BAM: Salmonella

December 2007 Edition*

Bacteriological Analytical Manual
Chapter 5
Salmonella

Authors

Chapter Contents

Introduction

Several changes are being introduced in this edition of BAM (8th Edition). The first change involves the expanded use of Rappaport-Vassiliadis (RV) medium for foods with both high and low levels of competitive microflora. In the previous edition, RV medium was recommended only for the analysis of shrimp. Based on the completion of AOAC precollaborative (5, 6) and collaborative (7, 8) studies, RV medium is now being recommended for the analysis of high microbial and low microbial load foods. RV medium replaces selenite cystine (SC) broth for the analysis of all foods, except guar gum. In addition, RV medium replaces lauryl tryptose broth for use with dry active yeast. Tetrathionate (TT) broth continues to be used as the second selective enrichment broth. However, TT broth is to be incubated at 43°C for the analysis of high microbial load foods and at 35°C for the analysis of low microbial load foods, including guar gum.

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

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 Unpublished data have demonstrated that the 48 h reading of LIA slants is without diagnostic value.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

The fourth change involves the procedure for surface disinfection of shell eggs. In the previous edition (BAM-7), egg shells were surface-disinfected by soaking in 0.1% mercuric chloride solution for 1 h followed by soaking in 70% ethanol for 30 min. Mercuric chloride is classified as a hazardous waste, and is expensive to dispose of according to Environmental Protection Agency guidelines. In this edition (BAM-8) egg shells are now surface-disinfected by soaking for at least 10 sec in a 3:1 solution consisting of 3 parts of 70% alcohol (ethyl or isopropyl) to 1 part of iodine/potassium iodide solution.

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 5l), 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 23-24 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. Sponges, non-bactericidal (Nasco cat # B01299WA), or equivalent.
26. Swabs, non-bactericidal, cotton-tipped.

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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). NOTE: RV medium must be made from its individual ingredients. Commercial formulations are not acceptable.
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)
13. Trypticase soy-tryptose broth (M160)
14. MR-VP broth (M104)
15. Simmons citrate agar (M138)
16. Urea broth (M171)
17. Urea broth (rapid) (M172)
18. Malonate broth (M92)
19. Lysine iron agar (LIA) (Edwards and Fife) (M89)
20. Lysine decarboxylase broth (M87)
21. Motility test medium (semisolid) (M103)
22. Potassium cyanide (KCN) broth (M126)
23. Phenol red carbohydrate broth (M121)
24. Purple carbohydrate broth (M130)
25. MacConkey agar (M91)
26. Nutrient broth (M114)
27. Brain heart infusion (BHI) broth (M24)
28. Papain solution, 5% (M56a)
29. Cellulase solution, 1% (M187)
30. Tryptose blood agar base (M166)
31. Universal preenrichment broth (M188)
32. Universal preenrichment broth (without ferric ammonium citrate) (M188a)
33. Buffered peptone water (M192)
34. Dey-Engley broth (M193)
35. Potassium sulfite powder, anhydrous
36. Chlorine solution, 200 ppm, containing 0.1% sodium dodecyl sulfate (R12a)
37. Ethanol, 70% (R23)
38. Kovacs' reagent (R38)
39. Voges-Proskauer (VP) test reagents (R89)
40. Creatine phosphate crystals
41. Potassium hydroxide solution, 40% (R65)
42. 1 N Sodium hydroxide solution (R73)
43. 1 N Hydrochloric acid (R36)
44. Brilliant green dye solution, 1% (R8)

45. Bromcresol purple dye solution, 0.2% (R9)
46. Methyl red indicator (R44)
47. Sterile distilled water
48. Tergitol Anionic 7 (R78)
49. Triton X-100 (R86)
50. Physiological saline solution, 0.85% (sterile) (R63)
51. Formalinized physiological saline solution (R27)
52. *Salmonella* polyvalent somatic (O) antiserum
53. *Salmonella* polyvalent flagellar (H) antiserum
54. *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
55. *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.** Remove any adherent material from the shell surface. Disinfect eggs with 3:1 solution consisting of 3 parts of 70% alcohol (ethyl or isopropyl) to 1 part iodine/potassium iodide solution. Prepare 70% alcohol solution either by diluting 700 ml 100% alcohol with sterile distilled water for a final volume of 1,000 ml or by diluting 700 ml 95% alcohol with sterile distilled water for a final volume of 950 ml. Prepare iodine/potassium iodide solution by dissolving 100 g potassium iodide in 200-300 ml sterile distilled water. Add 50 g iodine and heat gently with constant mixing until the iodine is dissolved. Dilute the iodine/potassium iodide solution to 1,000 ml with sterile distilled water. Store iodine/potassium iodide solution in amber glass-stoppered bottle in the dark. Prepare the disinfection solution by adding 250 ml iodine/potassium iodide solution to 750 ml 70% alcohol solution and mix well. Submerge eggs in disinfection solution for at least 10 seconds. Remove eggs and allow to air dry. Eggs with chipped, cracked, or

broken shells are not included in the sample. Each sample shall consist of twenty (20) eggs cracked aseptically into a Whirl-Pak bag, for a total of fifty (50) samples per poultry house. Eggs are cracked aseptically by gloved hands, with a change of gloves between samples. Mix samples thoroughly by gloved hands, with a change of gloves between samples. Mix samples thoroughly by hand until yolks are completely mixed with the albumen. Samples are held at room temperature (20-24°C) for 96 ± 2 h. After 96 ± 2 h, remove 25 ml portion from each sample of pooled eggs, and preenrich 25 ml test portion in 225 ml sterile trypticase 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).** Combine fifteen (15) 25 ml test portions into a 375 ml composite contained in a 6-liter Erlenmeyer flask. Composites are held at room temperature (20-24°C) for 96 ± 2 h. After 96 ± 2 h, add 3,375 ml sterile TSB supplemented with ferrous sulfate, as described above, 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.

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**
   a. **Lactic 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 Universal Preenrichment 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. **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.

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

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

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

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

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

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

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

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

11. **Candy and candy coating (including chocolate)**. Aseptically weigh 25 g sample into sterile blending container. Add 225 ml sterile, reconstituted nonfat dry milk and blend 2 min. Aseptically transfer homogenized mixture to sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container and let stand 60 ± 5 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Add 0.45 ml 1% aqueous brilliant green dye solution and mix well. Loosen jar caps 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.
12. **Coconut**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile lactose broth, shake well, and let stand 60 ± 5 min at room temperature with jar securely capped. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Add up to 2.25 ml steamed (15 min) Tergitol Anionic 7 and mix well. Alternatively, use steamed (15 min) Triton X-100. Limit use of these surfactants to minimum quantity needed to initiate foaming. For Triton X-100 this quantity may be as little as 2 or 3 drops. Loosen jar cap about l/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.
13. **Food dyes and food coloring substances**. For dyes with pH 6.0 or above (10% aqueous suspension), use method described for dried whole eggs (C-l, above). For laked dyes or dyes with pH below 6.0, aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml tetrathionate broth without brilliant green dye. Mix well and let stand 60 ± 5 min at room temperature with jar securely capped. Using pH meter, adjust pH to 6.8 ± 0.2. Add 2.25 ml 0.1% brilliant green dye solution and mix thoroughly by swirling. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 3-11, below.
14. **Gelatin**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile lactose broth and 5 ml 5% aqueous papain solution and mix well. Cap jar securely and incubate at 35°C for 60 ± 5 min. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.
15. **Meats, meat substitutes, meat by-products, animal substances, glandular products, and meals (fish, meat, bone)**. Aseptically weigh 25 g sample into sterile blending container. Add 225 ml sterile lactose broth and blend 2 min. Aseptically transfer homogenized mixture to sterile wide-mouth, screw-cap jar (500 ml) or other appropriate container and let stand 60 ± 5 min at room temperature with jar securely capped. If mixture is powder or is ground or comminuted, blending may be omitted. For samples that do not require blending, add lactose broth and mix thoroughly; let stand for 60 ± 5 min at room temperature with jar securely capped.

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

16. **Frog legs.** (This method is used for all domestic and imported frog legs.) Place 15 pairs of frog legs into sterile plastic bag and cover with sterile lactose broth at a 1:9 sample-to-

broth (g/ml) ratio (**see** A, 23-24, 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. 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 frog legs and 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 rabbit carcass into sterile plastic bag. Place bag in beaker or other suitable container. Add sterile lactose broth at a 1:9 sample-to-broth (g/ml) ratio to cover carcass (see A, 23-24, above). Mix well by swirling 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).
20. **Pig ears and other types of dog chew pieces.** Place 1 piece (or 2-3 pieces if smaller sizes) from each sample unit into sterile plastic bag. Place bag into large beaker or other suitable container. Add sterile lactose broth at a 1:9 sample-to-broth (g/ml) ratio to cover pieces (see A, 23-24, above). Mix well by swirling 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. Add either steamed (15 min) Tergitol Anionic 7 or steamed (15 min) Triton X-100 up to a 1% concentration. For example, if 225 ml lactose broth is added, the maximum volume of added surfactant is 2.25 ml. Limit use of these surfactants to minimum quantity to initiate foaming. Incubate 24 ± 2 h at 35° C. Continue examination as in D, 1-11, below.
21. **Cantaloupes**. 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.

    For comminuted or cut fruit, aseptically weigh 25 g sample into sterile blending container. Add 225 ml sterile Universal preenrichment broth (UP) 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. Do not adjust pH. Mix well and loosen jar cap about 1/4 turn. Incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

    For whole cantaloupes, do not rinse even if there is visible dirt. Examine the cantaloupes "as is".

    Place the cantaloupe into a sterile plastic bag. Add enough UP broth to allow the cantaloupe to float. The volume of UP broth may be 1.5 times the weight of the cantaloupes. For example, cantaloupes weighing 1500 g will probably need a volume of approximately 2250 ml UP broth to float. Add more broth, if necessary. Place the plastic bag, with cantaloupes and UP broth, into a 5 liter beaker, or other appropriate container,

for support during incubation. Allow the open-end flap of the plastic bag to "fold over" so as to form a secure, but not air-tight, closure during incubation.

Let stand for 60 ± 5 min at room temperature. Do not adjust pH. Incubate slightly opened bag, containing cantaloupe, for 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

22. **Mangoes**. 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.

    For comminuted or cut fruit, aseptically weigh 25 g sample into sterile blending container. Add 225 ml sterile buffered peptone water (BPW) 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.

    For whole mangoes, do not rinse even if there is visible dirt. Examine the mangoes "as is".

    Place the mango into a sterile plastic bag. Add enough BPW to allow the mango to float. The volume of BPW may be 1.0 times the weight of the mangoes. For example, mangoes weighing 500 g will probably need a volume of approximately 500 ml BPW broth to float. Add more broth, if necessary. Place the plastic bag, with mangoes and BPW broth, into a 5 liter beaker, or other appropriate container, for support during incubation.

    Let stand for 60 ± 5 min at room temperature. Adjust pH to 6.8 ± 0.2, if necessary. Incubate slightly opened bag for 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

23. **Tomatoes**. For comminuted or cut fruit, aseptically weigh 25 g sample into sterile blending container. Add 225 ml sterile buffered peptone water 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.

    For whole tomatoes, do not rinse even if there is visible dirt. Examine the tomatoes "as is".

    Place the tomato into a sterile plastic bag or other suitable container (sterile foil covered beaker can be used). Add enough UP broth to allow the tomato to float. The volume of UP broth may be 1.0 times the weight of the tomato. For example, tomatoes weighing 300 g will probably need a volume of approximately 300 ml UP broth to float. Add more, if necessary. Place the plastic bag (if used), with tomato and UP broth, into a sterile beaker (beaker size is dependent on the size of the tomato), or other appropriate container, for support during incubation. Allow the open-end flap of the plastic bag to "fold over" so as to form a secure, but not air-tight, closure during incubation.

    Let stand for 60 ± 5 min at room temperature. Do not adjust pH. Incubate slightly opened bag for 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

24. **Environmental testing**. Sample environmental surfaces with sterile swabs or sponges. Place the swab/sponge in a sterile Whirl-pak bag, or equivalent, that contains enough Dey-Engley (DE) broth to cover the swab/sponge.

    Transport swabs/sponges in an insulated transport container with frozen gel packs to keep the samples cold, but not frozen. If samples cannot be processed immediately, refrigerate at 4 ± 2°C. Start sample analysis within 48 ± 2 h of collection.

    Add swab/sponge to 225 ml lactose 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. 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.

25. **Alfalfa seeds and mung beans**. Aseptically weigh 25g alfalfa seeds or mung beans into a sterile 500 mL Erlenmeyer flask. Aseptically add 225 mL lactose broth to the test portion and swirl the Erlenmeyer flask. Cover the mouth of the Erlenmeyer flask with sterile aluminum foil and allow contents to stand at room temperature for 60 ± 5 min. Adjust the pH of the culture to 6.8 ± 0.2, if necessary. Incubate for 24 ± 2h at 35 ± 2°C. Continue as in D, 1-11, below (treat as high microbial load food).
26. **Mamey pulp**. 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.

    For mamey pulp, suspected to be contaminated with *S*. Typhi, aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile Universal Preenrichment broth without ferric ammonium citrate, mix by swirling, and let stand 60 ± 5 min at room temperature with jar securely capped. Do not adjust pH. Mix well and loosen jar cap about 1/4 turn. Incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below. Treat as a low microbial load food.

    For mamey pulp, NOT suspected to be contaminated with *S*. Typhi, aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. Add 225 ml sterile Universal Preenrichment broth, mix by swirling, and let stand 60 ± 5 min at room temperature with jar securely capped. Do not adjust pH. Mix well and loosen jar cap about 1/4 turn. Incubate 24 ± 2 h at 35°C. Continue as in D, 1-11, below.

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D. Isolation of *Salmonella*

1. Tighten lid and gently shake incubated sample.

   Guar gum and foods suspected to be contaminated with *S*. Typhi. Transfer 1 ml mixture to 10 ml selenite cystine (SC) broth and another 1 ml mixture to 10 ml TT broth [98]. 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 and foods suspected to be contaminated with *S.* Typhi). 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 and foods suspected to be contaminated with *S.* Typhi. 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:

d. **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.
e. **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. **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. **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. **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. **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. **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-l, above) and retested, beginning with E-2, above.

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

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

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

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

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

   c. **MR-VP broth**. Inoculate medium with small amount of growth from each unclassified TSI slant suspected to contain *Salmonella*. Incubate 48 ± 2 h at 35°C.
      - **1)** Perform Voges-Proskauer (VP) test at room temperature as follows: Transfer 1 ml 48 h culture to test tube and incubate remainder of MR-VP broth an additional 48 h at 35°C. Add 0.6 ml α-naphthol and shake well. Add 0.2 ml 40% KOH solution and shake. To intensify and speed reaction, add a few crystals of creatine. Read results after 4 h; development of pink-to-ruby red color throughout medium is positive test. Most cultures of *Salmonella* are VP-negative, indicated by absence of development of pink-to-red color throughout broth.
      - **2)** Perform methyl red test as follows: To 5 ml of 96 h MR-VP broth, add 5-6 drops of methyl red indicator. Read results immediately. Most *Salmonella* cultures give positive test, indicated by diffuse red color in medium. A distinct yellow color is negative test. Discard, as not *Salmonella*, cultures that give positive KCN and VP tests and negative methyl red test.

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

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

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

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

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

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

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

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

Table 2. Criteria for discarding non-*Salmonella* cultures	
Test or substrate	**Results**
1. Urease	positive (purple-red color)
2. Indole test and Polyvalent flagellar (H) test;	positive (violet color at surface) negative (no agglutination)
or Indole test and Spicer-Edwards flagellar test	positive (violet color at surface) negative (no agglutination)
3. Lysine decarboxylase and KCN broth	negative (yellow color) positive (growth)
4. Phenol red lactose broth	positive (yellow color and/or gas)[(a), (b)]
5. Phenol red sucrose broth	positive (yellow color and/or gas)[(b)]
6. KCN broth, Voges-Proskauer test, and Methyl red test	positive (growth) positive (pink-to-red color) negative (diffuse yellow color)
[a] Test malonate broth positive cultures further to determine if they are *S. arizonae*. [b] Do not discard positive broth cultures if corresponding LIA cultures give typical *Salmonella* reactions; test further to determine if they are *Salmonella* species.	

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 (I). 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

    FDA Denver Laboratory
    Attention Sample Custodian
    Denver Federal Center, Building 20
    6th Avenue & Kipling Streets
    Denver, CO 80225-0087

    (Above address effective October 1, 2004)

    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 a copy of the Collection Report, FD-464, or Import Sample Report, FD-784 for each sample. 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.

    Microbiology Field laboratories should follow the following guidance in sending Salmonella isolates for serotyping:

Isolates from NRL, WEAC, SRL and ARL will be serotyped in ARL:

Arkansas Regional Laboratory
3900 NCTR Road Building 26
Jefferson, AR 72079
Attention: Gwendolyn Anderson
Tel # 870-543-4621
Fax# 870-543-4041

Isolates from SAN, PRL-NW, PRL-SW and DEN will be serotyped in DEN

Denver District Laboratory
6th Avenue & Kipling Street
DFC Building 20
Denver, CO 80225-0087
Attention: Doris Farmer
Tel # 303-236-9604
Fax # 303-236-9675

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References

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Chapter 5 Contents

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October 25, 2001 - Extension of the applicability of the orange juice method in section C.19 to apple juice and apple cider.
April 2003 - Frog legs method, Lactic casein, Rennet casein, Sodium caseinate and Rabbit carcass methods revised, top ears and other dog chew toys added. Removed section A.25, Mechanical shaker.
June 2006 Edition - Eggs method revised for shell eggs and liquid whole eggs.
December 2007 - Mamey pulp method added, and Section D revised.

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