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U. S. Department of Health and Human Services
U.S. Food & Drug Administration
Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual *Online*

January 2001

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About the Bacteriological Analytical Manual

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

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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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March 2001

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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.

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

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April 2003

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.

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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.

In certain instances, it may not be possible to fully conform to the sampling plan. Nonetheless it is still important to ascertain whether or not Salmonella is present in the suspect food. Therefore, the analyst should still try to analyze as many analytical units as is required for the food of interest, i.e., 60 analytical units for Category I foods, 30 analytical units for Category II foods, and 15 analytical units for Category III foods. Individual 25 g analytical units may be combined into 375 g composites as described above unless otherwise indicated in Chapter 5 or the OMA. Below are examples of situations that might confront the analyst.

1) The number and weights of the sample units is correct.

Each sample should be mixed to ensure homogeneity before withdrawing a 25 g analytical unit. The analytical units can be composited (fifteen 25 g units into a 375 g composite), unless otherwise indicated in Chapter 5 or in the OMA. Samples should be preenriched at a 1:9 sample-to-broth ratio.

2) The number of sample units is correct, but several of the sample units have been damaged and are unusable.

For example, fifteen 1 lb bags of pasta have arrived for testing, but 5 of the bags are torn and unusable. In this case, the analyst should only sample from the 10 intact bags. The contents of each intact bag should be mixed to ensure homogeneity before the analytical units are withdrawn. Since the analyst needs one 375 g composite, ten 37.5 g analytical units, from the remaining 10 intact bags, should be used to form the composite. The composite should be combined with its preenrichment medium at a 1:9 sample-to-broth ratio (375 g sample/3375 ml preenrichment) as directed in Chapter 5 or the OMA.

3) The number of sample units is incorrect, but the total weight of the sample unit(s) is greater than what would be necessary to perform the sample analysis.

For example, a single 10 lb wheel of cheese has arrived for testing. Since cheese is a Category II food, thirty 25 g analytical units must be analyzed. These analytical units should be taken randomly from a wide variety of locations around the wheel. If *Salmonella* is present in a food, then the odds of detecting it will be enhanced if two 375 g composites are analyzed rather than a single 25 g analytical unit, as would be the case if the analyst were to treat the entire wheel as a single sample.

4) There is less sample available than is necessary to form the required number of composites.

For example, an 8 oz (226.8 g) bag of almonds has arrived for testing. Almonds are

a Category II food. Category II foods require thirty 25 g analytical units (750g), so it is impossible to analyze the amount of almonds required by the sampling plan. In this case, the analyst should analyze all of the almonds at a 1:9 sample-to-broth ratio (226.8g sample/2041 ml preenrichment medium).

If, in the above example, the total weight of the almonds had been less than 2 composites (750 g), but more than 1 composite, then the analyst should analyze both a whole and a partial composite. The analytical units comprising these composites should be taken randomly from a wide variety of locations in the lot of almonds. Both composites, should be preenriched at a 1:9 sample-to-broth ratio.

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

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

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

b. Sample analysis

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

Keep the remainder of the sample unit in a sterile container for compliance requirements as per section 702(b) of the Federal Food, Drug, and Cosmetic Act as amended through

February, 1993. Refrigerate perishable samples and samples supporting microbial growth. An analytical control is required for each sample tested. The sampled lot is acceptable only if analyses of all composite units are negative for *Salmonella*. If one or more composite units are positive for *Salmonella*, the lot is rejected, provided that the analytical control is negative for *Salmonella*. A lot will not be resampled unless the environmental control for *Salmonella* is positive. For all samples positive for *Salmonella*, determine the somatic group. See Chapter 5 for information on further handling of these cultures. Recommendations for regulatory action may be based on the identification of the *Salmonella* somatic group and will not require definitive serotyping before initiation of regulatory action.

c. Imports.

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

d. Classification of food products for sampling purposes

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

Industry Product Code

2	Milled grain products not cooked before consumption (bran and wheat germ)
3	Bread, rolls, buns, sugared breads, crackers, custard- and cream-filled sweet goods, and icings
5	Breakfast cereals and other ready-to-eat breakfast foods
7	Pretzels, chips, and other snack foods
9	Butter and butter products, pasteurized milk and raw fluid milk and fluid milk products for direct consumption, pasteurized and unpasteurized concentrated liquid milk products for direct consumption, dried milk and dried milk products for direct consumption, casein, sodium caseinate, and whey
12	Cheese and cheese products
13	Ice cream from pasteurized milk and related products that have been pasteurized, raw ice cream mix and related unpasteurized products for direct consumption
14	Pasteurized and unpasteurized imitation dairy products for direct consumption
15	Pasteurized eggs and egg products from pasteurized eggs, unpasteurized eggs and egg products from unpasteurized eggs for consumption without further cooking
16	Canned and cured fish, vertebrates, and other fish products; fresh and frozen raw

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

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

Industry Product Code

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

26	Vegetable oils, oil stock, and vegetable shortening
35	Dry dessert mixes, pudding mixes, and rennet products that are cooked before consumption

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

    a. Sample collection

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

    b. Sample analysis.

    Analyze samples as indicated in current compliance programs.

B. Equipment and materials

1. Mechanical blender. Several types are available. Use blender that has several operating speeds or rheostat. The term "high-speed blender" designates mixer with 4 canted, sharp-edge, stainless steel blades rotating at bottom of 4 lobe jar at 10,000-12,000 rpm or with equivalent shearing action. Suspended solids are reduced to fine pulp by action of blades and by lobular container, which swirls suspended solids into blades. Waring blender, or equivalent, meets these requirements.
2. Sterile glass or metal high-speed blender jar, 1000 ml, with cover, resistant to autoclaving for 60 min at 121°C
3. Balance, with weights; 2000 g capacity, sensitivity of 0.1 g
4. Sterile beakers, 250 ml, low-form, covered with aluminum foil
5. Sterile graduated pipets, 1.0 and 10.0 ml
6. Butterfield's phosphate-buffered dilution water (RII), 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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Center for Food Safety & Applied Nutrition

Bacteriological Analytical Manual *Online*

October 2001

Chapter 5

Salmonella

Authors

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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 ± 2 °C
7. Refrigerated incubator or laboratory refrigerator, 4 ± 2°C
8. Water bath, 49 ± 1°C
9. Water bath, circulating, thermostatically-controlled, 43 ± 0.2°C
10. Water bath, circulating, thermostatically-controlled,42 ± 0.2°C
11. Sterile spoons or other appropriate instruments for transferring food samples
12. Sterile culture dishes, 15 x 100 mm, glass or plastic
13. Sterile pipets, 1 ml, with 0.01 ml graduations; 5 and 10 ml, with 0.1 ml graduations
14. Inoculating needle and inoculating loop (about 3 mm id or 10 μl), nichrome, platinum-iridium, chromel wire, or sterile plastic
15. Sterile test or culture tubes, 16 x 150 mm and 20 x 150 mm; serological tubes, 10 x 75 mm or 13 x 100 mm
16. Test or culture tube racks
17. Vortex mixer
18. Sterile shears, large scissors, scalpel, and forceps
19. Lamp (for observing serological reactions)
20. Fisher or Bunsen burner
21. pH test paper (pH range 6-8) with maximum graduations of 0.4 pH units per color change
22. pH meter
23. Plastic bags, 28 x 37 cm, sterile, with resealable tape. (Items 23-25 are needed in the analysis of frog legs and rabbit carcasses.)
24. Plastic beakers, 4 liter, autoclavable, for holding plastic bag during shaking and incubation
25. Mechanical shaker, any model that can be adjusted to give 100 excursions/min with a 4 cm (1-1/2 inches) stroke, such as the Eberbach shaker with additional 33 and 48 cm clamp bars.

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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. Bromcresol purple dye solution, 0.2% (R9)
44. Methyl red indicator (R44)
45. Sterile distilled water
46. Tergitol Anionic 7 (R78)
47. Triton X-100 (R86)
48. Physiological saline solution, 0.85% (sterile) (R63)
49. Formalinized physiological saline solution (R27)
50. *Salmonella* polyvalent somatic (O) antiserum
51. *Salmonella* polyvalent flagellar (H) antiserum
52. *Salmonella* somatic group (O) antisera: A, B, C_1, C_2, C_3, D_1, D_2, E_1, E_2, E_3, E_4, F, G, H, I, Vi, and other groups, as appropriate
53. *Salmonella*Spicer-Edwards flagellar (H) antisera

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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 Cl^- solution containing 0.1% sodium dodecyl sulfate (SDS) for 30 min. Prepare the 200 ppm Cl^-/0.1% SDS solution by adding 8 ml commercial bleach (5.25% sodium hypochlorite) to 992 ml distilled water containing 1 g SDS. This disinfectant should be prepared immediately before analysis. Crack eggs aseptically into a sterile container and thoroughly mix egg yolks and egg whites with a sterile spoon or other sterile instrument. Aseptically weigh 25 g into sterile 500 ml Erlenmeyer flask or other appropriate container. Add 225 ml trypticase (tryptic) soy broth (TSB) supplemented with ferrous sulfate (35 mg ferrous sulfate added to 1000 ml TSB) and mix well by swirling. Let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

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

   c. **Hard-boiled eggs (chicken, duck, and others)**. If the egg shells are still intact, disinfect the shells as described above and aseptically separate the shells from the eggs. Pulverize

the eggs (egg yolk solids and egg white solids) aseptically and weigh 25 g into a sterile 500 ml Erlenmeyer flask or other appropriate container. Add 225 ml TSB (without ferrous sulfate) and mix well by swirling. Continue as described above.

3. **Nonfat dry milk**

   a. **Instant**. Aseptically weigh 25 g sample into sterile beaker (250 ml) or other appropriate container. Using sterile glass or paper funnel (made with tape to withstand autoclaving), pour 25 g analytical unit gently and slowly over surface of 225 ml brilliant green water contained in sterile 500 ml Erlenmeyer flask or other appropriate container. Alternatively, 25 g analytical units may be composited and poured over the surface of proportionately larger volumes of brilliant green water. Prepare brilliant green water by adding 2 ml 1% brilliant green dye solution per 1000 ml sterile distilled water. Let container stand undisturbed for 60 ± 5 min. Incubate loosely capped container, without mixing or pH adjustment, for 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

   b. **Non-Instant**. Examine as described for instant nonfat dry milk, except that the 25 g analytical units may not be composited.

4. **Dry whole milk**. Examine as described for instant nonfat dry milk, except that the 25 g analytical units may not be composited.

5. **Casein** UPDATED

   a. **Lactic casein and rennet casein.** Aseptically weigh 25 g sample into sterile beaker (250 ml) or other appropriate container. Using sterile glass or paper funnel (made with tape to withstand autoclaving), pour 25 g analytical unit gently and slowly over the surface of 225 ml lactose broth contained in sterile 500 ml Erlenmeyer flask or other appropriate container. Analytical units (25 g) may be composited. Let container stand undisturbed 60 ± 5 min. Incubate loosely capped container, without mixing or pH adjustment, for 24 ± 2 h at 35 °C. Continue as in D, 1-11, below.

   b. **Sodium caseinate**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile lactose broth and mix well. Analytical units may be composited. Let stand 60 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Loosen jar about 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

6. **Soy flour**. UPDATED Examine as described for lactic casein and rennet casein, except 25 g analytical units (25 g) may not be composited.

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

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

8. **Dried yeast (active and inactive yeast)**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile trypticase soy broth. Mix well to form smooth suspension. Let stand 60 ± 5 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2, mixing well before determining final pH. Loosen jar cap 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

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

10. **Spices**

    a. **Black pepper, white pepper, celery seed or flakes, chili powder, cumin, paprika, parsley flakes, rosemary, sesame seed, thyme, and vegetable flakes**.

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

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

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

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

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

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

12. **Coconut**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile lactose broth, shake well, and let stand 60 ± 5 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Add up to 2.25 ml steamed (15 min) Tergitol Anionic 7 and mix well. Alternatively, use steamed (15 min) Triton X-100. Limit use of these surfactants to minimum quantity needed to initiate foaming. For Triton X-100 this quantity may be as little as 2 or 3 drops. Loosen jar cap about l/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

13. **Food dyes and food coloring substances**. For dyes with pH 6.0 or above (10% aqueous suspension), use method described for dried whole eggs (C-l, above). For laked dyes or dyes with pH below 6.0, aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml tetrathionate broth without brilliant green dye. Mix well and let stand 60 ± 5 min at room temperature with jar securely capped. Using pH meter, adjust pH to 6.8 ± 0.2. Add 2.25 ml 0.1% brilliant green dye solution and mix thoroughly by swirling. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 3-11, below.

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

15. **Meats, meat substitutes, meat by-products, animal substances, glandular products, and meals (fish, meat, bone)**. Aseptically weigh 25 g sample into sterile blending container. Add 225 ml sterile lactose broth and blend 2 min. Aseptically transfer homogenized mixture to sterile wide-mouth, screw-cap jar (500 ml) or other appropriate container and let stand 60 ± 5 min at room temperature with jar securely capped. If mixture is powder or is ground or comminuted, blending may be omitted. For samples that do not require blending, add lactose broth and mix thoroughly; let stand for 60 ± 5 min at room temperature with jar securely capped.

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

16. **Frog legs**. (This method is used for all domestic and imported frog legs.) Place 15 pairs of frog

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legs into sterile plastic bag and cover with sterile lactose broth (**see** A, 23-25, above). If single legs are estimated to average 25 g or more, examine only one leg of each of 15 pairs. Place bag in large plastic beaker or other suitable container and shake 15 min on mechanical shaker set for 100 excursions/min with stroke of 4 cm (1-1/2 inches). Pour off lactose broth from bag into another sterile plastic bag and add more lactose broth to total volume of 3500 ml. Mix well and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Place plastic bag containing the lactose broth into plastic beaker or other suitable container. Incubate 24 ± 2 h at 35°C. Continue examination as in D, 1-11, below.

17. **Rabbit carcasses.** (This method is used for all domestic and imported rabbit carcasses.) Place each of 3 rabbit carcasses into sterile plastic bag and cover with sterile lactose broth (see A, 23-25, above). Place bag in large plastic beaker or other suitable container and shake 15 min on mechanical shaker set for 100 excursions/min with stroke of 4 cm (1-1/2 inches). Composite lactose broth rinsings by pouring into another sterile container and add more lactose broth to total volume of 3500 ml. Mix well and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Incubate 24 ± 2 h at 35°C. Continue examination as in D, 1-11, below.

18. **Guar gum**. Aseptically weigh 25 g sample into sterile beaker (250 ml) or other appropriate container. Prepare a 1.0% cellulase solution (add 1 g cellulase to 99 ml sterile distilled water). Dispense into 150 ml bottles. (Cellulase solution may be stored at 2-5°C for up to 2 weeks). Add 225 ml sterile lactose broth and 2.25 ml sterile 1% cellulase solution to sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. While vigorously stirring the cellulase/lactose broth with magnetic stirrer, pour 25 g analytical unit quickly through sterile glass funnel into the cellulase/lactose broth. Cap jar securely and let stand 60 ± 5 min at room temperature. Incubate loosely capped container without pH adjustment, for 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

19. **Orange juice (pasteurized and unpasteurized), apple cider (pasteurized and unpasteurized), and apple juice (pasteurized)** Aseptically add 25 ml sample to 225 ml Universal preenrichment broth in a sterile, wide mouth, screw-capped jar (500 ml) or other appropriate container. Swirl the flask contents thoroughly. Cap jar securely and let stand 60 ± 5 min at room temperature. Do not adjust pH. Incubate loosely capped container for 24 ± 2 h at 35°C. Continue as in D, 1-11, below (treat as a low microbial load food).

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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 ± 0.2°C (circulating, thermostatically-controlled, water bath). Incubate TT broth 24 ± 2 h at 43 ± 0.2°C (circulating, thermostatically-controlled, water bath).

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

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

3. Mix (vortex, if tube) and streak 3 mm loopful (10 µl) incubated TT broth on bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar. **Prepare BS plates the day before streaking and store in dark at room temperature until streaked.**

4. Repeat with 3 mm loopful (10 µl) of RV medium (for samples of high and low microbial load foods) and of SC broth (for guar gum).

5. Refer to 994.04 in *Official Methods of Analysis* (1) for option of refrigerating incubated sample preenrichments and incubated sample selective enrichments (SC and TT broths only) of low moisture foods. This option allows sample analyses to be initiated as late as Thursday while still avoiding weekend work.

6. Incubate plates 24 ± 2 h at 35°C.

7. Examine plates for presence of colonies that may be *Salmonella*.

   TYPICAL *Salmonella* COLONY MORPHOLOGY

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

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

   b. **Xylose lysine desoxycholate (XLD) agar**. Pink colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.

   c. **Bismuth sulfite (BS) agar**. Brown, gray, or black colonies; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation, producing the so-called halo effect.

   If typical colonies are present on the BS agar after 24 ± 2 h incubation, then pick 2 or more colonies. Irrespective of whether or not BS agar plates are picked at 24 ± 2 h, reincubate BS agar

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

ATYPICAL *Salmonella* COLONY MORPHOLOGY

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

a. **HE and XLD agars**. Atypically a few *Salmonella* cultures produce yellow colonies with or without black centers on HE and XLD agars. In the absence of typical *Salmonella* colonies on HE or XLD agars after 24 ± 2 h incubation, then pick 2 or more atypical *Salmonella* colonies.

b. **BS agar.** Atypically some strains produce green colonies with little or no darkening of the surrounding medium. If typical or suspicious colonies are not present on BS agar after 24 ± 2 h, then do not pick any colonies but reincubate an additional 24 ± 2 h. If typical or suspicious colonies are not present after 48 ± 2 h incubation, then pick 2 or more atypical colonies.

SUGGESTED CONTROL CULTURES

In addition to the positive control cultures (typical *Salmonella*), 3 additional *Salmonella* cultures are recommended to assist in the selection of atypical *Salmonella* colony morphology on selective agars. These cultures are a lactose-positive, H_2S-positive *S. diarizonae* (ATCC 12325) and a lactose-negative, H_2S-negative *S. abortus equi* (ATCC 9842); **OR** a lactose-positive, H_2S-negative *S. diarizonae* (ATCC 29934). These cultures may be obtained from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209.

8. Lightly touch the very center of the colony to be picked with sterile inoculating needle and inoculate TSI slant by streaking slant and stabbing butt. Without flaming, inoculate LIA slant by stabbing butt twice and then streaking slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm). Store picked selective agar plates at 5-8°C.

9. Incubate TSI and LIA slants at 35°C for 24 ± 2 h. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H_2S production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar) in TSI. In LIA, *Salmonella* typically produces alkaline (purple) reaction in butt of tube. Consider only distinct yellow in butt of tube as acidic (negative) reaction. Do not eliminate cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce H_2S in LIA. Some non- *Salmonella* cultures produce a brick-red reaction in LIA slants.

10. All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates and submitted for biochemical and serological tests. Cultures that

give an acid butt in LIA and an alkaline slant and acid butt in TSI should also be considered potential *Salmonella* isolates and should be submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella* . Test retained, presumed-positive TSI cultures as directed in D-11, below, to determine if they are *Salmonella* species, including *S. arizonae*. If TSI cultures fail to give typical reactions for *Salmonella* (alkaline slant and acid butt) pick additional suspicious colonies from selective medium plate not giving presumed-positive culture and inoculate TSI and LIA slants as described in D-8, above.

11. Apply biochemical and serological identification tests to:

    a. Three presumptive TSI cultures recovered from set of plates streaked from RV medium (or SC broth for guar gum), if present, and 3 presumptive TSI agar cultures recovered from plates streaked from TT broth, if present.

    b. If 3 presumptive-positive TSI cultures are not isolated from one set of agar plates, test other presumptive-positive TSI agar cultures, if isolated, by bioche mical 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 ± 0.5°C water bath. Discard all cultures giving positive test. Retain for further study all cultures that give negative test (no change in color of medium).

3. Serological polyvalent flagellar (H) test

   a. Perform the polyvalent flagellar (H) test at this point, or later, as described in E-5, below. Inoculate growth from each urease-negative TSI agar slant into either 1) BHI broth and incubate 4-6 h at 35°C until visible growth occurs (to test on same day); or 2) trypticase soy-tryptose broth and incubate 24 ± 2 h at 35°C (to test on following day). Add 2.5 ml formalinized physiological saline solution to 5 ml of either broth culture.

   b. Select 2 formalinized broth cultures and test with *Salmonella* polyvalent flagellar (H) antisera. Place 0.5 ml of appropriately diluted *Salmonella* polyvalent flagellar (H) antiserum in 10 x 75 mm or 13 x 100 mm serological test tube. Add 0.5 ml antigen to be tested. Prepare saline control by mixing 0.5 ml formalinized physiological saline solution with 0.5 ml formalinized antigen. Incubate mixtures in 48-50°C water bath. Observe at 15 min intervals and read final results in 1 h.

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

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

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

4. **Spicer-Edwards serological test**. Use this test as an alternative to the polyvalent flagellar (H) test. It may also be used with cultures giving nonspecific agglutination in polyvalent flagellar (H) test. Perform Spicer-Edwards flagellar (H) antisera test as described in E, 3b, above. Perform additional biochemical tests (E, 5a-c, below) on cultures giving positive flagellar test results. If both formalinized broth cultures are negative, perform serological tests on 4 additional broth cultures (E, 3a, above). If possible, obtain 2 positive cultures for additional biochemical testing E, 5a-c, below). If all urease-negative TSI cultures from sample give negative serological flagellar (H) test results, perform additional biochemical tests E, 5a-c, below).

5. Testing of urease-negative cultures

   a. **Lysine decarboxylase broth**. If LIA test was satisfactory, it need not be repeated. Use lysine decarboxylase broth for final determination of lysine decarboxylase if culture gives doubtful LIA reaction. Inoculate broth with small amount of growth from TSI slant suspicious for *Salmonella* . Replace cap tightly and incubate 48 ± 2 h at 35°C but examine at 24 h intervals. *Salmonella* species cause alkaline reaction indicated by purple color throughout medium. Negative test is indicated by yellow color throughout medium. If medium appears discolored (neither purple nor yellow) add a few drops of 0.2% bromcresol purple dye and re-read tube reactions.

   b. **Phenol red dulcitol broth or purple broth base with 0.5% dulcitol**. Inoculate broth with

small amount of growth from TSI culture. Replace cap loosely and incubate 48 ± 2 h at 35°C, but examine after 24 h. Most *Salmonella* species give positive test, indicated by gas formation in inner fermentation vial and acid pH (yellow) of medium. Production of acid should be interpreted as a positive reaction. Negative test is indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromcresol purple as indicator) color throughout medium.

c. Tryptone (or tryptophane) broth. Inoculate broth with small growth from TSI agar culture. Incubate 24 ± 2 h at 35°C and proceed as follows:

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

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

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

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

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

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 *Salmonella*

Test or substrate	Result		*Salmonella* species reaction[(a)]
	Positive	Negative	
1. Glucose (TSI)	yellow butt	red butt	+
2. Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3. H_2S (TSI and LIA)	blackening	no blackening	+
4. Urease	purple-red color	no color change	-

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

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

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

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

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

Test or substrate	Results
1. Urease	positive (purple-red color)
2. Indole test and Polyvalent flagellar (H) test;	positive (violet color at surface) negative (no agglutination)
or Indole test and Spicer-Edwards flagellar test	positive (violet color at surface) negative (no agglutination)
3. Lysine decarboxylase and KCN broth	negative (yellow color) positive (growth)
4. Phenol red lactose broth	positive (yellow color and/or gas)[a], [b]

5. Phenol red sucrose broth	positive (yellow color and/or gas)[b]
6. KCN broth, Voges-Proskauer test, and Methyl red test	positive (growth) positive (pink-to-red color) negative (diffuse yellow color)

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

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

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:

   a. Report as *Salmonella* those cultures classified as presumptive *Salmonella* with commercial biochemical kits when the culture demonstrates positive *Salmonella* somatic (O) test and positive *Salmonella* (H) test.

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

   c. For cultures that do not conform to a or b, classify according to additional tests specified in E, 2-7, above, or additional tests as specified by Ewing (2), or send to reference typing laboratory for definitive serotyping and identification.

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

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U.S. Department of Health and Human Services
U. S. Food and Drug Administration
Center for **F**ood **S**afety & **A**pplied **N**utrition

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Chapter 10

Detection and Enumeration of *Listeria monocytogenes* in Foods

Author

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The genus *Listeria* contains 6 species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi* (Table 1). *L. grayi* (29, 34) and *L. ivanovii* (12, 28) each contain two subspecies, which do not need to be specified in this analysis. A recent taxonomic review of the genus by Rocourt (35) updates the previous reviews (11, 37). *L. ivanovii* and *L. monocytogenes*are pathogenic for mice and other animals. However, only *L. monocytogenes* is commonly associated with human listeriosis. Listeriosis associated infection by *L. ivanovii*, and even *L. seeligeri*, is extremely rare in humans. The universal occurrence of *L. monocytogenes* in food (36) and the risk of contracting food-borne *L. monocytogenes* listeriosis (41b, http://www.foodsafety.gov/~dms/lmrisk.html) have been thoroughly reviewed recently. This chapter describes the detection and enumeration of *L. monocytogenes* in foods. Detection of this pathogen in the food processing environment, such as on food contact surfaces and equipment, is described elsewhere (41a).

The preferred standard methodology, and permitted alternative rapid methodologies, to be used for detection and isolation of *Listeria monocytogenes* are as follows. Presumptive contaminated food lots are sampled. Generally, sub-samples are composited if required by FDA field laboratory instructions. Analytical portions (25 g) are pre-enriched for *Listeria* species at 30° C for 4 h in buffered *Listeria* enrichment broth (BLEB), equivalent (26) to AOAC/IDF dairy products enrichment broth (4, 40) base containing sodium pyruvate (43). At the fourth hour of the incubation, the selective agents (acriflavin, 10 mg/L (4, 40); sodium nalidixate, 40 mg/L; optional antifungal, e.g. cycloheximide 50 mg/L) are added. Incubation for selective enrichment is continued at 30° C for a total of 48 h. The enrichment culture is streaked at 24 and 48 h on one of the prescribed differential selective-agars in order to isolate *Listeria* species.

Alternatively, prescribed rapid detection kits with their respective enrichment media may be conditionally used to screen for presence of *Listeria* contaminants. Putative listeria isolates on selective agars from standard or screen positive enrichments are purified on non-selective agar and confirmed by conventional

identification tests or by a battery of such tests in kit form. Isolates may be rapidly confirmed as *L. monocytogenes* (or not) by using specific test kits. Subtyping of *L. monocytogenes* isolates is optional except for FDA isolates, which have to be typed serologically, and by pulsed-field gel electrophoresis (PFGE) and by ribotyping. Non-obligatory pathogenicity testing of *L. monocytogenes* isolates is described.

Enumeration of *L. monocytogenes* in positive samples is performed on reserve sample by colony count on *L. monocytogenes* differential selective agar in conjunction with MPN enumeration using selective enrichment in BLEB with subsequent plating on ALOA or BCM differential selective agar.

The major revisions to the *Listeria* methodology follow.

1. Certain prescribed rapid detection kits and their enrichments are now authorized screening alternatives to the standard selective enrichment.

2. It is now necessary to use only one instead of two of the several prescribed selective isolation agars (Oxford agar, PALCAM, LPM plus esculin and ferric iron, MOX). Oxford agar is still the preferred standard selective isolation medium. MOX has been added to the list of prescribed selective agars and LPM without added esculin and ferric iron has been removed. Trial use of the new chromogenic differential selective agars, like BCM, ALOA, CHROMagar Listeria and Rapid' L. mono, is encouraged as long as it is in parallel with one of the prescribed selective agars. The new agar media differentiate *L. monocytogenes*/*L. ivanovii* colonies from those of other *Listeria* spp. and will greatly facilitating picking of *L. monocytogenes* colonies when colonies of more than one species are present on a plate.

3. The Henry illumination technique is de-emphasized because only differential selective isolation agars are prescribed.

4. The current enrichment medium, which resembles the step-1 enrichment of the internationally harmonized method proposed by Asperger *et al*. (10a), is basically unchanged. However, pimaricin (natamycin), a much less toxic compound than cycloheximide, is introduced as the alternative antifungal compound in the *Listeria* enrichment medium,

   http://www.cfsan.fda.gov/~ebam/cyclohex.html.

5. If *L. monocytogenes* is detected in a food sample, enumeration of the level of contamination in the food is required.

Table 1. Differentiation of *Listeria* species

		Acid produced from			
Species	**β-Hemolysis[a]**	**Mannitol**	**Rhamnose**	**Xylose**	**Virulence[b]**
L. monocytogenes	+	-	+	-	+
***L. ivanovii*[c]**	+	-	-	+	+
L. innocua	-	-	V[d]	-	-
L. welshimeri	-	-		+	-

			V[d]		
L. seeligeri	+	-	-	+	-
L. grayi[e]	-	+	V[d]	-	-

[a] Sheep blood agar stab.

[b] Mouse test.

[c] Ribose fermenting strains are classified as *L. ivanovii* subsp. *ivanovii* and ribose non-fermenters as *L. ivanovii* subsp. *londiniensis*.

[d] V, variable biotypes

[e] Includes two subspecies - *L. grayi* subsp. *murrayi* reduces nitrate *L. grayi* subsp. *grayi* does not reduce nitrate.

A. Equipment and materials

1. Balance for weighing sample to 0.1 g
2. Cover slip, glass
3. Erlenmeyer flask, 500 ml
4. Fermentation tubes (Durham)
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. Pipettes, 25, 10, and 1 ml
15. Tubes, 16 x 125 mm or other appropriate sizes, screw-cap
16. Blender motor and jars or Stomacher and bags
17. Tuberculin syringe, sterile, disposable

B. Media and Reagents

1. Acetic Acid, 5 N
2. Acriflavine monohydrochloride
3. Agar (Difco Laboratories, Detroit, MI)
4. N-(1-naphthyl) ethylene diamine (R48)
5. α-Naphthol reagent (R48)
6. Blood agar base No. 2 (Unipath)
7. Cycloheximide
8. Natamycin (pimaricin)

9. Sheep blood, defibrinated
10. Ethanol, absolute
11. Fluorescent antibody (FA) buffer (Difco)
12. Glycine anhydride
13. Gram stain kit
14. Hydrogen peroxide solution, 3% for catalase test (R12)
15. KOH 40% solution (R65)
16. *Listeria*-typing sera set (Difco)
17. Lithium chloride-phenylethanol-moxalactam (LPM) agar (M81) with added esculin and iron (M82)
18. Nalidixic acid (sodium salt)
19. Nitrate reduction medium (M108) and nitrate detection reagents (R48)
20. Nutrient broth (M114)
21. Physiological saline solution, 0.85% (R63)
22. Purple carbohydrate fermentation broth base (M130), containing 0.5% solutions of dextrose, esculin, maltose, rhamnose, mannitol, and xylose
23. SIM medium (Becton-Dickinson Microbiology Systems, M137) or motility test medium (MTM, Difco) (M103)
24. Sulfanilic acid reagent (R48)
25. Trypticase soy agar with 0.6% yeast extract (TSAye) (M153)
26. Trypticase soy broth with 0.6% yeast extract (TSBye) (M157)
27. Oxford medium (OXA) (M118)
28. Buffered *Listeria* Enrichment Broth (BLEB) (M52)
29. PALCAM agar (M118a)
30. Carageenan (Sigma type II)
31. BCM agar (M17a)
32. MOX agar (M103a)
33. ALOA agar (M10a)
34. Chromogenic *Listeria* Agar (M40b)
35. Rapid L'mono (M131a)
36. CHROMagar *Listeria* (M40a)
37. Tryptose broth and agar (Difco) (M167)

Note: Alternative companies may be used when the products are equivalent.

C. Sampling and Enrichment Procedures

1. **Sample treatment.** Sample 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 if other conditions permit. However, if the sample is already frozen, it should not be thawed until analysis.

2. **Composited samples**. Generally, composited samples are prepared as follows. A food lot sample is collected consisting of 10 sub-samples (liquid, cream or solid food) and 50-g or ml portions of each sub-sample are used to make two composite samples (250 g each). Take care to make sub-samples representative of a food's outer surface as well as its interior. For the first composite 5 x 50-g portions from 5 sub-samples are pooled and blended or stomached in 250 ml buffered *Listeria* enrichment broth base containing sodium pyruvate without selective agents (BLEB,

M52). The second composite is made from the remaining 5 sub-samples in the same way. Both blended composites contain 250-g food portions and 250 ml basal BLEB.

Normally 50 g of each composite blend (equivalent to 25g food plus 25 ml basal BLEB) is mixed with a 200 ml amount of basal BLEB. Since there are two composites, the end result is two 25-g analytical portions each contained in 225 ml amounts of basal BLEB. Thus, for each sub-sample a total of 50 g of composited food is analyzed. An aliquot (50 ml) of the composite blend should be retained, preferably at 5° C and not below 0° C, for possible pathogen enumeration.

3. **Non-composited samples**. If composite samples are not required, single 25-g analytical portions of food are simply blended or stomached in 225 ml of basal BLEB and pre-enriched/enriched as described later. A 25-g sample should be retained for possible pathogen enumeration. Store it at 5° C if it is not frozen or, if frozen, in a non-defrosting freezer.

4. **Pre-enrichment and enrichment.** Incubate for 4 h at 30° C, add the selective agents and continue incubating for a total time of 48 h at 30° C. If cycloheximide is unavailable, the preferred substitute is pimaricin (natamycin) at 25 mg/L (27). Natamycin is much safer to use than cycloheximide. Another possibility, if the matrices of interest (e.g. pasteurized milk and cream, yogurt, and precooked frozen seafood) are low in yeast and mold, is to do without an anti-fungal agent. This is not advisable for mold-ripened cheeses, smoked or dried seafood or fresh produce.

5. **Enrichment with enumeration**. Surveillance enumeration of *Listeria monocytogenes* levels in contaminated food is now required. Detection may be done first and if contamination is detected, a reserve sample portion can be enumerated. This is probably the preferable method as, generally, only a few percent of samples can be expected to be positive and then most often at a level of only about 1 cfu/25g. However, the option of combining regulatory detection and enumeration is provided in Enumeration.

6. **Prescribed Alternative Methods for Screening Enriched Samples.** For the prescribed methods kits listed in Table 2, follow the manufacturers' package insert instructions making certain they have not deviated from the approved versions of the AOAC INTERNATIONAL Official Methods Manual protocols (Table 2). The kits are only approved for the specified food matrices, listed in Chapter 10, Supplement 1A, which vary from kit to kit. For other food matrices listed in Chapter 10, Supplement 1B, in-house validation is necessary. The easiest way to validate is to streak all kit-associated 48-h enrichments, which give kit-negative results, on a plate of one of the prescribed standard method esculin agars. Even with the validated matrices the 48-h streaking of kit-negative enrichments to esculin selective agars is recommended for five or so different lots of a given food matrix until the analyst is confident of the kit's performance with that particular matrix. This is because conventional validation of matrices does not take into account qualitative and quantitative competitive microflora variation among different lots of a given food matrix. Strong competition will impact rapid kit methods more than cultural methods. Kit thresholds of detection (>10^4 cfu/ml of enrichment culture) are higher than that of the culture plate method (about 10^2 cfu/ml of enrichment culture) so that, with very competitive microflora, kits could give false negative results. Alternatively, the limit of detection (50% endpoint) by quantitative spiking with a single strain of *L. monocytogenes* should be determined. It should be

not more than 3 cfu per 25-g analytical portion. A value greater than this would only be acceptable if the conventional culture method's performance level is comparable. For all food matrices, a kit positive result must be supported by a culture of the *L. monocytogenes* isolate.

Table 2. *Listeria* Genus Detection Test Kits

A. **Prescribed for Regulatory Screening**

1. AOAC Official Method 993.09. 2000. *Listeria* in dairy products, seafoods, and meats. Colorimetric deoxyribonucleic acid hybridization method (GENE-TRAK *Listeria* Assay). (3, 14)

2. AOAC Official Method 994.03. 2000. *Listeria monocytogenes* in dairy products, seafoods, and meats. Colorimetric monoclonal enzyme-linked immunosorbent assay method (*Listeria*). (5, 15, 32)

3. AOAC Official Method 995.22. 2000. *Listeria* in foods. Colorimetric polyclonal enzyme immunoassay screening method (TECRA *Listeria* Visual Immunoassay [TLVIA]). (6, 30)

4. AOAC Official Method 996.14. 2000. Assurance (Polyclonal Enzyme Immunoassay Method). (7, 21)

5. AOAC Official Method 997.03. 2000. Visual Immunoprecipitate Assay (VIP). (8, 22)

6. AOAC Official Method 999.06. 2000. Enzyme Linked Immunofluorescent Assay (ELFA) VIDAS LIS Assay Screening Method. (9, 23)

B. **Not Currently Prescribed for Regulatory Screening**

1. CLEARVIEW *Listeria* RAPID TEST. (Oxoid Inc., Ogdensburg, NY).

2. AOAC Accredited Method. 2000. BAX *Listeria* test. (Qualicon, Inc., Wilmington, DE).

D. **Isolation procedure**

At 24 and 48 h, streak BLEB culture onto one of the following esculin-containing selective isolation agars: either OXA (16, M118) or PALCAM (42, M118a) or MOX (41, M103a) or LPM (31, M81) fortified with esculin and Fe^{3+} (M82). These esculin-containing media are listed in order of preferred use, subject to their availability. Incubate OXA, PALCAM or MOX plates at 35° C for 24-48 h and fortified LPM plates at 30° C for 24-48 h. It is strongly recommended that one of the *L. monocytogenes-L. ivanovii* differential selective agars, such as BCM (33, M117a), ALOA (M10a), *RapidL'mono* (M131a), or CHROMagar *Listeria* (M40a) be streaked at 48 h (optionally at 24 h, too) in addition to the chosen esculin-containing selective agar. This will reduce the problem of masking of *L. monocytogenes* by *L. innocua*. [Note: BCM has been collaboratively validated by FDA (26a). An ISO TC34 SC9 comparative validation showed that all the media (and a selective blood agar - LMBA, Sifin, Germany) inhibited *Listeria* competitors more or less equally well. ALOA was preferred only because

its formulation is public. Another differential selective medium, Chromogenic *Listeria* Agar (M40b) is due to be marketed in the future.]

Listeria colonies are black with a black halo on esculin-containing media. Certain other bacteria can form weakly brownish black colonies, but color development takes longer than 2 days. Transfer 5 or more typical colonies from OXA and PALCAM or modified LPM or MOX to Trypticase soy agar with yeast extract (TSAye), streaking for purity and typical isolated colonies. If BCM plates are streaked as recommended above and blue colonies are observed, they are presumptive *L. monocytogenes* colonies since *L. ivanovii* is not often reported in foods. *L. monocytogenes* and *L. ivanovii* colonies on ALOA are blue and have a zone of lipolysis around them. Purification on TSAye is a mandatory step in the conventional analysis because isolated colonies on selective agar media 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. Use of BCM and ALOA plates will help to reduce the number of colonies that need to be picked. *L. monocytogenes* and *L. ivanovii* can be distinguished using a commercial Confirmatory Medium (Biosynth International, Inc.) or by conventional rhamnose/xylose fermentation broths or agars. 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 wet-mount motility observations (see E-2, below). For the approved rapid methods (Table 2), use the selective isolation agar recommended by the manufacturer but, as noted above, auxiliary use of the new *L. monocytogenes-L.ivanovii* differential agars is also recommended.

E. Identification procedure

Identify purified isolates by the following classical tests (E, 1-11). 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. Observation with Henry oblique transmitted illumination can be helpful at this stage but is not mandatory (See ref. 25 for details).

2. Pick typical colony from culture plate incubated at 30°C or less and examine by wet mount, using 0.85% saline for suspending medium and oil immersion objective of 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 non-motile. *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- to 24-h cultures. All *Listeria* spp. are short, Gram-positive rods; however, with older cultures the Gram stain reaction can be variable and also cells may appear coccoidal. The cells have a tendency to palisade in thick-stained smears. This can lead 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. Commercial kits are available for isolate identification (see E-11)

6. Inoculate heavily (from TSAye colony) 5% sheep 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 24-48 h at 35° C. Attempt to stab as near to bottom of agar layer as possible, without actually touching bottom of agar layer and possibly fracturing the agar.

7. Examine blood agar plates containing culture stabs with bright light. *L.monocytogenes* and *L. seeligeri* produce a slightly cleared zone around the stab. *L. innocua* shows no zone of hemolysis, whereas *L. ivanovii* produces a well-defined clear zone around the stab. Do not try to differentiate species at this point, but note nature of hemolytic reaction. Resolve questionable reactions by the CAMP test. (Note: Hemolysis is more easily determined when the depth of the blood agar is thinner than the usual 5mm. Optionally, this may be achieved by use of a blood agar overlay (1-2 mm) technique).

8. Nitrate reduction test. This test is optional. Only *L. grayi* ssp. *murrayi* reduces nitrates. The test distinguishes *L. grayi* ssp. *murrayi* from *L. grayi* ssp. *grayi*. Use a 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 hold for 1 h. A developing red-violet color indicates that nitrate is still present and has not been reduced.

   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 provides the best defined umbrellas. Alternatively, observe the 30° C TSBye cultures, by phase contrast microscopy (x1000) for tumbling motility.

10. From TSBye culture, inoculate the following carbohydrates as 0.5% 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. Positively 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, MOX or LPM plus esculin/Fe^{3+} is unequivocal, the esculin test may be omitted.

11. Purified isolates may be rapidly identified by using commercial kits (additional tests may be needed to speciate completely): Vitek Automicrobic Gram Positive and Gram Negative Identification cards (bioMerieux, Hazelwood, MO) or API Listeria (bioMerieux, Marcy-l'Etoile, France) which does not require an additional CAMP test. The MICRO-ID™ kit (bioMerieux, Hazelwood, MO; 1, 24) permits speciation of *Listeria* isolates if their CAMP reactions are known. The Phenotype MicroArray for *Listeria* (BiOLOG, Hayward, CA) is another recently introduced kit.

12. Alternative rapid methods that are prescribed for identifying *Listeria* isolates as *L. monocytogenes* are listed in Table 3. Depending on the kit, isolates may be identified in pure culture or from OXA or the other selective isolation agars. Purified isolates identified as *Listeria monocytogenes* by these tests should be retained for regulatory reference.

Table 3. Test Kits Useful in Confirming *Listeria* Isolates as *Listeria monocytogenes* or not*

1. AccuProbe™ *Listeria monocytogenes* culture confirmation test (Gen-Probe, Inc, San Diego, CA; 10, 19).
2. GeneTrak *Listeria monocytogenes* test kit (GeneTrak, Framingham, MA; 19).
3. Probelia *Listeria monocytogenes* test kit (BioControl, Seattle, WA).
4. VIDAS *Listeria monocytogenes* test kit (bioMerieux).
5. Transia Plate *Listeria monocytogenes* (Diffchamb SA, Lyon, France)(38)
6. FDA, SRL application of Niederhauser *et al*. method for PCR detection and identification of *L. monocytogenes* (32a, 32b)

* These kits are in various stages of validation and when suitably validated can also be used to screen enrichments for *L. monocytogenes*. Presently, FDA only prescribes validated kits that screen for all *Listeria* species.

F. **The CAMP Test.**

The Christie-Atkins-Munch-Peterson (CAMP) test (Table 4 and Fig. 1) 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. *L. monocytogenes* and *L. seeligeri* hemolytic reactions are enhanced in the zone influenced by the *S. aureus* streak. The other species remain non-hemolytic. The *L. monocytogenes* reaction is often optimal at 24 h rather than 48 h. To obtain enough *R. equi* to provide a good streak of growth, incubate the slant culture 48 h rather than 24 h. Use of known control isolates of *Listeria* spp. on a separate sheep blood agar plate is recommended. Sheep blood agar plates should be as fresh as possible.

Table 4. CAMP test hemolytic enhancement of *Listeria* species

	Hemolysis enhancement with	
	***Staphylococcus aureus* (S)**	***Rhodococcus equi* (R)**
L. monocytogenes	+	-*
L. ivanovii	-	+

L. innocua	-	-
L. welshimeri	-	-
L. seeligeri	+	-

* Rare strains are S+ and R+. The R+ reaction is less pronounced than that of *L. ivanovii*.

CAMP test strains are available from culture collections, including the American Type Culture Collection (ATCC), Manassas, VA http://www.atcc.org

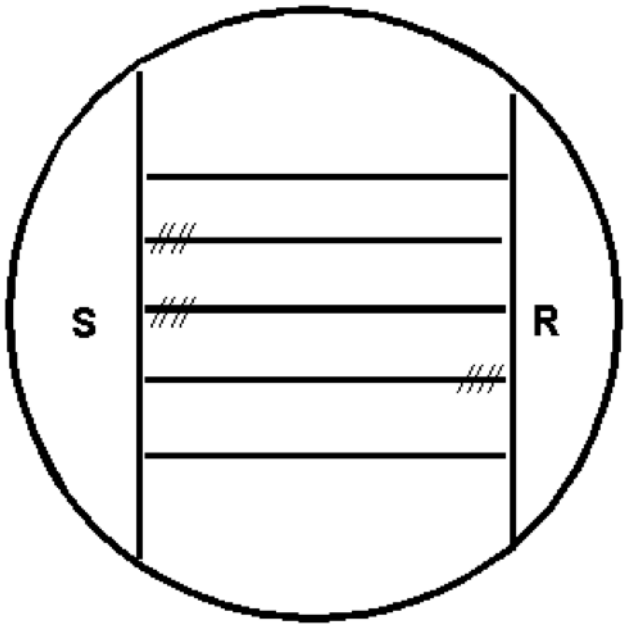


Figure 1. CAMP test for *Listeria monocytogenes*: Inoculation pattern of the sheep blood agar plate. Horizontal lines represent streak inoculations of 5 test strains. Vertical lines represent streak inoculations of *Staphylococcus aureus* (S) and *Rhodococcus equi* (R). Hatched lines indicate (diagrammatically only) the locations of hemolysis enhancement regions.

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 them. After 24- and 48-h incubation at 35° C, examine plates for hemolysis in the zone of influence of the vertical streaks. Figure 1 shows the arrangement of the culture streaks on a CAMP plate. Hemolysis of *L. monocytogenes* and *L. seeligeri* is enhanced near the *S. aureus* streak; *L. ivanovii* hemolysis is enhanced near the *R. equi* streak. Other species are non-hemolytic 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 non-hemolytic) from *L. welshimeri*. Isolates giving reactions typical for *L. monocytogenes* except for hemolysis should be CAMP-tested before they are characterizedas *L. innocua*. A factor easily prepared from *S. aureus* cultures can be used to enhance hemolysis by *L. monocytogenes* and *L. seeligeri* in sheep blood agar plates. Disks impregnated with

the β-lysin of *S. aureus* (REMEL, Lenexa, KS) can be used for the same purpose.

G. **Subtyping of *L. monocytogenes* isolates (Required)**

FDA isolates should be typed serologically and genetically.

1. **Serological typing**. Use commercial sera to characterize isolates as type 1, type 4 or not type 1 or 4 (types 3, 5, 6 etc.).

Table 5. Serotypes of *Listeria* species (37)

Listeria species	Serotypes
L. monocytogenes	1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b 4c, 4d, 4e, "7"
L. ivanovii	5
L. innocua	4ab, 6a, 6b, Un[a]
L. welshimeri	6a, 6b
L. seeligeri	1/2b, 4c, 4d, 6b, Un

[a] Un, undefined

Table 5 exhibits the serological relationships of *Listeria* spp. Most *L. monocytogenes* isolates obtained from patients and the environment are type 1 or 4. More than 90% of *L. monocytogenes* isolates 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*.

Serology is useful when epidemiological considerations are crucial. Use a TSBye culture to inoculate Tryptose broth. Incubate for 24 h at 35° C, at which temperature flagella (H) antigen expression is reduced. Transfer to Tryptose agar slants and incubate for 24 h at 35° C. Wash both slants in a total of 3 ml Difco fluorescent antibody (FA) buffer and transfer to a sterile 16 x 125-mm screw-cap tube. Heat in a water bath at 80°C for 1 h. Sediment cells by centrifugation at 1600 **g** for 30 min. Remove 2.2-2.3 ml of supernatant fluid and resuspend the pellet in the remainder of buffer. Follow manufacturer's recommendations for sera dilution and agglutination procedure. If flagella (H) or somatic (O) sub-factor serotyping is desired, see Chapter 11.

2. **Genetic subtyping**. Data from pulsed-field gel electrophoresis (PFGE) of DNA restriction fragments of FDA isolates should be submitted to PulseNet (CDC, Atlanta, GA). Isolates should also be ribotyped or sent to a ribotyping laboratory.

H. Immunocompromised Mouse Pathogenicity Test (optional).

The classical tests for *Listeria* pathogenicity are the Anton conjunctivitis test (rabbits), inoculation of mice, and inoculation of embryonated eggs. The immunocompromised mouse test, using intra-peritoneal (i.p.) injection, used here, is recommended because of its greatly improved sensitivity (39). Confirmation of *L. monocytogenes* animal pathogenicity is not needed for clinical isolates and is optional for food isolates. An isolate should be identified as *L. monocytogenes* if it meets all the other criteria outlined in this chapter.

About 4 mg of carrageenan (Sigma type II) dissolved in distilled water (40 mg/ml) is injected, i.p., into 18-20 g mice 24 h before the *Listeria* challenge. The exact volume of carrageenan solution injected, about 0.1 ml, depends on the mouse body weight. The target dose is 200 mg carrageenan per kg bodyweight. 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 a 16 x 125 mm tube and centrifuge at 1600 **g** for 30 min. Discard supernatant and resuspend pellet in 1 ml of phosphate buffered saline. This suspension will contain approximately 10^{10} bacteria/ml; dilute to 10^5 bacteria per ml and determine actual concentration by a pour or spread-plate count. Inject (i.p.) 16 to 18 g immunocompromised Swiss white mice (5 mice/culture) with 0.1 ml of the concentrated suspension, i.e. approximately 10^4 bacteria per mouse. Observe for death over 5-day period. Nonpathogenic strains will not kill, but 10^4 pathogenic cells will kill, usually within 3 days. Use known pathogenic and nonpathogenic strains and carrageenan-treated, uninoculated mice as controls. Use 5 mice per control group. Carrageenan controls should be challenged with 0.1 ml PBS.

I. Interpretation of test data

The importance of completely characterizing each isolate cannot be overemphasized. Partial characterization, even if accurate, may be misleading. Since all *Listeria* species test negative for indole, oxidase, urease, and H_2S production from organic sulfur compounds (H_2S is produced from thiosulfate in the MICRO-ID test kit) and test positive for methyl red and Voges-Proskauer, these tests are discretionary. *Brochothrix*, which is closely related phylogenetically to *Listeria*, is distinguishable from *Listeria* by its inability to grow at 35° C and by its lack of motility. Distinguishing features of the Gram-positive non-sporeforming rods, *Erysipelothrix* and *Kurthia*, which occur rarely in *Listeria* analysis, can be found elsewhere (11, 37).

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, and some species utilize mannitol, rhamnose, and xylose with production of acid. An isolate utilizing mannitol with acid production is *L. grayi*. *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 non-hemolytic species, *L. innocua* may provide the same rhamnose-xylose reactions as *L. monocytogenes* but it is negative in the CAMP test. *L. innocua* sometimes gives negative results for utilization of rhamnose . The significance of the undocumented reference (41) to hemolytic *L. innocua* isolates is unclear since it is commonly accepted that *L. innocua* is non-hemolytic and *L. monocytogenes* is hemolytic. A *L. welshimeri* isolate that is rhamnose-negative may be confused with a weakly hemolytic *L. seeligeri* isolate unless resolved by the CAMP test.

Sometimes aberrant listeria strains are isolated which are extremely difficult to speciate (27a). (See Guideline for BAM Users on Identification of Atypical Hemolytic Listeria Isolates.) If such an aberrant *Listeria* isolate is obtained, contact Anthony.Hitchins@cfsan.fda.gov [Note: The clinical significance of a strain of *L. monocytogenes* that is phenotypically hemolytic-negative is debatable. If it is due to a defect of the hemolysin gene, especially a deletion rather than a point mutation, it is likely clinically less significant than a normal strain would be, judging from laboratory studies of constructed hemolysin mutants in mice. However, if it is due to a regulatory defect that affects the expression of the hemolysin gene *in vitro*, the possibility of conditional expression *in vivo* is raised. Until convenient methods are devised to distinguish these structural and regulatory alternatives, the isolate need only be carefully confirmed as being a strain of *L. monocytogenes* phenotypically hemolysin-negative *in vitro* so that a soundly based regulatory decision can then be made, based upon all the relevant circumstances.]

Only after all other results are available does serotyping and other kinds of typing of *Listeria* isolates become meaningful. Biochemical, serological and pathogenicity data are summarized in Tables 1, 4 and 5. All data collection must be completed before species identities are determined. FDA no longer conducts routine bacteriophage-susceptibility typing of *L. monocytogenes* isolates.

J. Enumeration (required)

If a sample tests positive for *L. monocytogenes*, use a reserve portion of sample for enumeration. Current methods of enumeration are only presumptive for *Listeria monocytogenes* and some degree of further testing of isolated *Listeria* colonies is necessary. Conventional enumeration is described and alternative rapid methods are indicated. The proportion of presumptive isolates that are actually *L. monocytogenes* may be determined by conventional or rapid tests. Flexibility in choice of methods and adaptations of them is permitted but the observed count must be reported with 95% confidence limits, the method used named and any modifications indicated. The correction factor for converting the observed count to *L. monocytogenes* numbers must be reported as the whole number ratio of number of isolates identified as *L. monocytogenes* to the total number of *Listeria* isolates tested.

All enumeration methods, including microscopic, colony and Most Probable Number (MPN) counts are fundamentally governed by the Poisson distribution law of infrequent events. This describes the distribution of *Listeria* among the arrays of compartments (tubes, wells, counting chamber squares, filter grid squares, and virtual squares on culture agar surfaces). Compartmentalization separates or delineates colony-forming units in the various methods. In general, the confidence limits (CLs) of these estimates are considered proportional to the square root of the observed count. [The tabulated CLs for MPN results are asymmetric about the mean because they are usually obtained with low numbers of tubes (3 or 5) near the dilution endpoint.] As the count increases its confidence limits, expressed as a percentage of the count, decrease. Thus, choosing among methods largely reduces to a consideration of material and labor expenses and to how inoculation manipulations for an optimal number of compartments can be reduced by techniques such as filtration, semi-automation and robotics.

Surveillance Enumeration. This is required for accumulating data on cell numbers of *L. monocytogene*s in regulatory samples that test positive for the pathogen. Contact Anthony Hitchins@cfsan.fda.gov if you are unable to enumerate the reserve portion of a positive sample in order to try to arrange for its enumeration. To estimate the degree of sample contamination by presumptive *L. monocytogenes*, quantify the initial enrichment broth, before starting incubation, by direct spread plate count on ALOA, BCM or equivalent differential agar. Also, use a 3 or more-

tube/well MPN culture procedure on 1, 0.1, 0.01and 0.001-g samples in BLEB (30° C, 48 h, with or without pyruvate and without delayed addition of selective agents) followed by streaking on the chosen selective agar. If all the MPN tubes are *Listeria* positive, use reserve sample to repeat the MPN determination using an appropriate range of more dilute analytical portions, e.g. 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} g.

If selective agar plates are in short supply, an economic alternative to spreading dilution aliquots on individual selective agar plates is the drop plating method. Using a multi-channel pipette is well suited to this method. Decimally dilute 10 μl amounts of the contents of the enrichment containers in 90 μl amounts of TSBye in micro-titer plates with round-bottomed wells. Mix with a gentle circular motion of the micropipette tip before changing the tip for the next dilution. Carefully plate 10 μl of the dilutions as drops on plates of ALOA, BCM or equivalent agar. Let the droplets be absorbed before inverting the plates for incubation. Square plates are most convenient and efficient for this technique.

Table 6. Alternative rapid enumeration methods.

Method	Reference	Validation	Specificity Matrix
MPN filter	Entis & Lerner (20)	AOAC INTL.	All *Listeria,* FDA foods
Filter/colony-lift	Carroll *et al*. (13)	Peer review	*L. monocytogenes* Meat
DNA probe colony hybridization	BAM Chapter 24 (17,18)	FDA	*L. monocytogenes*, FDA foods

Alternatively, the methods shown in Table 6 may be used. Identify isolates by conventional or rapid methods. When all *Listeria* are enumerated estimate the proportion that is *L. monocytogenes* by determining the species of 10 typical *Listeria* colonies. For advice on using these techniques consult Anthony.Hitchins@cfsan.fda.gov . Grant (23a) has developed a filter enumeration method, based on the BAM *Listeria* enrichment and isolation method, which enumerates *Listeria* at cell numbers of >100 cfu/g.

Tolerance enumeration. Enumeration to determine if a regulated level of tolerance is being met is not needed with the current "zero-tolerance" policy of no detectable *L. monocytogenes* in 2 x 25-g analytical portions of food or beverage. It would require narrower confidence limits than does surveillance enumeration. Narrower confidence limits for tolerance and surveillance enumeration can be accomplished by counting more colony forming units, which can be accomplished by increasing the number of replicate tubes or other containers. For the current FDA method, the wells of one or more 96-well micro-titer plates, with round-bottomed wells, can be inoculated, by multi-channel pipette or robotically, with 0.1 ml of homogenate of complete BLEB and sample. After incubation at 30° C for 48 h, use the same kinds of transfer methods described in **Surveillance Enumeration** to inoculate enriched samples to ALOA, BCM or equivalent differential agar to determine which wells are positive. Using the proportion of *L. monocytogenes*-positive wells, the mean concentration can be calculated using the Poisson equation.

Alternatively, the 1600 filter grid compartments MPN method for *Listeria* (20) may be used for presumptive enumeration of *L. monocytogenes*.

Identify isolates by conventional methods, including the use of ALOA, BCM or equivalent agar, or by rapid methods. When necessary estimate the proportion of *L. monocytogenes* among 10 *Listeria* isolates.

Simultaneous detection and enumeration. Most samples are likely to be negative and thus it is efficient to delay enumeration of reserve samples until the *Listeria* detection stage is completed. Even then, most positive samples will only contain a few cfu/25g. Nevertheless it may sometimes be more convenient to do simultaneous detection and enumeration. To accomplish this, prepare the enrichment homogenate as described above and immediately spread 0.1 ml on ALOA, BCM or an equivalent *L. monocytogenes* selective agar. Incubate plates at 35° C for 24-48 h. The combined minimal method will allow the cell number of presumptive *L. monocytogenes* to be categorized as <0.04 cfu/g, 0.04 - 100 cfu/g, 100-250,000 cfu/g, or > 250,000 cfu /g. More replica plates and more decimal dilutions in TSBye are optional to obtain a more precise enumeration. Test 5 representative colonies for ability to ferment L-rhamnose by the conventional fermentation method, by the BCM rhamnose confirmatory agar or by a rapid *L. monocytogenes* identification kit to definitively rule out the uncommon occurrence of *L. ivanovii* in foods.

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SUPPLEMENT 1A.
Stated Food Matrix Applicability and Specific Food Matrices Used in Validating *Listeria* Rapid Screening Methods

Assurance EIA Kit and VIP Kit

(Applicable to dairy foods, red meats, pork, poultry products, fruits, nutmeats, seafood, pasta, vegetables, cheese, animal meal, chocolate, and eggs)

Milk, non-fat, dry
Ice cream
Poultry, raw
Shrimp, raw
Beef, cooked roast
Beans, green

GENE-TRAK *Listeria* Hybridization Assay

(Applicable to dairy products, meats, and seafoods)

Milk, 2%
Cheese, Brie

Crab meat, cooked
Frankfurters
Beef, roast
Pork, raw ground

Listeria -Tek ELISA Assay

(Applicable to dairy products, seafoods, meats)

Frankfurters
Beef, roast, vacuum-packed
Cheese, Brie
Milk, 2%, pasteurized
Shrimp, raw, shelled, frozen
Crab meat, cooked, frozen

TECRA *Listeria* Visual Immunoassay

(Applicable to dairy foods, seafoods, poultry, meats (not raw ground chuck), leafy vegetables)

Fish, fillets
Ice cream
Lettuce
Chicken
Turkey, ground

VIDAS LIS Assay Screening Method

(Applicable to dairy products, vegetables, seafoods, raw meats and poultry, and processed meats and poultry)

Ice cream
Green beans
Fish
Turkey
Cheese
Roast beef

SUPPLEMENT 1B.
Validated Application of *Listeria* Genus Rapid Methods to the Historical List of Food Matrices Analyzed by FDA

Abbreviations: P = Pre-collaborative study; C = Collaborative study, "." = No data.

The listed matrices are those encountered by FDA laboratory analysts as of January 1998.

	Rapid Test Kits				

Food Matrix		Assurance-EIA and VIP kits	Gene-Trak	ListeriaTek	Tekra VIA	VIDAS Listeria
Condiments	Mayonnaise	.	.	.	.	.
	Mustard	.	.	.	.	.
Crustacean	Crab	.	C	.	.	.
	Crab_cakes	.	.	.	.	.
	Crab_ck_fr	.	.	C	.	.
	Crab_claws	.	.	.	.	.
	Crab_meat	P	P	P	P	P
	Crab_meat_cocktail	.	.	.	.	.
	Crab_meat_deviled	.	.	.	.	.
	Crab_meat_imitation	.	.	.	.	.
	Crab_snow (3)	.	.	.	.	.
	Crab_stuffed	.	.	.	.	.
	Crabmeat	.	.	.	.	.
	Crabmeat_backfin	.	.	.	.	.
	Crawfish	.	.	.	.	.
	Crawfish_chinese	.	.	.	.	.
	Crawfish_tails (2)	.	.	.	.	.
	Extruded	.	.	.	.	.
	Lobster-imitation	.	.	.	.	.
	Lobster_meat (4)	.	.	.	.	.
	Lobster_meat_cooked	.	.	.	.	.
	Lobster_meat_imit.	.	.	.	.	.
	Prawns_cooked	.	.	.	.	.
	Shrimp	.	.	.	P	P
	Shrimp_breaded (2)	.	.	.	.	.
	Shrimp_cooked (3)	.	.	.	.	.
	Shrimp_peeled	.	.	.	.	.
	Shrimp_raw	C	P	C	.	.
	Shrimp_salad	.	.	.	.	.
Dairy	Cheese_unnamed (2)	.	.	.	.	CP
	Cheese_blue	.	.	.	.	.

Cheese_blue_cashel	.	.	.	.	.
Cheese_brie	.	C	C	.	.
Cheese_cheddar	.	P	.	.	.
Cheese_costello	.	.	.	.	.
Cheese_cottage	.	P	.	.	.
Cheese_cows-milk	.	.	.	.	.
Cheese_cream_lox	.	.	.	.	.
Cheese_gorgonzola	.	.	.	.	.
Cheese_ilha_azul	.	.	.	.	.
Cheese_korsholm	.	.	.	.	.
Cheese_kosher-lox	.	.	.	.	.
Cheese_mozzarella	.	.	.	.	.
Cheese_pimento	.	.	.	.	.
Cheese_queso-fr (2)	.	.	.	.	.
Cheese_reblochon	.	.	.	.	.
Cheese_ricotta_hard	.	.	.	.	.
Cheese_ricotta	.	.	.	.	.
Cheese_ricotta_pec	.	.	.	.	.
Cheese_salad-spread	.	.	.	.	.
Cheese_semi_soft	.	.	.	.	.
Cheese_soft	P	.	.	P	.
Cheese_soft_mexican	.	.	.	.	.
Cheese_spread	.	.	.	.	.
Cheese_swiss	.	.	.	.	.
Cheese_white	.	.	.	.	.
Cheese_white_soft	.	.	.	.	.
Cheese_Island	.	.	.	.	.
Cheese_Island_wheel	.	.	.	.	.
Cheese_St.Jorge(2)	.	.	.	.	.
Cheese_St.Jorge_Isl	.	.	.	.	.
Egg_dry	P	.	.	.	.
Egg_liq._frozen	P	.	.	.	.
Ice cream_unnamed	C	P	P	C	C

	Ice cream_chocolate	.	.	.	.	.
	Ice cream_cookie_flv	.	.	.	.	.
	Ice cream_peppermint	.	.	.	.	.
	Ice cream_raisin_Fr.	.	.	.	.	.
	Ice cream_rum_French	.	.	.	.	.
	Ice cream_sandwich	.	.	.	.	.
	Ice cream_strawberry	.	.	.	.	.
	Ice cream_vanil_crch	.	.	.	.	.
	Ice cream_vanl_Fr(2)	.	.	.	.	.
	Ice cream_vanilla(2)	.	.	.	.	P
	Milk	P	.	P	.	P
	Milk_chocolate	P	P	P	P	.
	Milk_nf_dry	C	P	P	P	P
	Milk_raw	.	.	.	.	.
	Milk_2%	.	C	C	.	.
	Sherbert_raspberry	.	.	.	.	.
	Yogurt_mix_bluebrry	.	.	.	.	.
	Yogurt_mix_vanilla	.	.	.	.	.
Fishery	Belt-fish (2)	.	.	.	.	.
	Calamari	.	.	.	.	.
	Catfish (2)	.	.	.	.	P
	Catfish_fillet	.	.	.	.	.
	Catfish_nuggets	.	.	.	.	.
	Chubs, raw	.	.	.	.	.
	Chum_roe_salted	.	.	.	.	.
	Cuttle-fish_salted	.	.	.	.	.
	Cuttlefish	.	.	.	.	.
	Cuttlefish_seasoned	.	.	.	.	.
	Eel_frozen	.	.	.	.	.
	Eel_broiled	.	.	.	.	.
	Eel_seasoned	.	.	.	.	.
	Eel_smoked	.	.	.	.	.
	File-fish_dried	.	.	.	.	.

Fish_barbequed	.	.	.	.	.
Fish_cake	.	.	.	.	.
Fish_cake_fried	.	.	.	.	.
Fish_fillet	.	P	.	C	.
Fish_nova_chips	.	.	.	.	.
Fish_raw	P	.	P	.	CP
Fish_salted	.	.	.	.	.
Fish_smoked (3)	.	.	.	.	.
Fish_stuffed	.	.	.	.	.
Flounder_stuffed	.	.	.	.	.
Flying-fish_roe_sd	.	.	.	.	.
Flying-fish_roe	.	.	.	.	.
Gefilte_fish	.	.	.	.	.
Halibut_fillet	.	.	.	.	.
Halibut_smoked	.	.	.	.	.
Halibut_smoked	.	.	.	.	.
Mackeral_dried	.	.	.	.	.
Pollack_roe (4)	.	.	.	.	.
Pollack_roe_salted	.	.	.	.	.
Pollack_roe_seasnd	.	.	.	.	.
Pollack_roe_spicy	.	.	.	.	.
Orange_roughy_stufd	.	.	.	.	.
Sable_fish_smoked	.	.	.	.	.
Salmon_caviar	.	.	.	.	.
Salmon_jerky	.	.	.	.	.
Salmon_pate	.	.	.	.	.
Salmon_patties (2)	.	.	.	.	.
Salmon_rainbow_smkd	.	.	.	.	.
Salmon_roe_caviar	.	.	.	.	.
Salmon_smoked (5)	.	.	.	.	.
Salmon_smoked_scot	.	.	.	.	.
Salmon_smoked_Atc	.	.	.	.	.
Salmon_sockeye_smok	.	.	.	.	.

	Sea-squirt	.	.	.	.	.
	Shad_cold-smoked	.	.	.	.	.
	Squid_rings	.	.	.	.	.
	Sturgeon_smoked	.	.	.	.	.
	Surimi	.	.	.	.	.
	Swordfish_smoked	.	.	.	.	.
	Trout_gldn_rainbow	.	.	.	.	.
	Trout_smoked	.	.	.	.	.
	Tuna_smoked (2)	.	.	.	.	.
	Walleye_fish-fillet	.	.	.	.	.
	White-bass	.	.	.	.	.
	White-fish_smoked	.	.	.	.	.
Fruit	Cantaloup_sliced	.	.	.	.	.
Meal	Bone_meal	P	.	.	.	.
Meat	Beef_cooked	.	.	P	.	.
	Beef_raw	P	.	.	P	P
	Beef_roast	C	C	P	P	CP
	Franks/Hotdog	.	C	C	P	P
	Ham_boiled_slices	.	.	.	.	.
	Ham_chopped	.	.	.	.	.
	Ham_cured	.	.	P	.	.
	Pork_raw	P	C	.	.	.
	Pork_raw_ground	.	P	.	.	.
	Sausage_bologna	.	.	.	P	P
	Sausage_fermented	.	P	.	.	.
	Sausage_pork	.	.	.	.	.
	Sausage_raw	.	.	P	.	P
Mollusks	Clam_baby_meat	.	.	.	.	.
	Clams _stuffed (2)	.	.	.	.	.
	Clams_baby	.	.	.	.	.
	Clams_jack-knife	.	.	.	.	.
	Mussells_green_shell	.	.	.	.	.

	Mussels_cooked	.	.	.	.	.
	Mussels_smoked	.	.	.	.	.
	Oysters	.	.	.	P	.
	Oysters_fresh	.	.	.	.	.
	Oysters_raw	.	.	P	.	.
	Scallop_imitat. (2)	.	.	.	.	.
	Scallops	P	.	.	.	.
Nuts	Chocolate	P	.	.	.	.
	Nuts	P	.	.	.	.
Pasta	Pasta_unnamed	P	.	.	.	.
	Rotini	.	.	.	.	.
	Tortellini_cheese	.	.	.	.	.
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	Chicken_raw	P	.	P	C	P
	Poultry_raw	C	.	.	.	.
	Turkey (2)	.	.	.	.	.
	Turkey_ground	.	.	.	C	.
	Turkey_raw	.	.	P	.	CP
	Turkey_raw_ground	.	P	.	.	.
Salads	Cole-slaw	.	.	.	.	.
	Crab_meat_imitation	.	.	.	.	.
	Crab_seafood	.	.	.	.	.
	Fish_whiting	.	.	.	.	.
	Fruit	.	.	.	.	.
	Ham	.	.	.	.	.
	Lobster	.	.	.	.	.
	Lobster_seafood	.	.	.	.	.
	Potato (3)	.	.	.	.	.
	Seafood (2)	.	.	.	.	.
	Toss_salad	.	.	.	.	.
Sandwiches	Beef	.	.	.	.	.
	Beef_roast (2)	.	.	.	.	.
	Beef_roast_po-boy	.	.	.	.	.

	Beef_rst/turkey/chs	.	.	.	.	.
	Cheese (2)	.	.	.	.	.
	Cheese_burger (2)	.	.	.	.	.
	Chicken & swiss	.	.	.	.	.
	Chicken_charboiled	.	.	.	.	.
	Chicken_salad	.	.	.	.	.
	Cold_cut	.	.	.	.	.
	Egg_sausage muffin	.	.	.	.	.
	Ham & cheese (6)	.	.	.	.	.
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	Ham & turkey (2)	.	.	.	.	.
	Ham/swiss_rye	.	.	.	.	.
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	Hamburger_artific.	.	.	.	.	.
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	Protein_vegetable	.	.	.	.	.
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Speciality	Capelin_dried	.	.	.	.	.
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	Beans_green	C	.	.	.	C

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	Broccoli_frozen	.	.	.	.	.
	Broccoli_normandy	.	.	.	.	.
	Cabbage_shredded	.	.	.	.	.
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	Salad_mix_veggies	.	.	.	.	.
	Salad_veggies_shred	.	.	.	.	.
	Spinach	.	.	.	.	.

Bad Bug Book:
Listeria monocytogenes

Hypertext Source: Bacteriological Analytical Manual, 8th Edition, Revision A, 1998. Chapter 10.
*Author: Anthony D. Hitchins
Draft Revision: 2002-September.

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Advisory for BAM Users on Reported Supply Problems for Cycloheximide

Guideline for BAM Users on Identification of Atypical Hemolytic *Listeria* Isolates

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