CHAPTER 4. ISOLATION AND IDENTIFICATION OF *SALMONELLA* FROM MEAT, POULTRY, AND EGG PRODUCTS (Revision # 1; 1/10/01)

Bonnie E. Rose

4.1 Introduction

The reference method found in the Microbiological Methods section of "Official Methods of Analysis of AOAC International" (1995) is the American standard and is the point of departure for the procedures given below. Follow AOAC (16th ed.) 17.9.01 et seq. with additions and exceptions as given below.

Success in isolating salmonellae 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 and 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.

In previous editions of the Microbiology Laboratory Guidebook, this chapter contained a rather lengthy discussion of the media used for *Salmonella* isolation and identification. It was intended to instruct laboratory workers who had no previous experience in this type of work. This section is omitted in the present edition but the material is available in manuals of microbiological culture media which can be obtained from various commercial sources of culture media.

4.2 Equipment, Reagents, Media and Test Kits

4.21 Equipment

a. Sterile tablespoons, scissors, forceps, knives, glass stirring rods, pipettes, petri dishes, test tubes, bent glass rods ("hockey sticks")
b. Sterile pint, quart, and two-quart Mason jars with screw-on lids
c. Sterile Stomacher 3500 bags (or equivalent) or plain, clear polypropylene autoclave bags (ca. 24" x 30 - 36")
d. Incubator, 35 ± 1°C
e. Incubator, 42 ± 0.5°C

f. Sterile Osterizer-type blender with sterilized cutting assemblies and adapters for use with Mason jars, or Stomacher™ (Tekmar) with sterile Stomacher bags
g. Whirl-Pak™ bags
h. Water bath, 48-50°C
i. Glass slides, glass plate marked off in one-inch squares or agglutination ring slides
j. Balance, 2000 g capacity, sensitivity of 0.1 g
k. Inoculating needles and loops
l. Vortex mixer

4.22 Reagents

a. Brilliant green dye, 1% aqueous solution, steamed
b. Crystal violet dye, 1% aqueous solution, steamed
c. Iodine solution for TT broth
d. Butterfield's phosphate diluent
e. Methyl red reagent
f. O'Meara's V-P reagent, modified
g. Kovac's reagent
h. Ferric chloride, 10% aqueous solution
i. Sterile mineral oil
j. Saline, 0.85%
k. Saline, 0.85% with 0.6% formalin
l. Calcium carbonate
m. *Salmonella* polyvalent O antiserum
n. *Salmonella* polyvalent H antiserum
o. *Salmonella* individual O grouping sera for groups A-I (antisera for further O groups are optional)
p. Oxoid *Salmonella* Latex Test (Unipath Company, Oxoid Division, Ogdensburg, NY) or equivalent

4.23 Media

a. Buffered peptone water
b. TT broth (Hajna)
c. Rappaport Vassiliadis (RV) broth - Merck Chemical Co., cat. # 7700 (GENE-TRAK Systems, Inc., Framingham, MA) or equivalent
d. Selenite brilliant green sulfa broth (SBGS; optional)
e. Brilliant green sulfa agar (BGS; contains 0.1% sodium sulfapyridine)
f. Xylose lysine tergitol 4 agar (XLT4)

or

g. Double modified lysine iron agar (DMLIA)
h. Triple sugar iron agar (TSI)
i. Lysine iron agar
j. MR-VP medium
k. Tryptone broth
l. Simmons citrate agar

m. Phenol red tartrate agar
n. Motility medium
o. Christensen's urea agar
p. Carbohydrate fermentation media with Andrade's indicator
q. Decarboxylase test media (Moeller)
r. Malonate broth
s. KCN broth†
t. Phenylalanine agar
u. Nutrient gelatin
v. Trypticase soy broth
w. Tryptose broth
x. Trypticase soy agar
y. Lactate broth (1/2 strength trypticase soy broth containing 10^{-2} M neutralized lactic acid)

4.24 Commercially Available Test Kits (optional)

Any screening method under consideration for *Salmonella* testing must meet or exceed the following performance characteristics: sensitivity $\geq$ 97%, specificity $\geq$ 90%, false-negative rate $\leq$ 3%, and false-positive rate $\leq$ 10%.

4.3 Tests for Efficiency of Plating Media

It is recommended that each new lot of plating medium be tested for its efficiency in growing those salmonellae that represent the spectrum of serotypes that can occur in animals. To do this, dilute 18 h broth cultures of *Salmonella* Typhimurium, *S*. Choleraesuis, *S*. Gallinarum, and *S*. Pullorum 10^{-5}, 10^{-6}, and 10^{-7} in Butterfield's phosphate diluent. Dilute *S*. Typhisuis 10^{-4}, 10^{-5}, and 10^{-6}. For each dilution of each culture use three plates of test medium and three plates of trypticase soy agar. Inoculate each plate with 0.1 ml of culture dilution and spread the inoculum with sterile glass hockey sticks. Complete the plating within 15 min. after a dilution is made. The dilutions suggested usually result in at least one countable set of plates. If they do not, repeat the test on a new 18 h culture. Incubate the plates at 35°C for 48 h, then count the colonies and also measure the colony size.

$$\% \text{ efficiency} = \frac{\text{no. of colonies on plating medium}}{\text{no. of colonies on trypticase soy agar}}$$

Test all *Salmonella* plating media in this manner. An efficiency of 75% with *S*. Typhimurium is satisfactory. Colonies of most salmonellae will be larger and more numerous on the non-selective medium. Data from the four additional strains are not used to calculate efficiency ratings but serve to orient the worker to the appearance of the more fastidious organisms on different media.

4.4 Isolation Procedures

4.41 Sample Pooling

NOTE: Follow sample pooling instructions in specific program protocols. Otherwise, do not use sample pooling.

When examining products that are expected to be salmonella-free, valuable time, labor, and materials can be saved by sample pooling. There are several ways this can be done. Pooling at the non-selective enrichment step is appropriate when the likelihood of finding salmonellae is nil or when, if a positive is found, it is not important to know which particular sample contained the organism.

a. Those samples that are to be examined only for salmonellae may be pooled in the blenders. Up to 30 samples may be combined, depending on the capacity of the blenders and culture flasks. The proportions of sample volume to broth must be maintained at the 1 to 10 ratio (1 part sample to 9 parts broth). The broth should be pre-warmed to the temperature of incubation.

b. When food homogenates (Section 4.48) are pooled, the culture flask should be warmed in a water bath to bring the contents up to incubation temperature before placing it in the incubator.

c. Incubation of large pools should be prolonged to two days if growth is not apparent in one day. Subculture 5 ml of the incubated non-selective broth pool in 100 ml of selective enrichment, and proceed as usual.

In cases in which it is important to identify particular samples that may contain salmonellae, it is still possible to take advantage of labor-saving by pooling. In such cases, the samples may be started in the usual way in non-selective broth. After incubation, up to ten of these cultures may be pooled in selective enrichment broth. Maintain the 0.5 to 10 ratio for inoculation of TT broth and the 0.1 to 10 ratio for inoculation of RV broth. The remaining non-selective broths (or portions of them) are refrigerated. The total volume of selective enrichment broth used will be the same, but the number of plates to be streaked is reduced. If a positive pool is found, all the pooled samples are started individually in selective enrichment broth by going back to the refrigerated non-selective broths.

4.42 Breading Mixes, Dehydrated Sauces and Dried Milk

Refer to AOAC 17.9.02A. Use paragraph (a) for dehydrated sauces and breading mixes; if either contains onion or garlic powder in appreciable quantity, refer to paragraph (d). See also Section 4.49.

4.43 Ready-to-Eat Foods

a. Using equal portions from each subsample, weigh a 325 g composite sample into a sterile blender jar or Stomacher bag. The number of subsamples is usually 5 or 6 but it may be less than 5 or 6. Add 2925 ml sterile buffered peptone water. Blend or stomach 2 minutes.

b. Incubate at 35 ± 1°C for 20-24 h.

c. Transfer 0.5 ml of incubated broth into 10 ml TT and 0.1 ml into 10 ml RV broth.

d. Incubate the enrichment broths at 42 ± 0.5°C for 22-24 h.

e. Streak above enrichments on BGS and either DMLIA or XLT4 agar plates. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.

f. Incubate at 35 ± 1°C.

g. Examine in 18-24 h. Reincubate all plates for an additional 18-24 h. Reexamine initially negative plates and those yielding non-confirmed *Salmonella* colonies from the initial selection.

h. Select colonies - see Section 4.5 et seq.

4.44 Fermented Products

Follow the procedure for ready-to-eat foods (Section 4.43) except:

a. Use culture jars that contain about 10 g of sterilized calcium carbonate.

b. Use buffered peptone water that contains 1 ml of a 1% aqueous solution of crystal violet per L.

4.45 Raw Meat

Ordinarily, inoculate raw meat directly into non-selective broth. If meat is expected to have high numbers of gram-negative bacteria or

is odorous, refer to Section 4.49 (b). Use buffered peptone water as the non-selective medium.

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. Avoid culturing samples in the collection bag, because there may be punctures in the plastic.

a. Weigh 25 g of meat into a sterile blender jar, other sterile jar, or a Whirl-Pak™ or Stomacher™ bag.

b. Add 225 ml buffered peptone water. Blend two minutes or shake thoroughly.

c. Incubate at 35 ± 1°C for 20-24 h.

d. Transfer 0.5 ml into 10 ml TT broth and 0.1 ml into 10 ml RV broth.

e. Incubate at 42 ± 0.5°C for 22-24 h.

f. Streak on DMLIA or XLT4 and BGS agar plates. Do not subdivide plates for streaking multiple samples; streak the entire agar plate with a single sample enrichment.

g. Incubate at 35 ± 1°C.

h. Examine in 18-24 h. Reincubate all plates for an additional 18-24 h. Reexamine initially negative plates and those yielding non-confirmed *Salmonella* colonies from the initial selection.

i. Select colonies. See Section 4.5 et seq.

4.46 Whole Bird Rinses

Also see Section 3.32.

Due to differences between sample types/sizes (e.g. chicken vs. turkey carcasses), follow instructions given in the specific program protocol. Aseptically transfer the carcass to a sterile Stomacher™ 3500 bag (or equivalent) or a plain, clear polypropylene autoclave bag (ca. 24" x 30-36"), draining any excess fluid. Add 400 ml (or other volume specified in program protocol) Butterfield's Phosphate Diluent (BPD) to the carcass contained in the bag. Pour approximately half of the liquid into the interior cavity and half onto the outside of the carcass.

Rinse 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 and the closed top of the bag with the other. Rock with a reciprocal motion in a 18-24 inch arc, assuring that all surfaces (interior and exterior of the carcass) are rinsed.

Aseptically remove the carcass from the bag, draining excess rinsate, and transfer the rinsate to a sterile bottle. Measure the volume of the rinsate and add an equal amount of 2X buffered peptone water. Incubate at 35 ± 1°C for 20-24 h, and then proceed according to 4.45 (d-i).

NOTE: If *Salmonella* is the only analysis to be performed, the carcass may be rinsed in 1X buffered peptone water instead of BPD.

4.47 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 buffered peptone water. If a special sample or specification requires a sample size greater than 100 g, the ratio of egg sample to buffered peptone water is to be maintained at 1:10.

c. Mix the inoculated buffered peptone water well by shaking, stomaching, or blending.

d. With dried egg samples, gradually add buffered peptone water to the sample. Add a small portion of sterile buffered peptone water and mix with a suitable sterile stirrer to obtain a homogeneous suspension. Repeat this procedure three times and then add the remainder of the buffered peptone water. Stir until a lump-free suspension is obtained.

e. Incubate at 35 ± 1°C for 20-24 h, and then proceed according to 4.45 (d-i).

4.48 Food Homogenates

To isolate salmonellae from food samples homogenized as outlined in Section 3.31, use the 10^{-1} food homogenate dilution (See also Section 4.41 Sample Pooling).

a. Weigh 250 g of food homogenate into a sterile jar (this contains 25 g of product).

b. Add 26 ml of 10x buffered peptone water (broth made to ten times normal strength).

c. Incubate a full 24 h at 35 ± 1°C.

d. Transfer 0.5 ml into 10 ml TT broth and 0.1 ml into 10 ml RV broth.

e. Incubate at 42 ± 0.5°C for 22-24 h.

f. Streak on DMLIA or XLT4 and BGS agar plates. 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 ± 1°C. Reincubate all plates for an additional 18-24 h. Reexamine initially negative plates and those yielding non-confirmed *Salmonella* colonies from the initial selection (see Section 4.5 et seq.).

4.49 Special Cases

a. Sublethally Injured Bacteria. Bacteria that have been sublethally injured by dehydration, heat, prolonged freezing or freeze-drying are harder to revive. In such cases microbiologists, at their discretion, may substitute lactate broth for buffered peptone water as the non-selective enrichment.

b. High Gram-Negative Competitive Flora. Meat containing high numbers of gram-negative spoilage bacteria presents a special problem and should be analyzed by one of the following procedures:

   i. Add 0.5 ml of 1% aqueous brilliant green per L of non-selective broth. Incubate at 42 ± 0.5°C.

   ii. Make two successive transfers into TT broth and into SBGS broth and incubate TT at 42 ± 0.5°C and SBGS at 35 ± 1°C.

c. If salmonellae are not expected to be in an injured state, direct enrichment in TT broth may be used. Incubate at 35 ± 1°C for 18-24 h, then streak selective/differential agar plates. As a "back-up", make a second transfer into two tubes of TT broth (1 ml added to 10 ml of fresh TT broth). Incubate overnight at 42 ± 0.5°C and 35 ± 1°C.

d. Most Probable Numbers (MPN) Determination. Due to

differences between sample types (e.g. whole chicken rinse vs. ground beef) and sample sizes (e.g. 25 g vs. 325 g), follow MPN instructions given in the specific program protocol.

4.5 Examination of and Picking Colonies From Plating Media

a. Pick typical well-isolated colonies. Reincubate negative plates and reexamine them the following day.

i. 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.
ii. XLT4. Select black colonies or red colonies with black centers. The rim of the colony may still be yellow in 24 h; later it should turn red.
iii. 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 three colonies from each plate. 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 no well-isolated colonies on a plate, restreak directly to selective agar plates. Alternatively, place a loopful of growth into a tube of TT or RV broth and incubate overnight, then restreak to selective agars.

4.51 Screening Media

a. Inoculation:

Inoculate TSI and lysine iron agar 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 ± 1°C for 24 ± 2 h.

b. Examination:

Examine TSI and lysine iron agar 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 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 the following chart for these tests:

Triple Sugar Iron Agar			Lysine Iron Agar		Polyvalent Sera		Disposal
Butt	Slant	H_2S	Butt	H_2S	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/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*

4.6 Biochemical Procedures

For biochemical testing, inoculate the following media first: tryptone broth, MR-VP medium, Simmons citrate agar, Christensen's urea agar, motility test medium, phenol red tartrate agar, and glucose, lactose, sucrose, salicin and dulcitol fermentation broths. Incubate at 35 or 37°C and record reactions the following day. Test tryptone broth with Kovac's reagent for indol production in 24 h and, if negative, again in 48 h. Do not perform the MR-VP test until 48 h have elapsed. If results are ambiguous, repeat MR test after five days of incubation. Hold negative carbohydrate fermentation tests for 14 days.

Refer to "Edwards and Ewing's Identification of Enterobacteriaceae", 4th Edition, for biochemical reactions of *Enterobacteriaceae* and for fermentation media and test procedures.

Discard all cultures that give positive urea or VP reactions. Discard any culture that has the following combination of characteristics: produces gas in glucose, produces indol but not H_2S, is MR positive, VP negative and citrate negative; it is *E. coli* regardless of whether it ferments lactose in 48 h.

Inoculate further biochemical tests as necessary to eliminate other *Enterobacteriaceae*. Refer to Edwards and Ewing for details. Eliminate *Providencia* spp. by a positive phenylalanine reaction. Eliminate *Hafnia alvei* on the basis of the following biochemical pattern: indol negative; MR negative and VP and citrate positive based on four days of incubation at 25 ± 1°C; fermentation of arabinose and rhamnose; failure to ferment adonitol, inositol, sorbitol, and raffinose.

Commercially available biochemical test kits, including automated systems, may be used as optional methods for biochemical identification. Follow the manufacturer's instructions.

4.7 Serological Tests

All isolates identified biochemically as *Salmonella* should be tested serologically.

4.71 O 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 which is typical of *Salmonella* (biochemically and poly H serologically) but non-reactive with antisera to groups A through I will be recovered; such an isolate should be reported as "*Salmonella* non A-I" or "*Salmonella* O group beyond I".

Follow the manufacturer's instructions that are enclosed with the antisera. Use growth from either the TSI or lysine iron agar slant. Test first with polyvalent O antiserum. Do not read agglutination tests with a hand lens. 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.

4.72 H Agglutination Tests

Inoculate trypticase soy broth or tryptose broth. Incubate at 35 ± 1°C overnight or until growth has an approximate density of three on McFarland's scale. Add an equal amount of saline containing 0.6% formalin and let sit one h. 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, or equivalent, may be used as an optional method for H agglutination testing. Follow the manufacturer's instructions. If a suspect *Salmonella* isolate is negative by the latex test, perform the poly H tube agglutination test described above.

4.8 Method Quality Control Procedures

It is recommended that a minimum of three method controls be analyzed whenever meat, poultry, or egg products are being examined for the presence of salmonellae. These controls should include *Salmonella* Typhimurium (H_2S-positive), *S.* Senftenberg (H_2S-negative), and an uninoculated media control. The inoculum level for the positive controls should approximate 30-300 cfu per container of enrichment medium. Incubate the three controls along with the samples, and analyze them in the same manner as the samples. Confirm at least one isolate recovered from each positive control sample.

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 ± 1°C overnight, and then store at 4-8°C.

For long-term storage of isolates, subculture *Salmonella* isolates by stabbing nutrient agar (0.75% agar). Incubate at 35 ± 1°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

cultures in the dark at room temperature. Such cultures will remain viable for several years.

Store "working" *Salmonella* stock cultures on nutrient agar slants. Transfer stocks monthly, incubate overnight at 35 ± 1°C, and then store them at 4-8°C.

† Safety Caution: **KCN is a deadly poison. Avoid inhalation of KCN vapors at all times by preparing media in a chemical fume hood. After autoclaving old inoculated KCN broth tubes, be sure an adequate, external exhaust system is turned on before opening the autoclave door and do not allow body contact with any vapors from the autoclave. Wear appropriate safety gloves.**

Consult a Material Safety Data Sheet (MSDS) before working with KCN.

Collect liquid KCN wastes in a separate container and dispose of in accordance with the standard chemical waste management procedures for your laboratory.

4.10 Selected References

Bailey, J. S., J. Y. Chiu, N. A. Cox, and R. W. Johnston. 1988. Improved selective procedure for detection of salmonellae from poultry and sausage products. J. Food Prot. 51:391-396.

Cunniff, P. (ed.). 1995. Official methods of analysis of AOAC International, 16th Edition. AOAC International Inc., Gaithersburg, MD 20877.

Ewing, W. H. 1986. Edwards and Ewing's Identification of Enterobacteriaceae, 4th Edition. Elsevier Science Publishing Co., Inc., New York.

Miller, R. G., C. R. Tate, and E. T. Mallinson. 1994. Improved XLT4 agar: small addition of peptone to promote stronger production of hydrogen-sulfide by salmonellae. J. Food Prot. 57:854-858.

Vassiliadis, P. 1983. The Rappaport-Vassiliadis (RV) enrichment medium for the isolation of salmonellas: an overview. J. Appl. Bacteriol. 54:69-76.