ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2002) - Part 5 - A method for the isolation and enumeration of enterococci by membrane filtration

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 a method for the isolation and enumeration of enterococci by membrane filtration

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 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
Part 10 - Methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment

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.

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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 this method 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 method for the isolation and enumeration of enterococci by membrane filtration

1 Introduction

In the United Kingdom, enterococci are regarded as secondary indicators of faecal pollution, and the main use of the test for enterococci is to assess the significance of coliform bacteria in a sample in the absence of *Escherichia coli* (*E. coli*). Occasionally, identification of the species of streptococci present in a sample may help to distinguish between human and animal pollution. The significance of enterococci in water treatment and supply are described elsewhere(1) in this series.

2 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).

3 Definitions

In the context of this method, presumptive enterococci reduce triphenyltetrazolium chloride after incubation to insoluble red formazan to produce red, maroon or pink colonies on membrane enterococcus agar(3). Some strains may produce very pale colonies. Confirmation is based on the organism being catalase-negative and on the demonstration of aesculin hydrolysis on bile aesculin agar or kanamycin aesculin azide agar incubated at 44 °C for up to 18 hours, although some strains of *Streptococcus bovis* and *Streptococcus equinus* may fail to grow at this temperature.

Enterococci are Gram-positive cocci which form pairs, or chains, and possess Lancefield's Group D antigen. The organism grows in the presence of bile salts, in concentrations of sodium azide that are inhibitory to coliform bacteria and most other Gram-negative bacteria, and at a temperature of 44 °C. Some species are resistant to heating at 60 °C for 30 minutes, to pH 9.6, and are able grow in nutrient broth containing 6.5 % sodium chloride.

4 Principle

Organisms are isolated on a membrane filter placed on the surface of an agar medium containing triphenyltetrazolium chloride. Enterococci usually produce pink, maroon or red colonies as a result of the formation of formazan.

5 Limitations

The 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. Accumulated deposit on the membrane filter may mask or

inhibit the growth of indicator organisms. The method also allows some other species (for example, *Aerococcus viridans* and species of *Staphylococcus* and *Bacillus*) to grow. The maximum number of colonies that should be counted from a single membrane filter is approximately 100.

6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[4] and appropriate assessments of risk should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

Some of the media described in this method contain sodium azide. As this substance is highly toxic, great care should be taken when these media are prepared, especially when powdered dehydrated ingredients are used. Sodium azide forms explosive compounds with metals, especially copper and lead. Waste material containing sodium azide should, therefore, be discarded into drains with care, preferably through plastic pipes. Azide compounds should be decomposed and rendered safe with excess sodium nitrite before disposal.

7 Apparatus

Standard laboratory equipment should be used which conforms to the performances criteria outlined elsewhere[2] in this series. Principally, appropriate membrane filtration apparatus and incubators (fan assisted, either static temperature or temperature cycling) are required. Other items include:

7.1 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).

7.2 Incubators capable of maintaining temperatures of 37.0 ± 1.0 °C and 44.0 ± 0.5 °C or cycling incubators, fitted with timers, capable of attaining these temperatures.

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

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

7.5 Smooth-tipped forceps.

8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. The performance of all media and reagents should be verified prior to their use in this method. Variations in the preparation and storage of media should also be verified.

8.1 *Membrane enterococcus agar*

Tryptose	20 g
Yeast extract	5 g
Glucose	2 g
Dipotassium hydrogen phosphate	4 g
Sodium azide	400 mg
Agar	12 g
2,3,5-triphenyltetrazolium chloride (1% m/v aqueous solution)	10 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients, except triphenyltetrazolium chloride, in the water either through steaming or bringing gently to the boil. The pH of the solution should be 7.2 ± 0.2. Sterilise the triphenyltetrazolium chloride by filtration, add to the solution, mix well and pour the medium directly into Petri dishes. The medium should not be stored and re-melted, but poured plates may be kept at a temperature between 2 - 8 °C for not more than 1 month, if protected against dehydration.

Special care should be taken to avoid overheating of the medium which may result in a deterioration of performance. If the medium is orange or pink when cooled to 50 °C, then consideration should be given whether it should be discarded.

8.2 *Kanamycin aesculin azide agar*

Tryptone	20 g
Yeast extract	5 g
Sodium chloride	5 g
Sodium citrate	1 g
Aesculin	1 g
Iron(III) ammonium citrate	500 mg
Sodium azide	150 mg
Kanamycin sulphate	20 mg
Agar	12 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and sterilise at 121 °C for 15 minutes. The final pH should be 7.0 ± 0.2. Sterile media can be stored for up to one month at a temperature between 2 - 8 °C, if protected against dehydration.

8.3 *Bile aesculin agar*

Peptone	8 g
Bile salts	20 g
Iron(III) citrate	500 mg
Aesculin	1 g
Agar	15 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the solution to a pH value of 7.1 ± 0.2. Sterilise at 121 °C for 15 minutes. The final pH should be 7.1 ± 0.2. Sterile media can be stored for up to one month at a temperature between 2 - 8 °C, if protected against dehydration.

8.4 *Other media*

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

9 Analytical procedure

9.1 *Sample preparation*

The volumes and dilutions of samples should be chosen so that the number of colonies to be counted on the membrane filter lies, if possible, between 20 and 80. With some waters, it may be advantageous to filter a selection of different volumes so that the number of colonies on any one of the membrane filters is likely to fall within this range. For treated waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes or dilute the sample with Ringer's solution or maximum recovery diluent before filtration.

9.2 *Sample processing*

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. When the volume of sample to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, quarter-strength Ringer's solution or maximum recovery diluent) to the funnel before addition of the sample. This aids the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the sample 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 transfer the membrane filter carefully to a well-dried membrane enterococcus agar plate. Ensure that no air bubbles are trapped between the membrane filter and the medium. 'Rolling' the membrane filter onto the medium minimises the likelihood of air bubbles becoming trapped. Cover the membrane filter with the lid of the Petri dish.

When the funnel is removed it 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 or highest dilution 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, 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.

Growth of enterococci on membrane enterococcus agar is better at 37 °C, although some organisms resembling enterococci may also grow on this medium. Selectivity is better at 44 °C although lower counts of enterococci may be obtained. It may however be more appropriate that membrane filters from samples of potable water are incubated at 37 °C for 48 hours, whilst membrane filters from untreated waters are incubated at 37 °C for 4 hours followed by 44 °C for 44 hours.

9.3 *Reading of results*

After incubation, count all red, maroon or pink colonies that are smooth and convex in shape. These are regarded as presumptive enterococci. Some types of enterococci may produce very pale colonies. Colonial size is variable but is usually not less than 0.5 mm. Some species of *Bacillus* may produce pink colonies but these are often rough, flat and sometimes spread. Some species of *Aerococcus* and *Staphylococcus* can also grow on membrane enterococcus agar producing red colonies.

9.4 *Confirmation tests*

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of red, maroon or pink colonies (however faint). If the aim is to estimate the number of organisms present, then for the greatest accuracy, all colonies should be sub-cultured if fewer than ten are present, or at least ten colonies should be sub-cultured if more than ten are present.

9.4.1 *Catalase test*

Enterococci are catalase-negative. For each colony to be tested, into a small screw-capped bottle, emulsify some of the isolated colony from the membrane enterococcus agar in approximately 0.1 ml of quarter strength Ringer's solution. Add approximately 0.05 ml of 3 % hydrogen peroxide solution and replace the cap. The immediate appearance of bubbles (of oxygen) indicates catalase activity. An alternative procedure is to add the hydrogen peroxide to an overnight culture of an isolate obtained from nutrient agar. The test should preferably not be performed on a slide because of the risk of aerosol formation.

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

On each occasion that catalase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *Staphylococcus aureus*) and one species is known to give a negative reaction (for example, *Enterococcus faecalis*).

9.4.2 *Aesculin hydrolysis*

From membrane enterococcus agar, subculture to bile aesculin agar or kanamycin aesculin azide agar and incubate at 44 °C for up to 18 hours. Enterococci should produce discrete colonies surrounded by a brown or black halo from aesculin hydrolysis. The development of this colour is usually evident within a few hours and should provide rapid confirmation. *Bacillus* species may produce some discoloration around the original inoculum site but should not develop discrete colonies.

9.5 *Additional differentiation tests for enterococci*

Although the possession of Lancefield's Group D antigen is referred to in the definition, serological methods of confirmation present many practical difficulties. Tolerance of 40 % bile is also characteristic of enterococci. Further tests with sub-cultures may be undertaken if necessary, partly as an aid to species differentiation. However, full identification depends on the demonstration of biochemical and other characteristics(5) or by means of one of the multi-test differential systems now available. Commercial biochemical and serological methods can be used, following appropriate verification of performance at the laboratory.

Enterococcus species are differentiated from other streptococci by their ability to grow in nutrient broth containing 6.5 % sodium chloride, and in glucose phenolphthalein broth(6) modified to pH 9.6.

9.5.1 *Bile tolerance*

From an overnight culture on nutrient agar incubated at 37 °C, sub-culture to a plate or tube of 40 % bile agar and incubate at 37 °C for 24 - 48 hours. Growth on this medium indicates tolerance of bile salts. Alternatively, use MacConkey agar to show growth in the presence of bile salts. Enterococci form small deep red colonies on MacConkey agar.

9.5.2 *Heat resistance*

Transfer 1 ml of a nutrient broth culture incubated at 37 °C for 24 hours to a small test tube. Place the test tube in a water bath at 60 °C for 30 minutes. Cool the tube rapidly and incubate at 37 °C for 24 hours. Subculture the broth to a blood agar plate or other non-selective medium. Incubate at 37 °C and examine for growth.

9.5.3 *Growth at pH 9.6*

From a nutrient agar plate, inoculate into a tube of glucose phenolphthalein broth modified to pH 9.6 and incubate at 37 °C for 24 hours. Tolerance of pH 9.6 is indicated by heavy growth and decolorisation of the medium.

9.5.4 *Salt tolerance*

From a nutrient agar plate, inoculate into a tube of nutrient broth containing 6.5 % of sodium chloride and incubate at 37 °C for 24 - 48 hours. Examine for growth.

10 Calculations

10.1 *Presumptive enterococci*

The number of presumptive enterococci is generally expressed as the number of colonies per 100 ml of sample. Calculate the presumptive count as follows:

$$\text{Presumptive count/100 ml} = \frac{\text{Number of colonies counted on membrane filter x 100}}{\text{Volume of sample filtered (ml)}}$$

10.2 *Confirmed enterococci*

The number of confirmed enterococci is calculated by multiplying the number of presumptive enterococci by the proportion of the isolates that are catalase-negative and able to hydrolyse aesculin on bile aesculin agar or kanamycin aesculin azide agar.

11 Expression of results

Presumptive and confirmed enterococci are expressed in colony forming units per volume of sample. For drinking water the volume is typically 100 ml.

12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *Enterococcus faecalis*) and non-target bacteria (for example, *Staphylococcus* species). Petri dishes should be incubated for 48 hours at 37 °C. Further details are given elsewhere(2) in this series.

13 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. Numbers of enterococci in water, sewage and faeces determined by the membrane filter technique with an improved medium. *Journal of Bacteriology*, Slanetz, L.W. & Bartley, C.H., 1957, **74**, 591-595.

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

5. *Cowan and Steels' Manual for the Identification of Medical Bacteria*, 3rd edition. (Editors, Barrow G.I. & Feltham R.K.A.). London, Cambridge University Press, 1993.

6. Growth of streptococci in a glucose phenolphthalein broth, *Journal of General Microbiology*, Clarke, P.H., 1953, **9**, 350-352.

Address for correspondence

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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