# Standing Committee of Analysts

## The Microbiology of Recreational and Environmental Waters (2016) – Part 9 – Methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment

Methods for the Examination of Waters and Associated Materials

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**Methods for the Examination of Waters and Associated Materials**

This booklet contains methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment and most probable number methods,

A The isolation of *Yersinia* species by selective enrichment, and a multiple tube most probable number technique

B The isolation of *Vibrio cholerae* and other V*ibrio* species by selective enrichment, and a multiple tube-most probable number technique

C The isolation of thermophilic *Campylobacter* species by selective enrichment, and a multiple tube-most probable number technique

This bluebook updates and replaces section 7.12 of the earlier version of The Microbiology of Recreational and Environmental Waters published in 2000 and adds methods for *Yersinia* and *Campylobacter*.

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products. They serve only as illustrative examples of the types of products available. Equivalent products may be available and it should be understood that the performance of the method might differ when other materials are used and all should be confirmed by validation of the method.

**Contents**

## About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soils (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Standing Committee of Analysts. At present, there are eight working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical, inorganic and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological, biodegradability and inhibition methods
8 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and strategic committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the SCA web-page (http://standingcommitteeofanalysts.co.uk/) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary. Users should ensure they are aware of the most recent version they seek.

Robert Carter
*Secretary*
June 2015

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## Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc. Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such resources are; HSE website HSE: Information about health and safety at work ; RSC website http://www.rsc.org/learn-chemistry/collections/health-and-safety ,"Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Biological Agents: Managing the Risks in Laboratories and Healthcare Premises", 2005 and "The Approved List of Biological Agents" 2013, produced by the Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE).

## A The isolation of *Yersinia* species by selective enrichment, and a multiple tube most probable number technique

### A1 Introduction

*Yersinia* species are environmental in origin and have been found in a wide range of foods. *Yersinia enterocolitica* can cause gastro-intestinal illness and is associated with the consumption of pig products. The significance of *Yersinia* bacteria in surface water and other environments are described elsewhere[(1)] in this series.

### A2 Scope

The method is suitable for the examination of surface water both fresh and saline, swimming pools, spa and hydrotherapy pools, primary and secondary wastewater effluents and sediments including sand. Clean water samples may be membrane filtered for either a presence absence or a multiple tube most probable number (MPN) test. Water samples with higher turbidities should be analysed using an appropriate (MPN) method by direct inoculation of material into bottles or tubes. Sediments may be suspended in a suitable diluent or inoculated directly into enrichment broths.

Users wishing to employ this method should verify its performance under their own laboratory conditions[(2)].

### A3 Definitions

In the context of the method, organisms are regarded as *Yersinia* species which, after enrichment and alkali treatment, form characteristic colonies on cefsulodin irgasan novobiocin (CIN) agar and then give positive reactions with triple sugar iron (TSI) agar and urea broth, and are non-motile at 37 °C after 4 - 6 hours. Further biochemical and serological identification can be carried out to speciate the isolates.

In addition, *Yersinia* species are Gram-negative rods, non-motile at 37 °C, catalase-positive, oxidase-negative and facultatively anaerobic. Sugars are attacked fermentatively with occasional gas production. Virulent strains are aesculin-negative and can bind crystal violet dye.

### A4 Principle

Samples are incubated in enrichment broth at 9 °C for up to two weeks followed by treatment with potassium hydroxide-sodium chloride solution and sub-culture to CIN agar incubated at 30 °C for 24 hours for the diagnostic detection of *Yersinia* species by a presence-absence determination or a multiple tube most probable number technique.

### A5 Limitations

This method is labour intensive and may require the preparation of large numbers of tubes or bottles of media and appropriate sub-cultures.

## A6 Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations[3] and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

## A7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators are required. Other items include:

A7.1 Sterile sample bottles of appropriate volume (at least 1 litre), made of suitable material. For swimming pools, spa pools and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

A7.2 Incubators capable of maintaining a temperature of 9 ± 1 °C, 30 ± 1 °C and 37 ± 1 °C.

A7.3 Filtration apparatus, filter funnels, (either sterilised or capable of being sterilised) and vacuum source.

A7.4 Sterile membrane filters, for example, white 47 mm diameter, cellulose-based 0.45 μm nominal pore size.

A7.5 Smooth-tipped forceps.

## A8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in the method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salt. If the pH of the media are not within the stated range, then, before heating, they should be adjusted accordingly. Sterilised media with a pH outside the required range should be discarded. Where media are stored in a refrigerator, they should be allowed to warm to room temperature before use.

A8.1 *Single strength Tris-buffered peptone broth*[4]

| | |
|---|---|
| Peptone | 10 g |

| | |
|---|---|
| Tris-(hydroxymethyl)aminomethane | 12.1 g |
| Sodium chloride | 5 g |
| Water | 1 litre |

Dissolve the ingredients in the water. Adjust the pH of the solution to 8.0 ± 0.2 with 10 M hydrochloric acid. Dispense the resulting solution, typically as 90 ml aliquots, into suitable screw-capped tubes or bottles and sterilise by autoclaving at 121 °C for 15 minutes. Autoclaved media may be stored in the dark at room temperature, for up to one month, if protected from dehydration.

Double-strength Tris-buffered peptone broth can be prepared using double the amounts of ingredients in the 1 litre of water.

A8.2 *Potassium hydroxide-sodium chloride solution*

| | |
|---|---|
| Potassium hydroxide | 500 mg |
| Sodium chloride | 500 mg |
| Water | 100 ml |

Dissolve the ingredients in the water. The solution can be stored in appropriate sized bottles at room temperature.

A8.3 *Cefsulodin irgasan novobiocin agar*[5, 6]

Basal medium:

| | |
|---|---|
| Peptone | 20 g |
| Yeast extract | 2 g |
| D (-) Mannitol | 20 g |
| Sodium pyruvate | 2 g |
| Sodium chloride | 1 g |
| Magnesium sulphate | 10 mg |
| Sodium desoxycholate | 500 mg |
| Neutral red | 30 mg |
| Crystal violet | 1 mg |
| Agar | 12.5 g |
| Water | 1 litre |

Dissolve the ingredients in the water by bringing slowly to the boil. Dispense in appropriate volumes into suitable containers and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, cool to 50 °C.

Antibiotic solution:

| | |
|---|---|
| Cefsulodin | 150 mg |
| Irgasan | 40 mg |
| Novobiocin | 25 mg |
| Ethanol | 20 ml |
| Water | 40 ml |

The ingredients are dissolved in the ethanol and water and made to 60 ml with water. The resulting solution is then filter-sterilised.

Complete medium

To each litre of cooled molten basal medium, add 6 ml of antibiotic solution and mix gently. The pH of the final medium should be 7.4 ± 0.2. Pour the complete medium at 50 °C and pour into sterile Petri dishes. Allow the medium to solidify. The medium may be stored at between 5 ± 3 °C, for up to seven days, if protected from dehydration.

A8.4 *Triple sugar iron agar*[7]

| | |
|---|---|
| Beef extract | 3 g |
| Yeast extract | 3 g |
| Peptone | 20 g |
| Sodium chloride | 5 g |
| Lactose | 10 g |
| Sucrose | 10 g |
| Glucose | 1 g |
| Iron(III) citrate | 300 mg |
| Sodium thiosulphate pentahydrate | 300 mg |
| Phenol red (0.4 % m/v aqueous solution) | 6 ml |
| Agar | 15 g |
| Water | 1 litre |

Dissolve the ingredients (except phenol red) in the water. To achieve this, it will be necessary to heat to boiling. Add the phenol red solution and mix well. Dispense the resulting solution in small volumes (typically, 5 -10 ml) into suitable containers and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored at between 5 ± 3 °C, for up to one month, if protected against dehydration.

A8.5 *Urea broth*

Broth base

| | |
|---|---|
| Peptone | 1.0 g |
| Glucose | 1.0 g |
| Disodium hydrogen phosphate | 1.0 g |
| Potassium dihydrogen phosphate | 800 mg |
| Sodium chloride | 5.0 g |
| Phenol red (0.4 % m/v aqueous solution) | 1.0 ml |
| Water | 1 litre |

Dissolve the ingredients in the water and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution, typically as 95 ml aliquots, into suitable screw-capped bottles and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. The prepared base medium may be

stored in the dark at room temperature, for up to one month.

Prior to use, add 5 ml of an aqueous 40 % m/v filter-sterilised solution of urea to each 95 ml of broth base and aseptically dispense in 2 - 3 ml volumes in sterile containers and cap. The complete medium should be prepared on the day of use.

A8.6 *Filter-aid*[8]

| | |
|---|---|
| Diatomaceous earth | 1 g (approximately) |
| Water | 15 ml |

Weigh out appropriate amounts of filter-aid into suitable bottles, add the water and cap. Sterilise by autoclaving at 121 °C for 15 minutes. The sterilised filter-aid may be stored in the dark at room temperature for up to 12 months.

A8.7 *Other media*

Standard and commercial formulations of other media and reagents used in this method include MacConkey agar, nutrient broth, quarter strength Ringer's solution and maximum recovery diluent.

## A9 Analytical procedure

A9.1 Sample preparation

A9.1.1 *Surface waters and sea water*

Due to the likelihood that, if present, the numbers of *Yersinia* in some surface waters and sea water are likely to be low, for presence absence, a sample volume of at least 1000 ml should be examined.

For the membrane filtration multiple tube technique, typically an 11-tube series can be used, i.e. the membrane filtration of 1 x 500 ml, 5 x 100 ml and 5 x 10 ml of sample. Alternatively, volumes of 500 ml and 100 ml can be filtered and the 10 ml volumes can be added directly to 10 ml volumes (or an equal volume) of double-strength Tris-buffered peptone broth. For a different series, smaller volumes of sample, for example 1 ml, may be appropriate and these can be added directly to 9 ml of single strength Tris-buffered peptone broth. Turbid waters, unsuitable for membrane filtration, may be filtered using filter aid.

A9.1.2 *Treated wastewater*

Treated wastewater may be analysed as described in A9.1.1 although several membranes may be required for presence absence determinations. A sample volume of at least 100 ml may need to be examined.

For the membrane filtration multiple tube technique, the volumes are typically reduced to 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 x 50 ml and 5 x 10 ml volumes can be membrane filtered or added to equal volumes of double-strength Tris-buffered peptone broth. The 5 x 1 ml volumes can be added to 9 ml of single-strength Tris-buffered peptone broth. To

represent smaller volumes of sample, a 1:10 dilution of the sample, for example 1.0 ml of sample diluted with quarter strength Ringer's solution or maximum recovery diluent, may be appropriate and 1 ml of these dilutions can be added directly to 9 ml of single strength Tris-buffered peptone broth.

A9.1.3 *Untreated wastewater*

For presence absence determinations, 100 ml of untreated wastewater may be required, as it may not be possible (owing to turbidity) to process larger volumes by membrane filtration.

For an 11-tube most probable number series, the volumes of untreated wastewater are usually 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 50 ml and 10 ml volumes can be filtered or added to an equal volume of double-strength Tris-buffered peptone broth. The 1 ml volumes can be added to 9 ml of single-strength Tris-buffered peptone broth. To represent smaller volumes, for example 0.1 ml or 0.01 ml volumes of sample, a 1:10 and 1:100 dilution of the sample may be appropriate. The diluted samples (1 ml volumes) can be added to 9 ml of single-strength Tris-buffered peptone broth.

A9.1.4 *Sediment and sand*

Solid material can be dispensed as a single weight for presence absence by weighing, for example 10 g into an appropriate volume (typically 100 ml) of single strength Tris-buffered peptone broth. For the multiple-tube technique, weigh 1 x 50 g, 5 x 10 g and 5 x 1 g into appropriate volumes (typically 1 x 450 ml, 5 x 100 ml and 5 x 10 ml) of single strength Tris-buffered peptone broth. For smaller quantities, for example 100 mg, these can be added directly to 10 ml of single strength Tris-buffered peptone broth.

A9.2 Sample processing

A9.2.1 *Membrane presence absence or filtration-multiple tube technique*

Appropriate volumes of sample are filtered through membrane filters.

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter onto the porous disc of the filter base. If a gridded membrane filter is used, place grid-side upwards. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the water slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and carefully transfer the membrane filter to a tube or bottle containing, typically, 10 - 15 ml of single strength Tris-buffered peptone broth, ensuring that the membrane filter is fully submerged. Record the volume filtered. Other volumes of sample should be similarly treated until all the filters are transferred to the corresponding tubes or bottles of single strength Tris-buffered peptone broth. The largest single volume of sample may require more than one membrane filter and, if so, all filters used for this

volume should be transferred to the same bottle or tube of single strength Tris-buffered peptone broth. Ensure that all membranes are fully submerged.

The funnel can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume of sample is filtered first. For different samples, a fresh pre-sterilised funnel should be taken or remove a funnel from the boiling water bath. Allow the funnel to cool and repeat the filtration process. If funnels are to be re-used, then after filtration of each sample, disinfect the funnel by immersing it in boiling distilled water for at least one minute. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When disinfected funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

A9.2.2 *Filter-aid*

The usual membrane filtration apparatus may be used but with a sterile absorbent pad in place of a membrane filter to act as a supporting base for the filter-aid. An aliquot of filter-aid (typically, 15 ml) should be filtered to form an initial layer on the absorbent pad. A second aliquot (typically, 15 ml) of filter-aid should be mixed with the volume of sample and then filtered. For turbid or dirty waters, additional aliquots of filter-aid may be required. When filtration is complete, remove the funnel carefully and transfer the absorbent pad and filter-aid to single strength Tris-buffered peptone broth. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 100 ml.

For presence absence place the culture vessel into an incubator and incubate. For a most probable number test, re-suspend the filter aid in the single strength Tris-buffered peptone broth and dispense in a multiple-tube most probable number series using 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 ml volumes are inoculated into 9 ml of fresh sterile single strength Tris-buffered peptone broth.

A9.2.3*Direct inoculation*

Where the numbers of *Yersinia* species in the sample are likely to be high, smaller volumes of sample, for example 50 ml and 10 ml can be inoculated directly into an equal volume of double strength Tris-buffered peptone broth. Volumes of 1 ml and subsequent dilutions of the sample can be inoculated directly into 9 ml of single strength Tris-buffered peptone broth.

A9.2.4*Sediment and sand*

Samples of sediment and sand may be analysed by weighing appropriate amounts, for example, a single aliquot of 10 g for presence absence or 1 x 5 g, into 90 ml and 5 x 1 g

and 5 x 0.1 g into 9 ml of single strength Tris-buffered peptone broth for a most probable number series. Larger weights of sample should be weighed into appropriately larger volumes of single strength Tris-buffered peptone broth.

A9.3 *Enrichment and sub-culture to selective agar*

The Tris-buffered peptone broth and membrane filters, and if appropriate filter-aid or sediment and sand, is mixed thoroughly and placed in an incubator and incubated aerobically at 9 °C for up to 2 weeks. After incubation for at least 48 hours, the tubes or bottles are examined for growth (demonstrated by the presence of turbidity in the Tris-buffered peptone broth). Following detection of turbidity or after 2 weeks incubation add 11 ml of potassium hydroxide-sodium chloride solution to each 100 ml of Tris-buffered peptone broth (or pro rata for smaller volumes of Tris-buffered peptone enrichment), mix well and plate out loopfuls of the Tris-buffered peptone broth into Petri dishes of cefsulodin igrasan novobiocin (CIN) agar. Incubate inoculated Petri dishes at 30 °C for 24 hours.

A9.4 *Reading of results*

After incubation, examine the CIN agar Petri dishes for small (1 - 2 mm diameter) typical “bullseye” colonies that are translucent with, or without, a dark-pink centre, occasionally with bile precipitation. These colonies can be regarded as presumptive *Yersinia* species (see Figures A1 and A2).

**Figure A1 Typical colonies of *Yersinia enterocolitica* on CIN agar**

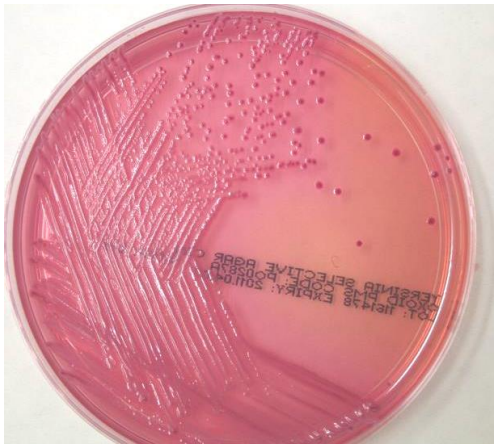
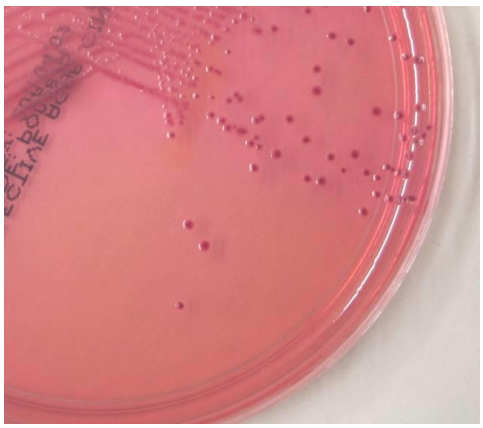


**Figure A2 Close up of typical 'bulls eye' colonies of *Yersinia enterocolitica* on CIN agar**



A9.5 *Confirmation tests*

Sub-culture characteristic colonies to each of the following media:

(i) triple sugar iron agar (smear on slope and spike butt) and incubate at 30 °C for 24 hours;
(ii) urea broth and incubate for at 30 °C for 24 to 72 hours; and
(iii) MacConkey agar and incubate at 30 °C for 24 hours.

Identify the isolates that produce acid (yellow colouration) but not gas (no bubbles in butt) and do not produce hydrogen sulphide (no blackening of medium) on triple sugar iron agar, and are urease-positive in urea broth (change of colour from beige to red) (see Figures A3 and A4).

**Figure A3 *Yersinia enterocolitica* in triple sugar iron with acid slope and butt (yellow with no gas bubbles).**

Front view

Side view

**Figure A4 *Yersinia enterocolitica* in urea broth**

*Yersinia enterocolitica*
Urease positive

*Escherichia coli*
Urease negative

Sub-culture from the MacConkey agar to nutrient broth for motility testing. Incubate the nutrient broth at 37 °C for 4 - 6 hours. Cultures that do not exhibit motility are assumed to be *Yersinia* species as demonstrated by lack of cloudiness in the whole medium. Some *Yersinia* species are motile at temperatures below 28 °C.

Speciation can be performed using commercially available biochemical or serological identification kits, following appropriate verification of performance in the laboratory.

## A10 Calculations

The basic test indicates the presence or absence of *Yersinia* species.

Multiple-tube confirmed *Yersinia* spp. are calculated by reference to the appropriate table in Appendix 1 for the number of tubes or bottles that yield typical *Yersinia* spp. colonies that produce an acid slope and butt in triple sugar iron agar, are urease positive and non-motile.

## A11 Expression of results

For presence-absence determinations, the tests indicate the presence or absence of *Yersinia* species in the volume or weight examined.

For the multiple tube most probable number method the number of Tris buffered peptone broth tubes or bottles of each volume of sample showing a positive reaction is counted, and then by reference to the appropriate tables in Appendix 1, the MPN of *Yersinia* species present in 100 ml of sample is determined. For example, if in a 15-tube test comprising 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample, the number of tubes showing positive reactions in each consecutive series is 3, 2 and 0 respectively, then, from Table 3, the MPN is 13 organisms per 100 ml.

## A12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains

of target bacteria (for example *Yersinia enterocolitica*) and non-target bacteria (*Escherichia coli*). Organisms should be incubated under the appropriate conditions. Further details of required quality control are given elsewhere[(2)] in this series.

## A13 References


1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

4. Comparison of enrichment at 9 °C and 21 °C for recovery of *Yersinia* species from food and milk, *Food Microbiology*, M H Greenwood, 1993, **10**, 23-30.

5. *Yersiniosis: Laboratory diagnosis, Clinical Features and Epidemiology*, London, Public Health Laboratory Service, N S Mair and E Fox, 1986.

6. Development of a two step enrichment procedure for recovery of *Yersinia enterocolitica* from food, *Applied and Environmental Microbiology*, D A Schiemann, 1982, **43**, 14-27.

7. Report of *Enterobacteriaceae* Subcommittee and the Nomenclature Committee of the International Association of Microbiological Societies, *International Bulletin of Bacterial Nomenclature and Taxonomy*, 1958, **8**, 25-70.

8. Concentration technique for demonstrating small amounts of bacteria in tap water, *Acta Pathologica et Microbiologica Scandinavia*, E Hammarstrom and V Ljutov, 1954, **35**, 365-369.

## B The isolation of *Vibrio cholerae* and other V*ibrio* species by selective enrichment, and a multiple tube most probable number technique

### B1 Introduction

*Vibrio* species occur naturally in brackish and saline waters but only a few species are capable of surviving in fresh water ecosystems. Most species, including the pathogenic species, are not, normally, able to grow except under highly eutrophic conditions. *Vibrio* species have been reported in tropical waters where the temperature remains reasonably constant at about 25 °C.

The species *Vibrio cholerae* can be divided into approximately 140 O-serovars. The organisms that usually produce outbreaks of epidemic cholera are toxin-producing strains of the O1 serovar and a more recently reported serovar, O139. Non-O1 *Vibrio cholerae* can also cause gastroenteritis. *Vibrio parahaemolyticus* causes diarrhoea, often through the consumption of raw, contaminated seafood. *Vibrio fluvialis* and *Vibrio mimicus* also cause diarrhoea. Outbreaks of cholera have been reported following consumption of crops irrigated with water contaminated with wastewater. The significance of *Vibrio* species in surface water and other environments are described elsewhere[(1)] in this series.

### B2 Scope

The method is suitable for the examination of surface water both fresh and saline, swimming pools, spa and hydrotherapy pools, primary and secondary wastewater effluents and sediments including sand. Clean water samples may be membrane filtered for either a presence absence or a multiple tube most probable number (MPN) test. Water samples with higher turbidities should be analysed using an appropriate (MPN) method by direct inoculation of material into bottles or tubes. Sediments may be suspended in a suitable diluent or inoculated directly into enrichment broths.

Users wishing to employ this method should verify its performance under their own laboratory conditions[(2)].

### B3 Definitions

For the purposes of this method, strains are identified as *Vibrio* species if organisms show growth at pH 8.6, are oxidase-positive and can grow within 24 hours at 37 °C in the presence of bile salts, 1 % sodium thiosulphate, and 1 % sodium citrate without the production of sulphide. In addition, *Vibrio* species are Gram-negative, can usually ferment glucose, without gas production, and are sensitive to the vibriostatic agent 0129 phosphate (2,4-diamino-6,7-diisopropylpteridine phosphate).

### B4 Principle

Samples are incubated in enrichment broth (alkaline peptone water at 25 °C for 14 hours) followed by sub-culture to thiosulphate citrate bile salt sucrose agar and incubation at 37 °C for 18 - 24 hours for the diagnostic detection of *Vibrio* species by a presence-absence determination or a multiple tube most probable number technique. Characteristic colonies are confirmed by biochemical and, if necessary, serological testing.

## B5 Limitations

The multiple tube most probable number part of this method is labour intensive and may require the preparation of large numbers of tubes or bottles of media and appropriate sub-cultures.

## B6 Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations[3] and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

## B7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators are required. Other items include:

B7.1 Sterile sample bottles of appropriate volume (at least 1 litre), made of suitable material. For swimming pools, spa pools and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

B7.2 Incubators capable of maintaining a temperature of 25 ± 1 °C and 37 ± 1 °C.

B7.3 Filtration apparatus, filter funnels, (either sterilised or capable of being sterilised) and vacuum source.

B7.4 Sterile membrane filters, for example, white 47 mm diameter, cellulose-based 0.45 μm nominal pore size.

B7.5 Smooth-tipped forceps.

## B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in the method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salt. If the pH of the media are not within the stated range, then, before heating, they should be adjusted accordingly. Sterilised media with a pH outside the required range should be discarded. Where media are stored in a refrigerator, they should be allowed to warm to room temperature before use.

B8.1 *Single-strength alkaline peptone water*

| | |
|---|---|
| Peptone | 10 g |
| Sodium chloride | 5 g |
| Water | 1 litre |

Dissolve the ingredients in the water. Adjust the pH of the solution to 8.6 ± 0.2. Dispense the resulting solution (typically, 90 ml) into suitable screw-capped tubes or bottles and sterilise by autoclaving at 121 °C for 15 minutes. Autoclaved media may be stored in the dark at room temperature, for up to one month, if protected from dehydration.

Double-strength alkaline peptone water can be prepared using double the amounts of ingredients in the 1000 ml of water.

B8.2 *Thiosulphate citrate bile salt sucrose (TCBS) agar*[4]

| | |
|---|---|
| Yeast extract | 5 g |
| Peptone | 10 g |
| Sodium thiosulphate pentahydrate | 10 g |
| Sodium citrate | 10 g |
| Ox bile | 8 g |
| Sucrose | 20 g |
| Sodium chloride | 10 g |
| Iron(III) citrate | 1 g |
| Bromothymol blue (1 % m/v aqueous solution) | 4 ml |
| Thymol blue (1 % m/v aqueous solution) | 4 ml |
| Agar | 14 g |
| Water | 1 litre |

Dissolve the ingredients in the water. This will require heating the solution to boiling. Do not autoclave. After boiling, cool the medium to approximately 50 °C and check the pH of the medium to confirm a pH of 8.6 ± 0.2. Pour the medium into sterile Petri dishes and allow the agar to solidify. Prepared Petri dishes can be stored between 5 ± 3 °C and used within one month if protected from dehydration.

B8.3 *Filter-aid*[5]

| | |
|---|---|
| Diatomaceous earth | 1 g (approximately) |
| Water | 15 ml |

Weigh out appropriate amounts of filter-aid into suitable bottles, add the water and cap. Sterilise by autoclaving at 121 °C for 15 minutes. The sterilised filter-aid may be stored in the dark at room temperature for up to 12 months.

B8.4 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar, oxidase reagent, quarter strength Ringer's solution and maximum recovery diluent.

## B9 Analytical procedure

### B9.1 Sample preparation

#### B9.1.1 *Surface waters and sea water*

Due to the likelihood that, if present, the numbers of *Vibrio* species in some surface waters and sea water are likely to be low, for presence absence, a sample volume of at least 1000 ml should be examined.

For the membrane filtration multiple tube technique, typically an 11-tube series can be used, i.e. the membrane filtration of 1 x 500 ml, 5 x 100 ml and 5 x 10 ml of sample. Alternatively, volumes of 500 ml and 100 ml can be filtered and the 10 ml volumes can be added directly to 10 ml volumes (or an equal volume) of double-strength alkaline peptone water. For a different series, smaller volumes of sample, for example 1 ml, may be appropriate and these can be added directly to 9 ml of single strength alkaline peptone water. Turbid waters, unsuitable for membrane filtration, may be filtered using filter aid.

#### B9.1.2 *Treated wastewater*

Treated wastewater may be analysed as described in B9.1.1 although several membranes may be required for presence absence determinations. A sample volume of at least 100 ml may need to be examined.

For the multiple tube technique, the volumes are typically reduced to 1 x 50 ml, 5 x 10 ml and 5 x 1 ml be used. The 50 ml and 10 ml volumes can be membrane filtered and filters added to 10 ml volumes of alkaline peptone water, or volumes of sample can be added directly to equal volumes of double-strength alkaline peptone water. The 5 x 1 ml volumes can be added to 9 ml of single-strength alkaline peptone water. To represent smaller volumes of sample, a 1:10 dilution of the sample, for example 1.0 ml of sample diluted with quarter strength Ringer's solution or maximum recovery diluent, may be appropriate and 1 ml of these dilutions can be added directly to 9 ml of single strength alkaline peptone water.

#### B9.1.3 *Untreated wastewater*

For presence absence determinations, 100 ml of untreated wastewater may be required, as it may not be possible (owing to turbidity) to process larger volumes by membrane filtration.

For an 11-tube most probable number series, the volumes of untreated wastewater are usually 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 50 ml and 10 ml volumes can be filtered or added to an equal volume of double-strength alkaline peptone water. The 1 ml volumes can be added to 9 ml of single-strength alkaline peptone water. To represent smaller volumes, for example 0.1 ml or 0.01 ml volumes of sample, a 1:10 and 1:100 dilution of the sample may be appropriate. The diluted samples (1 ml volumes) can be added to 9 ml of single-strength alkaline peptone water.

B9.1.4 *Sediment and sand*

Solid material can be dispensed as a single weight for presence absence by weighing, for example 10 g into an appropriate volume (typically 100 ml) of single strength alkaline peptone water. For the multiple-tube technique, weigh 1 x 50 g, 5 x 10 g and 5 x 1 g into appropriate volumes (typically 1 x 450 ml, 5 x 100 ml and 5 x 10 ml) of single strength alkaline peptone water. For smaller quantities, for example 100 mg, these can be added directly to 10 ml of single strength alkaline peptone water.

B9.2 Sample processing

B9.2.1 *Membrane presence absence or filtration-multiple tube technique*

Appropriate volumes of sample are filtered through membrane filters.
Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter onto the porous disc of the filter base. If a gridded membrane filter is used, place grid-side upwards. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the water slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and carefully transfer the membrane filter to a tube or bottle containing, typically, 10 - 15 ml of single strength alkaline peptone water, ensuring that the membrane filter is fully submerged. Record the volume filtered. Other volumes of sample should be similarly treated until all the filters are transferred to the corresponding tubes or bottles of single strength alkaline peptone water. The largest single volume of sample may require more than one membrane filter and, if so, all filters used for this volume should be transferred to the same bottle or tube of single strength alkaline peptone water. Ensure that all membranes are fully submerged.

The funnel can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume of sample is filtered first. For different samples, a fresh pre-sterilised funnel should be taken or remove a funnel from the boiling water bath. Allow the funnel to cool and repeat the filtration process. If funnels are to be re-used, then after filtration of each sample, disinfect the funnel by immersing it in boiling distilled water for at least one minute. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When disinfected funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

B9.2.2 *Filter-aid*

The usual membrane filtration apparatus may be used but with a sterile absorbent pad in place of a membrane filter to act as a supporting base for the filter-aid. An aliquot of filter-aid (typically, 15 ml) should be filtered to form an initial layer on the absorbent pad. A second aliquot (typically, 15 ml) of filter-aid should be mixed with the volume of sample and then filtered. For turbid or dirty waters, additional aliquots of filter-aid may be required. When filtration is complete, remove the funnel carefully and transfer the absorbent pad and filter-aid to single strength alkaline peptone water. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 100 ml.

For presence absence, place the culture vessel into an incubator and incubate. For a most probable number test, re-suspend the filter aid in the single strength alkaline peptone water and dispense in a multiple-tube most probable number series using 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 ml volumes are inoculated into 9 ml of fresh sterile single strength alkaline peptone water.

B9.2.3*Direct inoculation*

Where the numbers of *Vibrio* species in the sample are likely to be high, smaller volumes of sample, for example 50 ml and 10 ml can be inoculated directly into an equal volume of double strength alkaline peptone water. Volumes of 1 ml and subsequent dilutions of the sample can be inoculated directly into 9 ml of single strength alkaline peptone water.

B9.2.4*Sediment and sand*

Samples of sediment and sand may be analysed by weighing appropriate amounts, for example, a single aliquot of 10 g for presence absence or 1 x 5 g, into 90 ml and 5 x 1 g and 5 x 0.1 g into 9 ml of single strength alkaline peptone water for a most probable number series. Larger weights of sample should be weighed into appropriately larger volumes of single strength alkaline peptone water.

B9.3 Enrichment and sub-culture to selective agar

The tubes and bottles of alkaline peptone water and membrane filters, and if appropriate filter-aid or sediment and sand, is mixed thoroughly and placed in an incubator and incubated aerobically at 25 °C for 2 hours followed by 37 °C for 12 – 16 hours. After incubation, the tubes or bottles are examined for growth (demonstrated by the presence of turbidity in the alkaline peptone water. All tubes or bottles that exhibit positive growth within the medium are retained and sub-cultured to TCBS agar by plating out loopfuls of alkaline peptone water taken from the top of each positive broth. Inoculated plates are incubated at 37 °C for 21 ± 3 hours.

B9.4 Reading of results

Examine the Petri dishes of TCBS agar for colonies characteristic of *Vibrio* species. These are usually flat colonies 1 - 3 mm in diameter, and either yellow (i.e. sucrose-fermenting) or blue-green (i.e. non-sucrose-fermenting). *Vibrio cholerae*, *Vibrio fluvialis* and *Vibrio metschnikovii* appear as yellow colonies, 2 - 3 mm in diameter, (see Figures

B1 and B2), and *Vibrio mimicus* and *Vibrio parahaemolyticus* appear as blue-green colonies, 2 - 5 mm in diameter with a slightly darker centre, (see Figures B3 and B4). As yellow colonies may be *Vibrio cholerae* appropriate precautions should be taken with confirmations procedures.

**Figure B1 *Vibrio cholerae* (sucrose-fermenting) on TCBS**

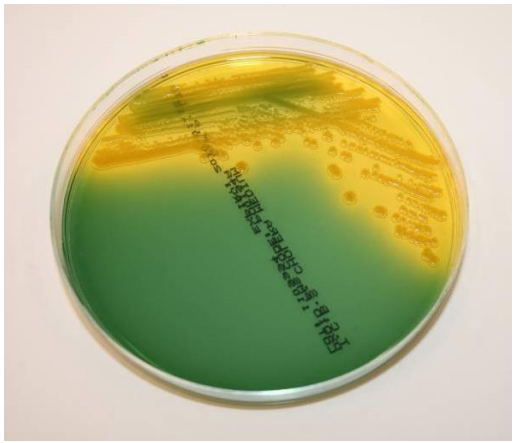


**Figure B2 *Vibrio furnissii* (sucrose-fermenting) on TCBS**

**Figure B3 *Vibrio parahaemolyticus* (non-sucrose-fermenting) on TCBS**

**Figure B4 *Vibrio parahaemolyticus* close up of colonies (non-sucrose-fermenting) on TCBS**

B9.5 Confirmation tests

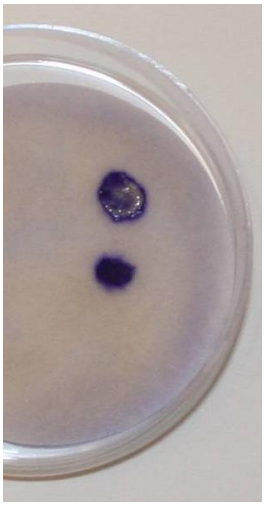
B9.5.1 *Oxidase test*

The oxidase test is carried out with pure cultures of presumptive *Vibrio* from TCBS agar plates subcultured onto nutrient agar (NA) and incubated at 37 C for 21 ± 3 h. Place 2 - 3 drops, typically 0.1 - 0.2 ml, (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from NA onto the treated filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction (see Figure B5).

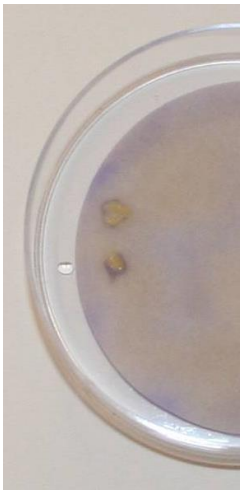
On each occasion where oxidase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example *Pseudomonas aeruginosa*) and one species is known to give a negative reaction (for example *Escherichia. coli*).

**Figure B5 Oxidase test**

*Vibrio cholerae*
oxidase-positive



*Escherichia coli*
oxidase-negative



Commercial test kits for oxidase testing are available and should be used in accordance with manufacturer's instructions and following appropriate performance verification at the laboratory.

B9.5.2 *Biochemical and serological tests*

Strains that are oxidase-positive may require further identification. If epidemic strains of *Vibrio cholerae* O1 are to be characterised, then colonies that are both sucrose-fermenting (i.e. yellow colonies on thiosulphate citrate bile salt sucrose agar) and oxidase-positive can be checked for agglutination with *Vibrio cholerae* O1 antiserum. An isolate that agglutinates can be regarded as presumptive *Vibrio cholerae* O1. Commercially available kits can be used to identify oxidase-positive, sucrose-fermenting and oxidase-positive, non-sucrose-fermenting isolates, following appropriate performance verification in the laboratory.

## B10 Calculations

The basic test indicates the presence or absence of *Vibrio* species.

Multiple-tube confirmed *Vibrio* spp. are calculated by reference to the appropriate table in Appendix 1 for the number of tubes or bottles that yield typical *Vibrio* spp. colonies that are oxidase positive.

## B11 Expression of results

For presence-absence determinations, the tests indicate the presence or absence of *Vibrio* species in the volume or weight examined.

For the multiple tube most probable number method the number of alkaline peptone water tubes or bottles of each volume of sample showing a positive reaction is counted, and

then by reference to the appropriate tables in Appendix 1, the MPN of *Vibrio* species present in 100 ml of sample is determined. For example, if in a 15-tube test comprising 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample, the number of tubes showing positive reactions in each consecutive series is 3, 2 and 0 respectively, then, from Table 3, the MPN is 13 organisms per 100 ml.

## B12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example *Vibrio cholerae* O24) and non-target bacteria (*Escherichia coli*). Organisms should be incubated under the appropriate conditions. Further details are given elsewhere[(2)] in this series.

## B13 References


1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and procedures for laboratories, *Methods for the Examination of Waters and Associated Materials*, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

4. A new selective medium for pathogenic vibrios, TCBS agar (modified Nakanishi's agar). *Japanese Journal of Bacteriology*, T Kobayashi, S Enomoto, R Sakazaki and S Kuwahara, 1963, **18,** 387-411.

5. Concentration technique for demonstrating small amounts of bacteria in tap water. *Acta Pathologica et Microbiologica Scandinavia*, E Hammarstrom and V Ljutov, 1954, **35**, 365-369.

## C The isolation of thermophilic *Campylobacter* species by selective enrichment, and a multiple tube most probable number technique

### C1 Introduction

Thermophilic *Campylobacter* species are not thought to be free living but are obligate parasites of humans, birds and other animals, particularly livestock. The organisms are found in the gastrointestinal system, and being excreted in faeces, can be isolated from wastewater and surface waters.

The thermophilic *Campylobacter* group is recognised as a common cause of enteritis in humans. *Campylobacter* infections give rise to a flu-like illness with malaise, fever and myalgia followed by diarrhoea. The incubation period varies between 1 - 7 days with an average of 3 days. Most cases occur from the consumption of contaminated raw or improperly cooked foods. Outbreaks of campylobacteriosis have been reported in relation to the use of recreational waters and the significance of *Campylobacter* bacteria in surface water and other environments are described elsewhere[1] in this series.

### C2 Scope

The method is suitable for the examination of surface water both fresh and saline, swimming pools, spa and hydrotherapy pools, primary and secondary wastewater effluents and sediments including sand. Clean water samples may be membrane filtered for either a presence absence or a multiple tube most probable number (MPN) test. Water samples with higher turbidities should be analysed using an appropriate (MPN) method by direct inoculation of material into bottles or tubes. Sediments may be suspended in a suitable diluent or inoculated directly into enrichment broths.

Users wishing to employ this method should verify its performance under their own laboratory conditions[2].

### C3 Definitions

In the context of this method, organisms which are Gram-negative, oxidase-positive, motile, form characteristic colonies on *Campylobacter* selective agar media, after culture in *Campylobacter* enrichment broth, and which produce the morphological, physiological and biochemical reactions described are regarded as *Campylobacter* species. These include *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari*. In addition, *Campylobacter* species grow under microaerophilic conditions, but not under aerobic conditions.

### C4 Principle

Samples are incubated in one of two enrichment broths (Bolton broth for clean water and Preston broth for more heavily contaminated water). Broths are incubated micro-aerobically at 37 °C for up to 24 hours, followed by inoculation onto a blood-free selective agar medium. Inoculated plates are incubated under microaerophilic conditions at 41.5 °C for 24 hours. Negative enrichment broths (lack of growth) are also incubated for a further 24 hours. Inoculated plates are examined for characteristic colonies after incubation for 24 hours and any positive enrichment broths may be discarded. Negative enrichment broths

are sub-cultured a second time onto blood-free medium and incubated together with any negative agar plates from the first sub-culture for a further 24 hours at 41.5 °C.

Confirmation of suspect colonies of *Campylobacter* species involves biochemical, morphological and physiological tests.

## C5 Limitations

This method is labour intensive and may require the preparation of large numbers of tubes or bottles of media and appropriate sub-cultures.

Note: The use of filter-aid is not recommended, as concentration of *Campylobacter* species via this procedure is not always effective.

## C6 Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations[3] and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

Extra care should be taken in the isolation and identification of *Campylobacter* species due to the pathogenic nature of the organisms. Staff should be adequately trained and supervised and work involving sub-culture and handling of cultures should be performed in a designated area of a properly equipped laboratory. Adequate facilities should be in place for disposal and sterilisation of test materials.

## C7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators are required. Other items include:

C7.1 Sterile sample bottles of appropriate volume (at least 1 litre), made of suitable material. For swimming pools, spa pools and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

C7.2 Incubators capable of maintaining a temperature of 37 ± 1 °C and 41.5 ± 1 °C.

C7.3 Filtration apparatus, filter funnels, (either sterilised or capable of being sterilised) and vacuum source.

C7.4 Sterile membrane filters, for example, white 47 mm diameter, cellulose-based 0.2 μm nominal pore size.

C7.5 Smooth-tipped forceps.

C7.6 Microaerophilic incubation jars, or equivalent and suitable atmosphere generating packs.

C7.7 Microscope capable of phase or differential interference contrast and/or dark ground illumination (optional).

## C8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in the method[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salt. If the pH of the media are not within the stated range, then, before heating, they should be adjusted accordingly. Sterilised media with a pH outside the required range should be discarded. Where media are stored in a refrigerator, they should be allowed to warm to room temperature before use.

C8.1 *Single-strength Campylobacter enrichment broth (Preston broth)*[4]

C8.1.1 *Broth base*

| | |
|---|---|
| Nutrient broth number 2 | 25 g |
| Iron(II) sulphate heptahydrate | 250 mg |
| Sodium metabisulphite | 250 mg |
| Sodium pyruvate | 250 mg |
| Trimethoprim | 10 mg |
| Polymixin | 5000 iu |
| Rifampicin | 10 mg |
| Amphotericin B | 10 mg |
| Lysed horse blood | 50 ml |
| Water | 950 ml |

Dissolve the nutrient broth number 2 in the water. Adjust the pH of the solution to 7.0 ± 0.2. Dispense the resulting solution (typically, 90 ml) into suitable screw-capped tubes or bottles and sterilise by autoclaving at 121 °C for 15 minutes. Autoclaved media may be stored in the dark at room temperature, for up to one month, if protected from dehydration.

Powdered charcoal added to the medium, prior to sterilisation, at a final concentration of 20 g/l, may improve recovery of *Campylobacter* [5].

Prior to use, add the lysed horse blood (C8.1.2), iron(II) sulphate, sodium metabisuphite and sodium pyruvate (C8.1.3). Antibiotic solutions (C8.1.4) are added after inoculation of the broth with the sample.

C8.1.2 *Blood supplement*

Add lysed horse blood to give a final concentration of 5 % (50 ml per litre). Lysed horse blood may be obtained commercially, but de-fibrinated horse blood can also be used. De-

fibrinated horse blood may be lysed by dispensing suitable volumes of horse blood into sterile containers which are then frozen. Blood, lysed in this way, can be kept frozen until required. The blood is thawed by warming to room temperature before use.

C8.1.3*Growth supplement*

Dissolve 1.25 g of iron(II) sulphate heptahydrate, 1.25 g of sodium metabisulphite and 1.25 g of sodium pyruvate in 20 ml of water. Add 4 ml of this filter-sterilised solution to 1 litre of medium to give a final concentration of each substance of 250 mg/l.

C8.1.4*Antibiotic solutions*

These solutions should be filter-sterilised and should be added to enrichment broth following inoculation with the sample.

(i) Trimethoprim solution: Dissolve 500 mg of trimethoprim lactate in 100 ml of water and add 2 ml of this filter-sterilised solution to 1 litre of the medium to give a final concentration of 10 mg/l.

(ii) Polymixin solution: Dissolve 500000 iu of polymixin B sulphate in 40 ml of water and add 0.4 ml of this filter-sterilised solution to 1 litre of the medium to give a final concentration of 5000 iu/l.

(iii) Rifampicin solution: Dissolve 200 mg in 20 ml of methanol and add 1 ml of this filter-sterilised solution to 1 litre of the medium to give a final concentration of 10 mg/l.

(iv) Amphotericin B solution: Dissolve 0.1 g in 10 ml of water and add 1 ml of this filter-sterilised solution to the 1 litre of medium to give a final concentration of 10 mg/l.

Dispense the fully prepared medium (typically, 90 ml) in screw capped bottles. Use on the day of preparation.

Antibiotic supplements and growth supplements are available commercially and should be prepared according to the manufacturer's instructions.

C8.1.5 *Double-strength Preston broth*

Double-strength Preston broth can be prepared using double the amounts of ingredients in the 900 ml of water.

C8.2 *Single-strength Bolton enrichment broth*[(6)]

C8.2.1*Broth base*

| | |
|---|---|
| Meat peptone | 10 g |
| Lactablumin hydrolysate | 5 g |
| Yeast extract | 5 g |
| Sodium chloride | 5 g |
| Alpha-ketoglutaric acid | 1 g |
| Sodium pyruvate | 0.5 g |

| | |
|---|---|
| Sodium metabisulphite | 0.5 g |
| Sodium carbonate | 0.6 g |
| Haemin | 10 mg |
| Cefoperazone | 20 mg |
| Vancomycin | 20 mg |
| Trimethoprim | 20 mg |
| Amphoteracin B | 10 mg |
| Lysed horse blood | 50 ml |
| Water | 950 ml |

Dissolve the peptone, lactalbumin hydrolysate, yeast extract, sodium chloride, alpha-ketoglutarate, sodium carbonate and haemin in 950 ml of water and adjust the pH to 7.4 ± 0.2. Dispense the resulting solution in appropriate volumes (typically 90 ml into suitable containers and sterilise by autoclaving at 121 °C for 15 minutes. Autoclaved media may be stored in the dark at room temperature, for up to one month, if protected from dehydration.

Prior to use, add the lysed horse blood (C8.2.2), sodium metabisuphite and sodium pyruvate (C8.2.3). Antibiotic solutions (C8.2.4) are added after inoculation of the broth with the sample.

Powdered charcoal added to the medium, prior to sterilisation, at a final concentration of 20 g/l, may improve recovery of *Campylobacter* [5].

C8.2.2*Blood supplement*

Add lysed horse blood to give a final concentration of 5 % (50 ml per litre). Lysed horse blood may be obtained commercially, but de-fibrinated horse blood can also be used. De-fibrinated horse blood may be lysed by dispensing suitable volumes of horse blood into sterile containers which are then frozen. Blood, lysed in this way, can be kept frozen until required. The blood is thawed by warming to room temperature before use.

C8.2.3*Growth supplement*

Dissolve 2.50 g of sodium metabisulphite and 2.50 g of sodium pyruvate in 20 ml of water. Add 4 ml of this filter-sterilised solution to 1 litre of medium to give a final concentration of each substance of 500 mg/l.

C8.2.4*Antibiotic solutions*

These solutions should be filter-sterilised and should be added to enrichment broth following inoculation with the sample.

(i) Cefoperazone solution: Dissolve 2.0 g of cefoperazone in 100 ml of water. Add 1 ml of this filter-sterilised solution to 1 litre of the cooled agar medium to give a final concentration of 20 mg/l.

(ii) Vancomycin solution: Dissolve 2.0 g of cefoperazone in 100 ml of distilled, deionised or similar grade water. Add 1 ml of this filter-sterilised solution to 1 litre of the cooled agar medium to give a final concentration of 20 mg/l.

(iii) Trimethoprim solution: Trimethoprim solution: Dissolve 500 mg of trimethoprim lactate in 100 ml of water and add 4 ml of this filter-sterilised solution to 1 litre of the medium to give a final concentration of 20 mg/l.

(iv) Amphotericin B solution: Dissolve 0.1 g in 10 ml of water and add 1 ml of this filter-sterilised solution to the 1 litre of medium to give a final concentration of 10 mg/l.

Antibiotic supplements and growth supplements are available commercially and should be prepared according to the manufacturer's instructions.

C8.2.5 *Double-strength Bolton broth*

Double-strength Bolton broth can be prepared using double the amounts of ingredients in the 900 ml of water.

C8.3 *Campylobacter selective agar*[7, 8]

| | |
|---|---|
| Nutrient broth No 2 | 25 g |
| Bacteriological charcoal | 4 g |
| Casein hydrolysate | 3 g |
| Sodium desoxycholate | 1 g |
| Iron(II) sulphate | 250 mg |
| Sodium pyruvate | 250 mg |
| Cefoperazone | 32 mg |
| Amphotericin B | 10 mg |
| Agar | 12 g |
| Water | 1 litre |

Dissolve the ingredients, except the cefoperazone and amphotericin B, in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable containers and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Cool the medium to approximately 50 °C.

Antibiotic solutions

(i) Cefoperazone solution: Dissolve 3.2 g cefoperazone in 100 ml of water. Add 1 ml of this filter-sterilised solution to 1 litre of the cooled agar medium to give a final concentration of 32 mg/l.

(ii) Amphotericin B solution: Dissolve 0.1 g amphotericin B in 10 ml of water. Add 1 ml of this filter-sterilised solution to 1 litre of the cooled agar medium to give a final concentration of 10 mg/l.

Pour the medium into sterile Petri dishes and allow the agar to solidify. Prepared Petri dishes can be stored between 5 ± 3 °C and used within one week if protected from dehydration. Alternatively, the bottled medium (without the antibiotic supplements) can be stored in the dark at room temperature and used within one month.

C8.4 *Other media*

Standard and commercial formulations of other media and reagents used in this method include blood-free agar, brain heart infusion broth, oxidase reagent, quarter strength Ringer's solution, maximum recovery diluent and Gram stain reagents.

## C9 Analytical procedure

C9.1 Sample preparation

C9.1.1 *Surface waters and sea water*

Due to the likelihood that, if present, the numbers of *Campylobacter* species in some surface waters and sea water are likely to be low, for presence absence, a sample volume of at least 1000 ml should be examined.

For the membrane filtration multiple tube technique, typically an 11-tube series can be used, i.e. the membrane filtration of 1 x 500 ml, 5 x 100 ml and 5 x 10 ml of sample. Alternatively, volumes of 500 ml and 100 ml can be filtered and the filters added to 10 ml volumes of Preston or Bolton broth and the 10 ml volumes can be added directly to 10 ml volumes of double-strength Preston or Bolton broth. For a different series, smaller volumes of sample, for example 1 ml, may be appropriate and these can be added directly to 9 ml of single strength Preston or Bolton broth. Turbid waters, unsuitable for membrane filtration, should be added directly to double or single-strength Preston broth.

C9.1.2 *Treated wastewater*

Treated wastewater may be analysed as described in C9.1.1 although several membranes may be required for presence absence determinations. A sample volume of at least 100 ml may need to be examined.

For the multiple tube technique, the volumes are typically reduced to 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 50 ml and 10 ml volumes can be membrane filtered or added to equal volumes of double-strength Preston broth. To represent smaller volumes of sample, a 1:10 dilution of the sample, for example 1.0 ml of sample diluted with quarter strength Ringer's solution or maximum recovery diluent, may be appropriate and 1 ml of these dilutions can be added directly to 9 ml of single-strength Preston broth.

C9.1.3 *Untreated wastewater*

For presence absence determinations, 100 ml of untreated wastewater may be required, as it may not be possible (owing to turbidity) to process larger volumes by membrane filtration.

For an 11-tube most probable number series, the volumes of untreated wastewater are usually 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 x 50 ml and 5 x 10 ml volumes can be membrane filtered or added to an equal volume of double-strength Preston broth. The 5 x 1 ml volumes can be added to 9 ml of single-strength Preston broth. To represent smaller volumes, for example 0.1 ml or 0.01 ml volumes of sample, a 1:10 and 1:100 dilution of the sample may be appropriate. The diluted samples (1 ml volumes) can be added to 9 ml

of single-strength Preston broth.

C9.1.4*Sediment and sand*

Solid material can be dispensed as a single weight for presence absence by weighing, for example 10 g into an appropriate volume (typically 100 ml) of single strength Preston broth. For the multiple-tube technique, weigh 1 x 50 g, 5 x 10 g and 5 x 1 g into appropriate volumes (typically 1 x 450 ml, 5 x 100 ml and 5 x 10 ml) of single strength Preston or Bolton broth. For smaller quantities, for example 100 mg, these can be added directly to 10 ml of single strength Preston or Bolton broth.

C9.2 Sample processing

C9.2.1*Membrane presence absence or filtration-multiple tube technique*

Appropriate volumes of sample are filtered through membrane filters. Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter onto the porous disc of the filter base. If a gridded membrane filter is used, place grid-side upwards. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the water slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and carefully transfer the membrane filter to a tube or bottle containing, typically, 10 - 15 ml of single strength Preston or Bolton broth, ensuring that the membrane filter is fully submerged. Record the volume filtered. Other volumes of sample should be similarly treated until all the filters are transferred to the corresponding tubes or bottles of single strength Preston or Bolton broth. The largest single volume of sample may require more than one membrane filter and, if so, all filters used for this volume should be transferred to the same bottle or tube of single strength Preston or Bolton broth. Ensure that all membranes are fully submerged.

The funnel can be placed in a boiling water bath if it is to be re-used. Alternatively, pre-sterilised filter funnels can be used for each sample. If different volumes of the same sample are to be examined, the funnel may be re-used without boiling provided that the smallest volume of sample is filtered first. For different samples, a fresh pre-sterilised funnel should be taken or remove a funnel from the boiling water bath. Allow the funnel to cool and repeat the filtration process. If funnels are to be re-used, then after filtration of each sample, disinfect the funnel by immersing it in boiling distilled water for at least one minute. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When disinfected funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible, and no longer than 2 hours.

C9.2.2*Direct inoculation*

Where the numbers of *Campylobacter* species in the sample are likely to be high, smaller volumes of sample, for example 50 ml and 10 ml can be inoculated directly into an equal volume of double strength Preston or Bolton broth. Volumes of 1 ml and subsequent dilutions of the sample can be inoculated directly into 9 ml of single strength Preston or Bolton broth.

C9.2.3*Sediment and sand*

Samples of sediment and sand may be analysed by weighing appropriate amounts, for example, a single aliquot of 10 g for presence absence or 1 x 5 g, into 90 ml and 5 x 1 g and 5 x 0.1 g into 9 ml of single strength Preston or Bolton broth for a most probable number series. Larger weights of sample should be weighed into appropriately larger volumes of single-strength Preston or Bolton broth.

C9.3 *Enrichment and sub-culture to selective agar*

The *Campylobacter* enrichment broth and membrane filters, and if appropriate, sediment and sand, is mixed thoroughly and placed in an incubator and incubated in a micro-aerobic atmosphere at 37 °C for 21 ± 3 hours. After incubation, each tube or bottle is sub-cultured to *Campylobacter* selective agar by plating out loopfuls of *Campylobacter* enrichment broth taken from just below the surface of each broth. Inoculated plates are incubated in a micro-aerobic atmosphere at 41.5 °C for 21 ± 3 hours. The micro-aerobic atmosphere should contain approximately 5 - 6% of oxygen, 10% of carbon dioxide and 84 - 85% of nitrogen. This may be achieved using commercially available gas generating kits. It is important that the kit used is appropriate to the volume of the jar.

Re-incubate the inoculated *Campylobacter* enrichment broth in a micro-aerobic atmosphere at 37 $\pm$ 1 °C for a further 21 ± 3 hours. After incubation, sub-culture loopfuls of *Campylobacter* enrichment broth to Petri dishes containing *Campylobacter* selective agar. Draw the inoculum from just below the surface of the enrichment broth. Incubate the Petri dishes micro-aerobically at 41.5 $\pm$ 1 °C for 21 ± 3 hours.

Examine the Petri dishes after 21 ± 3 hours incubation. Those showing no growth should be incubated for a further 21 ± 3 hours. All enrichment broths which are positive may be discarded. Negative enrichment broths should be sub-cultured onto fresh Petri dishes of *Campylobacter* selective agar and incubated as above.

C9.4 *Reading of results*

After incubation, examine the Petri dishes for *Campylobacter* colonies. Preliminary identification may be made on the basis of colonial morphology and Gram stain. Colonies of *Campylobacter* on the selective agar may vary in size from 0.05 - 4 mm in diameter. The size will depend, to some extent, on the number of competing organisms growing on the agar. Large numbers of *Proteus* or *Pseudomonas* can grow on the selective agar and may restrict the growth of *Campylobacter* and, under such circumstances, careful examination of each Petri dish, for example with a hand lens or microscope, is important.

Colonies of *Campylobacter* are typically small, flat and transparent, and may be circular or ovoid, and may extend along the line of inoculation (see Figures C1 and C2). They resemble small flat droplets of water. Where competing organisms are minimal, or absent, *Campylobacter* colonies are slightly convex, with an entire edge, and again, may extend along the line of inoculation. They are grey or buff in colour and may also exhibit swarming. Occasionally, two colony types occur in one culture. One is, typically, slightly convex as above, and the other is smaller and more dome-shaped. On moist plates, *Campylobacter* may spread as a thin film (see Figure C3).

**Figure C1 *Campylobacter* species colonies on *Campylobacter* selective agar from wastewater**

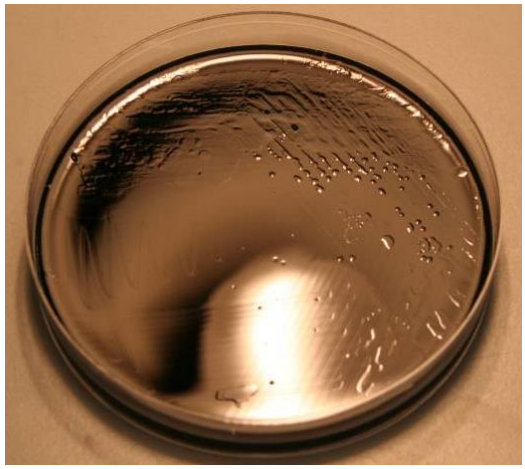


**Figure C2 *Campylobacter* species colonies from wastewater on *Campylobacter* selective agar**

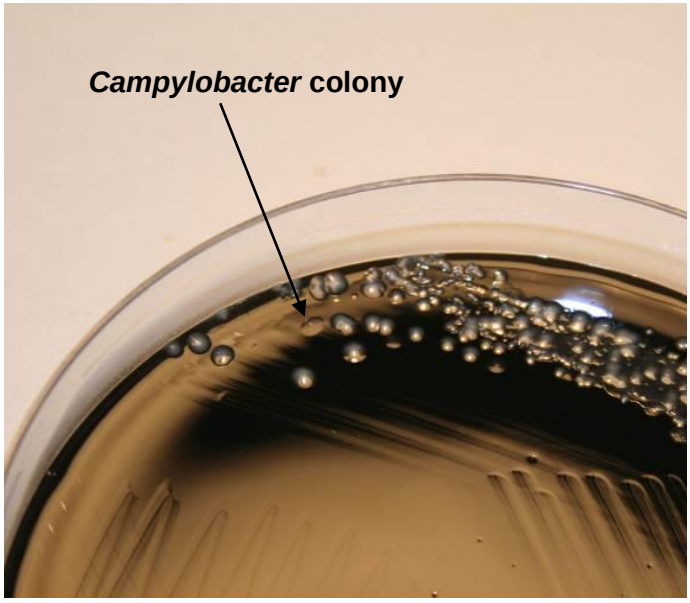


**Figure C3 Spreading colonies of *Campylobacter* spp. from wastewater on *Campylobacter* selective agar**

C9.5 Confirmation tests

C9.5.1*Micro-aerobic growth*

Sub-culture suspect colonies to two Petri dishes of a blood-free and antibiotic-free *Campylobacter* selective agar, or similar blood-free agar. Incubate one plate in an micro-aerobic atmoshpere and one plate aerobically at 37 °C for 24 hours. *Campylobacter* should grow in the modified atmosphere but not aerobically (see Figure C4).

**Figure C4 Growth of *Campylobacter* isolates on antibiotic-free *Campylobacter* selective agar**

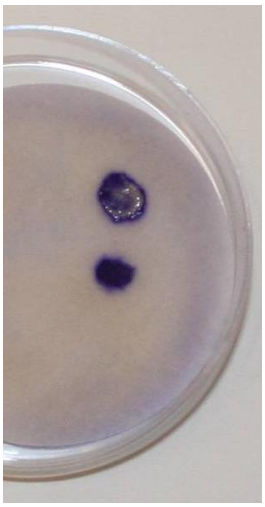
Incubated microaerophilically (growth) Incubated aerobically (no growth)

C.9.5.2 *Oxidase test*

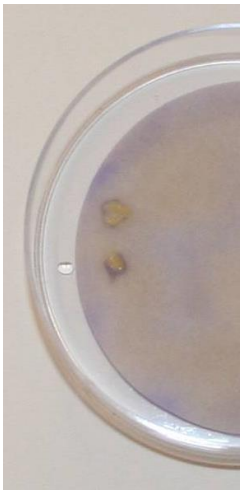
Using the micro-aerobic blood-free plate from 9.5.1, test the growth for oxidase. Place 2 - 3 drops, typically 0.1 - 0.2 ml, (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the micro-aerobic plate inoculated in C9.5.1 onto the treated filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction (see Figure C5).

**Figure C5 Oxidase test**

*Campylobacter* spp.
oxidase-positive



*Escherichia coli*
oxidase-negative



Commercial test kits for oxidase testing are available and should be used in accordance with the manufacturer's instructions and following appropriate performance verification in the laboratory.

On each occasion that oxidase reagent is used, conduct control tests with organisms of which one species is known to give a positive reaction (for example, *Pseudomonas aeruginosa*) and one species is known to give a negative reaction (for example, *Escherichia coli*).

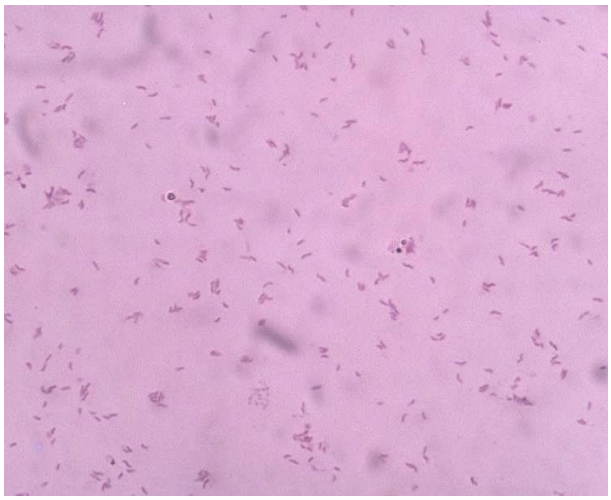
C9.5.3 *Cell morphology and motility*

Prepare a wet preparation from the micro-aerobic plate by emulsifying some of the suspect colony in a drop of brain heart infusion broth on a slide. Cover with a cover-slip and examine immediately, using either phase contrast or dark ground microscopy taking care when handling. *Campylobacter* species are highly motile slender rods with spiral morphology. Motility is characterised by darting or corkscrew like movements. Motility can also be determined by means of the hanging drop method and light microscopy.

C9.5.4G*ram stain*

To confirm cell morphology carry out a Gram-stain test on oxidase-positive cultures using either 1:20 carbol fuchsin or 0.5 % m/v safranin solutions as counter-stains. *Campylobacter* species are curved rods with typical “gull’s wings”, S-shapes or short spirals (see Figure C6).

**Figure C6 Gram stain of presumptive *Campylobacter* isolate**



An isolate which is oxidase-positive and Gram-negative, possesses S- or spiral-shaped cell morphology, shows characteristic motility and grows under micro-aerobic conditions (but not aerobic conditions) is confirmed as *Campylobacter* species.

If required, species and sub-species identification may be carried out, following appropriate performance verification in the laboratory, using biotyping schemes[9, 10, 11].

## C10 Calculations

The basic test indicates the presence or absence of *Campylobacter* species.

Multiple-tube confirmed *Campylobacter* spp. are calculated by reference to the appropriate table in Appendix 1 for the number of tubes or bottles that yield typical *Campylobacter* spp. colonies that grow under microaerophilic conditions, are oxidase positive and demonstrate typical morphology and motility.

## C11 Expression of results

For presence-absence determinations, the tests indicate the presence or absence of *Campylobacter* species in the volume or weight examined.

For the multiple tube most probable number method the number of Preston or Bolton bottles of each volume of sample showing a positive reaction is counted, and then by reference to the appropriate tables in Appendix 1, the MPN of *Campylobacter* species present in 100 ml of sample is determined. For example, if in a 15-tube test comprising 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample, the number of tubes showing positive

reactions in each consecutive series is 3, 2 and 0 respectively, then, from Table 3, the MPN is 13 organisms per 100 ml.

## C12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *Campylobacter jejuni*) and non-target bacteria (*Escherichia coli* or *Pseudomonas aeruginosa*). Organisms should be incubated under the appropriate conditions. Further details are given elsewhere[(2)] in this series.

## C13 References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## Appendix 1 Tables of most probable numbers

From the various combinations of positive and negative reactions for the different volumes examined, the following tables indicate the MPN of bacteria in 100 ml of sample. It is important to realise that the MPN is only an estimate, based on statistical probabilities and that the actual number may lie within a range of values. Approximate 95 % confidence intervals, which demonstrate the range of possible numbers (the MPR) which could yield the number of positive reactions, have been published[1]. A procedure for estimating these confidence intervals for other dilution series has also been published[2]. These confidence intervals are seldom of practical use when reporting results because they apply to the accuracy of the method and not the likely variability of organisms at the sampling source[3]. The MPR in tables 1 - 3 illustrates those situations where the method becomes relatively imprecise, particularly when nearly all the tubes show growth within the medium. In these situations, further dilutions should have been prepared and added to tubes of medium.

Table 1 gives the MPN (and where applicable the MPR) for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample. Similarly table 2 gives the MPN (and where applicable the MPR) for an 11-tube series comprising 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample. Table 3 shows data for a 15-tube series of 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of samples but gives only those values of the more likely combinations of positive and negative reactions. For example, positive reactions in the 0.1 ml tubes would not be expected if all of the 10 ml and 1 ml tubes were negative. Hence, MPN and MPR values for a combination of results like for instance 0, 0, 2 etc are not tabulated. If these unlikely combinations are observed in practice with greater than expected frequencies, then this might indicate that the statistical assumptions underlying the MPN estimation are not correct[1, 4, 5]. For example, the organisms may not have been uniformly distributed throughout the sample, or toxic substances may have been present.

### Calculation of MPN

The number of positive reactions for each set of tubes is recorded and, from the relevant table, the MPN of organisms present in 100 ml of the sample is determined.

Where a series of dilutions of the sample is used, then the following rules should be applied, as illustrated by the numbers in bold, underlined, italic type in table 4.

(i) Use only three consecutive sets of dilutions for calculating the MPN.

(ii) Wherever possible, select three consecutive dilutions where the results are neither all positive nor all negative. The most efficient statistical estimate will result when about half the tubes are positive (see examples (a), (b) and (c) in table 4).

(iii) If less than three sets of dilutions give positive results, begin with the set containing the largest volume of sample (see example (d) in table 4).

(iv) If only one set of tubes gives a positive reaction, use this dilution and the one higher and one lower (see example (e) in table 4).

**Table 1 MPN and MPR per 100 ml of sample for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample**

| Number of tubes giving a positive reaction | | MPN per 100 ml | MPR* per 100 ml |
|---|---|---|---|
| 1 x 50 ml | 5 x 10 ml | | |
| 0 | 0 | None found | |
| 0 | 1 | 1 | |
| 0 | 2 | 2 | |
| 0 | 3 | 3 | |
| 0 | 4 | 4 | 4-5 |
| 0 | 5 | 6 | |
| 1 | 0 | 1 | |
| 1 | 1 | 2 | |
| 1 | 2 | 5 | 4-5 |
| 1 | 3 | 9 | 8-10 |
| 1 | 4 | 15 | 13-18 |
| 1 | 5 | >18** | |

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 18.

**Table 2 MPN and MPR per 100 ml of sample for an 11-tube series of 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample**

| Number of tubes giving a positive reaction | | | MPN per 100 ml | MPR* per 100 ml |
|---|---|---|---|---|
| 1 x 50 ml | 5 x 10 ml | 5 x 1 ml | | |
| 0 | 0 | 0 | None found | |
| 0 | 0 | 1 | 1 | |
| 0 | 1 | 0 | 1 | |
| 0 | 1 | 1 | 2 | |
| 0 | 2 | 0 | 2 | |
| 0 | 2 | 1 | 3 | |
| 0 | 3 | 0 | 3 | |
| 1 | 0 | 0 | 1 | |
| 1 | 0 | 1 | 2 | |
| 1 | 1 | 0 | 2 | |
| 1 | 1 | 1 | 4 | |
| 1 | 1 | 2 | 6 | |
| 1 | 2 | 0 | 4 | 4-5 |
| 1 | 2 | 1 | 7 | 6-7 |
| 1 | 2 | 2 | 9 | 9-10 |
| 1 | 3 | 0 | 8 | 7-9 |
| 1 | 3 | 1 | 10 | 10-11 |
| 1 | 3 | 2 | 13 | 12-13 |
| 1 | 3 | 3 | 17 | 15-18 |
| 1 | 4 | 0 | 12 | 11-14 |
| 1 | 4 | 1 | 16 | 15-19 |
| 1 | 4 | 2 | 21 | 19-24 |
| 1 | 4 | 3 | 27 | 24-30 |
| 1 | 4 | 4 | 33 | 30-38 |
| 1 | 5 | 0 | 23 | 20-27 |
| 1 | 5 | 1 | 33 | 29-40 |
| 1 | 5 | 2 | 53 | 44-65 |
| 1 | 5 | 3 | 91 | 75-110 |
| 1 | 5 | 4 | 160 | 134-190 |
| 1 | 5 | 5 | >180** | |

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 180.

## Table 3 MPN and MPR per 100 ml of sample for a 15-tube series containing 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample

| Number of tubes giving a positive reaction | | | MPN per 100 ml | MPR* per 100 ml |
|---|---|---|---|---|
| 5 x 10 ml | 5 x 1 ml | 5 x 0.1 ml | | |
| 0 | 0 | 0 | None found | |
| 0 | 0 | 1 | 2 | |
| 0 | 1 | 0 | 2 | |
| 1 | 0 | 0 | 2 | |
| 1 | 0 | 1 | 4 | |
| 1 | 1 | 0 | 4 | |
| 2 | 2 | 0 | 4 | |
| 2 | 0 | 1 | 5 | |
| 2 | 1 | 0 | 5 | |
| 2 | 1 | 1 | 7 | |
| 2 | 2 | 0 | 7 | 7-9 |
| 2 | 3 | 0 | 11 | |
| 3 | 0 | 0 | 7 | |
| 3 | 0 | 1 | 9 | |
| 3 | 1 | 0 | 9 | |
| 3 | 1 | 1 | 13 | |
| 3 | 2 | 0 | 13 | |
| 3 | 2 | 1 | 16 | 14-16 |
| 3 | 3 | 0 | 16 | 14-16 |
| 4 | 0 | 0 | 11 | 11-13 |
| 4 | 0 | 1 | 14 | 14-16 |
| 4 | 1 | 0 | 16 | 14-16 |
| 4 | 1 | 1 | 20 | 18-20 |
| 4 | 2 | 0 | 20 | 18-22 |
| 4 | 2 | 1 | 25 | 23-27 |
| 4 | 3 | 0 | 25 | 23-27 |
| 4 | 3 | 1 | 31 | 29-34 |
| 4 | 4 | 0 | 32 | 29-34 |
| 4 | 4 | 1 | 38 | 34-41 |
| 5 | 0 | 0 | 22 | 20-23 |
| 5 | 0 | 1 | 29 | 25-34 |
| 5 | 0 | 2 | 41 | 36-50 |
| 5 | 1 | 0 | 31 | 27-36 |
| 5 | 1 | 1 | 43 | 36-50 |
| 5 | 1 | 2 | 60 | 50-70 |
| 5 | 1 | 3 | 85 | 70-95 |
| 5 | 2 | 0 | 50 | 40-55 |
| 5 | 2 | 1 | 70 | 60-80 |
| 5 | 2 | 2 | 95 | 80-110 |
| 5 | 2 | 3 | 120 | 105-135 |
| 5 | 3 | 0 | 75 | 65-90 |
| 5 | 3 | 1 | 110 | 90-125 |
| 5 | 3 | 2 | 140 | 120-160 |
| 5 | 3 | 3 | 175 | 155-200 |
| 5 | 3 | 4 | 210 | 185-240 |
| 5 | 4 | 0 | 130 | 110-150 |
| 5 | 4 | 1 | 170 | 150-200 |
| 5 | 4 | 2 | 220 | 190-250 |
| 5 | 4 | 3 | 280 | 240-320 |
| 5 | 4 | 4 | 345 | 300-390 |
| 5 | 5 | 0 | 240 | 200-280 |
| 5 | 5 | 1 | 350 | 290-420 |
| 5 | 5 | 2 | 540 | 450-600 |
| 5 | 5 | 3 | 910 | 750-1100 |
| 5 | 5 | 4 | 1600 | 1350-1900 |
| 5 | 5 | 5 | >1800** | |

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 1800.

**Table 4 Examples of the derivation of the MPN from the numbers of positive reactions in a series of dilutions***

| Example in text | Volume of sample (ml) | | | | | MPN per 100 ml |
|---|---|---|---|---|---|---|
| | 10 | 1 | 0.1 | 0.01 | 0.001 | |
| (a) | ***5*** | ***3*** | ***2*** | 0 | | 140 |
| (b) | 5 | ***5*** | ***3*** | ***2*** | 0 | 1400 |
| (c) | 5 | ***5*** | ***2*** | ***0*** | 0 | 500 |
| (d) | ***3*** | ***1*** | ***0*** | 0 | | 9 |
| (e) | ***0*** | ***1*** | ***0*** | 0 | | 2 |

* Numbers in bold, underlined, italic type indicate which results should be used in determining the MPN.

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