

Australian Standard[®]

AS 4276.19:2014

Water microbiology

Method 19: Examination for thermophilic *Campylobacter* spp.—Membrane filtration

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 4276.19:2001, *Water microbiology*, Method 19: *Examination for thermophilic Campylobacter* spp.—*Membrane filtration*.

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 Standard and consider ISO 17995:2005, *Water quality—Detection and enumeration of thermotolerant Campylobacter species*, and research over the last 10 years into methods and media for the detection of *Campylobacter*.

ISO 17995:2005 has not been adopted due to this method's age and that work has commenced to technically review the first edition. In addition, laboratories in Australia require some level of speciation and ISO 17995:2005 does not include speciation nor a resuscitation step at enrichment prior to antibiotic addition. A review of recent publications suggests that mExeter broth is the enrichment medium of choice for waters with expected high background counts as it is more inhibitory compared to Bolton broth.

The genus *Campylobacter* comprises two main groups according to growth temperature. Those in the first group will grow at 42°C and are referred to as thermophilic *Campylobacter* spp. The second group will grow at 25°C but not at 42°C.

The thermophilic *Campylobacter* spp. are not thought to be free living but are obligate parasites of humans, animals and birds. *Campylobacter jejuni*, (*C. jejuni*) and *Campylobacter coli* (*C. coli*) are common causes of intestinal infections in humans. *Campylobacter upsaliensis* (*C. upsaliensis*) may be of like importance. *Campylobacter lari* (*C. lari*) is less frequently associated with human infections. *Campylobacter* infections give rise to a flu-like illness with malaise, fever and myalgia followed by diarrhoea. The vehicles for *Campylobacter* infections are usually food, farm animals, pets and person-to-person contact; water is also important. They can also be isolated from waters contaminated with human or animal faeces such as wastewater and surface waters. Outbreaks of campylobacteriosis have been reported in relation to the use of contaminated drinking water and from recreational water use.

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 detection and speciation of thermophilic *Campylobacter* in water using culture in enrichment broth and on selective agar media.

NOTE: Due to the selective nature of the isolation media used and its requirements for a hydrogen enhanced microaerobic atmosphere, some strains of *C. upsaliensis* may not be detected.

2 REFERENCED DOCUMENTS

The following documents are referred to in this Standard:

AS

2031 Water quality—Sampling for microbiological analysis (ISO 19458:2006, MOD)

4276 Water microbiology

4276.1 Method 1: General information and procedures

3 PRINCIPLE

Campylobacter spp. are microaerophilic microorganisms requiring reduced oxygen tension of approximately 5% and an increased carbon dioxide tension of approximately 10%. Isolation of thermophilic *Campylobacter* spp. is achieved by concentration of organisms on a membrane filter followed by broth enrichment and subculture onto a selective agar. The broth and agar are both incubated microaerobically at 41.5°C. Confirmation of *Campylobacter* spp. involves biochemical, morphological and physiological tests. Alternative confirmation may be achieved using genotypic tests.

NOTE: This method does not include enumeration. If enumeration of thermophilic *Campylobacter* spp. in water is required, a method that may be employed is the most probable number (MPN) method using the multiple tube dilution technique. 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 Bolton or mExeter broth, and then proceeds according to this standard. Calculation of the most probable number per given volume requires the use of MPN tables.

4 CULTURE MEDIA AND REAGENTS

4.1 Culture media

4.1.1 *Modified Exeter broth (mExeter)*

4.1.2 *Bolton broth*

4.1.3 *Modified charcoal cefoperazone dextrochole agar (mCCD)*

4.1.4 *Blood agar*

4.2 Reagents

4.2.1 *Kovacs' oxidase reagent*

4.2.2 *Dilute carbol fuchsin*

4.2.3 *Physiological saline solution, 0.85% aqueous solution*

4.2.4 *Sodium hippurate, 10 g/L aqueous solution*

4.2.5 *Ninhydrin solution, 3.5% in 1:1 mixture of acetone and butanol*

4.2.6 *Catalase reagent, 3% hydrogen peroxide (H₂O₂)*

4.2.7 *Indoxyl acetate discs*

5 REFERENCE CULTURES

5.1 *Campylobacter jejuni*, WDCM 00156 or WDCM 00005 or NCTC 11351 or a culture traceable to any of these strains.

This organism gives positive reactions for thermophilic *Campylobacter* spp. and is hippurate positive.

5.2 *Campylobacter coli*, WDCM 00072 or WDCM 00004 or a culture traceable to any of these strains.

This organism gives positive reactions for thermophilic *Campylobacter* spp. and is hippurate negative.

NOTE:

WDCM—World Data Centre for Microorganisms

NCTC—National Collection of Type Cultures

5.3 Use of reference cultures

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

As a minimum, the reference cultures shall be used to assure that the method has been carried out correctly. Each day samples are processed by this method, at least one of the reference cultures is submitted to the test procedure. Growth of the reference culture(s) obtained is taken through the procedure as far as any isolates obtained from the samples, including confirmation tests. If speciation is carried out, both of the reference cultures shall be submitted to the hippurate hydrolysis or equivalent genotypic test.

If the reference cultures do 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.

6 APPARATUS

6.1 Gas cylinders or kits

To produce a microaerobic incubation atmosphere with approximately 5% oxygen, 10% carbon dioxide, optional 10% hydrogen, with the balance being nitrogen.

6.2 Filtration apparatus

6.3 Filter membranes 0.2 or 0.22 µm pore size

6.4 Sterile diatomaceous earth ('filter-aid') plus sterile absorbent pads (optional)

6.5 Incubators

Operating at $37 \pm 1^\circ\text{C}$ and $41.5 \pm 1^\circ\text{C}$

7 SAMPLING

See AS 2031. The sample volume may vary according to the source and level of sensitivity desired, however, at least a 1 L water sample is recommended for most investigations.

8 PROCEDURES

8.1 Microaerobic incubation

Campylobacter spp. are microaerophiles and shall be incubated in an atmosphere with approximately 5% oxygen, 10% carbon dioxide, optional 10% hydrogen, with the balance being nitrogen.

8.2 Membrane filtration

The membrane filtration procedure shall be as set out in this Clause 8.2.

Filter the appropriate volumes of sample through sterile filter membranes. Minimize unnecessary exposure to air of the membrane filter post filtration.

With turbid samples, it will be necessary to use two or more filters for the larger sample volumes to avoid clogging. All these filters are transferred to the same portion of enrichment broth.

The use of diatomaceous earth (SiO_2), also known as 'filter aid', will facilitate the filtration of turbid samples. Sterile diatomaceous earth can be added to the sample and the sample filtered through a sterile absorbent pad, for example glass fibre or cellulosic filter support pads. The pad acts as a supporting base and replaces the membrane filter. Alternatively, the membrane filter can be retained, with the absorbent pad being placed over the filter.

Diatomaceous earth is used as follows. Prepare a filtration setup by adding an aliquot of diatomaceous earth, typically 1–3 g, to 15–50 mL sterile water. Mix thoroughly and filter to form an even initial layer on the absorbent pad. Prepare a second suspension of diatomaceous earth in 15 mL of sterile water and add to the sample so that approximately 1 g of diatomaceous earth is added per litre of sample. Filter the diatomaceous earth/sample through the prepared filtration setup. For highly turbid samples, separate aliquots of the sample can be filtered through individual filtration setups. Rinse the funnel to collect residual diatomaceous earth and sample. When filtration is complete, remove the funnel and carefully transfer the absorbent pad and diatomaceous earth, and filter if used, to the enrichment medium. If more than one filtration setup is used, all of the material from the filters is added to the one enrichment.

8.3 Enrichment

8.3.1 General

For waters with an expected low background count it is appropriate to use Bolton broth. Where the background count is expected to be high, mExeter broth is more appropriate.

The enrichment procedures shall be as set out in this Clause 8.3.

8.3.2 Waters with low background counts

Place membrane(s) in 100 mL of Bolton enrichment broth in a suitable screw-capped container. Incubate microaerobically at $37 \pm 1^\circ\text{C}$ for 44 ± 4 h.

NOTE: Thermophilic *Campylobacter* spp. may die or grow too slowly if the incubation temperature is below 36°C .

8.3.3 Waters with high background counts

Place membrane(s) in 100 mL of mExeter enrichment broth (with defibrinated horse blood but without antibiotic supplement) in a suitable screw-capped container. Resuscitate by incubating aerobically at $37 \pm 1^\circ\text{C}$ for 4 to 6 h.

Add the antibiotic supplement at the end of the resuscitation period. Mix well. Incubate the selective broth in a microaerobic condition at $41.5 \pm 1^\circ\text{C}$ for 44 ± 4 h.

NOTE: As an alternative to incubation in a microaerobic atmosphere, the enrichment broths may be incubated in screw-capped bottles or flasks filled with enrichment broth, leaving a headspace of less than 2 cm, and tightly closing the caps. Successful application of this procedure is demonstrated by growth of the reference culture(s) used.

8.4 Subculture onto selective medium

After incubation for 21 ± 3 h, subculture approximately 10 μ L of enrichment culture from beneath the broth surface to mCCD selective agar. Incubate the plate and the enrichment broth in a microaerobic atmosphere at $41.5 \pm 1^\circ\text{C}$ for up to 44 ± 4 h.

Examine plates after 21 ± 3 h. If colonies are too small, re-incubate plates microaerobically for a total of 44 ± 4 h.

If no growth is observed on mCCD agar after 21 ± 3 h, subculture a further 10 μ L of enrichment culture from beneath the surface of the re-incubated enrichment culture onto mCCD selective agar. Incubate the mCCD plates for a total of 44 ± 4 h.

8.5 Examination of selective agar plates

Examine plates for characteristic colonies, using a hand lens if necessary. Typical colonies of *Campylobacter* spp. vary in size and on mCCD plate appear greyish, flat and moistened, with a tendency to spread. They may have a metallic sheen. Variation in colonial morphology is not uncommon, so it is advisable to perform confirmation tests on several colonies. Select three isolated colonies from each plate showing suspect *Campylobacter* colonies. If there are less than three such colonies on any one plate, confirm the identity of each.

9 CONFIRMATION TESTS

9.1 General

Confirmation tests shall be carried out as set out in this Clause 9.

9.2 Screening of colonies

Subculture each selected colony onto two blood agar plates. Incubate one aerobically and another microaerobically at $41.5 \pm 1^\circ\text{C}$ for up to 44 ± 4 h.

Disregard all those colonies which grow both aerobically as well as microaerobically as they are not *Campylobacter* spp.

Subject all those colonies which grow only microaerobically on blood agar at $41.5 \pm 1^\circ\text{C}$ within 44 ± 4 h to further confirmatory tests as follows.

9.3 Oxidase test

Perform an oxidase on the blood agar cultures.

Campylobacter spp. are oxidase positive.

9.4 Gram stain

Perform a Gram stain (using dilute carbol fuchsin as counterstain) on the blood agar cultures which are oxidase positive.

Campylobacter spp. are Gram negative, small, curved, spiral, seagull or 'S' shaped.

9.5 Motility

Inoculate colonies that are Gram negative and oxidase positive into a tube of nutrient broth and incubate microaerobically at $41.5 \pm 1^\circ\text{C}$ for 21 ± 3 h. Examine a wet mount under the microscope.

Campylobacter spp. demonstrate corkscrew or darting motility.

9.6 Confirmed thermophilic *Campylobacter* spp.

An isolate which grows at $41.5 \pm 1^\circ\text{C}$, is strictly microaerobic, oxidase positive, Gram negative, small, curved, spiral, seagull or 'S' shaped, and demonstrates corkscrew or darting motility is confirmed as thermophilic *Campylobacter* spp.

9.7 Genotypic tests

Genotypic based testing is an alternative to phenotypic based testing for the identification and characterization of *Campylobacter* spp. Laboratories must demonstrate that a nucleic acid amplification testing procedure is equivalent to the phenotypic based identification and characterization in this Standard.

The following reference may be consulted:

RICE, EW, BAIRD, RB, EATON, AD and CLESCERI, LS. (Eds): *Standard Methods for the Examination of Water and Wastewater*. 22nd ed., Washington DC: American Public Health Association, American Water Works Association and Water Environment Federation, 2012.

10 SPECIATION TESTS (OPTIONAL)

10.1 General

For most practical purposes, Gram stain, motility and an oxidase test are adequate for identification. If required, species differentiation may be carried out as set out in this Clause 10.

10.2 Hippurate hydrolysis

Inoculate a 21 ± 3 h blood agar culture into 0.4 mL sterile sodium hippurate solution to obtain a turbid suspension in a small screw-capped tube. Incubate tubes (including an uninoculated control) at $37 \pm 1^\circ\text{C}$ for 2 h. Add 0.2 mL ninhydrin solution. Do not shake. Development of a deep purple colour within 5 min indicates a positive result; development of a slight bluish colour is regarded as negative.

10.3 Catalase test

Perform a catalase test using a fresh colony from the non-selective agar and catalase reagent.

Campylobacter spp. are catalase-positive. Strains of *C. upsaliensis* are reported to be catalase-negative or weakly positive.

NOTE: Blood agar may give rise to a false positive catalase reaction if blood is present with the colony material tested.

10.4 Indoxyl acetate

Place a selected colony on an indoxyl acetate disc and add a drop of sterile water. A loopful of colony material is required for a strong reaction. If the indoxyl acetate is hydrolysed, a colour change to dark blue occurs within 5 min to 10 min. No colour change indicates that hydrolysis has not taken place.

Campylobacter spp. growing at $41.5 \pm 1^\circ\text{C}$ may be identified at a species level according to Table 2.

TABLE 2
CHARACTERISTICS OF *CAMPYLOBACTER* SPP.

Characteristic	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. lari</i>	<i>C. upsaliensis</i>
Hydrolysis of hippurate	+	–	–	–
Catalase	+	+	+	– or slight
Indoxyl acetate	+	+	–	+

LEGEND:

+ Positive
– Negative

11 TEST REPORT

The report shall contain the following information:

- (a) Reference to this Australian Standard, i.e. AS 4276.19.
- (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 thermophilic *Campylobacter* spp. and if required, the species identified in the sample volume tested.
- (e) Any circumstances or conditions which may have influenced the result.

12 BIBLIOGRAPHY

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