

Australian Standard®

AS 5013.18—2010

Food microbiology

Method 18: Examination for specific organisms—*Vibrio parahaemolyticus*

PREFACE

This Standard was prepared by the Standards Australia Committee FT-024, Food Products and Subcommittee FT-024-01, Food Microbiology (Constituted) to supersede AS/NZS 1766.2.9:1997, *Food microbiology, Method 2.9: Examination for specific organisms—Vibrio parahaemolyticus*.

The Committee reviewed ISO 8914:1990, *Microbiology—General guidance for the detection of Vibrio parahaemolyticus*, for adoption as the revision of AS/NZS 1766.2.9—1997. However, adoption of ISO 8914:1990 cannot be recommended because—

- (a) ISO methodology is to determine the presence or the absence of the organism, which is inadequate for regulatory purposes; and
- (b) ISO methodology is a qualitative method whereas an Australian Standard should be a quantitative and reference method.

This Standard is one of a series of methods for the microbiological examination of foods for quality control and investigative purposes and, where applicable, for checking that foods comply with regulatory specifications.

This edition confirms the method without technical change but updates the referenced documents and reflects current editorial style.

The laboratory should have a clearly defined quality control system to ensure that the apparatus, culture media, reagents and technique are suitable for the test. The use of positive controls is part of this system.

The term ‘normative’ has been used in this Standard to define the application of the appendix to which it applies. A ‘normative’ appendix is an integral part of a Standard.

METHOD

1 SCOPE

This Standard sets out a method for estimating the most probable number (MPN) of *Vibrio parahaemolyticus* in foods.

2 REFERENCED DOCUMENTS

The following documents are referred to in this Standard.

AS

5013 Food microbiology

5013.3 Method 3: Microbiology of food and animal feeding stuffs—Horizontal method for the detection and enumeration of coliforms—Most probable number technique

5013.11.1 Method 11.1: Microbiology of food and animal feeding stuffs—Preparation of test samples, initial suspension and decimal dilutions for microbiological examination—General rules for the preparation of the initial suspension and decimal dilutions

5013.11.3 Method 11.3: Microbiology of food and animal feeding stuffs—Preparation of test samples, initial suspension and decimal dilutions for microbiological examination—Specific rules for the preparation of fish and fishery products

3 PRINCIPLE

Two sequential liquid selective enrichments and solid selective media are used for the isolation of presumptive *V. parahaemolyticus* and for their subsequent biochemical confirmation.

NOTE: A flow diagram of the examination procedure is shown in Appendix A.

4 CULTURE MEDIA, REAGENTS AND REFERENCE CULTURES

4.1 General

The formulations for culture media and reagents specified in this Clause are given in Appendix B. The culture media listed in Clause 4.3 require an appropriate addition of sodium chloride to provide a concentration of 30 g/L NaCl after sterilization.

4.2 Media

4.2.1 *Alkaline peptone water*

4.2.2 *Thiosulfate citrate bile-salts sucrose (TCBS) agar*

4.2.3 *Salt tolerance media*—containing 0 g/L, 80 g/L and 110 g/L NaCl.

4.2.4 *Decarboxylase broth base (Moeller's) and decarboxylase broths containing lysine, ornithine and arginine*—with 10 g/L NaCl added.

4.2.5 *Physiological saline*

4.2.6 *V. parahaemolyticus preservation medium*

4.3 Media containing 30 g/L sodium chloride (see Clause 4.1)

4.3.1 *Tryptone water*—containing 30 g/L NaCl.

4.3.2 *Buffered glucose broth*—containing 30 g/L NaCl.

4.3.3 *Nutrient agar*—containing 30 g/L NaCl.

4.3.4 *Triple sugar iron (TSI) agar*—containing 30 g/L NaCl.

4.3.5 *Bromcresol purple cellobiose broth*—containing 30 g/L NaCl.

4.3.6 *Peptone solution, 0.1%*—containing 30 g/L NaCl.

4.4 Reagents

4.4.1 *Voges-Proskauer reagents*

4.4.1.1 *α-naphthol*—alpha-naphthol, 5% w/v in absolute alcohol

4.4.1.2 *Potassium hydroxide solution*—potassium hydroxide, 40% w/v in distilled water

4.4.2 *Oxidase reagent (Kovacs)*

4.4.3 *Toluene*

4.4.4 *Buffered ONPG solution 0.0133 M*

4.5 Reference cultures

V. parahaemolyticus NCTC 10884 or UQM 2200

4.5.1 Additional control cultures—optional

V. cholerae (non-O1) NCTC 4711, ATCC 14730

V. vulnificus ATCC 27562.

See Appendix C for procedures for the long-term preservation of reference cultures.

5 PREPARATION OF DILUTIONS OF FOOD PRODUCT

5.1 Diluent

Peptone solution containing 30 g/L NaCl (4.3.6) should be used to prepare dilutions of the test sample.

5.2 Procedure

The procedure shall be as follows:

- (a) Prepare the sample of food product for testing in accordance with the instructions given for that product in the appropriate AS 5013 Standard.
- (b) Prepare the required decimal dilutions of the prepared sample by the procedure described in AS 5013, Methods 11.1 and 11.3, as appropriate.

6 TEST PROCEDURES

6.1 Isolation of presumptive *V. parahaemolyticus*

The procedure shall be as follows:

- (a) Using the most probable number (MPN) method (3-tube technique) described in AS 5013.3, inoculate tubes containing 10 mL of alkaline peptone water (4.2.1) with appropriate dilutions of the test sample (primary enrichment).
- (b) Prepare a set of controls for each series of tests by inoculating tubes of alkaline peptone water (4.2.1) with the reference culture(s) specified in Clause 4.5.
- (c) Incubate tests and control at $36 \pm 2^\circ\text{C}$ for 6 h to 8 h.
- (d) Transfer 1 mL from each tube to a fresh tube of alkaline peptone water (4.2.1) (secondary enrichment). Streak a loopful of each primary enrichment culture on to a prepared plate of TCBS agar (4.2.2) to obtain isolated colonies.

Each individual primary and secondary enrichment culture shall carry a unique identification code in order to determine which secondary enrichment corresponds to each primary enrichment culture when selecting colonies for confirmation and when calculating the most probable number of *V. parahaemolyticus* present.

- (e) Incubate the secondary enrichments and the TCBS agar plates at $36 \pm 2^\circ\text{C}$ for 18 ± 2 h.
- (f) Streak a loopful of each secondary enrichment on to a prepared plate of TCBS agar (4.2.2) to obtain isolated colonies.
- (g) Incubate the TCBS agar plates at $36 \pm 2^\circ\text{C}$ for 18 ± 2 h.

- (h) At the end of their respective incubation periods, examine the plates for typical colonies of *V. parahaemolyticus*.

6.2 Confirmation of *V. parahaemolyticus*

The procedure shall be as follows:

- (a) Select three typical *V. parahaemolyticus* colonies from the TCBS agar plates streaked from each pair of primary and secondary enrichment cultures. Subculture each colony into a tube of tryptone water containing 30 g/L NaCl (4.3.1).

Typical colonies of *V. parahaemolyticus* are blue/green in colour and 3–5 mm in diameter.

It is not necessary to inoculate into tryptone broth prior to inoculation of biochemical tests if well isolated colonies on the selective agar plates are selected for confirmation. However, the nutrient agar containing 30 g/L NaCl plate inoculated at the time of biochemical confirmation shall be examined to confirm that the inocula were pure. Step (b) will not apply if this approach is used.

- (b) Incubate at $36 \pm 2^\circ\text{C}$ until faintly turbid, usually 2 h to 4 h.
- (c) Inoculate each of the following with a drop of the culture from Step (b):
- (i) Lysine, ornithine and arginine decarboxylase broths (4.2.4) and decarboxylase broth base (as a control).
 - (ii) Buffered glucose broth containing 30 g/L NaCl (4.3.2).
 - (iii) Bromcresol purple cellobiose broth containing 30 g/L NaCl (4.3.5).
 - (iv) Salt tolerance media (4.2.3).
 - (v) Tryptone water containing 30 g/L NaCl (4.3.1).
- (d) Using the culture from Step (b), inoculate a slope of TSI agar containing 30 g/L NaCl (4.3.4) by stabbing the butt and streaking the slope with a straight wire.
- (e) Using a loopful of the culture from Step (b), streak a prepared plate of nutrient agar containing 30 g/L NaCl (4.3.3) to obtain isolated colonies.
- (f) Incubate the inoculated media from Steps (c), (d) and (e) as follows:
- (i) Tryptone water containing 30 g/L NaCl..... $42 \pm 1^\circ\text{C}$ for 18 h to 24 h.
 - (ii) Decarboxylase broths $36 \pm 2^\circ\text{C}$ for 4 d.
 - (iii) Bromcresol purple cellobiose broth..... $36 \pm 2^\circ\text{C}$ for 4 d.
 - (iv) Salt tolerance media $36 \pm 2^\circ\text{C}$ for 48 ± 2 h.
 - (v) All other media $36 \pm 2^\circ\text{C}$ for 18 h to 24 h.
- (g) Perform an oxidase test on the nutrient agar culture by removing some growth from the agar surface with a platinum loop and smearing it on a filter paper moistened with oxidase reagent (4.4.2).
- NOTE: Development of a purple colour within 15 s is a positive reaction.
- (h) After TSI slope has been incubated and the result recorded, perform ONPG test as follows:
- (i) In a small sterile tube containing 0.25 mL physiological saline (4.2.5), emulsify a large loopful of culture growth from the TSI slope to form a heavy suspension.
 - (ii) Add 1 drop of toluene (4.4.3) to the tube and shake well to liberate enzyme.

(iii) Let tube stand 5 min at $36 \pm 2^\circ\text{C}$. Add 0.25 mL Buffered 0.0133M ONPG solution (4.4.4) to each suspension. Incubate at 37°C .

(iv) Examine at intervals up to 24 h.

NOTE: Development of a yellow colour is a positive result; colourless is negative.

(i) Perform a Voges-Proskauer test on the buffered glucose broth as follows:

(i) Mix 1 mL of broth with 0.6 mL of α -naphthol solution (4.4.1.1), then add 0.2 mL of potassium hydroxide solution (4.4.1.2).

(ii) Mix well and stand the tube in a slanted position at room temperature for 15 min.

NOTE: Development of a red colour at the surface of the medium is a positive reaction.

(j) Examine the incubated media. Typical reactions of *Vibrio* controls are given in Table 1.

TABLE 1
TYPICAL REACTIONS OF SELECTED *VIBRIO* SPP

Medium	Test	Reactions		
		<i>V. parahaemolyticus</i>	<i>V. cholerae</i>	<i>V. vulnificus</i>
Nutrient agar (4.3.3)	Oxidase	+	+	+
Buffered glucose broth (4.3.2)	Voges-Proskauer	—	Variable	—
ONPG test (4.3.4, 4.4.3)	beta-D-galactosidase	—	+	+
Tryptone water (4.3.1)	Growth at 42°C	+	+	+
Bromcresol purple cellobiose broth (4.3.5)	Cellobiose	—	—	+
	Fermentation			
Salt tolerance medium with 0 g/L NaCl (4.2.3)	Growth	—	+	—
Salt tolerance medium with 80 g/L NaCl (4.2.3)	Growth	+	—	—
Salt tolerance medium with 110 g/L NaCl (4.2.3)	Growth	—	—	—
TSI agar (4.3.4)	Glucose fermentation	Acid, no gas	Acid, no gas	Acid, no gas
TSI agar (4.3.4)	Lactose fermentation	—	—	+
TSI agar (4.3.4)	Sucrose fermentation	—	+	—
TSI agar (4.3.4)	Hydrogen sulphide	—	—	—
Decarboxylase broths (4.2.4)	Lysine	Alkaline	Alkaline	Alkaline
	Ornithine	Alkaline	Alkaline	Alkaline
	Arginine	Acid	Acid	Acid
	Base	Acid	Acid	Acid

NOTE: Atypical reaction can occur if the results are inconclusive, additional tests should be carried out or the culture forwarded to a reference laboratory for further tests.