ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2010) - Part 5 -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, and both of the confirmation procedures previously described in the 2002 and 2006 versions of this document which are now replaced.

Whilst specific commercial products may be referred to in this document, this does not constitute an endorsement of these products but serves only as illustrative examples of the type of products available. Equivalent products are 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.

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

The Microbiology of Drinking Water (2002)
Part 1 - Water quality and public health
Part 2 - Practices and procedures for sampling (currently undergoing revision)
Part 3 - Practices and procedures for laboratories (currently undergoing revision)
Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment.

The Microbiology of Drinking Water (2004)
Part 11 - Taste, odour and related aesthetic problems
Part 12 - Methods for micro-organisms associated with taste, odour and related aesthetic problems.

The Microbiology of Drinking Water (2006)
Part 9 - The isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube-most probable number techniques

The Microbiology of Drinking Water (2007)
Part 7 - Methods for the enumeration of heterotrophic bacteria (currently undergoing revision)
Part 13 - The isolation and enumeration of aerobic spore-forming bacteria by membrane filtration

The Microbiology of Drinking Water (2009)
Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)
Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

The Microbiology of Drinking Water (2010)
Part 5 - 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 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration

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.

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.

In the procedures described in each method any reference to the tolerances to be adopted with respect to, for example the amount or volume of reagents to be used is left to the discretion of the laboratory. These tolerances should be as low as possible in order to satisfy stringent performance criteria. Tolerances of between 1 - 5 % have been shown to be satisfactory for most purposes. Lower tolerances should result in improved precision.

In the methods described, for example for wavelengths, storage conditions, concentrations of the same or similar reagents, etc, differences may be noted. This information is provided by individual laboratories operating under their own management systems and is dependent on specific conditions pertaining to each laboratory. It is assumed this information is supported by sufficient data to justify its inclusion. Users intending to use or vary the quoted wavelengths, storage conditions, concentrations, etc, should ensure they are appropriate to their own laboratory and verify their application to demonstrate appropriate performance of the method. 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.

Methods are produced by panels of experts in the appropriate field, often in co-operation with working groups and the main committee. The names of those members principally associated with these methods are listed at the back of this booklet. A report describing all SCA activities for the period 1 July to 30 June is produced annually and is available from the Agency's web-page (www.environment-agency.gov.uk/nls).

Users should ensure they are aware of the most recent version of the draft they seek. If users wish to receive copies or advance notice of forthcoming publications, or obtain details of the index of methods then contact the Secretary on the Agency's internet web-page or by post, see address listed at the back of this booklet.

Great efforts are made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary
February 2010

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 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.

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 enterococci or streptococci 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

This 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 (after incubation) triphenyltetrazolium chloride to insoluble red formazan to produce red, maroon or pink colonies on membrane enterococcus agar (mEA)[3] when incubated at 37 °C or 44 °C. Some strains may produce colonies which are very small and/or pale in colour. Confirmation is based[4] on the demonstration of aesculin hydrolysis on bile aesculin agar (BAA) or kanamycin aesculin azide agar (KAAA) by sub-culturing colonies from mEA to BAA or KAAA and incubating at 44 °C for up to 18 hours, or alternatively, by transferring the membrane filter from mEA to BAA or KAAA, pre-warmed to room temperature, and incubating at 44 °C for 4 hours. Some strains of enterococci and some strains of *Streptococcus bovis* and *Streptococcus equinus*, whilst growing at 37 °C on mEA may fail to grow at 44 °C.

Enterococci are Gram-positive cocci which form pairs or chains, possess Lancefield's Group D antigen and are catalase-negative. 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 display other characteristics useful for identification, such as resistance to heating at 60 °C for 30 minutes, tolerance to pH 9.6, and the ability to 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 red, maroon or pink 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 growth of

organisms. Other bacteria (for example, *Aerococcus viridans* and species of *Staphylococcus* and *Bacillus*) are also able to grow under the conditions described. The maximum number of typical and non-typical colonies that should be present on a single membrane filter from which direct counts are made should be approximately 200[5]. However, this would need to be reduced if several large colonies are present. For this method, the enterococci may grow as very small colonies, allowing counts in excess of 200 organisms per membrane filter to be estimated. If the number of colonies exceeds 200, and an attempt is made to count the target or total colonies present, the report of the results should contain a statement that the counts are estimates, and may not reflect the true number of colonies.

6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[6] and appropriate risk assessments 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. This substance is highly toxic, and 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 with care, preferably through plastic pipes. Azide compounds may be decomposed and rendered safe with excess sodium nitrite, before disposal.

7 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 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 sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

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, filter funnels (either sterilised or capable of being sterilised, 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[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar quality. Unless otherwise stated chemical constituents should be added as anhydrous salts. If the pH of media is not within the stated range, then, before heating, they should be adjusted accordingly.

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 (TTC) (1 % m/v aqueous solution)	10 ml
Water	1 litre

Dissolve the ingredients, except triphenyltetrazolium chloride, in the water either by steaming or bringing gently to the boil. The pH of the solution should be 7.2 ± 0.2. Filter-sterilise the TTC solution through a nominal 0.2 µm membrane filter. Cool the medium to 50 °C and add the sterilised TTC solution and mix well. The medium should not be stored and re-melted. Pour appropriate amounts of the complete medium directly into Petri dishes. Poured plates may be kept at a temperature of 5 ± 3 °C for up to one month, if protected against dehydration.

Commercial formulations which contain TTC should not be overheated when dissolving the ingredients as this may result in a deterioration of the performance of the medium. This is due to the breakdown of TTC and if the medium is orange or pink when cooled to 50 °C, then consideration should be given to discarding the medium.

8.2 *Bile aesculin agar*

Peptone	8 g
Bile salts	20 g
Iron(III) citrate	500 mg
Aesculin	1 g
Agar	15 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH value of the solution to 7.1 ± 0.2. Sterilise the solution at 121 °C for 15 minutes. The final pH of the cooled solution should be 7.1 ± 0.2. Sterile media may be stored for up to one month at a temperature of 5 ± 3 °C, if protected against dehydration.

8.3 *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
Water	1 litre

Dissolve the ingredients in the water and sterilise at 121 °C for 15 minutes. The pH of the cooled solution should be 7.0 ± 0.2. Sterile media may be stored for up to one month at a temperature of 5 ± 3 °C, if protected against dehydration.

8.4 *Other media*

Standard and commercial formulations of other media and reagents used in this method may include nutrient agar, brain heart infusion agar, Mueller Hinton agar, MacConkey agar, nutrient broth, blood agar, bile agar, catalase reagent, quarter strength Ringer's solution and maximum recovery diluent.

9 Analytical procedure

9.1 *Sample preparation*

For treated waters, filter 100 ml of the sample. For polluted waters, filter smaller volumes, or dilute the sample with maximum recovery diluent or quarter strength Ringer's solution before filtration. 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 single membrane filter lies within this range.

9.2 *Sample processing*

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum. The stopcock should be in the closed position. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter, for example 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 or diluted sample into the funnel. When the volume to be filtered is less than 10 ml, add 10 - 20 ml of sterile diluent (for example, maximum recovery diluent or quarter-strength Ringer's solution) 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.

Remove the funnel and transfer the membrane filter carefully to a Petri dish containing mEA. The surface of the medium should be dry and free of any surplus water. 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 it being placed in boiling water, provided that the smallest volume or highest dilution of sample is filtered first. For different samples, a fresh pre-sterilised funnel should be used, or remove a funnel from the boiling water bath, allow the funnel to cool and carry out the filtration process. If funnels are to be re-used, after filtration of each sample, disinfect the funnel by immersing it in boiling water for at least one minute. During the filtration of a series of samples the filter base need not be sterilised unless it becomes or is suspected of being 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.

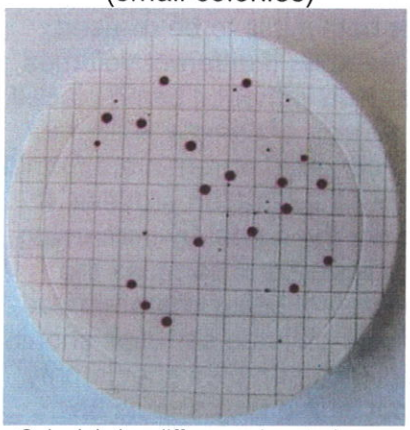
Encouraging the growth of enterococci is a balance between selectivity and sensitivity. The growth of enterococci on mEA is better at 37 °C, although some organisms resembling enterococci may also grow on this medium at this temperature. Selectivity is better at 44 °C although lower counts of enterococci may be obtained, as some strains do not grow or do not grow well at 44 °C. It may be more appropriate if membrane filters from samples of potable water are incubated at 37.0 ± 1.0 °C for 44 ± 4 hours, whilst membrane filters from samples of untreated water are incubated at 37.0 ± 1.0 °C for 4.0 ± 0.5 hours followed by 44.0 ± 0.5 °C for 40 ± 4 hours.

9.3 *Reading of results*

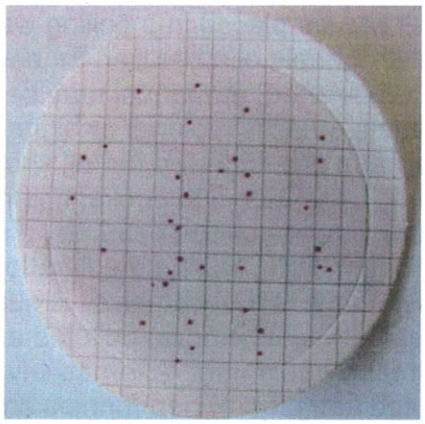
After incubation, count all red, maroon or pink colonies on mEA that are smooth and convex in shape (see Figure 1). These colonies are regarded as presumptive enterococci. Some types of enterococci may produce very pale colonies. Colonial size is variable. Some species of *Bacillus* may produce pink colonies but these are often rough, flat and sometimes spread over the surface of the agar. Some species of *Aerococcus* and *Staphylococcus* can also grow on mEA producing red colonies.

Figure 1 Colonies on membrane enterococcus agar

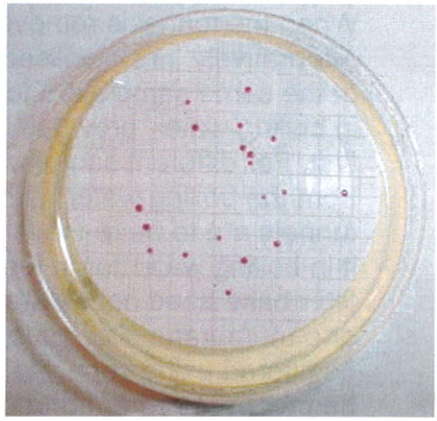
Enterococcus faecalis (large colonies) with *Aerococcus viridans* (small colonies)



Enterococcus casseliflavus



Enterococcus faecalis



Colonial size difference is not always apparent.

9.4 *Confirmation tests*

Presumptive enterococci may be confirmed by sub-culturing from mEA to BAA or KAAA and incubating at 44 ± 0.5 °C for up to 18 hours, or alternatively, by transferring the membrane filter from mEA to BAA or KAAA, pre-warmed to room temperature, and incubating at 44.0 ± 0.5 °C for 4 hours ± 5 minutes. See flow chart.

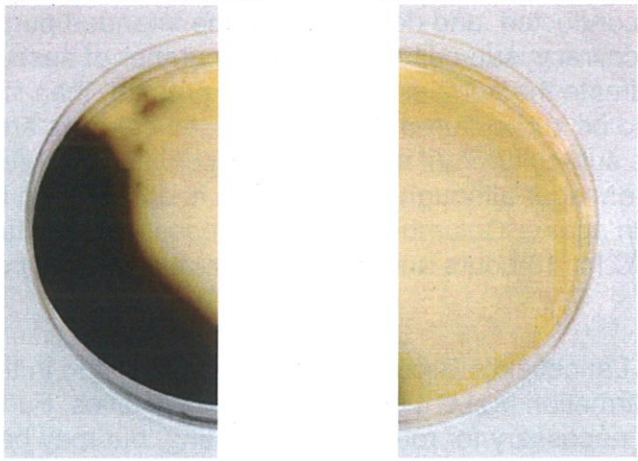
9.4.1 Aesculin hydrolysis by sub-culture

Depending on the intended purpose of the analysis and the required accuracy, sub-culture from mEA a suitable number of red, maroon or pink colonies (however faint) to BAA or KAAA. 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.

From mEA, subculture to BAA or KAAA and incubate at 44 °C for up to 18 hours. Enterococci should produce discrete colonies surrounded by a brown or black halo resulting from aesculin hydrolysis. See Figure 2. 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.

Figure 2 Colonies on kanamycin aesculin azide agar

Enterococcus faecalis *Aerococcus viridans*

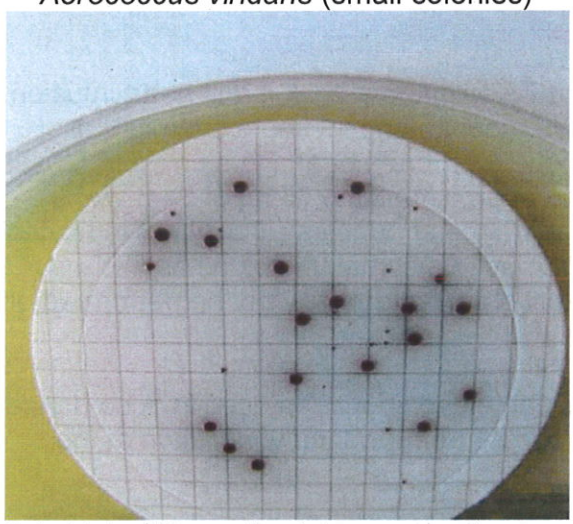


9.4.2 Aesculin hydrolysis by membrane transfer

Transfer the membrane filter from mEA carefully to a Petri dish containing BAA or KAAA, pre-warmed to room temperature, and incubate at 44.0 ± 0.5 °C for 4 hours ± 5 minutes. Data on the verification of the performance of this confirmation procedure are given in appendix 1. The surface of the medium should be dry and free of any surplus water. 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. Colonies that are surrounded (after incubation) by a brown or black halo, resulting from the aesculin hydrolysis process, are regarded as confirmed enterococci (see Figure 3). The development of this halo is often evident within 2 hours and may provide rapid confirmation, but see appendix 1. However, some strains may take longer to produce the colouration, and hence the need for further incubation.

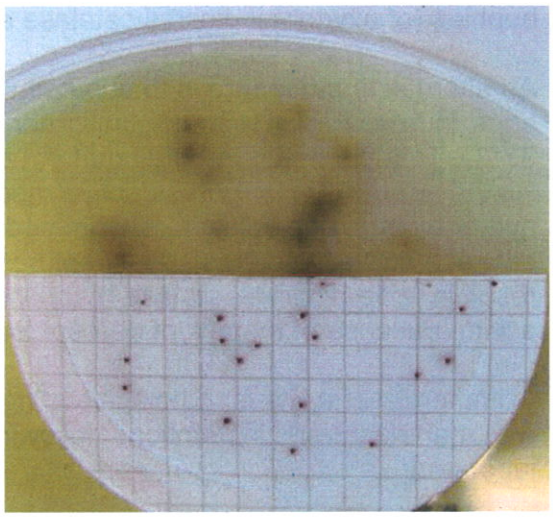
Figure 3 Colonies on bile aesculin agar

Enterococcus faecalis (large colonies) with *Aerococcus viridans* (small colonies)



Colonial size difference is not always apparent.

Enterococcus casseliflavus



9.5 *Additional differentiation tests for enterococci*

If additional tests are to be conducted, and depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of aesculin-positive colonies. 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 colonies are present) or at least ten colonies should be sub-cultured (if more than ten colonies are present). Colonies can be sub-cultured to nutrient agar although better growth is obtained on brain heart infusion agar, Mueller Hinton agar or Columbia blood agar base. Sub-cultured Petri dishes should be incubated at 37 °C for 18 hours and checked to make sure that cultures are pure.

Although the possession of Lancefield's Group D antigen is referred to in the definition, serological methods of confirmation present many practical difficulties. Further differentiation is not usually necessary for routine water testing, but may be appropriate where particular problems are encountered. Tolerance of 40 % bile is also characteristic of enterococci, as is a negative catalase reaction. Further tests with appropriate sub-cultures previously obtained from BAA or KAAA may be undertaken if necessary, partly as an aid to species differentiation. However, full identification depends on the demonstration of biochemical and other characteristics[7]. This may be achieved 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 can also be differentiated from other streptococci by their ability to grow in nutrient broth containing 6.5 % sodium chloride, and in glucose phenolphthalein broth[8] modified to pH 9.6, and their resistance to heating at 60 °C.

9.5.1 *Catalase test*

Enterococci are catalase-negative. For each sub-culture to be tested, from the sub-cultured BAA or KAAA Petri dish (9.4.1) or the membrane filter incubated on BAA or KAAA (9.4.2) into a small screw-capped bottle, emulsify some of the isolated colony in approximately 0.1 ml of quarter strength Ringer's solution. Add approximately 0.05 ml of 5 - 6 v/v % hydrogen peroxide solution and replace the cap. The immediate appearance of bubbles (of oxygen) indicates catalase activity.

An alternative procedure is to sub-culture from mEA to nutrient agar, brain heart infusion agar, Mueller Hinton agar or similar non-selective medium and incubate overnight at 37 °C. To a pure culture obtained from the nutrient agar, brain heart infusion agar, Mueller Hinton agar or similar non-selective medium add approximately 0.05 ml of 5 - 6 v/v % hydrogen peroxide solution. The immediate appearance of bubbles (of oxygen) indicates catalase activity. Isolates should not be taken from media containing blood as this may result in false-positive catalase reactions. 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.

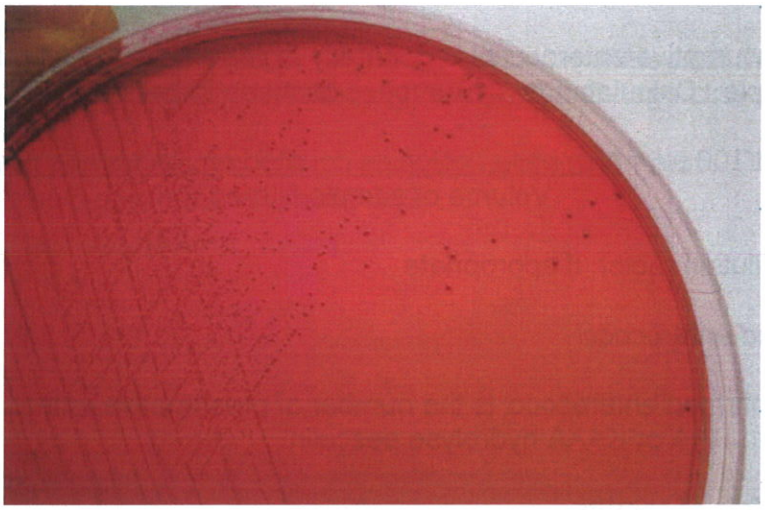
On each occasion that catalase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example, *E. coli*,

Pseudomonas aeruginosa or *Staphylococcus aureus*) and one species is known to give a negative reaction (for example, *Enterococcus faecalis*).

9.5.2 *Bile tolerance*

From an overnight culture (previously isolated from BAA or KAAA) on nutrient agar, incubated at 37 °C, sub-culture to a Petri dish or tube containing 40 % bile agar and incubate at 37 °C for 24 - 48 hours. Growth on this medium indicates tolerance of bile salts. Alternatively, sub-culture to a Petri dish or tube containing MacConkey agar and incubate at 37 °C for 24 - 48 hours to show growth in the presence of bile salts. Enterococci form small deep red colonies on MacConkey agar (see Figure 4). Include control tests with organisms, of which one species is known to grow in the presence of 40 % bile (for example, *Enterococcus faecalis*) and one species is known not to grow in the presence of 40 % bile (for example, *Streptococcus pneumoniae*).

Figure 4 Colonies of *Enterococcus faecalis* on MacConkey agar



9.5.3 *Heat resistance*

From BAA or KAAA, transfer appropriate colonies to nutrient broth and incubate at 37 °C for 21 ± 3 hours. Transfer 1 ml of the nutrient broth culture 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 21 ± 3 hours. Sub-culture the broth to a Petri dish containing blood agar or other non-selective medium. Incubate overnight at 37 °C and examine for growth. Include control tests with organisms, of which one species is known to survive this heat treatment (for example, *Enterococcus faecalis*) and one species is known not to survive (for example, *Streptococcus bovis* or *Streptococcus equinus*).

9.5.4 *Growth at pH 9.6*

From BAA or KAAA, transfer appropriate colonies to a Petri dish containing nutrient agar and incubate overnight at 37 °C. Transfer a colony from the nutrient agar and inoculate into a tube of glucose phenolphthalein broth modified to pH 9.6 and incubate at 37 °C for 21 ± 3 hours. Tolerance to this solution (at pH 9.6) is demonstrated by the heavy growth of organisms and by the decolourisation of the medium from pink (red) to colourless.

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