of *E. coli* must also be considered, additional tests may need to be performed. Some of these new or modified reactions are discussed here.

1. **Primary screening.** Transfer suspicious colonies to TSI agar, BAB slant, tryptone broth, arabinose broth, and urea broth. Incubate 20 h at 35°C. Reject H₂S-positive, urease-positive, arabinose non-fermenting, and indole-negative strains. To test for the ONPG reaction, suspend growth from TSI in 0.85% saline to give detectable turbidity. Add an ONPG-impregnated disk and incubate 6 h at 35°C. Yellow color indicates positive reaction. Reject ONPG-negative, aerogenic cultures. Some *Alkalescens-Dispar* strains (i.e., anaerogenic *Escherichia*) are ONPG-negative.
2. **Secondary screening** (48 h incubation at 35°C unless otherwise specified). To identify cultures, test additional reactions shown in Table 1, Chapter 4, to subdivide *Escherichia* spp. Since it is not known whether these additional species are of pathogenic significance to humans, strains giving typical

reactions for *E. coli* should be further investigated. To differentiate *E. coli* from *Shigella*, examine anaerogenic, non-motile, slow lactose fermenters for lysine decarboxylase, mucate, and acetate reactions. *Shigella sonnei*, which may grow in the same enrichment conditions, is anaerogenic and non-motile. It also produces a negative indole reaction and shows slow or non-fermentation of lactose. The biochemical-physiological characteristics of *E. coli* are summarized in Table 2, chapter 4.

3. Alternatively, use API20E or the automated VITEK biochemical assay to identify the organism as *E. coli*.

H. Tests for Enterotoxigenic *E. coli* (ETEC)

When *E. coli* levels in foods exceed 10^4 cells/g, perform enumeration for ETEC by colony hybridization analysis using DNA probes for LT and ST (Chapter 24). If biological activity assays are necessary, LT can be detected by the Y-1 tissue culture test (20) and ST can be detected by the infant mouse test (20) (For detailed procedures of these assays, see Chapter 4, BAM, Edition 8, Revision A /1998). There are also commercially available RPLA and ELISA tests to detect LT and ST toxins (Appendix 1) as well as PCR assays (30).

I. Tests for Enteroinvasive *E. coli* (EIEC)

If an isolate is suspected to be EIEC, the invasive potential of the isolates may be tested by the Sereny test or the Guinea pig keratoconjunctivitis assay (20) (For detailed procedures on Sereny test, see Chapter 4, BAM, Edition 8, Revision A /1998). Invasive potential of the isolates can also be determined by the HeLa tissue culture cell assay as described (18), or with the *in vitro* staining technique using acridine orange to stain intracellular bacteria in HeLa monolayers (19). Alternatively, since the *invA* gene sequence of EIEC closely resembles that of *Shigella*, DNA probe (Chapter 24) and PCR (Chapter 28) assays for *Shigella* will also work for EIEC.

Caution: Since both EIEC and *Shigella* will give positive probe and PCR reactions, it is critical that the organisms are identified first as *E. coli*.

J. Tests for Enteropathogenic *E. coli* (EPEC)

EPEC strains are identified based on 3 key traits: attachment and effacing lesion (A/E), localized adherence on cells and the lack of Shiga toxin (Stx) production. This last trait is also used to distinguish strains of EPEC from EHEC. Phenotypically, A/E and localized adherence are tested using Hep-2 or HeLa tissue cells. Absence of Stx can be determined using tests outlined for EHEC (see below). There are also PCR and probes for the EAF plasmid that encodes for localized adherence and the *eae* gene that encodes for the intimin that causes the A/E phenotype. **Caution:** There are several variants of *eae* gene and some EPEC strains carry *eae* variants identical to EHEC serotypes; hence, these tests will detect strains from both pathogenic groups. For specific virulence assays for EPEC, see Nataro and Kaper, 1998 (20) for detailed procedures.

K. Enrichment and isolation of *E. coli* Serotype O157:H7 from Foods

An enrichment/isolation procedure using EHEC Enrichment broth (EEB) followed by plating on TCSMAC agar has been developed for detecting O157:H7 in foods. Both the enrichment and the selective medium contain several anti-microbial reagents that effectively suppress normal flora growth and interference.

Comparative analysis showed that the EEB-TCSMAC method was superior to the mTSB-HC agar (chapter 24) method in the recovery of O157:H7 bacteria in contaminated and seeded foods examined (29).

L. Isolation of O157:H7 with Tellurite-Cefixime-Sorbitol MacConkey (TC SMAC) agar

Sorbitol MacConkey (SMAC) agar is effectively used in clinical testing for isolating O157:H7 from bloody stool samples. However, SMAC is not very selective and therefore, less effective for food testing, because normal flora bacteria can easily outgrow O157:H7 strains that may be present. Similarly, the HC agar developed for food analysis, though slightly more selective, is also susceptible to normal flora overgrowth. By addition of potassium tellurite and cefixime to SMAC, a very selective TC SMAC agar was developed for isolation of *E. coli* O157:H7. Caution: Although most non-O157:H7 *E. coli* ferment sorbitol, about 6% of the isolates do not (7). These atypical strains along with other sorbitol non-fermenting bacteria such as *Morganella* and *Hafnia* appear identical to O157:H7 colonies on TC SMAC agar. Hence, confirmatory tests must be performed to distinguish these from the O157:H7 isolates. For additional information on the TCSMAC procedure, contact Steve Weagant, 425-483-4873, FDA, Bothell, WA, sweagant@ora.fda.gov.

M. *E. coli* O157:H7 Media Preparation

EHEC Enrichment Broth (EEB) - same as mTSB (M156) but without novobiocin. Instead, add the following filter-sterilized antibiotics after autoclaving and tempering.

Cefixime*	0.0125 mg/L
Cefsulodin	10.00 mg/L
Vancomycin	8.00 mg/L

*Available from Dynal Inc., Lake Success, NY (800) 638-9416.

NOTE: The level of cefixime shown above has been reduced to ¼ strength from that described in BAM, Edition 8, Revision A /1998, because the original concentration of 0.05 mg/L was found to be inhibitory to the growth of O157:H7 positive control strains in the absence of competing microflora.

Tellurite Cefixime Sorbitol-MacConkey agar (TC SMAC) - Prepare Sorbitol MacConkey Agar (M139) and add the following filter-sterilized additives after autoclaving and tempering:

Potassium tellurite*	2.5mg/L
Cefixime*	0.05mg/L

*Available from Dynal Inc., Lake Success, NY (800) 638-9416.

N. *E. coli* O157:H7 Enrichment Procedure

1. Weigh 25 g of food into 225 mL of EEB, blend or stomach briefly as necessary.
2. Incubate at $37 \pm 0.5^{\circ}\text{C}$ with shaking for 24 h (see O.1. below).

NOTE: An abbreviated 6 h incubation may suffice in some instances to enable rapid isolation of O157:H7 from foods, but the 24 h enrichment was found to be more sensitive and therefore is recommended.

OPTIONAL. Additional selective enrichment by use of immunomagnetic separation (IMS) has been found to be useful in the analysis of some foods, particularly those with high levels of competing microbial flora (29). Anti-O157 immunomagnetic beads (Dynabeads) are available commercially (Dynal Inc., see address above). Perform IMS on 1 mL of enrichment broth, following manufacturer's instructions. Cell-bound Dynabeads are plated on TC SMAC agar as described below.

***O. E. coli* O157:H7 Isolation Procedure**

1. After incubation, spread plate 0.1 mL of appropriately diluted enrichment broth to TC SMAC agar plates to obtain 100-300 isolated colonies. Streak one loop-full of the enrichment to one additional TC SMAC plate. Incubate plates at $35-37^{\circ}\text{C}$ for 18 - 24 h.
2. Sorbitol-fermenting bacteria appear as pink to red colonies on TC SMAC. Typical O157:H7 colonies are colorless or neutral/gray with a smoky center and 1-2 mm in diameter. Pick up to 5 typical O157:H7 colonies from TC SMAC onto TSAYE (M153) plates and incubate at 35°C for 18 - 24 h.

***P. E. coli* O157:H7 Presumptive Screening and Confirmation**

1. Spot indole - Spot some growth from TSAYE to a filter wetted with Kovac's reagent. *E. coli* O157:H7 are indole-positive. Discard indole-negative strains.
2. L-EMB and MUG - Test indole-positive isolates by streak plating some growth from TSAYE onto EMB agar and another TSAYE plate. On the latter, place a ColiComplete disc (Biocontrol, Bellview, WA) in the area of heaviest streak. Similarly prepare another TSAYE plate using a known MUG-positive *E. coli* strain as positive control. Incubate the plates overnight at $35-37^{\circ}\text{C}$. On EMB, *E. coli* O157:H7 should appear as typical *E. coli* colonies showing dark centers with or without metallic green sheen. Examine the TSAYE plates under long wave UV [365 nm] light. The positive control should be MUG-positive as evidenced by blue fluorescence around the disc that is indicative of β -glucuronidase activity on MUG. Strains of *E. coli* O157:H7 are MUG-negative. Eliminate isolates that give positive MUG reactions.

***Q. E. coli* O157:H7 Confirmation**

Test the above presumptive-positive isolates with the following tests to confirm the isolates as *E. coli* O157:H7, with potential to produce shiga toxin.

1. Test for the presence of the O157 antigen using 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. **Caution:** Use single colonies for latex agglutination tests, as the use of excessive colony growth (i.e.: sweeps) may result in

false positive reactions due to autoagglutination (24).

NOTE: The RIM *E. coli* O157:H7 Latex Test kit also includes the H7 latex reagent. It is highly recommended to also test the O157 positive isolates with the H7 reagent. If the isolate is H7 positive, it is good evidence that the isolate is of the O157:H7 serotype. But, if the O157 isolate is H7 negative, proceed with steps 2 and 3 below, as it may be a toxigenic, non-motile, variant of O157:H7 serotype (8). Do not use H7 latex reagent without testing first with the O157 reagent.

2. Using growth from TSAYE plate, perform API20E or VITEK to identify the isolates as *E. coli*.
3. If the isolate is O157 positive and identified as *E. coli*, test for the presence of *stx1* and *stx2* genes by DNA probe (see, Chapter 24) or preferably by PCR. (12)

Additional Information - There is also O157:H7-specific DNA probe and multiplex PCR (5P PCR) (9) assays that can specifically detect O157:H7 serotype and its toxigenic non-motile variants. The probe is directed at the +93 base mutation in the *uidA* gene that encodes for β -glucuronidase. The mutation is highly conserved in O157:H7 and its toxigenic, non-motile variants; therefore specific only for O157:H7 strains of public health concern. The 5P PCR couples primers to the *uidA* with primers specific for *stx1*, *stx2*, gamma variant of *eae* that is found in O157:H7 and the *ehxA* enterohemolysin genes to form an assay that simultaneously detect these virulence traits in Stx-producing *E. coli* and determines whether it is of O157:H7 serotype. The *stx* gene PCR primers used in this assay are also different from those described in LIB 3811; hence, this assay can be used to verify toxigenic potential of isolates, should the results of the LIB 3811 PCR assay be inconclusive. For information on the probe and 5P PCR, contact Peter Feng, 301-436-1650, CFSAN, FDA, College Park, MD Pfeng@cfsan.fda.gov For protocol on using the probe, see Chapter 24. For information purposes only, a partial list of commercially available rapid methods for detecting *E. coli* O157:H7 is shown in Appendix 1.

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Bacteriological Analytical Manual *Online*

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Chapter 5 *Salmonella*

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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.

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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, $35 \pm 2^\circ\text{C}$
7. Refrigerated incubator or laboratory refrigerator, $4 \pm 2^\circ\text{C}$
8. Water bath, $49 \pm 1^\circ\text{C}$
9. Water bath, circulating, thermostatically-controlled, $43 \pm 0.2^\circ\text{C}$
10. Water bath, circulating, thermostatically-controlled, $42 \pm 0.2^\circ\text{C}$
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.

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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. Bromocresol 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, C₁, C₂, C₃, D₁, D₂, E₁, E₂, E₃, E₄, F, G, H, I, Vi, and other groups, as appropriate
53. *Salmonella* Spicer-Edwards flagellar (H) antisera

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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 \$\text{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 $2-5^{\circ}\text{C}$.

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 1/4 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 1/4 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-1](#), 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 $2-5^{\circ}\text{C}$ 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).

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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 $42 \pm 0.2^\circ\text{C}$ (circulating, thermostatically-controlled, water bath). Incubate TT broth 24 ± 2 h at $43 \pm 0.2^\circ\text{C}$ (circulating, thermostatically-controlled, water bath).

Foods with a low microbial load (except guar gum). Incubate RV medium 24 ± 2 h at $42 \pm 0.2^\circ\text{C}$ (circulating, thermostatically controlled, water bath). Incubate TT broth 24 ± 2 h at $35 \pm 2.0^\circ\text{C}$.

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

3. Mix (vortex, if tube) and streak 3 mm loopful ($10 \mu\text{l}$) 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 ($10 \mu\text{l}$) 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, H₂S-positive *S. diarizonae* (ATCC 12325) and a lactose-negative, H₂S-negative *S. abortus equi* (ATCC 9842); **OR** a lactose-positive, H₂S-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 H₂S production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H₂S (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 H₂S 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.

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E. 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 $37 \pm 0.5^\circ\text{C}$ 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 $48\text{--}50^\circ\text{C}$ 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% bromocresol 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 bromocresol 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 $\pm$.
- **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.

6. 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.
7. **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-1](#), 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.

8. **Classification of cultures.** Classify, as *Salmonella*, cultures that have reaction patterns of [Table 1](#). 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 1 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 <i>Salmonella</i>			
Test or substrate	Result		<i>Salmonella</i> species reaction ^(a)
	Positive	Negative	
1. Glucose (TSI)	yellow butt	red butt	+
2. Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3. H ₂ S (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
<p>^a +, 90% or more positive in 1 or 2 days; -, 90% or more negative in 1 or 2 days; v, variable.</p> <p>^b Majority of <i>S. arizonae</i> cultures are negative.</p> <p>^c Majority of <i>S. arizonae</i> cultures are positive.</p>			

Table 2. Criteria for discarding non- <i>Salmonella</i> 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)
<p>^a Test malonate broth positive cultures further to determine if they are <i>S. arizonae</i>.</p> <p>^b Do not discard positive broth cultures if corresponding LIA cultures give typical <i>Salmonella</i> reactions; test further to determine if they are <i>Salmonella</i> species.</p>	

9. **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 (1). 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:

- 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.
 - Discard cultures presumptively classified as not *Salmonella* with commercial biochemical kits when cultures conform to AOAC criteria (1) for classifying cultures as not *Salmonella*.
 - 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.
10. **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.

11. **Submission of cultures for serotyping.** Submit 1 isolate 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.

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[Bad Bug Book:](#)
[Salmonella](#)

[Chapter 5 Contents](#)

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998. Chapter 5.

Authors: [Wallace H. Andrews](#) and [Thomas S. Hammack](#)

Revisions: 1999-DEC, 2000-MAR, and 2000-AUG Final revision on 2000-NOV-14 (see the Introduction for a summary of changes).

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

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

Bacteriological Analytical Manual *Online*

August 2002

Chapter 10

Listeria monocytogenes

[Author](#)

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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)
<i>L. monocytogenes</i>	+	-	-	+	-	+
<i>L. ivanovii</i>	+	-	-	-	+	+
<i>L. innocua</i>	-	-	-	V ^(b)	-	-
<i>L. welshimeri</i>	-	-	-	V ^(b)	+	-
<i>L. seeligeri</i>	+	-	-	-	+	-
<i>L. grayi</i> ^(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 Fe³⁺ 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/Fe³⁺ agars. PALCAM agar (17) may be substituted for LPM agars. Incubate OXA and PALCAM plates (use of a CO₂-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.

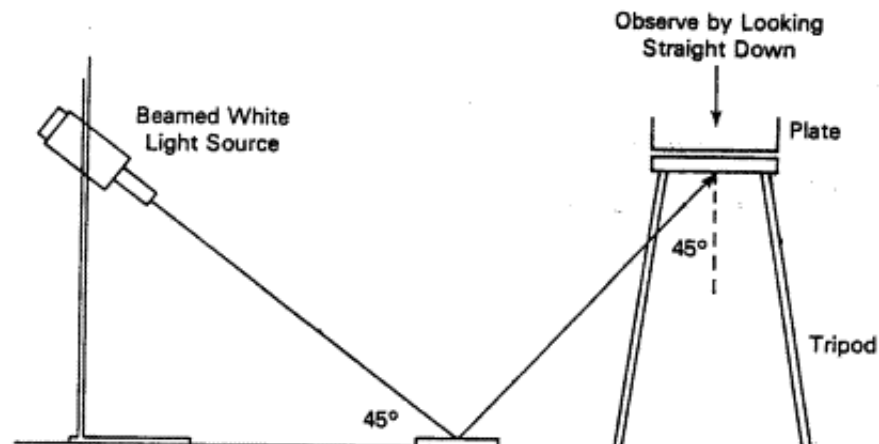


Figure 1. Examination of plates by Henry illumination for suspect colonies of *Listeria* spp. Light source used, Bausch & Lomb Nicholas Illuminator; see current Fisher Catalog. Reference: H.P.R. Seeliger, *Listeriosis*. Hafner Publishing Co., New York (1961). NOTE: Some observers prefer a concave mirror.

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/Fe³⁺ 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
<i>L. monocytogenes</i>	1/2A, 1/2B, 1/2C, 3A, 3B, 3C, 4A, 4AB, 4B, 4C, 4D, 4E, "7"
<i>L. ivanovii</i>	5
<i>L. innocua</i>	4AB, 6A, 6B, Un ^(a)
<i>L. welshimeri</i>	6A, 6B
<i>L. seeligeri</i>	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 10¹⁰ bacteria/ml; dilute to 10⁵ 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 10⁴ bacteria per mouse. Observe for death over 5-day period. Nonpathogenic strains will not kill, but 10⁴ 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 β -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](https://www.atcc.org/)), 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.

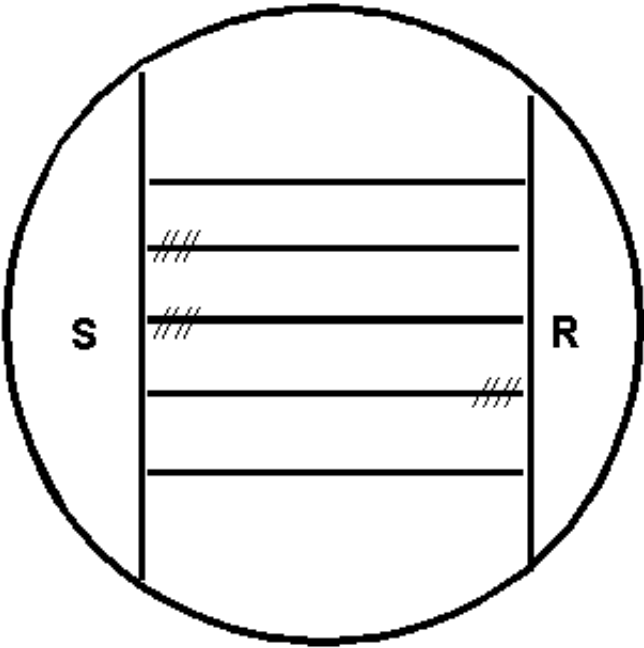


Figure 2. CAMP test for *L. monocytogenes*: Inoculation pattern of sheep blood agar plate. Horizontal lines represent streak inoculations of 5 test strains. Vertical lines represent streak inoculations of *S. aureus* (S) and *R. equi* (R). Hatched lines indicate (diagrammatically only) locations of hemolysis 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	
	<i>Staphylococcus aureus</i> (S)	<i>Rhodococcus equi</i> (R)
<i>L. monocytogenes</i>	+	_(a)
<i>L. ivanovii</i>	-	+
<i>L. innocua</i>	-	-
<i>L. welshimeri</i>	-	-
<i>L. seeligeri</i>	+	-

^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 H₂S 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). If all the MPN tubes are *Listeria* positive, use reserve sample to repeat the MPN determination using an appropriate range of smaller analytical portions. Alternatively, perform a plate count (for details contact Anthony.Hitchins@cfsan.fda.gov). 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 10⁶ 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:
Listeria monocytogenes

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2001-April. Section H. CAMP test: Updated address for ATCC and added a link to its web page.

2002-August: Section J. Enumeration: Added instructions for positive result on all MPN tubes.

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U.S. Food & Drug Administration
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Bacteriological Analytical Manual *Online*

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Appendix 1

Rapid Methods for Detecting Foodborne Pathogens

[Author](#)

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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	<i>Enterobacteriaceae, Listeria, Staphylococcus, Campylobacter, Non-fermenters, anaerobes</i>
Cobas IDA	biochemical	Hoffmann LaRoche	<i>Enterobacteriaceae</i>
Micro-ID ^b	biochemical	REMEL	<i>Enterobacteriaceae, Listeria</i>
EnterotubeII	biochemical	Roche	<i>Enterobacteriaceae</i>
Spectrum 10	biochemical	Austin Biological	<i>Enterobacteriaceae</i>
RapID	biochemical	Innovative Diag.	<i>Enterobacteriaceae</i>
BBL Crystal	biochemical	Becton Dickinson	<i>Enterobacteriaceae, Vibrionaceae, Non-fermenters, anaerobes</i>
Minitek	biochemical	Becton Dickinson	<i>Enterobacteriaceae</i>
Microbact	biochemical	Microgen	<i>Enterobacteriaceae, Gram negatives, Non-fermenters, Listeria</i>
Vitek ^b	biochemical ^a	bioMerieux	<i>Enterobacteriaceae, Gram negatives, Gram positives</i>
Microlog	C oxidation ^a	Biolog	<i>Enterobacteriaceae, Gram negatives, Gram positives</i>
MIS ^b	Fatty acid ^a	Microbial-ID	<i>Enterobacteriaceae, Listeria, Bacillus, Staphylococcus, Campylobacter</i>
Walk/Away	biochemical ^a	MicroScan	<i>Enterobacteriaceae, Listeria, Bacillus, Staphylococcus, Campylobacter</i>
Replianalyzer	biochemical ^a	Oxoid	<i>Enterobacteriaceae, Listeria, Bacillus, Staphylococcus, Campylobacter</i>
Riboprinter	nucleic acid ^a	Qualicon	<i>Salmonella, Staphylococcus, Listeria, Escherichia coli</i>
Cobas Micro-ID	biochemical ^a	Becton Dickinson	<i>Enterobacteriaceae, Gram negatives, Non-fermenters</i>
Malthus ^b	conductance ^a	Malthus	<i>Salmonella, Listeria, Campylobacter, E. coli, Pseudomonas, coliforms</i>
Bactometer	impedance ^a	bioMerieux	<i>Salmonella</i>

* 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
<i>Clostridium botulinum</i>	Probelia	PCR	BioControl
<i>Campylobacter</i>	AccuProbe	probe	GEN-PROBE
	GENE-TRAK	probe	GENE-TRAK
<i>Escherichia coli</i>	GENE-TRAK	probe	GENE-TRAK
<i>E. coli</i> O157:H7	BAX	PCR ^a	Qualicon
	Probelia	PCR	BioControl
<i>Listeria</i>	GENE-TRAK ^c	probe	GENE-TRAK
	AccuProbe	probe	GEN-PROBE
	BAX	PCR	Qualicon
	Probelia	PCR	BioControl
<i>Salmonella</i>	GENE-TRAK ^c	probe	GENE-TRAK
	BAX	PCR	Qualicon
	BIND ^b	phage	BioControl
	Probelia	PCR	BioControl
<i>Staphylococcus aureus</i>	AccuProbe	probe	GEN-PROBE
	GENE-TRAK	probe	GENE-TRAK
<i>Yersinia enterocolitica</i>	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
<i>Bacillus cereus</i> diarrhoeal toxin	TECRA	ELISA	TECRA
	BCET	RPLA	Unipath

<i>Campylobacter</i>	Campyslide	LA	Becton Dickinson
	Meritec-campy	LA	Meridian
	MicroScreen	LA	Mercia
	VIDAS	ELFA ^b	bioMerieux
	EiaFOSS	ELISA ^b	Foss
	TECRA	ELISA	TECRA
<i>Clostridium botulinum</i> toxin	ELCA	ELISA	Elcotech
<i>C. perfringens</i> enterotoxin	PET	RPLA	Unipath
<i>Escherichia coli</i>			
EHEC ^{**c} O157:H7	RIM	LA	REMEL
	<i>E. coli</i> O157	LA	Unipath
	Prolex	LA	PRO-LAB
	Ecolex O157	LA	Orion Diagnostica
	Wellcolex O157	LA	Murex
	<i>E. coli</i> 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
	<i>E. coli</i> O157	ELISA	LMD Lab
	Premier O157	ELISA	Meridian
	<i>E. coli</i> O157:H7	ELISA	Binax
	<i>E. coli</i> Rapitest	ELISA	Microgen
	Transia card	ELISA	Transia
	<i>E. coli</i> 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
<i>Listeria</i>	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
<i>Salmonella</i>	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
<i>Shigella</i>	Bactigen	LA	Wampole Labs
	Wellcolex		Laboratoire Wellcome
<i>Staphylococcus aureus</i>	Staphyloslide	LA	Becton Dickinson
	AureusTest ^e	LA	Trisum
	Staph Latex	LA	Difco
	<i>S. aureus</i> 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

<i>Vibrio cholera</i>	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. hermannii* 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/ <i>E. coli</i>	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
<i>E. coli</i>	MUG disc	MUG	REMEL
	CHROMagar	Medium	CHROMagar
EHEC ^d	Rainbow Agar	Medium	Biolog
	BCMO157:H7	Medium	Biosynth
	Fluorocult O157:H7	Medium	Merck
<i>Listeria monocytogenes</i>	BCM	Medium	Biosynth
<i>Salmonella</i>	Isogrid ^b	HGMF	QA Labs
	OSRT	Medium/ motility	Unipath (Oxoid)
	Rambach	Medium	CHROMagar
	MUCAP	C8esterase	Biolife
	XLT-4	Medium	Difco
	MSRV ^b	Medium	
<i>Yersinia</i>	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-β-D-glucuronide; ONPG, O-nitrophenyl β-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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