Published, Nov 19, 2012
MOHWM0022.01

Methods of Test for Food Microorganisms-Test of Fecal Streptococci in Bottled and Packaged Drinking Water

1. Scope

This method is applicable to examine fecal streptococci in bottled and packaged drinking water.

2. Method

Filter water sample through a membrane, and put the membrane on selective medium, then count the colonies after incubation.

2.1. Working environment: The working platform needs to be spacious, clean and well-lit with illumination of cabinet over 100 cd. The air in the 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. Dry heat sterilizer.

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

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

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

2.2.5. Water bath: capable of controlling water temperature at ± 0.2 °C.

2.2.6. Balance: weighting up to 2,000 g with sensitivity of 0.1 g, weighting up to 120 g with sensitivity of 5 mg.

2.2.7. Sterile filter membrane: hydrophilic cellulose acetate membrane with pore size 0.2 μm.

2.2.8. Membrane filtration device: the funnel and vacuum holder base can be placed for filtering film, and the funnel should be sterile or sterilizable.

2.2.9. Vortex mixer

2.2.10. Filter membrane: pore size 0.45 μm nitrocellulose filter film (for water quality inspection, white, latticed, sterile) or equivalent, suitable for the membrane filter device in section 2.2.8.

2.2.11. pH meter.

2.2.12. Pipette aid.

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

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

2.2.15. Container: sterile bag or 1000 mL, 500 mL, 99 mL and 90 mL sterilizable wide-mouth bottles with labeled caps (plugs).

2.2.16. Spatula, scissors, knives and forceps: sterilizable or disposable.

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

2.2.18. pH test paper: range 6-8.

2.2.19. Chemicals: 30% hydrogen peroxide solution, 2,3,5-triphenyltetrazolium chloride, crystal violet, 95% ethanol, ammonium oxalate, potassium iodide, iodine, safranin O, sodium chloride, sodium azide (NaN_3), sodium glycerophosphate, maltose, lactose, bromcresol purple, esculin, ferric citrate, glucose (dextrose) and disodium hydrogen phosphate (Na_2HPO_4) are of reagent grade. Proteose peptone, polypeptone, yeast extract, agar, beef extract, peptone, oxgall, calf brain infusion and beef heart infusion are of microbiological grade.

2.2.20. Reagent

2.2.20.1. 3% hydrogen peroxide solution: take 1 mL of 30% hydrogen peroxide solution, add distilled water to make 10 mL, and prepare it freshly before use.

2.2.20.2. 1% 2,3,5-triphenyltetrazolium chloride solution: take 1 g of 2,3,5-triphenyltetrazolium chloride, add 100 mL of distilled water, stir to dissolve, filter with a sterile filter, and store in the dark before use. Discard and don't use the solution while it turns pink.

2.2.21. Gram stain solution(note)

2.2.21.1. 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 with solution B, let it stand for 24 hr, filter it with filter paper, and take the filtrate as a primary dye.

2.2.21.2. Gram's iodine solution (mordant)

Weigh 2 g of potassium iodide and 1 g of iodine and put it in the mortar and grind for 5 to 10 s~~ec~~. Add 1 mL of distilled water to grind, and then add 5 mL of distilled water for grinding, and add 10 mL of distilled water to grind until potassium iodide and iodine are

completely dissolved in water. Pour this solution into a brown bottle, then wash the mortar and pestle with an appropriate amount of distilled water, and incorporate these washing distilled water into amber bottle to make 300 mL.

2.2.21.3. Hucker's counterstain solution (counterstain)

Dissolve 2.5 g of safranin O in 100 mL of 95% ethanol as a counterstain stock solution. When using, take 10 mL of the stock solution and 90 mL of distilled water as the counterstain solution.

Note: Gram stain solution may be invalid due to long-term storage, so if purchase the commercial product, should pay attention to the shelf life; if use the self-prepared, should check the staining effect.

2.2.22. Media

2.2.22.1. KF Streptococcus agar

Proteose peptone or polypeptone……..……..…..10 g
Yeast extract ……..……..10 g
Sodium chloride…….……..……...5 g
Sodium azide, NaN_3 …….…….……0.4 g
Sodium glycerophosphate …….……10 g
Maltose …….……..……..20 g
Lactose ……..1 g
Bromcresol purple …….....0.015 g
Agar……..……..……...20 g
Distilled water……..……..……1000 mL

Heat to dissolve, continue heating for 5 min, cool to 50 or 60°C, add 10 mL of sterilized 1% 2,3,5-triphenyltetrazolium chloride solution, and the final pH is 7.2 ± 0.2, aliquot into Petri dishes.

2.2.22.2. Bile esculin agar

Beef extract .....3 g
Peptone.....5 g
Esculin.....1 g
Oxgall.....40 g
Ferric citrate.....0.5 g
Agar .....15 g
Distilled water.....1000 mL

After heating and dissolving, sterilize at 121°C for 15 min. The final pH is 6.6 ± 0.2., aliquot into Petri dishes.

2.2.22.3. Brain-heart infusion broth, BHI

Calf brain infusion.....200 g
Beef heart infusion.....250 g
Proteose peptone.....10 g
Dextrose.....2 g
Sodium chloride…….....5 g
Disodium phosphate (Na_2HPO_4) .....2.5 g
Distilled water.....1000 mL

After heating and dissolving, aliquot into test tubes and sterilize at 121°C for 15 min. The final pH is 7.4 ± 0.2.

2.2.22.4. Brain-heart infusion agar, BHA

Calf brain infusion.....200 g
Beef heart infusion .....250 g
Proteose peptone .....10 g
Dextrose .....2 g
Sodium chloride…….....5 g
Disodium phosphate (Na_2HPO_4) .....2.5 g
Agar .....15 g
Distilled water.....1000 mL

Heat to boiling to completely dissolve the culture medium, then distribute it into test tubes and sterilize at 121°C for 15 min to make a slant medium with a final pH of 7.4 ± 0.2.

2.3. Sampling

Take 100 mL of sample from a sterilized container or a sterile bag, and filter it; if the sample is in severe contamination, then filtering 10 mL of sample is available. Examine at least two replicates for each sample.

2.4. Filtration

Filter the sample under reduced pressure with a 0.45 μm nitrocellulose membrane.

2.5. Culture

Take out the membrane in section 2.4, and place it on KF Streptococcus agar, invert and incubate at 35°C for 48 hr.

2.6. Observation

After incubation, observe whether colonies are formed. Dark red or pink colonies are typical colonies.

2.7. Confirmation tests

2.7.1. Select 3 to 5 typical colonies for confirmation tests.

2.7.2. Inoculate the typical bacterial colonies isolated in section 2.6. into brain heart infusion slant medium and culture at 35°C for 48 hr. If it grows, perform the following tests.

2.7.3. Catalase test: take (hook) bacteria from the brain heart infusion slant medium, smear it on a glass slide, add 1 to 2 drops of 3% hydrogen peroxide solution, and observe whether bubbles are generated or not, the one that generates bubbles is a positive reaction, otherwise it is a negative reaction. Fecal streptococci are negative.

2.7.4. Gram stain

2.7.4.1. Add an appropriate amount of sterile saline solution on the glass slide, use an inoculation needle (or loop) to pick up an appropriate amount of bacteria from the brain heart infusion slant medium, spread it evenly into a thin smear, and quickly dry it in the air. Fix with gentle heat 3 to 4 times over a flame, do not bake directly on the fire.

2.7.4.2. Primary staining: stain the fixed smear with Hacker's crystal violet solution for 1 min and wash with water.

2.7.4.3. Mordant: add Gram's iodine solution for 1 min and wash with water.

2.7.4.4. Decolorization: wash with 95% ethanol until the purple color no longer fades, then wash with water. This step only takes about 30 sec, but it depends on the thickness of the smear.

2.7.4.5. Counterstaining: counterstain with Hacker's counterstain solution for 30 sec and wash with water.

2.7.4.6. Air dry.

2.7.4.7. Microscopic examination: Those that appear dark purple are Gram-positive bacteria, and those that appear light red are Gram-negative bacteria. Fecal streptococci should be Gram-positive, cocci, 0.5 to 1.0 μm in diameter, in pairs or short chains.

2.7.5. Take a loop of bacteria from the brain heart infusion slant medium and inoculate into brain heart infusion broth, and culture it at 45°C for 24 to 48

hr. Inoculate another loop of bacteria into bile esculin agar and culture it at 35°C for 24 to 48 hr. Observe for growth. Fecal streptococci can grow in these two media.

2.8. Counting

Calculate the number of fecal streptococci according to the ratio of the confirmation tests, and the bacteria count is expressed as CFU/100 mL.

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

Test flow chart