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Methods of Test for Food Microorganisms - Test of *Clostridium perfringens*

Part I: Isolation, plate count and identification of *Clostridium perfringens*

1. Scope: This method is applicable to examine and enumerate *Clostridium perfringens* (*C. perfringens*) 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. Working environment: The working platform needs to be spacious, clean and well-lit with illumination of cabinet at 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. Incubator: capable of controlling temperature at ± 1°C.

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

2.2.7. Blender or stomacher: appropriate for aseptic operation.

2.2.8. Hot plate: with magnetic stirring.

2.2.9. Microscope: a phase contrast microscope or an optical microscope with least 1000x magnification.

2.2.10. pH meter.

2.2.11. Water bath: capable of controlling water temperature at ± 0.5°C.

2.2.12. Vortex mixer.

2.2.13. Anaerobic jar or anaerobic incubator.

2.2.14. Pipette aid or micro-dispenser.

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

2.2.16. Tips: sterile, 100 μL and 1000 μL.

2.2.17. 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.

2.2.18. Test tube: with screw caps, 16 × 125 mm, 16 × 150 mm, 20 × 150 mm or a comparable product.

2.2.19. Gas bag: appropriate for anaerobic bacteria culture.

2.2.20. Durham fermentation tube: outer diameter 9 × 22 mm or a comparable product.

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

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

2.2.23. Scissors, spatula, knife and forceps: sterilizable.

2.2.24. Spreading rod: sterilizable or disposable.

2.2.25. Sterile membrane filter: hydrophilic membrane with 0.45 μm size or less.

2.2.26. Slides and coverslips: suitable for dyeing and microscopy.

2.2.27. Filter paper and brown reagent bottle.

2.2.28. Crayons or markers: mark or label on the slide.

2.2.29. Mortar and pestle.

2.2.30. Chemicals: 95% ethanol, neutral red, salicin, galactose, soluble starch, glycerol, glacial acetic acid, sodium ascorbate, safranin O, lactose, sodium bisulfite ($NaHSO_3$), resazurin sodium salt, L-cystine, ammonium oxalate, sodium hydroxide, ammonium hydroxide, thioglycollic acid, sodium thioglycollate, ferrous sulfate ($FeSO_4 \cdot 7H_2O$), magnesium sulfate ($MgSO_4 \cdot 7H_2O$), phenol red, raffinose, sodium chloride, cobalt chloride hexahydrate, anhydrous magnesium sulfate ($MgSO_4$), potassium nitrate (KNO_3), crystal violet, *N*-(1-naphthyl)ethylenediamine dihydrochloride, bromothymol blue, iodine, potassium iodide, glucose), sulfanilic acid, sodium carbonate, ammonium acetate (CH_3COONH_4), zinc powder, D-cycloserine, potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$), and ferric ammonium citrate, reagent grade. Soytone, beef heart, beef extract, gelatin, proteose peptone, agar, tryptose, tryptone, peptone, neopeptone, polypeptone and yeast extract, microbiological grade.

2.2.31. Reagents

2.2.31.1. 0.1% Peptone diluent

Dissolve 1 g of peptone with 1000 mL of distilled water, aliquot into containers, and autoclave at 121 °C for 15 min. Final pH is 7.0 ± 0.2.

2.2.31.2. Gram stain solution (Note1)

(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 h, 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 300 mL.

(3) Hucker's counterstain solution (counterstain)

Dissolve 2.5 g of safranin O in 100 mL of 95% ethanol as counterstain stock solution. When to use, dilute 10 mL of the stock solution with 90 mL of distilled water.

Note 1: 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.**

2.2.31.3. Nitrite detection reagents

Solution A: dissolve 1 g of sulfanilic acid in 125 mL of 5 N acetic acid.

Solution B: dissolve 0.25 g of *N*-(1-naphthyl)ethylenediamine dihydrochloride in 200 mL of 5 N acetic acid.

2.2.31.4. 0.66 M Sodium carbonate solution

Dissolve 7.0 g of sodium carbonate with distilled water to 100 mL, and sterilize with a sterile membrane filter.

2.2.31.5. 0.32% $CoCl_2 \cdot 6H_2O$ solution

Dissolve 0.32 g of cobalt chloride with distilled water to 100 mL, and sterilize with a sterile membrane filter.

2.2.31.6. 1.5% Sodium ascorbate solution

Dissolve 1.5 g of sodium ascorbate with distilled water to 100 mL, and sterilize with a sterile membrane filter.

2.2.31.7. Bromothymol blue test paper

Dissolve 2 g of bromothymol blue with distilled water to 1000 mL, make it slightly alkaline with ammonium hydroxide, immerse a filter paper with a diameter of 15 cm in the solution, take it out and dry naturally, and store for later use.

2.2.31.8. 0.04% Bromothymol blue solution

Dissolve 0.2 g of bromothymol blue in 32 mL of 0.01 N sodium hydroxide solution, and then add distilled water to 500 mL.

2.2.31.9. 10% Salicin solution

Dissolve 10 g of salicin with distilled water to 100 mL, and sterilize with a sterile membrane filter.

2.2.31.10. 10% Raffinose solution

Dissolve 10 g of raffinose with distilled water to 100 mL, and sterilize with a sterile membrane filter.

2.2.31.11. 0.85% saline

Dissolve 8.5 g of sodium chloride with distilled water to 1000 mL, aliquot into test tubes, and autoclave at 121°C for 15 min.

2.2.31.12. Reversed passive latex agglutination (RPLA) test kit: It can detect the enterotoxins of *C. perfringens*.

2.2.31.13. 70% Ethanol solution

Dilute 737 mL of 95% ethanol with distilled water to 1000 mL.

2.2.31.14. 2 M Sodium carbonate solution

Dissolve 21.2 g of sodium carbonate with distilled water to 100 mL.

2.2.32. Media

2.2.32.1. Tryptose-sulfite-cycloserine agar (TSC)

(a) Basal medium

Tryptose	15 g
Yeast extract	5 g

Soytone	5 g
Ferric ammonium citrate	1 g
$NaHSO_3$	1 g
Agar	20 g
Distilled water	900 mL

Dissolve ingredients by heating, adjust pH to 7.6 ± 0.2, aliquot 250 mL into the container, and autoclave at 121 °C for 15 min.

(b) 0.5% D-cycloserine solution

Dissolve 1 g of D-cycloserine in 200 mL of distilled water, sterilize with a sterile membrane filter, and refrigerate for later use.

(c) Egg yolk-free tryptose-sulfite-cycloserine agar, EY-free TSC

Keep the sterilized basal medium at 50°C, slowly add 20 mL of 0.5% D-cycloserine solution per 250 mL, and mix well. Before pour medium into the petri dish, shake and mix to disperse the flocculent sediment evenly, and avoid bubbles during shaking. Pour about 6-7 mL (for double-layer medium) into each petri dish, and let dry overnight at room temperature. Dry the surface of the medium and refrigerate for later use. Before use, check for microbial contamination.

(d) 50% Egg yolk emulsion

Wash fresh eggs, soak eggs in 70% ethanol solution for 1 h. Aseptically, take egg yolks with sterile syringe, add an equal amount of 0.85% saline, mix well, and refrigerate for later use.

(e) Egg yolk tryptose-sulfite-cycloserine agar (EY-TSC)

Keep the sterilized EY-free TSC medium at 45-50°C, and slowly add 20 mL of 50% egg yolk emulsion per 250 mL, and mix well. Before pour medium into the petri dish, shake and mix to disperse the flocculent sediment evenly, and avoid bubbles during shaking. Pour about 6-7 mL (for double-layer medium) or 18 mL (for monolayer medium) into each petri dish, and the following operation is the same as this section (c).

2.2.32.2. Cooked meat medium, modified

(a) Cooked meat medium

Beef heart	454 g

Proteose peptone	20 g
Glucose	2 g
NaCl	5 g

(b) Diluent

Tryptone	10 g
Sodium thioglycollate	1 g
Soluble starch	1 g
Glucose	2 g
1% Neutral red	5 mL
Distilled water	1000 mL

Dissolve ingredients by heating, adjust pH to 6.8 ± 0.2, and dispense 15 mL into 20 × 150 mm test tubes. Take 1 g of cooked meat medium (a), add 15 mL of diluent (b), mix well and let stand until all granular components are soaked, then autoclave at 121°C for 15 min.

2.2.32.3. Fluid thioglycollate medium (FTG)

L-cystine	0.5 g
NaCl	2.5 g
Glucose	5 g
Yeast extract	5 g
Tryptone	15 g
Sodium thioglycollate or thioglycollic acid	0.5 g
Resazurin, sodium solution 1:1000	1 mL
Agar	0.75 g
Distilled water	1000 mL

Add 1000 mL of distilled water to L-cystine, NaCl, glucose, yeast extract, trypsin and agar, heat and boil to dissolve, then add sodium thioglycollate or thioglycollic acid, adjust pH after dissolving, add sodium resazurin solution, mix well. Dispense 10 mL into 16 × 150 mm test tubes, and autoclave at 121 °C for 20 min, then rapidly cool. Final pH is 7.1 ± 0.2, and modulate when use.

2.2.32.4. Iron milk medium, modified

Fresh whole milk	1000 mL
$FeSO_4 \cdot 7H_2O$	1 g

Distilled water	50 mL

Dissolve ferrous sulfate in distilled water, slowly add fresh whole milk, and mix well with magnetic stirrer at the same time. Dispense 11 mL into 16 × 150 mm test tubes, autoclave at 118 °C for 12 min. Prepare fresh medium before use.

2.2.32.5. Lactose-gelatin medium

Tryptose	15 g
Yeast extract	10 g
Lactose	10 g
Phenol red	0.05 g
Gelatin	120 g
Distilled water	1000 mL

Add 400 mL of distilled water to tryptose, yeast extract and lactose, heat to dissolve. Add 600 mL of distilled water to gelatin, and stir at 50-60°C to dissolve. Mix the two solutions, adjust pH to 7.5 ± 0.2, and then add phenol red. Dispense 10 mL into 16 × 150 mm test tubes with a fermentation tube, autoclave at 121°C for 10 min. If the medium is not used within 8 h, deaerate by heating at 50-70°C for 2-3 h before use.

2.2.32.6. Sporulation broth

Polypeptone	15 g
Yeast extract	3 g
Soluble starch	3 g
$MgSO_4$	0.1 g
Sodium thioglycollate	1 g
Na_2HPO_4	11 g
Distilled water	1000 mL

Dissolve ingredients by heating, adjust pH to 7.8 ± 0.1, then dispense 15 mL into 20 × 150 mm test tubes, and autoclave at 121 °C for 15 min.

2.2.32.7. Motility-nitrate medium, buffered

Beef extract	3 g
Peptone	5 g
KNO_3	1 g

Na_2HPO_4	2.5 g
Galactose	5 g
Glycerol	5 mL
Agar	3 g
Distilled water	1000 mL

Dissolve all ingredients except agar by heating, adjust pH to 7.3 ± 0.1, then add agar, heat to boiling until dissolve completely and mix well, then dispense 11 mL into 16 × 150 mm test tubes, and autoclave at 121°C for 15 min. If the medium is not used within 4 h, heat 10 min in boiling water or flowing steam, and chill in cold water before use.

2.2.32.8. Spray's fermentation medium

Tryptone	10 g
Neopeptone	10 g
Sodium thioglycollate	0.25 g
Agar	2 g
Distilled water	1000 mL

Dissolve all ingredients except agar by heating, adjust pH to 7.4 ± 0.2, then add agar, heat to boiling until dissolve completely and mix well, then dispense 9 mL into 16 × 125 mm test tubes with fermentation tubes, and autoclave at 121°C for 15 min.

2.2.32.9. AE sporulation medium, modified

Polypeptone	10 g
Yeast extract	10 g
Na_2HPO_4	4.36 g
KH_2PO_4	0.25 g
CH_3COONH_4	1.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Distilled water	1000 mL

Dissolve all ingredients by heating, adjust pH to 7.5 ± 0.1 with 2 M sodium carbonate solution, then dispense 15 mL into 20 × 150 mm test tubes, and autoclave at 121°C for 15 min.

After sterilization, add 0.6 mL of filter-sterilized 10% raffinose solution, 0.2 mL of 0.66 M sodium carbonate solution and 0.2 mL of 0.32% cobalt chloride solution into each test tube. Check pH of one or two

tubes; it should be 7.8 ± 0.1. Just before use, steam medium for 10 min; after cooling, add 0.2 mL of filter-sterilized 1.5% sodium ascorbate to each test tube.

2.2.32.10. Duncan-Strong sporulation medium, modified

Proteose peptone	15 g
Yeast extract	4 g
Sodium thioglycollate	1 g
$Na_2HPO_4 \cdot 7H_2O$	10 g
Raffinose	4 g
Distilled water	1000 mL

Dissolve all ingredients by heating, and autoclave at 121°C for 15 min. Adjust pH to 7.8 ± 0.1 with filter-sterilized 0.66 M sodium carbonate solution before use.

2.3. Preparation of test solution (Note2,3)

2.3.1. Solid samples

Mince and mingle the sample properly, take 25 g of the sample, and mix well with 225 mL of 0.1% peptone diluent 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 0.1% peptone diluent 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 0.1% peptone diluent as the 10-fold diluted test solution.

2.3.4. 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 h), 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 0.1% peptone diluent as

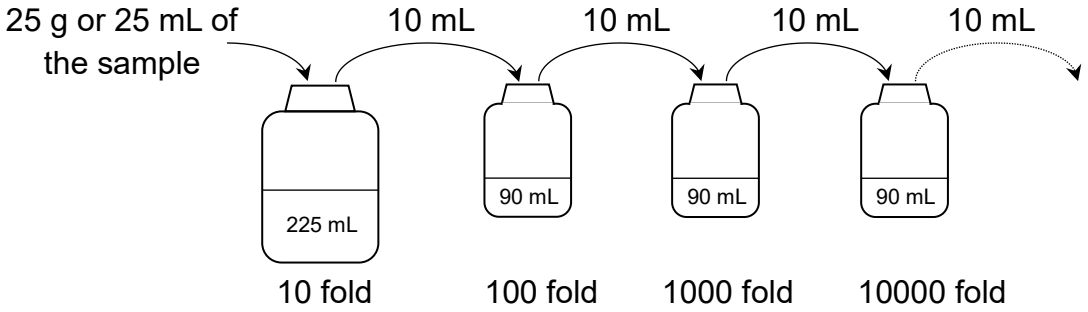
the 10-fold diluted test solution.

2.3.5. 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 0.1% peptone diluent 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.



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 25 g (mL), add an appropriate amount of 0.1% peptone diluent, 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. Direct plate count method

2.4.1.1.1. Shake the diluted test solution and/or sample solution thoroughly in section 2.3. Pipette 1 mL onto the petri dish already filled with EY-free TSC medium, and repeat at least twice for each diluted test solution and/or the sample solution. Then pour 15 mL of EY-free TSC medium that has been cooled to 45-50 °C into each petri dish, and then shake gently to mix the diluted test solution and/or sample

solution with the medium evenly. Make a double-layer medium, and anaerobic culture at 35°C for 20 to 24 h, then observe the morphology of the colonies. If the colony is black, it is suspected of *C. perfringens*. Pick the suspected colony and inoculate in the liquid thioglycolate medium that has just sterilized and rapidly cooled, and anaerobic culture at 35°C for 18 to 24 h.

2.4.1.1.2. An alternative plating method which described in section 2.4.1.1.1. Shake the diluted test solution and/or sample solution thoroughly in section 2.3. Pipette 0.1 mL onto the petri dish already filled with EY-TSC medium, and repeat at least twice for each diluted test solution and/or the sample solution. Spread evenly on the surface of the medium with sterile rod spreader, after about 5 min, overlay plates with 10 mL of EY-free TSC medium that has been cooled to 45-50 °C, let stand for the medium to solidify, and anaerobic culture at 35°C for 20 to 24 h, then observe the morphology of the colonies. The colony is black, usually with a 2-4 mm opaque white ring around the periphery, which is suspected of *C. perfringens*. Pick the suspected colony and inoculate in the liquid thioglycolate medium that has just sterilized and rapidly cooled, and anaerobic culture at 35°C for 18 to 24 h.

2.4.1.2. Enrichment

Heat the sterilized modified cooked meat medium with steam or boiling water for 10 min, and let stand for rapid cooling. Immediately shake the 10-fold diluted test solution and/or sample solution in section 2.3. and pipette 2 mL of each into 3 or 4 of the above-mentioned modified cooked meat medium, anaerobic culture at 35°C for 24-48 h. This section can be omitted if suspect colonies of *C. perfringens* grow on the isolation medium described in section 2.4.1.1.

2.4.2. Strain purification

Take an inoculation loop of culture medium from the liquid thioglycollate medium in section 2.4.1.1. or the modified cooked meat medium in section 2.4.1.2. and streak the surface of the EY-TSC medium, anaerobic culture at 35°C for 24 ± 2 h, observe the growth state of the colonies. Pick the colony that are round, about 1-2 mm in diameter, yellowish-gray with a 2-

4 mm opaque white zone surrounding the colony, inoculate in freshly sterilized and rapidly cooled liquid thioglycollate medium, and anaerobic culture at 35°C for 24 h.

2.4.3. Gram stain

(1) Place a drop of sterile 0.85% saline on a glass slide. Pick a part of the isolated colonies from EY-TSC medium in Section 2.4.2 using an inoculating needle (or loop), mix with 0.85% saline solution on the slide, and then smear the mixture into a thin film. After air-dry, heat-fix 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) Decolorizing

Decolorize 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. *C. perfringens* is Gram-positive, hypertrophic, short rod-shaped.

2.4.4. Iron-milk presumptive test

Pipette 1 mL of actively growing fluid thioglycollate culture in section 2.4.2. and inoculate in modified iron-milk medium, incubate at 46°C in a water bath for 2 h, and observe whether " vigorous fermentation " occurs every 1 h. Within 5 h, if the milk rapidly agglutinated and fragmented into spongy mass and rose to the surface of the medium, which is a positive reaction, otherwise it is a negative reaction. *C. perfringens* show positive reaction

(when "vigorous fermentation" occurs, remove the test tube immediately to avoid spilling into the water bath).

2.4.5. Confirmation test

2.4.5.1. Motility test

Pick the colony from EY-TSC medium of section 2.4.2., stab in about 1/3 deep of motility-nitrate medium, and incubate anaerobically at 35°C for 24 ± 2 h. Diffuse growth along stab line or turbid medium, which is a positive reaction, otherwise, it is a negative reaction. *C. perfringens* shows negative reaction.

2.4.5.2. Nitrate reduction test

Add 0.5 mL of nitrite test reagent A and 0.2 mL of reagent B into the motility-nitrate medium incubated for 24 h in section 2.4.5.1. Shake gently and observe the result, if it turns purple within 5 min, which is a positive reaction. When the color does not change, add a little zinc powder and let stand for a few minutes. When the purple color appears, which is a negative reaction, otherwise, it is a positive reaction. *C. perfringens* shows positive reaction.

2.4.5.3. Lactose fermentation test

Pick the colonies from EY-TSC medium in section 2.4.2, inoculate into lactose-gelatin medium, and culture anaerobically at 35°C for 24 h. If the color changes from red to yellow and there is gas production, which is a positive reaction, otherwise, it is a negative reaction. *C. perfringens* shows positive reaction.

2.4.5.4. Gelatin liquefaction test

Place the lactose-gelatin medium cultivated for 24 h in section 2.4.5.3. in a refrigerator at 5°C for 1 h. If there is a liquefaction phenomenon, it is a positive reaction, otherwise, it is a negative reaction. In the case of negative reaction, anaerobic culture an additional 24 h at 35°C, then place in a refrigerator at 5°C for 1 h before observation. *C. perfringens* shows positive reaction.

2.4.5.5. Sporulation test

Take (Pipette) 1 mL of the culture medium from section 2.4.2. and inoculate into the sporulation broth. After anaerobic culture at 35°C for 24 h, Gram stain and microscopic examination show that the spores

are oval and locate in the middle are suspected to be *C. perfringens*.

2.4.5.6. Carbohydrate fermentation test

After the tests in sections 2.4.5.1. to 2.4.5.4., if any of the test results cannot be judged as suspected *C. perfringens*, the carbohydrate fermentation test shall be carried out. Take three sterilized Spray's fermentation medium by boiling water or steam to remove air and cool rapidly. Immediately add 1 mL of sterile 10% salicin solution to the first test tube, add 1 mL of sterile 10% raffinose solution to the second test tube, and add no solution to the third test tube (for the control group). Respectively, take 0.1 mL of the liquid thioglycollate medium in section 2.4.2. into the above three test tubes, anaerobic culture at 35°C for 24 h, conduct section 2.4.5.6.1. or section 2.4.5.6.2.

2.4.5.6.1. Transfer a loop of the culture solution from each of the three test tubes in section 2.4.5.6. to bromothymol blue test paper. If the color does not change or appears slight green, it means acid production, and there is gas in the fermentation tube, which is a positive reaction, otherwise it is a negative reaction. The color of the control group should be unchanged and no gas should be produced. If the reaction is negative, continue to culture for 48 h, and then observe the results. The reaction of *C. perfringens* to salicin is neither acid nor gas production, and the reaction to raffinose is acid production.

2.4.5.6.2. Transfer 1 mL of the culture solution from each of the three test tubes in section 2.4.5.6. into another test tube, and then add 1 or 2 drops of 0.04% bromothymol blue solution. A yellow or light green color indicates acid production, and there is gas in the fermentation tube, which is a positive reaction, otherwise it is a negative reaction. The color of the control group should be unchanged and no gas should be produced. If the reaction is negative, continue to culture for 48 h, and then observe the results. The reaction of *C. perfringens* to salicin is neither acid nor gas production, and the reaction to raffinose is acid production.

2.5. Determination

2.5.1. Positive of *C. perfringens* should in accordance with the results listed in the following table.

Test or substrate	Positive (+)	Negative (−)	Reaction of *C. perfringens* [a]
Gram stain	Dark purple, short rod-shaped, hypertrophic	Light red	+
Iron-milk presumptive test	Vigorous fermentation, milk rapidly agglutinate and fragment into spongy mass and rise above medium surface	No severe fermentation	+
Motility test	Diffuse growth, cloudy medium	No diffuse growth or transparent medium	−
Nitrate reduction test	Purple	Original color [b]	+
Lactose fermentation test	Yellow and gas production	Original color or no gas production	+
Gelatin liquefaction test	Liquefaction	No liquefaction	+
Sporulation test	The spores are oval and in the middle space	Vegetative bacteria	+
Salicin fermentation test	Acid production and/or gas production	No acid and gas production	−
Raffinose fermentation test	Acid production	No acid production	+

[a] +: 90% or more positive; −: 90% or more negative.

[b] When there is no purple color, add a little zinc powder, and the purple color appears, which is a negative reaction, otherwise it is a positive reaction.

2.5.2. For those who are determined to be positive for *C. perfringens* according to section 2.5.1, the bacterial count is calculated according to section 2.6.

2.6. Counting

2.6.1. Select all the plates in section 2.4.1.1.1. or section 2.4.1.1.2. which contain 20 to 200 suspect colonies at the same dilution. When there are different suspect colonies, should also count separately. Express as CFU/g or CFU/mL.

2.6.2. Select 10 suspect colonies from each plate according to section 2.6.1. and determine whether as *C. perfringens* according to section 2.5.1. Calculate positive ratio of colonies tested that are determined as *C. perfringens*. If only one dilution factor of plates whose colonies are between 20-200, the number of *C. perfringens* is the result of the average number of colonies on all plates multiply the dilution factor and the ratio. If there are two dilution factor of plates whose colonies are between 20-200, calculate the number of *C. perfringens* according to the following formula. The number of *C. perfringens* should do Banker's rounding to the second 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 *C. perfringens* in the sample (CFU/g or CFU/mL)

$$= \frac{\left(\frac{Aa + Ab}{2}\right) \times A \times \frac{Ya}{Xa} + \left(\frac{Ba + Bb}{2}\right) \times B \times \frac{Yb}{Xb}}{2} \times F$$

A, B：Dilution factor

Aa, Ab：The number of colonies on each plate of dilution factor A

Ba, Bb：The number of colonies on each plate of dilution factor B

Xa, Xb：The number of suspect colonies on each plate of dilution factor A and B

Ya, Yb：The number of suspect colonies on each plate of dilution factor A and B, those were determined to be positive for *C. perfringens* according to section 2.5.1.

F (Dilution factor)：When the plate of section 2.4.1.1.1. is selected for counting, F is 1; and when the plate of section 2.4.1.1.2. is selected for counting, F is 10 (because the plate of section 2.4.1.1.2, only add 0.1 mL of diluted test solution or original sample solution)

2.7. Testing for enterotoxins

2.7.1. Strains to be stored or transported to other laboratories for testing should be inoculated into modified cooked meat medium, anaerobic cultured at 35°C for 24 h, then anaerobic cultured at room temperature for 24 h, and stored at 4 °C. During the inspection, mix the above-mentioned culture medium by vortex, and transfer 0.5 mL into each of two tubes containing 10 mL of fluid thioglycollate medium. Heat one tube at 75°C for 10 min and then incubate anaerobically at 35°C for 18 h. Incubate the second tube anaerobically at 35°C for 4 h. Inoculate the above medium into 15 mL of modified AE or Duncan-Strong sporulation medium.

2.7.2. If the isolated and purified strains are to be directly tested for sporulation and enterotoxin production, inoculate the strains into liquid thioglycollate medium, culture at 35°C for 4 h, and then inoculate 0.75 mL of the above medium into 15 mL of modified AE or Duncan-Strong sporulation medium.

2.7.3. Cultur anaerobically the inoculated sporulation medium at 35°C for 18-24 h. Confirm spore formation by phase contrast microscopy or smear staining. Take part of the sporulated culture medium and centrifuge at 10,000 ×g for 15 min. Test the supernatant by reversed passive latex agglutination reagent kit. The operation method is in accordance with the instructions of each product.

Remark

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.

Part II: Real-time PCR detection of *Clostridium perfringens*

1. Scope

This method is suitable for the identification of the gene of the *Clostridium perfringens* strain.

2. Method

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

2.1. Working 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 biological safety cabinet.

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. Spectrophotometer: with wavelengths of 260 nm and 280 nm.

2.2.8. Refrigeration and freezing equipment: with refrigeration and freezing (-20 °C) functions.

2.2.9. Vortex mixer.

2.2.10. pH meter.

2.2.11. Balance: weighing up to 2000 g with sensitivity of 0.1 g；weighing up to 100 g with sensitivity of 1 mg.

2.3. Reagents

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

2.3.2. Real-time PCR (Note 1)

2.3.2.1. Primers and probes sequences

C. perfringens identification gene (Target gene: *cpa* gene)

Primer F：5’-AAAAGAAAGATTTGTAAGGCGCTTAT-3’
Primer R：5’-CCCAAGCGTAGACTTTAGTTGATG-3’
Probe P：5’-(FAM)-TGCCGCGCTAGCAACTAGCCTATGG-(BHQ1)-3’
PCR amplification size: 85 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. The 5’ end of the probe was labeled with 6-carboxy-fluorescein (FAM), and the 3’ end was labeled with black hole quencher-1 (BHQ1).

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

2.3.3. Reference material: *C. perfringens* reference strain or its DNA template.

2.4. Equipment and materials (Note 2)

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

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

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

2.4.4. Real-time PCR reaction tube: 100 μL.

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

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

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

2.5. Preparation of the solution of real-time PCR (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
10 μM primer P	0.5 μL
TaqMan® Fast Reagents Starter Kit	13.0 μL
Sample DNA solution	2.0 μL
Sterile deionized water	5.5 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 solution

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, or store it at -20°C.

2.6.2. Preparation of DNA solutions for isolated strains

Transfer 1 loop of isolate strain from the culture medium 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 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 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

Properly dilute the sample DNA stock solution, primers and probes with sterile deionized water for later use. Take the sterilized centrifuge tube, prepare the real-time PCR solution according to section 2.5., and inject into the reaction well of the real-time PCR reaction plate. Then place the PCR reaction tubes in a centrifuge, and instantaneously centrifuge at 200 ×g. Transfer into the real-time PCR reactor, and perform the reaction according to the following conditions (Note 4). Both positive and negative controls should be used in real-time PCR experiments.

Steps	Temperature	Time
1. Activation	95°C	2 min
2. Denaturation	95°C	15 sec
3. Annealing, extension	60°C	30 sec
Repeat step 2 to step 3 for 40 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 *C. perfringens*, the sample can be confirmed to contain *C. perfringens*.

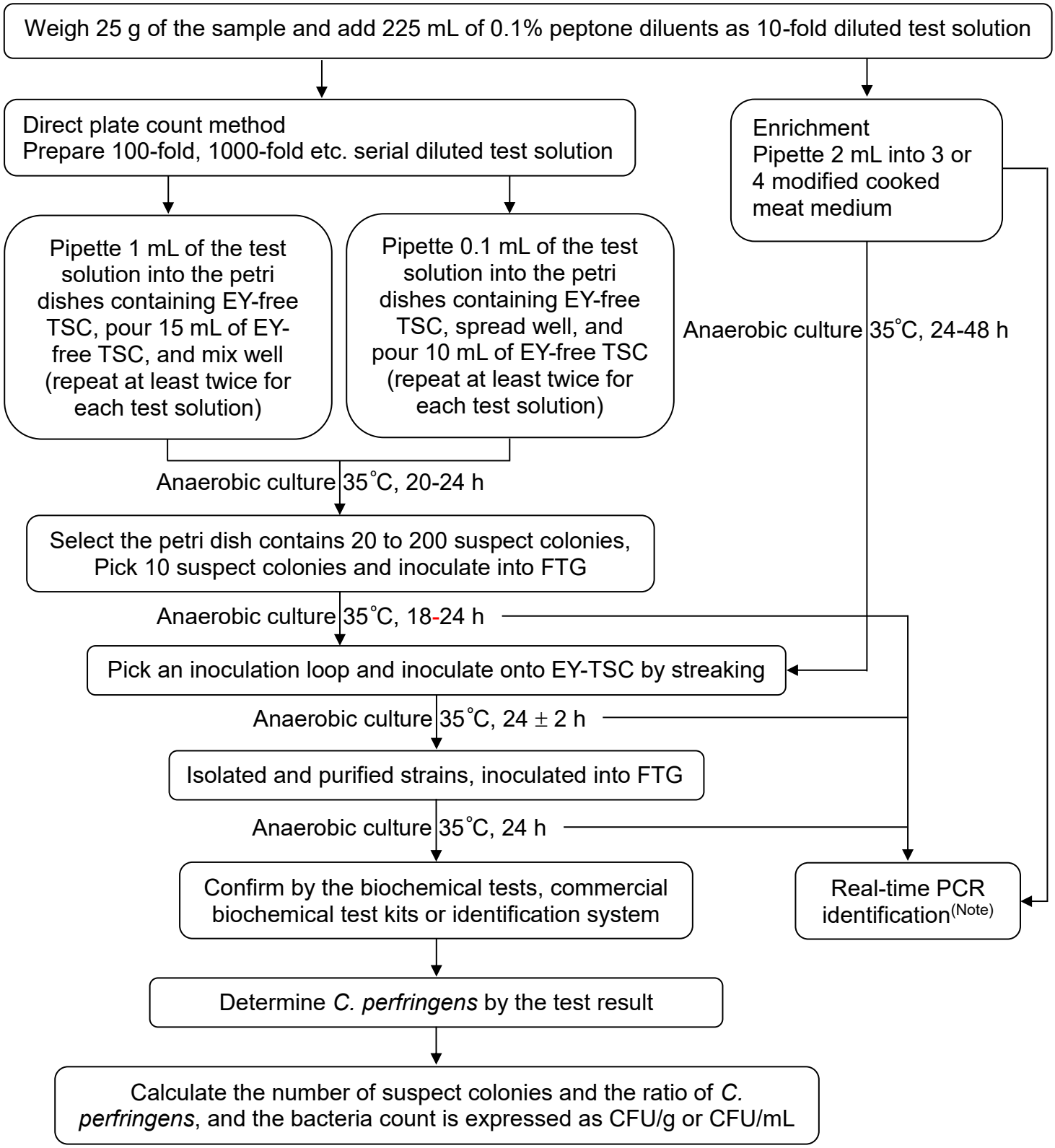
Remark:

Determination of *C. perfringens* by real-time PCR assay in Part II can be performed as needed.

Reference

1. Rhodehamel, E. J. and Harmon, S. M. 2001. Chapter 16 *Clostridium perfringens*. Bacteriological Analytical Manual. [https://www.fda.gov/food/laboratory-methods-food/bam-chapter-16 clostridium-perfringens].
2. Chon, J. W., Park, J. S., Hyeon J. Y., Park, C., Song, K. Y., Hong, K. W., Hwang I. G., Kwak, H. S. and Seo, K. H. 2012. Development of real-time PCR for the detection of *Clostridium perfringen*s in meats and vegetables. J. Microbiol. Biotechnol. 22: 530-534.

Test flow chart



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.