GB

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National food safety standard - Food microbiology test

Listeria monocytogenes test

食品安全国家标准 食品微生物学检验 单核细胞增生李斯特氏菌检验

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National food safety standards

Food microbiology test

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1 Scope

This Standard specifies the test methods for *Listeria monocytogenes* in food.

The first method of this Standard applies to the qualitative test of *Listeria monocytogenes* in food; the second method applies to the counting of *Listeria monocytogenes* in food with a high content of *Listeria monocytogenes*; the third method applies to the counting of *Listeria monocytogenes* in food with a low content of *Listeria monocytogenes*.

2 Equipment and materials

In addition to routine sterilization and culture equipment of microbiology laboratories, other equipment and materials are as follows.

2.1 Refrigerator: 2 ℃ ~ 8 ℃.

2.2 Constant temperature incubator: 30 ℃ ± 1 ℃, 36 ℃ ± 1 ℃, 25 ℃ ~ 30 ℃.

2.3 Homogenizer.

2.4 Microscope: 100× ~ 1000×.

2.5 Electronic balance: the sensitivity is 0.1 g, 0.1 mg.

2.6 Conical flask: 100 mL, 500 mL.

2.7 Sterile pipette: 1 mL (with 0.01 mL scale), 10 mL (with 0.1 mL scale) or pipette (with a scale of 0.1 mL, 1 mL, 10 mL) and sterile pipette tip.

2.8 Sterile culture dish: diameter of 90 mm.

2.9 Sterile test tube: 16 mm × 160 mm.

2.10 Centrifuge: 4000 r/min.

2.11 Sterile centrifuge tube: 30 mm × 100 mm.

2.12 Sterile syringe: 1 mL.

2.13 Oil immersion lens or phase contrast microscope.

2.14 Sterile coating stick.

2.15 *Listeria monocytogenes* ATCC 19111 or CMCC 54004 or other equivalent strains.

2.16 *Listeria innocua* ATCC 33090 or other equivalent strains.

2.17 *Listeria ivanovii* ATCC 19119 or other equivalent strains.

2.18 *Listeria seeligeri* ATCC 35967 or other equivalent strains.

2.19 *Staphylococcus aureus* ATCC 25923 or other equivalent strains, requiring the production of *β*-hemolytic ring.

2.20 *Rhodococcus equi* ATCC 6939 or NCTC 1621 or other equivalent strains.

2.21 Mice: ICR strain, weight 18 g ~ 22 g.

2.22 Microbial biochemical identification system.

2.23 Sterile filtration device.

3 Culture medium and reagents

3.1 Tryptic soy broth with 0.6 % yeast extract powder (TSB-YE): see A.1 in Annex A.

3.2 Tryptic soy agar with 0.6 % yeast extract powder (TSA-YE): see A.2.

3.3 Fraser enrichment broth (FB_1, FB_2): see A.3.

3.4 Agar Listeria according to Ottaviani and Agosti: see A.4.

3.5 PALCAM medium: see A.5.

3.6 Gram staining solution: see A.6.

3.7 SIM motility medium: see A.7.

3.8 Buffered glucose peptone water [for methyl red (MR) and acetyl methyl alcohol (VP) tests]: see A.8.

3.9 Sheep blood agar: see A.9.

3.10 Sterile phosphate buffer: see A.10.

3.11 Sterile normal saline: see A.11.

3.12 Sugar fermentation tube: see A.12.

5 Operation steps

5.1 Enrichment

Take 25 g (mL) of sample by aseptic operation, place it in a sterile homogenizing cup containing 225 mL of FB_1 enrichment broth, and homogenize at 8000 r/min ~ 10000 r/min for 1 min ~ 2 min, or place it in a sterile homogenizing bag containing 225 mL of FB_1 enrichment broth, and beat it with a slapping homogenizer for 1 min ~ 2 min to make a 1:10 sample homogenate. If the sample is in liquid form, it can also be mixed by oscillation or stirring. Culture at 30 ℃ ± 1 ℃ for 24 h ± 2 h. Mix well, pipette 0.1 mL of FB_1 enrichment broth, and inoculate in 10 mL of FB_2 enrichment broth. Culture at 30 ℃ ± 1 ℃ for 24 h ± 2 h.

5.2 Separation

Take the mixed FB_2 enrichment broth and inoculate on agar Listeria according to Ottaviani and Agosti (or other equivalent Listeria chromogenic medium) plates and PALCAM medium plates respectively, culture at 36 ℃ ± 1 ℃ for 24 h ~ 48 h, and observe the colonies grown on each plate. Typical colonies form round blue-green colonies with a diameter of 1 mm ~ 3 mm on agar Listeria according to Ottaviani and Agosti plates, surrounded by opaque halos. Typical colonies form round gray-green colonies with a diameter of 1 mm ~ 3 mm on PALCAM medium plates, surrounded by brown-black hydrolysis circles. After 48 h of culture, some colonies form black spots and depressions in the center. The characteristics of colonies on other equivalent Listeria chromogenic medium plates shall be determined according to the product instructions.

NOTE 1: Some *Listeria monocytogenes* colonies on agar Listeria according to Ottaviani and Agosti have weak or no halos around them. There are also some *Listeria monocytogenes* colonies on agar Listeria according to Ottaviani and Agosti have halos that appear slowly, sometimes taking more than 4 days to appear.

NOTE 2: The colony morphology of *Listeria ivanovii* on agar Listeria according to Ottaviani and Agosti is similar to that of *Listeria monocytogenes*.

5.3 Pure culture

Pick 3 to 5 typical or suspicious colonies (select all if less than 3) from each plate (plate that meet the requirements of 5.2), streak on TSA-YE plates or sheep blood plates, and culture at 36 ℃ ± 1 ℃ for 18 h ~ 24 h. *Listeria monocytogenes* on TSA-YE plates or sheep blood plates are grayish white, translucent, with neat edges, dew drop-shaped colonies, and a diameter of 1 mm ~ 2 mm.

5.4 Preliminary identification

Pick an individual colony on TSA-YE plates or sheep blood plates, inoculate in xylose and rhamnose fermentation tubes, and culture at 36 ℃ ± 1 ℃ for 24 h ± 2 h; at the same time, streak on TSA-YE plates or sheep blood plates, and culture at 36 ℃ ± 1 ℃ for 18 h ~ 24 h to obtain pure culture for the next step of identification. Then select the pure culture that is xylose negative and rhamnose positive to continue identification.

5.5 Identification

NOTE: It can first pick one strain from each plate (plate that meets the requirements of 5.2) for identification test. If it is identified as *Listeria monocytogenes*, the result of "detected" can be directly reported according to the provisions of "6 Results and reports"; the result of "not detected" can be reported only after 3 to 5 typical or suspicious colonies (select all if less than 3) selected according to the requirements of 5.3 are identified as non- *Listeria monocytogenes*.

5.5.1 Microscopic examination: Pick an individual colony of pure culture for 18 h ~ 24 h for Gram staining microscopic examination. Listeria is a Gram-positive brevibacterium with a size of (0.4 μm ~ 0.5 μm) × (0.5 μm ~ 2.0 μm). Use normal saline to make a bacterial suspension, observe under an oil immersion lens or phase contrast microscope, the bacteria show slight rotation or tumbling motion.

5.5.2 Motility test: Pick an individual colony of pure culture for 18 h ~ 24 h and puncture the semi-solid or SIM motility culture medium. Culture at 25 ℃ ~ 30 ℃ for 48 h ± 2 h. Listeria grows in an irregular cloud-like manner along the puncture line, and an umbrella-like (or fusiform) interface is formed 3 mm ~ 5 mm below the surface of the culture medium. If the umbrella-like (or fusiform) growth is not obvious, continue to culture and observe the results once a day for 5 days.

5.5.3 Biochemical identification: Pick an individual colony of pure culture for 18 h ~ 24 h and perform a catalase test. The colonies with positive catalase reactions continue to undergo sugar fermentation tests and MR-VP tests. The main biochemical characteristics of *Listeria monocytogenes* are shown in Table 1.

5.5.4 Hemolysis test: Divide the bottom of a fresh sheep blood agar plate into 20 ~ 25 small grids, pick an individual colony of pure culture for 18 h ~ 24 h and inoculate on the blood plate, inoculate one colony per grid, and inoculate positive control bacteria (*Listeria monocytogenes*, *Listeria ivanovii* and *Listeria seeligeri*) and negative control bacteria (*Listeria innocua*). When puncturing, try to get as close to the bottom as possible, but do not touch the bottom surface, and avoid agar rupture. Culture at 36 ℃ ± 1 ℃ for 24 h ~ 48 h, and observe in a bright place. *Listeria monocytogenes* presents a narrow, clear, and bright hemolysis zone, *Listeria seeligeri* produces a narrow, weak hemolysis zone around the inoculation point, *Listeria innocua* has no hemolysis zone, and *Listeria ivanovii* produces a wide, clearly outlined β-hemolysis zone.

5.5.5 Cooperative hemolysis test CAMP (optional): Streak *Staphylococcus aureus* and *Rhodococcus equi* in parallel lines on sheep blood agar plates. Pick an individual colony of pure culture for 18 h ~ 24 h and streak vertically between the parallel lines, the two

homogenizing bag containing 225 mL of the above diluent, and beat it with a slapping homogenizer for 1 min ~ 2 min to make a 1:10 sample homogenate. If the sample is in liquid form, it can also be mixed by oscillation or stirring to make a 1:10 sample homogenate.

8.1.2 Use a 1 mL sterile pipette to pipette 1 mL of the 1:10 sample homogenate, and slowly pour it along the tube wall into a sterile test tube containing 9 mL of phosphate buffer or Fraser enrichment broth without additives (be careful not to let the tip of the pipette touch the liquid surface of the diluent), mix thoroughly, and make a 1:100 sample homogenate.

8.1.3 According to the operating procedure in 8.1.2, prepare sample homogenates of 10-fold serial dilutions. For each incremental dilution, use a 1 mL sterile pipette or pipette tip.

8.2 Sample inoculation

8.2.1 Based on the estimation of the sample contamination status, select 2 to 3 sample homogenates with appropriate serial dilutions (liquid samples may include stock solution), pipette 0.1 mL of sample homogenate for each dilution, and inoculate on 1 agar Listeria according to Ottaviani and Agosti (or other equivalent Listeria chromogenic medium) plate. Use a sterile coating stick to coat the entire plate, being careful not to touch the edge of the plate. Before use, if there are water droplets on the surface of the agar plate, place it in an incubator at 25 ℃ ~ 50 ℃ to dry until the water droplets on the surface of the plate disappear.

NOTE: If necessary, each dilution can be tested repeatedly.

8.2.2 For food samples with low content of *Listeria monocytogenes*, pipette 1 mL of the sample homogenate with lowest dilution and coat it on 3 agar Listeria according to Ottaviani and Agosti (or other equivalent Listeria chromogenic medium) plates with inoculation volumes of 0.3 mL, 0.3 mL, and 0.4 mL respectively. The coating method is the same as 8.2.1.

8.2.3 It shall not exceed 45 min from sample dilution to sample inoculation.

8.3 Culture

8.3.1 Under normal circumstances, after coating, place the plate upright on a horizontal table for 10 min ~ 20 min, then turn the plate over and place it in an incubator to culture at 36 ℃ ± 1 ℃ for 24 h ~ 48 h.

8.3.2 If the sample homogenate is not easily absorbed, place the plate upright in an incubator to culture at 36 ℃ ± 1 ℃ for 1 h. After the sample homogenate is absorbed, turn the plate over and place it upside down in an incubator to culture at 36 ℃ ± 1 ℃ for another 24 h ~ 48 h.

8.4 Count and confirmation of typical colony

8.4.1 Select plates with typical or suspicious *Listeria monocytogenes* colonies. If:

a) the total number of typical or suspicious colonies on plates of only one dilution is between 15 CFU and 150 CFU, it shall count the typical or suspicious colonies on the plates of that dilution;

b) the total number of typical or suspicious colonies on plates of all dilutions is less than 15 CFU, it shall count the typical or suspicious colonies on the plates of the lowest dilution;

c) the total number of typical or suspicious colonies on plates of all dilutions is more than 150 CFU, it shall count the typical and suspicious colonies on the plates of the highest dilution;

d) the total number of typical or suspicious colonies of plates of all dilutions is not between 15 CFU and 150 CFU, and some of them are less than 15 CFU or more than 150 CFU, it shall count the typical or suspicious colonies on the plates of the dilution closest to 15 CFU or 150 CFU;

those that meet a) ~ d) shall be calculated according to formula (1) in 9.1.1.

e) the total number of typical or suspicious colonies on the plates of two consecutive dilutions is between 15 CFU and 150 CFU, calculate according to formula (2) in 9.1.2.

8.4.2 Select 5 typical or suspicious colonies from each plate (plate that meet the requirements of 5.2) (select all if less than 5), and identify them according to 5.3, 5.4, and 5.5.

9 Results and report

9.1 Count method

9.1.1 Formula (1):

$$T = \frac{AB}{CVd} \qquad \cdots\cdots(1)$$

where:

T - the number of colonies of *Listeria monocytogenes* in the sample [CFU/g(mL)];

A - the total number of typical or suspicious colonies in the counting dilution;

B - the number of colonies confirmed to be *Listeria monocytogenes* in the counting dilution;

C - the number of colonies in the counting dilution used for the confirmation test of *Listeria monocytogenes*;

V - the inoculum volume of the counting dilution, in milliliters (mL);

d - the dilution factor.

9.1.2 Formula (2):

$$T = \frac{A_1 B_1 / C_1 + A_2 B_2 / C_2}{Vd} \qquad \cdots\cdots (2)$$

where:

T - the number of colonies of *Listeria monocytogenes* in the sample [CFU/g (mL)];

A_1 - the total number of typical or suspicious colonies of *Listeria monocytogenes* at the first counting dilution (low dilution multiple);

A_2 - the total number of typical or suspicious colonies of *Listeria monocytogenes* at the second counting dilution (high dilution multiple);

B_1 - the number of colonies identified as *Listeria monocytogenes* at the first counting dilution (low dilution multiple);

B_2 - the number of colonies identified as *Listeria monocytogenes* at the second counting dilution (high dilution multiple);

C_1 - the number of colonies used for the identification of *Listeria monocytogenes* at the first counting dilution (low dilution multiple);

C_2 - the number of colonies used for the identification of *Listeria monocytogenes* at the second counting dilution (high dilution multiple);

V - the inoculum volume of the first counting dilution plus 1/10 of the inoculum volume of the second counting dilution, in milliliters (mL), e.g.: the *V* value is 0.11 for the method using the 0.1 mL inoculum volume; the *V* value is 1.1 for the method using the 0.3 mL, 0.3 mL, 0.4 mL inoculum volumes;

d - the dilution factor (first dilution)

9.2 Result report

9.2.1 When the number of colonies is less than 100 CFU, it shall be rounded off according to the principle of "rounding off" and reported as an integer.

9.2.2 When the number of colonies is greater than or equal to 100 CFU, the third digit shall be rounded off according to the principle of "rounding off", and the first two digits shall be taken, and the digits behind shall be replaced by 0. It can also be expressed in

11 Operation steps

11.1 Sample dilution

Phosphate buffer is used as sample diluent. The sample dilution method is the same as 8.1.

11.2 Inoculation and culture

11.2.1 Based on the estimation of sample contamination status, select 3 sample homogenates (liquid samples may include stock solution) with appropriate serial dilutions and inoculate them in 10 mL of FB_1 enrichment broth. Inoculate 3 tubes for each dilution, and inoculate 1 mL in each tube. If the inoculation volume is 10 mL, inoculate it in 10 mL of double-mix FB_1 enrichment broth. Culture at 30 ℃ ± 1 ℃ for 24 h ± 2 h. Pipette 0.1 mL from each tube, inoculate to 10 mL of FB_2 enrichment broth, and culture at 30 ℃ ± 1 ℃ for 24 h ± 2 h.

11.2.2 Use inoculation loops to transfer 1 loop from each tube of FB_2 enrichment broth, inoculate on agar Listeria according to Ottaviani and Agosti (or other equivalent Listeria chromogenic medium) plates, and culture at 36 ℃ ± 1 ℃ for 24 h ~ 48 h.

11.3 Confirmation test

Pick 3 ~ 5 typical or suspicious colonies (select all if less than 3) from each plate (plate that meets the requirements of 5.2) and identify them according to 5.3, 5.4, and 5.5.

12 Results and report

Based on the number of test tubes confirmed to be positive for *Listeria monocytogenes*, lookup the MPN retrieval table (see Annex B) and report the most probable number of *Listeria monocytogenes* per gram (ml) of sample, expressed as MPN/g (mL).

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