

**United States Department of Agriculture
Agricultural Marketing Service, Science & Technology
Microbiological Data Program**

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Title: <i>Salmonella</i> Cultural Method		
Revision: 02	Replaces: 08/15/03	Effective: 01/01/04

1. Purpose:

To standardize the cultural procedures for the identification and confirmation of *Salmonella* serotypes isolated from fruit and vegetables for all laboratories participating in the Microbiological Data Program (MDP).

2. Scope:

This standard operating procedure (SOP) shall be followed by all laboratories conducting microbiological studies for MDP, including support laboratories conducting non-routine activities that may impact the program.

3. Principle

This is a general method for testing *Salmonella* and is detailed in Chapter 5 of the Food and Drug Administration, Bacteriological Analytical Manual (BAM). It is presented here as the method to be followed when confirming samples which are presumptive positive for *Salmonella* by other means such as PCR-BAX or VIDAS.

4. Outline of Procedures:

- 4.1. Media and Reagents 6.1
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 - 4.3. Isolation of *Salmonella* Procedure 6.3
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- 4.8. Interpretation of Analyses 6.8
- 4.9. Quality Assurance 6.9
- 4.10. Transport and Storage of Cultures 6.10

5. References:

- 5.1. Andrews WH, and Hammack TS. *Salmonella* (Chapter 5). BAM online. FDA. April 2003. <http://www.cfsan.fda.gov/~ebam/bam-5html>
 - 5.2. Maturin LJ and Peeler JT. Aerobic Plate count (Chapter 3). BAM online, FDA. January 2002. <http://www.cfsan.fda.gov/~ebam/bam-3.html>
 - 5.3. June, G.A., Sherrod, P.S., Hammack, T.S.; Amaguana, R.M., and Andrews, W.H. 1995. Relative effectiveness of selenite cystine broth, tetrathionate broth, and Rappaport-Vassiliadis medium for the recovery of *Salmonella* from raw flesh and other highly contaminated foods: Precollaborative study. J. AOAC Int. 78:375- 380.
 - 5.4. Rose BE. Isolation and identification of Salmonella from meat, poultry, and egg products. Chapter 4, revision 1, effective 10-25-02. USDA/FSIS. <http://www.fsis.usda.gov/ophs/microlab/mlg4.02.pdf>
 - 5.5. MDP-SHIP-01 Procedures for Packaging and Shipping Microbiological Cultures for detailed procedures on subsequent handling of isolates.
 - 5.6. Maijala R, Johansson T, and Hirn J. 1992. Growth of *Salmonella* and competing flora in five commercial RV media. International Journal of Food Microbiology. Vol.17, pp.1-8.
6. **Specific Procedures:** Identification of isolates obtained from the selective and differential agar plates may also be achieved using VITEK.
- 6.1. Media and Reagents
 - 6.1.1. Rappaport-Vassiliadis (RV) medium - 16 x 150 mm sterile test tubes containing 10 mL aliquots. (NOTE: RV broth by Oxoid has been shown to provide better enrichment of *Salmonella* compared to RV produced by other manufacturers. Therefore, Oxoid RV broth is recommended.)

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- 6.1.2. Tetrathionate (TT) broth (with iodine and Brilliant Green Dye .1%) - 16 x 150 mm sterile test tubes containing 10 mL aliquots (on the day of use add 20 mL iodine and 10 mL of Brilliant Green solution per 1 liter basal broth)
 - 6.1.3. Iodine solution for basal TT broth (see BAM reference M145)
 - 6.1.4. Brilliant Green Dye solution for basal TT broth (see BAM reference M145)
 - 6.1.5. *Salmonella enterica* serovar Poona strain carrying a pKT-kan plasmid that contains a gene coding for green fluorescent protein (GFP) as a positive culture control (CDHS/MDL #00A 3563; USDA/ARS/PW #RM235)
 - 6.1.6. *Enterobacter aerogenes* as negative culture control
 - 6.1.7. *Proteus vulgaris* ATCC 13315 or equivalent stock bacterial culture to be used as the positive control for the urease production test. Urea broth medium is positive for *Proteus*, *Morganella morganii*, *Providencia rettgeri* and a few *Providencia stuartii* strains.
 - 6.1.8. Xylose lysine deoxycholate (XLD) agar
 - 6.1.9. Hektoen enteric (HE) agar
 - 6.1.10. Bismuth sulfite (BS) agar - prepare plates 24 hours before use
 - 6.1.11. Triple sugar iron agar (TSI)
 - 6.1.12. Lysine iron agar (LIA)
 - 6.1.13. Trypticase (tryptic) soy broth (TSB)
 - 6.1.14. Tryptone (tryptophane) broth
 - 6.1.15. MR-VP broth
 - 6.1.16. Malonate broth
 - 6.1.17. Simmons citrate agar
 - 6.1.18. Urea broth
 - 6.1.19. Rapid urea broth (optional)
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- 6.1.20. Dulcitol (0.5% w/v) carbohydrate fermentation broth (purple broth base)
 - 6.1.21. Lactose (1.0% w/v) carbohydrate fermentation broth (purple broth base)
 - 6.1.22. Sucrose (1.0% w/v) carbohydrate fermentation broth (purple broth base)
 - 6.1.23. MacConkey agar
 - 6.1.24. Brain heart infusion (BHI) broth (optional)
 - 6.1.25. Kovac's indole reagent: 5 grams p-dimethylaminobenzaldehyde, 75 mL amyl alcohol, and 25 milliliters concentrated hydrochloric acid. Dissolve the p-dimethylaminobenzaldehyde in the amyl alcohol and slowly add the concentrated hydrochloric acid. Store at 4°C. Discard if color is darker than light straw. May be commercially purchased.
 - 6.1.26. BD DrySlide™ Indole (optional): a disposable slide containing a 4-segmented reagent cassette impregnated with 5% p-dimethylaminobenzaldehyde (DMABA). Store slides at 2-8°C.
 - 6.1.27. Methyl red-Voges-Proskauer (MR-VP) medium or buffered glucose broth
 - 6.1.28. Methyl red indicator (MR): Methyl red, 0.10 g; ethanol, 95%, 300 mL. Dissolve 0.1 gram methyl red in 300 mL ethanol, and make up to 500 mL with purified water. May be commercially purchased.
 - 6.1.29. Sterile purified water (deionized, reverse osmosis (RO), or distilled)
 - 6.1.30. 40% aqueous potassium hydroxide (KOH) solution for Voges-Proskauer (VP) test
 - 6.1.31. Creatine phosphate crystals for VP test
 - 6.1.32. 1-naphthol solution for VP test: 5% (w/v) 1-naphthol in absolute ethanol.
 - 6.1.33. Motility test medium (semisolid or 0.3 % w/v agar)
 - 6.1.34. *Salmonella* polyvalent somatic (O) antiserum, Poly AI and Vi (poly O antiserum): Contains agglutinins for at least the following somatic (O) antigens: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 22, 23, 24, 25, 34, and Vi. They are agglutinins for somatic (O) groups: A, B, C₁, C₂, D, E₁, E₂, E₃, E₄, F, G₁, G₂, H, I, and Vi.
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6.1.35. *Salmonella* polyvalent flagellar (H), Poly a-z (poly H antiserum): Contains agglutinins for at least the following flagellar (H) antigens: a, b, c, d, e, f, g, h, i, k, l, m, n, p, q, r, s, t, u, v, w, x, y, z, Z₄, Z₆, Z₁₀, Z₁₃, Z₁₅, Z₂₃, Z₂₄, Z₂₈, Z₂₉, Z₃₂, 1, 2, 5, 6, 7.

6.1.36. McFarland three (3.0) turbidity standard or commercial equivalent: Prepare this barium sulfate (BaSO₄) opacity standard by adding 3.0 mL of 0.048 M barium chloride solution (1.175% w/v BaCl₂ · 2H₂O) to 97.0 mL of 0.36 N sulfuric acid solution (1% v/v H₂SO₄ solution). Distribute 4 to 6 mL into a sterile 13 x 100 mm glass tube with screw-cap. Tightly seal this tube and store in the dark at room temperature. A new standard should be prepared every six months.

6.1.37. Formalinized physiological saline solution, 0.6% (v/v): Prepare by adding 6 mL formaldehyde (HCHO) solution (36-38%) to 1,000 mL of sterile 0.85% w/v saline (NaCl) solution.

6.1.38. Physiological saline solution, sterile 0.85% w/v saline (NaCl) solution

6.1.39. Lysine decarboxylase broth

6.1.40. Nutrient agar

6.1.41. Bromcresol purple dye solution, 0.2% w/v: Dissolve 0.2 g bromcresol purple dye in 100 mL sterile water.

6.2. Apparatus

6.2.1. Balance, top loading, minimum 1,000 g capacity, sensitive to 0.1 g

6.2.2. Incubator –at constant temperature 35±1°C.

6.2.3. Water bath and cover – thermostatically controlled and constant temperature 35±0.5°C and 42±0.5°C . The water level should be above the level of the medium in immersed tubes.

6.2.4. Sterile culture dishes, 15 x 100 mm, glass or plastic

6.2.5. Sterile test or culture tubes, 16 x 150 mm and 20 x 150 mm

6.2.6. Serological tubes, 10 x 75 mm and 13 x 100 mm

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- 6.2.7. Durham fermentation tubes, to be used in fermentation broth to capture gas.
 - 6.2.8. Sterile glass or plastic pipets, cotton plugged, 1 mL and 2 mL, with 0.01 mL graduations; 5 and 10 mL, with 0.1 mL graduations. May use adjustable volume micropipetter with disposable tips if desired.
 - 6.2.9. Test tube caps, plastic or metal
 - 6.2.10. Test or culture tube racks
 - 6.2.11. A pipet aide or mechanical pipetting device is required for transfer pipets.
 - 6.2.12. Grease pencil or permanent ink magic marker
 - 6.2.13. Inoculating needle and inoculating loop (about 3 mm id or 10 μ L), 24 gauge nichrome, platinum-iridium, chromel wire, or sterile plastic
 - 6.2.14. Frosted disposable glass slides, 3"x1"
 - 6.2.15. Burner, Bunsen, Fisher or high temperature incinerator (optional)
 - 6.2.16. Lamp for observing serological reactions
 - 6.2.17. Vortex mixer
 - 6.2.18. Agglutination ring slide, typing plate, Technical Glass Products (from Fisher)
- 6.3. Isolation of *Salmonella* Procedure
- 6.3.1. Transfer 25mL of produce sample wash to 225mL of lactose broth. Incubate at $35\pm 2^{\circ}\text{C}$ for 18-24 hours.
 - 6.3.2. Transfer 1mL of lactose culture into 10mL tetrathionate broth (TT broth) and incubate at $42\pm 0.5^{\circ}\text{C}$ for 18-24 hours. Also, transfer 0.1mL of lactose broth into 10mL of RV (Rappaport-Vassiliadis) broth and incubate for 18-24 hours at $42\pm 0.5^{\circ}\text{C}$.
 - 6.3.3. Carry forward the two original positive and negative culture controls also grown in lactose broth into RV and TT enrichment broths through all the remaining procedural steps.
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6.3.4. Streak 3 mm (10 µL) loopful incubated RV broth on selective and differential plates of bismuth sulfite (BS) agar, Hektoen enteric (HE) agar, and xylose lysine desoxycholate (XLD) agar for colony isolation. Repeat with 3 mm (10 µL) loopful incubated TT broth on plates of BS agar, HE agar, and XLD agar. **Note:** Do not subdivide the selective and differential agar plates for streaking multiple samples; streak the entire agar plate with only one sample enrichment.

6.3.5. Incubate BS, HE, and XLD agar plates for 18 to 24 hours at 35±1°C in an incubator. Bismuth sulfite (BS) agar plates should be reincubated an additional 24 hours at 35° ±1° C.

6.3.6. After 18 to 24 hours incubation examine BS, HE, and XLD agar plates for presence of colonies that may be *Salmonella*. Bismuth sulfite (BS) agar plates should also be reexamined after 42 to 48 hours for *Salmonella* growth. *Salmonella* colony growth on selective and differential agar plates is recognized as follows:

6.3.6.1. HE agar - Typical *Salmonella* colonies are blue-green to blue 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. Atypically, a few *Salmonella* cultures produce yellow colonies with or without black centers (lactose or sucrose fermenters).

6.3.6.2. XLD agar - Typical *Salmonella* appear as pink or red colonies with or without black centers. Many cultures of *Salmonella* have colonies with large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* cultures produce yellow colonies with or without black centers (lactose or sucrose fermenters).

6.3.6.3. BS agar - Typical *Salmonella* may appear as brown, gray, or black colonies; usually they exhibit a metallic sheen. Surrounding media to the colonies is usually brown at first, but may turn black in time with increased incubation. Some atypical *Salmonella* strains with little or no darkening of the surrounding medium produce green colonies.

6.4. Initial Identification Procedures

6.4.1. After incubation, pick suspect *Salmonella* isolated colonies from BS, HE, and XLD agar plates, according to the section 6.4 (d) *Salmonella* colony morphology descriptions, and

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inoculate triple sugar iron (TSI) agar tubes and lysine iron agar (LIA) tubes. Inoculate TSI and then LIA in tandem without re-flaming the needle or without using another sterile needle with a sole pick from an isolated colony by stabbing the butts and streaking the slants in one continuous operation. **Note:** When picking isolated colonies with the sterile needle, lightly touch the center surface of the colony without touching the agar media. Twelve suspect *Salmonella* colonies are to be picked, if available. When possible, pick at least one suspect *Salmonella* colony from each of the differential and selective agar plates. Besides the presumptive positive *Salmonella* samples, include colony pickings to TSI and lysine iron agar slants from the growth on differential and selective agar plates for the known positive culture control and the known negative culture (*Enterobacter aerogenes*) control. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm or 1½-in.). A 5 cm (2-in.) slant is required for the TSI in order to provide adequate growth for subsequent tests. Store picked selective and differential agar plates at 2-8°C.

- 6.4.2. Incubate the TSI and LIA slants for 18 to 24 hours at 35±1°C in a incubator. Cap tubes loosely to maintain aerobic conditions while incubating slants.
- 6.4.3. Examine TSI and lysine iron agar (LIA) slants as sets. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H₂S (blackening of agar) in TSI. In LIA, *Salmonella* typically produces alkaline (purple) reaction in slant and butt of tube. Consider only distinct yellow in LIA butt as acidic (negative) reaction. However, do not discard cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce H₂S in LIA. Some non-*Salmonella* cultures produce a brick-red deamination reaction in LIA slants. All cultures that give an alkaline butt (purple) in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates to apply biochemical and serological identification tests. Cultures that give an acid butt (yellow) in LIA and an alkaline slant (red) and acid butt (yellow) in TSI should also be considered potential *Salmonella* isolates to apply biochemical and serological identification tests. Cultures that give an acid butt in LIA (yellow) and an acid slant (yellow) and acid butt (yellow) with or without H₂S in TSI may be discarded as not being *Salmonella*. Also discard isolates with TSI that are without

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change in color (red slant/red butt), with or without H₂S, when comparing to uninoculated TSI medium.

6.5. Pure Culture Attainment Techniques

- 6.5.1. From 18 to 24-hour growth on TSI agar slants, streak the cultures onto appropriate media for isolation as described below.
- 6.5.2. Incubate agar plates 18 to 24 hours at 35° ±1° C.
- 6.5.3. Examine plates for purity of cultures, and the presence of isolated colonies suspected of being *Salmonella*.
- 6.5.4. The serological and biochemical identification tests require the use of a pure culture.

6.6. Biochemical Tests

- 6.6.1. *Note: An AOAC International approved or MDP accepted commercial biochemical kit or system such as Vitek (biomerieux) may be used as an alternative to conventional biochemical testing if the following conditions are met*
 - 6.6.1.1. The analyst follows all the current and appropriate manufacturer's instructions for the test kit.
 - 6.6.1.2. The commercial biochemical kits are not used as a substitute for serological tests.
 - 6.6.1.3. The analyst adds reagents, observes, and records results as required for the test kit.
- 6.6.2. Urease test
 - 6.6.2.1. With a sterile 3 mm loop, transfer inoculum from each presumptive positive TSI culture into a sterile tube containing 1.5 to 3 mL of urea broth and incubate 18 to 24 hours at 35° ±1° C or inoculate 1.5 to 3 mL of rapid urea broth and incubate 2 hours in a 35±0.2°C water bath.
 - 6.6.2.2. Since sometimes uninoculated tubes of urea broth turn purplish red or cerise (positive test) on standing, include uninoculated tube of this broth as control. Incubate 18 to 24 hours at 35° ±1° C.

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6.6.2.3. Discard all cultures giving positive test (pink-red or cerise in color of medium). Retain for further testing all cultures that give negative test (orange-yellow or no change in color of medium).

6.6.3. Lysine decarboxylase test

6.6.3.1. With a sterile 3 mm loop, inoculate lysine decarboxylase broth in a screw-capped tube with a small amount of 18 to 24-hour growth from a TSI slant presumptive positive for *Salmonella*. Overlay with sterile mineral oil.

6.6.3.2. Cap the tube tightly and incubate 42 to 48 hours at $35^{\circ} \pm 1^{\circ} \text{C}$ but examine at 18 to 24-hour intervals. *Salmonella* species cause alkaline reaction indicated by purple color throughout medium.

6.6.3.3. Negative test is indicated by yellow color throughout medium. If medium appears discolored (gray color) add a few drops of 0.2% (w/v) bromocresol purple dye and re-read tube reactions

6.6.4. Carbohydrate fermentation broth tests

6.6.4.1. Purple fermentation broth base with 0.5% dulcitol. Inoculate broth with small amount of slant growth from an 18 to 24-hour TSI culture. Cap tube loosely and incubate 42 to 48 hours at $35^{\circ} \pm 1^{\circ} \text{C}$ but examine after 18 to 24 hours. The majority of *Salmonella* species give positive test, indicated by gas formation in inner inverted Durham tube and acid medium (yellow). Production of acid should be interpreted as a positive reaction. Negative test is indicated by no gas formation in inner inverted Durham tube and purple color throughout the medium.

6.6.4.2. Purple fermentation broth base with 1.0% lactose. Inoculate broth with small amount of slant growth from an 18 to 24-hour TSI culture. Cap tube loosely and incubate 42 to 48 hours at $35^{\circ} \pm 1^{\circ} \text{C}$, but examine after 18 to 24 hours. The majority of *Salmonella* species give a negative test, indicated by no gas formation in inner inverted Durham tube and purple color throughout the medium. Production of acid (yellow) with or without gas formation in inner inverted Durham tube should be interpreted as a positive reaction. Discard as non-*Salmonella*, cultures that give positive lactose tests,



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except those that gave acid (yellow) slants in TSI and positive alkaline (purple) reactions in LIA.

6.6.4.3. Purple fermentation broth base with 1.0% sucrose. Inoculate broth with small amount of slant growth from an 18 to 24-hour TSI culture. Cap tube loosely and incubate 42 to 48 hours at $35^{\circ} \pm 1^{\circ}$ C but examine after 18 to 24 hours. The majority of *Salmonella* species give a negative test, indicated by no gas formation in inner inverted Durham tube and purple color throughout the medium. Production of acid (yellow) with or without gas formation in inner inverted Durham tube should be interpreted as a positive reaction. Discard as non- *Salmonella*, cultures that give positive sucrose tests, except those that gave acid (yellow) slants in TSI and positive alkaline (purple) reactions in LIA.

6.6.5. Malonate broth

6.6.5.1. With a sterile 3 mm loop, inoculate malonate broth with a small amount of 18 to 24-hour growth from a TSI slant.

6.6.5.2. Incubate malonate broth 42 to 48 hours at $35^{\circ} \pm 1^{\circ}$ C but examine after 18 to 24 hours.

6.6.5.3. Some malonate positive bacteria produce only slight alkalinity (blue colored broth) due to the formation of small amounts of sodium hydroxide. Compare any tube in question with an uninoculated malonate broth tube. Any trace of blue in medium after a 42 to 48-hour incubation denotes a positive test.

6.6.5.4. The majority of *Salmonella* species cultures give a negative test (green or unchanged color) in the malonate broth after 42 to 48 hours incubation.

6.6.6. Indole, Voges-Proskauer (VP), methyl red, and citrate testing for *Salmonella* identification

6.6.6.1. Indole production test.

6.6.6.1.1. With a sterile 3 mm loop, inoculate tryptone broth with a small amount of 18 to 24-hour growth from a TSI slant. Incubate broth 18 to 24 hours at $35^{\circ} \pm 1^{\circ}$ C

6.6.6.1.2. Transfer 5 mL of 18 to 24-hour tryptone broth culture to empty test tube. Test for indole production by adding 0.2 to 0.3 mL Kovac's reagent.

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6.6.6.1.3. Most *Salmonella* cultures give negative test (lack of deep red color at surface of tryptone broth). Appearance of distinct red color in upper broth layer is positive test.

6.6.6.2. DrySlide™ indole test (optional)

6.6.6.2.1. With a sterile 3 mm loop, smear a suspected colony from the an appropriate agar plate (section 6.6) on one unused reaction cell area of the DrySlide™ indole containing a 5% p-dimethylaminobenzaldehyde (DMABA) formulation.

6.6.6.2.2. Most *Salmonella* cultures give negative test (no color change or light gray color). Appearance of distinct red color change within 30 seconds indicates an indole positive test.

6.6.6.3. Testing for Voges-Proskauer-reactive compounds and methyl red-reactive compounds

6.6.6.3.1. With a sterile 3 mm loop, inoculate a tube of MR-VP broth with a small amount of 18 to 24-hour growth from a TSI slant. Incubate broth 42 to 48 hours at 35° ±1° C.

6.6.6.3.2. Perform Voges-Proskauer (VP) test at room temperature by transferring 1 mL of 42 to 48-hour MR-VP broth culture to empty 13 x 100 mm tube. Test for VP-reactive compounds by adding 0.6 mL " -naphthol solution and 0.2 mL 40% KOH solution to broth, followed by shaking. Add a few crystals of creatine. Shake and let stand 4 hours. The development of pink-to-ruby red color or eosin pink color throughout medium is a positive test. The majority of *Salmonella* cultures are VP-negative. Incubate remainder of MR-VP broth an additional 48 hours at 35° ±1° C for methyl red test.

6.6.6.3.3. Perform methyl red test by transferring 5 mL of 90 to 96-hour MR-VP broth culture to empty 13 x 100 mm tube. Test for methyl red (MR)- reactive compounds by adding 5-6 drops of methyl red to MR-VP broth in tube. Read results immediately. Most *Salmonella* cultures give positive test, indicated by diffuse and distinct red color in broth. A distinct yellow color is a negative test.

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6.6.6.4. Testing for citrate utilization

6.6.6.4.1. With a sterile needle, inoculate Simmons citrate agar by streaking slant and stabbing butt with 18 to 24-hour growth from a TSI slant. Incubate Simmons citrate agar slant 96 ± 2 hours at $35^\circ \pm 1^\circ$ C with tube cap loosen.

6.6.6.4.2. After 96-hours incubation read results. A negative reaction is indicated by no growth to very little growth without change in medium color (remains green). A positive reaction is indicated by the presence of growth, usually accompanied by color change of medium from green to blue (alkaline reaction). Most cultures of *Salmonella* are citrate-positive (intense blue color).

6.7. Serological Tests

6.7.1. Polyvalent somatic (O) test

6.7.1.1. Use *Salmonella* polyvalent somatic (O) antiserum, Poly A-I and Vi for the poly O test and follow the manufacturer's instructions that are enclosed with the antiserum which include the procedures for proper rehydration.

6.7.1.2. Using a wax marking pencil, make two circles approximately 2 cm in diameter on a glass slide. An agglutination ring slide or Petri dish may also be used.

6.7.1.3. Place 1 drop (approximately 3 μ L) of sterile 0.85% physiological saline in the upper part of each circle. Transfer a portion of culture growth from an 18 to 24-hour nutrient slant to both drops and mix each thoroughly. (Be sure to test a positive and negative culture along with the unknowns.)

6.7.1.4. Add 1 drop of sterile saline solution to lower part of one circle only. Add 1 drop of *Salmonella* polyvalent somatic (O) antiserum to other circle only. With clean sterile transfer loop or needle, mix culture suspension with saline solution for one circle and repeat for other circle containing antiserum. The circle containing only saline and culture will serve as an autoagglutination control.



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6.7.1.5. Rock the mixtures in a back-and-forth motion on the slide for 1 minute and observe against dark background in good illumination. Consider any degree of agglutination as a positive reaction. All results must be read within 1 minute and classify polyvalent somatic (O) test reactions as follows:

- 6.7.1.5.1. Positive - agglutination in test mixture and positive culture control and no agglutination in saline control or negative culture control.
- 6.7.1.5.2. Negative - no agglutination in test mixture and no agglutination saline control; agglutination in positive culture control and no agglutination in negative culture control.
- 6.7.1.5.3. Nonspecific - agglutination in both test and saline control mixtures. Purify such cultures on appropriate agar; repeat TSI/LIA, and agglutination tests. If the repeat isolate produces the same reaction, the culture is autoagglutinable and is most likely a rough strain. A further repeat of the poly O test has no value, so identify such a culture by the biochemical reactions and the polyvalent H agglutination testing.

6.7.2. Polyvalent flagellar (H) test.

- 6.7.2.1. Use *Salmonella* polyvalent flagellar (H) antiserum, Poly a-z for the poly H test. Follow the manufacturer's instructions that are enclosed with the antiserum which include the procedures for properly rehydrating, for preparing the antiserum dilution, and for determining the antiserum volume required for testing.
 - 6.7.2.2. Perform the polyvalent flagellar (H) test by inoculating growth from each 18 to 24-hour TSI agar slant into either (a) Brain-Heart Infusion (BHI) broth for same day testing or (b) trypticase soy broth (TSB) for next day testing.
 - 6.7.2.3. Incubate BHI cultures at $35^{\circ} \pm 1^{\circ}$ C for 4-6 hours or until growth has a density to match a three (3.0) McFarland turbidity standard following thorough vortex mixing
 - 6.7.2.4. Incubate TSB cultures at $35^{\circ} \pm 1^{\circ}$ C for 18 to 24 hours or until growth has a density to match a three (3.0) McFarland turbidity standard following thorough vortex mixing.
-

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6.7.2.5. Add 2.5 ml formalinized physiological saline solution to 5 ml of either BHI broth or TSB culture following incubation. The formalinized broth culture (antigen) must stand for 30 minutes to 1 hour prior to continuing this test.

6.7.2.6. 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 and add 0.5 mL antigen to be tested. (Be sure to set up tubes for the positive and negative control cultures.) Prepare saline control in another test tube by mixing 0.5 mL formalinized physiological saline solution with 0.5 mL formalinized antigen.

6.7.2.7. Incubate mixtures in test tubes at 48 to 50°C in water bath. Observe reactions in tubes at 15-minute intervals and read final results in 1 hour.

6.7.2.8. **Caution:** Do not shake the tubes. Record at the end of 1 hour the “positive” and “negative” results based upon the following guidelines for interpretation:

6.7.2.8.1. Positive -- agglutination in test mixture and no agglutination in saline control; agglutination in positive control culture and no agglutination in negative control culture.

6.7.2.8.2. Negative -- no agglutination in test mixture and no agglutination in saline control; agglutination in positive control culture and no agglutination in negative control culture.

6.7.2.8.3. Nonspecific - agglutination in both test and control mixtures. Purify cultures on appropriate agar; repeat TSI/LIA, and agglutination tests.

6.8. Interpretation of Analyses

6.8.1. An isolated culture identification as a confirmed positive *Salmonella* is based on all biochemical and serological reactions obtained through testing in sections 6.5, 6.7, and 6.8. These biochemical and serological reactions and the guidelines for classification of cultures as typical *Salmonella* are summarized in the following table:



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Table 1. Biochemical and serological reactions of *Salmonella*

Test or substrate	Result		<i>Salmonella</i> species reaction ^a
	Positive	Negative	
1. Glucose (TSI)	yellow butt	red butt	+
2. Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3. H ₂ S (TSI and LIA)	blackening	no blackening	+
4. Urease	purple-red color	no color change	-
5. Lysine decarboxylase broth	purple color	yellow color	+
6. Purple 0.5% dulcitol broth	yellow color and/or gas	no gas; no color change	+ ^b
7. Malonate broth	blue color	no color change	- ^c
8. Indole test	violet color at surface	yellow color at surface	-
9. Polyvalent flagella test	agglutination	no agglutination	+
10. Polyvalent somatic test	agglutination	no agglutination	+
11. Purple 1% lactose broth	yellow color and/or gas	no gas; no color change	- ^c
12. Purple 1% sucrose broth	yellow color and/or gas	no gas; no color change	-
13. Voges-Proskauer test	Pink-to-red color	no color change	-
14. Methyl red test	diffuse red color	diffuse yellow color	+
15. Simmons citrate	growth; blue color	no growth; no color	v

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Test or substrate	Result		<i>Salmonella</i> species reaction ^a
	Positive	Negative	
		change	
16. DrySlide™ indole test (optional)	red color within 30 sec.	no color change or light gray color	
^a +, 90% or more positive in 1 or 2 days; -, 90% or more negative in 1 or 2 days; v, variable. ^b Majority of <i>S. arizonae</i> cultures are negative. ^c Majority of <i>S. arizonae</i> cultures are positive.			

6.8.2. The following will serve as a guide for interpretation of biochemical and serological results for cultures:

6.8.2.1. Both *Salmonella* polyvalent “H” and polyvalent “O” antisera have agglutination and the biochemicals are typical: The culture is a *Salmonella*.

6.8.2.2. The *Salmonella* polyvalent “H” antiserum agglutinates, but not the polyvalent “O” and the biochemicals are typical: The culture is a *Salmonella*.

6.8.2.3. The *Salmonella* polyvalent “O” antiserum agglutinates, but not the polyvalent “H” and the biochemicals are typical: The culture is a *Salmonella*.

6.8.2.4. Both *Salmonella* polyvalent “H” and polyvalent “O” antisera do not agglutinate and the biochemicals are not typical: The culture is not a *Salmonella*.

6.8.2.5. Both *Salmonella* polyvalent “H” and polyvalent “O” antisera do not agglutinate and the biochemicals are typical confirm by molecular methods.

6.9. Quality Assurance

6.9.1. Allow all refrigerated or frozen cultures, media, and reagents to come to room temperature prior to use.

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- 6.9.2. Store all media and reagents under the proper conditions as specified in SOP LABOP-03.
 - 6.9.3. Adhere to aseptic technique when handling all samples, cultures, reagents, media, and materials in the laboratory.
 - 6.9.4. Always run a positive, negative, and an uninoculated media control to ensure all media and method performance. Use a bacterial culture control that provides a reliable positive urease production test in urea broth or rapid urea broth. *Proteus vulgaris* ATCC 13315 or equivalent stock bacterial culture is to be used as the positive control for the urease production test. Urea broth medium is positive for *Proteus*, *Morganella morganii*, *Providencia rettgeri* and a few *Providencia stuartii* strains.
 - 6.9.5. Always run a positive and negative culture control to ensure the test performances of both *Salmonella* polyvalent “H” and *Salmonella* polyvalent “O” antisera.
 - 6.9.6. Make sure that all waste biohazardous products generated from this testing procedure are properly treated.
- 6.10. Transport and Storage of Cultures
- 6.10.1. Refer to appropriate SOPs on archiving and shipping of isolates.
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Shanker Reddy

12/18/03

Prepared By: Shanker Reddy, Ph.D.
Microbiologist, Monitoring Programs Office
8609 Sudley Road, Suite 206
Manassas, VA 20110
(703) 330-2300

Date

Grace Hall

12/22/03

Approved by: Grace Hall, Chairperson
MDP Technical Advisory Committee
Florida Department of Agricultural and Consumer Services
Food Laboratory, Bldg. 9
3125 Conner Blvd.
Tallahassee, Florida 32399-1650
(850) 488-4407

Date

Diana Haynes

12/29/03

Approved By: Diana Haynes
Technical Director, Microbiological Data Program
8609 Sudley Road, Suite 206
Manassas, VA 20110
(703) 330-2300

Date

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Revision 01

May 15, 2003

Technical Advisory Committee

- Updated references
 - Changed from selenite cystine (SC) broth to Rappaport-Vassiliadis (RV) medium; added recommendation for use of Oxoid RV Broth
 - Removed reference to SOP MDP-QA-01
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