United States Department of Agriculture
Food Safety and Inspection Service
Office of Public Health Science
Laboratory QA/QC Division
950 College Station Road
Athens, GA 30605

Laboratory Guidebook
Notice of Change

Chapter new, **revised**, or archived: MLG 4.05

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

Effective Date: 1/20/11

Description and purpose of change(s):

MLG 4.04 Section 4.1.1 *General* was renamed Section 4.1 *Introduction* and Section 4.1.2 *Limits of Detection* was removed. Instructions for analysis of raw catfish products were added as Section 4.5.8. Additional guidance to reference the optional rapid screen test was added to isolation procedures in Section 4.5. Enrichment and plating media instructions were combined in Section 4.6. An additional test kit, described in Section 4.9, was validated as an option for performing biochemical confirmation. Section references were corrected.

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.

MLG 4.05		Page 1 of 16
Title: Isolation And Identification of *Salmonella* From Meat, Poultry, Pasteurized Egg and Catfish Products		
Revision:05	Replaces: MLG 4.04	Effective: 1/20/11

Procedure Outline

4.1 Introduction

This method describes the analysis of various meat, poultry and catfish products, sponge and rinse samples, 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 samples, the competitive flora may be the most important factor. It varies from sample to sample and from one kind of matrix 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 ± 2%.

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. The Material Safety Data Sheet (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 the MSDS prior to startup.

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_2S-negative culture, a *Salmonella* spp. H_2S-positive culture and an uninoculated media control. To facilitate identification of control isolates, the

laboratory may use strains of uncommonly found serogroups. *S. Abaetetuba* is suggested as a readily available, H_2S-positive culture that is not commonly found in meats or meat products. *S. Choleraesuis* is typically negative for H_2S production. These cultures may be obtained from ATCC. Other serotypes may be found that have aberrant H_2S-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 cultures or 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

Not all of the materials listed below may 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 4.9.

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 equivalent and adapters for use with Mason jars; or a Stomacher® (Tekmar or equivalent)
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, VITEK® 2 Compact System, or equivalent

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 and *Salmonella* individual O grouping sera for groups A-I (antisera for further O groups are optional)
g. *Salmonella* polyvalent H antiserum, Slide Agglutination H Antisera from Statens Serum Institut (SSI), or (Optional) Oxoid *Salmonella* Latex Test (Unipath Company, Oxoid Division, Ogdensburg, NY) or equivalent
h. Additional reagents as needed for biochemical tests: e.g. GNI cards for VITEK® or GN cards for VITEK® 2 Compact 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)
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

4.5 Sample Preparation

Sample preparation and enrichment incubation times may vary by matrix. Refer to Table 1 and the following sample preparation sections for additional details.

Table 1. Sample Preparation and Enrichment Guide for Cultural and PCR Methods

Product	Sample Preparation		Incubation Time
	Portion Size	BPW Amount	Cultural or PCR rapid screen
Breading Mixes, Dehydrated Sauces and Dried Milk	325 ± 6.5 g	2925 ± 58.5 ml	18-24 h
Ready-to-Eat Foods	325 ± 6.5 g	2925 ± 58.5 ml	18-24 h
Fermented Products	325 ± 6.5 g + 10 g of sterilized calcium carbonate	2925 ± 58.5 ml of BPW that contains 1 ml of a 1% aqueous solution of crystal violet per liter	18-24 h
Raw Meat Products	25 ± 0.5 g HACCP: 25 ± 2.5 g	225 ± 22.5 ml	20-24 h
Carcass Sponge and Environmental Swabs	1 sponge	50 ± 1 ml (brings total volume to 60 ml)	20-24 h
Whole Bird Rinses	30 ± 0.6 ml sample rinse fluid	30 ± 0.6 ml	20-24 h
Pasteurized Liquid, Frozen or Dried Egg Products	100 ± 2 g	900 ± 18 ml	18-24 h
Raw Catfish Products	25 ± 2.5 g	225 ± 22.5 ml	22-26 h

4.5.1 Breading Mixes, Dehydrated Sauces and Dried Milk

a. Weigh 325 ± 6.5 g of product.

b. Add a small portion of 2925 ± 58.5 ml of ambient temperature sterile BPW and mix to obtain a homogeneous suspension. Add the remainder of the BPW. Mix until a lump-free suspension is obtained.

c. Incubate at 35 ± 2°C for 18-24 h.

d. Proceed to Section 4.6 to continue the cultural analysis or refer to MLG 4C for use of the BAX® PCR Assay.

4.5.2 Ready-to-Eat Foods

Follow additional program requirements for preparing sample and sub-sample composites. Outbreak samples may require a different sample preparation. Follow customer specifications.

a. Weigh 325 ± 6.5 g of the composite sample into a Stomacher bag (or sterile blender jar if required by the customer or sample type).

Instructions for multi-component RTE products:

i. If the meat or poultry component is separate and distinct from other non-meat ingredient, analyze only the representative meat/poultry portion of the RTE product.
ii. When meat/poultry is combined with other ingredients to form the product, analyze representative meat/poultry portions in combination with other ingredients.

b. Add approximately one third to one-half of 2925 ± 58.5 ml of ambient temperature sterile BPW. Blend or stomach approximately 2 minutes then add the remainder of the 2925 ml of BPW.

c. Incubate at 35 ± 2°C for 18-24 h.

d. Proceed to Section 4.6 to continue the cultural analysis or refer to MLG 4C for use of the BAX® PCR Assay.

4.5.3 Fermented Products

Follow the procedure for ready-to-eat foods in Section 4.5.2 except:

a. Blend/stomach the sample with 10 ± 0.2 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 Products

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 ± 0.5 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 ± 2.5 g.

b. Add 225 ± 22.5 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.

c. Incubate at 35 ± 2°C for 20-24 h.

d. Proceed to Section 4.6 to continue the cultural analysis or refer to MLG 4C for use of the BAX® PCR Assay.

4.5.5 Carcass Sponges and Environmental Swabs

a. Add 50 ± 1 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 ± 2°C for 20-24 h.

c. Proceed to Section 4.6 to continue the cultural analysis or refer to MLG 4C for use of the BAX® PCR Assay.

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.

a. For chicken carcasses, aseptically drain excess fluid from the carcass and transfer the carcass to a sterile Stomacher™ 3500 bag, or equivalent.

b. Pour 400 ml (or other volume specified in program protocol) of BPW into the cavity of the carcass contained in the bag.

c. 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 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.

d. Transfer the sample rinse fluid to a sterile container.

e. Use 30 ± 0.6 ml of the sample rinse fluid obtained above for *Salmonella* analysis. Add 30 ± 0.6 ml of sterile BPW and mix well.

f. Incubate at 35 ± 2°C for 20-24 h.

g. Proceed to Section 4.6 to continue the cultural analysis or refer to MLG 4C for use of the BAX® PCR Assay.

NOTE: If analyses other than *Salmonella* are to be performed, the carcass may be rinsed in BPW and dilutions made directly from the BPW rinse. Alternatively, 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 Pasteurized Liquid, Frozen, or Dried Egg Products

a. Mix the sample with a sterile spoon, spatula, or by shaking.

b. Aseptically weigh a minimum of 100 ± 2 g of egg sample into a sterile blender jar, other sterile jar, or a Whirl-Pak™ or Stomacher™ bag containing 900 ± 18

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 ± 2°C for 18-24 h.

f. Proceed to Section 4.6 to continue the cultural analysis or refer to MLG 4C for use of the BAX® PCR Assay.

4.5.8 Raw Catfish Products

Follow program requirements for preparing sample and sub-sample composites.

a. Weigh 25 ± 2.5g of tissue into a sterile blender jar, other sterile jar or a Whirl-Pak™ or Stomacher™ bag.

b. Add 225 ± 22.5 ml of BPW. Stomach or blend, as required, for approximately two minutes or shake thoroughly.

c. Incubate at 35 ± 2°C for 22-26 h.

d. Proceed to Section 4.6 to continue the cultural analysis or refer to MLG 4C for use of the BAX® PCR Assay.

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 Selective Enrichment and Plating Media

a. Transfer 0.5 ± 0.05 ml of sample into 10 ml TT broth and 0.1 ± 0.02 ml into 10 ml mRV broth.

b. Incubate at 42 ± 0.5°C for 22-24 h or in a water bath at 42 ± 0.5°C for 18-24 h.

c. Carefully mix contents of tube by vortexing or equivalent means. Streak to BGS and either DMLIA or XLT4 agar plates using a 10 ul loopful of inoculum for each plate. Streak the entire agar plate with a single sample enrichment.

d. Incubate at 35 ± 2°C for 18-24 h.

e. Select typical colonies.

4.7 Examination of and Picking Colonies from Plating Media

4.7.1 Picking Colonies

a. After the recommended incubation interval, examine the selective-differential agar plates and controls 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.
- XLT4. Select black colonies (H_2S-positive) or red colonies with (H_2S-positive) or without (H_2S-negative) black centers. The rim of the colony may still be yellow in 24 h; later it should turn red.
- DMLIA. Select purple colonies with (H_2S-positive) or without (H_2S-negative) black centers. Since *Salmonella* 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,

transfer typical colonies into a tube of TT or mRV broth and incubate overnight, then re-streak to selective agars.

c. Re-incubate all plates for an additional 18-24 h at 35 ± 2°C.

d. Reexamine initially negative plates and pick colonies as above. After 48-hour incubation, plates with no typical colonies may be discarded as negative. Plates with colonies undergoing confirmation testing should be stored at 2-8°C until testing is complete. If suspect *Salmonella* colonies do not confirm, reexamine the plates from which they were picked, and if appropriate, re-pick colonies for confirmation following Section 4.7.1.b.

4.7.2 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 ± 2°C for 24 ± 2 h.

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. A typical control on LIA should produce a purple butt with (H_2S-positive) or without (H_2S-negative) blackening of the media. A typical control on TSI should produce a yellow butt and red slant, with (H_2S-positive) or without (H_2S-negative) blackening of the media.

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 that are suggestive but not typical of *Salmonella* spp. should be confirmed by a combination of biochemical and serological procedures. Refer to Table 2 for a summary of TSI-LIA reactions.

b. The motility testing in the last column of the table is optional. Refer to "Edwards and Ewing's Identification of Enterobacteriaceae" (Ewing, 1986) for additional information.

c. (Optional: for some biochemical test kits) For the VITEK system, streak a TSA + 5% sheep blood agar plate from either the TSI or LIA slant. Incubate 18-24 h

at 35 ± 2°C. Some commercial biochemical test systems may require streaking to other non-selective medium prior to inoculation of their test kit. Follow manufacturer's instructions.

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 test the isolate using individual *Salmonella* antisera for O groups A through I. Additional individual O groups may be tested. Testing for O groups A through I should encompass the majority of the *Salmonella* serotypes commonly recovered from meat and poultry products.

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.

Typically, a positively identified isolate and/or control is identified by the individual O group result, but an isolate can be identified by the poly group if it tests as positive for multiple individual O groups or it can be noted as an auto agglutinator if it reacts with saline.

Occasionally, an isolate will be recovered which is typical of *Salmonella* biochemically and is serologically poly H-positive, but is non-reactive with any of the available O group antisera. Report these isolates as "*Salmonella* non A-I" or "*Salmonella* O group beyond I" if no further testing is performed. Further tested isolates are reported by their specific serotype.

4.8.2 Flagellar (H) Antigen Agglutination Tests

The Oxoid *Salmonella* Latex Test, SSI H Antisera for Slide Agglutination, or equivalent, may be used for H antigen agglutination testing. Follow the manufacturer's instructions for performing the test.

Alternatively, use growth from either the TSI or LIA slant to inoculate a tube of trypticase soy broth or tryptose broth. Incubate at 35 ± 2°C overnight. 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 hr without mixing or shaking during incubation. Record the 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).

4.9 Biochemical Procedures

Commercially available biochemical test kits, including automated systems may be used for biochemical identification. If the VITEK® or VITEK® 2 Compact 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 2 is a list of potential biochemical reactions. Additional culture work may be required and other factors should be considered before discarding any sample.

Table 2. Potential *Salmonella* Biochemical Reactions

Triple Sugar Iron Agar			Lysine Iron Agar		Polyvalent Sera		Further Testing/ 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	-	-	-	** B. & M. T.
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 (optional) motility tests;
NC = No change in color from uninoculated medium.

* *Salmonella* Typhisuis (found seldom in swine in U.S.)
** *Salmonella Para-typhi A* (example)
*** *Salmonella enterica* subsp. *arizonae* or *S. enterica* subsp. *diarizonae* (occasional reaction)

4.10 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 ± 2°C overnight and then store at 2-8°C.

Store "working" *Salmonella* stock cultures on nutrient agar slants or equivalent. Transfer stocks monthly onto duplicate nutrient agar slants, incubate overnight at 35 ± 2°C, and then store them at 2-8°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, lyophilize cultures or freeze using cryo-beads, i.e. Cryostor™ or equivalent.

4.11 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.

Centers for Disease Control and Prevention and National Institutes of Health (CDC/NIH). 2007. BioSafety in Microbiological and Biomedical Laboratories, 5th ed. U.S. Government Printing Office, Washington, D.C. (internet site: http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm)

Horowitz, William. (ed.). 2000. Official methods of analysis of AOAC International, 17th 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.

Federal Register, Vol. 61, No. 144, Thursday, July 25, 1996, Appendix E, pp. 38917 – 38925.

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., D. Trichopoulos, A. Kalandidi, and E. Xirouchaki. 1978. Isolation of salmonellae from sewage with a new procedure of enrichment. J. Appl. Bacteriol. **66**:523-528.

Insert No. 15983: *Salmonella* Antisera for *in vitro* diagnostic use, Statens Serum Institut, Denmark, December 2005

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