SALMONELLA SPECIES
PRESUMPTIVE AND CONFIRMATION TESTS

PRINCIPLE

Enrichment and selective procedures are used to provide a reasonably sensitive, definitive and versatile means of qualitatively isolating all members of the genus *Salmonella*. The presumed positive isolates are then confirmed by biochemical and serological tests. Caution must be exercised when applying the method since *Salmonella* are pathogenic bacteria.

SCOPE

The method is applicable to starches, syrups and sugars and most coproducts of the corn wet milling industry.

MEDIA AND REAGENTS

1. Lactose Broth (LB)

2. Selenite-Cystine (SC) Broth

3. Tetrathionate (TT) Broth Base with iodine. Dissolve 5 g of potassium iodine in 5 mL of sterile distilled water. Add 6 g of iodine crystals and stir to dissolve completely. Dilute to 20 mL with sterile water.

4. Xylose-Lysine-Deoxycholate (XLD) Agar

5. Bismuth Sulfite (BS) Agar

6. Hektoen Enteric (HE) Agar

7. Triple Sugar Iron (TSI) Agar

8. Lysine Iron Agar (LIA)

9. Urea Broth Concentrate (10x)

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10. Brain Heart Infusion (BHI) Broth

11. *Salmonella* Polyvalent Somatic (O) Antiserum

12. *Salmonella* Polyvalent Flagellar (H) Antiserum

13. Saline Solution (sterile). 0.85% Sodium Chloride

14. Formalinized Saline Solution. Add 6 mL formaldehyde solution (36-38%) to 1 L sterile Saline Solution. Do not autoclave.

Prepare the above media following the manufacturer's directions.

PROCEDURE

A. Sample Preparation and Pre-enrichment:

Weigh 25 g of sample into a 500 mL flask containing 225 mL of sterile Lactose Broth (1000 mL flask and 450 mL Lactose for a pre-gelatinized starch). Shake the contents until thoroughly dispersed. Let stand at room temperature for 60 mins. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.6 to 7.0 with sterile 1*N* NaOH or HCl. Mix well, and determine final pH. Loosen cap and incubate at 35-37°C for 22-26 hrs.

B. Selective Enrichment:

Mix the flask by gentle shaking. Pipet 1 mL portions into two tubes containing, respectively, 10 mL of Selenite-Cystine Broth and Tetrathionate Broth, mix and incubate at 35-37°C for 22-26 hrs.

C. Isolation:

Vortex-mix and streak portions from both the Selenite-Cystine and Tetrathionate Broths onto the surface of XLD Agar, Hektoen Enteric Agar, and Bismuth Sulfite Agar with a 3 mm inoculating loop. Cover and invert

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the dishes, and incubate at 35-37°C for 22-26 hrs.

D. Examination for Typical or Suspicious *Salmonella* Colonies:

After incubation examine the dishes for colonies which conform to the following descriptions.

1. XLD Agar - Pink colonies with or without black centers. Many *Salmonella* colonies may have 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.

2. HE Agar - Blue-green to blue colonies with or without black centers. Many *Salmonella* cultures may have large glossy black centers or may appear as almost completely black colonies.

3. BS Agar - Brown, gray or black colonies sometimes with metallic sheen. Surrounding medium is usually brown at first, turning black with increasing incubation time. Some strains produce green colonies with little or no darkening of surrounding medium. If BS plates have no colonies typical of *Salmonella* or no growth, incubate the plates for an additional 24 hrs. and examine again (Note 1).

E. Treatment of Typical or Suspicious Colonies:

Select two or more suspicious colonies, if present, from each XLD, HE and BS plate having growth. Inoculate TSI slant with a portion of each colony by streaking slant and stabbing butt. Without flaming, inoculate LIA slant by stabbing butt twice and then streaking slant. Retain plates at 5-8°C. Incubate TSI Agar at 35-37°C for 22-26 hrs. Incubate LIA at 35-37°C for 46-50 hrs. (Note 2).

F. Examination of TSI and LIA Slants for Presumptive (+) Cultures:

1. TSI Agar: Presumptive (+) cultures have alkaline (red) slants and acid (yellow) butts, with or without H_2S production (blackened

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agar). Do not exclude H_2S negative slants.

2. LIA: Presumptive (+) cultures have an alkaline (purple) slants and alkaline butts. Consider only a distinct yellow coloration in the butt as an acid (negative) reaction.

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 alkaline slant and acid butt in TSI should also be considered potential *Salmonella* isolates. Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as non-*Salmonella* (Note 1).

G. Confirmation of *Salmonella*:

If slant reactions are presumed-positive, confirm all suspected colonies (6 minimum) by the biochemical and serological tests as positive *Salmonella*. However, a complete serological typing of the isolates is not required.

1. Urease Test: Transfer all presumed-positive cultures grown on TSI slants to tubes of urea broth and incubate at 35-37 °C for 22-26 hrs. Cultures that are urease positive (purple-red), regardless of other reactions, are not *Salmonella* suspects. Retain all urease negative cultures for further study.

2. Biochemical Test: Biochemical confirmation of *Salmonella* on all urease negative cultures may be carried out by the alternative biochemical identification kits approved by AOAC.

3. Polyvalent Flagellar (H) Test: Inoculate growth from each urease-negative TSI slant into 5 mL BHI broth and incubate 4-6 hrs. at 35-37°C until visible growth occurs. Add 2.5 mL formalinized saline solution to the broth culture.

   Select two formalinized broth cultures and test with *Salmonella* polyvalent flagellar (H) antisera. Add 0.5 mL of formalinized culture to 0.5 mL of polyvalent flagellar (H) antiserum in a small test

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tube (10 × 75 mm). Prepare saline control by mixing 0.5 mL formalinized saline with 0.5 mL antiserum.

Incubate mixtures in a 48-50°C water bath and observe agglutination at 15 min. intervals. Read final results after one hr.

Result	Test	Control
Positive	Agglutination	No agglutination
Negative	No agglutination	No agglutination
Non-specific	Agglutination	Agglutination

4. Polyvalent somatic (O) Test: Using a wax pencil mark off test and control sections (about 1 cm square) on a glass slide. Prepare a heavy suspension by emulsifying a loopful of culture from the presumed-positive TSI slant in 1 mL saline solution.

   Place 1 drop of the polyvalent O antiserum on the test section and 1 drop of the saline solution on the control section. Transfer a loopful of culture suspension to the saline drop. Flame the loop and transfer a second loopful of the suspension to the antiserum section. Tilt the slide in a back-and-forth motion for 1 min. Read the slide and consider any degree of agglutination as a positive reaction. Classify polyvalent somatic (O) test results as described above for the polyvalent II test.

Once a single colony has been identified as *Salmonella* by the biochemical and serological tests, additional colonies need not be tested (Note 3).

NOTES AND PRECAUTIONS

1. Isolates should be sent out to an experienced microbiological lab for confirmation. In the absence of typical colonies, several atypical colonies should be picked from plates and sent to a qualified experienced lab for confirmation as recommended by *BAM* (current edition).

2. If cultures appear to be mixed, they must be restreaked and isolated after incubation at 35-37°C for 24 hrs.

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3. No isolate is considered as *Salmonella* unless it contains somatic O and/or flagellar H antigens. Biochemical reactions alone are not sufficient for classifying an isolate as *Salmonella.*

REFERENCE

Bacteriological Analytical Manual, http://www.cfsan.fda.gov.

Compendium of Methods for the Microbiological Examination of Foods, Current Edition, American Public Health Association.