GB

NATIONAL STANDARD OF THE

PEOPLE'S REPUBLIC OF CHINA

GB 4789.10-2016

National food safety standard - Food microbiological examination - *Staphylococcus aureus*

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Issued by: National Health and Family Planning Commission of the People's Republic of China;

China Food and Drug Administration.

Table of Contents

Foreword

This Standard replaces GB 4789.10-2010 “National food safety standard - Food microbiological examination: *Staphylococcus aureus*”, SN/T 0172-2010 “Determination of *Staphylococcus aureus* in foods for import and export”, SN/T 2154-2008 “Determination of coagulase-positive staphylococci in import and export food - Technique using rabbit plasma fibrinogen agar medium”.

Compared with GB 4789.10-2010, the main changes of this standard are as follows:

- The enrichment fluid for test is modified to 7.5 % sodium chloride broth.

National food safety standard - Food microbiological examination – *Staphylococcus aureus*

1 Scope

This Standard specifies the examination method for *Staphylococcus aureus* in foods.

Method I of this Standard applies to the qualitative examination of *Staphylococcus aureus* in foods; Method II applies to the counting of *Staphylococcus aureus* in foods with high *Staphylococcus aureus* content; Method III applies to the counting of *Staphylococcus aureus* in foods with low *Staphylococcus aureus* content.

2 Equipment and materials

In addition to routine sterilization and culture equipment for microbiological laboratories, other equipment and materials are as follows:

2.1 Constant temperature incubator: 36 °C ± 1 °C.

2.2 Refrigerator: 2 °C ~ 5 °C.

2.3 Constant temperature water bath: 36 °C ~ 56 °C.

2.4 Balance: with division of 0.1 g.

2.5 Homogenizer.

2.6 Oscillator.

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

2.8 Sterile Erlenmeyer flask: with capacity of 100 mL and 500 mL.

2.9 Sterile Petri dish: with diameter of 90 mm.

2.10 Spreader.

2.11 pH meter or pH colorimetric tube or precision pH test paper.

5.4 Preliminary identification

Staphylococcus aureus on the Baird-Parker plate is round, smooth-surface, raised, moist, with colony diameter of 2 mm ~ 3 mm, color of gray black to black color, shiny, and usually light (non-white) edge, around the opaque circle (precipitation) and a clear band outside. When the inoculating needle touches the colony, there is a butter-like sticky feeling. Sometimes strains of fats can be decomposed can be seen, except opaque circle and clear band, the appearance is basically the same. Colonies isolated from long-term stored frozen or dehydrated foods are often with lighter black than typical colonies, and more rough appearance and more dry texture. On the blood plate, the colony is larger, round, smooth raised, moist, golden (sometimes white), with transparent hemolytic circle seen around the colony. Pick the above-mentioned suspicious colonies for Gram's stain microscopic examination and plasma coagulase test.

5.5 Confirmation identification

5.5.1 Stain microscopic examination: *Staphylococcus aureus* is Gram's positive cocci, arranged in grape-like, no spores, no capsule, with the diameter of about 0.5 μm ~ 1 μm.

5.5.2 Plasma coagulase test: PICK at least 5 suspicious colonies (select all when there are less than 5) on Baird-Parker plate or blood plate; INOCULATE them to 5 mL of BHI and nutrient agar slants respectively; CULTURE at 36 °C ± 1 °C for 18 h ~ 24 h.

TAKE 0.5 mL of newly prepared rabbit plasma into a small test tube, ADD 0.2 mL ~ 0.3 mL of BHI culture, SHAKE well; PLACE it in temperature box or water bath box at 36 °C ± 1 °C; OBSERVE every half hour for a total of 6 h; if there is solidification (that is, when the test tube is tilted or inverted, there are clots) or if the solidification volume is greater than half the original volume, it is determined to be positive results. While the broth culture of the positive and negative *Staphylococcus aureus* strains of plasma coagulase test is used as a control. It can also use commercial reagents, operate according to the instructions, carry out the plasma coagulase test.

If the result is suspicious, pick the colonies on the nutrient agar slant into 5 mL of BHI and culture at 36 °C ± 1 °C for 18 h ~ 48 h.

5.6 Staphylococcal enterotoxin examination (optional)

For identification of suspicious food poisoning samples or *Staphylococcus aureus* strains producing staphylococcal enterotoxin, Staphylococcal enterotoxin shall be tested according to Annex B.

T - the number of *Staphylococcus aureus* colonies in the sample;

A - the total number of typical colonies of a certain dilution;

B - the number of colonies identified as positive of a certain dilution;

C - the number of colonies used for identification test of a certain dilution;

d - the dilution factor.

Equation (2):

$$T = \frac{A_1 B_1 / C_1 + A_2 B_2 / C_2}{1.1d} \qquad \cdots\cdots (2)$$

where:

T - the number of *Staphylococcus aureus* colonies in the sample;

A_1 - the total number of typical colonies of the first dilution (low dilution factor);

B_1 - the number of colonies identified as positive of the first dilution (low dilution factor);

C_1 - the number of colonies used for identification test of the first dilution (low dilution factor);

A_2 - the total number of typical colonies of the second dilution (high dilution factor);

B_2 - the number of colonies identified as positive of the second dilution (high dilution factor);

C_2 - the number of colonies used for identification test of the second dilution (high dilution factor);

1.1 - the calculation coefficient;

d - the dilution factor (first dilution).

10 Report

According to the calculation results of the equations in Clause 9, report the number of *Staphylococcus aureus* in per g (mL) of sample, expressed as CFU/g (mL); if the *T* value is 0, report the lowest dilution factor multiplies by less than 1.

95 % ethanol	20.0 mL
1 % aqueous ammonium oxalate solution	80.0 mL

A.8.1.2 Preparation method

Completely DISSOLVE the crystal violet in ethanol, and then MIX with ammonium oxalate solution.

A.8.2 Gram's liquid iodine

A.8.2.1 Ingredients

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 mL

A.8.2.2 Preparation method

MIX iodine and potassium iodide first; ADD a small amount of distilled water and SHAKE well; after completely dissolved, ADD distilled water to 300 mL.

A.8.3 Safranin counter stain

A.8.3.1 Ingredients

Safranin	0.25 g
95 % ethanol	10.0 mL
Distilled water	90.0 mL

A.8.3.2 Preparation method

DISSOLVE safranin in ethanol, and then DILUTE with distilled water.

A.8.4 Staining method

a) FIX the smear on the flame, DROP crystal violet stain, STAIN for 1 min, RINSE.

b) DROP Gram's liquid iodine, REACT for 1 min, RINSE.

c) DROP 95 % ethanol to decolorize for about 15 s ~ 30 s, until the stain is rinsed away, do not over-decolorize, RINSE.

d) DROP counter stain to stain for 1 min, RINSE and DRY for microscopic

Annex B

Staphylococcal enterotoxin detection

B.1 Reagents and materials

Unless otherwise specified, the reagents used are analytical regents, and the test water shall comply with the provisions of Grade I water in GB/T 6682.

B.1.1 A, B, C, D, E-type Staphylococcal enterotoxin ELISA kit.

B.1.2 pH test paper, with the range of 3.5 ~ 8.0, and the accuracy of 0.1.

B.1.3 0.25 mol/L Tris buffer with pH 8.0: DISSOLVE 121.1 g of Tris in 800 mL of deionized water; after cooling to room temperature, ADD 42 mL of concentrated HCL to pH 8.0.

B.1.4 Phosphate buffer with pH 7.4: WEIGH 0.55 g of $NaH_2PO_4 \cdot H_2O$ (or 0.62 g of $NaH_2PO_4 \cdot 2H_2O$), 2.85 g of $Na_2HPO_4 \cdot 2H_2O$ (or 5.73 g of $Na_2HPO_4 \cdot 12H_2O$), 8.7 g of NaCl to dissolve in 1000 mL of distilled water, MIX well.

B.1.5 Heptane.

B.1.6 10 % sodium hypochlorite solution.

B.1.7 Enterotoxin production medium

B.1.7.1 Ingredients

Peptone	20.0 g
Pancreas digestion casein	200 mg (amino acid)
Sodium chloride	5.0 g
Dipotassium phosphate	1.0 g
Potassium dihydrogen phosphate	1.0 g
Calcium chloride	0.1 g
Magnesium sulfate	0.2 g
Nicotinic acid	0.01 g
Distilled water	1000 mL

The method can be completed by using A, B, C, D, E-type Staphylococcal enterotoxin enzyme linked immunosorbent assay kits. The method is based on enzyme linked immunosorbent assay (ELISA) reaction. A, E, C, D, E-type Staphylococcal enterotoxin antibodies are coated by A ~ E pores on each of the microporous strips of the 96-pore ELISA plate. H pores are positive quality controls, coating mixed Staphylococcal enterotoxin antibodies; F and G pores are negative quality controls, coating antibodies of non-immunized animals. If the staphylococcal enterotoxin is present in the sample, the free staphylococcal enterotoxin binds to the specific antibody coated by each micropore to form an antigen-antibody complex, and the remaining unbound ingredients are washed off during the plate washing process; the antigen-antibody complex binds to the peroxidase enzyme label (secondary antibody), the unbound enzyme labels are washed off during the plate washing process. The enzyme substrate and the color developer are added and incubated, so that the enzyme-catalyzed substrate on the enzyme label is decomposed to make the colorless color developer blue; adding the reaction stop solution to make the color change from blue to yellow and terminate the enzyme reaction. Measure the absorbance value of the microporous solution with ELISA with 450 nm wavelength, and the staphylococcal enterotoxin in the sample is proportional to the absorbance value.

B.4 Detection procedure

B.4.1 Method for detection of staphylococcal enterotoxin from isolated strains cultures

INOCULATE the test strains on the nutrient agar slant (test tube of 18 mm × 180 mm) at 36 °C for 24 h; WASH away the colonies with 5 mL of physiological saline; POUR them into 60 mL of the producing medium; SHAKE CULTURE at 36 °C for 48 h at a rate of 100/min. PIPETTE the bacterial liquid to centrifuge at 8000 r/min for 20 min; HEAT at 100 °C for 10 min; TAKE the supernatant. TAKE 100 μL of diluted sample solution for test.

B.4.2 Method for extraction and detection of staphylococcal enterotoxin from foods

B.4.2.1 Milk and milk powder

DISSOLVE 25 g of milk powder in 125 mL of Tris buffer with 0.25 M, pH 8.0, MIX well and prepare in the following procedure as same as liquid milk. CENTRIFUGE the milk at 15 °C and 3500 g for 10 min. REMOVE a layer of fat layer formed on the surface to make it into skim milk. DILUTE with distilled water (1:20). TAKE 100 μL of diluted sample solution for test.

B.4.2.2 Foods with fat content of not more than 40 %

WEIGH 10 g of sample to grind; ADD 15 mL of PBS solution with pH 7.4 for

MEASURE the OD value of each microporous solution with ELISA at 450 nm in 30 min.

B.4.4 Calculation and expression of results

B.4.4.1 Quality control

For the test result, the OD value of positive quality controls shall be greater than 0.5, and the OD value of negative quality controls shall be less than 0.3. If the above requirements cannot be met at the same time, the test result is not recognized. For positive results, it shall exclude interference of endogenous peroxidase.

B.4.4.2 Calculation of critical value

The F pore and G pore of each microporous strip are negative quality controls, and the mean of the OD values of two negative quality controls plus 0.15 is the critical value.

Example: Negative quality control 1 = 0.08

Negative quality control 2 = 0.10

Mean = 0.09

Critical value = 0.09 + 0.15 = 0.24

B.4.4.3 Result expression

The sample pore with OD value less than the critical value is determined negative, expressed that a certain type of *Staphylococcus aureus* enterotoxin is not detected in the sample; the sample pore with OD value greater than or equal to the critical value is determined positive, expressed that a certain type of *Staphylococcus aureus* enterotoxin is detected in the sample.

B.5 Biosecurity

Because it does not rule out the existence of other potentially infectious substances in the sample, the waste shall be disposed in strict accordance with GB 19489 “Laboratories - General requirements for biosafety”.

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