Chapter 2

Traditional Methods for Isolation of *Listeria monocytogenes*

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Abstract

Conventional methods for the detection of *Listeria monocytogenes* in foods and environmental samples relies on selective pre-enrichment, enrichment, and plating. This is followed by confirmation of suspected colonies by testing a limited number of biochemical markers.

Key words Culture methods, Enrichment, Detection, Enumeration, Confirmation, Selective media, ISO standards, Most Probable Number

1 Introduction

Detection and identification of *Listeria monocytogenes* in food and environmental samples traditionally involve culture methods based on selective pre-enrichment, enrichment, and plating. This is followed by confirmation of suspected colonies using colony morphology, sugar fermentation pattern, and hemolytic properties (Fig. 1). *L. monocytogenes* is a non-spore forming, catalase-positive, Gram-positive rod-shaped bacterium that shows hemolytic activity on blood agar.

On this basis, several methods were developed worldwide for the detection and/or enumeration of this pathogen. FDA-BAM [1], USDA [2] methods, and ISO 11290 standards [3, 4] are probably the most commonly used reference methods. The criteria of the EU Regulation 20073/2005 [5] define ISO 11290-1 [3] and ISO 11290-2 [4] as the reference methods for detection and enumeration, respectively, of *L. monocytogenes*. Negative results can be confirmed in 3–4 days, the time for a positive result is usually 5–7 days from sample collection.

It is well known that microorganisms in foods are often injured so that they become sensitive to the presence of selective agents

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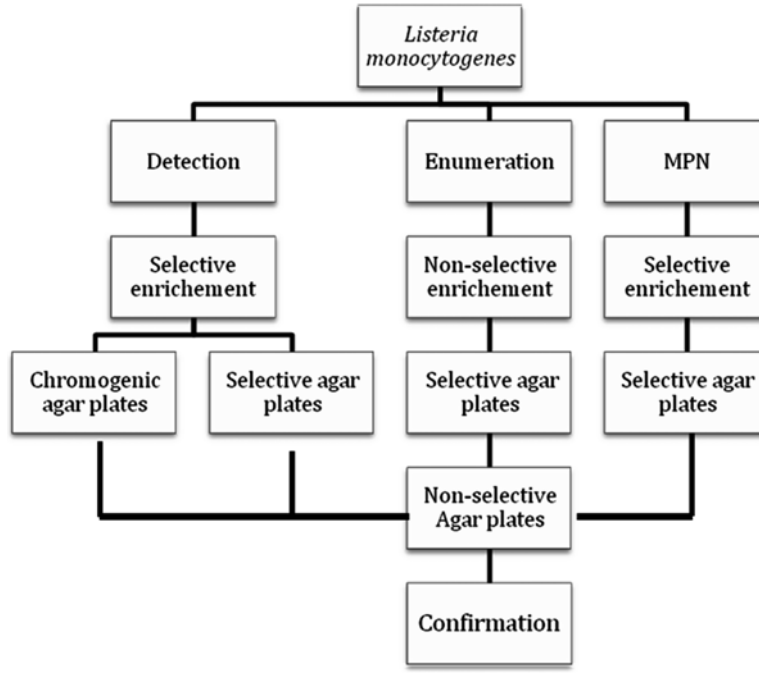

Fig. 1 Conventional approaches for the detection and enumeration of *Listeria monocytogenes*

present in media recommended for their isolation [6, 7]. In order to overcome this limitation, recovery of stressed cells is promoted by a pre-enrichment step in a non-selective broth prior to the selective enrichment and isolation on selective/differential agar media. Most conventional selective enrichment broths contain selective agents: nalidixic acid that inhibits growth of Gram-negative organisms; acriflavine that inhibits Gram-positive bacteria; cycloheximide that inhibits the growth of saprophytic fungi; and lithium chloride (LiCl) that inhibits enterococci. The selective agents commonly used in *L. monocytogenes* isolation media are acriflavine, LiCl, polymyxin B, and cephalosporins.

Detection of *L. monocytogenes* after enrichment is hindered by several factors namely the high population of competitive microflora, the low levels of the pathogen, and the interference of inhibitory food components. The higher growth rate of *L. innocua* in selective liquid media compared with *L. monocytogenes* can result in a high number of false-negative results on Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM) and Oxford (OXA) agars, the media initially recommended by ISO [8, 9]. Differentiation of colonies of *L. monocytogenes* from other nonpathogenic species of *Listeria* is not possible on these media—detection based on the hydrolysis of aesculin. Johansson [10] demonstrated that the selection of five colonies for confirmation

from these media might not be sufficient if other *Listeria* species were present. In 2004, ISO modified the isolation as well as enumeration media for *L. monocytogenes*. The chromogenic medium Agar *Listeria* according to Ottaviani and Agosti (ALOA) was adopted as an obligatory selective and differential medium for the isolation of *Listeria* spp. and presumptive identification and enumeration of *L. monocytogenes* [3, 4]; detection based on the activity of the enzymes phosphatidylinositol phospholipase C and β-glucosidase. Lecithin present in the agar is hydrolyzed by phospholipase enzyme synthesized only by *L. monocytogenes* and *L. ivanovii* forming a white precipitation zone around the colony. β-Glucosidase cleaves the chromogenic substrate producing green-blue colonies [11]. In addition to ALOA, another selective medium at the choice of the laboratory (e.g., PALCAM or OXA) must be used [3, 4]. It is likely that the more selective/indicator media or methods that are used in the examination of a sample the more likely it is that the results obtained are representative of the true status of the pathogen in the sample.

In addition to the standard method for the enumeration of *L. monocytogenes* in food and environmental samples [4], the Most Probable Number (MPN) technique might be used to estimate the cell density in a test sample; it is particularly useful when low numbers of organisms are present. Generally, three tenfold serial dilutions are used in either a three or five tubes MPN series. Based on positive results achieved, an MPN table is used to infer the cell numbers in the original sample [12].

2 Materials

2.1 Media Preparation

Before sample examination for *L. monocytogenes*, microbiological media and all materials coming into contact with it must be sterile. During any subsequent handling of the bacterial cultures, unwanted or contaminant organisms must be excluded employing aseptic techniques. Complete instructions for the preparation of culture media (namely quantity of powder per liter and sterilization conditions) are given on the label of each bottle. Appropriate precautions must be taken when preparing media that contain toxic agents, particularly antibiotics. They should be handled with care avoiding dispersion of powder which can give rise to allergic or other reactions in laboratory personnel.

1. Rinse all glassware with the distilled/deionized water and make sure that the vessels are clean and free from toxic chemicals which may be absorbed on the surface of the glass.
2. Use freshly prepared distilled water. Use warm (50 °C) water to hasten the solution of the powder.

3. Prepare the medium in a flask about twice the final volume of the medium to allow adequate mixing.
4. With a clean spatula accurately weigh the prescribed amount of medium powder, avoiding inhaling the powder and prolonged skin contact. Close the medium container as soon as possible.
5. Pour half the required volume of distilled water in the flask, then the weighed quantity of medium. Add a stir bar and stir for a few minutes. Pour the rest of the distilled water, washing the sides of the flask to remove any adherent powder.
6. Agar-free media will usually dissolve on gentle agitation. Media containing agar should be heated to dissolve the agar before autoclaving. The media should clarify near boiling (90–100 °C). Do not allow to boil over.
7. Prior to sterilization, after the medium has been cooled to 25 °C, the final pH of the prepared medium must be checked to guarantee that it conforms to the label specification.
8. Most culture media will require final sterilization in an autoclave. Broth media can be distributed into individual lab tubes in the desired amount prior to sterilization. Place dissolved, loosely capped media in the autoclave. If using dehydrated commercial media, follow carefully the manufacture instructions for media preparation, sterilization (time/temperature), and storage conditions.
9. Carefully remove from autoclave and allow cooling to 50–60 °C.
10. For agar culture media, open a sterile package of Petri-dishes preserving the bag for later storage. Mark the sides of the dishes to indicate the type of media and pour about 15–20 mL of the medium, using aseptic technique. When plates have solidified, invert, place in 37 °C incubator for 24–48 h to check for sterility. Store in labelled plastic bag at 4 °C. Pre-warm before using.

2.2 Selective Enrichment Broth Media

1. Buffered *Listeria* Enrichment Broth: Buffered *Listeria* Enrichment Broth (BLEB) base is used in the FDA/BAM recommendations for selective enrichment procedure for isolation of *L. monocytogenes*. The medium BLEB is a modification of the initial formula developed by Lovett et al. [13], by the addition of disodium phosphate, which results in an increased buffering capacity of the medium and improvement of the enrichment properties. Selective agents can be added after an initial 4 h period to facilitate resuscitation, repair, and growth of injured *Listeria* cells.

   Composition: casein enzymic hydrolysate, 17.0 g/L; dextrose, 2.5 g/L; dipotassium hydrogen phosphate, 2.5 g/L; disodium phosphate, anhydrous, 9.6 g/L; monopotassium phosphate, anhydrous, 1.35 g/L; papaic digest of soyabean

meal, 3.0 g/L; sodium chloride, 5.0 g/L; sodium pyruvate, 1.0 g/L; yeast extract, 6.0 g/L. Selective agents include: acriflavine hydrochloride, 10 mg/L; nalidixic acid, 40 mg/L; and cycloheximide, 50 mg/L.

Preparation: dissolve the base components or commercial dehydrated medium base in distilled water, by heating if necessary. Adjust the pH if necessary, so that after sterilization it is 7.3 at 25 °C. Sterilize in the autoclave for 15 min at 121 °C.

The following filter sterilized supplements are aseptically added to the basal media at 47 °C immediately prior to use: 10 mg/L acriflavine hydrochloride (0.5 % aqueous solution); 40 mg/L nalidixic acid sodium salt (0.5 % aqueous solution); 50 mg/L cycloheximide (1 % solution in 40 % ethanol).

Appearance of prepared medium: clear, medium amber with none to moderate precipitate.

2. University of Vermont Medium: University of Vermont Medium (UVM) *Listeria* selective enrichment broth is based on the formula described by Donnelly and Baigent [14], and it is the media recommended in the USDA-FSIS method for isolation of *L. monocytogenes*. UVMI broth has been recommended as a primary enrichment broth for recovery of heat-injured *L. monocytogenes*.

   Composition: beef extract, 5.0 g/L; casein enzymic hydrolysate, 5.0 g/L; disodium hydrogen phosphate, 12.0 g/L; aesculin, 1.0 g/L; monopotassium hydrogen phosphate, 1.35 g/L; proteose peptone, 5.0 g/L; sodium chloride, 20.0 g/L; yeast extract, 5.0 g/L. Selective agents for UVMI include: nalidixic acid, 20 mg/L; acriflavine hydrochloride, 12 mg/L.

   Preparation: dissolve the base components or commercial dehydrated medium base in the distilled water, by heating if necessary. Adjust the pH if necessary, so that after sterilization it is 7.4 at 25 °C. Sterilize in the autoclave for 15 min at 121 °C.

   The following filter sterilized supplements are aseptically added to the basal media at 47 °C immediately prior to use: 12 mg/L acriflavine hydrochloride (0.5 % aqueous solution); 20 mg/L nalidixic acid sodium salt (0.5 % aqueous solution).

   Appearance of prepared medium: medium amber colored, slightly opalescent solution with a bluish tinge.

3. Fraser broth: Fraser broth base is recommended by the ISO 11290-1 [3], for the selective enrichment and enumeration of *L. monocytogenes* and other *Listeria* species in food and environmental samples, based on the formula described by Fraser and Sperber [15]. The base formula of the medium already includes antibiotics, but it is necessary to add the ferric ammonium citrate supplement. Half Fraser broth is used as the primary enrichment broth in the ISO methodology and consists of a modification of Fraser broth which contains half of the

concentration of nalidixic acid and acriflavine hydrochloride to aid in the recovery of stressed cells.

Composition: meat peptone, 5.0 g/L; tryptone, 5.0 g/L; beef extract, 5.0 g/L; yeast extract, 5.0 g/L; sodium chloride, 20.0 g/L; disodium hydrogen phosphate dehydrated, 12.0 g/L; potassium dihydrogen phosphate, 1.35 g/L; aesculin, 1.0 g/L; lithium chloride, 3.0 g/L. Selective agents for Fraser broth and half Fraser broth include nalidixic acid, acriflavine hydrochloride, and ferric ammonium citrate at different concentrations. Nalidixic acid sodium salt solution may be added to the base before autoclaving.

Preparation: dissolve the base components or commercial dehydrated medium base in the distilled water, by heating if necessary. Adjust the pH if necessary, so that after sterilization it is 7.2 at 25 °C. Sterilize in the autoclave for 15 min at 121 °C.

For half Fraser broth preparation, the following filter sterilized supplements are aseptically added to the basal medium at 47 °C immediately prior to use: ferric ammonium citrate 500 mg/L (5 % aqueous solution); nalidixic acid sodium salt, 10 mg/L (1 % in 0.05 M sodium hydroxide solution); 12.5 mg/L acriflavine hydrochloride (0.25 % aqueous solution).

For Fraser broth preparation, the following filter sterilized supplements are aseptically added to the basal medium at 47 °C immediately prior to use: ferric ammonium citrate 500 mg/L (5 % aqueous solution); nalidixic acid sodium salt, 20 mg/L (1 % in 0.05 M sodium hydroxide solution); 25 mg/L acriflavine hydrochloride (0.25 % aqueous solution).

Appearance of prepared medium: Straw colored solution.

2.3 Isolation Selective Media

Selective isolation media can be divided into two categories: aesculin-containing media and chromogenic media. The characteristic of colonies of *Listeria* spp. and *L. monocytogenes* are summarized in Table 1.

1. Aesculin containing media: Aesculin offers differential properties to the media. It is hydrolyzed by β-D-glucosidase, resulting in the formation of 6,7-dihydroxycoumarin that reacts with the ferric ions. All colonies of *Listeria* spp. are greyish-green with brown-black surrounding halos.
2. Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol Agar: Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol Agar (PALCAM) is based on the formulation of van Netten et al. [16], who developed this medium, highly selective due to the presence of LiCl, ceftazidime, polymyxin B, and acriflavine. The double indicator system (aesculin and ferrous iron and mannitol and phenol red) allows the easy differential between *L. monocytogenes*, which does not ferment mannitol, from contaminants, such as enterococci and staphylococci.

Table 1
Characteristics of typical colonies of *Listeria* species and *L. monocytogenes* on isolation media

	Medium	Characteristics of *Listeria* spp. colonies	Characteristics of *L. monocytogenes* colonies
Based on the activity of phosphatidylinositol phospholipase C	ALOA	*L. ivanovii*: blue-green regular round colonies with halo Other *Listeria*: blue-green regular round colonies with or without halo	Blue-green colonies with an opaque halo
	BCM	*L. ivanovii*: turquoise convex colonies with turquoise halos Other *Listeria*: white convex colonies; 2.0 mm without precipitates or halos	Turquoise convex colonies with turquoise halos
	Rapid'*L.mono*	*L. ivanovii*: blue-green colonies with a yellow halo Other *Listeria*: white, with or without a yellow halo	Blue (pale blue, grey-blue to dark blue) colonies
	CHROMagar *Listeria*	*L. ivanovii*: blue with white halo Other *Listeria*: blue without halo	Blue with white halo
Based on the hydrolysis of aesculin	OXA/MOX	At 24 h black with black halos After 48 h remain black with a black halo, but with a sunken center	At 24 h olive-green with black halo After 48 h become darker with a hollow black center surrounded by black zones
	PALCAM	Grey-green with a black halo	Grey-green with a black zone

Composition: protease peptones, 23.0 g/L; starch, 1.0 g/L; sodium chloride, 5.0 g/L; yeast extract, 3.0 g/L; D-glucose, 0.5 g/L; D-mannitol, 10.0 g/L; aesculin, 0.8 g/L; ferric ammonium citrate, 0.5 g/L; phenol red, 0.08 g/L; lithium chloride, 15 g/L; agar, 9–18 g/L. PALCAM selective supplement includes: polymyxin B, 10 mg/L; acriflavine, 5 mg/L; ceftazidime, 20 mg/L.

Preparation: dissolve the base components or commercial dehydrated medium base in the distilled water, by boiling. Adjust the pH if necessary, so that after sterilization it is 7.2 at 25 °C. Sterilize in the autoclave for 15 min at 121 °C.

The following filter sterilized supplements are aseptically added to the basal medium at 47 °C immediately prior to use:

10 mg/L of polymyxin B sulfate solution (1 % aqueous solution), 5 mg/L of acriflavine hydrochloride solution (0.05 % aqueous solution), and 20 mg/L of sodium ceftazidime pentahydrate solution (0.1 % aqueous solution). Mix gently before pour the medium into sterile Petri-dishes.

Appearance of prepared medium: Red gel.

3. Oxford Agar: Oxford *Listeria* Agar (OXA) is prepared according to the formulation of Curtis et al. [17] and is a specified plating medium in the FDA/BAM isolation procedure. Selectivity is increased by adding various antimicrobial agents (acriflavine, colistin sulfate, cefotetan, cycloheximide, and fosfomycin) to the Oxford *Listeria* Agar base.

   Composition: protease peptones, 23.0 g/L; starch, 1.0 g/L; sodium chloride, 5.0 g/L; aesculin, 1.0 g/L; ferric ammonium citrate, 0.5 g/L; lithium chloride, 15.0 g/L; agar, 15.0 g/L. Selective supplements: acriflavine, 5 mg/L; cefotetan, 2 mg/L; colistin sulfate, 20 mg/L; cycloheximide, 400 mg/L; fosfomycin, 10 mg/L.

   Preparation: dissolve the base components or commercial dehydrated medium base in the distilled water by boiling. Adjust the pH if necessary, so that after sterilization it is 7.0 at 25 °C. Sterilize in the autoclave for 15 min at 121 °C.

   Then, after cooling to 47 °C, and immediately before use, aseptically add 10 mL of a filtered sterilized supplement solution containing: 0.4 g of cycloheximide, 0.02 g of colistin sulfate, 0.005 g of acriflavine hydrochloride, 0.002 g of cefotetan, 0.01 g of fosfomycin (dissolved in 5 mL of distilled water and 5 mL of ethanol). Mix gently before pour the medium into sterile Petri dishes.

   Appearance of prepared medium: pale green-colored gel.

4. Modified Oxford Agar: Modified Oxford Agar (MOX) is a modification of the Oxford Agar medium referred above. MOX is recommended for isolating and identifying *L. monocytogenes* from processed meat and poultry products, while OXA is recommended for isolating *Listeria* from enrichment broth cultures. The difference between the two media relies on the selective supplements that are added to the oxford agar base formula: colistin and moxalactam.

   The supplement for MOX includes colistin sulfate, 10 mg/L; and moxalactam, 20 mg/L.

2.4 Chromogenic Media

Culture media utilizing virulence factors of pathogenic *Listeria* spp. for selectivity are an attractive alternative to the conventional methods due to a more rapid detection of pathogenic *Listeria* spp. These types of media are available commercially in powder or ready-to-use agar plates.

1. Agar Listeria according to Ottaviani and Agosti: Agar *Listeria* according to Ottaviani and Agosti (ALOA) is a selective and differential medium for the isolation of *Listeria* spp. from foodstuffs and other samples and for the identification of *L. monocytogenes*. The selectivity of the medium is due to LiCl and to the addition of antimicrobial selective mixture containing ceftazidime, polymyxin B, nalidixic acid, and cycloheximide. The differential activity is due to the presence in the medium of the chromogenic compound for the detection of β-glucosidase, common to all *Listeria* species. The specific differential activity is obtained by means of a substrate (L-α-phosphatidylinositol) for a phospholipase C enzyme that is present in *L. monocytogenes* and in some strains of *L. ivanovii*. The combination of both substrates permits the differentiation of *Listeria* spp., which grow with a green-blue color, from the colonies of *L. monocytogenes*, which grow with a green-blue color surrounded by an opaque halo. Occasionally, some non-*Listeria* spp. appear green-blue with a halo, so confirmation of suspect colonies is necessary.
2. CHROMOagar: CHROMOagar *Listeria* easily differentiates *L. monocytogenes* from other *Listeria* spp. Colonies of *L. monocytogenes* appear a blue color, regular with a white halo. Other microorganisms are blue, colorless, other color, or inhibited. Some strains of *L. ivanovii* may also give blue colonies with a white halo. Some strains of *Bacillus cereus* can also grow as blue colonies but can easily be distinguished as they are much larger with an irregular edge to the colony and very large white halo.
3. Rapid'*L.mono* agar: The principle of RAPID'*L.mono* chromogenic agar medium relies on the specific detection of the phosphatidylinositol phospholipase C activity of *L. monocytogenes* and the inability of this species to metabolize xylose. The addition of xylose to the medium allows for differentiation of *L. monocytogenes* that form characteristic blue, pale blue, grey-blue to dark blue colonies without a yellow halo from *L. ivanovii* that produces blue-green colonies with a distinct yellow halo. Other *Listeria* spp. produce white colonies with or without a yellow halo. The selective supplement inhibits the majority of interfering flora, including Gram-positive and Gram-negative bacteria, yeasts and moulds.
4. Biosynth Chromogenic Medium: The Biosynth Chromogenic Medium I (BCMI) is based on the activity of phosphatidylinositol phospholipase C. The medium contains a novel enzyme substrate 5-Bromo-4-chloro-3-indoxyl-myo-inositol-1-phosphate, which enzymatic cleavage by *L. monocytogenes* and *L. ivanovii* leads to turquoise colonies, easy to enumerate. Non-pathogenic *Listeria* spp. appear clearly distinguishable

as white colonies. The Biosynth Chromogenic Medium II (BCMII) additionally combines the cleavage of X-phos-Inositol in forming turquoise colonies with the production of a white precipitate surrounding the colonies due to lecithinase activity. The inhibition of contaminants is increased by the addition of antibiotics and LiCl.

2.5 Nonselective Media

1. Tryptic Soy Agar Yeast Extract: Tryptic Soy Agar supplemented with 0.6 % of Yeast Extract (TSAYE) is a general purpose plating medium used for the isolation, cultivation, and maintenance of *Listeria* spp., namely for purification of colonies isolated on selective media (e.g., OXA or PALCAM). TSAYE plates can be examined for typical colonies under an obliquely transmitted light—Henry illumination test. Using a powerful source of beamed white light, striking the bottom of the plate in a 45° angle *Listeria* spp. colonies appear blue-grey to blue color and a granular surface.

   Composition: tryptone, 17 g/L; soya peptone, 3 g/L; sodium chloride, 5 g/L; dipotassium phosphate, 2.5 g/L; glucose, 2.5 g/L; yeast extract, 6 g/L; agar, 15 g/L.

   Preparation: Dissolve the components or the commercial dehydrated medium by boiling. Adjust the final pH 7.3 at 25 °C. Autoclave for 15 min at 121 °C.

   Appearance of prepared medium: Prepared medium is trace to slight hazy and yellow beige color.

2. Carbohydrate utilization Broth: This medium is used to differentiate *Listeria* species based on carbohydrate fermentation. This is a carbohydrate-free medium with bromocresol purple as pH indicator. Specific carbohydrates are added to the basal medium, and when inoculated with an organism that has the capacity to ferment the carbohydrate present, acid is produced and the indicator changes the medium color from purple to yellow. If the carbohydrate is not fermented, the color will remain unchanged.

   Composition: enzymatic digest of animal tissues, 10 g/L; meat extract, 1 g/L; sodium chloride, 5 g/L; bromocresol purple.

   Preparation: Dissolve the components or the commercial dehydrated medium by heating if necessary. Adjust the final pH 6.8 at 25 °C. Dispense appropriate amounts of the medium into tubes. Autoclave for 15 min at 121 °C.

   Carbohydrate solutions: dissolve 5 g of the carbohydrate (D-mannitol or L-rhamnose or D-xylose) in 100 mL of distilled water. Sterilize by filtration. For each carbohydrate add aseptically 1 mL of the carbohydrate solution to 9 mL of the medium base.

   Appearance of prepared medium: purple.

3 Methods

Samples should be examined as soon as possible after receipt, preferably within 24 h. If they are highly perishable products (such as shellfish), testing should commence within 24 h of sampling.

In the case of impossibility of initiate the testing at time mentioned, the samples may be frozen at below −15 °C, preferably −18 °C, if the recovery of *L. monocytogenes* is not significantly impaired with the sample matrix concerned. Frozen samples should not be thawed until analysis.

3.1 Detection of L. monocytogenes

1. Weigh 25 g of analytical portions of solid food or 25 mL liquid foods into a sterile plastic bag. Add 225 mL of pre-enrichment medium broth (half Fraser base, BLEB or UVM I). Homogenize the mixture in a Blender or Stomacher for 1–3 min (*see* **Note 1**).
2. Incubate for 24 h at 30 °C.
3. After incubation transfer 0.1 mL of the pre-enrichment broth culture to the 10 mL of enrichment broth medium (Fraser).
4. Incubate for 24 h at 37 °C.
5. Streak a loop of pre-enrichment broth culture onto two selective solid media (*see* **Note 2**).
6. Incubate at 37 °C for 24–48 h (*see* **Note 3**).
7. Streak a loop of enrichment broth culture onto two selective solid media.
8. Incubate at 37 °C for 24–48 h.
9. Examine the dishes for the presence of typical colonies of *Listeria* spp. (*see* Table 1) and proceed to confirmation.

3.2 Enumeration of L. monocytogenes

1. Initial suspension (10^{-1} dilution)—Weigh 10 g of analytical portions of solid food or 10 mL liquid foods into a sterile plastic bag. Add 90 mL or g of diluent medium broth (Buffered peptone water or half Fraser base without the addition of selective agents) (*see* **Note 4**).
2. Homogenize the mixture in a Blender or Stomacher for 1–3 min.
3. Incubate for 1 h at 20 °C.
4. Prepare tenfold dilutions.
5. Transfer 0.1 mL of the liquid test sample or 0.1 mL of the initial suspension and dilutions onto dried ALOA plate (*see* **Note 5**).
6. Spread the inoculum over the surface of the agar plate with the aid of a sterile spreader (*see* **Note 6**).
7. Let the plates on the bench for 15 min for the inoculum to be absorbed into the agar.

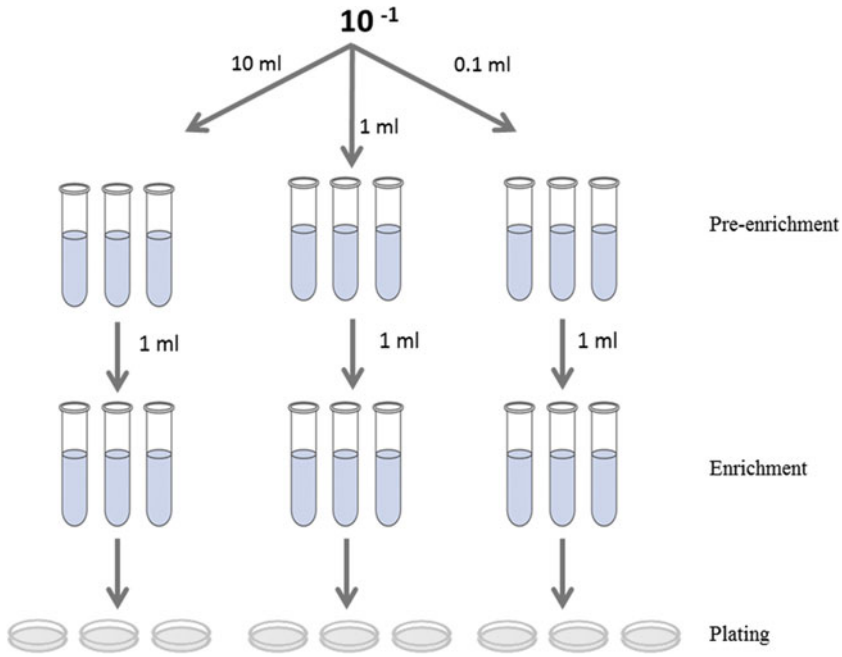

Fig. 2 Schematic representation of MPN method with three tubes dilutions

8. Invert dishes and incubate at 37 °C for 48 h.
9. Count all characteristic colonies presumed to be *L. monocytogenes* and proceed to confirmation (*see* **Note 7**).

3.3 Most Probable Number (MPN) of L. monocytogenes

1. Initial suspension (10^{-1} dilution). Weigh 10 g of analytical portions of solid food into a sterile plastic bag. Add 90 mL or g of diluent media broth (half Fraser base, BLEB or UVM I) (*see* Fig. 2).
2. Homogenize the mixture in a Blender or Stomacher for 1–3 min.
3. Transfer 10 mL of the liquid analytical portion or 10 mL of the initial suspension to three tubes containing 10 mL of double strength pre-enrichment (half Fraser base, BLEB or UVM I) (*see* **Note 8**).
4. Transfer 1 and 0.1 mL of the liquid analytical portion or 1 and 0.1 mL of the initial suspension to three tubes containing 10 mL of single strength pre-enrichment (half Fraser base, BLEB, or UVM I).
5. Incubate for 24 h at 30 °C.
6. Transfer 1 mL from each tube to 10 mL of enrichment media broth (Fraser broth).
7. Incubate for 24 h at 37 °C.
8. Streak a loop of the enrichment broth culture onto chromogenic selective solid medium.

Table 2
Biochemical tests to differentiate *Listeria* species

Species	Phospholipase C	Hemolysis	Production of acid from			CAMP test	
			D-Mannitol	L-Rhamnose	D-Xylose	*S. aureus*	*R. equi*
L. monocytogenes	+	+	–	+	–	+	–
L. innocua	–	–	–	V	–	–	–
L. ivanovii	+	++	–	–	+	–	+
L. seeligeri	–	(+)	–	–	+	(+)	–
L. welshimeri	–	–	–	V	+	–	–
L. grayi subsp. *Grayi*	–	–	+	–	–	–	–
L. grayi subsp. *Murrayi*	–	–	–	V	–	–	–

V: variable; (+): weak reaction; ++: strong positive reaction; +: >90 % positive reactions; –: negative reaction

9. Incubate at 37 °C for 24–48 h.
10. Examine the dishes for the presence of typical colonies of *Listeria* spp. (*see* Table 2) and proceed to confirmation.

3.4 Confirmation of Isolates

1. Select five colonies for confirmation that are representative of suspect colony types and isolate onto TSAYE (*see* **Note 9**).
2. Incubate TSAYE plates at 37 °C for 18–24 h.
3. For biochemical confirmation use only pure cultures. Perform the following classical tests: Gram stain, catalase, hemolysis, and carbohydrate fermentation (*see* Table 2).
4. Test typical colonies for catalase and Gram stain.
5. Inoculate carbohydrate broth (mannitol, rhamnose, and xylose).
6. Incubate at 37 °C 24–48 h (*see* **Note 10**).
7. Perform CAMP test as follows: streak a β-hemolytic *Staphylococcus aureus* and a *Rhodococcus equi* culture in parallel and diametrically opposite each other on a 5 % sheep blood agar plate (*see* **Note 11**).
8. Streak test cultures parallel to one another, but at right angles to and between the *S. aureus* and *R. equi* streaks (but not touching them).
9. Incubate at 37 °C for 24–48 h (*see* **Note 12**).
10. Read tests and interpret the results (*see* Table 2).
11. Report as present/absent in the case of *L. monocytogenes* detection; give a number of *L. monocytogenes* as cfu/g or mL; or as most probable number/g or mL in the case of the MPN method (*see* **Note 13**).

Alternatively, confirmation or identification of *Listeria* species can be performed using commercial kits: API *Listeria* (bioMerieux, Marcy-l'Etoile, France), MICRO-ID™ kit (bioMerieux, Hazelwood, MO; 1, 24), Phenotype MicroArray for *Listeria* (BiOLOG, Hayward, CA), or by Polymerase Chain Reaction (PCR; *see* Chapter 3).

4 Notes

1. If a different amount of sample is used, add a quantity of diluent equal to $9 \times m$ g or $9 \times V$ mL of pre-enrichment medium.
2. Choose media that are complementary, i.e., one chromogenic and one aesculin-containing medium.
3. In the case of use of chromogenic media, follow the manufacturer's instructions.
4. If a different amount of sample is used add a quantity of diluent equal to $9 \times m$g or $9 \times V$mL of pre-enrichment medium. Liquid samples could be inoculated directly onto selective agar.
5. If the sample has low numbers of *Listeria*, distribute 1 mL of the liquid test sample or the initial suspension on the surface of the agar medium in a 140 mm Petri dish or over the surface of three small Petri dishes. Other equivalent media can be used instead of ALOA. In this case follow the recommendations of manufacturer. Agar plates should be dried in an oven or in a laminar-flow cabinet between 25 and 50 °C until the droplets have disappeared from the surface of the medium.
6. It is possible to use the same spreader for the same sample if spreading is started from the higher dilution.
7. Count plates containing less than 150 characteristic or non-characteristic colonies.
8. Five tubes for each dilution can also be used; in case of liquid products prepare the first serial dilution in single strength pre-enrichment medium.
9. For confirmation of the typical colonies it is prescribed to streak isolated colonies from the selective plating medium onto TSAYE agar before performing the biochemical confirmation. However, this step is not necessary if well-isolated colonies (of a pure culture) are available on the selective plating medium. If this is the case, perform the biochemical confirmation directly on a typical (suspect), well-isolated colony of each selective plating medium.
10. Fermentation of carbohydrates usually occurs in 24 h. However, there are *Listeria* species that require more time of incubation so it is advisable to incubate up to 5 days.

11. Instead of the CAMP test commercially available lysin discs could be used.
12. The hemolytic activity of *L. monocytogenes* and to a lesser extent *L. seeligeri* is enhanced in the zone influenced by the *S. aureus* streak. The other species remain non-hemolytic. *L. ivanovii* hemolysis is enhanced in the vicinity of *R. equi*.
13. Use MPN tables to determine MPN value [12].

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