ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2002) - Part 10 - Methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment

Methods for the Examination of Waters and Associated Materials

The Microbiology of Drinking Water (2002) - Part 10 - Methods for the isolation of *Yersinia, Vibrio* and *Campylobacter* by selective enrichment

Methods for the Examination of Waters and Associated Materials

This booklet contains three membrane filtration methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

Within this series there are separate booklets dealing with different topics concerning the microbiology of drinking water. Other booklets include

Part 1 - Water quality and public health
Part 2 - Practices and procedures for sampling
Part 3 - Practices and procedures for laboratories
Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)
Part 5 - A method for the isolation and enumeration of enterococci by membrane filtration
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 7 - Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube most probable number techniques

Whilst specific commercial products may be referred to in this document this does not constitute an endorsement of these particular materials. Other similar materials may be suitable and all should be confirmed as such 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 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 Environment Agency. At present, there are nine 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 and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological methods
8 Biodegradability and inhibition methods
9 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 main 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. An index of methods is available from the Secretary.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary

January 2002

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 1999 (SI 1999/437). 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 publications are; "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 "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

A The isolation of *Yersinia* species by selective enrichment

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 water treatment and supply are described elsewhere[1] in this series.

A2 Scope

The method is suitable for the examination of drinking waters including samples from all stages of treatment and distribution, and those source waters of moderate turbidity.

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 agar and then give positive reactions with triple sugar iron 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 (although some species are motile at temperatures below 28 °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

Bacteria are concentrated on membrane filters, followed by incubation and alkali treatment of the enrichment broth. This is followed by subculture to cefsulodin irgasan novobiocin agar. Characteristic colonies are confirmed by biochemical and serological tests.

A5 Limitations

This method is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter. This will limit the volume of sample that can be filtered. In such instances, the use of several membrane filters is recommended. When low numbers of organisms are present, detection is dependent only on the volume of sample that can be filtered and tested. High numbers of competing organisms may inhibit the growth, or detection, of target organisms.

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, made of suitable material, containing 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 $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

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

A7.3 Filtration apparatus, sterile or sterilisable filter funnels, and sources of vacuum.

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. Variations in the preparation and storage of media should also be verified.

A8.1 *Tris-buffered peptone*[4]

Peptone	10.0 g
Tris-(hydroxymethyl)aminomethane	12.1 g
Sodium chloride	5.0 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water. Adjust the pH of the solution to 8.0 ± 0.2 with 10M hydrochloric acid. Dispense in aliquots (typically, 100 ml) in suitable screw-capped containers and sterilise by autoclaving at 121 °C for 15 minutes. Prepared media may be stored in the dark at room temperature for up to one month.

A8.2 *Potassium hydroxide-sodium chloride solution*

Potassium hydroxide	500 mg
Sodium chloride	500 mg
Distilled, deionised or similar grade water	100 ml

Dissolve the ingredients in the water.

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

Peptone	20.0 g
Yeast extract	2.0 g
D (-) Mannitol	20.0 g
Sodium pyruvate	2.0 g
Sodium chloride	1.0 g
Magnesium sulphate	10 mg
Sodium desoxycholate	500 mg
Neutral red	30 mg
Crystal violet	1 mg
Agar	12.5 g
Distilled, deionised or similar grade 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
Distilled, deionised or similar grade 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 appropriate amounts into Petri dishes and allow the medium to solidify. Allow the medium to dry prior to use. Prepared Petri dishes may be stored between 2 - 8 °C, protected against dehydration, and used within seven days.

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

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g

Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Iron(III) citrate	300 mg
Sodium thiosulphate pentahydrate	300 mg
Phenol red (0.4 % m/v aqueous solution)	6 ml
Agar	15.0 g
Distilled, deionised or similar grade 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 (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 between 2 - 8 °C, protected from dehydration, and used within one month.

8.5 *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
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution (typically, 95 ml) 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. Prepared base medium may be stored in the dark at room temperature and used within one month. Prior to use, add 5 ml of an aqueous 40 % m/v filter-sterilised solution of urea to 95 ml of broth base and aseptically dispense in 2 - 3 ml volumes in sterile containers.

A8.6 *Other media*

Standard and commercial formulations of other media and reagents used in this method include MacConkey agar and nutrient broth.

A9 Analytical procedure

A9.1 *Sample preparation*

Due to the likelihood that, if present, numbers of *Yersinia* species in drinking water are likely to be low, a sample volume of at least 1000 ml should be examined. Smaller volumes may be appropriate for polluted source waters.

A9.2 *Sample processing*

A9.2.1 *Membrane filtration*

Filter an appropriate volume of sample. If the sample is turbid, several membrane filters may be required.

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, grid side upwards, onto the porous disc of the filter base. 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 so that as little air as possible is drawn through the membrane filter.

Remove the funnel and carefully transfer the membrane filter to 100 ml of tris-buffered peptone in screw-capped containers. If more than one membrane filter is required, all filters are transferred to the 100 ml of tris-buffered peptone. Mix well. 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, take a fresh pre-sterilised funnel 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, deionised or similar grade 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 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 *Incubation of enrichment broth and subculture to selective agar*

Incubate the tris-buffered peptone at 9 °C for two weeks. After incubation, add 11 ml of potassium hydroxide-sodium chloride solution to each 100 ml of tris-buffered peptone, mix well and plate out loopfuls of the treated tris-buffered peptone culture into Petri dishes of cefsulodin irgasan novobiocin agar. Incubate the Petri dishes at 30 °C for 24 hours.

A9.3 *Reading of results*

After incubation, examine the cefsulodin irgasan novobiocin 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.

A9.4 *Confirmation tests*

Subculture characteristic colonies to each of the following media:

(i) triple sugar iron agar and incubate at 30 °C for 24 hours;
(ii) urea broth and incubate for at 30 °C 1 to 72 hours; and
(iii) MacConkey agar and incubate at 30 °C for 24 hours.

Identify the isolates that produce acid but not gas and do not produce hydrogen sulphide on triple sugar iron agar, and are urease-positive in urea broth.

Subculture 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. 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

This test indicates the presence or absence of *Yersinia* species.

A11 Expression of results

Presumptive and confirmed *Yersinia* species are reported as being detected, or not detected, in the volume of sample examined.

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 are given elsewhere[(2)] in this series.

A13 References

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality 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*, in this series, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

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

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

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

B The isolation of *Vibrio cholerae* and other V*ibrio* species by selective enrichment

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 sewage-contaminated water. The significance of *Vibrio* species in water treatment and supply are described elsewhere[(1)] in this series.

B2 Scope

The method is suitable for the examination of drinking waters including samples from all stages of treatment and distribution, and those source waters of moderate turbidity.

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

Bacteria are concentrated on membrane filters followed by semi-selective enrichment with subculture to selective agar containing ox bile and sodium citrate. Characteristic colonies are confirmed by biochemical and, if necessary, serological testing. Alternatively, isolation may be performed by the addition of known volumes of sample to equal volumes of double strength enrichment broth with subculture to selective agar and testing of colonies.

B5 Limitations

The membrane filtration procedure is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter. This will

limit the volume of sample that can be filtered. In such instances, the use of several membrane filters is recommended. When low numbers of organisms are present, detection is dependent only on the volumes of sample that can be filtered and tested. High numbers of competing organisms may inhibit the growth, or detection, of target organisms.

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 (fan assisted, either static or temperature cycling) are required. Other items include:

B7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing 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 $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

B7.2 Incubators capable of maintaining temperatures of 25 ± 1 °C and 37 ± 1 °C, or cyclical incubators fitted with timers, capable of attaining these temperaures.

B7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.

B7.4 Sterile membrane filters, for example, white 47 mm diameter, cellulose-based 0.2 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. Variations in the preparation and storage of media should also be verified.

B8.1 *Alkaline peptone water*

Peptone	10.0 g
Sodium chloride	5.0 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH to 8.6 ± 0.2. Dispense (typically, 100 ml) into screw capped bottles 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 8.6 ± 0.2. The medium can be stored at room temperature, protected against dehydration, and used within one month.

Double-strength medium is prepared by adding the ingredients to 500 ml of the water and treating as above.

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

Yeast extract	5.0 g
Peptone	10.0 g
Sodium thiosulphate pentahydrate	10.0 g
Sodium citrate	10.0 g
Ox bile	8.0 g
Sucrose	20.0 g
Sodium chloride	10.0 g
Iron(III) citrate	1.0 g
Bromothymol blue (1 % m/v aqueous solution)	4 ml
Thymol blue (1 % m/v aqueous solution)	4 ml
Agar	14.0 g
Distilled, deionised or similar grade 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 2 - 8 °C, protected from dehydration, and used within one month.

B8.3 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar and oxidase reagent.

B9 Analytical procedure

B9.1 *Sample preparation*

Sample volumes of between 100 - 1000 ml should be examined depending upon the source water and the number of *Vibrio* species expected to be present.

B9.2 *Sample processing*

B9.2.1 *Membrane filtration*

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, grid-side upwards, onto the porous disc of the filter base. 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 so that as little air as possible is drawn through the membrane.

Remove the funnel and carefully transfer the membrane filter to 100 ml of alkaline peptone water. If more than one membrane filter is required, all filters are transferred to the 100 ml of alkaline peptone water. Mix well. 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, take a fresh pre-sterilised funnel or remove a funnel from the boiling water bath. Allow the funnel to cool and repeat the filtration process. If funnels are re-used, then after filtration of each sample, disinfect the funnel by immersing it in boiling distilled, deionised or similar grade 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 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 *Double strength enrichment broth*

Add at least a 100 ml volume of sample to an equal volume of double strength alkaline peptone water.

B9.2.3 *Incubation of enrichment broth and subculture to selective agar*

Incubate the alkaline peptone water at 25 °C for 2 hours followed by 37 °C for 12 - 16 hours. After incubation, plate out loopfuls of alkaline peptone water taken from the top of the broth onto Petri dishes of thiosulphate citrate bile salt sucrose agar and incubate at 37 °C for 16 - 24 hours.

B9.3 *Reading of results*

Examine the Petri dishes of thiosulphate citrate bile salt sucrose agar for colonies of characteristic of *Vibrio* species. These are usually flat colonies 1 - 3 mm in diameter, and either yellow (ie, sucrose-fermenting) or blue-green (ie, non-sucrose-fermenting). *Vibrio cholerae*, *Vibrio fluvialis* and *Vibrio metschnikovii* appear as yellow colonies, 2 - 3 mm in diameter, and *Vibrio mimicus* and *Vibrio parahaemolyticus* appear as blue-green colonies, 2 - 5 mm in diameter.

B9.4 *Confirmation tests*

B9.4.1 *Oxidase test*

Subculture suspect colonies to nutrient agar and incubate at 37 °C for 24 hours.

Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth on the prepared filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction.

Commercial test kits for oxidase testing are available and should be used in accordance with 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*).

B9.4.2 *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 (ie, 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

This test indicates the presence or absence of *Vibrio* species.

B11 Expression of results

Vibrio species are reported as being detected, or not detected, in the volume of sample examined.

B12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example *Vibrio cholerae* 024) 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 Drinking Water (2002) - Part 1 - Water Quality 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*, in this series, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

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

C The isolation of thermophilic *Campylobacter* species by selective enrichment

C1 Introduction

Thermophilic *Campylobacter* species are not thought to be free living but are obligate parasites of humans, animals and birds. The organisms are found in the gastrointestinal system, and being excreted in faeces, can be isolated from sewage 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. Waterborne outbreaks of campylobacteriosis have been reported and the significance of *Campylobacter* bacteria in water treatment and supply are described elsewhere[(1)] in this series.

C2 Scope

The method is suitable for the examination of drinking waters including samples from all stages of treatment and distribution, and those source waters of moderate turbidity.

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-postive, 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 micro-aerobic conditions, but not under aerobic conditions.

C4 Principle

Two enrichment procedures may be used for the detection of *Campylobacter* species in water. The first procedure involves filtration of a known volume of sample, usually one litre, and the placement of the membrane filter in a selective enrichment broth. Alternatively, the second procedure used where higher numbers of *Campylobacter* species may be expected, a volume of water is added to nine volumes of selective enrichment broth. After selective enrichment at 37 °C for 24 hours followed by 42 °C for 24 hours, an enrichment culture is inoculated onto a blood-free selective agar medium, which is incubated micro-aerobically at 37°C for 48 hours, and examined for characteristic colonies.

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

C5 Limitations

The membrane filtration procedure is suitable for most types of aqueous samples except those with high turbidities, which tend will block the membrane filter. This will limit the volume of sample that can be filtered. In such instances, the use of several membrane filters is recommended. The use of filter-aid is not recommended, as concentration of *Campylobacter* species via this procedure is not always effective. When low numbers of organisms are present, detection is dependent only on the volumes of sample that can be filtered and tested.

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 subculture 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 (fan assisted, either static or temperature cycling) are required. Other items include

C7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing 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 $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

C7.2 Incubators capable of maintaining temperatures of 37 ± 1 °C and 42 ± 0.5 °C, or cyclical incubators fitted with timers, capable of attaining these temperatures.

C7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.

C7.4 Thick glass fibre pre-filter, 40 mm diameter

C7.5 Sterile membrane filters, for example, white 47 mm diameter, cellulose-based, 0.2 μm nominal pore size.

C7.6 Smooth-tipped forceps.

C7.7 Micro-aerobic incubation jars, or equivalent.

C7.8 Microscope capable of phase 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. Variations in the preparation and storage of media should also be verified.

C8.1 *Campylobacter enrichment broth*

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
Distilled, deionised or similar grade water	950 ml

Dissolve the nutrient broth in 950 ml of the water and adjust the pH to 7.0 ± 0.2. 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 7.0 ± 0.2. Autoclaved base may be stored in the dark at room temperature and used within one month. Prior to use, add the lysed horse blood, iron(II) sulphate, sodium metabisuphite and sodium pyruvate. Antibiotic solutions are added after inoculation of the broth with the sample.

Originally, cycloheximide, which is an anti-fungal agent, was used in the preparation of the medium; however, cycloheximide is not widely available, and alternative anti-fungal agents (for example, amphotericin B) may be used.

Powdered charcoal added to the medium, prior to sterilisation, at a final concentration of 20 g/l, may improve recovery of *Campylobacter* [(4)].

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.

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 distilled, deionised or similar grade 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.

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 distilled, deionised or similar grade 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 distilled, deionised or similar grade 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 solution to 1 litre of the medium to give a final concentration of 10 mg/l.

(iv) Amphotericin B solution: Dissolve 0.2 g in 10 ml of distilled, deionised or similar grade water and add 1 ml of this solution to the 1 litre of medium to give a final concentration of 10 mg/l.

Dispense the final solution (typically, 90 ml) in screw capped bottles. Use on the day of preparation.

C8.2 *Campylobacter selective agar*[5, 6]

Nutrient broth	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
Distilled, deionised or similar grade 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

These solutions should be filter-sterilised.

(i) Cefoperazone solution: Dissolve 3.2 g 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 32 mg/l.

(ii) Amphotericin B solution: Dissolve 1.0 g amphotericin B 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 10 mg/l.

Pour the medium into Petri dishes and allow the agar to solidify. Poured Petri dishes may be stored between 2 - 8 °C and used within one week if protected against dehydration. Alternatively, the bottled medium (without the antibiotic supplements) can be stored in the dark at room temperature and used within one month.

C8.3 *Other media*

Standard and commercial formulations of other media and reagents used in this method include blood agar, blood-free agar, brain heart infusion broth, oxidase reagent and Gram stain reagents.

C9 Analytical procedure

C9.1 *Sample preparation*

Due to the likelihood that, if present, numbers of *Campylobacter* species in drinking water are likely to be low, a sample volume of 100 - 1000 ml should be examined.

C9.2 *Sample processing*

C9.2.1 *Membrane filtration*

Filter an appropriate volume of sample. If the sample is turbid, several membrane filters may be required. A thick glass fibre pre-filter may be placed on top of the membrane filter to aid filtration.

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, grid-side upwards, onto the porous disc of the filter base. 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 so that as little air as possible is drawn through the membrane.

Remove the funnel and transfer the membrane filter (and pre-filter if used) carefully to 90 ml of *Campylobacter* enrichment broth which has been allowed to equilibrate at room temperature. If more than one membrane filter is required, all filters are transferred to the 90 ml of *Campylobacter* enrichment broth. The filter-sterilised antibiotic solutions are now added and the caps screwed down tightly. Mix well. Alternatively, the caps on inoculated bottles can be left loose and the bottles incubated in micro-aerobic conditions.

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, take a fresh pre-sterilised funnel 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, deionised or similar grade 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 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 enrichment*

For environmental waters where numbers of *Campylobacter* species may be higher, add 10 ml of sample to 90 ml of *Campylobacter* enrichment broth that has been allowed to equilibrate at room temperature. The filter-sterilised antibiotic solutions are then added and the caps screwed down tightly. Alternatively, the caps on inoculated bottles can be left loose and the bottles incubated in micro-aerobic conditions.

C9.2.3 *Incubation and subculture to selective agar*

Incubate the inoculated *Campylobacter* enrichment broth at 37 °C for 24 hours, and then at 42°C and for a further 24 hours. After incubation, subculture loopfuls of *Campylobacter* enrichment broth to Petri dishes containing *Campylobacter* selective agar. Incubate the Petri dishes micro-aerobically at 37 °C for 48 hours in an anaerobic jar containing 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.

If required, for example in outbreak situations, Petri dishes can be examined after 18 - 24 hours, and if no growth is noted, returned to the incubator.

C9.3 *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 may vary in size from 0.05 - 0.1 mm to 2 - 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* will 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 are typically small, flat and transparent, and may be circular or ovoid, and may extend along the line of inoculation. 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.

C9.4 *Confirmation tests*

C9.4.1 *Oxidase test*

Subculture suspect colonies to a blood-free and antibiotic-free *Campylobacter* selective agar, or similar blood-free agar, and incubate micro-aerobically at 42 °C for 48 hours.

Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth on the prepared filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction.

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*).

C9.4.2 *Micro-aerobic growth*

Subculture suspect colonies from the *Campylobacter* selective agar onto two Petri dishes of blood agar. Incubate one Petri dish micro-aerobically at 37 °C for 24 hours and the other Petri dish, aerobically at 37 °C for 24 hours.

C9.4.3 *Cell morphology and motility*

Prepare a wet preparation 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.

C9.4.4 G*ram stain*

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.

An isolate which is oxidase-positive and Gram-negative, possesses S- or spiral-shaped cell morphology, is typically motile 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[7, 8, 9].

C10 Calculations

This test indicates the presence or absence of *Campylobacter* species.

C11 Expression of results

Presumptive and confirmed *Campylobacter* species are reported as being detected, or not detected, in the volume of sample examined.

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

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality 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*, in this series, Environment Agency.

3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.

4. The isolation and identification of thermotolerant *Campylobacter* spp. from sewage and river waters. *Journal of Applied Bacteriology*, Marcola, B., Watkins, J. & Riley, A., 1981, **51**, xii.

5. A selective medium for isolating *Campylobacter jejuni*/*coli. Journal of Clinical Pathology*, Bolton, F. J. & Robertson, L., 1982, **35,** 462-467.

6. Comparison of three selective agars for isolation of *Campylobacter*. *European Journal of Clinical Microbiology*, Bolton, F. J., Hutchinson, D. N. & Coates, D., 1986, **5**, 466-468.

7. *Campylobacter* biotyping scheme of epidemiological value. *Journal of Clinical Pathology*, Bolton, F. J., Holt, A. V. & Hutchinson, D. N., 1984, **37**, 677-681.

8. Application of three typing schemes (Penner, Lior, Preston) to strains of *Campylobacter* spp. isolated from three outbreaks. *Epidemiology and Infection*, Hutchinson, D. N., Bolton, F. J., Jones, D. M., Sutcliffe, E. M. & Abbott, J. D., 1987, **98**, 139-144.

9. New, extended biotyping scheme for *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter laridis*. *Journal of Clinical Microbiology*, Lior, H., 1984, **20**, 4, 636-640.

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However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below.

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