Standing Committee of Analysts

The Microbiology of Recreational and Environmental Waters (2016) – Part 10 – A method for the isolation and enumeration of sorbitol-fermenting bifidobacteria 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 sorbitol-fermenting bifidobacteria by membrane filtration.

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

Contents

About this series

Introduction

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

Performance of methods

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

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

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

Standing Committee of Analysts

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

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

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

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

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

Robert Carter
Secretary
June 2015

Warning to users

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

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

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

A A method for the isolation and enumeration of sorbitol-fermenting bifidobacteria by membrane filtration

A1 Introduction

Bifidobacteria represent a major component of human gut microflora, occurring in numbers up to 10^{10} per gram of faeces. They occur less frequently and in much lower numbers in warm blooded animals. The sorbitol-fermenting bifidobacteria (primarily *Bifidobacterium adolescentis* and *Bifidobacterium breve*) are a sub-group of the bifidobacteria that are specific for human faecal waste and have not been found in a wide range of domesticated and wild mammals and birds. These bacteria are strict anaerobes and do not multiply below 30 °C and, consequently, are unlikely to multiply once released into surface waters. They do, however, have only a relatively limited survival capability in the environment, and are thus considered to be indicators of recent (temporal or spatial) contamination with human faecal waste. The significance of sorbitol-fermenting bifidobacteria in recreational and other waters are described elsewhere(1) in this series.

A2 Scope

The method is suitable for the examination of fresh and saline surface waters, swimming pools, spa pools and hydrotherapy pools and primary and secondary wastewater effluents.

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

A3 Definitions

In the context of this method, presumptive sorbitol-fermenting bifidobacteria are defined as those bacteria which produce domed and/or mucoid yellow colonies on human bifid sorbitol agar (HBSA)(3) after anaerobic incubation at 37 °C.

Confirmed sorbitol-fermenting bifidobacteria are characteristic colonies from HBSA which grow only under anaerobic conditions, and when Gram-stained appear as Gram-variable bifurcating groups of rod-shaped bacteria, typically showing Y and V forms.

A4 Principle

Organisms are isolated on a membrane filter placed on the surface of an agar medium containing nalidixic acid and polymyxin B as selective agents and sorbitol as the fermentable carbohydrate, and incubated under anaerobic conditions. Sorbitol-fermenting bifidobacteria produce yellow domed and/or mucoid colonies.

A5 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 organisms. Where high numbers of organisms may be expected (for example, primary wastewater effluent) serial ten-fold dilutions should be made to

obtain a countable number of colonies. The ideal range of numbers of typical and non-typical colonies that should be present on a single membrane filter from which counts are estimated is 20 – 80 with the maximum being approximately 100[2]. However, this would need to be reduced if several large colonies are present. If the number of colonies exceeds 100, 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.

A6 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 risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2,4] 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 (fan assisted) are required. Other items include:

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

A7.2 Incubators capable of maintaining temperatures of 37.0 ± 1.0 °C.

A 7.3 Anaerobic jars, or similar equipment, and anaerobic gas-generating system (for generating atmospheres of approximately 9 - 13 % carbon dioxide).

A7.4 Filtration apparatus, filter funnels (either sterilised or capable of being sterilised, and vacuum source).

A7.5 Sterile, membrane filters, for example, white, 47 mm diameter, cellulose-based 0.45 µm nominal pore size.

A7.6 Smooth-tipped forceps.

A8 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 grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salts. If the pH of media are not within the stated range, then, before heating, they should be adjusted accordingly. Sterilised media with a pH outside the required range should be discarded.

A8.1 *Human bifid sorbitol agar (HBSA)*[3]

Polypeptone	10 g
Yeast extract	20 g
Casamino acids	8 g
Sorbitol	10 g
Sodium chloride	3.2 g
Bromocresol purple	80 mg
Cysteine hydrochloride	0.4 g
Nalidixic acid	30 mg
Polymyxin B	10 IU
Agar	15 g
Water	1 litre

Dissolve the ingredients except the cysteine hydrochloride, nalidixic acid and polymyxin B in the water, either by steaming or bringing gently to the boil. Allow to cool and add the cysteine hydrochloride and nalidixic acid. Adjust the pH to 6.9 ± 0.2. Dispense the resulting solution in appropriate volumes into suitable screw-capped bottles and sterilise by autoclaving at 121 ºC for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.9 ± 0.2. Cool to approximately 50 ºC and add 1 ml of a filter sterilised solution of 10 IU/ml polymyxin B. Mix well and pour into Petri dishes. The Petri dishes should be the vented type to ensure anaerobic conditions for the medium during storage and incubation.

Performance of the medium deteriorates during storage due to exposure to oxygen. Prepared media should be stored in a refrigerator under anaerobic conditions at a temperature between 5 ± 3 °C for up to one week. However, some anaerobic generating systems may not work satisfactorily at this temperature. Medium in Petri dishes, once removed from the refrigerator, should be discarded if not used.

A8.2 Reinforced clostridial agar

Peptone	10 g
Meat extract	10 g
Yeast extract	3 g
Glucose	5 g
Soluble starch	1 g
Sodium chloride	5 g
Sodium acetate	3 g
Cysteine hydrochloride	0.5 g
Agar	15 g
Water	1 litre

Dissolve the ingredients in the water, either by steaming or bringing gently to the boil. Dispense the resulting solution in appropriate volumes into suitable screw-capped bottles and sterilise by autoclaving at 121 ºC for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. Cool to approximately 50 ºC, mix well and pour into Petri dishes. The Petri dishes should be the vented type to ensure anaerobic conditions for the medium during storage and incubation.

Performance of the medium deteriorates during storage due to exposure to oxygen. Prepared media should be stored in a refrigerator under anaerobic conditions at a

temperature between 5 ± 3 °C for up to one month. However, some anaerobic generating systems may not work satisfactorily at this temperature. When fresh medium is used, the colony characteristics that are observed tend to be more defined. Medium in Petri dishes, once removed from the refrigerator, should be discarded if not used.

A8.3 *Other media*

Standard and commercial formulations of other media and reagents used in this method may include quarter strength Ringer's solution or maximum recovery diluent (both supplemented with 0.5 g/l cysteine hydrochloride) and Gram staining reagents.

A9 Analytical procedure

A9.1 *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 of sample so that the number of colonies on at least one of the membrane filters is likely to fall within this range. For swimming pool, spa pool and hydrotherapy pool waters, filter 100 ml of the sample. For polluted waters either filter smaller volumes, or dilute the sample with quarter strength Ringer's solution or maximum recovery diluent (both supplemented with 0.5 g/l cysteine hydrochloride) before filtration.

A9.2 *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, 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 of sample 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 supplemented with 0.5 g/l cysteine hydrochloride) 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 filtered.

Remove the funnel and transfer the membrane filter carefully to a Petri dish containing HBSA. 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.

Incubate the Petri dishes at 37 °C in an anaerobic jar or similar system containing an indicator of anaerobiosis and an atmosphere containing 9 - 13 % carbon dioxide. Examine the dishes after 44 ± 4 hours incubation.

A9.3 *Reading of results*

After incubation, examine the filters under good light and count deep yellow colonies, 1 – 2 mm in diameter, which are domed and/or mucoid in appearance. These are regarded as presumptive sorbitol-fermenting bifidobacteria.

A9.4 *Confirmation tests*

Depending upon the intended purpose of the analysis, and the required accuracy, sub-culture a suitable number of yellow colonies onto reinforced clostridial agar. Two plates are inoculated for each isolate. If the aim is to estimate the number of organisms present, then for the greatest accuracy, all yellow colonies should be sub-cultured if fewer than ten are present. At least ten yellow colonies should be sub-cultured if more than ten are present. A pair of reinforced clostridia agar plates is also streaked with a stock culture of *Bifidobacterium breve* as a positive control. The time between the exposure of the HBSA plates to ambient air conditions and the beginning of the incubation of the inoculated reinforced clostridial agar plates should be as short as possible and no longer than 2 hours. One of each pair of inoculated plates are inverted and incubated at 37 ºC under aerobic conditions, whilst the second is inverted and incubated at 37 °C under anaerobic conditions. Both sets of plates are incubated for 44 ± 4 hours.

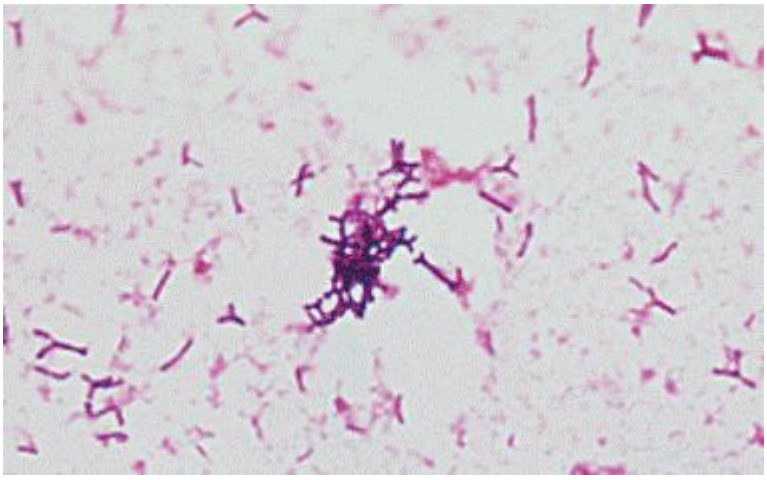
A9.4.1 *Aerobic/anaerobic growth*[5]

After incubation, examine the pairs of plates and record those isolates that grew under anaerobic conditions with growth equivalent to the positive control but with no growth on the plate incubated under aerobic conditions. These isolates only are then subjected to Gram-staining.

A9.4.2 *Gram stain*

Using a sterile loop pick a discrete colony from the anaerobic plate culture and perform a Gram-stain[2] . Bifidobacteria are observed as Gram-variable bifurcating groups of rod-shaped bacteria, typically showing Y and V forms (see Figure 1).

Figure 1 Gram stain of *Bifidobacterium* spp.



A10 Calculations

A10.1 *Presumptive sorbitol-fermenting bifidobacteria*

The number of presumptive sorbitol-fermenting bifidobacteria colonies is generally quoted as the number of colonies per 100 ml. Calculate the presumptive count as follows:

$$\text{Presumptive count/100 ml} = \frac{\text{Number of colonies counted} \times 100 \times \text{DF}}{\text{Volume of sample filtered (ml)}}$$

Where DF is dilution factor if appropriate.

A10.2 *Confirmed sorbitol-fermenting bifidobacteria*

The number of confirmed sorbitol-fermenting bifidobacteria is calculated by multiplying the number of presumptive sorbitol-fermenting bifidobacteria by the proportion of the isolates which are strictly anaerobic and show typical morphology with Gram-staining.

A11 Expression of results

The number of presumptive and confirmed sorbitol-fermenting bifidobacteria is expressed in colony forming units per volume of sample. For most samples the volume is typically 100 ml.

A12 Quality assurance

New batches of isolation medium should be tested with appropriate reference strains of target bacteria (for example *Bifidobacterium breve*) and non-target bacteria (for example *Escherichia coli*). Petri dishes should be incubated for 44 ± 4 hours at 37 °C. New batches of confirmatory media and reagents should be tested with appropriate reference strains of bacteria chosen to verify positive and negative reactions in each case. Petri dishes should be incubated for 44 ± 4 hours at 37 °C. Further details of required quality control are given elsewhere[2] in this series.

13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2016) - Part 1 - Water quality, epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

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

3. Sorbitol-fermenting bifidobacteria as specific indicators of human faecal pollution, *Journal of Applied Bacteriology,* D D Mara, and J I Oragui, 1983, **55**, 349-357.

4. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677, The Stationery Office.

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

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. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

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Standing Committee of Analysts

Members assisting with this method

Without the good will and support given by these individuals and their respective organisations SCA would not be able to continue and produce the highly valued and respected blue book methods.

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