NATIONAL STANDARD OF THE

PEOPLE'S REPUBLIC OF CHINA

GB 4789.13-2012

Replacing GB/T 4789.13-2003

National food safety standard - Food microbiological examination - Examination of Clostridium perfringens

食品安全国家标准 食品微生物学检验 产气荚膜梭菌检验

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1 Scope

The standard lays down the method for the examination of Clostridium perfringens in food.

It applies to the examination of Clostridium perfringens in food.

2 Equipment and materials

Apart from the sterilisation and incubation equipment in routine use in a microbiological laboratory, the other equipment and materials are as follows:

a) constant-temperature incubator: 36 °C +/- 1 °C; b) refrigerator: 2 °C to 5 °C; c) constant-temperature water baths: 50 °C +/- 1 °C and 46 °C +/- 0.5 °C; d) balance: readability 0.1 g; e) homogeniser; f) microscope: 10 times to 100 times; g) sterile pipettes: 1 mL graduated in 0.01 mL, 10 mL graduated in 0.1 mL, or micropipettes and tips; h) sterile test tubes: 18 mm by 180 mm; i) sterile Petri dishes: diameter 90 mm; j) pH meter or pH colorimetric tube or precision pH test paper; k) anaerobic incubation device.

3 Culture media and reagents

3.1 Tryptose-sulfite-cycloserine (TSC) agar: see A.1 in Annex A. 3.2 Fluid thioglycollate medium (FTG): see A.2 in Annex A. 3.3 Buffered motility-nitrate medium: see A.3 in Annex A. 3.4 Lactose-gelatin medium: see A.4 in Annex A. 3.5 Iron milk medium: see A.5 in Annex A.

3.6 0.1 % peptone water: see A.6 in Annex A. 3.7 Gram staining solution: see A.7 in Annex A. 3.8 Nitrate reduction reagent: see A.8 in Annex A. 3.9 Buffered glycerol-sodium chloride solution: see A.9 in Annex A.

The text of Annex A itself lies beyond the pages examined and has not been read.

4 Examination procedure

The examination procedure for Clostridium perfringens is given in Figure 1.

Figure 1, 'Examination procedure for Clostridium perfringens', is a flow chart that runs: test sample, 25 g (mL) of test sample plus 225 mL of 0.1 % peptone water; homogenise and dilute; 1 mL each of the dilutions from 10 to the minus 1 down to 10 to the minus 6, mixed with TSC agar; anaerobic incubation at 36 °C +/- 1 °C for 20 h to 24 h, counting the black colonies; any 5 black colonies chosen and each inoculated into FTG medium; confirmation test, which branches into microscopic morphology, milk fermentation, motility-nitrate and lactose-gelatin; and finally the report.

5 Operating steps

5.1 Sample preparation. 5.1.1 Samples are to be examined as soon as possible after collection; if they cannot be examined in time they may be kept at 2 °C to 5 °C. If examination cannot be carried out within 8 h, 25 g (mL) of sample is to be weighed out aseptically, an equal quantity of buffered glycerol-sodium chloride solution added (a double quantity for liquid samples), and the sample placed as soon as possible in a low-temperature refrigerator at minus 60 °C for frozen storage, or kept with dry ice.

5.1.2 Aseptically weigh out 25 g (mL) of sample and place it in a homogenisation bag containing 225 mL of 0.1 % peptone water (for a sample frozen under 5.1.1, thaw at room temperature and add 200 mL of 0.1 % peptone water); homogenise continuously for 1 min to 2 min in a paddle-type homogeniser; or place it in a homogenisation cup holding 225 mL of 0.1 % peptone water and homogenise at 8 000 r/min to 10 000 r/min for 1 min to 2 min. This is the 1:10 dilution. 5.1.3 Take 1 mL of the above 1:10 dilution, add 9 mL of 0.1 % peptone water and prepare the series of dilutions from 10 to the minus 2 down to 10 to the minus 6.

5.2 Incubation. 5.2.1 Pipette 1 mL of each dilution into a sterile Petri dish, two plates per dilution. Pour into each plate 15 mL of TSC agar cooled to 50 °C (it may be held in a constant-temperature water bath at 50 °C +/- 1 °C) and rotate the plate slowly so that the dilution and the agar mix thoroughly. 5.2.2 After the agar plate has set, add a further 10 mL of TSC agar cooled to 50 °C to cover the surface layer evenly. 5.2.3 Once the agar has set, place the plates the right way up in the anaerobic incubation device and incubate at 36 °C +/- 1 °C for 20 h to 24 h. 5.2.4 Typical Clostridium perfringens forms black colonies on the TSC agar plate.

5.3 Confirmation test. 5.3.1 From a single plate choose any 5 black colonies (all of them if there are fewer than 5), inoculate each into FTG medium and incubate at 36 °C +/- 1 °C for 18 h to 24 h. 5.3.2 Prepare a smear from the culture, Gram stain it and examine it under the microscope for purity. Clostridium perfringens is a Gram-positive thick short rod and spores

may sometimes be seen. If the culture is not pure, streak it on a TSC agar plate for purification, incubate anaerobically at 36 °C +/- 1 °C for 20 h to 24 h, pick a single typical black colony, inoculate it into FTG medium and incubate at 36 °C +/- 1 °C for 18 h to 24 h for the subsequent confirmation tests.

5.3.3 Take 1 mL of vigorously growing FTG culture, inoculate it into iron milk medium, incubate for 2 h in a water bath at 46 °C +/- 0.5 °C and then observe once an hour whether 'stormy fermentation' occurs; the phenomenon is characterised by the milk curd breaking up and a sponge-like substance forming rapidly, which usually rises to the surface of the medium. A culture that does not ferment within 5 h is negative. Clostridium perfringens ferments lactose, coagulates casein and produces gas abundantly, showing the 'stormy fermentation' phenomenon, but does not blacken the medium.

5.3.4 With an inoculating loop (needle), take FTG culture and stab-inoculate buffered motility-nitrate medium; incubate at 36 °C +/- 1 °C for 24 h. Examine the growth of the bacteria along the stab line under transmitted light to judge whether motility is present: motile strains grow diffusely along the stab line, non-motile strains grow only along it. Then add 0.5 mL of reagent A and 0.2 mL of reagent B to check for the presence of nitrite. A red colour appearing within 15 min shows that nitrate has been reduced to nitrite; if no colour change appears, add a little zinc powder, leave for 10 min, and a red colour then shows that the strain cannot reduce nitrate. Clostridium perfringens is non-motile and can reduce nitrate to nitrite.

5.3.5 With an inoculating loop (needle), take FTG culture and stab-inoculate lactose-gelatin medium; incubate at 36 °C +/- 1 °C for 24 h and observe the result. Gas production and a change of the medium from red to yellow show that lactose has been fermented with acid production. Place the tube at about 5 °C for 1 h and check whether the gelatin has liquefied. If the medium is solid, incubate again at 36 °C +/- 1 °C for 24 h and check the liquefaction of the gelatin once more. Clostridium perfringens ferments lactose and liquefies gelatin.

6 Results and report

6.1 Counting of typical colonies. Select the plates whose number of typical colonies lies between 20 CFU and 200 CFU and count the typical colonies. If: a) the typical colonies of only one dilution plate lie between 20 CFU and 200 CFU, count the typical colonies on that dilution plate; b) the typical colonies of the lowest dilution plate are all fewer than 20 CFU, count the typical colonies on that dilution plate; c) the typical colonies of one dilution plate are all more than 200 CFU but the next dilution plate has no typical colony, count the typical colonies on that dilution plate; d) the typical colonies of one dilution plate are all more than 200 CFU and the next dilution plate has typical colonies but their number does not lie between 20 CFU and 200 CFU, count the typical colonies on that dilution plate; e) the typical colonies of two consecutive dilution plates both lie between 20 CFU and 200 CFU, count the typical colonies on each of the two dilution plates separately.

6.2 Calculation of the result. The document numbers the following item '6.1 The counting result is calculated by formula (1)'. The symbols of formula (1) are: T, the colony count of Clostridium perfringens in the sample; A, the number of typical colonies on a single plate; B, the number of colonies on a single plate confirmed by the confirmation test to be Clostridium perfringens; C, the number of colonies on a single plate used for the confirmation test; n1, the number of plates of the first dilution (low dilution factor) having Clostridium perfringens after the confirmation test; n2, the number of plates of the second dilution (high dilution factor) having Clostridium perfringens after the confirmation test; 0.1, the dilution coefficient; d, the dilution factor (first dilution). The equation itself is not reproduced here.

6.3 Report. On the basis of the number of typical Clostridium perfringens colonies on the TSC agar plate, calculate according to formula (1) in 6.2 and report the number of Clostridium perfringens per g (mL) of sample, the unit of the report being expressed as CFU/g (mL); if the value of T is 0, report it as less than 1 multiplied by the lowest dilution factor.

Note Relationship to previous standards

The foreword states that the standard replaces GB/T 4789.13-2003 'Microbiological examination of food hygiene - Examination of Clostridium perfringens' and lists the main changes with respect to it: the Chinese name of the standard was modified; the sample preparation process was modified; the culture media and reagents were modified; the operating steps were modified; the colony counting part was modified; and Annex A was added.

The replacement statement appears in the foreword; the frontispiece of this printing carries no replacement note.