Testing Methodology for *Listeria* species or *L. monocytogenes* in Environmental Samples

October 2015, Version 1

U.S Food and Drug Administration
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I. Introduction

The method described here is for the purpose of testing environmental samples for *Listeria* species or *L. monocytogenes*. This method has been utilized by FDA's Compliance Program for testing the environmental samples and is based on procedures described in the Microbiology Laboratory Guidebook (MLG) of U.S. Department of Agriculture (USDA)[1] and FDA's Bacteriological Analytical Manual (BAM)[2].

A. Equipment and materials

1. Electronic top-loading balance capable of weighing a minimum of 25 ± 0.1 g (500 g capability recommended)
2. Incubator, 30 ± 2°C
3. Incubator, 35 ± 2°C
4. Incubator, 20 or 25 ± 2°C
5. Vortex mixer
6. Phase-contrast microscope (40X and 100X objectives)
7. Fluorescent desk lamp or natural spectrum light source
8. Sterile sample preparation supplies: scalpels, chisels, knives, scissors, spatulas, forceps, disposable or reusable dishes, pans or trays
9. Sterile, filter or non-filter bags
10. Pipets (e.g., 1 ml)
11. Pippettor and sterile disposable tips for dispensing 100 µl
12. Microscope slides, cover slips and immersion oil
13. Disposable plastic and/or platinum inoculating needles and loops
14. Wax and waterproof ink marker
15. Sterile cotton-tipped applicators (i.e., swabs)
16. Non-bactericidal sampling sponges, polyurethane, or cellulose

[1] U.S. Department of Agriculture (USDA), Microbiology Laboratory Guidebook (MLG) 8.09, effective date: 05/01/2013
[2] Bacteriological Analytical Manual, Chapter 10. Detection and Enumeration of *Listeria monocytogenes* in Foods, Version of April 2011, *Current* content as of September 25, 2015

B. Media and Reagents

1. Modified University of Vermont broth (UVM, also known as UVM1)
2. Fraser broth (FB)
3. Modified Oxford agar (MOX)
4. Horse blood overlay agar (HL, also known as HBO)
5. Trypticase soy agar with 5% sheep blood (TSA-SB, also known as CAMP test agar)
6. Brain heart infusion broth (BHI broth)
7. Dey-Engley (D/E) neutralizing broth
8. β-lysin CAMP factor discs
9. Biochemical test panel (MICRO-ID® *Listeria*, *Listeria* API® system, VITEK® 2 compact or equivalent)

C. Sampling

Aseptically pour 9-10 ml of sterile Dey-Engley (D/E) broth into the bag to hydrate the swabs and sponges. Close the bag and evenly moisten the swabs or sponges by hand message. Position the swab/sponge so that the handle is sticking out of the bag and close the bag around the handle. Through the bag, squeeze the excess broth gently out of the swab/sponge. Carefully take the swab/sponge out of the bag by grasping the handle and swab the area selected using firm and even pressure. Swab/sponge vertically (approximately 10 times); then flip the swab/sponge and use the other side to swab/sponge horizontally (approximately 10 times); then swab/sponge diagonally, using the same surface side as you used for horizontal (approximately 10 times). Swab areas of food contact or environmental surface at least 1" x 1" in size (1' x 1' for sponges or according to manufacturer's instructions). Open the bag and insert the swab/sponge portion into the bag. Break the handle and close the bag according to manufacturer's instructions.

For shipping samples to labs, the samples need to be placed in a pre-chilled shipping container by placing pre-frozen gel packs at the bottom with a cool board placed on top of them. The samples will then be placed in the container followed by another layer of foam plugs or cool board. The samples should be shipped on the same day. When this is not possible, the samples should be stored refrigerated while waiting to be shipped. Contact the appropriate laboratory in advance to inform the shipment and expected arrival of the samples.

D. Primary enrichment

Add 225 ± 5 ml UVM broth to each bagged single swab/sponge sample. Stomach for 2 ± 0.2 min, or briefly massage each sample by hand to expel the collection broth into the UVM broth. Incubate at 30 ± 2°C for 20 - 26 h.

E. Secondary enrichment

1. Transfer 0.1 ± 0.02 ml of the UVM enrichment to 10 ± 0.5 ml of Fraser broth (FB) containing appropriate supplements. Incubate inoculated FB tubes at 35 ± 2°C for 26 ± 2 h.
2. On the same day of transferring to FB, streak a loopful or a drop approximating 0.1 ml of the UVM over the surface of a MOX plate. Incubate the MOX at 35 ± 2°C for 26 ± 2 h.

F. Exanimation of UVM-streaked MOX and interpretation/plating of 26 h Fraser broth

1. Examine the UVM-streaked MOX for colonies with morphology typical of *Listeria* spp. at 26 ± 2 h, suspect colonies are typically small (ca. 1 mm) and are surrounded by a zone of darkening due to esculin hydrolysis. If suspect colonies are present on MOX, transfer suspect colonies to HL agar as described below in H.1. If no suspect colonies are evident, re-incubate the MOX plate for an additional 26 ± 2 hour.
2. After 26 ± 2 h of incubation, examine the FB for the potential presence of *L. monocytogenes* by visual examination and checking for darkening of the broth due to esculin hydrolysis. If any degree of FB darkening is evident, aseptically dispense a drop approximating 0.1 ± 0.02 ml of FB onto a MOX plate. Streak 25-40% of the surface of the MOX plate with the FB inoculum. Use a loop to streak for isolation from the initial swab/streak quadrant onto the remainder of the plate. Incubate the MOX plate at 35 ± 2°C for 26 ± 2 h; if no FB darkening is evident, re-incubate the FB at 35 ± 2°C until a total incubation time of 48 ± 2 h has been achieved. Proceed to Section G.2.

G. Examination of MOX Plates and Interpretation/Plating of 48 h Fraser broth

1. Examine and select suspect colonies from any MOX agar plate awaiting analysis (i.e., MOX plates streaked from 26 ± 2 h FB and/or UVM cultures).
2. Re-examine the FB for evidence of darkening after 48 ± 2 h of total incubation. If any degree of darkening is evident, streak the culture onto a MOX plate and incubate the plate according to the procedures described above. If no darkening of FB is evident and no suspect MOX and/or HL colonies are seen, the sample is considered negative for *L. monocytogenes*.

H. Isolation procedures

1. If suspect colonies are present on MOX from any sample, use a loop or equivalent sterile device to touch a minimum of 20 (if available) suspect colonies and collectively streak for isolation on one or more HL agar plates. Alternatively, a swipe of suspect growth representing at least 20 colonies may be used. Incubate the streaked HL plates at 35 ± 2°C for 22 ± 4 h.
2. After incubation, examine the HL plate(s) against backlight for translucent colonies surrounded by a small zone of β-hemolysis. If at least one suspect colony is clearly isolated, proceed to confirmatory testing. Store all HL plates containing suspect colonies

(at room temperature or under refrigeration) until confirmatory testing is complete. If suspect colonies or β-hemolytic growth are present on HL but not clearly isolated, re-streak representative suspect colonies/growth onto one or more fresh HL plates and incubate at 35 ± 2°C for 22 ± 4 h. If no suspect isolates are present on HL, pursue follow-up of MOX and/or HL isolates from other follow-up analysis (e.g. FB follow-up vs. UVM Primary Enrichment streak follow-up). If none of the follow-up analyses produces suspect β-hemolytic colonies on HL, the sample is considered negative for *L. monocytogenes*.

I. Confirmation and identification procedures

1. **Preliminary confirmation tests for *Listeria* spp.** A minimum of one colony must be confirmed. If the first selected suspect HL colony does not confirm as *L. monocytogenes*, confirmation must be attempted for additional suspect HL colonies, if available, until at least three isolates from the test portion have failed confirmation.

   a. Tumbling motility test (optional). Use one isolated HL colony to inoculate one aliquot of BHI broth. Incubate at 18-25°C for 16-18 h. From the enriched culture, prepare a wet mount. Using the 100× oil immersion objective lens (phase contrast microscopy recommended), examine the wet mount culture for small rods that exhibit an active end-over-end tumbling/rotating movement characteristic of *Listeria* spp. If cell morphology and motility are not characteristic of *Listeria* spp., and the culture appears pure, report the sample as negative for *L. monocytogenes*; if a mixture of typical *Listeria* cells and cells that do not have the characteristic *Listeria* morphology (i.e. small rods) are present, streak a loopful of the contaminated BHI broth onto fresh HL agar for further purification; if no growth is evident at 16-18 h, re-incubate at 18-25°C until growth is evident or up to a total of 48 h; if cell morphology is typical, tumbling motility is evident and the culture appears pure, proceed with biochemical confirmation.

   b. Media required for inoculation of biochemical test systems must be inoculated from the same colony or growth sub-cultured from that colony.

2. **Biochemical tests**

   Using a pure culture, perform confirmatory biochemical tests. Commercially available test systems (MICRO-ID® *Listeria*, *Listeria* API® test system or VITEK® 2) or validated equivalent systems, including well-established schemes involving traditional tube biochemical media (e.g., FDA *Bacteriological Analytical Manual*), may be employed. However, exercise caution in interpreting the identification of atypical *Listeria* spp. isolates when using these biochemical test systems. Cultures identified as "*L. monocytogenes*/*innocua*" or any β-hemolytic *Listeria* spp. that is biochemically indeterminate or identified as *L. innocua* must be further characterized using additional tests.

   a. **MICRO-ID® *Listeria* test system.** Follow the instructions provided by the manufacturer for inoculation and interpretation of the test pane. A CAMP/CAMP factor test must be performed to augment MICRO-ID results.

b. ***Listeria* API® test system.** Follow the instructions provided by the manufacturer for inoculation and interpretation of the test panel.

c. **VITEK® 2 Compact.** Follow the instructions provided by the manufacturer for inoculation and loading the VITEK 2 test system. A CAMP/CAMP factor test must be performed to augment VITEK 2 results.

3. **CAMP/CAMP Factor Test**

A CAMP/CAMP factor test is required to augment traditional biochemical test (MICRO-ID® or VITEK® 2) results to resolve the hemolytic capability of indeterminate strains. Either of the following two test options may be employed:

a. **β-lysin CAMP factor test.** This test system may provide results that are easy to interpret compared to the traditional CAMP test. Therefore, the β-lysin CAMP factor test is recommended over the traditional test. Aseptically place a β-lysin disc in the approximate center of a TS-SBA plate. Individually and aseptically inoculate and streak up to eight sample isolates per plate as straight lines radiating away from the disc. The inoculation line should almost but not quite touch the disc. Be sure to include positive and negative control cultures. A non-hemolytic *L. innocua* is an appropriate negative control. Incubate at 35 ± 2°C for 24 ± 2 h. An arrowhead-shaped zone of β-hemolysis surrounding the inoculation line proximal to the disc indicates a positive CAMP factor reaction. *L. monocytogenes*, *L. seeligeri* and *L. ivanovii* are CAMP factor-positive by this test. However, *L. ivanovii* demonstrates relatively intense β-hemolysis distal to the disk and, therefore, can be distinguished from the other two species. Non-hemolytic *Listeria* spp. are CAMP factor-negative. If a suspected β-hemolytic *Listeria* spp. does not produce a CAMP factor-positive reaction at 24 ± 2 h, continue to incubate the culture at 35 ± 2°C until a total incubation time of 48 ± 2 h has been achieved. Re-examine the plate. If a CAMP factor-positive reaction is still not evident at 48 ± 2 h, additional testing described in I.2 that does not require CAMP reactions may be required to determine the identity of the isolate.

b. **Traditional CAMP test.** If the CAMP test is necessary and β-lysin discs are not available, perform the traditional culture CAMP test as follows: apply single-line streaks of *S. Pseudintermedius* ATCC® 49444 or *S. aureus* ATCC®25923 and *R. equi* (ATCC®6939) reference cultures on a TS-SBA plate in parallel and 3-4 cm apart. Streak test cultures between and perpendicular to the two reference cultures (i.e. like rungs of a ladder). The test culture streak must be 2-4 mm from each reference culture streak. Test and reference cultures must not touch or be cross contaminated in any manner. Incubate the plate for 24 ± 2 h at 35 ± 2°C. Examine the test culture streaks for enhanced β-hemolysis at both ends proximal to the reference cultures. The zone of enhanced β-hemolysis may resemble an arrowhead, circle or rectangle. The presence of this zone indicates a CAMP-positive reaction. Absence of enhanced β-hemolysis indicates a CAMP-negative reaction. *L. monocytogenes* and *L. seeligeri* are CAMP-positive to the *Staphylococcus* reference strain and CAMP-negative to *R. equi*. In contrast, *L. ivanovii* is CAMP-positive to the *R. equi* reference strain and CAMP-negative to the *Staphylococcus* reference strains. If a suspected β-hemolytic *Listeria*

spp. does not produce a CAMP-positive reaction with either reference culture at 24 ± 2 h, continue to incubate the culture at 35 ± 2°C until a total incubation time of 48 ± 2 h has been achieved. Re-examine the plate. If the culture does not produce a CAMP-positive reaction with the *Staphylococcus* culture, but clearly demonstrates β-hemolysis on HL agar, additional testing described in I.2 that does not require CAMP reactions maybe required to determine the genetic identity of the isolate.

J. Media preparations

1. Modified Oxford agar (MOX)

Columbia Blood Agar Base (depending on brand)	38 - 44.0 g
Esculin	1.0 g
Ferric Ammonium Citrate	0.5 g
Lithium Chloride (Sigma L0505)	15.0 g
Colistin	0.01g
Microbiologically Suitable (MS) water	1.0 L

Rehydrate commercial Modified Oxford Agar Base with constant stirring using a magnetic mixer. Autoclave this base at 121 ºC for 15 minutes, mix again, and cool to 45ºC to 50ºC in a water bath. Add 2 ml of 1% filter sterilized Moxalactam solution to make the complete MOX medium, mix well, and pour 12 ml per plate.

Final pH 7.0 ± 0.2 at 25ºC.

1% Moxalactam Solution or use commercially available supplement at same level

Sodium (or Ammonium) Moxalactam (Sigma)	1.0 g
0.1 M Potassium Phosphate Buffer, pH 6.0	100.0 ml

Dissolve, sterilize by filtration, dispense in small quantities for use and store in freezer at -10ºC or below. Refreezing may decrease potency.

CAUTION: DO NOT use the Modified Oxford Antibiotic Supplement since it contains both moxalactam and colistin.

2. Modified UVM Broth

Proteose Peptone	5.0 g
Tryptone	5.0 g
Lab Lemco Powder (Oxoid)	5.0 g
Yeast Extract	5.0 g
NaCl	20.0 g
KH_2PO4	1.35 g
Na_2HPO4	12.0 g
Esculin	1.0 g
Naladixic Acid (2% in 0.1 M NaOH)	1.0 ml
Acriflavin	12.0 mg
MS water	1.0 L

Sterilize at 121ºC for 15 minutes. Store in the refrigerator.

Final pH 7.2 ± 0.2 at 25ºC.

3. **Fraser Broth**

Proteose Peptone	5.0 g
Tryptone	5.0 g
Beef Extract (Oxoid LabLemco)	5.0 g
Yeast Extract	5.0 g
NaCl	20.0 g
KH_2PO_4	1.35 g
Na_2HPO_4	12.0 g
Esculin	1.0 g
Naladixic Acid (2% in 0.1 M NaOH)	1.0 ml
Acriflavin	25.0 mg
Lithium Chloride	3.0 g
MS water	1.0 L

Acriflavin Stock

Acriflavin Hydrochloride (Sigma)	13mg
MS water	10 ml

Dissolve and add to 1 L of Fraser Broth.

Ammonium iron (III) citrate (Ferric Ammonium Citrate)

Ammonium iron (III) citrate (Sigma)	5 g
MS water	100 ml

In a 100 ml volumetric flask, dissolve 5 g of ammonium iron (III) citrate (Sigma) in MS water. Bring to volume and filter sterilize. Store at 2-8°C.

Fraser Broth may be prepared from commercially available Modified UVM by adding the appropriate amounts of lithium chloride and acriflavin before sterilizing and ammonium iron (III) citrate after sterilization.

Mix well to resuspend the media and dispense into test tubes. Sterilize at 121ºC for 15 minutes. Store in the refrigerator.

Just before use, 0.1 ml of ammonium iron (III) citrate in MS water to each 10 ml tube. Final pH 7.2 ± 0.2 at 25ºC.

4. **Horse Blood Overlay Medium (HL)**

a. Base Layer

Columbia Blood Agar Base 1.0 L

Prepare according to manufacturer's specifications and sterilize at 121ºC for 15 minutes. Pour 10 ml per 100 mm diameter Petri dish. Allow to solidify, overlay with blood agar as escribed below.

b. Top Layer

Add 4 ml of sterile horse blood to each 100 ml of melted/tempered Columbia Blood Agar Base which has been cooled to 46ºC.

Stir or swirl to mix evenly. Quickly place 5 to 6 ml on top of the base layer and tilt the plates to spread top layer evenly.

Store plates refrigerated and use within 2 weeks.

Discard any plates which become discolored.

Final pH 7.2 ± 0.2 at 25ºC.

5. **Dey-Engley (D/E) Neutralizing Broth**

Tryptone	5.0 g
Yeast Extract	2.5 g
Glucose	10.0 g
Sodium thioglycollate	1.0 g
Sodium thiosulfate	6.0 g
Sodium bisulfite	2.5 g
Polysorbate 80	5.0 g
Lecithin (soy bean)	7.0 g
Brom cresol purple	0.02 g
MS water	1.0 L

Heat to dissolve ingredients in MS water, dispense into appropriate containers and sterilize in the autoclave at 121ºC for 15 minutes.

Final pH 7.6 ± 0.2 at 25ºC.

6. **Brain Heart Infusion (BHI) Broth**

Pancreatic digest of gelatin	14.5 g
Brain heart, solids form	6.0 g
Peptic digest of animal tissue	6.0 g
NaCl	5.0 g
Glucose	3.0 g
Na_2HPO_4	2.5 g
MS water	1.0 L

Dissolve ingredients in MS water. Heat the mixture until it is well dissolved. Dispense as desired and autoclave at 121ºC for 15 minutes. This may also be prepared by adding 15 g of agar to each liter of BHI broth.

Final pH 7.4 ± 0.2 at 25°C.

7. **Trypticase Soy Agar with 5% Sheep Blood (TSA-SB, Sheep Blood Agar or SBA)**

Trypticase (Tryptic)	15.0 g
Phytone	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
MS water	1.0 L

Suspend ingredients in water. Heat the mixture until it is well dissolved. Sterilize at 121°C for 15 minutes. If desired, cool to approximately 50ºC, add 5% sterile, defibrinated, sheep blood and swirl. Avoid bubble formation. Pour 15 ml into each sterile

100 x 15 mm Petri dish. When used for the *Listeria monocytogenes* CAMP test, pour 9 ± 1 ml into each sterile 100 x 15 mm Petri dish for ease of test interpretation.

Final pH 7.3 ± 0.2 at 25°C.