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Water quality — Detection and enumeration of thermotolerant *Campylobacter* spp

Qualité de l'eau — Recherche et dénombrement d'espèces thermotolérantes du genre Campylobacter

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

This second edition cancels and replaces the first edition (ISO 17995:2005), which has been technically revised. The main changes compared to the previous edition are as follows:

— the inclusion of direct inoculation of enrichment broths in addition to membrane filtration with additional information about sample processing.

— methods for the speciation of *Campylobacter*.

— performance testing for the quality assurance of culture media has been added to Annex D.

— performance characteristics of the method have been added as an Annex E.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The thermotolerant *Campylobacter* species are not thought to propagate as free living but are zoonotic pathogenic bacteria of mammals and birds and which cause disease in humans. *Campylobacter jejuni* subsp. *jejuni* and *Campylobacter coli* are common causes of intestinal infections in humans. *Campylobacter upsaliensis* is found mainly in cats and dogs and is of minor importance for human infections. *Campylobacter 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 be isolated from waters contaminated with human or animal faeces such as wastewater and surface waters. The bacteria have been demonstrated to survive within amoebae. Outbreaks of campylobacteriosis have been reported in relation to the use of contaminated drinking water and sporadic cases from recreational water use.

Water quality — Detection and enumeration of thermotolerant *Campylobacter* spp

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably trained staff.

1 Scope

This document specifies a method for the detection, semi-quantitative and quantitative (MPN) enumeration of thermotolerant *Campylobacter* species.

The method can be applied to all kinds of waters including: drinking water, ground water and well water, fresh, brackish and saline surface water, swimming pools, spa and hydrotherapy pools, recreational waters, agricultural waters and runoff, untreated and treated wastewater and also sand and other sediments.

This method can be used for the detection of *Campylobacter* species in a specified sample volume. Clean water samples with low turbidity can be membrane filtered for either a qualitative method, semi-quantitative or quantitative (MPN) method. Water samples with higher turbidity, such as primary and secondary wastewater effluents and sediments, are analysed using the same qualitative, semi-quantitative or quantitative MPN method by direct inoculation of material into bottles or tubes. Sediments can be suspended in a suitable diluent or inoculated directly into enrichment broths.

Users wishing to employ this method are expected to verify its performance for the particular matrix under their own laboratory conditions.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 8199, *Water quality — General requirements and guidance for microbiological examinations by culture*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

ISO 19458, *Water quality — Sampling for microbiological analysis*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org./obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
Campylobacter
microorganisms forming characteristic colonies on solid selective media when incubated in a microaerobic atmosphere at 41,5 °C and which possess the characteristic morphology, motility, biochemical and growth properties described when tests are conducted in accordance with ISO 17995

Note 1 to entry: Thermotolerant *Campylobacter* species of relevance in human infections include *Campylobacter jejuni* subsp. *jejuni* (hereafter referred to as *Campylobacter jejuni*), *Campylobacter coli*, *Campylobacter lari* and possibly *Campylobacter upsaliensis*. *Campylobacter upsaliensis* is found mainly in cats and dogs and is of minor importance for human infection.

3.2
detection of *Campylobacter*
determination of the presence or absence, semi-quantitative enumeration or quantitative enumeration using a most probable number (MPN) system of *Campylobacter* in water and solid materials when the test is conducted in accordance with ISO 17995

4 Principle

4.1 General

In general, the detection of *Campylobacter* requires enrichment followed by isolation of colonies and their confirmation. The flow diagram of the procedure in Annex A shall be applied.

4.2 Inoculation and enrichment in selective liquid medium

Samples are inoculated either directly or after concentration using membrane filtration into one of two selective enrichment broths depending on the expected level of background microorganisms: Bolton broth for clean water and Preston broth for more heavily contaminated water. A single sample volume is processed for *Campylobacter* detection and, where necessary, at least three 10-fold volumes (for example 10 ml, 100 ml and 1 000 ml) are used for a semi-quantitative determination (Annex B shall be applied). For a quantitative (MPN) determination, volumes of 500 ml, 5 × 100 ml, 5 × 10 ml and, where counts may be high, smaller volumes are used or the initial sample is diluted. The broths are then incubated microaerobically at (37 ± 1) °C for (44 ± 4) h.

The ratio of sample to enrichment broth shall be 10 % or less if single strength broth is used.

4.3 Isolation and selection for confirmation

From the enrichment broth cultures in 4.2, liquid selective media are inoculated onto modified charcoal cefoperazone deoxycholate agar (mCCDA).

The mCCDA plates are then incubated at (41,5 ± 1) °C for (44 ± 4) h in a microaerobic atmosphere and examined for characteristic colonies after incubation. Colonies with typical characteristics are presumed to be *Campylobacter* (see 9.3). It is necessary to confirm at least one colony from each plate to demonstrate that the corresponding enrichment culture is positive for the presence of *Campylobacter* species.

4.4 Confirmation

Confirmation of suspect colonies of *Campylobacter* species involves biochemical, morphological and physiological tests. See flow diagram in Annex A.

The colonies presumed to be *Campylobacter* are inoculated on the non-selective Columbia agar or other appropriate solid non-selective media, and are then confirmed by means of microscopic examination, failure to grow aerobically at 25 °C and appropriate biochemical tests. Optionally, *Campylobacter* species are further identified by specific biochemical tests.

For species identification, it is recommended that more than one isolate per enrichment culture is tested.

If typical *Campylobacter* species are confirmed or identified, the result is given as the qualitative, semi-quantitative or quantitative (MPN) estimate per volume of sample.

5 Culture media and reagents

For current laboratory practice see ISO 8199 and ISO 11133.

The composition and preparation of the culture media and reagents given in Annex C shall be used. The performance testing requirements given in Annex D shall be applied.

6 Apparatus

Usual microbiological laboratory equipment (as specified in ISO 8199) and, in particular, the following:

6.1 Water baths or incubators, capable of operating at (37 ± 1) °C and (41,5 ± 1) °C.

6.2 Water bath, capable of operating between 47 °C and 50 °C.

6.3 Membrane filtration equipment, as specified in ISO 8199.

6.4 Membrane filters, sterile membrane filters made of cellulose ester with a diameter of 45 mm to 50 mm and a pore size of 0,45 µm. Larger diameter membranes may be used with suitable filter holders provided they have a pore size of 0,45 µm.

6.5 pH-meter, accurate to within 0,2 pH units at 25 °C.

6.6 Apparatus suitable for achieving a microaerobic atmosphere, with oxygen content of (5 ± 2) %, carbon dioxide (10 ± 3) %, optional hydrogen at ≤10 %, with the balance being nitrogen.

Appropriate gastight containers of, for example, 3,5 l capacity are used to hold Petri dishes and/or flasks or bottles for the enrichment broth, such as bacteriological anaerobic jars. The appropriate microaerobic atmosphere can be obtained using commercially available gas-generating kits, following precisely the manufacturer's instructions, particularly those relating to the volume of the jar and the capacity of the gas-generating kit. The jar may be flushed and filled with an appropriate gas mixture prior to incubation. Alternatively, an incubator with appropriately modified atmosphere (approximately 5 % oxygen and 10 % carbon dioxide) can also be used.

Gas-generating pouches can be used if they are able to maintain an atmosphere with approximately 5 % oxygen and approximately 10 % carbon dioxide.

6.7 Microscope, preferably with phase contrast or differential interference contrast. Dark ground illumination can also be used.

6.8 Bottles, 100 ml to 250 ml, with screw caps for the selective enrichments.

6.9 Vented Petri dishes, sterile, 90 mm.

6.10 Disinfected forceps, for handling membrane filters.

6.11 Refrigerator, capable of operating at (5 ± 3) °C.

7 Sampling, transport and storage

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage. Be aware that campylobacters are very sensitive to adverse conditions. Keep samples cool (5 ± 3) °C and in the dark until they are processed. The temperature should not be above that at which the sample was taken, nor should the sample be allowed to freeze. Avoid unnecessary mixing with air. Process the samples as soon as possible after collection, but within a maximum of 30 h. In all other aspects of sampling, transport and storage, follow the instructions given in ISO 19458.

NOTE 1 Campylobacters survive well in clean water at (3 ± 2) °C. At higher temperatures or in other media, they can quickly deteriorate.

NOTE 2 It is important that sample bottles are filled to the rim. This avoids air spaces in the container and helps to ensure *Campylobacter* survival.

8 Sample processing

8.1 General

Depending on the sample properties and expected level of faecal contamination, use direct inoculation (see 8.2) or membrane filtration (see 8.3) for sample processing. Direct inoculation is suitable for samples with a high contamination level such as wastewater, whereas membrane filtration is suitable for surface water, groundwater and drinking water samples. Direct inoculation is only suitable for relatively small volumes, for example 100 ml or less and membrane filtration is suitable for larger volumes.

The ratio of a maximum of 10% of sample to enrichment broth is used (for example 100 ml of sample to 1 L of broth) to avoid significant change to the composition of the medium. In addition, background microorganisms present in the sample are diluted sufficiently to avoid their inhibition of the growth of campylobacters during enrichment.

Throughout the processing steps, care should be taken to minimise the risk of cross-contamination of enrichment broths. The use of pipette tips with sterile filters is essential if automated pipettes are used. In addition any positive controls should be processed separately.

8.2 Direct inoculation into enrichment broth

Samples with expected high contamination levels are inoculated directly into Preston broth (see C.2). Where the expected level of background microorganisms is low and samples cannot be processed by membrane filtration, Bolton broth (see C.3) may be used. If no information about the contamination level is available, both broths should be used.

Double strength broth should be used for large volumes. Single strength broth should be used for relatively small volumes of water where the ratio of sample to enrichment broth shall be 10 % or less. Generally, volumes of 100 ml, 50 ml and 10 ml are inoculated into an equal volume of double strength broth. Volumes of 1 ml and 0,1 ml if required are inoculated into 10 ml of single strength broth.

Solid material can be weighed directly into single strength enrichment broths. Aliquots of 10 g can be weighed into 100 ml of enrichment broth, 1 g and smaller aliquots should be weighed into 10 ml of enrichment broth.

NOTE When high numbers of campylobacters are expected, the sample can also additionally be streaked directly onto mCCDA (see C.4) without prior selective enrichment.

8.3 Membrane filtration

Samples with expected low levels of contamination are filtered using membrane filters with a pore size of 0,45 µm (see 6.4). Select one of the two enrichment broths depending on the expected level of background

microorganisms and use 100 ml of single strength broth for the enrichment of each sample volume. Bolton broth (see C.3) is used for clean samples and Preston broth (see C.2) for heavily contaminated water. If no information about the contamination level is available, both broths should be used.

For detection of campylobacters in drinking water, other treated waters and clean surface waters, a 1 000 ml sample is filtered. If required, larger volumes may be processed using more filters or, where necessary, larger diameter filters may be used. Alternative concentration methods may be used, for example ultra-filtration or centrifugation providing that they are verified for recovery by the user. For more contaminated water, smaller volumes should be used and, for samples containing more particulate material, several membrane filters may be used to filter the required volume. All membrane filters from one sample volume shall be placed in the same container of enrichment broth.

For a semi-quantitative determination, volumes of 10 ml, 100 ml and 1 000 ml are filtered (see Annex B). The smaller volumes of 10 ml can also be pipetted directly into 100 ml of single strength medium or 10 ml of double strength medium.

For quantitative estimates, an MPN series can be used. In an MPN series, 100 ml and larger volumes can be filtered and membrane filters from different volumes are placed into 100 ml of separate single strength broth. Smaller volumes of 10 ml can be pipetted into 100 ml of single strength medium or 10 ml of double strength medium, and 1 ml can be pipetted into 10 ml of single strength medium. For example, volumes of 5 × 100 ml and 500 ml can be filtered and membranes placed into 100 ml of single strength broth. Volumes of 10 ml can be directly inoculated into 5 × 100 ml of single strength medium or 5 × 10 ml of double strength medium. Volumes of 5 × 1 ml can be inoculated directly into 10 ml of single strength broth.

After filtration, the membranes are transferred using disinfected forceps (see 6.10) into enrichment broths.

9 Procedure

9.1 Enrichment

For samples known to have low concentrations of background microorganisms, use the less selective Bolton broth for enrichment and for samples expected to have a high concentration of background microorganisms use the highly selective Preston broth. For samples where the background microbial concentration is unknown, enrich parts of each sample in both Preston and Bolton broths. Preston broth may be too selective to allow the recovery of some strains of *C. coli*. Bolton broth may not be selective enough to counteract the growth of non-campylobacters in some samples. If the available sample size is limited, chose one of the enrichment broths which is considered to be the most appropriate.

NOTE 1 The amount of sample (volume or weight) to be analysed can vary with the sample material and the scope of the investigation.

Bring enrichment broths to room temperature prior to inoculation.

Transfer the samples (see 8.2 and 8.3) to bottles with the appropriate volumes of enrichment broth(s). Put the inoculated broths in jars (6.6) or in an incubator with modified atmosphere. Leave the caps loosely placed on the inoculated broths during incubation to allow the modified atmosphere to reach the broths. Apply the modified atmosphere to the jars (6.6) and incubate at (37 ± 1) °C for (44 ± 4) h.

NOTE 2 The Preston campylobacter-selective supplement (see C.2.2) contains antibiotics (polymyxin B and rifampicin) known to be rather toxic towards *C. coli* and towards sub-lethally injured *C. jejuni*. Accordingly, pre-enrichment for 4 h in Preston broth without the selective supplement prior to the enrichment in the complete Preston broth (see C.2) has been found by some laboratories to increase the recovery of campylobacters from waters with low numbers of other microorganisms. The Bolton broth selective supplement (see C.3.2) does not include antibiotics known to be toxic towards campylobacters.

NOTE 3 Some campylobacters can die or grow too slowly if the incubation temperature is below 36 °C.

9.2 Plating on solid, selective medium

After incubation for (44 ± 4) h, remove the broths carefully from the jars to avoid re-suspension of sedimented material including background microorganisms. With a sterile loop, transfer approximately 10 µl of enrichment culture onto the surface of an mCCDA (C.4) plate. Draw the inocula from just below the surface of the broths and streak onto the surface of the plates. Without delay, incubate the inoculated plates in jars with a modified atmosphere (see 6.6) at (41,5 ± 1) °C for (44 ± 4) h.

NOTE 1 Bolton broth (see C.3) contains cefoperazone. The use of a second plating medium with selective agents different from those in mCCDA (see C.4) could improve *Campylobacter* detection, especially in the presence of background microflora resistant to third generation β-lactams like cefoperazone.

NOTE 2 The use of additional mCCDA plates for spread-plating 100 µl of enrichment broths can enhance the detection of low counts or injured *Campylobacter*.

9.3 Reading of presumptive results

Examine the inoculated mCCDA plates for visible growth after incubation.

Typical colonies of campylobacters are small, flat or convex with a greyish glossy surface often with a metallic sheen. They have a tendency to spread along the inoculation tracks. Well-spaced colonies resemble droplets of fluid. On moist agar, a thin, spreading film may be seen. Colony mass collected on a loop has a tan or creamy colour. See Annex F for additional guidance. Images of *Campylobacter* colonies on mCCDA are available at https://standards.iso.org/iso/17995/ed-2/en.

In cases of doubt, collect colony material from the surface of the plates and check under the microscope for typical appearance (see 9.4.4).

With continued incubation, colonies become flat or convex with a dull surface. A metallic sheen may develop. The colour of the colonies varies from transparent to greyish or whitish.

NOTE After the first examination of plates, the prolonged incubation of negative plates for up to 92 h might enhance the detection of injured *Campylobacter*, for example, from disinfected waters.

9.4 Confirmation

9.4.1 General

Throughout all tests, be aware that cultures may deteriorate quickly in light and air and follow the procedures in 9.4 without delay. At least one colony from each plate needs to be confirmed to say the corresponding enrichment broth was positive.

If speciation is required, several colonies should be subcultured and, if necessary, the cultures sent to an appropriate reference laboratory.

Alternative methods for confirmation and species identification such as commercially available biochemical test kits, gene amplification using genus and species specific PCR assays or matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) may be used provided they have been verified by the user.

9.4.2 Selection of colonies for confirmation

For confirmation, take from each plate of mCCDA (see 9.3) at least one colony considered to be typical or suspected of being *Campylobacter*. One confirmed isolate per sample is sufficient. If the first colony is negative, select up to four more colonies.

If needed, store the original isolation plates (see 9.3) under microaerobic conditions at (5 ± 3) °C for use in further confirmation and /or identification. *Campylobacter* isolates can loose their culturability when stored aerobically at 5 °C.

