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National food safety standard - Food microbiology testing - Shiga toxin-producing Escherichia coli test

食品安全国家标准　食品微生物学检验　产志贺毒素大肠埃希氏菌检验

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1 Scope

This standard specifies the testing methods for Shiga toxin-producing Escherichia coli (STEC) in food.

This standard is applicable to the testing for Shiga toxin-producing Escherichia coli in food.

2 Equipment and materials

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

2.1 Constant temperature incubator: 36 ℃±1 ℃, 44 ℃±1 ℃.

2.2 Refrigerator: 2 ℃~5 ℃, -18 ℃~-20 ℃.

2.3 Balance: The sensitivity is 0.001 g.

2.4 Homogenizer.

2.5 Vortex mixer.

2.6 Constant temperature water bath of 50 ℃~55 ℃, and constant temperature metal bath of 99 ℃±1 ℃.

2.7 Centrifuge: The maximum speed is at least 16000 *g*.

2.8 Real-time fluorescence quantitative PCR instrument.

2.9 Mixer.

2.10 Sterile pipette: 1 mL (with a scale of 0.01 mL), 10 mL (with a scale of 0.1 mL).

2.11 Electronic pipette or ear bulb.

2.12 Sterile conical flask with stopper: The capacities are 250 mL, 500 mL, and 1000 mL.

2.13 Sterile culture dish: 90 mm in diameter.

2.14 Sterile homogenizing bag: with a filter and a sterile homogenizing cup.

2.15 Flat-cap 8-tube strips or 96-well PCR microplates.

2.16 Magnetic bead separation and enrichment device.

2.17 Micropipette and tip: 0.2 μL~2 μL, 2 μL~20 μL, 20 μL~200 μL, and 100 μL~1000 μL.

2.18 10 μL sterile inoculating loop.

2.19 1.5 mL sterile EP tubes, sterile microcentrifuge tubes, sterile 15 mL centrifuge tubes.

2.20 0.22 μm pore size sterile filter membrane.

2.21 Fully automatic microbial biochemical identification system.

2.22 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

3 Culture media and reagents

3.1 Modified tryptone soya broth medium (mTSB): see A.1.

3.2 EC broth medium (EC): See A.2.

3.3 Tryptone soya agar medium (TSA): See A.3.

3.4 STEC chromogenic medium: see A.4.

3.5 MacConkey agar medium (MAC): See A.5.

3.6 Brain heart infusion broth (BHI)-50% glycerol: see A.6.

3.7 0.85% sterile saline.

3.8 5% Chelex-100 solution: see A.7.

3.9 E-buffer solution: see A.8.

3.10 1 mol/L hydrochloric acid solution.

3.11 1×TE buffer: see A.9.

3.12 Sterile PBS buffer: see A.10.

5 Operating steps

5.1 Sample preparation

5.1.1 Sample storage

After sampling, the sample shall be sent to the laboratory for testing as soon as possible. If it cannot be tested in time, the sample to be tested shall be stored according to the sample storage requirements. If there are no special requirements, it shall be refrigerated in a refrigerator at 2 ℃~5 ℃, and the test shall be completed within 24 hours.

5.1.2 Solid or semi-solid samples

Aseptically weigh and take 25 g of the test sample, add it into a sterile homogenizing bag with a filter containing 225 mL of EC (or mTSB), and homogenize with a slap homogenizer for 1 to 2 minutes; or put it into a sterile homogenizing cup containing 225 mL of EC (or mTSB), use a rotating blade homogenizer to homogenize at 8000 r/min~10000 r/min for 1 min~2 min to make a 1:10 homogeneous sample solution.

5.1.3 Liquid samples

Aseptically take 25 mL of the test sample and add it into a sterile homogenizing bag or sterile conical flask containing 225 mL of EC (or mTSB), and mix thoroughly to make a 1:10 sample solution.

5.2 Enrichment

Culture the sample solution prepared in 5.1 at 44 ℃±1 ℃ for 18 h~24 h. At the same time, set up a control test; use the standard strain of Escherichia coli carrying *stx1* and/or *stx2* to be cultured in EC (or mTSB) as a positive control, and the standard strain of Escherichia coli not carrying *stx1* and *stx2* to be cultured in EC (or mTSB) as a negative control, and carry out a blank medium control.

5.3 Initial screening of *stx* virulence genes

5.3.1 Test environment and process control

The environmental conditions and process control of real-time fluorescence quantitative PCR experiments shall be carried out in accordance with the requirements specified in GB/T 27403-2008.

5.3.2 DNA template preparation

in fluorescence, and the corresponding Ct values are <30.

5.3.3.6 Real-time fluorescence quantitative PCR result determination

In compliance with the principle of result validity in 5.3.3.5, when the sample to be tested is tested,

a) If the Ct values of *stx1* and *stx2* are ≥40, it is judged as *stx*-negative;

b) If the Ct value of *stx1* or *stx2* is <35, and the Ct value of 16S rDNA gene is <35, it is judged as *stx*-positive;

c) If the Ct values of *stx1* and *stx2* are both ≥35 and <40, the real-time fluorescence quantitative PCR amplification experiment shall be repeated. After the second amplification experiment, if the Ct value of *stx1* or *stx2* is <40 and the Ct value of the 16S rDNA gene is <35, it is judged as *stx*-positive; if the Ct values of *stx1* and *stx2* are ≥40, it is judged as *stx*-negative.

For the sample judged as *stx*-negative, the result is reported as "No Shiga toxin-producing Escherichia coli is detected in the 25 g(mL) of the sample" according to the process.

The sample enrichment solution judged as *stx*-positive shall be subjected to acid treatment and immunomagnetic bead enrichment treatment respectively according to the provisions of 5.4 and 5.5.

5.4 Acid treatment for enrichment solution

Pipette 450 μL of *stx*-positive sample enrichment solution confirmed in 5.3 into a 1.5 mL sterile EP tube, centrifuge at 10000g for 2 min, discard the supernatant, and resuspend the bacterial precipitate in 450 μL of E-buffer solution; add 25 μL of 1 mol/L hydrochloric acid (pH 2.0~2.5), shake at room temperature for 1 hour, streak inoculate on STEC chromogenic medium or MacConkey agar medium plate with 10 μL sterile inoculation loop, and culture at 36 ℃±1 ℃ for 18 h~24 h.

Pipette 100 μL of the above acid treatment solution to 900 μL of the E-buffer solution to dilute it, vortex and mix, then use a 10 μL sterile inoculation loop to inoculate it on the STEC chromogenic medium or MacConkey agar medium plate, and incubate at 36 ℃±1 ℃ for 18 h~ 24 h.

5.5 Immunomagnetic bead enrichment processing for 7 important serotypes of STEC

5.5.1 In addition to the acid treatment described in 5.4, the enrichment solution of the sample judged as *stx*-positive in 5.3 also needs to be enriched with immunomagnetic

beads at the same time. According to the instructions provided by the manufacturer, 7 important serotypes (O26, O45, O103, O111, O121, O145, O157) STEC are enriched, and standard strains with corresponding serotypes are set as positive controls. When there is a deviation between the manufacturer's instructions and the following description, operate according to the manufacturer's instructions and use one pipette for each sample to avoid cross-contamination.

5.5.2 Number the microcentrifuge tubes according to the sample and standard strain. Use 7 microcentrifuge tubes for each sample (one tube for each of the 7 types of immunomagnetic beads) and place them on the magnetic plate rack. Mix the 7 STEC immunomagnetic bead suspension reagents by gentle shaking, then add them to the corresponding numbered microcentrifuge tubes.

5.5.3 Pipette 8 mL of *stx*-positive enrichment solution into a 15 mL centrifuge tube, centrifuge at 10,000 *g* for 3 min, and discard the supernatant; add 3 mL of sterile PBS to resuspend the precipitate and vortex to mix, centrifuge at 10,000 *g* for 3 min, repeat the PBS washing step 2 to 3 times, discard the supernatant, and set aside the bacterial precipitate for later use.

5.5.4 Add 800 μL of sterile PBS buffer to resuspend the bacterial precipitate produced in 5.5.3, vortex and mix thoroughly, then pipette 100 μL of the bacterial suspension into the 7 microcentrifuge tubes with immunomagnetic beads mentioned in 5.5.2, and cover the tubes. Change the pipette tip for each sample, and the standard strain must be tested separately from the sample to avoid cross-contamination. Each sample needs to be enriched with 7 serotypes of immunomagnetic beads.

5.5.5 Binding: Invert and mix several times. Incubate on a mixer at 37 °C for 60 min, with a gentle speed setting to allow the target bacteria to fully contact with the immunomagnetic beads.

5.5.6 Capture: After the incubation, insert the magnetic plate into the magnetic plate rack to capture the magnetic beads. Keep tilting the magnetic plate rack within 3 minutes to ensure that all the immunomagnetic beads in the suspension and on the lid are collected, and round or oval brown aggregates can be seen in the middle of the wall of the microcentrifuge tube.

5.5.7 Magnetic bead purification: Carefully open the centrifuge tube cover and gently remove the supernatant from the opposite side of the immunomagnetic bead aggregates. When the liquid level is close to the immunomagnetic bead aggregates, the liquid shall be slowly sucked away until the liquid level is away from the vicinity of the aggregates to ensure that the immunomagnetic bead aggregates are not sucked away. If the drawn supernatant contains magnetic beads, put it back into the microcentrifuge tube and repeat step 5.5.6. This step is a critical step in purification and needs to be completed

Cultures that produce acid or not on the triple sugar iron slant, produce acid on the bottom layer, are indole-positive, and are H_2S-negative and urease-negative, are Escherichia coli. Cultures that do not produce acid on the bottom layer of the triple sugar iron slant, or are positive for any of H_2S, KCN, and urease, are not Escherichia coli.

For strain identification, a fully automatic microbial biochemical identification system, a microbial biochemical identification reagent strip (box), or a matrix-assisted laser desorption ionization time-of-flight mass spectrometer can be chosen for identification, and the operation and judgment are carried out according to the instructions of the instrument or kit.

5.7 Serological test (optional)

The capable laboratories can conduct serological tests on isolated *stx*-positive Escherichia coli. The O-antigen and H-antigen identification are carried out according to the operating instructions provided by manufacturers.

6 Results and reports

If the initial screening result is *stx*-negative, the report is "No Shiga toxin-producing Escherichia coli is detected in the 25 g(mL) of the sample."

If the initial screening result is *stx*-positive, but no *stx*-positive Escherichia coli is isolated, the report is "No Shiga toxin-producing Escherichia coli is detected in the 25 g(mL) of the sample."

If the initial screening result is *stx*-positive, and an *stx*-positive strain is isolated and identified as Escherichia coli, the report is "Shiga toxin-producing Escherichia coli is detected in 25 g(mL) of sample."

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