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

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

Reference number
ISO 17995:2005(E)

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 17995 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

Introduction

Campylobacter jejuni subsp. *jejuni* and *Campylobacter coli* are common causes of intestinal infections in humans. *Campylobacter upsaliensis* may be of like importance. *Campylobacter lari* is less frequently associated with human infections. The vehicles for campylobacter infections are usually food, farm animals, pets and person-to-person contact, but water is also important.

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

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This standard 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 and to ensure compliance with any national regulatory conditions.

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

1 Scope

This International Standard specifies a method for the detection and semiquantitative enumeration of thermotolerant *Campylobacter* species. The method can be applied to all kinds of filterable waters.

NOTE 1 The method can also be used as a presence/absence test for *Campylobacter* species in a specified sample volume.

NOTE 2 A more quantitative result can be obtained using a most probable number (MPN) set-up (see ISO 8199).

2 Normative references

The following referenced documents are indispensable for the application 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 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 8199, *Water quality — General guidance on the enumeration of micro-organisms by culture*

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

3 Terms and definitions

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

3.1
thermotolerant *Campylobacter* species
bacteria retained on filters during the filtration described in 8.2, multiplying during the selective enrichment described in 8.3, forming typical colonies during incubation at elevated temperatures on the selective medium described in 8.4, forming no visible colonies during incubation in air under the conditions specified in 8.6, being highly motile, slender rods with spiral morphology and a motility characterized by darting or corkscrew-like movements.

NOTE 1 Thermotolerant *Campylobacter* species of relevance in human infections include *Campylobacter jejuni* subsp. *jejuni* (hereafter referred to as *C. jejuni*), *C. coli*, *C. lari* and possibly *C. upsaliensis*.

NOTE 2 Thermotolerant *Campylobacter* species are Gram-negative, oxidase-positive and catalase-positive (strains of *C. upsaliensis* are reported to be catalase-negative or weakly positive) curved or spiral-shaped rods with a characteristic darting, often rotating, motility. In older cultures, coccoid forms occur.

NOTE 3 *Campylobacter* species require enriched media for optimum growth. They are very sensitive to toxic oxygen derivatives like peroxides and superoxide anions which can arise in media exposed to light and oxygen. They are microaerophilic and prefer an atmosphere containing approximately 5 % oxygen and approximately 10 % carbon dioxide (CO_2). Some *Campylobacter* species may require an atmosphere containing hydrogen.

NOTE 4 Most *C. jejuni*, *C. coli* and *C. lari* grow at temperatures between 32 °C and 45 °C, but some strains will not grow below 35 °C. Other strains may not grow above 43 °C.

NOTE 5 *C. upsaliensis* may not grow under the conditions described in this International Standard.

4 Principle

The sample is filtered through membrane filters with a pore size of 0,45 µm. The filters are transferred to selective enrichment broths and incubated for (44 ± 4) h at (37 ± 1) °C in a microaerobic environment. Following incubation, inoculums from each broth are streaked onto a solid medium, modified charcoal cefoperazone desoxycholate agar (mCCDA), and incubated for (44 ± 4) h at (41,5 ± 1) °C in a microaerobic environment. Colonies resembling campylobacters are tested for growth aerobically and, if negative, examined by microscopy. If necessary, further biochemical reactions are performed. See flow diagram in Annex A.

If typical campylobacters are found, the sample is positive for thermotolerant campylobacters. The result is given as the semiquantitative estimate per volume of sample (see Annex B).

Non-filterable waters can be analysed by direct inoculation of sample into enrichment broths. The ratio of sample to enrichment broth shall be 10 % or less.

NOTE When sufficient numbers of campylobacters are present, the sample can be streaked directly onto a solid medium, mCCDA, without prior selective enrichment.

5 Apparatus

5.1 Incubators, thermostatically controlled at (37 ± 1) °C and at (41,5 ± 1) °C.

5.2 Equipment for membrane filtration, as specified in ISO 8199.

5.3 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.

Similar filters with a pore size of 0,22 µm are recommended for sterilization of supplements.

5.4 Equipment for microaerobic incubation: Jars able to maintain a modified atmosphere during incubation, fitted with valves for outlet and inlet of gases; vacuum pump; monitor for gas composition; and a suitable source of nitrogen, oxygen, carbon dioxide and preferably also hydrogen.

NOTE Commercially available equipment (like ANOXOMAT[1)]) can reproducibly deliver the modified atmosphere to the jars.

1) ANOXOMAT is a trade name. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

Alternatively, gas-generating pouches can be used if they are able to maintain an atmosphere with approximately 5 % oxygen, approximately 10 % carbon dioxide and preferably also approximately 10 % hydrogen.

5.5 Microscope, preferably with phase contrast.

5.6 Bottles, 150 ml to 250 ml, with airtight screw caps for the selective enrichments.

5.7 Vented Petri dishes, sterile, 9 cm.

5.8 Usual laboratory equipment.

6 Culture media and reagents

All ingredients and chemicals shall be of recognized quality, “for microbiology” or better. Water used shall be distilled or of like quality, as specified for ISO 3696:1987, grade 3. Follow the instructions given in Annex C.

Use of commercially available dehydrated substrates is encouraged, provided they comply with the descriptions in Annex C. They shall be prepared in accordance with the manufacturer's instructions.

Other grades of chemicals may be used provided they can be shown to lead to the same results.

7 Sampling, transport and storage

In addition to the instructions given in ISO 19458, be aware that campylobacters are very sensitive to adverse conditions. Keep samples cool (3 ± 2) °C and in the dark until the filtrations have been done. Avoid unnecessary mixing with air. Filter the samples as soon as possible after collection. Store for a maximum of 30 h prior to analysis.

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

8 Procedure

8.1 General

Parallel to samples, run a positive control spiked in sample material or in sterile water through all the steps of the procedure to demonstrate the proper functioning of the apparatus, culture media and procedure, and to facilitate recognition of campylobacters (8.5 to 8.6).

Parts of each sample shall be enriched in the highly selective Preston broth (C.1.1) and parts in the less selective Bolton broth (C.1.2).

NOTE 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, the most appropriate enrichment broth should be used. For waters with an expected high background count, it is more appropriate to use Preston broth, and for clean waters or where the background count is likely to be low Bolton broth is more appropriate.

NOTE 2 The amount of sample to be analysed varies with the sample material and the scope of the investigation. In Annex B, sample volumes for the analyses of drinking water are proposed.

8.2 Membrane filtration

Filter the appropriate volumes of sample through sterile membrane filters (5.3). Avoid unnecessary exposure to air.

With turbid samples, it may be necessary to use two or more filters for the largest sample volumes to counteract clogging. All these filters are transferred to the same portion of enrichment broth. The use of a "filter aid" will facilitate the filtration of turbid samples. For a short description of filter aids, see ISO 8199.

8.3 Enrichment

Preheat enrichment broths (C.1.1, C.1.2) to 20 °C to 30 °C before inoculation.

Transfer the filters (see 8.2) to bottles with 100 ml of enrichment broth immediately after filtration. Put the inoculated broths in jars (see 5.4). Leave the caps off or loosely placed on the inoculated broths during incubation to allow the modified atmosphere to reach the broths. Apply the modified atmosphere (see 5.4) and incubate at (37 ± 1) °C for (44 ± 4) h.

NOTE 1 100 ml volumes of enrichment broth are used to make sure that the contaminating bacteria present on the filters are diluted sufficiently to avoid their inhibition of the growth of campylobacters during the enrichment.

NOTE 2 Some laboratories report successful isolation of campylobacters without the use of a modified atmosphere, closed bottles or tubes with only a small headspace of air being used instead. This procedure may need careful standardization to avoid false negative results due to sub-optimum conditions during enrichment.

NOTE 3 The Preston campylobacter-selective supplement (C.1.1.2) 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 (C.1.1) 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 does not include antibiotics known to be toxic towards campylobacters.

NOTE 4 Thermotolerant campylobacters may die or grow too slowly if the incubation temperature is below 36 °C.

8.4 Plating on solid, selective medium

After incubation, remove the broths carefully from the jars to avoid resuspension of sedimented material like contaminating bacteria.

NOTE 1 When high numbers of campylobacters are expected, a rapid result is obtained by subculture after only (21 ± 3) h of enrichment.

With a sterile loop, transfer approximately 10 µl of enrichment culture onto the surface of an mCCDA (C.2) plate. Draw the inoculums from just below the surface of the broths and streak onto the surface of the plates.

As soon as possible, incubate the inoculated plates in jars with a modified atmosphere (see 5.4) at (41,5 ± 1) °C for (44 ± 4) h.

NOTE 2 Inoculated plates will usually show visible growth after (21 ± 3) h of incubation. If negative, reincubate without delay the plates for a further 24 h in the modified atmosphere.

NOTE 3 Techniques have been described whereby the motility of the campylobacters is used to selectively isolate them from concomitant bacterial flora. One or several drops of enrichment culture is placed on a cellulose acetate membrane filter with a pore size of 0,45 µm or 0,65 µm that is laid on a solid, non-selective medium (e.g. the basal medium of mCCDA, see C.2.1). The filter is incubated on this plate at 37 °C for 0,5 h to 24 h in a modified atmosphere (see 5.4). The filter is then removed and the plate incubated for (21 ± 3) h to allow the campylobacters that have passed through the membrane filter to develop into visible colonies. Alternatively, the enrichment culture can be centrifuged and the concentrate placed on the membrane filter instead of the one or several drops of enrichment culture.

8.5 Reading of results

Check the inoculated mCCDA plates prepared in 8.4 for visible growth after incubation.

Typical colonies of campylobacters are small, flat or convex with a glossy surface. 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.

In cases of doubt, collect colony material from the surface of the plates and check under the microscope for typical appearance (see 8.6.2) or extend the incubation of the plates for one day more.

Colony mass collected on a loop has a tan or creamy colour.

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

8.6 Confirmation

8.6.1 Growth on non-selective agar plates

Throughout all tests, beware that cultures may deteriorate quickly in light and air.

Subculture suspect colonies from mCCDA on two rich, non-selective agar plates [mCCDA basal medium without supplements (C.2.1), nutrient agar plates with or without 5 % lysed blood (C.1.1.4), Mueller-Hinton agar plates or other non-selective agar plates which have been demonstrated to support the growth of campylobacters]. Incubate one of the two plates in the modified atmosphere (see 5.4) and the other aerobically, both at (41,5 ± 1) °C for (21 ± 3) h.

Campylobacters grow in the modified atmosphere, but not aerobically.

Confirm the presence of campylobacters by microscopy (see 8.6.2). If typical campylobacters are present, report this as indicated in Clause 10. In cases of doubt, proceed with the supplementary tests described in Annex D.

8.6.2 Motility and cell morphology

Suspend material from a suspect colony in a rich broth like Preston basal broth (C.1.1.1) on a slide. Cover with a cover slip and examine immediately under the microscope, preferably using phase contrast.

Campylobacters are highly motile, slender rods with a spiral morphology. Motility is characterized by darting or corkscrew-like movements.

Campylobacters can be immobilized prior to microscopy for typical appearance by suspending in water instead of nutrient broth.

NOTE As an alternative to phase contrast microscopy for examining the appearance, campylobacters can be studied by dark-field microscopy or after staining for 5 min with 2 % carbolfuchsin. Gram staining often gives unsatisfactory results with campylobacters.

8.7 Further verification

If further verification is necessary, send isolates to an experienced laboratory for species identification and typing by phenotypic or molecular methods.

Be sure to adhere strictly to the instructions for transport given by this laboratory in order to avoid the campylobacters dieing due to adverse conditions during transport.

9 Quality assurance

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

Campylobacter jejuni ATCC 35918 or ATCC 35919 and *Campylobacter coli* ATCC 33559 or ATCC 43133 are suitable positive controls for this method.

10 Expression of results

If the presence of typical campylobacters is confirmed (see 8.6), report the result as *Campylobacter* detected in the sample volume examined.

Campylobacters are reported as detected in a given test volume of sample, whether the campylobacters were demonstrated in one or both of the selective enrichment broths used for that test volume.

A semiquantitative estimate of the numbers present in the sample is made from results with different test volumes (see Annex B).

11 Test report

The test report shall include at least the following information:

a) a reference to this International Standard (ISO 17995);

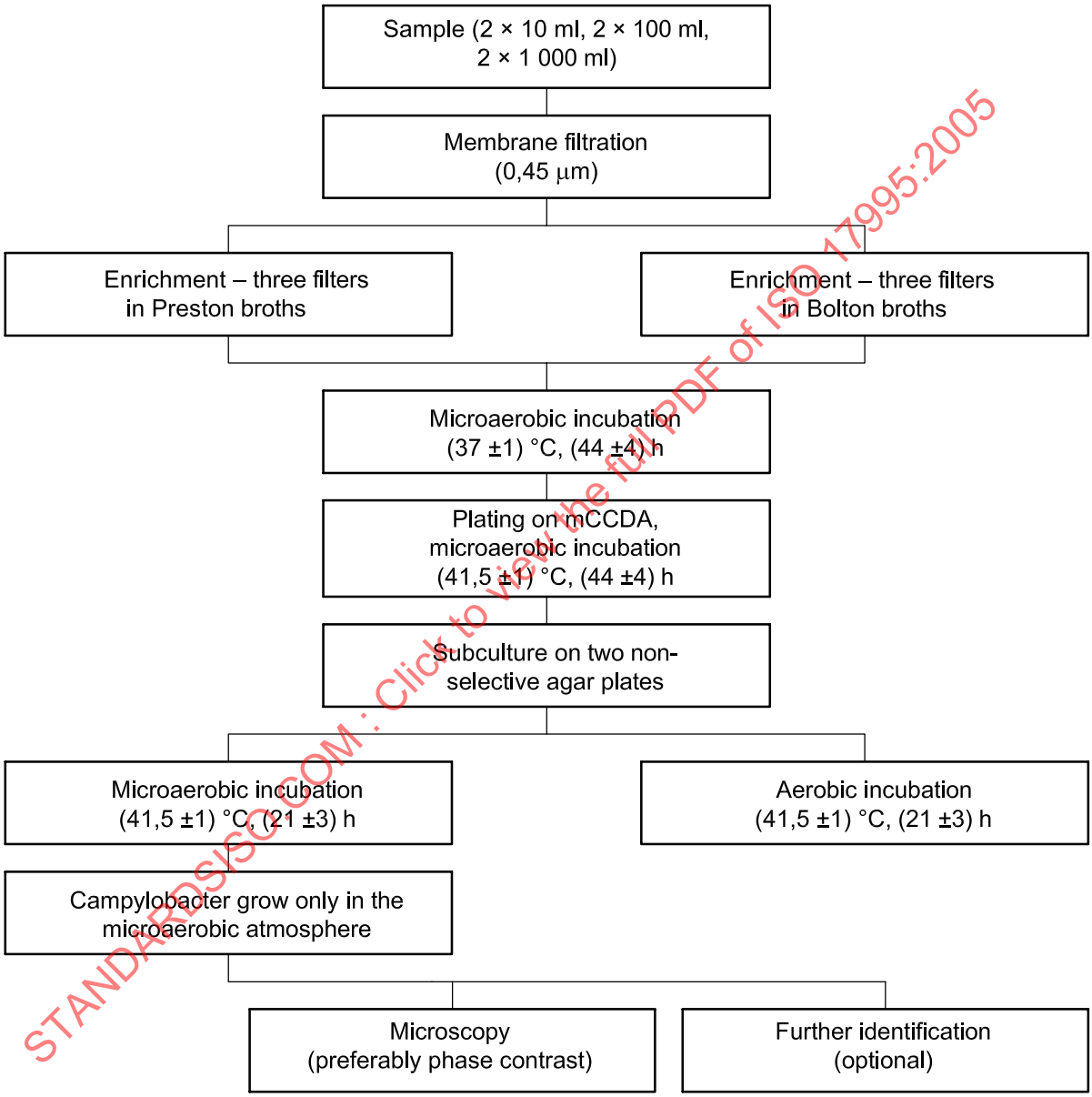
b) all details necessary for the complete identification of the sample;

c) the result of the test, as specified in Clause 10;

d) details of any particular phenomena observed during the analysis and any operation not specified in the method, or considered optional, that could have modified the results.

Annex A
(informative)

Flow diagram of method



Annex B
(informative)

Semiquantitative analysis

The volume of sample filtered will depend on the number of campylobacters expected in the water. For drinking water, volumes of 10 ml, 100 ml and 1 000 ml are recommended. The smallest volume is filtered first.

For water thus analysed, the result is given as a semiquantitative estimate per 1 000 ml. Count as positive any test volume which results in the demonstration of campylobacters present in at least one of the two enrichments. Estimate the number of campylobacters present in the sample material from Table 1.

Table B.1 — Numbers of campylobacters present in sample as a function of the results obtained on the test volumes

Sample volume tested			Semiquantitative estimate per 1 000 ml
1 000 ml	100 ml	10 ml	
–	–	–	<1 cfu[a]
+	–	–	⩾ 1 cfu but < 10 cfu
–	+	–	⩾ 1 cfu but < 10 cfu[b]
+	+	–	⩾ 10 cfu but < 100 cfu
+	–	+	⩾ 10 cfu but < 100 cfu[b]
+	+	+	⩾ 100 cfu
–	+	+	⩾ 100 cfu[c]
–	–	+	⩾ 100 cfu[c]

\+ Test volume campylobacter-positive.

– Test volume campylobacter-negative.

[a] cfu = colony-forming units.

[b] Depending on the number of campylobacters in the sample, the probability of this atypical outcome can be up to 10 %, caused by mere coincidence.

[c] This outcome can be seen when large numbers of competing microorganisms in the sample inhibit the campylobacters.

An atypical outcome should always result in a scrutiny of all the steps of the analysis to check for possible sources of error.

If further verification (see 8.7) has been carried out, the semiquantitative estimate can be given for a specific species and serotype of campylobacter. In an outbreak situation, multiple colonies have to be verified.

Annex C
(normative)

Culture media and reagents

C.1 Enrichment broths

C.1.1 Complete Preston broth

Composition:

Special meat extract[2)]	10 g
Peptone	10 g
Sodium chloride, NaCl	5 g
Polymyxin B	5 000 IU
Rifampicin	10 mg
Trimethoprim	10 mg
Amphotericin B	10 mg
Sodium pyruvate	0,25 g
Sodium metabisulfite	0,25 g
Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	0,25 g
Lysed blood	50 ml
Water	950 ml

NOTE Amphotericin B is used instead of cycloheximide.

C.1.1.1 Preston basal broth

Special meat extract[2)]	10 g
Peptone	10 g
Sodium chloride, NaCl	5 g
Water	940 ml

Suspend in water. Dispense 94 ml aliquots in 150 ml to 250 ml bottles with screw caps. Make allowance for losses during sterilization.

Sterilize in an autoclave at (121 ± 3) °C for (15 ± 1) min. Open the autoclave as soon as it has cooled to a safe temperature (e.g. 70 °C) and tighten the screw caps to avoid inclusion of air.

After sterilization, the pH at 25 °C shall be 7,4 ± 0,2.

In airtight bottles, the basal broth will keep for up to three weeks in the dark at (5 ± 3) °C.

2) E.g. Lab-Lemco powder (OXOID), which is a trade name. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

C.1.1.2 Solution of Preston campylobacter-selective supplement

A solution containing:

Polymyxin B	1 250 IU per ml
Rifampicin	2,5 mg per ml
Trimethoprim	2,5 mg per ml
Amphotericin B	2,5 mg per ml

C.1.1.3 Solution of Preston campylobacter growth supplement

A solution containing:

Sodium pyruvate	0,062 5 g per ml
Sodium metabisulfite	0,062 5 g per ml
Ferrous sulfate, $FeSO_4 \cdot 7H_2O$	0,062 5 g per ml

C.1.1.4 Preparation of lysed blood

Use defibrinated or citrate-stabilized blood from cattle, sheep or horses. The blood can be stored for 1 month at (5 ± 3) °C, provided it shows no signs of deterioration.

The blood is lysed by freezing at (–20 ± 5) °C overnight. Portions of lysed blood can be stored in tight vials at (5 ± 3) °C for up to 3 days.

C.1.1.5 Preparation of complete Preston broth

Broth and supplements are mixed immediately prior to carrying out inoculations. The campylobacter growth supplement (C.1.1.3) and the lysed blood (C.1.1.4) are the last ingredients to be added. After tightening the screw caps to minimize uptake of oxygen, the complete broths are gently rotated.

Preston broth is made by gently mixing the following ingredients:

Basal broth (C.1.1.1)	94 ml
Preston campylobacter-selective supplement (C.1.1.2)	0,4 ml
Preston campylobacter growth supplement (C.1.1.3)	0,4 ml
Lysed blood (C.1.1.4)	5 ml

C.1.2 Complete Bolton broth

Composition:

Meat peptone	10 g
Lactalbumin hydrolysate	5 g
Yeast extract	5 g
Sodium chloride, NaCl	5 g
Alpha-ketoglutaric acid	1 g
Sodium pyruvate	0,5 g
Sodium metabisulfite	0,5 g
Sodium carbonate	0,6 g

Haemin	10 mg
Cefoperazone	20 mg
Vancomycin	20 mg
Trimethoprim	20 mg
Amphotericin B	10 mg
Lysed blood	50 ml
Water	950 ml

NOTE Amphotericin B is used instead of cycloheximide.

C.1.2.1 Bolton basal broth

Meat peptone	10 g
Lactalbumin hydrolysate	5 g
Yeast extract	5 g
Sodium chloride, NaCl	5 g
Alpha-ketoglutaric acid	1 g
Sodium pyruvate	0,5 g
Sodium metabisulfite	0,5 g
Sodium carbonate	0,6 g
Haemin	10 mg
Water	950 ml

Suspend in water. Dispense 95 ml aliquots in 150 ml to 250 ml bottles with screw caps. Make allowance for losses during sterilization.

Sterilize in an autoclave at (121 ± 3) °C for (15 ± 1) min. Open the autoclave as soon as it has cooled to a safe temperature (e.g. 70 °C) and tighten the screw caps to avoid inclusion of air.

After sterilization, the pH at 25 °C shall be 7,4 ± 0,2.

In airtight bottles, the basal broth will keep for up to three weeks in the dark at (5 ± 3) °C.

C.1.2.2 Solution of Bolton broth selective supplement

A solution containing:

Cefoperazone	5 mg per ml
Vancomycin	5 mg per ml
Trimethoprim	5 mg per ml
Amphotericin B	2,5 mg per ml

C.1.2.3 Preparation of complete Bolton broth

Broth and supplements are mixed immediately prior to carrying out inoculations. The lysed blood (C.1.1.4) is the last ingredient to be added. After tightening the screw caps to minimize uptake of oxygen, the complete broths are gently rotated.