U.S. Food and Drug Administration
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CENTER FOR FOOD SAFETY AND APPLIED NUTRITION



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## Chapter 10

# Detection and Enumeration of *Listeria monocytogenes* in Foods

Authors

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The genus *Listeria* contains 6 species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi* (Table 1). *L. grayi* (29, 34) and *L. ivanovii* (12, 28) each contain two subspecies, which do not need to be specified in this analysis. A recent taxonomic review of the genus by Rocourt (35) updates the previous reviews (11, 37). *L. ivanovii* and *L. monocytogenes*are pathogenic for mice and other animals. However, only *L. monocytogenes* is commonly associated with human listeriosis. Listeriosis associated infection by *L. ivanovii*, and even *L. seeligeri*, is extremely rare in humans. The universal occurrence of *L. monocytogenes* in food (36) and the risk of contracting food-borne *L. monocytogenes* listeriosis (41b, http://www.foodsafety.gov/~dms/lmrisk.html) have been thoroughly reviewed recently. This chapter describes the detection and enumeration of *L. monocytogenes* in foods. Detection of this pathogen in the food processing environment, such as on food contact surfaces and equipment, is described elsewhere (41a).

The preferred standard methodology, and permitted alternative rapid methodologies, to be used for detection and isolation of *Listeria monocytogenes* are as follows. Presumptive contaminated food lots are sampled. Generally, sub-samples are composited if required by FDA field laboratory instructions. Analytical portions (25 g) are pre-enriched for *Listeria* species at 30° C for 4 h in buffered *Listeria* enrichment broth (BLEB), equivalent (26) to AOAC/IDF dairy products enrichment broth (4, 40) base containing sodium pyruvate (43). At the fourth hour of the incubation, the selective agents (acriflavin, 10 mg/L (4, 40); sodium nalidixate, 40 mg/L; optional antifungal, e.g. cycloheximide 50 mg/L) are added. Incubation for selective enrichment is continued at 30° C for a total of 48 h. The enrichment culture is streaked at 24 and 48 h on one of the prescribed differential selective-agars in order to isolate *Listeria* species.

Alternatively, prescribed rapid detection kits with their respective enrichment media may be conditionally used to screen for presence of *Listeria* contaminants. Putative listeria isolates on selective agars from standard or screen positive enrichments are purified on non-selective agar

and confirmed by conventional identification tests or by a battery of such tests in kit form. Isolates may be rapidly confirmed as *L. monocytogenes* (or not) by using specific test kits. Subtyping of *L. monocytogenes* isolates is optional except for FDA isolates, which have to be typed serologically, and by pulsed-field gel electrophoresis (PFGE) and by ribotyping. Non-obligatory pathogenicity testing of *L. monocytogenes* isolates is described.

Enumeration of *L. monocytogenes* in positive samples is performed on reserve sample by colony count on *L. monocytogenes* differential selective agar in conjunction with MPN enumeration using selective enrichment in BLEB with subsequent plating on ALOA or BCM differential selective agar.

The major revisions to the *Listeria* methodology follow.

1. Certain prescribed rapid detection kits and their enrichments are now authorized screening alternatives to the standard selective enrichment.

2. It is now necessary to use only one instead of two of the several prescribed selective isolation agars (Oxford agar, PALCAM, LPM plus esculin and ferric iron, MOX). Oxford agar is still the preferred standard selective isolation medium. MOX has been added to the list of prescribed selective agars and LPM without added esculin and ferric iron has been removed. Trial use of the new chromogenic differential selective agars, like BCM, ALOA, CHROMagar Listeria and Rapid' L. mono, is encouraged as long as it is in parallel with one of the prescribed selective agars. The new agar media differentiate *L. monocytogenes*/*L. ivanovii* colonies from those of other *Listeria* spp. and will greatly facilitating picking of *L. monocytogenes* colonies when colonies of more than one species are present on a plate.

3. The Henry illumination technique is de-emphasized because only differential selective isolation agars are prescribed.

4. The current enrichment medium, which resembles the step-1 enrichment of the internationally harmonized method proposed by Asperger *et al.* (10a), is basically unchanged. However, pimaricin (natamycin), a much less toxic compound than cycloheximide, is introduced as the alternative antifungal compound in the *Listeria* enrichment medium,

   http://www.cfsan.fda.gov/~ebam/cyclohex.html.

5. If *L. monocytogenes* is detected in a food sample, enumeration of the level of contamination in the food is required.

**Table 1. Differentiation of *Listeria* species**

| | | Acid produced from | | | |
|---|---|---|---|---|---|
| **Species** | **β-Hemolysis[a]** | **Mannitol** | **Rhamnose** | **Xylose** | **Virulence[b]** |
| ***L. monocytogenes*** | + | - | + | - | + |

| | | | | | |
|---|---|---|---|---|---|
| ***L. ivanovii***[c] | + | - | - | + | + |
| ***L. innocua*** | - | - | V[d] | - | - |
| ***L. welshimeri*** | - | - | V[d] | + | - |
| ***L. seeligeri*** | + | - | - | + | - |
| ***L. grayi***[e] | - | + | V[d] | - | - |

[a] Sheep blood agar stab.

[b] Mouse test.

[c] Ribose fermenting strains are classified as *L. ivanovii* subsp. *ivanovii* and ribose non-fermenters as *L. ivanovii* subsp. *londiniensis*.

[d] V, variable biotypes

[e] Includes two subspecies - *L. grayi* subsp. *murrayi* reduces nitrate *L. grayi* subsp. *grayi* does not reduce nitrate.

## A. Equipment and materials

1. Balance for weighing sample to 0.1 g
2. Cover slip, glass
3. Erlenmeyer flask, 500 ml
4. Fermentation tubes (Durham)
5. Grease pencil or magic marker
6. Incubators, 30 and 35°C
7. Immersion oil
8. Inoculating loops
9. Inoculating needle
10. Microscope slides
11. Needle, 26 gauge, 3/8 inch
12. Phase-contrast microscope with oil immersion phase objective (100X)
13. Petri plates
14. Pipettes, 25, 10, and 1 ml
15. Tubes, 16 x 125 mm or other appropriate sizes, screw-cap
16. Blender motor and jars or Stomacher and bags
17. Tuberculin syringe, sterile, disposable

## B. Media and Reagents

1. Acetic Acid, 5 N
2. Acriflavine monohydrochloride
3. Agar (Difco Laboratories, Detroit, MI)
4. N-(1-naphthyl) ethylene diamine (R48)
5. α-Naphthol reagent (R48)
6. Blood agar base No. 2 (Unipath)
7. Cycloheximide

8. Natamycin (pimaricin)
9. Sheep blood, defibrinated
10. Ethanol, absolute
11. Fluorescent antibody (FA) buffer (Difco)
12. Glycine anhydride
13. Gram stain kit
14. Hydrogen peroxide solution, 3% for catalase test (R12)
15. KOH 40% solution (R65)
16. *Listeria*-typing sera set (Difco)
17. Lithium chloride-phenylethanol-moxalactam (LPM) agar (M81) with added esculin and iron (M82)
18. Nalidixic acid (sodium salt)
19. Nitrate reduction medium (M108) and nitrate detection reagents (R48)
20. Nutrient broth (M114)
21. Physiological saline solution, 0.85% (R63)
22. Purple carbohydrate fermentation broth base (M130), containing 0.5% solutions of dextrose, esculin, maltose, rhamnose, mannitol, and xylose
23. SIM medium (Becton-Dickinson Microbiology Systems, M137) or motility test medium (MTM, Difco) (M103)
24. Sulfanilic acid reagent (R48)
25. Trypticase soy agar with 0.6% yeast extract (TSAye) (M153)
26. Trypticase soy broth with 0.6% yeast extract (TSBye) (M157)
27. Oxford medium (OXA) (M118)
28. Buffered *Listeria* Enrichment Broth (BLEB) (M52)
29. PALCAM agar (M118a)
30. Carageenan (Sigma type II)
31. BCM agar (M17a)
32. MOX agar (M103a)
33. ALOA agar (M10a)
34. Chromogenic *Listeria* Agar (M40b)
35. Rapid L'mono (M131a)
36. CHROMagar *Listeria* (M40a)
37. Tryptose broth and agar (Difco) (M167)

Note: Alternative companies may be used when the products are equivalent.

## C. Sampling and Enrichment Procedures

1. **Sample treatment.** Sample refrigeration at 4°C is recommended for handling, storing, and shipping materials to be analyzed for *L. monocytogenes*, which will grow, although slowly, at this temperature if other conditions permit. However, if the sample is already frozen, it should not be thawed until analysis.

2. **Composited samples**. Generally, composited samples are prepared as follows. A food lot sample is collected consisting of 10 sub-samples (liquid, cream or solid food) and 50-g or ml portions of each sub-sample are used to make two composite samples (250 g each). Take care to make sub-samples representative of a food's outer surface as well as its interior. For the first composite 5 x 50-g portions from 5 sub-samples are pooled and blended or stomached in 250 ml buffered *Listeria*

enrichment broth base containing sodium pyruvate without selective agents (BLEB, M52). The second composite is made from the remaining 5 sub-samples in the same way. Both blended composites contain 250-g food portions and 250 ml basal BLEB.

Normally 50 g of each composite blend (equivalent to 25g food plus 25 ml basal BLEB) is mixed with a 200 ml amount of basal BLEB. Since there are two composites, the end result is two 25-g analytical portions each contained in 225 ml amounts of basal BLEB. Thus, for each sub-sample a total of 50 g of composited food is analyzed. An aliquot (50 ml) of the composite blend should be retained, preferably at 5° C and not below 0° C, for possible pathogen enumeration.

3. **Non-composited samples**. If composite samples are not required, single 25-g analytical portions of food are simply blended or stomached in 225 ml of basal BLEB and pre-enriched/enriched as described later. A 25-g sample should be retained for possible pathogen enumeration. Store it at 5° C if it is not frozen or, if frozen, in a non-defrosting freezer.

4. **Pre-enrichment and enrichment.** Incubate for 4 h at 30° C, add the selective agents and continue incubating for a total time of 48 h at 30° C. If cycloheximide is unavailable, the preferred substitute is pimaricin (natamycin) at 25 mg/L (27). Natamycin is much safer to use than cycloheximide. Another possibility, if the matrices of interest (e.g. pasteurized milk and cream, yogurt, and precooked frozen seafood) are low in yeast and mold, is to do without an anti-fungal agent. This is not advisable for mold-ripened cheeses, smoked or dried seafood or fresh produce.

5. **Enrichment with enumeration**. Surveillance enumeration of *Listeria monocytogenes* levels in contaminated food is now required. Detection may be done first and if contamination is detected, a reserve sample portion can be enumerated. This is probably the preferable method as, generally, only a few percent of samples can be expected to be positive and then most often at a level of only about 1 cfu/25g. However, the option of combining regulatory detection and enumeration is provided in Enumeration.

6. **Prescribed Alternative Methods for Screening Enriched Samples.** For the prescribed methods kits listed in Table 2, follow the manufacturers' package insert instructions making certain they have not deviated from the approved versions of the AOAC INTERNATIONAL Official Methods Manual protocols (Table 2). The kits are only approved for the specified food matrices, listed in Chapter 10, Supplement 1A, which vary from kit to kit. For other food matrices listed in Chapter 10, Supplement 1B, in-house validation is necessary. The easiest way to validate is to streak all kit-associated 48-h enrichments, which give kit-negative results, on a plate of one of the prescribed standard method esculin agars. Even with the validated matrices the 48-h streaking of kit-negative enrichments to esculin selective agars is recommended for five or so different lots of a given food matrix until the analyst is confident of the kit's performance with that particular matrix. This is because conventional validation of matrices does not take into account qualitative and quantitative competitive microflora variation among different lots of a given food matrix. Strong competition will impact rapid kit methods more than cultural

methods. Kit thresholds of detection (>$10^4$ cfu/ml of enrichment culture) are higher than that of the culture plate method (about $10^2$ cfu/ml of enrichment culture) so that, with very competitive microflora, kits could give false negative results. Alternatively, the limit of detection (50% endpoint) by quantitative spiking with a single strain of *L. monocytogenes* should be determined. It should be not more than 3 cfu per 25-g analytical portion. A value greater than this would only be acceptable if the conventional culture method's performance level is comparable. For all food matrices, a kit positive result must be supported by a culture of the *L. monocytogenes* isolate.

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**Table 2. *Listeria* Genus Detection Test Kits Prescribed for Regulatory Screening**

1. AOAC Official Method 993.09. 2000. *Listeria* in dairy products, seafoods, and meats. Colorimetric deoxyribonucleic acid hybridization method (GENE-TRAK *Listeria* Assay). (3, 14)

2. AOAC Official Method 994.03. 2000. *Listeria monocytogenes* in dairy products, seafoods, and meats. Colorimetric monoclonal enzyme-linked immunosorbent assay method (*Listeria* Tek). (5, 15, 32)

3. AOAC Official Method 995.22. 2000. *Listeria* in foods. Colorimetric polyclonal enzyme immunoassay screening method (TECRA *Listeria* Visual Immunoassay [TLVIA]). (6, 30)

4. AOAC Official Method 996.14. 2000. Assurance (Polyclonal Enzyme Immunoassay Method). (7, 21)

5. AOAC Official Method 997.03. 2000. Visual Immunoprecipitate Assay (VIP). (8, 22)

6. AOAC Official Method 999.06. 2000. Enzyme Linked Immunofluorescent Assay (ELFA) VIDAS LIS Assay Screening Method. (9, 23)

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D. **Isolation procedure**

At 24 and 48 h, streak BLEB culture onto one of the following esculin-containing selective isolation agars: either OXA (16, M118) or PALCAM (42, M118a) or MOX (41, M103a) or LPM (31, M81) fortified with esculin and $Fe^{3+}$ (M82). These esculin-containing media are listed in order of preferred use, subject to their availability. Incubate OXA, PALCAM or MOX plates at 35° C for 24-48 h and fortified LPM plates at 30° C for 24-48 h. It is strongly recommended that one of the *L. monocytogenes-L. ivanovii* differential selective agars, such as BCM (33, M117a), ALOA (M10a), *RapidL'mono* (M131a), or CHROMagar *Listeria* (M40a) be streaked at 48 h (optionally at 24 h, too) in addition to the chosen esculin-containing selective agar. This will reduce the problem of masking of *L. monocytogenes* by *L. innocua*. [Note: BCM has been collaboratively validated by FDA

(26a). An ISO TC34 SC9 comparative validation showed that all the media (and a selective blood agar - LMBA, Sifin, Germany) inhibited *Listeria* competitors more or less equally well. ALOA was preferred only because its formulation is public. Another differential selective medium, Chromogenic *Listeria* Agar (M40b) is due to be marketed in the future.]

*Listeria* colonies are black with a black halo on esculin-containing media. Certain other bacteria can form weakly brownish black colonies, but color development takes longer than 2 days. Transfer 5 or more typical colonies from OXA and PALCAM or modified LPM or MOX to Trypticase soy agar with yeast extract (TSAye), streaking for purity and typical isolated colonies. If BCM plates are streaked as recommended above and blue colonies are observed, they are presumptive *L. monocytogenes* colonies since *L. ivanovii* is not often reported in foods. *L. monocytogenes* and *L. ivanovii* colonies on ALOA are blue and have a zone of lipolysis around them. Purification on TSAye is a mandatory step in the conventional analysis because isolated colonies on selective agar media may still be in contact with an invisible weak background of partially inhibited competitors. At least 5 isolates are necessary because more than one species of *Listeria* may be isolated from the same sample. Use of BCM and ALOA plates will help to reduce the number of colonies that need to be picked. *L. monocytogenes* and *L. ivanovii* can be distinguished using a commercial Confirmatory Medium (Biosynth International, Inc.) or by conventional rhamnose/xylose fermentation broths or agars. Incubate TSAye plates at 30° C for 24-48 h. The plates may be incubated at 35° C if colonies will not be used for wet-mount motility observations (see E-2, below). For the approved rapid methods (Table 2), use the selective isolation agar recommended by the manufacturer but, as noted above, auxiliary use of the new *L. monocytogenes-L.ivanovii* differential agars is also recommended.

## E. Identification procedure

Identify purified isolates by the following classical tests (E, 1-11). Rapid kits are available to facilitate biochemical testing to genus or species level (see E-11 and E-12).

1. Examine TSAye plates for typical colonies. Observation with Henry oblique transmitted illumination can be helpful at this stage but is not mandatory (See ref. 25 for details).

2. Pick typical colony from culture plate incubated at 30°C or less and examine by wet mount, using 0.85% saline for suspending medium and oil immersion objective of phase-contrast microscope. Choose a colony with enough growth to make a fairly heavy suspension; emulsify thoroughly. If too little growth is used, the few cells present will stick to the glass slide and appear non-motile. *Listeria* spp. are slim, short rods with slight rotating or tumbling motility. Always compare with known culture. Cocci, large rods, or rods with rapid, swimming motility are not *Listeria* spp. Alternatively, use the 7-day motility test medium (see E-9).

3. Test typical colony for catalase. *Listeria* species are catalase-positive.

4. Gram stain 16- to 24-h cultures. All *Listeria* spp. are short, Gram-positive rods; however, with older cultures the Gram stain reaction can be variable and also cells may appear coccoidal. The cells have a tendency to palisade in thick-stained smears.

This can lead to false rejection as a diphtheroid.

5. Pick typical colony to a tube of TSBye for inoculating carbohydrate fermentation and other test media. Incubate at 35° C for 24 h. This culture may be kept at 4°C several days and used repeatedly as inoculum. Commercial kits are available for isolate identification (see E-11)

6. Inoculate heavily (from TSAye colony) 5% sheep blood agar by stabbing plates that have been poured thick and dried well (check for moisture before using). Draw grid of 20-25 spaces on plate bottom. Stab one culture per grid space. Always stab positive controls (*L. ivanovii* and *L. monocytogenes*) and negative control (*L. innocua*). Incubate for 24-48 h at 35° C. Attempt to stab as near to bottom of agar layer as possible, without actually touching bottom of agar layer and possibly fracturing the agar.

7. Examine blood agar plates containing culture stabs with bright light. *L.monocytogenes* and *L. seeligeri* produce a slightly cleared zone around the stab. *L. innocua* shows no zone of hemolysis, whereas *L. ivanovii* produces a well-defined clear zone around the stab. Do not try to differentiate species at this point, but note nature of hemolytic reaction. Resolve questionable reactions by the CAMP test. (Note: Hemolysis is more easily determined when the depth of the blood agar is thinner than the usual 5mm. Optionally, this may be achieved by use of a blood agar overlay (1-2 mm) technique).

8. Nitrate reduction test. This test is optional. Only *L. grayi* ssp. *murrayi* reduces nitrates. The test distinguishes *L. grayi* ssp. *murrayi* from *L. grayi* ssp. *grayi*. Use a TSBye culture to inoculate nitrate broth (M108). Incubate at 35° C for 5 days. Add 0.2 ml reagent A, followed by 0.2 ml reagent B (R48). A red-violet color indicates presence of nitrite, i.e. nitrate has been reduced. If no color develops, add powdered zinc and hold for 1 h. A developing red-violet color indicates that nitrate is still present and has not been reduced.

   As an alternative procedure (R48), add 0.2 ml reagent A followed by 0.2 ml reagent C. An orange color indicates reduction of nitrate. If no color develops, add powdered zinc as above. Development of an orange color indicates unreduced nitrate.

9. Inoculate SIM or MTM from TSBye. Incubate for 7 days at room temperature. Observe daily. *Listeria* spp. are motile, giving a typical umbrella-like growth pattern. MTM provides the best defined umbrellas. Alternatively, observe the 30° C TSBye cultures, by phase contrast microscopy (x1000) for tumbling motility.

10. From TSBye culture, inoculate the following carbohydrates as 0.5% solutions in purple carbohydrate broth (the use of Durham tubes is optional): dextrose, esculin, maltose, rhamnose, mannitol, and xylose. Incubate 7 days at 35° C. Positively reacting *Listeria* spp. produce acid with no gas. Consult Table 1 for xylose-rhamnose reactions of *Listeria* spp. All species should be positive for dextrose, esculin, and maltose. All *Listeria* spp. except *L. grayi* should be mannitol-negative. If pigmentation of the isolate on OXA, PALCAM, MOX or LPM plus esculin/$Fe^{3+}$ is

unequivocal, the esculin test may be omitted.

11. Purified isolates may be rapidly identified by using commercial kits (additional tests may be needed to speciate completely): Vitek Automicrobic Gram Positive and Gram Negative Identification cards (bioMerieux, Hazelwood, MO) or API Listeria (bioMerieux, Marcy-l'Etoile, France) which does not require an additional CAMP test. The MICRO-ID™ kit (bioMerieux, Hazelwood, MO; 1, 24) permits speciation of *Listeria* isolates if their CAMP reactions are known. The Phenotype MicroArray for *Listeria* (BiOLOG, Hayward, CA) is another recently introduced kit.

12. Alternative rapid methods that are prescribed for identifying *Listeria* isolates as *L. monocytogenes* are listed in Table 3. Depending on the kit, isolates may be identified in pure culture or from OXA or the other selective isolation agars. Purified isolates identified as *Listeria monocytogenes* by these tests should be retained for regulatory reference.

**Table 3. Test Kits Useful in Confirming *Listeria* Isolates as *Listeria monocytogenes* or not***

1. AccuProbe™ *Listeria monocytogenes* culture confirmation test (Gen-Probe, Inc, San Diego, CA; 10, 19).

2. GeneTrak *Listeria monocytogenes* test kit (Neogen, Lansing, MI; 19).

3. Probelia *Listeria monocytogenes* test kit (BioControl, Seattle, WA).

4. VIDAS *Listeria monocytogenes* test kit (bioMerieux).

5. Transia Plate *Listeria monocytogenes* (Diffchamb SA, Lyon, France)(38)

6. FDA, SRL application of Niederhauser *et al*. method for PCR detection and identification of *L. monocytogenes* (32a, 32b)

7. BAX *Listeria monocytogenes* test. (Qualicon, Inc., Wilmington, DE) (9a).

* These kits are in various stages of validation and when suitably validated can also be used to screen enrichments for *L. monocytogenes*. Presently, FDA only prescribes validated kits that screen for all *Listeria* species.

F. **The CAMP Test.**

The Christie-Atkins-Munch-Peterson (CAMP) test (Table 4 and Fig. 1) is useful in confirming species particularly when blood agar stab test results are equivocal. To perform the test, streak a β-hemolytic *Staphylococcus aureus* and a *Rhodococcus equi* culture in parallel and diametrically opposite each other on a sheep blood agar plate. Streak several test cultures parallel to one another, but at right angles to and between the *S. aureus* and *R. equi* streaks. After incubation at 35° C for 24-48 h, examine the plates for hemolysis. *L.*

*monocytogenes* and *L. seeligeri* hemolytic reactions are enhanced in the zone influenced by the *S. aureus* streak. The other species remain non-hemolytic. The *L. monocytogenes* reaction is often optimal at 24 h rather than 48 h. To obtain enough *R. equi* to provide a good streak of growth, incubate the slant culture 48 h rather than 24 h. Use of known control isolates of *Listeria* spp. on a separate sheep blood agar plate is recommended. Sheep blood agar plates should be as fresh as possible.

**Table 4. CAMP test hemolytic enhancement of *Listeria* species**

| | **Hemolysis enhancement with** | |
|---|---|---|
| | ***Staphylococcus aureus* (S)** | ***Rhodococcus equi* (R)** |
| *L. monocytogenes* | + | -* |
| *L. ivanovii* | - | + |
| *L. innocua* | - | - |
| *L. welshimeri* | - | - |
| *L. seeligeri* | + | - |

* Rare strains are S+ and R+. The R+ reaction is less pronounced than that of *L. ivanovii*.

CAMP test strains are available from culture collections, including the American Type Culture Collection (ATCC), Manassas, VA http://www.atcc.org

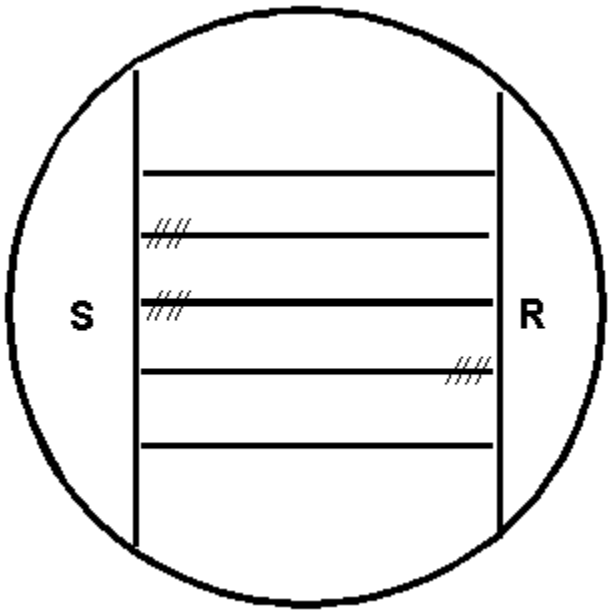


Figure 1. CAMP test for *Listeria monocytogenes*: Inoculation pattern of the sheep blood agar plate. Horizontal lines represent streak inoculations of 5 test strains. Vertical lines represent streak inoculations of *Staphylococcus aureus* (S) and *Rhodococcus equi* (R).

Hatched lines indicate (diagrammatically only) the locations of hemolysis enhancement regions.

Streak weakly β-hemolytic *S. aureus* FDA strain ATCC 49444 (CIP 5710; NCTC 7428) or strains ATCC 25923 and *R. equi* (ATCC 6939; NCTC 1621) vertically on sheep blood agar. Separate vertical streaks so that test strains may be streaked horizontally between them without quite touching them. After 24- and 48-h incubation at 35° C, examine plates for hemolysis in the zone of influence of the vertical streaks. Figure 1 shows the arrangement of the culture streaks on a CAMP plate. Hemolysis of *L. monocytogenes* and *L. seeligeri* is enhanced near the *S. aureus* streak; *L. ivanovii* hemolysis is enhanced near the *R. equi* streak. Other species are non-hemolytic and do not react in this test. The CAMP test differentiates *L. ivanovii* from *L. seeligeri* and can differentiate a weakly hemolytic *L. seeligeri* (that may have been read as non-hemolytic) from *L. welshimeri*. Isolates giving reactions typical for *L. monocytogenes* except for hemolysis should be CAMP-tested before they are characterizedas *L. innocua*. A factor easily prepared from *S. aureus* cultures can be used to enhance hemolysis by *L. monocytogenes* and *L. seeligeri* in sheep blood agar plates. Disks impregnated with the β-lysin of *S. aureus* (REMEL, Lenexa, KS) can be used for the same purpose.

## G. Subtyping of *L. monocytogenes* isolates (Required)

FDA isolates should be typed serologically and genetically.

1. **Serological typing**. Use commercial sera to characterize isolates as type 1, type 4 or not type 1 or 4 (types 3, 5, 6 etc.).

**Table 5. Serotypes of *Listeria* species (37)**

| *Listeria* species | Serotypes |
|---|---|
| *L. monocytogenes* | 1/2a, 1/2b, 1/2c,<br>3a, 3b, 3c,<br>4a, 4ab, 4b<br>4c, 4d, 4e, "7" |
| *L. ivanovii* | 5 |
| *L. innocua* | 4ab, 6a, 6b, Un[a] |
| *L. welshimeri* | 6a, 6b |
| *L. seeligeri* | 1/2b, 4c, 4d, 6b, Un |

[a] Un, undefined

Table 5 exhibits the serological relationships of *Listeria* spp. Most *L. monocytogenes* isolates obtained from patients and the environment are type 1 or 4. More than 90% of *L. monocytogenes* isolates can be serotyped with commercially available sera. All nonpathogenic species, except *L. welshimeri*, share one or more somatic antigens

with *L. monocytogenes*. Serotyping alone without thorough characterization, therefore, is not adequate for identification of *L. monocytogenes*.

Serology is useful when epidemiological considerations are crucial. Use a TSBye culture to inoculate Tryptose broth. Incubate for 24 h at 35° C, at which temperature flagella (H) antigen expression is reduced. Transfer to Tryptose agar slants and incubate for 24 h at 35° C. Wash both slants in a total of 3 ml Difco fluorescent antibody (FA) buffer and transfer to a sterile 16 x 125-mm screw-cap tube. Heat in a water bath at 80°C for 1 h. Sediment cells by centrifugation at 1600 **g** for 30 min. Remove 2.2-2.3 ml of supernatant fluid and resuspend the pellet in the remainder of buffer. Follow manufacturer's recommendations for sera dilution and agglutination procedure. If flagella (H) or somatic (O) sub-factor serotyping is desired, see Chapter 11.

2. **Genetic subtyping**. Data from pulsed-field gel electrophoresis (PFGE) of DNA restriction fragments of FDA isolates should be submitted to PulseNet (CDC, Atlanta, GA). Isolates should also be ribotyped or sent to a ribotyping laboratory.

## H. Immunocompromised Mouse Pathogenicity Test (optional).

The classical tests for *Listeria* pathogenicity are the Anton conjunctivitis test (rabbits), inoculation of mice, and inoculation of embryonated eggs. The immunocompromised mouse test, using intra-peritoneal (i.p.) injection, used here, is recommended because of its greatly improved sensitivity (39). Confirmation of *L. monocytogenes* animal pathogenicity is not needed for clinical isolates and is optional for food isolates. An isolate should be identified as *L. monocytogenes* if it meets all the other criteria outlined in this chapter.

About 4 mg of carrageenan (Sigma type II) dissolved in distilled water (40 mg/ml) is injected, i.p., into 18-20 g mice 24 h before the *Listeria* challenge. The exact volume of carrageenan solution injected, about 0.1 ml, depends on the mouse body weight. The target dose is 200 mg carrageenan per kg bodyweight. Grow isolate for 24 h at 35° C in TSBye. Transfer to 2 tubes of TSBye for another 24 h at 35° C. Place a total of 10 ml culture broth from both tubes into a 16 x 125 mm tube and centrifuge at 1600 **g** for 30 min. Discard supernatant and resuspend pellet in 1 ml of phosphate buffered saline. This suspension will contain approximately $10^{10}$ bacteria/ml; dilute to $10^5$ bacteria per ml and determine actual concentration by a pour or spread-plate count. Inject (i.p.) 16 to 18 g immunocompromised Swiss white mice (5 mice/culture) with 0.1 ml of the concentrated suspension, i.e. approximately $10^4$ bacteria per mouse. Observe for death over 5-day period. Nonpathogenic strains will not kill, but $10^4$ pathogenic cells will kill, usually within 3 days. Use known pathogenic and nonpathogenic strains and carrageenan-treated, uninoculated mice as controls. Use 5 mice per control group. Carrageenan controls should be challenged with 0.1 ml PBS.

## I. Interpretation of test data

The importance of completely characterizing each isolate cannot be overemphasized. Partial characterization, even if accurate, may be misleading. Since all *Listeria* species test negative for indole, oxidase, urease, and $H_2S$ production from organic sulfur compounds

($H_2S$ is produced from thiosulfate in the MICRO-ID test kit) and test positive for methyl red and Voges-Proskauer, these tests are discretionary. *Brochothrix*, which is closely related phylogenetically to *Listeria*, is distinguishable from *Listeria* by its inability to grow at 35° C and by its lack of motility. Distinguishing features of the Gram-positive non-sporeforming rods, *Erysipelothrix* and *Kurthia*, which occur rarely in *Listeria* analysis, can be found elsewhere (11, 37).

All *Listeria* spp. are small, catalase-positive, Gram-positive rods that are motile in wet mounts and in SIM. They utilize dextrose, esculin, and maltose, and some species utilize mannitol, rhamnose, and xylose with production of acid. An isolate utilizing mannitol with acid production is *L. grayi. L. monocytogenes, L. ivanovii*, and *L. seeligeri* produce hemolysis in sheep blood stabs and consequently are CAMP test-positive. Of the three, only *L. monocytogenes* fails to utilize xylose and is positive for rhamnose utilization. The difficulty in differentiating *L. ivanovii* from *L. seeligeri* can be resolved by the CAMP test. *L. seeligeri* shows enhanced hemolysis at the *S. aureus* streak. *L. ivanovii* shows enhanced hemolysis at the *R. equi* streak. Of the non-hemolytic species, *L. innocua* may provide the same rhamnose-xylose reactions as *L. monocytogenes* but it is negative in the CAMP test. *L. innocua* sometimes gives negative results for utilization of rhamnose . The significance of the undocumented reference (41) to hemolytic *L. innocua* isolates is unclear since it is commonly accepted that *L. innocua* is non-hemolytic and *L. monocytogenes* is hemolytic. A *L. welshimeri* isolate that is rhamnose-negative may be confused with a weakly hemolytic *L. seeligeri* isolate unless resolved by the CAMP test. Sometimes aberrant listeria strains are isolated which are extremely difficult to speciate (27a). (See Guideline for BAM Users on Identification of Atypical Hemolytic Listeria Isolates.) If such an aberrant *Listeria* isolate is obtained, contact Anthony.Hitchins@cfsan.fda.gov [Note: The clinical significance of a strain of *L. monocytogenes* that is phenotypically hemolytic-negative is debatable. If it is due to a defect of the hemolysin gene, especially a deletion rather than a point mutation, it is likely clinically less significant than a normal strain would be, judging from laboratory studies of constructed hemolysin mutants in mice. However, if it is due to a regulatory defect that affects the expression of the hemolysin gene *in vitro*, the possibility of conditional expression *in vivo* is raised. Until convenient methods are devised to distinguish these structural and regulatory alternatives, the isolate need only be carefully confirmed as being a strain of *L. monocytogenes* phenotypically hemolysin-negative *in vitro* so that a soundly based regulatory decision can then be made, based upon all the relevant circumstances.]

Only after all other results are available does serotyping and other kinds of typing of *Listeria* isolates become meaningful. Biochemical, serological and pathogenicity data are summarized in Tables 1, 4 and 5. All data collection must be completed before species identities are determined. FDA no longer conducts routine bacteriophage-susceptibility typing of *L. monocytogenes* isolates.

J. **Enumeration (required)**

If a sample tests positive for *L. monocytogenes*, use a reserve portion of sample for enumeration. Current methods of enumeration are only presumptive for *Listeria monocytogenes* and some degree of further testing of isolated *Listeria* colonies is necessary. Conventional enumeration is described and alternative rapid methods are

indicated. The proportion of presumptive isolates that are actually *L. monocytogenes* may be determined by conventional or rapid tests. Flexibility in choice of methods and adaptations of them is permitted but the observed count must be reported with 95% confidence limits, the method used named and any modifications indicated. The correction factor for converting the observed count to *L. monocytogenes* numbers must be reported as the whole number ratio of number of isolates identified as *L. monocytogenes* to the total number of *Listeria* isolates tested.

All enumeration methods, including microscopic, colony and Most Probable Number (MPN) counts are fundamentally governed by the Poisson distribution law of infrequent events. This describes the distribution of *Listeria* among the arrays of compartments (tubes, wells, counting chamber squares, filter grid squares, and virtual squares on culture agar surfaces). Compartmentalization separates or delineates colony-forming units in the various methods. In general, the confidence limits (CLs) of these estimates are considered proportional to the square root of the observed count. [The tabulated CLs for MPN results are asymmetric about the mean because they are usually obtained with low numbers of tubes (3 or 5) near the dilution endpoint.] As the count increases its confidence limits, expressed as a percentage of the count, decrease. Thus, choosing among methods largely reduces to a consideration of material and labor expenses and to how inoculation manipulations for an optimal number of compartments can be reduced by techniques such as filtration, semi-automation and robotics.

**Surveillance Enumeration**. This is required for accumulating data on cell numbers of *L. monocytogene*s in regulatory samples that test positive for the pathogen. Contact Anthony Hitchins@cfsan.fda.gov if you are unable to enumerate the reserve portion of a positive sample in order to try to arrange for its enumeration. To estimate the degree of sample contamination by presumptive *L. monocytogenes*, quantify the initial enrichment broth, before starting incubation, by direct spread plate count on ALOA, BCM or equivalent differential agar. Also, use a 3 or more-tube/well MPN culture procedure on 1, 0.1, 0.01and 0.001-g samples in BLEB (30° C, 48 h, with or without pyruvate and without delayed addition of selective agents) followed by streaking on the chosen selective agar. If all the MPN tubes are *Listeria* positive, use reserve sample to repeat the MPN determination using an appropriate range of more dilute analytical portions, e.g. $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, and $10^{-8}$ g.

If selective agar plates are in short supply, an economic alternative to spreading dilution aliquots on individual selective agar plates is the drop plating method. Using a multi-channel pipette is well suited to this method. Decimally dilute 10 µl amounts of the contents of the enrichment containers in 90 µl amounts of TSBye in micro-titer plates with round-bottomed wells. Mix with a gentle circular motion of the micropipette tip before changing the tip for the next dilution. Carefully plate 10 µl of the dilutions as drops on plates of ALOA, BCM or equivalent agar. Let the droplets be absorbed before inverting the plates for incubation. Square plates are most convenient and efficient for this technique.

**Table 6. Alternative rapid enumeration methods.**

| Method | Reference | Validation | Specificity Matrix |
|---|---|---|---|

| | | | |
|---|---|---|---|
| MPN filter | Entis & Lerner (20) | AOAC INTL. | All *Listeria,* FDA foods |
| Filter/colony-lift | Carroll *et al.* (13) | Peer review | *L. monocytogenes* Meat |
| DNA probe colony hybridization | BAM Chapter 24 (17,18) | FDA | *L. monocytogenes*, FDA foods |

Alternatively, the methods shown in Table 6 may be used. Identify isolates by conventional or rapid methods. When all *Listeria* are enumerated estimate the proportion that is *L. monocytogenes* by determining the species of 10 typical *Listeria* colonies. For advice on using these techniques consult Anthony.Hitchins@cfsan.fda.gov . Grant (23a) has developed a filter enumeration method, based on the BAM *Listeria* enrichment and isolation method, which enumerates *Listeria* at cell numbers of >100 cfu/g.

**Tolerance enumeration.** Enumeration to determine if a regulated level of tolerance is being met is not needed with the current "zero-tolerance" policy of no detectable *L. monocytogenes* in 2 x 25-g analytical portions of food or beverage. It would require narrower confidence limits than does surveillance enumeration. Narrower confidence limits for tolerance and surveillance enumeration can be accomplished by counting more colony forming units, which can be accomplished by increasing the number of replicate tubes or other containers. For the current FDA method, the wells of one or more 96-well micro-titer plates, with round-bottomed wells, can be inoculated, by multi-channel pipette or robotically, with 0.1 ml of homogenate of complete BLEB and sample. After incubation at 30° C for 48 h, use the same kinds of transfer methods described in **Surveillance Enumeration** to inoculate enriched samples to ALOA, BCM or equivalent differential agar to determine which wells are positive. Using the proportion of *L. monocytogenes*-positive wells, the mean concentration can be calculated using the Poisson equation.

Alternatively, the 1600 filter grid compartments MPN method for *Listeria* (20) may be used for presumptive enumeration of *L. monocytogenes*.

Identify isolates by conventional methods, including the use of ALOA, BCM or equivalent agar, or by rapid methods. When necessary estimate the proportion of *L. monocytogenes* among 10 *Listeria* isolates.

**Simultaneous detection and enumeration**. Most samples are likely to be negative and thus it is efficient to delay enumeration of reserve samples until the *Listeria* detection stage is completed. Even then, most positive samples will only contain a few cfu/25g. Nevertheless it may sometimes be more convenient to do simultaneous detection and enumeration. To accomplish this, prepare the enrichment homogenate as described above and immediately spread 0.1 ml on ALOA, BCM or an equivalent *L. monocytogenes* selective agar. Incubate plates at 35° C for 24-48 h. The combined minimal method will allow the cell number of presumptive *L. monocytogenes* to be categorized as <0.04 cfu/g, 0.04 - 100 cfu/g, 100-25,000 cfu/g, or > 25,000 cfu /g. More replica plates and more decimal dilutions in TSBye are optional to obtain a more precise enumeration. Test 5

representative colonies for ability to ferment L-rhamnose by the conventional fermentation method, by the BCM rhamnose confirmatory agar or by a rapid *L. monocytogenes* identification kit to definitively rule out the uncommon occurrence of *L. ivanovii* in foods.

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## SUPPLEMENT 1A.
## Stated Food Matrix Applicability and Specific Food Matrices Used in Validating *Listeria* Rapid Screening Methods

### Assurance EIA Kit and VIP Kit

(Applicable to dairy foods, red meats, pork, poultry products, fruits, nutmeats, seafood, pasta, vegetables, cheese, animal meal, chocolate, and eggs)

Milk, non-fat, dry
Ice cream
Poultry, raw
Shrimp, raw
Beef, cooked roast

Beans, green

### GENE-TRAK *Listeria* Hybridization Assay

(Applicable to dairy products, meats, and seafoods)

Milk, 2%
Cheese, Brie
Crab meat, cooked
Frankfurters
Beef, roast
Pork, raw ground

### *Listeria* -Tek ELISA Assay

(Applicable to dairy products, seafoods, meats)

Frankfurters
Beef, roast, vacuum-packed
Cheese, Brie
Milk, 2%, pasteurized
Shrimp, raw, shelled, frozen
Crab meat, cooked, frozen

### TECRA *Listeria* Visual Immunoassay

(Applicable to dairy foods, seafoods, poultry, meats (not raw ground chuck), leafy vegetables)

Fish, fillets
Ice cream
Lettuce
Chicken
Turkey, ground

### VIDAS LIS Assay Screening Method

(Applicable to dairy products, vegetables, seafoods, raw meats and poultry, and processed meats and poultry)

Ice cream
Green beans
Fish
Turkey
Cheese
Roast beef

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# SUPPLEMENT 1B.
# Validated Application of *Listeria* Genus Rapid Methods to the Historical List of Food Matrices Analyzed by FDA

Abbreviations: P = Pre-collaborative study; C = Collaborative study, "." = No data.

The listed matrices are those encountered by FDA laboratory analysts as of January 1998.

| Food Matrix | | Rapid Test Kits | | | | |
|---|---|---|---|---|---|---|
| | | Assurance-EIA and VIP kits | Gene-Trak | ListeriaTek | Tekra VIA | VIDAS Listeria |
| *Condiments* | Mayonnaise | . | . | . | . | . |
| | Mustard | . | . | . | . | . |
| *Crustacean* | Crab | . | C | . | . | . |
| | Crab_cakes | . | . | . | . | . |
| | Crab_ck_fr | . | . | C | . | . |
| | Crab_claws | . | . | . | . | . |
| | Crab_meat | P | P | P | P | P |
| | Crab_meat_cocktail | . | . | . | . | . |
| | Crab_meat_deviled | . | . | . | . | . |
| | Crab_meat_imitation | . | . | . | . | . |
| | Crab_snow (3) | . | . | . | . | . |
| | Crab_stuffed | . | . | . | . | . |
| | Crabmeat | . | . | . | . | . |
| | Crabmeat_backfin | . | . | . | . | . |
| | Crawfish | . | . | . | . | . |
| | Crawfish_chinese | . | . | . | . | . |
| | Crawfish_tails (2) | . | . | . | . | . |
| | Extruded | . | . | . | . | . |
| | Lobster-imitation | . | . | . | . | . |
| | Lobster_meat (4) | . | . | . | . | . |
| | Lobster_meat_cooked | . | . | . | . | . |
| | Lobster_meat_imit. | . | . | . | . | . |
| | Prawns_cooked | . | . | . | . | . |
| | Shrimp | . | . | . | P | P |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Shrimp_breaded (2) | . | . | . | . | . |
| | Shrimp_cooked (3) | . | . | . | . | . |
| | Shrimp_peeled | . | . | . | . | . |
| | Shrimp_raw | C | P | C | . | . |
| | Shrimp_salad | . | . | . | . | . |
| *Dairy* | Cheese_unnamed (2) | . | . | . | . | CP |
| | Cheese_blue | . | . | . | . | . |
| | Cheese_blue_cashel | . | . | . | . | . |
| | Cheese_brie | . | C | C | . | . |
| | Cheese_cheddar | . | P | . | . | . |
| | Cheese_costello | . | . | . | . | . |
| | Cheese_cottage | . | P | . | . | . |
| | Cheese_cows-milk | . | . | . | . | . |
| | Cheese_cream_lox | . | . | . | . | . |
| | Cheese_gorgonzola | . | . | . | . | . |
| | Cheese_ilha_azul | . | . | . | . | . |
| | Cheese_korsholm | . | . | . | . | . |
| | Cheese_kosher-lox | . | . | . | . | . |
| | Cheese_mozzarella | . | . | . | . | . |
| | Cheese_pimento | . | . | . | . | . |
| | Cheese_queso-fr (2) | . | . | . | . | . |
| | Cheese_reblochon | . | . | . | . | . |
| | Cheese_ricotta_hard | . | . | . | . | . |
| | Cheese_ricotta | . | . | . | . | . |
| | Cheese_ricotta_pec | . | . | . | . | . |
| | Cheese_salad-spread | . | . | . | . | . |
| | Cheese_semi_soft | . | . | . | . | . |
| | Cheese_soft | P | . | . | P | . |
| | Cheese_soft_mexican | . | . | . | . | . |
| | Cheese_spread | . | . | . | . | . |
| | Cheese_swiss | . | . | . | . | . |
| | Cheese_white | . | . | . | . | . |
| | Cheese_white_soft | . | . | . | . | . |
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|---|---|---|---|---|---|---|
| | Cheese_Island | . | . | . | . | . |
| | Cheese_Island_wheel | . | . | . | . | . |
| | Cheese_St.Jorge(2) | . | . | . | . | . |
| | Cheese_St.Jorge_Isl | . | . | . | . | . |
| | Egg_dry | P | . | . | . | . |
| | Egg_liq._frozen | P | . | . | . | . |
| | Ice cream_unnamed | C | P | P | C | C |
| | Ice cream_chocolate | . | . | . | . | . |
| | Ice cream_cookie_flv | . | . | . | . | . |
| | Ice cream_peppermint | . | . | . | . | . |
| | Ice cream_raisin_Fr. | . | . | . | . | . |
| | Ice cream_rum_French | . | . | . | . | . |
| | Ice cream_sandwich | . | . | . | . | . |
| | Ice cream_strawberry | . | . | . | . | . |
| | Ice cream_vanil_crch | . | . | . | . | . |
| | Ice cream_vanl_Fr(2) | . | . | . | . | . |
| | Ice cream_vanilla(2) | . | . | . | . | P |
| | Milk | P | . | P | . | P |
| | Milk_chocolate | P | P | P | P | . |
| | Milk_nf_dry | C | P | P | P | P |
| | Milk_raw | . | . | . | . | . |
| | Milk_2% | . | C | C | . | . |
| | Sherbert_raspberry | . | . | . | . | . |
| | Yogurt_mix_bluebrry | . | . | . | . | . |
| | Yogurt_mix_vanilla | . | . | . | . | . |
| *Fishery* | Belt-fish (2) | . | . | . | . | . |
| | Calamari | . | . | . | . | . |
| | Catfish (2) | . | . | . | . | P |
| | Catfish_fillet | . | . | . | . | . |
| | Catfish_nuggets | . | . | . | . | . |
| | Chubs, raw | . | . | . | . | . |
| | Chum_roe_salted | . | . | . | . | . |
| | Cuttle-fish_salted | . | . | . | . | . |
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|---|---|---|---|---|---|
| Cuttlefish | . | . | . | . | . |
| Cuttlefish_seasoned | . | . | . | . | . |
| Eel_frozen | . | . | . | . | . |
| Eel_broiled | . | . | . | . | . |
| Eel_seasoned | . | . | . | . | . |
| Eel_smoked | . | . | . | . | . |
| File-fish_dried | . | . | . | . | . |
| Fish_barbequed | . | . | . | . | . |
| Fish_cake | . | . | . | . | . |
| Fish_cake_fried | . | . | . | . | . |
| Fish_fillet | . | P | . | C | . |
| Fish_nova_chips | . | . | . | . | . |
| Fish_raw | P | . | P | . | CP |
| Fish_salted | . | . | . | . | . |
| Fish_smoked (3) | . | . | . | . | . |
| Fish_stuffed | . | . | . | . | . |
| Flounder_stuffed | . | . | . | . | . |
| Flying-fish_roe_sd | . | . | . | . | . |
| Flying-fish_roe | . | . | . | . | . |
| Gefilte_fish | . | . | . | . | . |
| Halibut_fillet | . | . | . | . | . |
| Halibut_smoked | . | . | . | . | . |
| Halibut_smoked | . | . | . | . | . |
| Mackeral_dried | . | . | . | . | . |
| Pollack_roe (4) | . | . | . | . | . |
| Pollack_roe_salted | . | . | . | . | . |
| Pollack_roe_seasnd | . | . | . | . | . |
| Pollack_roe_spicy | . | . | . | . | . |
| Orange_roughy_stufd | . | . | . | . | . |
| Sable_fish_smoked | . | . | . | . | . |
| Salmon_caviar | . | . | . | . | . |
| Salmon_jerky | . | . | . | . | . |
| Salmon_pate | . | . | . | . | . |
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|---|---|---|---|---|---|---|
| | Salmon_patties (2) | . | . | . | . | . |
| | Salmon_rainbow_smkd | . | . | . | . | . |
| | Salmon_roe_caviar | . | . | . | . | . |
| | Salmon_smoked (5) | . | . | . | . | . |
| | Salmon_smoked_scot | . | . | . | . | . |
| | Salmon_smoked_Atc | . | . | . | . | . |
| | Salmon_sockeye_smok | . | . | . | . | . |
| | Sea-squirt | . | . | . | . | . |
| | Shad_cold-smoked | . | . | . | . | . |
| | Squid_rings | . | . | . | . | . |
| | Sturgeon_smoked | . | . | . | . | . |
| | Surimi | . | . | . | . | . |
| | Swordfish_smoked | . | . | . | . | . |
| | Trout_gldn_rainbow | . | . | . | . | . |
| | Trout_smoked | . | . | . | . | . |
| | Tuna_smoked (2) | . | . | . | . | . |
| | Walleye_fish-fillet | . | . | . | . | . |
| | White-bass | . | . | . | . | . |
| | White-fish_smoked | . | . | . | . | . |
| *Fruit* | Cantaloup_sliced | . | . | . | . | . |
| *Meal* | Bone_meal | P | . | . | . | . |
| *Meat* | Beef_cooked | . | . | P | . | . |
| | Beef_raw | P | . | . | P | P |
| | Beef_roast | C | C | P | P | CP |
| | Franks/Hotdog | . | C | C | P | P |
| | Ham_boiled_slices | . | . | . | . | . |
| | Ham_chopped | . | . | . | . | . |
| | Ham_cured | . | . | P | . | . |
| | Pork_raw | P | C | . | . | . |
| | Pork_raw_ground | . | P | . | . | . |
| | Sausage_bologna | . | . | . | P | P |
| | Sausage_fermented | . | P | . | . | . |
| | Sausage_pork | . | . | . | . | . |
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|---|---|---|---|---|---|---|
| | Sausage_raw | . | . | P | . | P |
| *Mollusks* | Clam_baby_meat | . | . | . | . | . |
| | Clams _stuffed (2) | . | . | . | . | . |
| | Clams_baby | . | . | . | . | . |
| | Clams_jack-knife | . | . | . | . | . |
| | Mussells_green_shell | . | . | . | . | . |
| | Mussels_cooked | . | . | . | . | . |
| | Mussels_smoked | . | . | . | . | . |
| | Oysters | . | . | . | P | . |
| | Oysters_fresh | . | . | . | . | . |
| | Oysters_raw | . | . | P | . | . |
| | Scallop_imitat. (2) | . | . | . | . | . |
| | Scallops | P | . | . | . | . |
| *Nuts* | Chocolate | P | . | . | . | . |
| | Nuts | P | . | . | . | . |
| *Pasta* | Pasta_unnamed | P | . | . | . | . |
| | Rotini | . | . | . | . | . |
| | Tortellini_cheese | . | . | . | . | . |
| *Poultry* | Chicken | . | . | . | P | . |
| | Chicken_raw | P | . | P | C | P |
| | Poultry_raw | C | . | . | . | . |
| | Turkey (2) | . | . | . | . | . |
| | Turkey_ground | . | . | . | C | . |
| | Turkey_raw | . | . | P | . | CP |
| | Turkey_raw_ground | . | P | . | . | . |
| *Salads* | Cole-slaw | . | . | . | . | . |
| | Crab_meat_imitation | . | . | . | . | . |
| | Crab_seafood | . | . | . | . | . |
| | Fish_whiting | . | . | . | . | . |
| | Fruit | . | . | . | . | . |
| | Ham | . | . | . | . | . |
| | Lobster | . | . | . | . | . |
| | Lobster_seafood | . | . | . | . | . |
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|---|---|---|---|---|---|---|
| | Potato (3) | . | . | . | . | . |
| | Seafood (2) | . | . | . | . | . |
| | Toss_salad | . | . | . | . | . |
| *Sandwiches* | Beef | . | . | . | . | . |
| | Beef_roast (2) | . | . | . | . | . |
| | Beef_roast_po-boy | . | . | . | . | . |
| | Beef_rst/turkey/chs | . | . | . | . | . |
| | Cheese (2) | . | . | . | . | . |
| | Cheese_burger (2) | . | . | . | . | . |
| | Chicken & swiss | . | . | . | . | . |
| | Chicken_charboiled | . | . | . | . | . |
| | Chicken_salad | . | . | . | . | . |
| | Cold_cut | . | . | . | . | . |
| | Egg_sausage muffin | . | . | . | . | . |
| | Ham & cheese (6) | . | . | . | . | . |
| | Ham & salmon sub. | . | . | . | . | . |
| | Ham & turkey (2) | . | . | . | . | . |
| | Ham/swiss_rye | . | . | . | . | . |
| | Ham_egg_chse_muff. | . | . | . | . | . |
| | Ham_salad | . | . | . | . | . |
| | Hamburger_artific. | . | . | . | . | . |
| | Hamburger_cheese | . | . | . | . | . |
| | Hot_dog_submarine | . | . | . | . | . |
| | Perogie | . | . | . | . | . |
| | Potato/cheese | . | . | . | . | . |
| | Protein_vegetable | . | . | . | . | . |
| | Rib_barbeque | . | . | . | . | . |
| | Steak | . | . | . | . | . |
| | Turkey (4) | . | . | . | . | . |
| | Turkey & cheese | . | . | . | . | . |
| | Turkey_club | . | . | . | . | . |
| *Speciality* | Capelin_dried | . | . | . | . | . |
| | Chicken_frd_mtless | . | . | . | . | . |
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|---|---|---|---|---|---|---|
| | Guacamole (2) | . | . | . | . | . |
| | Guacomole_chili | . | . | . | . | . |
| | Tofu | . | . | . | . | . |
| *Vegetables* | Avocado_pulp (2) | . | . | . | . | . |
| | Beans_green | C | . | . | . | C |
| | Blend_frozen | . | . | . | . | . |
| | Broccoli_frozen | . | . | . | . | . |
| | Broccoli_normandy | . | . | . | . | . |
| | Cabbage_shredded | . | . | . | . | . |
| | Carrots_crinkle-cut | . | . | . | . | . |
| | Coleslaw | P | . | . | . | . |
| | Lettuce | . | . | . | C | . |
| | Mixed_rotini | . | . | . | . | . |
| | Salad_mix_veggies | . | . | . | . | . |
| | Salad_veggies_shred | . | . | . | . | . |
| | Spinach | . | . | . | . | . |

Bad Bug Book:
*Listeria monocytogenes*

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*Author: Anthony D. Hitchins
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