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Methods of Test for Food Microorganisms - Test of *Listeria monocytogenes* in Foods

Part 1: Isolation and identification of *Listeria monocytogenes* in foods

1. Scope： This method is applicable to examine and enumerate *Listeria monocytogenes* in foods.

2. Method： Samples are serially diluted. Culture appropriate diluted test solution with selected media, and then enumerate the colonies. Or enrich the test solution, then isolate and identify the colonies.

2.1. Work environment： The working platform needs to be spacious, clean and well-lit with illumination of cabinet over 100 cd. The air in closed room is well-ventilated, with as little dust and flowing air as possible. Colonies must not exceed 15 CFU/dish for every 15 min.

2.2. Equipment and materials

2.2.1. Biological safety cabinet (BSC): class II or above.

2.2.2. Sterilization oven (Hot air sterilizer): capable of operating at 170 ± 10°C.

2.2.3. Autoclave: capable of operating at 121°C or higher temperature.

2.2.4. Refrigerator: capable of operating at 5 ± 3 °C.

2.2.5. Freezer: capable of operating at –20 ± 3°C.

2.2.6. Ultra-low temperature freezer: capable of operating at –70 ± 5°C.

2.2.7. Water bath: capable of controlling water temperature at ± 1.0°C.

2.2.8. Incubator: capable of controlling temperature at ± 1.0°C.

2.2.9. Blender or stomacher: appropriate for aseptic operation.

2.2.10. Balance: weighting up to 2,000 g with sensitivity of 0.1 g ; weighting up to 100 g with sensitivity of 1 mg.

2.2.11. Microscope: capable of magnification least 1000 times.

2.2.12. pH meter.

2.2.13. Vortex mixer.

2.2.14. Centrifuge.

2.2.15. Hot plate.

2.2.16. Blender.

2.2.17. Shaker.

2.2.18. Light source: general lamp.

2.2.19. Petri dishes: sterile, 90 × 15 mm, surface of the dish should be flat and contain no bubbles or scratches.

2.2.20. Container: screw-capped of flasks, glass bottles or wide-mouth jars made of glass, polyethylene, Teflon or other materials that can be

sterilized at 121°C for more than 20 min, or sterilized bags.

2.2.21. Test tube: 13 × 100 mm,16 × 150 mm or a comparable product.

2.2.22. Inoculating needle and inoculating loop (3 mm i.d.): made of nichrome, platinum-iridium or chromel wire material, or a disposable product.

2.2.23. Spreading rod: 3~4 mm diameter with 45~55 mm spreading area, sterile or disposable.

2.2.24. Sterile membrane filter: 0.45 μm or less, hydrophilic acetate cellulose.

2.2.25. pH test paper: with pH range 6~8.

2.2.26. Spatula, scissors, knife, forceps, mortar and pestle: sterilizable or disposable.

2.2.27. Slides and cover slips: suitable for dyeing and microscopy.

2.2.28. Sterile cotton swab.

2.2.29. Pipette aid or micro-dispenser.

2.2.30. Pipette: sterile, 1 mL tip with scale of 0.01 mL; 5 and 10 mL with scale of 0.1 mL.

2.2.31. Micropipette: 10, 20, 200 and 1000 μL.

2.2.32. Tip: sterile, 10, 20, 200 and 1000 μL.

2.2.33. McFarland nephelometer standard units.

2.2.34. Filter paper.

2.2.35. Crayon or marker: mark or label on the slide.

2.2.36. Aseptic freezing tube.

2.2.37. Brown reagent bottle.

2.2.38. Test strains

Staphylococcus pseudintermedius (ATCC49444; BCRC14980)
Staphylococcus aureus (ATCC25923; BCRC10781)
Rhodococcus equi (ATCC6939; BCRC12859)
Listeria monocytogenes (ATCC19111; BCRC14845、ATCC19115; BCRC15352).

2.2.39. Chemicals

Potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), dipotassium hydrogen phosphate (K_2HPO_4), sodium pyruvate, esculin, ferric ammonium citrate, lithium chloride, nalidixic acid (sodium salt), cycloheximide, colistin sulfate, acriflavin-HCl, cefotean, fosfomycin, 95% ethanol, sodium chloride, mannitol, glucose, phenol red, polymyxin B sulfate, ceftazidime, potassium nitrate (KNO_3 ; nitrite-free), bromocresol purple, starch, rhamnose, xylose, maltose, crystal

violet, ammonium oxalate, potassium iodide, iodine, safranin O, 30% sodium peroxide solution, sulfanilic acid, glacial acetic acid, [*N*-(1-naphthyl) ethyl enediamine dihydrochloride], methyl red, α-naphthol, dehydrated ethanol, colistin methanesulfonate, sodium moxalactam, potassium hydroxide, zinc powder, creatine, *N,N,N′,N′*- tetramethyl-*p*-phenylenediamine dihydrochloride, polysorbate 80 (Tween 80), and glycerol, reagent grade;

yeast extract, beef extract, peptone, agar, trypticase peptone, phytone peptone, proteose peptone No.3, buffered peptone-water powder, Columbia blood agar base, blood agar base, and defibrinated sheep blood, microbiological grade.

2.2.40. Reagent

2.2.40.1. Gram stain solutions

(1) Hucker's crystal violet solution (primary dye)

Solution A: dissolve 2 g of crystal violet in 20 mL of 95% ethanol.

Solution B: dissolve 0.8 g of ammonium oxalate in 80 mL of distilled water.

Mix solution A and solution B, filter with a paper filter after standing for 24 hr, and take the filtrate as the primary dye.

(2) Gram iodine solution (mordant)

Put 2 g of potassium iodide and 1 g of iodine into a mortar. Grind the crystals initially with a pestle for 5 to 10 sec, and then grind with sequential addition of 1 mL, 5 mL, and 10 mL of distilled water until the crystals are completely dissolved. Pour the solution into a brown bottle, and wash the mortar and pestle with an appropriate amount of distilled water. Pool the washings and the initial solution and add distilled water to bring the volume to 300 mL.

(3) Hucker's counterstain solution (counterstain)

Dissolve 2.5 g of safranin O in 100 mL of 95% ethanol to make counterstain stock solution. Working solution is

prepared by diluting 10 mL of the stock solution with 90 mL of distilled water.

Note1: Gram stain solutions deteriorate after prolonged storage. Note the expiry date of the commercial products. Users preparing the staining solutions themselves should regularly check the staining quality.

2.2.40.2. 3% Hydrogen peroxide

Mix 5 mL of 30% hydrogen peroxide with 45 mL of sterile distilled water in a brown reagent bottle. Store it in a refrigerator.

2.2.40.3. 0.85 % Physiological saline solution

Dissolve 8.5 g of sodium chloride in 1000 mL of distilled water in a container, and autoclave at 121^{o}C for 15 min.

2.2.40.4. 0.5 % Acriflavin solution

Dissolve 0.5 g of acriflavine -HCl in 100 mL of distilled water, sterilize with a sterile membrane filter. Store it in a refrigerator.

2.2.40.5. 0.5 % Nalidixic acid

Dissolve 0.5 g of nalidixic acid in 100 mL of distilled water, sterilize with a sterile membrane filter. Store it in a refrigerator.

2.2.40.6. 10% Sodium pyruvate solution

Dissolve 10 g of nalidixic acid in 100 mL of distilled water, sterilize with a sterile membrane filter. Store it in a refrigerator.

2.2.40.7. 1% Cycloheximide in 40% ethanol solution

Dissolve 1 g cycloheximide in 100 mL of dehydrated ethanol and distilled water with ratio of 2:3, sterilize with a sterile membrane filter. Store it in a refrigerator.

2.2.40.8. Nitrite detection reagents

Reagent A: dissolve 1g of sulfanilic acid in 125 mL of 5 N acetic acid. Store it in a refrigerator.

Reagent B: dissolve 0.25 g of N-(1-naphthyl)ethylenediamine dihydrochloride in 200 mL of 5 N acetic acid. Store it in a refrigerator.

2.2.40.9. Methyl red indicator

Dissolve 0.1 g of methyl red in 300 mL of 95% ethanol, dilute with distilled water to 500 mL.

2.2.40.10. Voges-Proskauer reagents (VP reagents)

Reagent A: dissolve 5 g of α-naphthol in 100 mL of absolute ethanol.

Reagent B: dissolve 40 g of potassium hydroxide in 100 mL of distilled water.

2.2.40.11. Oxidase reagent

Dissolve 1 g of *N,N,N',N'*-tetramethyl-p-phenylenediamine dihydrochloride in 100 mL of distilled water. Pour into a brown reagent bottle. Store it in a refrigerator not more than one week before use.

2.2.40.12. 0.1 M Potassium phosphate buffer solution

Dissolve 17.4 g of K_2HPO_4 in 500 mL of distilled water, adjust to pH 6.0, dilute with distilled water to 1000 mL, and autoclave at 121°C for 15 min. Store it in a refrigerator.

2.2.40.13. 1 % Colistin solution

Dissolve 1 g of colistin in 100 mL of 0.1 M potassium phosphate buffer solution. Store it in a refrigerator.

2.2.40.14. Moxalactam solution

Dissolve 1g of moxalactam in 100 mL of 0.1 M potassium phosphate buffer solution, sterilize with a sterile membrane filter, and dispense 2 mL into each tube. Store it in a refrigerator.

2.2.40.15. 5 N Acetic acid

Dilute 286 mL of acetic acid with distilled water to 1000 mL.

2.2.40.16. 5% Rhamnose solution

Dissolve 25 g of rhamnose in 500 mL of distilled water, and sterilize with a sterile membrane filter. Store it in a refrigerator.

2.2.40.17. 5% Xylose solution

Dissolve 25 g of xylose in 500 mL of distilled water, and sterilize with a sterile membrane filter. Store it in a refrigerator.

2.2.41. Media

2.2.41.1. Trypticase soy broth (TSB)

Trypticase peptone ····· 17 g
Phytone peptone ····· 3 g
NaCl ····· 5 g
K_2HPO_4 ····· 2.5 g
Glucose ····· 2.5 g
Distilled water ····· 1000 mL

Dissolve ingredients by heating, and autoclave at 121°C for 15 min. Final pH is 7.3 ± 0.2.

2.2.41.2. Buffered listeria enrichment broth (BLEB)

Trypticase peptone ····· 17 g
Phytone peptone ····· 3 g
NaCl ····· 5 g
K_2HPO_4 ····· 2.5 g
Glucose ····· 2.5 g
Yeast extract ····· 6 g
KH_2PO_4 ····· 1.35 g
Na_2HPO_4 ····· 9.6 g
Distilled water ····· 1000 mL

Dissolve ingredients by heating, and autoclave at 121°C for 15 min. Add 11.1 mL of 10% Sodium pyruvate solution sterilized by filtration before enrichment incubation and mix evenly. Final pH is 7.3 ± 0.2.

2.2.41.3. Oxford medium (OXA)

Columbia blood agar base 39～44 g(Depends on brands)
Esculin ····· 1 g
Ferric ammonium citrate ····· 0.5 g
Lithium chloride ····· 15 g
Distilled water ····· 1000 mL

Dissolve ingredients by heating, and autoclave at 121°C for 15 min. Cool to 50°C and aseptically add supplement, mix, and pour into sterile petri dishes.

To prepare supplement, dissolve cycloheximide 0.4 g、colistin sulfate 0.02 g、acriflavin 0.005 g、cefotetan 0.002 g and

fosfomycin 0.01 g in 10 mL of 1:1 mixture of dehydrated ethanol and distilled water. Filter-sterilize supplement before use.

2.2.41.4. PALCAM Listeria selective agar (PALCAM)

Peptone ……11.5 g
Starch……0.5 g
NaCl……2.5 g
Mannitol ……5 g
Esculin……0.4 g
Glucose…… 0.25 g
Ferric ammonium citrate …… 0.25 g
Lithium chloride……7.5 g
Phenol red …… 0.04 g
Agar ……6.5 g
Distilled water……500 mL

Dissolve ingredients by heating, and autoclave at 121°C for 15 min. Cool to 50°C and aseptically add supplement, mix, and adjust to pH 7. 2 ± 0.1, then pour into sterile petri dishes. To prepare supplement, dissolve polymyxin B sulfate 0.005 g、acriflavin 0.0025 g and ceftazidine 0.01 g in 1 mL distilled water. Filter-sterilize supplement before use.

2.2.41.5. Modified Oxford medium, MOX

Columbia blood agar base 39～44 g(depends on brands)
Aagar……2 g
Esculin……1 g
Ferric ammonium citrate …… 0.5 g
Lithium chloride……15 g
1% colistin solution …… 1 mL
Distilled water…… 1000 mL

Dissolve ingredients by heating, and autoclave at 121°C for 10 min. Final pH is 7.2 ± 0.1. Cool to 46°C quickly in a water bath at constant temperature, and add 2 mL of moxalactam solution sterilized by filtration. Mix evenly, dispense into petri dishes. (Do not need to add any further supplement to MOX).

2.2.41.6. Trypticase soy broth with 0.6% yeast extract (TSBYE)

Trypticase peptone ····· 17 g
Phytone peptone ····· 3 g
NaCl ····· 5 g
K_2HPO_4 ····· 2.5 g
Glucose ····· 2.5 g
Yeast extract ····· 6 g
Distilled water ····· 1000 mL

Dissolve ingredients by heating, and autoclave at 121°C for 15 min. Final pH is 7.3 ± 0.2.

2.2.41.7. Trypticase soy agar with 0.6% yeast extract (TSAYE)

Trypticase peptone ····· 15 g
Phytone peptone ····· 5 g
NaCl ····· 5 g
Agar ····· 15 g
Yeast extract ····· 6 g
Distilled water ····· 1000 mL

Dissolve ingredients by heating, and autoclave at 121°C for 15 min. Final pH is 7.3 ± 0.2.

2.2.41.8. Motility test medium (MTM)

Beef extract ····· 3 g
Peptone ····· 10 g
NaCl ····· 5 g
Agar ····· 4 g
Distilled water ····· 1000 mL

Dissolve ingredients by heating. Dispense 5 mL into tubes, and autoclave at 121°C for 15 min. Cool and make it ready to use. Final pH is 7.4 ± 0.2.

2.2.41.9. Sheep blood agar

Blood agar base ····· 33～44 g(Depends on brands)
Distilled water ····· 1000 mL

Dissolve ingredients by heating, and autoclave at 121°C for 15 min. Cool to 45~46°C. Add 50 mL of defibrinated sheep blood. Mix evenly, dispense into petri dishes.

2.2.41.10. Nitrate broth

Beef extract……3 g
Peptone ……5 g
KNO_3 (nitrite-free)……1 g
Distilled water…… 1000 mL
Dissolve ingredients by heating. Dispense 5 mL into tubes, and autoclave at 121°C 15 min. Final pH is 7.0 ± 0.2.

2.2.41.11. MR-VP broth

Buffered peptone-water powder……7 g
Glucose……5 g
K_2HPO_4 ……5 g
Distilled water…… 1000 mL
Dissolve ingredients by heating. Dispense 5 mL into tubes, and autoclave at 121°C for 15 min. Final pH is 6.9 ± 0.2.

2.2.41.12. Purple carbohydrate broth

Proteose peptone No.3…… 10 g
Beef extract……1 g
NaCl ……5 g
Bromocresol purple…… 0.02 g
Distilled water…… 1000 mL
Carbohydrate of glucose, esculin, maltose, and mannitol test medium:
Dissolve 5 g of glucose, esculin, maltose and mannitol in the purple carbohydrate broth. Dispense 2.5 mL of broth into tubes, and autoclave at 118°C for 10 min. Final pH is 6.8 ± 0.2.
Carbohydrate of rhamnose and xylose test medium:
Dispense 5% rhamnose and 5% xylose solution sterilized by filtration into steriled purple carbohydrate broth, respectively. Final concentration is 0.5 %. Dispense 2.5 mL of broth into tubes. Final pH is 6.8 ± 0.2.

2.3. Preparation of the test solution

2.3.1. Solid samples

Mince and mingle the sample properly, take 25 g of the sample, and mix well with 225 mL of BLEB as the 10-fold diluted test

solution.

2.3.2. Powder, granule or other easily smashed samples

Smash the sample with a sterilized spatulas or other utensils and mix well. Take 25 g of the sample, and mix well with 225 mL of BLEB as the 10-fold diluted test solution.

2.3.3. Liquid samples

Mix the sample thoroughly, take 25 mL of the sample, and mix well with 225 mL of BLEB as the 10-fold diluted test solution.

2.3.4. Condensed and thick liquid samples

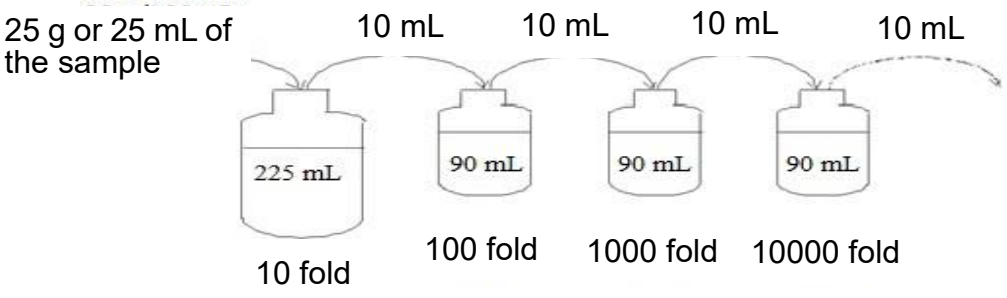
Stir and mix thoroughly the sample, such as pudding, condensed milk, etc. Take 25 g of the sample, and mix well with 225 mL of BLEB as the 10-fold diluted test solution.

2.3.5. Frozen samples

Completely thaw the sample, such as frozen fish meat, poultry meat, fruits, vegetables and dumplings, etc. at refrigerated temperatures (e.g. at 2-5°C within 18 hr), or quickly thaw at higher temperatures (e.g. in a water bath at 45°C for the sample could be thawed within 15 min). Shake the sample frequently to accelerate thawing. After thawing, chop the sample, and mix evenly. Make the sample which do not need to be thawed, such as ice cubes, popsicles and ice cream, etc., into appropriate small pieces. Take 25 g of the sample, and mix well with 225 mL of BLEB as the 10-fold diluted test solution.

2.3.6. Serial diluted test solution

Using a sterilize pipette, pipette 10 mL of the 10-fold diluted test solution into 90 mL of the diluent. Prepare a series of decimal dilutions, such as 100-fold, 1000-fold test solution, and 10000-fold as shown in the following figure.



2.3.7. Swab sample

Aseptically remove the top of swab into a sterile tube, and fold (cut) off the wooden handle of the swab. Add 5 mL of BLEB into the tube. Cap the tube securely, and shake vigorously by tapping with palm 50 times per 10 sec (vibration amplitude must be 15 cm), or by vortexing the tube to loosen the cotton of the swab. Take the eluate as a test solution.

Note 2: In the case of the greasy sample, difficult to disperse and easy to foam, it is necessary to add appropriate amount of sterilized emulsifier (such as Tween 80 at the final concentration in the test solution of 1%), and shake well to emulsify.

Note 3: As the total amount of the sample is less than 50 g (mL), add an appropriate amount of BLEB, according to the amount of the sample, to prepare a 10-fold diluted test solution.

2.4. Identification

2.4.1. Isolation and culture

2.4.1.1. Enrichment

Shake the diluted test solution and/or sample solution thoroughly in section 2.3. and mix well. Incubate at 30°C for 4 hr. Filter-sterilize 0.5 mL of 0.5% acriflavine-HCl solution, 2 mL of 0.5% nalidixic acid sodium salt solution, and 1.25 mL of 1% cyclohexylamine (dissolved in 40% ethanol solution), then add to sample solution respectively. Keep incubating them at 30°C for remainder 44 hr. At 24 and 48 hr, follow the procedure described in section 2.4.2. and swab BLEB enrichment solution respectively onto selective media. Then incubate at 35°C for 24~48 hr.

2.4.1.2. Direct plate count method

2.4.1.2.1. Shake the diluted test solution and/or sample solution thoroughly in section 2.3. and dilute appropriately.

2.4.1.2.2. Pipette 1mL of each diluted test solution and/or the original sample solution onto 3-5 selective media (e.g., 0.3 mL, 0.3

mL and 0.4 mL, make the total volume be 1 mL), and repeat at least twice for each test solution.

2.4.1.2.3. Spreading evenly with the spreading rod and incubate at 35°C for 24 to 48 hr, then observe the morphology of the colonies.

2.4.1.2.4. Select the plates containing 25 to 250 suspect colonies and count these colonies. Follow the procedure described in section 2.4.2.3.

Note 4: When the number of typical colonies at each dilution is less than 25 or greater than 250, the plates with the lowest or highest dilution is counted.

2.4.2. Selective media

2.4.2.1. Choose of selective media: select one of the selective media containing esculin such as OXA, PALCAM and MOX. Recommend to use a commercial chromogenic differential selective agars at the same time. The discoloration caused by the decomposition of the chromogen of the medium due to the biochemical properties of *L. monocytogenes* is helpful to distinguish *L. monocytogenes* from another *Listeria* spp.

2.4.2.2. Spread test solution from section 2.4.1.1. with sterile cotton swab onto about 1/2 of the area of the selective agar then streak onto second section of the plate by inoculate loop (Fig 1), then incubate at 35°C for 24~48 hr. If inoculate onto OXA, PALCAM, and MOX. Typical *L. monocytogenes* show black ring around the colonies after incubating 24~48 hr, and the typical colony diameter should be around 1 mm. it would need incubating additional 24 hr for the slow growth strains.

2.4.2.3. Select up to 5 typical colonies from each plate, and streak onto TSAYE. Then incubate them at 30°C for 24~48 hr. After colony formed repeat inoculate into TSBYE and incubate at 30°C for 24~48 hr for the following identification. If inoculate onto chromogenic differential selective agars, follow the manufacture to pick typical colonies, and inoculate onto TSAYE and into TSBYE for identification.

2.4.3. Identification

2.4.3.1. Gram stain

(1) Preparation of a smear

Place a drop of sterile 0.85% physiological saline solution on a glass slide. Pick a part of the isolated colonies from TSAYE using an inoculating needle (or loop), mix it with saline on the slide, and then smear the mixture into a thin film. Allow the smear to air dry and heat-fix it by quick passing over a flame 3-4 times. Don't put the slide on the fire.

(2) Primary Staining

Stain the fixed smear with Hucker's crystal violet solution for 1 min and wash with water.

(3) Mordant Staining

Cover the smear with Gram iodine solution for 1 min and wash with water.

(4) Decoloring

Decolor with 95% ethanol until no further fading of the blue-violet stains (about 30 sec, longer for thicker smears), and then rinse with water.

(5) Counterstaining

Stain with Hucker's counterstain solution for 30 sec and wash with water.

(6) Air-dry

(7) Microscopic examination

Under the microscope, Gram-positive bacteria appear dark purple and Gram-negative bacteria appear pink. *L. monocytogenes* is Gram-positive and rod- or short rod-shaped without spores.

2.4.3.2. Catalase test

Pick the colonies from TSAYE and smear on the slide, then add 1~2 drop of 3% hydrogen peroxide solution. The bubbles are generated, which is a positive reaction, otherwise it is a negative reaction. *L. monocytogenes* shows positive reaction.

2.4.3.3. Umbrella motility test

Stab in 1/2 deep of MTM from TSAYE and incubate at 20~25°C. Observe every 24 hr for up to 7 days. The MTM shows a umbrella-like growth pattern at 3~5 mm below the upper edge (Fig. 2), which is a positive reaction, otherwise it is a negative reaction. *L. monocytogenes* shows positive reaction.

2.4.3.4. β-hemolysis test

Pick the colony from TSAYE and inoculate into sheep blood agar, then incubate at 35°C for 48 hr. The agar is produced cleared zone around the colony, which is a positive reaction, otherwise it is a negative reaction. *L. monocytogenes* produces a slightly cleared zone so is a positive reaction.

2.4.3.5. Carbohydrate utilization test

Pick colony from TSBYE broth and respectively inoculate the following carbohydrates in 0.5% solutions in purple carbohydrate broth rhamnose, xylose, mannitol, glucose, maltose, or esculin. then incubate at 35°C. Observe once for every 24 hr until 7 days. The test solution turns into yellow, which is a positive reaction, otherwise it is a negative reaction. *L. monocytogenes* shows positive in rhamnose, glucose, maltose and esculin, shows negative in xylose and mannitol.

2.4.3.6. Voges-Proskauer (VP) test

Pick the colony from TSAYE and inoculate into MR-VP broth, and incubate at 35°C for 48 ± 2 hr. Pipette 1 mL of the broth into another sterilized tube, add 0.6 mL of VP solution A and 0.2 mL of VP solution B, then add a few creatine. Shake gently, and let stand for 4 hr. The test solution turns into pink, which is a positive reaction, otherwise it is a negative reaction. *L. monocytogenes* shows positive reaction.

2.4.3.7. Methyl red test (MR test)

Incubate the remaining of MR-VP broth in section 2.4.3.6. at 35°C for 48 ± 2 hr. Add 0.3 mL of methyl red indicator and shake gently. The test solution turns into red, which is a positive reaction, turning yellow is a negative reaction. *L.*

monocytogenes shows positive reaction.

2.4.3.8. Oxidase test

Pick the colony from TSAYE and smear on the filter paper with oxidase reagent. The test paper turns into deep purple after 10~15 seconds, which is a positive reaction, otherwise it is a negative reaction (Avoid to use nickel/chromium loop or wire). *L. monocytogenes* shows a negative reaction.

2.4.3.9. CAMP test

Respectively inoculate *R. equi* and *S. aureus* (or *S. pseudintermedius*) on TSAYE and incubate at 35°C for 24 hr. Pick the colony from TSAYE and dissolve it with 0.85 % Physiological saline solution, the final turbidity more than 1.0 McFarland. Then parallel streak the *R. equi* and *S. aureus* (or *S. pseudintermedius*) with the swab on the fresh sheep blood agar. And vertically streak the test strains (the final turbidity both is McFarland) with the swab between the *R. equi* and *S. aureus* (or *S. pseudintermedius*). Also streak *L. monocytogenes* (ATCC19111; BCRC14845、ATCC19115; BCRC15352) as positive control. Do not overlap the streaks and the gap is about 2~3 mm (Fig.3). Incubate at 35 °C for 24~48 hr. There is an arrow-shaped hemolytic zone near *S. aureus*, but the hemolysis is not obvious near *R. equi*, which is a positive reaction, otherwise it is a negative reaction. *L. monocytogenes* shows positive reaction.

2.4.3.10. Nitrate reduction test

Pick colonies from TSBYE and inoculate into a nitrate broth, and incubate at 35°C for 5 days. Add 0.2 mL each of solution A and solution B of nitrate test reagent in sequence and shake gently. The test solution turns into red-violet, which is a positive reaction. If there is no change in the color of the test solution, a little zinc powder is added and the test solution turns into red-violet, it is a negative reaction. *L. monocytogenes* shows a negative reaction.

2.4.3.11. Culture storage

For long-term storage, Pipette 1 mL of TSB culture solution incubated 6~12 hr into sterile frozen tubes, add 0.1 mL of glycerol sterilized at 121°C for 15 min, and then frozen in liquid nitrogen or immediately keep in ultra-low temperature freezer.

2.5. Determination

Positive of *L. monocytogenes* should in accordance with the results listed in the following table.

Test	Positive (+)	Negative (-)	Reaction of *L. monocytogenes*
1. Catalase test	The bubbles are generated	No bubble	＋
2. Gram stain	Positive (dark purple), coccobacilli and non-sporeforming	Without positive reaction	＋
3. Umbrella motility test	umbrella-like growth pattern at 3~5 mm below the upper edge	Without positive reaction	＋
4. CAMP test	Hemolysis zone near *S. aureus*; not obvious near *R. equi*	Without positive reaction	＋
5. Nitrate reduction test	red-violet	Original color	－
6. Oxidase test	deep purple	Original color	－
7. VP test	pink	Original color	＋
8. Methyl Red test	Red	Yellow	＋
9. Rhamnose utilization test	Yellow	Purple	＋
10. Xylose utilization test	Yellow	Purple	－
11. Mannitol utilization test	Yellow	Purple	－
12. Esculin utilization test	Yellow	Purple	＋
13. Glucose utilization test	Yellow	Purple	＋
14. Maltose utilization test	Yellow	Purple	＋
15. β-hemolysis test	Weak hemolysis	Without positive reaction	＋

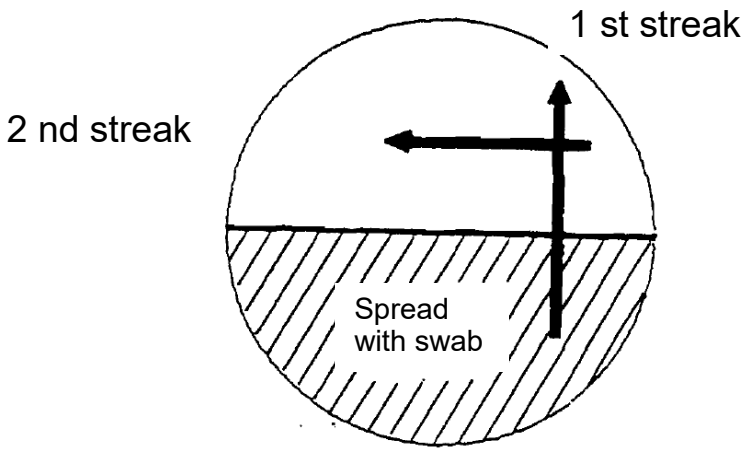


Figure 1. Streak method of OXA, MOX, and PALCAM agar.

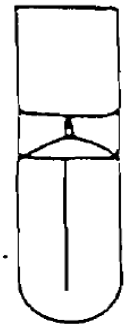


Figure 2. Umbrella motility typical positive reaction

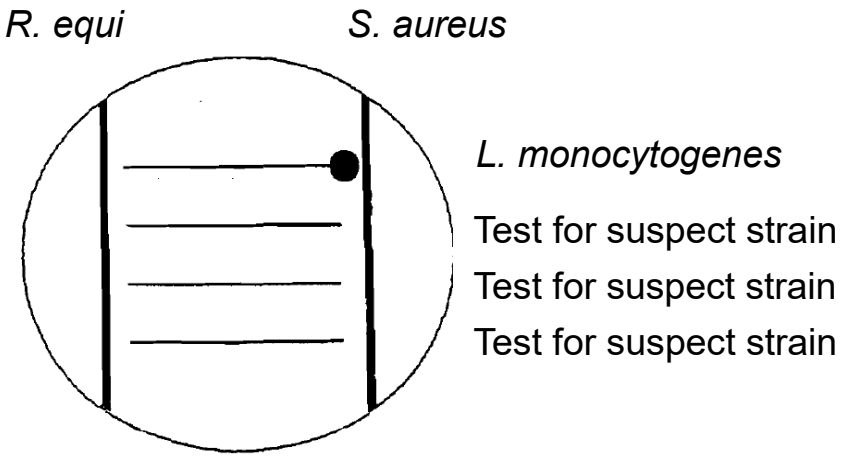


Figure 3. Distribution of strains of CAMP test

2.6. Counting

2.6.1. Calculation of the direct plate method.

2.6.1.1. Calculate the ratio of *L. monocytogenes* in suspect colonies according to section 2.5. (R, see formula below) and calculate the number of *L. monocytogenes* in the sample by the formula of section 2.6.1.2 or 2.6.1.3.

$$\text{Ratio(R)} = \frac{N_1}{N_0}$$

N_0：The number of suspect colonies in all of the plates.

N_1：The number of colonies determined to be *L. monocytogenes* in the identification tests.

2.6.1.2. If only one dilution factor of plates whose colonies are between 25-250 colonies, the total number of suspect colonies in all plates of this dilution factor should be counted, and calculate the number of *L. monocytogenes* in CFU/g or CFU/mL according to the following formula. The number of *L. monocytogenes* should do Banker's rounding to the third digit (When the third digit is five, round up if the second digit is odd, and round off if the second digit is even.) The significant digit is two.

The number of *L. monocytogenes* (CFU/g or CFU/mL)

$$= (\sum a) \times \frac{A}{V_A} \times R$$

Σa：The sum of the number of suspect colonies in all plates of A dilution factor

V_A：The sum of the volume of test solution in all plates of A dilution factor

A：Dilution factor

R：Ratio

2.6.1.3. If there are two dilution factor of plates whose colonies are between 25-250 colonies, calculate the number of *L. monocytogenes* in each dilution factor, and then take the average value according to the following formula.

The number of *L. monocytogenes* (CFU/g or CFU/mL)

$$= \left[(\sum a) \times \frac{A}{V_A} + (\sum b) \times \frac{B}{V_B} \right] \times \frac{R}{2}$$

Σa：The sum of the number of suspect colonies in all plates of A dilution factor

Σb：The sum of the number of suspect colonies in all plates of B dilution factor

VA：The sum of the volume of test solution in all plates of A dilution factor

V_B：The sum of the volume of test solution in all plates of B dilution factor

A & B：Dilution factor

R：Ratio

2.7. It is allowed to use validated commercial media, biochemical test kits or biochemical identification systems. However, when the test results are disputed, the test method shall prevail.

Remark : Pregnant women or potential immunodeficiency individuals are prohibited from accessing *L. monocytogenes* test area.

Part II: Determination of *L. monocytogenes* by real-time PCR assay

1. Scope

This method is applicable to the identification of *L. monocytogenes* in foods.

2. Method

The DNA was extracted from the enrichment broth or isolate culture and identify *L. monocytogenes* by real-time polymerase chain reaction (real-time PCR).

2.1. Work environment: The working platform should be spacious, clean, and well-lit. Procedures of sample pretreatment, sample DNA extraction, real-time PCR reagent preparation and assay require separate compartments to avoid cross-contamination. The preparation of real-time PCR reagents should be carried out in a laminar flow hood.

2.2. Equipment

2.2.1. Real-time polymerase chain reactor: Applied Biosystems 7500 Real-Time PCR System, or equivalent product.

2.2.2. Autoclave: capable of operating at 121°C or higher temperature.

2.2.3. Biological safety cabinet (BSC): class II or above.

2.2.4. Thermomixer: with temperature control and oscillation function.

2.2.5. Refrigerated microcentrifuge: centrifugal force ≥ 20000 ×g, with temperature control at 4°C.

2.2.6. Centrifuge: appropriate for each volume of centrifuge tubes.

2.2.7. Refrigerator: capable of operating at 5 ± 3 °C.

2.2.8. Freezer: capable of operating at –20 ± 3°C.

2.2.9. Vortex mixer.

2.2.10. pH meter.

2.2.11. Spectrophotometry: with wavelengths of 260 nm and 280 nm.

2.2.12. Balance：weighting up to 2,000 g with sensitivity of 0.1 g；weighting up to 100 g with sensitivity of 1 mg.

2.3. Reagent

2.3.1. DNA extraction: Commercial kits for Gram-positive bacteria DNA extraction.

2.3.2. Real-time PCR(Note1)

2.3.2.1. Primers and probes sequences

2.3.2.1.1. *Listeria monocytogenes* (Target gene: *iap* gene)

Primer F：Lm835F

5′- AACTGGTTTCGTTAACGGTAAATACTTA-3′

Primer R：Lm998R

5′- TAGGCGCAGGTGTAGTTGCT-3′

Probe P：Lm918P

5′-FAM-CTACTACTCAACAAGCTGCACCTGCTGC-BHQ-3′

PCR amplicon size :163 bp

2.3.2.1.2. *Listeria* spp. (Target gene: *iap* gene)

Primer F：Lall1055F

5′- GTTAAAAGCGGTGACACTATTTGG-3′

Primer R：Lall1163R

5′-TTTGACCTACATAAATAGAAGAAGAAGATAA-3′

Probe P：Lall1118P

5′-FAM-ATGTCATGGAATAAT-MGB-3′

PCR amplicon size :108 bp

Note 1: After unpacking, dilute the synthesized primers and probes with sterile deionized water to an appropriate concentration. After aliquoting, store at -20°C. In addition, Keep the probes from light. Identification of *L. monocytogenes* of the 5’ end of the probe was labeled with 6-carboxy-fluorescein (FAM), and the 3’ end was labeled with black hole quencher-3 (BHQ3). Identification of *Listeria* spp. of the 5’ end of the probes was labeled with 6-carboxy-fluorescein (FAM), and 3’ end was labeled with minor groove binders (MGB).

2.3.2.2. TaqMan® Fast Reagents Starter Kit (applicable to Applied Biosystems 7500 Real-Time PCR System), this reagent contains deoxyribonucleotide triphosphate, polymerase, etc. When to use, add primers, probes, and the sample DNA.

2.3.3. Reference material: *L. monocytogenes* reference strain or its DNA template.

2.4. Equipment and materials (note 2)

2.4.1. Micropipette: 10, 20, 200, and 1000 μL.

2.4.2. Tip: sterile, 10, 20, 200 and 1000 μL.

2.4.3. Microcentrifuge tube: 200 μL, 600 μL, 1.5 mL, and 2 mL.

2.4.4. Real-time PCR tube: 100 μL.

2.4.5. Real-time PCR plate: 96 wells, applicable to Applied Biosystems 7500 Real-Time PCR System

2.4.6. Glass or plastic bottle: 50, 100, 250, 500, 1000, and 2000 mL.

Note 2: The plastic or glassware used are DNase-free.

2.5. Real-time PCR solution (Note 3)

Applied Biosystems 7500 Real-Time PCR System for identification test

5 μM Primer F	2.0 μL
5 μM Primer R	2.0 μL
5 μM Prober	1.0 μL
TaqMan® Fast Reagents Starter Kit	12.5 μL
Sample DNA solution	5.0 μL
Sterile deionized water	2.5 μL
Total volume	25.0 μL

Note 3: Real-time PCR solution should be prepared in an ice bath.

2.6. Sample DNA solution preparation

2.6.1. Preparation of DNA solution from enrichment broth

Transfer 1 mL of enrichment broth from Part I. section 2.4.1. in 1.5 mL of sterile microcentrifuge tube. Centrifuge at 15000 ×g for 3 min, and discard the supernatant.

2.6.1.1. Direct boiling

Resuspend pellet in 1 mL of sterile deionized water and vortex, then centrifuge at 15000 ×g for 3 min and discard the supernatant. Resuspend pellet in 1 mL of sterile deionized water and vortex again. Boil on the shaker for 10 min, and as the sample DNA stock solution, or store it at -20°C.

2.6.1.2. DNA extraction

Use commercial DNA kits suitable for Gram-positive bacteria DNA extraction, and extract the sample DNA according to the operating instruction. Collect the extracted sample DNA

solution into to a 1.5 mL sterilized centrifuge tube as the sample DNA stock solution. After determining the DNA concentration according to the procedure in section 2.6.3., store it at -20°C.

2.6.2. Preparation of DNA solution from isolate strain

Transfer 1 loop of isolate strain in 1 mL of sterile deionized water in 1.5 mL sterile centrifuge tube and vortex, and mix well. Boil on the shaker for 10 min and centrifuge at 15000 ×g for 3 min, then pipette the supernatant into another 1.5 mL sterile centrifuge tube as the sample DNA stock solution, or store it at -20°C. Preparation of DNA solution also can follow the procedure described in section 2.6.1.2.

2.6.3. Measurement of DNA concentration and purity

Take an appropriate amount of the sample DNA stock solution, dilute it with sterile deionized water to an appropriate dilution, and measure the absorbance (O.D.) at wavelengths of 260 nm and 280 nm, respectively. Multiply the absorbance at wavelength of 260 nm by 50 ng/μL and the dilution factor to obtain the concentration of the sample DNA stock solution. The purity of the DNA solution is judged by the O.D.260/280 ratio, and the ratio should be between 1.7 and 2.0.

2.7. Real-time PCR identification test

2.7.1. Real-time PCR operating procedure (Note 4)

Properly dilute the sample DNA stock solution, primers and probes with sterile deionized water for later use. Take a 1.5 mL sterile centrifuge tube, and prepare the real-time PCR solution according to the composition in section 2.5. Add the TaqMan® Fast Reagents Starter Kit, diluted primers and probes, and sterile deionized water in sequence. Mix well, and aliquot 20 μL into each PCR reaction tubes. Add 5 μL of the sample DNA solution, then place the PCR reaction tubes in a centrifuge, and instantaneously centrifuge at 200 ×g. Transfer them into the real-time PCR reactor, and perform the reaction according to the following conditions. Both positive and negative controls should be used in real-time PCR

experiments.

Steps	Temperature(℃)	Time(sec)
1. Activation	95	20
2. Denaturation	95	5
3. Annealing, extension	60	30
Repeat step 2 to step 3 for 45 cycles		

Note 4: The reaction conditions can be adjusted depending on the instruments used if the above reaction conditions are not applicable.

2.7.2. Real-time PCR fluorescence analysis

After the sample DNA is analyzed by the real-time PCR, the results of the reaction can be interpreted by evaluating the fluorescence amplification curve generated by the probe from the screen on the real-time PCR reactor. Both positive and negative controls should be used in real-time PCR experiments.

2.7.3. Confirmation

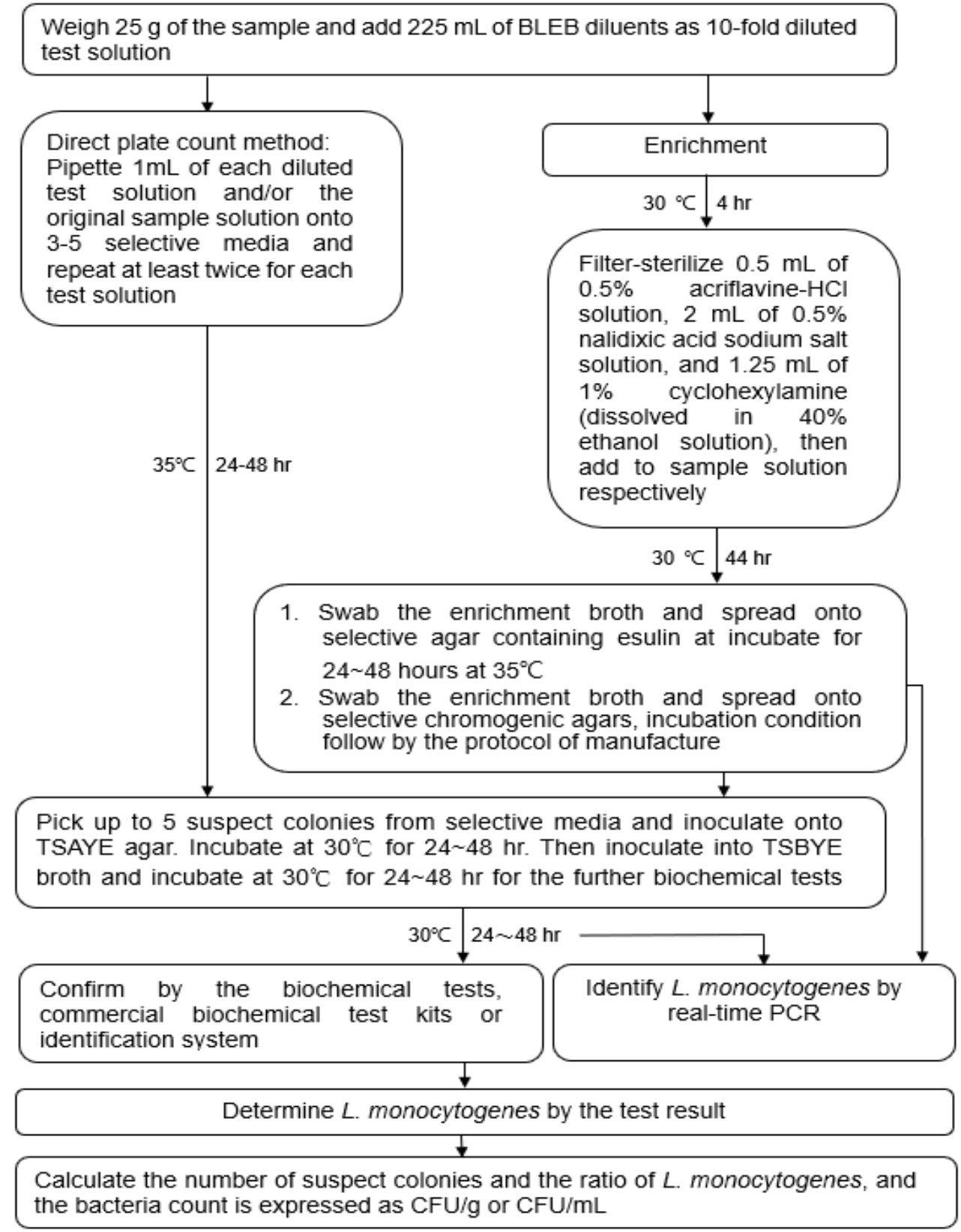
Compare the fluorescence amplification curve of the sample DNA by the real-time PCR with that of positive control. When the sample DNA and the positive control both show the fluorescence amplification curves generated by probe, which means that the real-time PCR amplification product of the sample is the target gene fragment of *Listeria* spp. or *L. monocytogenes*, the sample can be confirmed to contain *Listeria* spp. or *L. monocytogenes.*

Remark: Determination of *L. monocytogenes* by real-time PCR assay in Part II can be performed as needed.

References

Hitchins, A. D., Jinneman, K. and Chen, Y. 2022. Chapter 10 Detection of *Listeria monocytogenes* in foods and environmental samples, and enumeration of *Listeria monocytogenes* in foods. Bacteriological Analytical Manual.
[https://www.fda.gov/food/laboratory-methods-food/bam-detection-and-enumeration-listeria-monocytogenes]

Experimental flow



Note: The real-time PCR steps and the enrichment time can be developed according to the bacteria content of the sample for rapid identification purposes.