ENVIRONMENT AGENCY

The Microbiology of Drinking Water (2002) - Part 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)

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 six methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157).

A The enumeration of coliform bacteria and *Escherichia coli* by a two membrane filtration technique.
B The enumeration of coliform bacteria and *Escherichia coli* by a single membrane filtration technique.
C The enumeration of coliform bacteria and *Escherichia coli* by a multiple tube most probable number technique.
D The enumeration of coliform bacteria and *Escherichia coli* by a defined substrate most probable number technique.
E The detection of coliform bacteria and *Escherichia coli* by a presence-absence technique.
F The detection of *Escherichia coli* O157:H7 by selective enrichment and immuno-magnetic separation.

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 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
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.

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 enumeration of coliform bacteria and *Escherichia coli* by a two membrane filtration technique

A1 Introduction

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. They provide the most sensitive means for detecting faecal contamination, for assessing the effectiveness of water treatment and disinfection, and for monitoring water quality in distribution. The significance of *E. coli* and coliform 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. Water samples with higher turbidities should be analysed using an appropriate multiple tube most probable number (MPN) method.

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

A3 Definitions

In the context of this method, organisms which are oxidase-negative, produce acid from lactose, and form all shades and sizes of yellow colonies on membrane filters (after incubation for 4 hours at 30 °C followed by 14 hours at 37 °C) are regarded as coliform bacteria.

Coliform bacteria are considered to be members of genera or species within the Family Enterobacteriaceae, capable of growth at 37 °C, that possess β-galactosidase. This definition includes anaerogenic (ie non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Citrobacter, Enterobacter, Escherichia, Hafnia, Klebsiella, Serratia, Yersinia, Buttiauxella* and *Leclercia.*

Organisms which are oxidase-negative, produce acid from lactose, and indole from tryptophan, and form all shades and sizes of yellow colonies on membrane filters (after incubation for 4 hours at 30 °C followed by 14 hours at 37 °C or 44 °C) are regarded as *E. coli.*

For the purposes of water examination, *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44 °C with the production of acid within 24 hours, and which produce indole from tryptophan. Most strains produce β-glucuronidase. Strains which possess these characteristics at 37 °C but do not express them at 44 °C may also be *E. coli.* When identified as *E. coli* they have the same sanitary and operational significance with regard to their faecal origin.

A4 Principle

Paired aliquots of sample are filtered, and the membrane filters (with isolated bacteria) placed on absorbent pads saturated with broth, or placed on agar, containing lactose and phenol red as an indicator of acidity. Isolation of presumptive colonies is followed by confirmation tests for the production of acid from lactose, negative oxidase reaction and, where necessary, indole formation.

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 indicator organisms. The method also allows species of non-coliform organisms to grow, high numbers of which may inhibit growth of coliform bacteria. The maximum number of colonies that should be counted from a single membrane filter is approximately 100.

For treated drinking water it may be convenient to incubate a single membrane filter at 37 °C. In this case, an immediate operational response should be made to any presumptive positive result on the assumption that any colonies isolated might be *E. coli*. Operational decisions should, therefore, not be delayed until confirmation tests for coliform bacteria and *E. coli* have been completed. As presumptive *E. coli* cannot be distinguished from other presumptive coliform bacteria on a single membrane, and may be present in much lower numbers than the other coliform bacteria, all presumptive colonies should be subject to confirmation testing to ensure detection of any *E. coli* that may be present.

A6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[3] 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.

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, either static temperature or temperature cycling) 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 temperatures of 30.0 ± 1.0 °C, 37.0 ± 1.0 °C and 44.0 ± 0.5 °C, or cyclical incubators fitted with timers, capable of attaining these temperatures.

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

A7.4 Sterile membrane filters, for example, white, 47 mm diameter, cellulose-based, 0.45 µm nominal pore size. If broth medium is used then appropriate absorbent pads are required.

A7.5 Smooth-tipped forceps.

A7.6 Water baths (or incubators) set at 37.0 ± 1.0 °C or 44.0 ± 0.5 °C and test tube racks.

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

A8.1 *Membrane lauryl sulphate broth*[4]

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red (0.4% m/v aqueous solution)	50 ml
Sodium lauryl sulphate - specially pure	1 g
Distilled, deionised or similar grade water	1 litre

Add the ingredients to the water and mix gently to avoid the formation of froth. The final pH of the sterile medium should be 7.4 ± 0.2 and, to achieve this, it may be necessary to adjust the pH to about 7.6 before sterilisation. The detection of acid production is influenced by the pH of the medium, thus, it is important that the medium is of the correct pH. Distribute the medium in loosely sealed or screw-capped bottles and autoclave at 115 °C for 10 minutes. The bottles may need to be removed from the autoclave as soon as possible after autoclaving in order to avoid possible breakdown of the lactose and reduction in the pH. When cooled, the screw cap should be more tightly sealed.

The media may be used in an agar form, as membrane lauryl sulphate agar, by the addition of agar (usually 10 - 13 g of a suitable quality) to the above formulation before autoclaving begins. Petri dishes containing the agar medium can be stored for up to one week at temperatures between 2 - 8 °C, protected against dehydration. Storage beyond this time may result in a deterioration of performance of the medium.

The broth medium should be used as soon as possible but can be stored for up to one month at temperatures between 2 - 8 °C. During refrigerated storage, sodium lauryl

sulphate may precipitate out of solution. Before use, allow the broth to come to room temperature and mix well before dispensing into Petri dishes.

A8.2 *Lactose peptone water*

Peptone	10 g
Sodium chloride	5 g
Lactose	10 g
Phenol red (0.4 % m/v aqueous solution)	2.5 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients, except the phenol red indicator solution, in the water and adjust the pH so that the pH of the sterile medium is 7.5 ± 0.2. Add the indicator solution and distribute in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

A8.3 *Tryptone water for the indole test*

The use of certain peptones that give satisfactory results in tests carried out at 37 °C may not be satisfactory for the indole test at 44 °C[5]. Care should, therefore, be taken in the appropriate selection of reagents.

Tryptone	20 g
Sodium chloride	5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.5 ± 0.2. Distribute in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

A8.4 *Kovacs' reagent for the indole test*[6]

p-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol (3-methylbutan-1-ol) (analytical grade reagent free from organic bases)	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the p-dimethylaminobenzaldehyde in the amyl alcohol and slowly add the hydrochloric acid. Protect from light and store at temperatures between 2 - 8 °C. The reagent should be pale yellow or straw-coloured when freshly prepared. Some types of amyl alcohol are unsatisfactory and give a dark colour with the aldehyde.

A8.5 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar (NA), MacConkey agar (MA), oxidase reagent, Ringer's solution and maximum recovery diluent.

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 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. Paired volumes of each sample are filtered and incubated separately at 37 °C and at 44 °C.

A9.2 *Sample processing*

If membrane lauryl sulphate broth is used, for each sample, place an absorbent pad into each of two empty sterile Petri dishes. Aseptically, add sufficient membrane lauryl sulphate broth to saturate the pad, allow the medium to soak into the pad and pour off and discard any excess medium. If this is not done, confluent growth may result.

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 one of the pads saturated with membrane lauryl sulphate broth, or to a Petri dish containing well-dried membrane lauryl sulphate agar (for example, Petri dishes left at room temperature for 2 hours or at 37 °C for 30 minutes, prior to use). 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. Repeat the process with the second volume of sample, transferring the membrane filter to the other saturated pad or second Petri dish containing well-dried membrane lauryl sulphate agar.

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.

Where broth medium is used the dishes should be placed in a sealed container to prevent drying out of the medium. The Petri dishes are inverted and placed in an incubator at 30 °C for 4 hours. One dish is then transferred to an incubator at 37 °C for 14 hours (for coliform bacteria) and the other dish to an incubator at 44 °C for 14 hours (for *E. coli*). Alternatively, cyclical temperature incubators can be used. Accurate temperature control and even temperature distribution are essential. False positive results may be obtained if lower incubation temperatures are used and some organisms may fail to multiply at higher incubation temperatures. If an early indication of a result is required for any reason, the membrane filters may be examined after a total incubation time of 12 hours but must be returned to the incubator for the full incubation period of 18 hours.

A9.3 *Reading of results*

After the total incubation period of 18 hours, examine the membrane filters under good light, if necessary with a hand lens. Colours are liable to change on cooling and standing, hence, within 15 minutes of being removed from the incubator, count all yellow colonies (however faint) irrespective of size. The number of colonies counted on the membrane filter incubated at 37 °C is regarded as the number of presumptive coliform bacteria and the number of colonies counted on the membrane filter incubated at 44 °C is regarded as the number of presumptive *E. coli*. It is important to note whether pink colonies (from non-target organisms) are present in numbers that may interfere with the growth of coliform bacteria. If the growth of pink colonies is considered to be such that they may be obscuring lactose-fermenting colonies, a further sample should be taken and re-submitted for examination. Any portion of sample retained in the refrigerator may be re-examined using an appropriate dilution of the sample, to enable isolated colonies to develop. However, a count of the number of colonies on the membrane filter should not be reported of the re-examined sample, as this may not reflect the number of colonies in the original sample when first examined. In addition, appropriate dilution of the sample, and MPN or presence-absence methods should also be considered.

A9.4 *Confirmation tests*

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of yellow 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. Colonies should always be chosen at random, but to avoid any bias from, for example, unconscious choice of similar colonies, all the colonies in a randomly chosen segment of appropriate size should be sub-cultured. Where a number of colonies of different appearance are clearly distinguishable, a note of the number of each morphological type should also be made. If only a single membrane, incubated at 37 °C, has been analysed, then all presumptive colonies should be sub-cultured for confirmation testing, as the presence of low numbers of *E. coli* could be missed if only a representative number of colonies are tested. The data and information obtained from the sub-cultured isolates are then used to calculate the confirmed counts of coliform bacteria and *E. coli.*

When colonies are sub-cultured for confirmation, they should be tested for confirmation as coliform bacteria and as *E. coli,* whether initially isolated at 37 °C or at 44 °C. This is important because presumptive coliform colonies isolated at 37 °C may confirm as *E. coli*. Conversely, presumptive *E. coli* colonies isolated at 44 °C may not confirm as *E. coli* but may confirm as coliform bacteria. This is particularly important when colonies are isolated on only one of the two membranes incubated at 37 °C and at 44 °C. *E. coli* are coliform bacteria, and if the confirmed count for *E. coli* from the 44 °C membrane filter is greater than the confirmed count for coliform bacteria from the 37 °C membrane filter, then the higher count must be recorded as the confirmed count for coliform bacteria. In the case where, for example, zero coliform bacteria have been isolated at 37 °C but 2 *E. coli* have been isolated at 44 °C then results should be reported as 2 coliform bacteria and 2 *E. coli.*

Colonies for confirmation tests should be sub-cultured as soon as practicable, preferably within 60 minutes, as colony colours can fade after removal of the Petri dishes from the incubator. After counting, Petri dishes may be stored in the appropriate incubator prior to sub-culturing to allow retention of colour and identification of colony.

A9.4.1 *Confirmation for coliform bacteria*

The confirmation procedure outlined is based upon the demonstration of lactose fermentation as being indicative of the possession of β-galactosidase enzyme. Alternative procedures based upon the direct detection of this enzyme, for example using the substrate, ortho-nitrophenyl-β-D-galactopyranoside, may be more appropriate.

From the membrane filter incubated at 37 °C, sub-culture to lactose peptone water (LPW) each colony to be tested and incubate at 37 °C. After 6 hours, the LPW cultures may be sub-cultured to MA and NA in order to check for purity and colonial appearance, and then returned for a further incubation period of 18 hours. Alternatively, large isolated colonies may be sub-cultured to MA and NA direct from

the membrane filter incubated at 37 °C. Examine the LPW after 24 hours for acid production and, if the results are negative, re-examine after a further 24 hour period of incubation. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Incubate the MA and NA plates at 37 °C for 24 hours and carry out an oxidase test on colonies only from the NA plate. Pure cultures are essential for the oxidase test and it may be necessary to make further sub-cultures. These should be made from characteristic coliform colonies obtained from the MA plate.

Typically, coliform bacteria produce pink to red, mucoid or non-mucoid, colonies on MA, often with a halo of precipitation of bile salts. *In situ* oxidase tests carried out directly on colonies on the membrane filter may not be suitable. Oxidase may diffuse from oxidase-positive colonies to adjacent oxidase-negative colonies and oxidase production may be inhibited by acid produced from lactose. Also, further sub-culture may be unsuccessful due to the toxic effects of the oxidase reagent. Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests[7]. Commercial test kits may be used following appropriate performance verification at the laboratory.

Some species of *Bacillus* and *Staphylococcus* may grow on membrane lauryl sulphate broth producing yellow colonies. These can be readily recognised by colony characteristics on MA, and by Gram staining.

A9.4.2 *Confirmation of E. coli*

From the membrane filter incubated at 44 °C sub-culture yellow colonies to two tubes of LPW and one tube of tryptone water (TW). Incubate one of the LPW tubes and the TW tube at 44 °C for 24 hours. After 24 hours, examine for the production of acid in the LPW tube and indole in the TW tube. The other LPW tube is incubated at 37 °C for 6 hours. After the 6 hour incubation, the 37 °C LPW cultures may be sub-cultured to MA and NA in order to check for purity and colonial appearance, and then returned for a further incubation period of 18 hours. Alternatively, large isolated colonies may be sub-cultured to MA and NA direct from the membrane filter incubated at 44 °C. Examine the 37 °C LPW after 24 hours for acid production, and if the results are negative, re-examine after a further 24 hour period of incubation. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Incubate the MA and NA at 37 °C for 24 hours and carry out an oxidase test on colonies only from the NA plate.

In addition, from the membrane filter incubated at 37 °C sub-culture yellow colonies to one tube of LPW and one tube of TW and incubate at 44 °C for 24 hours. After 24 hours, examine for the production of acid in the LPW tube and indole in the TW tube.

Typically, *E. coli* colonies are oxidase-negative, produce acid in LPW at 37 °C and at 44 °C, and produce indole in TW at 44 °C. Tests for β-glucuronidase may assist in the early confirmation of *E. coli* [8, 9]. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

A9.4.2.1 *Indole test*

After incubation of the TW tubes at 44 °C, add 0.2 - 0.3 ml of Kovacs' reagent. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer.

A9.4.3 *Oxidase test*

Some organisms that are found in water may conform to the definition of coliform bacteria in most respects, but are able to produce acid from lactose only at temperatures below 37 °C. *Aeromonas* species, which occur naturally in water, possess optimum growth at temperatures between 30 - 35 °C but may produce acid from lactose at 37 °C. These organisms are of uncertain public health significance and are distinguishable from coliform bacteria by a positive oxidase reaction. The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA. Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the NA onto the treated 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 at the laboratory.

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

A10 Calculations

A10.1 *Presumptive coliform bacteria and E. coli*

The number of presumptive coliform bacteria and *E. coli* is generally expressed as the number of colonies per 100 ml of sample. Calculate the presumptive count as follows:

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

The count from the 37 °C incubation is regarded as presumptive coliform bacteria and that from the 44 °C incubation as presumptive *E. coli*.

A10.2 *Confirmed coliform bacteria and E. coli*

The number of confirmed coliform bacteria is calculated by multiplying the number of presumptive coliform bacteria by the proportion of the isolates that are both lactose-positive (in LPW) and oxidase-negative.

The number of confirmed *E. coli* is calculated by multiplying the number of presumptive *E. coli* by the proportion of the isolates that are lactose-positive (in LPW), produce indole from TW at 44 °C and are oxidase-negative.

On rare occasions, a significant number of isolates from the 37°C incubation may confirm as *E. coli* and the count calculated may be higher that that calculated for the 44 °C incubation. For these examples, the higher count from the 37°C incubation should be reported.

A11 Expression of results

Counts for presumptive and confirmed coliform bacteria and *E. coli* are expressed in colony forming units per volume of sample. For drinking water, the volume is typically 100 ml.

A12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example *E. coli* and *Enterobacter aerogenes*) and non-target bacteria (for example *Pseudomonas aeruginosa*). Petri dishes should be incubated for 24 hours at 37 °C or 44 °C as appropriate. 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.

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5. The standardisation and selection of bile salt and peptone for culture media used in the bacteriological examination of water. *Proceedings of the Society for Water Treatment and Examination*, Burman, N.P., 1955, **4**, 10-26.

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7. *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.

8. Fluorogenic assay for immediate confirmation of *Escherichia coli. Applied and Environmental Microbiology*, Feng, P.C.S. & Hartman, P.A., 1982, **43**, 1320-1329.

9. Glycosidase profiles of members of the family Enterobacteriaceae. *Journal of Clinical Microbiology*, Kampfer, P., Rauhoff, O. & Dott, W., 1991, **29**, 2877-2879.

B The enumeration of coliform bacteria and *Escherichia coli* by a single membrane filtration technique

B1 Introduction

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. They provide the most sensitive means for detecting faecal contamination, for assessing the effectiveness of water treatment and disinfection, and for monitoring water quality in distribution. The significance of *E. coli* and coliform bacteria 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. Water samples with higher turbidities should be analysed using an appropriate multiple tube most probable number (MPN) method.

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

B3 Definitions

In the context of this method, organisms which are oxidase-negative, produce acid from lactose, and form all shades and sizes of yellow colonies on membrane filters (after incubation for 4 hours at 30 °C followed by 14 hours at 37 °C) are regarded as coliform bacteria. In addition, organisms, which conform to the definition of *E. coli* below, are also coliform bacteria.

Coliform bacteria are considered to be members of genera or species within the Family Enterobacteriaceae, capable of growth at 37 °C, that possess β-galactosidase. This definition includes anaerogenic (ie non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Citrobacter, Enterobacter, Escherichia, Hafnia, Klebsiella, Serratia, Yersinia, Buttiauxella* and *Leclercia.*

Isolates which produce acid from lactose and produce β-glucuronidase forming green colonies after incubation for 4 hours at 30 °C followed by 14 hours at 37 °C are regarded as *E. coli*. Most strains of *E. coli* express β-glucuronidase, as do some strains of *Shigella* and *Salmonella*.

For the purposes of water examination *E. coli* have historically been regarded as members of the Family Enterobacteriaceae which ferment lactose or mannitol at 44 °C with the production of acid within 24 hours, and which produce indole from tryptophan. Strains which possess these characteristics at 37 °C but do not express them at 44 °C may also be *E. coli.* When identified as *E. coli* they have the same sanitary and operational significance with regard to their faecal origin.

B4 Principle

Organisms are isolated on a membrane filter placed on an agar medium containing lactose, phenol red as an indicator of acidity, and the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) either as the cyclohexylammonium or sodium salt for the indication of the production of β-glucuronidase. Isolation of colonies is followed by confirmation tests for acid production from lactose, negative oxidase reaction and, where necessary, indole formation.

B5 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. Accumulated deposit on the membrane filter may mask or inhibit the growth of indicator organisms. The method also allows species of non-coliform organisms to grow, high numbers of which may inhibit growth of coliform bacteria. The maximum number of colonies that should be counted from a single membrane is 100. The growth of high numbers of coliform and non-coliform bacteria from untreated waters may inhibit the production of β-glucuronidase by *E. coli*.

B6 Health and safety

Media, reagents and bacteria used in this method are covered by the Control of Substances Hazardous to Health Regulations[3] 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.

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 temperature 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 30.0 ± 1.0 °C and 37.0 ± 1.0 °C, or cycling incubators, fitted with timers, capable of attaining these temperatures.

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 0.45 μm nominal pore size.

B7.5 Smooth-tipped forceps.

B7.6 Water baths (or incubators) set at 37.0 ± 1.0 °C or 44.0 ± 0.5 °C and test tube racks.

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

B8.1 *Membrane lactose glucuronide agar*[4, 5]

Peptone	40 g
Yeast extract	6 g
Lactose	30 g
Phenol red (0.4% m/v solution)	50 ml
Sodium lauryl sulphate	1.0 g
Sodium pyruvate	0.5 g
Agar	10.0 g
BCIG	0.2 g
Distilled, deionised or similar grade water	1 litre

Suspend the ingredients, except BCIG, in the water and bring to the boil to dissolve. Dissolve the cyclohexylammonium salt of BCIG in 3 ml of solution consisting of 2.5 ml of 95% v/v aqueous ethanol and 0.5 ml of 1 molar sodium hydroxide solution. Add this solution to the medium. The sodium salt of BCIG can be added directly to the medium. Mix the solution well and autoclave at 121 °C for 15 minutes. Allow the solution to cool, distribute in Petri dishes and allow to solidify. Petri dishes containing the agar medium may be stored at temperatures between 2 - 8 °C for up to one week, protected against dehydration. Storage beyond this time may result in a deterioration of performance of the medium. The pH after sterilisation should be 7.4 ± 0.2. The detection of acid production is influenced by the pH of the medium, thus, it is important that the medium is of the correct pH.

B8.2 *Lactose peptone water*

Peptone	10 g
Sodium chloride	5 g
Lactose	10 g
Phenol red (0.4 % m/v aqueous solution)	2.5 ml
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients, except the phenol red indicator solution, in the water and adjust the pH so that the pH of the sterile medium is 7.5 ± 0.2. Add the indicator solution and distribute in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

B8.3 *Tryptone water for the indole test*

The use of certain peptones that give satisfactory results in tests carried at 37 °C may not be satisfactory for the indole test at 44 °C[6]. Care should, therefore, be taken in the appropriate selection of reagents.

Tryptone	20 g
Sodium chloride	5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH so that the pH of the sterile medium is 7.5 ± 0.2. Distribute in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored for up to one month at temperatures between 2 - 8 °C.

B8.4 *Kovacs' reagent for the indole test*[7]

p-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol (3-methylbutan-1-ol)	75 ml
(analytical grade reagent free from organic bases)	
Hydrochloric acid (concentrated)	25 ml

Dissolve the aldehyde in the amyl alcohol and slowly add the acid. Protect from light and store at 2 - 8 °C. The reagent should be pale-yellow or straw-coloured after preparation. Some types of amyl alcohol are unsatisfactory and give a dark colour with the aldehyde.

B8.5 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar (NA), MacConkey agar (MA), oxidase reagent, Ringer's solution and maximum recovery diluent.

B9 Analytical procedure

B9.1 *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 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.

B9.2 *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 Petri dish containing well-dried membrane lactose glucuronide agar (for example, Petri dishes left at room temperature for 2 hours or at 37 °C for 30 minutes, prior to use). 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.

The Petri dishes are inverted and placed in an incubator at 30 °C for 4 hours then transferred to an incubator at 37 °C for 14 hours. Alternatively, a cycling temperature incubator can be used. Accurate temperature control and even temperature distribution are essential. False positive results may be obtained if lower incubation temperatures are used and some organisms may fail to multiply at higher incubation temperatures. If an early indication of a result is required for any reason, the membrane filter may be examined after a total incubation time of 12 hours but must be returned to the incubator for the full incubation period of 18 hours.

B9.3 *Reading of results*

After the total incubation period of 18 hours, examine the membrane filters under good light, if necessary with a hand lens. Count all yellow and green colonies (however faint) irrespective of size within 15 minutes of being removed from the incubator, as the yellow coloration may change on cooling and standing. All yellow colonies are presumptive non-*E. coli* coliform bacteria and green colonies are *E. coli*. The total combined count of yellow and green colonies is regarded as the number of coliform bacteria. It is important to note whether pink colonies (from non-target organisms) are present in numbers that may interfere with the growth of coliform bacteria and *E. coli*. If the growth of pink colonies is considered to be such that they may be obscuring lactose-fermenting colonies, a further sample should be taken and re-submitted for examination. Any portion of sample retained in the refrigerator may be re-examined using an appropriate dilution of the sample, to enable isolated colonies to develop. However, a count of the number of colonies on the membrane filter should not be reported of the re-examined sample, as this may not reflect the number of colonies in the original sample when first examined. In addition, appropriate dilution of the sample and MPN or presence-absence methods should also be considered.

B9.4 *Confirmation tests*

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of yellow and green colonies (however faint). The specificity of the green colonies on membrane lactose glucuronide agar being *E. coli* is very high, and, following suitable confirmation of performance within the laboratory, confirmation of green colonies may not be needed. Occasionally, blue colonies may be noted and recorded. These colonies may be lactose-negative *E. coli*, but are more commonly strains of *Aeromonas*. Blue colonies should, therefore, initially be classed as presumptive coliform bacteria and be subjected to confirmatory testing. 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 are present. Colonies should always be chosen at random, but to avoid any bias from, for example, unconscious choice of similar colonies, all the colonies in a randomly chosen segment of appropriate size should be sub-cultured. Where a number of colonies of different appearance are clearly distinguishable, a note of the number of each morphological type should also be made. The data and information from the sub-cultured isolates are then used to calculate the confirmed counts of coliform bacteria and *E. coli*.

When colonies are sub-cultured for confirmation, they should be tested for confirmation as coliform bacteria and as *E. coli*. This is important because yellow colonies may confirm as *E. coli* (as some strains do not express β-glucuronidase, and other strains appear negative when first isolated). Occasionally, green (presumptive *E. coli*) colonies may not confirm as *E. coli* but may, nevertheless, confirm as coliform bacteria.

Colonies for confirmation tests should be sub-cultured as soon as practicable, preferably within 60 minutes, as colony colours can fade after removal of the Petri

dishes from the incubator. After counting, Petri dishes may be stored in the incubator prior to sub-culturing to allow retention of colour and identification of colony.

B9.4.1 *Confirmation for coliform bacteria*

The confirmation procedure outlined is based upon demonstration of lactose fermentation as being indicative of possession of the β-galactosidase enzyme. Alternative procedures based upon the direct detection of this enzyme, for example using the substrate, ortho-nitrophenyl-β-D-galactopyranoside, may be more appropriate.

Sub-culture to lactose peptone water (LPW) each colony to be tested and incubate at 37 °C. After 6 hours, the LPW cultures may be sub-cultured to MA and NA in order to check for purity and colonial appearance, and then returned for a further incubation period of 18 hours. Alternatively, large isolated colonies may be sub-cultured to MA and NA direct from the membrane filter. Examine the LPW after 24 hours for acid production and, if the results are negative, re-examine after a further 24 hour period of incubation. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Incubate the MA and NA plates at 37 °C for 24 hours and carry out an oxidase test on colonies only from the NA plate. Pure cultures are essential for the oxidase test and it may be necessary to make further sub-cultures. These should be made from characteristic coliform colonies obtained from the MA plate.

Typically, coliform bacteria produce pink to red, mucoid or non-mucoid, colonies on MA, often with a halo of precipitation of bile salts. *In situ* oxidase tests carried out directly on colonies on the membrane filter may not be suitable. Oxidase may diffuse from oxidase-positive colonies to adjacent oxidase-negative colonies and oxidase production may be inhibited by acid produced from lactose. Also, further sub-culture may be unsuccessful due to the toxic effects of the oxidase reagent. Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests[8]. Commercial test kits may be used following appropriate performance verification at the laboratory.

Some species of *Bacillus* and *Staphylococcus* may grow on membrane lactose glucuronide agar producing yellow colonies. These can be readily recognised by colony characteristics on MA, and by Gram staining.

B9.4.2 *Confirmation of E. coli*

The specificity of membrane lactose glucuronide agar for *E. coli* is such that, following performance verification within the laboratory, confirmation of green colonies as *E. coli* may not be required.

Sub-culture yellow and, if required, green colonies from the membrane filter to two tubes of LPW and one tube of tryptone water (TW). Incubate one of the LPW tubes and the TW tube at 44 °C for 24 hours. After 24 hours, examine for the production of acid in the LPW tube and indole in the TW tube. The other LPW tube is incubated at 37 °C for 6 hours. After the 6 hour incubation, the 37 °C LPW cultures may be sub-cultured to MA and NA in order to check for purity and colonial appearance, and then

returned for a further incubation period of 18 hours. Alternatively, large isolated colonies may be sub-cultured to MA and NA direct from the membrane filter. Examine the 37 °C LPW after 24 hours for acid production, and if the results are negative, re-examine after a further 24 hour period of incubation. Confirmation of acid production is demonstrated by the change of colour from red to yellow. Incubate the MA and NA at 37 °C for 24 hours and carry out an oxidase test on colonies only from the NA plate.

Typically, *E. coli* colonies are oxidase-negative, produce acid in LPW at 37 °C and at 44 °C, and indole in TW at 44 °C. Tests for β-glucuronidase may assist in the early confirmation of *E. coli* [9, 10]. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

B9.4.2.1 *Indole test*

After incubation of the TW tubes at 44 °C add 0.2 - 0.3 ml of Kovacs' reagent. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer.

B9.4.3 *Oxidase test*

Some organisms that are found in water may conform to the definition of coliform bacteria in most respects, but are able to produce acid from lactose only at temperatures below 37 °C. *Aeromonas* species, which occur naturally in water, possess optimum growth at temperatures between 30 - 35 °C but may produce acid from lactose at 37 °C. These organisms are of uncertain public health significance and are distinguishable from coliform bacteria by a positive oxidase reaction. The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA. Place 2 - 3 drops (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the NA onto the treated 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 manufacturers' instructions and following appropriate performance verification at the laboratory.

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

B10 Calculations

B10.1 *Presumptive coliform bacteria and E. coli*

The number of presumptive coliform bacteria and *E. coli* is generally expressed as the number of colonies per 100 ml of sample. Calculate the presumptive count as follows: