

Australian Standard[®]

Water microbiology

Method 15: Examination for *Vibrio cholerae*

PREFACE

This Standard was prepared by the Australian members of Joint Standards Australia/Standards New Zealand Committee FT-020, Water Microbiology, to supersede AS/NZS 4276.15:1999, *Water microbiology*, Method 15: *Examination for Vibrio cholerae*.

After consultation with stakeholders in both countries, Standards Australia and Standards New Zealand decided to develop this Standard as an Australian Standard rather than an Australian/New Zealand Standard.

The objective of this revision is to update the method to reflect current practice according to recognized reference methods, as well as any developments in the relevant area of science. This has included the International Technical Specification for the detection of *Vibrio parahaemolyticus* and *Vibrio cholerae* in food, ISO/TS 21872-1. The bibliography lists the methods and references taken into account in the preparation of this Standard.

During the review, the Committee noted there is no published ISO method for the detection of *V. cholerae* in water to be considered for adoption, or in the pipeline.

This method is designed to detect *V. cholerae* in water using conventional culture-based techniques. The enumeration of this microorganism is not normally carried out because its presence, irrespective of numbers, is significant from a public health point of view.

The *V. cholerae* species is composed of numerous O-serotypes. Toxin producing strains of the O1 serotype are responsible for epidemic cholera. However, another serotype, O139, has relatively recently been identified as an epidemic producing strain. Other strains belonging to different serotypes may cause sporadic cases of diarrhoeal illness. This method will detect all strains of *V. cholerae* and allow identification of the cholera epidemic producing O1 and O139 serotypes.

It should be emphasized that the finding of *V. cholerae* O1 or O139 in a water sample would necessitate the prompt notification of the relevant health authority.

In addition, isolates of *V. cholerae* O1 and O139 are now subject to an Australian Commonwealth security sensitive biological agent (SSBA) regulatory scheme (the *National Health Security Act 2007* and the *National Health Security Regulations 2008*). The aim of the SSBA regulatory scheme is to limit the opportunities for acts of bioterrorism or biocrime to occur using harmful biological agents and to provide a legislative framework for managing the security of SSBA. The regulatory scheme imposes statutory obligations on laboratories that isolate or receive strains of *V. cholerae* O1 or O139. Laboratories that have an involvement with *V. cholerae*, in whatever form, can familiarize themselves with this regulatory scheme by accessing the Department of Health website: www.health.gov.au/SSBA.

The term 'informative' has been used in this Standard to define the application of the appendix to which it applies. An 'informative' appendix is only for information and guidance.

METHOD

1 SCOPE

This Standard sets out a method for detecting *V. cholerae* in water using culture in enrichment broth and on selective agar media.

NOTES:

- 1 A flow diagram of the procedure is shown in Appendix A.
- 2 This method is suitable for the isolation of other *Vibrio* species besides *V. cholerae*. However, the NaCl concentration in the enrichment broth should be increased to 1% for isolation of such species.

2 REFERENCED DOCUMENTS

The following documents are referred to in this Standard:

AS

4276 Water microbiology

4276.1 Part 1: General information and procedures (ISO 8199:2005, MOD)

3 DEFINITION

For the purposes of this Standard the following definition applies:

3.1 *V. cholerae*

V. cholerae is a member of the family Vibrionaceae. It is a facultatively anaerobic, gram-negative, non-spore-forming curved rod, about 1.4–2.6 µm long, capable of respiratory and fermentative metabolism. It is oxidase-positive, reduces nitrate, and is motile by means of a single, sheathed, polar flagellum.

V. cholerae is able to grow at pH 8.6 in the presence of bile salts, 1% sodium thiosulphate, and 1% sodium citrate and ferment sucrose without the production of sulphide.

4 PRINCIPLE

V. cholerae is tolerant of alkaline conditions and will grow rapidly in peptone water at an alkaline pH and with 0.5% sodium chloride. The water sample is enriched in alkaline peptone water and plated onto thiosulphate citrate bile salts sucrose agar. Typical colonies are confirmed as *V. cholerae* by biochemical tests. The serotypes responsible for epidemic cholera, O1 and O139, are identified by slide agglutination with specific antisera.

NOTE: This method does not include enumeration. If enumeration of *V. cholerae* in water is required, the most probable number (MPN) procedure may be employed.

See AS 4276.1. The volumes tested depend on the degree of contamination of the water, but the set of 50 mL, 10 mL and 1 mL is a useful starting point. The method commences with enrichment using APW, and then proceeds according to this standard. Calculation of the most probable number per given volume requires the use of MPN tables.

5 CULTURE MEDIA AND REAGENTS

5.1 Culture media

- 5.1.1 *Alkaline peptone water (APW)*
- 5.1.2 *Thiosulphate citrate bile salts sucrose agar (TCBS)*
- 5.1.3 *Nutrient agar*
- 5.1.4 *8% NaCl tryptone water (8% NaCl TW)*
- 5.1.5 *3% NaCl tryptone water (3% NaCl TW)*
- 5.1.6 *0% NaCl tryptone water (0% NaCl TW)*
- 5.1.7 *Tryptone water*
- 5.1.8 *Buffered glucose broth*
- 5.1.9 *Arginine dihydrolase broth*
- 5.1.10 *Lysine decarboxylase broth*
- 5.1.11 *Ornithine decarboxylase broth*
- 5.1.12 *Control decarboxylase broth*
- 5.1.13 *Glucose fermentation broth*
- 5.1.14 *Arabinose fermentation broth*
- 5.1.15 *Cellobiose fermentation broth*
- 5.1.16 *Lactose fermentation broth*
- 5.1.17 *Maltose fermentation broth*
- 5.1.18 *Mannitol fermentation broth*
- 5.1.19 *Salicin fermentation broth*
- 5.1.20 *Sucrose fermentation broth*
- 5.1.21 *Nitrate reduction broth*
- 5.1.22 *O-nitrophenyl- β -D-galactopyranoside broth (ONPG)*
- 5.1.23 *Cellobiose-colistin (CC) Agar*
- 5.1.24 *Modified cellobiose polymyxin B-colistin (mCPC) Agar*
- 5.1.25 *Sodium dodecyl sulphate polymyxin sucrose (SDSPS) Agar*

5.2 Reagents

- 5.2.1 *Kovacs' oxidase reagent*
- 5.2.2 *Kovacs' indole reagent*
- 5.2.3 *5% α naphthol in ethanol*
- 5.2.4 *40% KOH*
- 5.2.5 *Nitrate reagent A (0.8% w/v sulphanilic acid in 5N acetic acid)*
- 5.2.6 *Nitrate reagent B (0.6% v/v N,N-Dimethyl- α -naphthylamine in 5N acetic acid)*

NOTE: Composition and preparation of culture media and reagents are given in Appendix B.

6 APPARATUS

6.1 Apparatus and materials for membrane filtration

As described in AS 4276.1.

6.2 Incubators or water baths

Able to maintain incubation temperatures of $36 \pm 2^\circ\text{C}$ and $41.5 \pm 1^\circ\text{C}$.

7 REFERENCE CULTURES

7.1 Positive culture

Positive culture to be used shall be *V. cholerae* non-O1 WDCM 00203, NCTC 4711, ATCC 14730 or a culture traceable to any of these strains. This culture will biochemically confirm as *V. cholerae* but does not agglutinate with *V. cholerae* O1 or O139 antisera.

NOTE:

WDCM—World Data Centre for Microorganisms

NCTC—National Collection of Type Cultures

ATCC—American Type Culture Collection

7.2 Use of reference culture

The reference culture is used in media quality control and analytical quality control procedures relevant to this method. The purpose of the reference culture is to demonstrate and ensure that typical growth characteristics and test reactions are exhibited on the media and in the tests used in this method.

As a minimum, the reference culture shall be used to assure that the method has been carried out correctly. Each day samples are processed by this method, the positive reference culture is submitted to the test procedure. Growth of the positive reference culture obtained is taken through the procedure as far as any isolates obtained from the samples, including confirmation tests.

If the reference culture does not give appropriate results, then an investigation shall be undertaken. Test results shall be regarded as invalid unless the investigation reveals reasons that invalidate the quality control procedures, e.g. prepared suspension shown not to contain target bacteria.

8 SAMPLES

8.1 Laboratory sample

Sample refrigeration or transport on ice may result in *V. cholerae* O1 or O139 entering into a viable but non-culturable (VBNC) state. Samples shall not be placed on ice or held at $<10^\circ\text{C}$ during transport or in the laboratory. Test samples as soon as possible after collection, ideally within 6 hours.

8.2 Sample volume

The sample volume may vary according to the source and level of sensitivity desired, however a 1 L sample is recommended for most investigations.

8.3 Concentration techniques

8.3.1 Moore swab

This is the classical method of concentrating pathogens and tracing their origin in a water or waste water system. Sterile gauze swabs made with absorbent cotton are suitably anchored in the water to be tested. After two days the swabs with entrapped water are collected in a sterile jar.

8.3.2 Membrane filtration

Samples treated in this manner shall be low in turbidity so that water flow through the membrane is not seriously impeded. The samples are filtered through membranes of 0.45 µm average porosity. More than one membrane may be needed to filter a sample.

For turbid samples, the use of filter aids may allow them to be successfully membrane filtered. See Appendix C.

9 PROCEDURE

9.1 Enrichment

The procedure for enrichment in APW shall be as follows:

- (a) The volume and strength of APW used depends on the sample type or sample pretreatment.
 - (i) *Membrane filter* Place membrane filters in 100 mL APW.
 - (ii) *Turbid water* Add sample to an equal volume of double strength APW.
 - (iii) *Moore swab* Place the Moore swab in APW at a 1:1 weight/volume ratio.

NOTE: It is desirable that the containers used for the APW enrichments are such that there is a reasonably large liquid surface area. This ensures a large volume of oxygenated medium where the growth of *V. cholerae* is enhanced.

- (b) Incubate the APW culture at $36 \pm 2^\circ\text{C}$. At 6 to 8 hours subculture 1 mL from the surface of the APW into 10 mL APW. Do not shake or mix the APW culture. This is the second APW culture.
- (c) Incubate both APW cultures overnight at $41.5 \pm 1^\circ\text{C}$ such that the first APW culture is incubated for no longer than 24 h and second APW culture incubated for 18 ± 1 h.

9.2 Selective agar culture

NOTE: Additional selective media may be used alongside TCBS. Examples are: cellobiose colistin (CC) agar, modified cellobiose-polymyxin B-colistin (mCPC) agar, and sodium dodecyl sulphate polymyxin sucrose (SDSPS) agar. Formulations and preparation procedures for these media are found in Appendix B.

There are also commercially available chromogenic media. These chromogenic media are acceptable additional selective media to TCBS provided documentation is available that demonstrates their reliability for identifying strains of *V. cholerae* and following suitable performance verification in the laboratory.

The isolation procedure using TCBS as the selective medium shall be as follows:

- (a) After 6 to 8 hours incubation, inoculate a plate of TCBS with one or two drops or a 10 µL loopful taken from the surface of the first APW culture. Do not shake or mix cultures. Streak the enrichment broth to obtain isolated colonies.
- (b) After overnight incubation, inoculate plates of TCBS with one or two drops or a 10 µL loopful taken from the surface of each of the first and second APW cultures. Do not shake or mix cultures. Streak the enrichment broth to obtain isolated colonies.
- (c) Incubate the TCBS plates at $36 \pm 2^\circ\text{C}$ for 21 ± 3 h.
- (d) Examine the plates for typical *V. cholerae* colonies. Typical colonies are smooth, yellow (sucrose positive) and 2 mm to 3 mm in diameter.

NOTE: Cultures grown on TCBS should be examined immediately after removal from an incubator because the yellow colonies may revert to a green colour when left at room temperature.

9.3 Presumptive identification of *Vibrio cholerae*

V. cholerae shall be identified to a presumptive stage as follows:

- (a) Select at least three colonies of each colony type typical of *V. cholerae* and subculture each to 3% NaCl TW. Incubate at $36 \pm 2^\circ\text{C}$ overnight.
- (b) When growth becomes evident, inoculate a 0% NaCl TW and a nutrient agar plate from each 3% NaCl TW.

NOTE: 0% NaCl TW is not strictly salt free, as it may contain other salts, both organic and inorganic, as well as trace amounts of NaCl, due to the chemical composition of tryptone. However, this does not affect the performance of this medium.

- (c) After overnight incubation check the nutrient agar plate cultures for purity. Perform oxidase tests on pure cultures. Presumptive *V. cholerae* is oxidase positive and shows growth in the 0% NaCl TW.

NOTE: *Aeromonas* spp. capable of growing on TCBS may presumptively mis-identify as *V. cholerae*.

9.4 Confirmation of *Vibrio cholerae*

NOTE: Isolates of *V. cholerae* O1 and O139 are subject to an Australian Commonwealth security sensitive biological agent (SSBA) regulatory scheme. Under this regulatory scheme these microorganisms are listed as Tier 2 SSBA's. This imposes statutory obligations in relation to the management of these microorganisms on laboratories that isolate or receive them. Laboratories that have an involvement with *V. cholerae*, in whatever form, can familiarise themselves with the SSBA regulatory scheme by accessing the Department of Health website: www.health.gov.au/SSBA.

9.4.1 Phenotypic identification and serological characterization

V. cholerae may be confirmed biochemically and serologically characterized as follows:

- (a) If the purpose of the investigation is to identify the serotypes of *V. cholerae* responsible for epidemic cholera, slide agglutination using O1 and O139 specific antisera on a formol saline suspension prepared from the nutrient agar plate is performed. Isolates that agglutinate with O1 antiserum may be further subtyped with Ogawa and Inaba antisera.

NOTES:

- 1 The species *V. cholerae* comprises numerous O-serotypes. Serotypes other than O1 are conventionally referred to as *V. cholerae* non-O1. However, this convention does not accommodate the emergence of the epidemiologically significant O139 serotype. Isolates that are shown to be negative to both O1 and O139 antisera are referred to as *V. cholerae* non-O1 non-O139.
- 2 It is recommended that all isolates of *V. cholerae* be tested with O1 antiserum. Cultures should be sent to a reference laboratory if this antiserum is not available in the testing laboratory.

- (b) From the 3% NaCl TW culture inoculate the biochemical tests listed in Table 1. Incubate at $36 \pm 2^\circ\text{C}$ for 48 ± 2 h.

NOTES:

- 1 All the biochemical tests, except Voges-Proskauer, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and nitrate, can be read after 24 hours incubation if desired.
- 2 If there is an interest in identifying other *Vibrio* species, then the media for the following tests require a NaCl concentration of 1%: indole, Voges-Proskauer and nitrate reduction.

- (c) After the addition of the required reagents to the indole, Voges-Proskauer and nitrate tests, the biochemical tests are read and the results compared with the expected results given in Table 1. Isolates that produce results in agreement with the expected results are confirmed as *V. cholerae*.

NOTES:

- 1 Instructions on performing the indole, Voges-Proskauer and nitrate reduction tests are given in Appendix D.
- 2 Atypical test results may occur and additional tests and information can be found in References [1] and [2]. If different media formulations are used other than those given in Appendix B, then the negative and positive reactions indicated in Table 1 may not be the same.
- 3 Further tests allow classification of *V. cholerae* O1 isolates as Eltor or classical biotypes. However, these tests are normally carried out in specialized reference laboratories and are beyond the scope of this Standard.
- 4 *V. cholerae* is included in the databases of several commercial identification systems. These systems are an acceptable alternative to conventional tests provided documentation is available that demonstrates their reliability for identifying both clinical and environmental strains of *V. cholerae* and following suitable performance verification in the laboratory.

TABLE 1
BIOCHEMICAL REACTIONS OF *VIBRIO CHOLERAE*

Test	Negative reaction	Positive reaction	Expected results
Indole (tryptone water)	Kovacs' reagent remains yellow	Kovacs' reagent turns red	+
Voges-Proskauer (buffered glucose broth)	No colour change	Medium turns red	V*
Arginine dihydrolase	Medium turns yellow and remains yellow	Medium turns yellow initially but reverts to purple	–
Lysine decarboxylase	Medium turns yellow and remains yellow	Medium turns yellow initially but reverts to purple	+
Ornithine decarboxylase	Medium turns yellow and remains yellow	Medium turns yellow initially but reverts to purple	+
Glucose – acid	Medium remains colourless	Medium turns pink/red	+
Glucose – gas	No gas in Durham tube	Gas in Durham tube	–
Arabinose	Medium remains colourless	Medium turns pink/red	–
Cellobiose	Medium remains colourless	Medium turns pink/red	–
Lactose	Medium remains colourless	Medium turns pink/red	–
Maltose	Medium remains colourless	Medium turns pink/red	+
Mannitol	Medium remains colourless	Medium turns pink/red	+
Salicin	Medium remains colourless	Medium turns pink/red	–
Sucrose	Medium remains colourless	Medium turns pink/red	+
Nitrate reduced to nitrite	No colour change	Medium turns red	+
ONPG	Medium remains colourless	Medium turns yellow	+
8% NaCl tryptone water	No growth	Growth	–

* 75% of strains positive. See Reference [1].

9.4.2 Genotypic tests

Genotypic based testing may be used as an alternative to phenotypic based testing for the identification and characterization of *V. cholerae*. Laboratories must demonstrate that a nucleic acid amplification testing procedure is equivalent to the phenotypic based identification and characterization in this Standard.

NOTE: Appendix E provides an informative example.

Vibrio isolates of uncertain identification, either biochemical, serological or genotypic, should be sent to a reference laboratory:

- (a) MDU Public Health Laboratory
Level 1
Peter Doherty Institute for Infection and Immunity
Corner Grattan & Elizabeth Streets
The University of Melbourne VIC 3010
- (b) Public Health Microbiology Laboratory
Queensland Health Forensic & Scientific Services
39 Kessels Road
COOPERS PLAINS QLD 4108

10 TEST REPORT

The report shall contain the following information:

- (a) Reference to this Australian Standard, i.e. AS 4276.15.
- (b) All details necessary for complete identification of the sample.
- (c) Dates of sample collection, receipt and test commencement.
- (d) The presence or absence of presumptive or confirmed *V. cholerae* in the sample volume or Moore swab tested.
- (e) The serotype of the isolate(s) if this was determined. That is, *V. cholerae* non-O1, non-O139, O1 or O139. If serotyping has not been carried out this is stated in the report.
- (f) Any circumstances or conditions which may have influenced the result.