

Australian/New Zealand Standard™

Water microbiology

Method 15: Examination for *Vibrio cholerae*

PREFACE

This Standard was prepared by the Joint Standards Australia/Standards New Zealand Committee FT/20, Water Microbiology, as part of a series of methods for the microbiological examination of waters for domestic and industrial use.

This method is designed to detect *Vibrio 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 *Vibrio 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 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 *Vibrio cholerae* and allow identification of the cholera epidemic producing O1 and O139 serotypes. It should be emphasised that the finding of *Vibrio cholerae* O1 or O139 in a water sample would necessitate the prompt notification of the relevant health authority.

The development and availability of PCR systems for the detection and identification of vibrio may lead to the use of a standardized PCR technique as an adjunct to this method. These tests would not be within the capability of an otherwise well-equipped microbiological laboratory and are not included in this Standard. Laboratories that cannot perform these specialized tests should consult a laboratory with the necessary facilities if further testing, beyond the scope of this Standard, is required to establish the identity of an organism isolated by this method.

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 *Vibrio 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%.

2 PRINCIPLE

Vibrio cholerae is tolerant of alkaline conditions and will grow rapidly in simple 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 salt 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.

3 CULTURE MEDIA, REAGENTS AND REFERENCE CULTURES

3.1 Culture media

3.1.1 *Alkaline peptone water (APW)*

3.1.2 *Thiosulphate citrate bile salt sucrose agar (TCBS)*

3.1.3 *Nutrient agar*

3.1.4 *3% NaCl tryptone water (3% NaCl TW)*

3.1.5 *0% NaCl tryptone water (0% NaCl TW)*

3.1.6 *Tryptone water*

3.1.7 *Buffered glucose broth*

3.1.8 *Arginine dihydrolase broth*

3.1.9 *Lysine decarboxylase broth*

3.1.10 *Ornithine decarboxylase broth*

3.1.11 *Control decarboxylase broth*

3.1.12 *Glucose fermentation broth*

3.1.13 *Arabinose fermentation broth*

3.1.14 *Cellobiose fermentation broth*

3.1.15 *Lactose fermentation broth*

3.1.16 *Maltose fermentation broth*

3.1.17 *Mannitol fermentation broth*

3.1.18 *Salicin fermentation broth*

3.1.19 *Sucrose fermentation broth*

3.1.20 *Nitrate reduction broth*

3.1.21 *O-nitrophenyl- β -D-galactopyranoside broth (ONPG)*

3.1.22 *8% NaCl tryptone water*

3.2 Reagents

3.2.1 *Kovacs' oxidase reagent*

3.2.2 *Kovacs' indole reagent*

3.2.3 *5% alpha naphthol in ethanol*

3.2.4 *40% KOH*

3.2.5 *Nitrate reagent A (0.8% w/v sulphanilic acid in 5N acetic acid)*

3.2.6 *Nitrate reagent B (0.6% v/v N,N - Dimethyl-1-naphthylamine in 5N acetic acid)*

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

3.3 Reference cultures

3.3.1 Positive culture

Positive culture to be used shall be *Vibrio cholerae* non-O1 NCTC 4711, ATCC 14730, ACM 2934. This culture will biochemically confirm as *V. cholerae* but does not agglutinate with *V. cholerae* O1 antiserum.

NOTE: NZRM does not contain an equivalent culture.

3.3.2 Negative culture

Negative culture to be used shall be *Vibrio fluvialis* NCTC 11218, ACM 2944, NZRM 2605. This culture is a halophillic *Vibrio* which does not biochemically identify as *V. cholerae*, either with the presumptive or confirmatory tests.

3.3.3 Use of reference cultures

When testing a sample of water by this standard method, cultures of the positive (3.3.1) and negative (3.3.2) reference cultures shall be submitted to the test procedures at the same time to demonstrate and ensure that typical positive and negative growth characteristics and test reactions are exhibited by the respective reference microorganisms.

NOTES:

- 1 NCTC—National Collection of Type Cultures
ATCC—American Type Culture Collection
ACM—Australian Collection of Microorganisms
NZRM—New Zealand Reference Culture Collection (Medical Section)
- 2 These cultures are obtainable from the following laboratories:

In Australia:
Australian Collection of Microorganisms (ACM)
Department of Microbiology
The University of Queensland
BRISBANE QLD 4072

In New Zealand:
New Zealand Reference Culture Collection (Medical Section) (NZRM)
ESR Kenepuru Science Centre
Kenepuru Drive
Porirua

4 SAMPLES

4.1 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.

4.2 Concentration techniques

4.2.1 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.

4.2.2 Membrane filtration

Samples treated in this manner must be low in turbidity so that water flow through the membrane is not seriously impeded. However, the addition of sterile diatomaceous earth to turbid samples may allow them to be successfully membrane filtered. The samples are filtered through membranes of 0.45 µm average porosity.

5 PROCEDURE

5.1 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 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 ±2°C. At 6–8 hours subculture 1 mL from the surface of the APW into 10 mL APW. This is the second APW culture.
- (c) Incubate both APW cultures overnight at 36 ±2°C such that the first APW culture is incubated for no longer than 24 hours and second APW culture incubated for 16 to 18 hours.

5.2 Selective agar cultures

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

- (a) Inoculate a plate of TCBS with one or two drops taken from the surface of the first APW culture after 6–8 hours incubation. Streak out for isolated colonies.
- (b) Inoculate plates of TCBS with one or two drops taken from the surface of each of the first and second APW cultures after overnight incubation (16 to 24 hours). Streak out for isolated colonies.
- (c) Incubate the TCBS plates at 36 ±2°C for 16–24 hours.
- (d) Examine the plates for typical *Vibrio cholerae* colonies. Typical colonies are smooth and yellow.

5.3 Presumptive identification of *Vibrio cholerae*

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

- (a) Select at least two colonies of each colony type typical of *V. cholerae* and subculture to a 3% NaCl TW. Incubate at 36 ±2°C.
- (b) After 4 hours, inoculate a 0% NaCl TW and a nutrient agar plate.

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 perform an oxidase test from the nutrient agar plate and check the same plate for purity. Presumptive *V. cholerae* is oxidase positive and shows growth in the 0% NaCl TW.

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

5.4 Confirmation of *Vibrio cholerae*

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 and a formol saline suspension prepared from the nutrient agar plate can be 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.
- 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 held 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 hours.

NOTE: All the biochemical tests, except Voges-Proskauer, Arginine Dihydrolase, Lysine Decarboxylase, Ornithine Decarboxylase and Nitrate, can be read after 24 hours incubation, if desired.

- (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 C.
- 2 Atypical test results may occur and additional tests and information can be found in references [1] and [3] (see Clause 8).
- 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*.
- 5 *Vibrio* isolates of uncertain identification, biochemical or serological, should be sent to a Reference Laboratory.

In Australia
Melbourne Diagnostic Unit
University of Melbourne
PARKVILLE VIC 3052

Public Health Microbiology
Queensland Health Scientific Services
39 Kessels Road
COOPERS PLAINS QLD 4108

In New Zealand
ESR Kenepuru Science Centre
Kenepuru Drive
PORIRUA

- 6 If different media formulations are used than those given in Appendix B, then the negative and positive reactions indicated in Table 1 may not be the same.

6 TEST REPORT

The report shall contain the following information:

- (a) Reference to this Australian/New Zealand Standard, i.e. AS/NZS 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 *Vibrio cholerae* in the sample volume or Moore swab tested.
- (e) The serotype of the isolate(s) if this was determined. That is, *Vibrio cholerae* O1, non-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.