



## Laboratory Guidebook Notice of Change

Chapter new, **revised**, or archived: MLG 4.04

Title: Isolation and Identification of *Salmonella* from Meat, Poultry, and Egg Products

Effective Date: 2/4/08

Description and purpose of change(s):

An alternative test for performing the flagellar (H) antigen agglutination test was validated. A section describing sample pooling was removed.

**The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use.**

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## **4.1 Introduction**

### **4.1.1 General**

This method describes the analysis of various meats, meat products, sponge and rinse samples, eggs, and egg products for *Salmonella*. It is not intended for the isolation and identification of *Salmonella* Typhi.

Success in isolating *Salmonella* from any food can be related to a number of factors including food preparation procedures, the number of organisms present, sample handling after collection, etc. With raw meat samples the competitive flora may be the most important factor. It varies from sample to sample and from one kind of meat to another.

Another consideration is whether the examination is for routine monitoring or epidemiological purposes. The analyst may choose to augment the method for epidemiological purposes with additional enrichment procedures and culture media, two temperatures of incubation, intensified picking of colonies from plates, and/or rapid screening methods.

All isolates must be identified as *Salmonella* biochemically and serologically.

Unless otherwise stated all measurements cited in this method have a tolerance range of  $\pm 2\%$ .

### **4.1.2 Limits of Detection**

The *Salmonella* detection limit for this method has been determined to be less than 1 colony forming unit (cfu)/g in a 25 g sample.

## **4.2 Safety Precautions**

*Salmonella* are generally categorized as BioSafety Level 2 pathogens. CDC guidelines for manipulating Biosafety Level 2 pathogens should be followed whenever live cultures of *Salmonella* are used. A Class II laminar flow biosafety cabinet is recommended for procedures in which infectious aerosols or splashes may be created. All available Material Safety Data Sheets (MSDS) must be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the material should read all MSDS sheets.

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### **4.3 Quality Control Procedures**

#### **4.3.1 Method Controls**

Include at least three method controls in all analyses. These controls must include a *Salmonella* spp. H<sub>2</sub>S-negative culture, a *Salmonella* spp. H<sub>2</sub>S-positive culture and an uninoculated media control. To facilitate identification of control isolates, the laboratory may use strains of uncommonly found serogroups. *S. Abaetetuba*, serogroup F, is suggested as a readily available, H<sub>2</sub>S-positive culture that is not commonly found in meats or meat products. *Salmonella* serotype Choleraesuis is typically negative for H<sub>2</sub>S production. These cultures may be obtained from ATCC. Other serotypes may be found that have aberrant H<sub>2</sub>S-negative strains. The control cultures should be inoculated into either a meat matrix or the matrix that is being analyzed. Incubate the controls along with the samples, and analyze them in the same manner as the samples. Confirm at least one isolate from each positive control sample. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses.

#### **4.3.2 Specific Procedure Controls**

The biochemical and serological tests used for confirmation of the sample isolates require the use of appropriate controls to verify that the results are valid. *Salmonella* 'O' antisera should be tested with QC control sera before initial use, and with a saline control for each test. Biochemical kit and rapid test manufacturers may specify control cultures for use with their products. If not specified, quality control procedures for biochemical tests and test media should include cultures that will demonstrate pertinent characteristics of the product.

### **4.4 Equipment, Reagents, Media and Test Kits**

All of the materials listed below may not be needed. Media and reagents specific to the biochemical test method that is used will be needed in addition to the materials listed below. See Section 4.7.

#### **4.4.1 Equipment**

- a. Sterile tablespoons, scissors, forceps, knives, glass stirring rods, pipettes, petri dishes, test tubes, bent glass rods ("hockey sticks") as needed
- b. Blending/mixing equipment: Sterile Osterizer-type blender with sterilized cutting assemblies, and blender jars or Mason jars and adapters for use with Mason jars; *or* a Stomacher™ (Tekmar or equivalent) with sterile Stomacher bags

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- c. Sterile Stomacher™ 3500 bags, plain, clear polypropylene autoclave bags (ca. 24" x 30 - 36"), or Whirl-Pak™ bags (or equivalent)
- d. Incubator, 35 ± 2°C
- e. Incubator or water bath, 42 ± 0.5°C
- f. Water bath, 48-50°C
- g. Glass slides, glass plate marked off in one-inch squares or agglutination ring slides
- h. Balance, 2000 g capacity, sensitivity of 0.1 g
- i. Inoculating needles and loops
- j. Vortex mixer
- k. VITEK® system

#### **4.4.2 Reagents**

- a. Crystal violet dye, 1% aqueous solution, steamed
- b. Butterfield's phosphate diluent
- c. Saline, 0.85%
- d. Saline, 0.85% with 0.6% formalin for flagellar antigen tests
- e. Calcium carbonate, sterile
- f. *Salmonella* polyvalent O antiserum
- g. *Salmonella* polyvalent H antiserum, Slide Agglutination H Antisera from Statens Serum Institut (SSI), or equivalent.
- h. *Salmonella* individual O grouping sera for groups A-I (antisera for further O groups are optional)
- i. (Optional) Oxoid *Salmonella* Latex Test (Unipath Company, Oxoid Division, Ogdensburg, NY) or equivalent
- j. Additional reagents as needed for biochemical tests: GNI cards for VITEK® system

#### **4.4.3 Media**

- a. Buffered peptone water (BPW)
- b. TT broth (Hajna)
- c. Modified Rappaport Vassiliadis (mRV) broth, Rappaport-Vassiliadis R10 broth, or Rappaport-Vassiliadis Soya Peptone Broth (RVS)
- d. Brilliant green sulfa agar (BGS; contains 0.1% sodium sulfapyridine)
- e. Xylose lysine Tergitol™ 4 agar (XLT4) or Double modified lysine iron agar (DMLIA)
- f. Triple sugar iron agar (TSI)

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- g. Lysine iron agar (LIA)
- h. Trypticase soy broth (TSB) or Tryptose broth
- i. Trypticase soy agar (TSA)
- j. Nutrient agar slants
- k. Nutrient broth, semi-solid
- l. Tryptic soy agar with 5% sheep blood agar
- m. Additional media as needed for biochemical tests

#### **4.4.4 Cultures**

At least one H<sub>2</sub>S-positive strain of *Salmonella* and one H<sub>2</sub>S-negative strain of *Salmonella* are required for method controls.

### **4.5 Isolation Procedures**

#### **4.5.1 Breeding Mixes, Dehydrated Sauces and Dried Milk**

For dehydrated sauces, dried milk, and breeding mixes add BPW as described for powdered egg in Section 4.5.8.

#### **4.5.2 Ready-to-Eat Foods**

Follow program requirements for preparing sample and sub-sample composites. Outbreak investigation requirements may differ; in which case, follow the client specifications for those samples.

- a. Weigh 325 g of the composite sample into a Stomacher bag (or sterile blender jar if required by the client or sample type).
- b. Add approximately one third to half of 2925 ml of ambient temperature sterile buffered peptone water. Blend or stomach approximately 2 minutes, and then add the remainder of the 2925 ml of BPW.
- c. Incubate at 35 ± 2°C for 20-24 h.
- d. Transfer 0.5 ± 0.05 ml of incubated broth into 10 ml TT and 0.1 ± 0.02 ml into 10 ml of mRV broth.
- e. Incubate the enrichment broths at 42 ± 0.5°C for 22-24 h.

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- f. Streak above enrichments on BGS and either DMLIA or XLT4 agar plates. Use one 10-microliter loopful for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.
- g. Incubate at  $35 \pm 2^{\circ}\text{C}$ .
- h. Examine in 18-24 h. Select colonies. Refer to Section 4.6 et seq.
- i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies as above. Reserve, under refrigeration, all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

#### **4.5.3 Fermented Products**

Follow the procedure for ready-to-eat foods (Section 4.5.3) except:

- a. Blend/stomach the sample with 10 g of sterilized calcium carbonate.
- b. Use buffered peptone water that contains 1 ml of a 1% aqueous solution of crystal violet per liter.

#### **4.5.4 Raw Meat**

If the sample is not already ground, in some cases it may be best to mince it with scissors or leave it whole (e.g. chicken wings) to avoid jamming blender blades with skin or connective tissue. Whirl-Pak™ bags can be used in culturing these samples.

- a. Weigh  $25 \pm 0.5\text{g}$  of meat into a sterile blender jar, other sterile jar or a Whirl-Pak™ or Stomacher™ bag. HACCP program samples collected using a sampling ring are allowed a weight range of  $25 \pm 2.5\text{ g}$ .
- b. Add 225 ml of BPW. Stomach or blend, as required, for approximately two minutes or shake thoroughly. Alternatively, ground beef samples may be briefly stomached or hand mixed to disperse clumps.

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- c. Incubate at  $35 \pm 2^{\circ}\text{C}$  for 20-24 h.
- d. Transfer  $0.5 \pm 0.05$  ml into 10 ml TT broth and  $0.1 \pm 0.02$  ml into 10 ml mRV broth.
- e. Incubate at  $42 \pm 0.5^{\circ}\text{C}$  for 22-24 h or in a water bath at  $42 \pm 0.5^{\circ}\text{C}$  for 18-24 h.
- f. Streak on DMLIA or XLT4 and BGS agar plates. Use one loopful of inoculum for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.
- g. Incubate at  $35 \pm 2^{\circ}\text{C}$ .
- h. Examine in 18-24 h. Select colonies. See Section 4.6 et seq.
- i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies as above. Reserve, under refrigeration, all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

#### **4.5.5 Carcass Sponge Samples**

- a. Add 50 ml of BPW to the sample bag containing the moistened sponge to bring the total volume to 60 ml. Mix well.
- b. Incubate at  $35 \pm 2^{\circ}\text{C}$  for 20-24 h.
- c. Follow the procedures in Section 4.5.5, d-i.

#### **4.5.6 Whole Bird Rinses**

Due to differences between sample types/sizes (e.g. chicken vs. turkey carcasses), follow instructions given in the specific program protocol. For chicken carcasses, aseptically drain excess fluid from the carcass and transfer the carcass to a sterile Stomacher™ 3500 bag, a plain, clear polypropylene bag (ca. 24" x 30-36"), or equivalent. Pour 400 ml (or other volume specified in program protocol) of Buffered Peptone Water (BPW) into the cavity of the carcass contained in the bag. Rinse the bird inside and out with a rocking motion for one minute (ca. 35 RPM). This is done by grasping the broiler carcass in the bag with one hand



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and the closed top of the bag with the other. Rock with a reciprocal motion in about an 18-24 inch arc, assuring that all surfaces (interior and exterior of the carcass) are rinsed. Transfer the sample rinse fluid to a sterile container.

Use 30 ml of the sample rinse fluid obtained above for *Salmonella* analysis. Add 30 ml of sterile BPW, and mix well. Incubate at  $35 \pm 2^{\circ}\text{C}$  for 20-24 h, and then proceed according to 4.5.5 (d-i).

NOTE: If analyses other than *Salmonella* are to be performed, the carcass may be rinsed in Butterfield's Phosphate Diluent instead of BPW. In this case, add 30 ml of 2X BPW to 30 ml of carcass-rinse fluid, mix well, and continue as above.

#### **4.5.7 Liquid, Frozen, Cooked or Dried Egg Samples**

- a. Mix the sample with a sterile spoon, spatula, or by shaking.
- b. Aseptically weigh a minimum of 100 g of egg sample into a sterile blender jar, other sterile jar, or a Whirl-Pak™ or Stomacher™ bag containing 900 ml of sterile BPW. If a special sample or specification requires a sample size other than 100 g, the ratio of egg sample to BPW is to be maintained at 1:10.
- c. Mix the inoculated BPW well by shaking, stomaching, or blending.
- d. With dried egg samples, gradually add BPW to the sample. Add a small portion of sterile BPW and mix to obtain a homogeneous suspension. Add the remainder of the BPW. Mix until a lump-free suspension is obtained.
- e. Incubate at  $35 \pm 2^{\circ}\text{C}$  for 20-24 h, and then proceed according to 4.5.5 (d-i).

#### **4.5.8 Sanitation Series Food Homogenates (optional)**

To isolate salmonellae from food samples homogenized as outlined in MLG 3 Section 3.3.1, as part of a sanitation test series, use the  $10^{-1}$  food homogenate dilution (See also this chapter, Section 4.5.1 Sample Pooling).

- a. Weigh 250 g of food homogenate into a sterile jar (this contains 25 g of product).
- b. Add 25 ml of 10x BPW (broth made to ten time's normal strength).

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- c. Incubate 24-26 h at  $35 \pm 2^{\circ}\text{C}$ .
- d. Transfer  $0.5 \pm 0.05$  ml into 10 ml of TT broth and  $0.1 \pm 0.02$  ml into 10 ml of mRV broth.
- e. Incubate at  $42 \pm 0.5^{\circ}\text{C}$  for 22-24 h.
- f. Streak on DMLIA or XLT4 and BGS agar plates. Use one loopful for each plate. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.
- g. Incubate 18-24 h at  $35 \pm 2^{\circ}\text{C}$ .
- h. Select colonies. See Section 4.6 et seq.
- i. Re-incubate all plates for an additional 18-24 h. Reexamine initially negative plates and pick colonies per Section 4.6. Reserve all plates from which colonies were picked. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation. See Section 4.6.1.b.

#### **4.5.9 Most Probable Numbers (MPN) Determination**

Follow MPN instructions given in the specific program protocol or see MLG Appendix 2, Most Probable Number Procedure and Tables.

### **4.6 Examination of and Picking Colonies from Plating Media**

#### **4.6.1 Picking Colonies**

- a. After the recommended incubation interval, examine the selective-differential agar plates for the presence of colonies meeting the description for suspect *Salmonella* colonies. Pick well-isolated colonies.
  - BGS. Select colonies that are pink and opaque with a smooth appearance and entire edge surrounded by a red color in the medium. On very crowded plates, look for colonies that give a tan appearance against a green background.

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- XLT4. Select black colonies or red colonies with or without black centers. The rim of the colony may still be yellow in 24 h; later it should turn red.
  - DMLIA. Select purple colonies with or without black centers. Since salmonellae typically decarboxylate lysine and ferment neither lactose nor sucrose, the color of the medium reverts to purple.
- b. Pick up to three colonies from each plate, if available. (NOTE: Before any sample is reported as *Salmonella*-negative, a total of three typical colonies, if available, from each selective agar plate must be examined). Pick only from the surface and center of the colony. Avoid touching the agar because these highly selective media suppress growth of many organisms that may be viable.

If there are typical colonies on a plate, that are not well isolated, pick from the typical colonies and re-streak directly to selective agar plates. Alternatively, place a loopful of growth into a tube of TT or mRV broth and incubate overnight, then re-streak to selective agars.

#### **4.6.2 Screening Media**

- a. Inoculate TSI and LIA slants in tandem with a single pick from a colony by stabbing the butts and streaking the slants in one operation. If screw cap tubes are used, the caps must be loosened. Incubate at  $35 \pm 2^\circ\text{C}$  for  $24 \pm 2$  h.
- b. Examine TSI and LIA slants as sets. Note the colors of butts and slants, blackening of the media and presence of gas as indicated by gas pockets or cracking of the agar. Note also the appearance of the growth on the slants along the line of streak. Discard, or re-streak for isolation, any sets that show "swarming" from the original site of inoculation. Discard sets that show a reddish slant in lysine iron agar. Isolates giving typical *Salmonella* spp. reactions and isolates which are suggestive, but not typical of *Salmonella* spp. should be confirmed by a combination of biochemical and serological procedures. Refer to Table 1 for a summary of TSI-LIA reactions. The motility testing in the last column of the table is optional.
- c. (Optional: for some biochemical test kits) Streak a TSA + 5% sheep blood agar plate from either the TSI or LIA slant. Incubate 18-24 h at  $35 \pm 2^\circ\text{C}$ .

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#### 4.7 Biochemical Procedures

Commercially available biochemical test kits, including automated systems may be used for biochemical identification. If the VITEK<sup>®</sup> system is used, the cytochrome oxidase and gram stain tests are optional. Alternatively, use traditional methods of biochemical identification. Refer to AOAC Official Method 967.27 or "Edwards and Ewing's Identification of Enterobacteriaceae", 4th Edition, for biochemical reactions of *Enterobacteriaceae* and for fermentation media and test procedures.

Table 1.

Triple Sugar Iron Agar			Lysine Iron Agar		Polyvalent Sera		Disposal
Butt	Slant	H <sub>2</sub> S	Butt	H <sub>2</sub> S	O	H	
Y	R	+	P	+	+	+	B. & M. T.
Y	R	+	P	+	+	-	B. & M. T.
Y	R	-	P	-			B. & M. T.
Y	R	-	Y	-	+	+	* B. & M. T.
Y	R	-	Y	-	-	-	Discard
Y	R	+	Y	+/-			B. & M. T.
Y	Y	-	Y or P	-			Discard
Y	Y	+	P	+			** B. & M. T.
NC	NC						Discard

Y = Yellow; R = Red; P = Purple; B. & M. T. = Biochemical and motility tests; NC = No change in color from uninoculated medium.

\* *Salmonella* Typhisuis (found seldom in swine in U.S.)

\*\* *Salmonella enterica* subsp. *arizonae* or *S. enterica* subsp. *diarizonae*

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## **4.8 Serological Tests**

### **4.8.1 Somatic (O) Antigen Agglutination Tests**

At a minimum, isolates should be tested with polyvalent O antiserum reactive with serogroups A through I. Following a positive reaction with polyvalent O antiserum, it is necessary to type the isolate using individual *Salmonella* antisera for O groups A through I. Testing for O groups A through I should encompass the majority of the *Salmonella* serotypes commonly recovered from meat and poultry products. Occasionally, however, an isolate will be recovered which is typical of *Salmonella* biochemically and is serologically poly H-positive, but is non-reactive with antisera to groups A through I. Such an isolate should be reported as "*Salmonella* non A-I" or "*Salmonella* O group beyond I".

Use growth from either the TSI or LIA slant. Test first with polyvalent O antiserum. Include a saline control with each isolate. If there is agglutination with the saline control alone (autoagglutination), identify such a culture by biochemical reactions only. If the saline control does not agglutinate and the polyvalent serum does, test the culture with *Salmonella* O grouping antisera. Record positive results and proceed to H agglutination tests.

### **4.8.2 Flagellar (H) Antigen Agglutination Tests**

Inoculate trypticase soy broth or tryptose broth. Incubate at  $35 \pm 2^{\circ}\text{C}$  overnight or until growth has an approximate density of three on the McFarland scale. Add an equal amount of saline containing 0.6% formalin and let sit one hour. Remove one ml to each of two 13 x 100 mm test tubes. To one of the tubes, add *Salmonella* polyvalent H serum in an amount indicated by the serum titer or according to the manufacturer's instructions. The other tube serves as an autoagglutination control. Incubate both tubes at 48-50°C in a water bath for up to 1 h. Record presence or absence of agglutination.

If desired, use Spicer-Edwards pooled serum or H typing serum. Find details in "Edwards and Ewing's Identification of Enterobacteriaceae" (Ewing, 1986).

The Oxoid *Salmonella* Latex Test, SSI H Antisera for Slide Agglutination, or equivalent, may be used as an optional method for H antigen agglutination testing. Follow the manufacturer's instructions. If a suspect *Salmonella* isolate is negative by the agglutination test, perform the poly H tube agglutination test described above.

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#### **4.9 Storage of Cultures**

Do not store cultures on TSI agar because this tends to cause roughness of O antigens. For short-term (2-3 months) storage, inoculate a nutrient agar slant, incubate at  $35 \pm 2^{\circ}\text{C}$  overnight and then store at  $2-8^{\circ}\text{C}$ .

Store "working" *Salmonella* stock cultures on nutrient agar slants. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at  $35 \pm 2^{\circ}\text{C}$ , and then store them at  $2-8^{\circ}\text{C}$ . Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the opportunity for contamination. Cultures may be subcultured up to 5 times. After this period the culture must be re-confirmed biochemically or a new culture initiated.

For long term storage freeze cultures using cryo-beads, i.e. Cryostor™ or equivalent, lyophilize or use the procedure that follows. Subculture *Salmonella* isolates by picking a colony with an inoculating needle and stabbing it into semi-solid nutrient broth (0.75% agar). Incubate at  $35 \pm 2^{\circ}\text{C}$  overnight, and then seal with hot, paraffin-soaked corks. Household wax is better than embedding paraffin because it stays relatively soft at room temperature making the corks easy to remove. Store the cultures in the dark at room temperature. Such cultures will remain viable for several years.

#### **4.10 Selected References**

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