NATIONAL STANDARD OF THE

PEOPLE'S REPUBLIC OF CHINA

GB 4789.44-2020

National food safety standard - Food microbiological examination - Vibrio vulnificus

Issued on: September 11, 2020 **Implemented on: March 11, 2021**

Issued by: National Health Commission and State Administration for Market Regulation

Table of Contents

1 Scope

This Standard specifies the examination method for Vibrio vulnificus in aquatic products.

This Standard applies to the examination of Vibrio vulnificus in aquatic products such as fish, shrimp, crab, shellfish.

2 Apparatus and materials

In addition to the conventional sterilization and culture apparatus in the microbiology laboratory, other apparatus and materials are as follows:

2.12 Micro-adjustable pipette (range 2.5 uL, 10 uL, 100 uL, 1000 uL) and

matching tips.

2.13 Precision pH test paper or pH meter.

be stored at 7 °C~10 °C (because Vibrio vulnificus is very easy to form a living and uncultivable state at 4 °C, samples must not be placed at 4 °C). And, as far as possible, complete the examination within 24 h. Frozen samples shall be thawed under warm conditions not exceeding 45 °C. The thawing time shall not exceed 15 min.

5.1.2 Sampling: For fish and cephalopods, take surface tissues, intestines and gills. For shellfish, take the entire contents (including meat and body fluids). For crustaceans, take the whole animal or its central part (including intestines and gills). For shellfish or hard shell crustaceans, it shall first use running tap water to rinse the shell; use filter paper to absorb the surface moisture. Then the shell shall be opened aseptically; the corresponding part shall be taken according to the above requirements.

5.1.3 USE aseptic operation to take 25 g of the above-treated sample; ADD 225 mL of

PNCC enrichment broth; USE a rotating blade homogenizer to homogenize at 8000 r/min for 1 min. Or use a flapping homogenizer to homogenize for 2 min; mix thoroughly to prepare a 1:10 sample homogenate.

If there is no homogenizer, put the sample in a sterile mortar and grind it thoroughly. TAKE 25 g of the ground sample; TRANSFER it into a 500 mL sterile conical flask; ADD 225 mL of PNCC enrichment broth and shake and mix well, to prepare a sample homogenate of 1:10.

5.2 Enrichment Incubate the 1:10 sample homogenate prepared in 5.1.3 at 36 °C+/-1 °C for 18 h+/-1 h.

5.3 PCR testing The environmental conditions and process control of PCR test shall be implemented in accordance with the provisions of GB/T 27403 "Criterion on quality control of laboratories - Molecular biological testing of food"; the same below.

5.3.1 DNA template preparation At a distance of 1 cm below the surface of the PNCC enrichment broth, pipette

1 mL into a 1.5 mL EP tube; CENTRIFUGE at 9000 r/min for 3 min and discard the supernatant. ADD 1 mL of PBS to the precipitate to suspend it and wash it thoroughly; CENTRIFUGE at 9000 r/min for 3 min and discard the supernatant.

FOLLOW this step to repeatedly wash the precipitate 2 to 3 times; DISCARD the last supernatant. ADD 1 mL of sterile ultrapure water; BOIL at 100 °C for 10 min; then centrifuge at 12000 r/min for 5 min; the supernatant is used for PCR analysis. If it cannot be analyzed in time, store it at -20 °C for later use.

5.3.3 Electrophoresis Gel electrophoresis is used to detect PCR products. USE 0.5xTBE buffer to prepare 1.5% agarose gel (containing DNA dyes such as EB 0.5 ug/mL or Goldview 5 uL/100 mL or Gelred 5 uL/50 mL). TAKE 5 uL of PCR amplification product; MIX it with 1 uL of 6x nucleic acid electrophoresis loading buffer; apply the sample; at the same time, add a DNA molecular weight standard (range 100 bp~1000 bp) to a hole. According to the following formula: the distance between the positive and negative electrodes of the electrophoresis tank (cm)x5 V/cm, calculate and set the voltage. USE 0.5xTBE buffer constant-pressure electrophoresis. According to the moving position of bromophenol blue, determine the electrophoresis time. USE the gel imaging system or ultraviolet detector to observe and record the results.

5.3.4 Result determination Quality control system: Negative control and blank control have no amplified bands; and, the positive control has amplified bands of expected size (519 bp);

then the detection system is normal. Otherwise, if any kind of control has abnormal results, the test shall be repeated. The pollution factor shall be eliminated at the same time.

Positive result: When the quality control system is normal, an amplified band of the

expected size (519 bp) appears in the sample to be tested, then the PCR result is determined to be positive.

Negative result: When the quality control system is normal, the sample to be tested does not show an amplified band of the expected size (519 bp), then the PCR result is determined to be negative.

5.4 Separation USE a 10 uL inoculation loop to dip a loop of enrichment broth at 1 cm below the surface of the PNCC enrichment broth. Respectively streak-inoculate on CC and mCPC plates; incubate at 36 °C+/-1 °C for 18 h+/-1 h. The typical morphology of Vibrio vulnificus on CC and mCPC plates is: round and flat;

yellow to orange colonies which are transparent, or are opaque in center but transparent at edges under light. The diameter of the colony is 1 mm~2 mm. A yellow halo may appear (or not appear) around the colony.

5.5 Isolated pure culture PICK at least 5 suspicious Vibrio vulnificus colonies from each of the CC plate and mCPC plate (select all if less than 5); respectively inoculate them on 3% sodium chloride tryptone soy agar plates; incubate at 36 °C+/-1 °C for 18 h+/-1 h for subsequent identification. The colony morphology of Vibrio vulnificus on the

Appendix A Media and reagents

A.1 Peptone-sodium chloride-cellobiose-polymyxin E (PNCC) enrichment broth A.1.1 Solution 1 A.1.1.1 Composition Peptone: 50.0 g Sodium chloride: 10.0 g Distilled water: 900.0 mL A.1.1.2 Preparation method DISSOLVE the components in A.1.1.1 in distilled water; USE 1 mol/L hydrochloric acid solution and 1 mol/L sodium hydroxide solution to adjust the pH to 8.5+/-0.2; autoclave at 121 °C for 10 min.

A.1.2 Solution 2 A.1.2.1 Composition Cellobiose: 0.8 g Polymyxin E: 1000 U Distilled water: 100.0 mL A.1.2.2 Preparation method DISSOLVE cellobiose in distilled water; HEAT it slightly to completely dissolve;

after cooling, add antibiotics; USE a 0.22 um microporous membrane to filter and sterilize for later use.

PNCC enrichment broth is obtained by mixing solution 1 with solution 2.

A.2 Cellobiose-polymyxin E (CC) agar medium A.2.1 Solution 1 A.2.1.1 Composition Sodium chloride: 20.0 g Bromothymol blue: 0.04 g Cresol red: 0.04 g Agar: 15.0 g Distilled water: 900.0 mL A.3.1.2 Preparation method DISSOLVE the components in A.3.1.1 in distilled water; HEAT and boil until completely dissolved; USE 1 mol/L hydrochloric acid solution and 1 mol/L sodium hydroxide solution to adjust the pH to 7.6+/-0.2; COOL to 48 °C~55 °C for later use.

A.3.2 Solution 2 A.3.2.1 Composition Cellobiose: 10.0 g Polymyxin B: 100000 U Polymyxin

E: 400000 U Distilled water: 100.0 mL A.3.2.2 Preparation method DISSOLVE cellobiose in 100.0 mL of distilled water; HEAT it slightly until it is completely dissolved; after cooling, add antibiotics; USE a 0.22 um microporous membrane to filter and sterilize for use.

MIX solution 2 and solution 1 and pour on the plate.

A.4 3% sodium chloride tryptone soy agar A.4.1 Composition Tryptone: 15.0 g Soy peptone: 5.0 g Sodium chloride: 30.0 g Agar: 15.0 g Sodium thiosulfate ($Na_2S_2O_3$): 0.3 g Agar: 12.0 g Distilled water: 1000.0 mL A.6.2 Preparation method DISSOLVE the various components in A.4.1 in distilled water; USE 1 mol/L hydrochloric acid solution and 1 mol/L sodium hydroxide solution to adjust the pH to 7.4+/-0.2. After autoclaving at 121 °C for 15 min, separate them into test tubes, to make a high-level slant with a length of 4 cm~5 cm and a depth of 2 cm~3 cm for use.

A.7 Halophilic test medium A.7.1 Composition Tryptone: 10.0 g Sodium chloride: ADD the corresponding amount in turn according to the different concentration Distilled water: 1000.0 mL A.7.2 Preparation method DISSOLVE the various components in A.5.1 in distilled water. After using 1 mol/L hydrochloric acid solution and 1 mol/L sodium hydroxide solution to adjust the pH to 7.2+/-0.2, dispense into 300 mL conical flasks. Each flask contains 100 mL. A total of 5 flasks are prepared. ADD different amounts of sodium chloride:

(1) 0 g; (2) 3 g; (3) 6 g; (4) 8 g; (5) 10 g. Then, the sodium chloride solution of each concentration is divided into test tubes of appropriate capacity. Each tube contains 10 mL. Autoclave at 121 °C for 15 min for later use.

A.8 3% sodium chloride lysine decarboxylase test medium A.8.1 Composition Peptone: 5.0 g Yeast powder: 3.0 g Glucose: 1.0 g Bromocresol purple: 0.02 g L-lysine: 5.0 g A.11.1 Composition N,N,N',N'-tetramethylp-phenylenediamine hydrochloride: 1.0 g Distilled water: 100.0 mL A.11.2 Preparation method Oxidase reagent shall be prepared in small amounts. After preparation, it shall be stored in a refrigerator at 2 °C~5 °C in the dark; and be used within 7 d.

A.12 Gram staining solution A.12.1 Crystal violet staining solution A.12.1.1 Composition Crystal violet: 1.0 g 95% ethanol: 20.0 mL 1% ammonium oxalate aqueous solution: 80.0 mL A.12.1.2 Preparation method Completely dissolve the crystal violet in ethanol; then mix it with the ammonium oxalate solution.

A.12.2 Gram iodine solution A.12.2.1 Composition Iodine: 1.0 g Potassium iodide: 2.0 g Distilled water: 300.0 mL A.12.2.2 Preparation method MIX iodine and potassium iodide first; ADD a little distilled water and shake well.

When it is completely dissolved, add distilled water to 300.0 mL.

A.12.3 Safranin counterstaining solution A.12.3.1 Composition Safranin: 0.25 g