ENVIRONMENT AGENCY

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)

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:H7).

A The enumeration of coliform bacteria and *Escherichia coli* by a two membrane filtration technique using membrane lauryl sulphate broth or agar incubated at 37 °C and 44 °C.

B The enumeration of coliform bacteria and *Escherichia coli* by a single membrane filtration technique using membrane lactose glucuronide agar incubated at 37 °C.

C The enumeration of coliform bacteria and *Escherichia coli* by a multiple tube most probable number technique using minerals modified glutamate medium incubated at 37 °C.

D The enumeration of coliform bacteria and *Escherichia coli* by a defined substrate most probable number technique incubated at 37 °C.

E The detection of coliform bacteria and *Escherichia coli* by a presence-absence technique using minerals modified glutamate medium incubated at 37 °C.

F The detection of *Escherichia coli* O157:H7 by selective enrichment and immuno-magnetic separation.

This bluebook updates and replaces the earlier version published in 2002. Included in the annex of this document are details of confirmation techniques for coliform bacteria and *Escherichia coli* using a tryptone nutrient agar plate method compared with sub-culture to lactose peptone water and tryptone water.

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

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
Part 3 - Practices and procedures for laboratories
Part 5 - The isolation and enumeration of enterococci by membrane filtration
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
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 5 - The isolation and enumeration of enterococci by membrane filtration
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
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 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 14 - Methods for the isolation, identification and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts

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. Users should ensure they are aware of the most recent version of the draft they seek.

Dr D Westwood
Secretary
January 2006

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.

A The enumeration of coliform bacteria and *Escherichia coli* by a two membrane filtration technique using membrane lauryl sulphate broth or agar incubated at 37 °C and 44 °C

A1 Introduction

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. These tests provide a sensitive means for detecting faecal contamination, for assessing raw water quality, 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 or express β-galactosidase, and form all shades and sizes of yellow colonies on membrane filters (after incubation at 30 °C for 4 hours followed by incubation at 37 °C for 14 hours) 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 (i.e. non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia, Klebsiella*, *Leclercia*, *Pantoea*, *Raoultella*, *Serratia*, and *Yersinia.*

Organisms which are oxidase-negative, produce acid from lactose or express β-galactosidase, and produce indole from tryptophan, and form all shades and sizes of yellow colonies on membrane filters (after incubation at 30 °C for 4 hours followed by incubation at 37 °C or 44 °C for 14 hours) 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 (together with the filtered 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 (or expression of β-galactosidase) 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 some species of non-coliform bacteria to grow, which when present in high numbers may inhibit the 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, rather than two membrane filters at 37 °C and 44 °C. In these cases, an immediate operational response should be made to any presumptive result. It should be assumed that any colonies isolated might be *E. coli*. Operational decisions should not be delayed whilst awaiting completion of confirmatory tests for coliform bacteria and/or *E. coli*. 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 other coliform bacteria, all presumptive colonies should be subject to confirmatory 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 sodium thiosulphate pentahydrate 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/or 44.0 ± 0.5 °C, or cyclical incubators fitted with timers, capable of attaining these temperatures.

A7.3 Filtration apparatus, sterile filter funnels or filter funnels that can be sterilised, and vacuum source.

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

A7.5 Smooth-tipped forceps.

A7.6 Water baths (or incubators) set at 30.0 ± 1.0 °C, 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 formulation. Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the 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 the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

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
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 value. Distribute the medium in screw-capped bottles, loosely seal 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 value. When cooled, the screw cap should be tightened.

The media may be used in an agar form, as membrane lauryl sulphate agar, by dissolving bacteriological agar (usually 10 - 13 g) in the above formulation before autoclaving begins. Petri dishes containing the agar medium can be stored in the range 5 ± 3 °C for up to one week, protected against dehydration. Storage beyond this time may result in deterioration of the performance of the medium.

The broth medium should be used as soon as possible but can be stored in the range 5 ± 3 °C for up to one month. During refrigerated storage, sodium lauryl sulphate may precipitate out of solution. Before use, allow the broth to reach 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
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, mix well and distribute the medium in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored in the range 5 ± 3 °C for up to one month.

A8.3 *Tryptone water*

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 the tryptone used.

Tryptone	20 g
Sodium chloride	5 g
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 the medium in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored in the range 5 ± 3 °C for up to one month.

A8.4 *Tryptone nutrient agar*

Beef extract powder	1 g
Yeast extract	2 g
Peptone	5 g
Tryptone	20 g
Sodium chloride	5 g
Agar	15 g
Water	1 litre

Dissolve the ingredients in the water. The final pH of the sterile medium should be 7.4 ± 0.2. Sterilise by autoclaving at 121 °C for 15 minutes. Bulk sterile medium may be stored in the dark at room temperature for up to one month. Alternatively, allow the medium to cool to about 50 °C, distribute it in Petri dishes and allow the medium to solidify. Petri dishes containing tryptone nutrient agar (TNA) medium may be stored in the range 5 ± 3 °C for up to one month, protected against dehydration. If bulk medium is used, heat the medium to melt the agar, distribute it into Petri dishes and allow the medium to solidify.

Alternative nutrient media may be used, for example yeast extract agar or blood agar base, and supplemented with tryptone (to a concentration of 20 g/l) but nutrient media containing fermentable carbohydrates should not be used. Alternative media would need to be validated accordingly.

A8.5 *Kovàcs' reagent*[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. Mix well. The reagent should be pale yellow or straw-coloured when freshly prepared and may be stored in the dark in the range 5 ± 3 °C for up to six months. Depending on the grade, some batches of amyl alcohol may be unsatisfactory and produce a dark colour with the p-dimethylaminobenzaldehyde.

A8.6 *Modified Kovàcs' reagent*[7]

p-Dimethylaminobenzaldehyde	5.0 g
Ethyl alcohol	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the p-dimethylaminobenzaldehyde in the ethyl alcohol and slowly add the hydrochloric acid. Mix well. The reagent should be a pale yellow colour when freshly prepared and may be stored in the dark in the range 5 ± 3 °C for up to six months.

A8.7 *ONPG discs*

These are small discs impregnated with ortho-nitrophenol-β-D-galactopyranoside (ONPG). Not all commercially available ONPG discs may be suitable for this method and discs should be validated before use. See Appendix 1.

A8.8 *Other media*

Standard and commercial formulations of other media and reagents used in this method include Gram stain reagents, nutrient agar (NA), MacConkey agar (MA), oxidase reagent, Ringer's solution, quarter strength 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 MLSB or MLSA 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 filter smaller volumes, or dilute the sample with Ringer's solution or maximum recovery diluent before filtration. The minimum volume filtered should not be less than 1 ml. This is to ensure that colonies are evenly spread across the membrane filter and the volume filtered is still representative of the sample submitted. If smaller volumes are required to ensure the count of colonies lies within the above range, then appropriate dilutions should be made. Paired volumes of each sample should be 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 discard any excess medium. If this is not carried out, confluent growth on the membrane filter 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, 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 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 the 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 one of the pads saturated with membrane lauryl sulphate broth, or to a Petri dish containing membrane lauryl sulphate agar, which should be well-dried (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 incubated at 30 °C for 4.00 ± 0.25 hours. One dish is then incubated at 37 °C for a minimum of 14 hours (to assess growth of coliform bacteria). The other dish is incubated at 44 °C for a minimum of 14 hours (to assess growth of *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, for any reason, an early indication of a result is required, the membrane filters incubated at 37 °C or 44 °C may be examined after approximately 8 hours, but following this examination membrane filters must be returned to the incubator at 37 °C or 44 °C for the full minimum incubation period of 14 hours prior to counting.

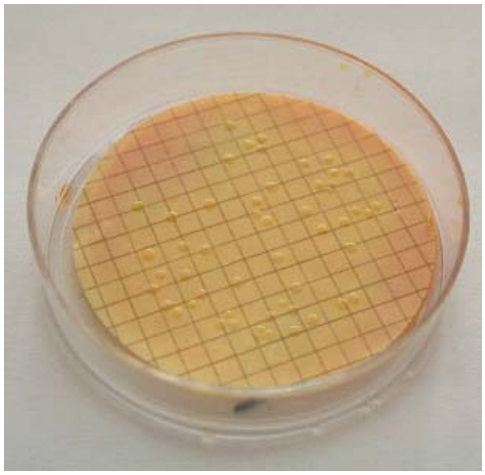
A9.3 *Reading of results*

After the total incubation period of 18 hours, examine the MLSB or MLSA 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 (see Figure A1). The number of colonies counted on the MLSB or MLSA membrane filter incubated at 37 °C is regarded as the number of presumptive coliform bacteria and the number of colonies counted on the MLSB or MLSA 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 presumptive coliform bacteria and *E. coli*. If the growth of pink colonies is considered to be such that they obscure lactose-fermenting colonies, a further sample should be taken and re-submitted for examination and this fact recorded. Alternatively, any portion of sample retained in a refrigerator may be re-examined using an appropriate dilution of the sample, to enable isolated colonies to develop. However, whilst a count of the number of colonies (of the re-examined sample) on the MLSB or MLSA membrane filter may be made, this value should not be reported and used to assess water quality, as this may not reflect the number of colonies in the original sample when first examined. In addition to appropriate dilution of the sample, MPN or presence-absence methods should also be considered.

Figure A1 Yellow colonies on membrane lauryl sulphate broth

Yellow colonies of presumptive coliform bacteria incubated at 37 °C

Yellow colonies of presumptive *E. coli* incubated at 44 °C



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 of the examination 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 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. Where one type of colonial species greatly exceeds another type, random choosing of colonies may result in the failure to culture the less frequently occurring species. In these cases, additional considerations should be given to choosing all colonial species. If only a single membrane, incubated at 37 °C, is to be or has been analysed, then all colonies should be sub-cultured for confirmatory testing, as the presence of low numbers of *E. coli* could be missed if only a representative number of all the colonies present 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 confirmatory testing, 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 confirmatory testing 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. Petri dishes should be stored in the appropriate incubator prior to sub-culturing, if confirmation is not undertaken immediately after counting.

Coliform bacteria can be confirmed by testing for lactose fermentation and the production of acid in lactose peptone water (LPW) at 37 °C and at 44 °C, as being indicative of the possession of the β-galactosidase enzyme, and for the absence of the oxidase enzyme. Also, *E. coli* can be similarly confirmed but with the inclusion of a test for indole production in tryptone water (TW) at 44 °C. See flow chart A1. Alternatively, these bacteria can be confirmed by testing directly for β-galactosidase at 37 °C and at 44 °C and indole production at 44 °C from growth on nutrient agar supplemented with tryptone (i.e. TNA) with a disc containing ortho-nitrophenyl-β-D-galactopyranoside (ONPG) on the agar surface. See flow chart A2.

A9.4.1 *Confirmation of coliform bacteria and E. coli using LPW and TW*

From the MLSB or MLSA membrane filters incubated at 37 °C and 44 °C, sub-culture each colony to be tested to

(i) LPW and incubate at 44 °C for 21 ± 3 hours, and
(ii) TW and incubate at 44 °C for 21 ± 3 hours.
(iii) If colonies on MLSB or MLSA membrane filters are small, sub-culture to LPW and incubate at 37 °C for approximately 6 hours. After approximately 6 hours of incubation, from the LPW tube or bottle incubated at 37 °C, sub-culture to MA and NA. Incubate the MA and NA at 37 °C for up to 24 hours. This is to check for purity and colonial appearance. After sub-culturing, the LPW is returned to the incubator at 37 °C for a further incubation period of 15 hours. Alternatively, if colonies on MLSB or MLSA membrane filters are large, sub-culture directly to MA and NA, and to LPW. Incubate the MA and NA at 37 °C for up to 24 hours. This is to check for purity and colonial appearance. Incubate the LPW at 37 °C for 21 hours.

After 21 hours of incubation at 37 °C, the LPW is examined for acid production. If no colour change is exhibited, i.e. the results are negative, the LPW is returned to the incubator at 37 °C and incubated for a further 24 hour period, and then re-examined for acid production. Confirmation of acid production is demonstrated by a change of colour from red to yellow (see Figure A2).

Figure A2 Demonstration of lactose fermentation in lactose peptone water

No fermentation exhibited, i.e. no colour change, regard as negative

Fermentation exhibited, i.e. colour change from red to yellow, regard as positive

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, exhibit 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.

Following incubation of MA and NA Petri dishes at 37 °C for up to 24 hours, examine the MA and NA to ascertain whether pure isolates develop. If the culture is pure, carry out an oxidase test on colonies only from NA. Pure cultures are essential for the oxidase test and it may be necessary to make further sub-cultures. If MA or NA exhibit mixed colonies, sub-culture a representative number of each lactose-fermenting colony from MA to NA. Incubate the NA at 37 °C for up to 24 hours.

In situ oxidase tests carried out directly on colonies on the MLSB or MLSA membrane filter may not be suitable. Oxidase may diffuse from oxidase-positive colonies to adjacent

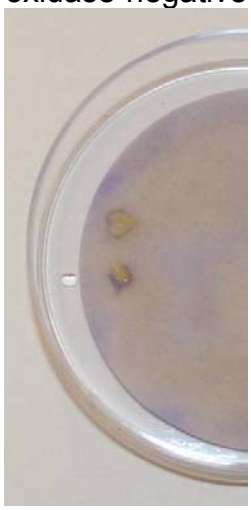
oxidase-negative colonies and oxidase production may be inhibited by acid produced from lactose. Also, further sub-culture may be unsuccessful due to the toxic effects of the oxidase reagent.

Typically, coliform bacteria produce large pink to red, mucoid or non-mucoid, colonies on MA, often with a halo of precipitation of bile salts. Some species of *Bacillus*, *Enterococcus* and *Staphylococcus* may grow on MLSB or MLSA membrane filters producing very small opaque yellow colonies. *Bacillus*, *Enterococcus* and *Staphylococcus* can be readily recognised by colony characteristics on MA and by Gram staining. If *Bacillus*, *Enterococcus* and *Staphylococcus* grow on MA, they produce very small opaque red or colour-less colonies. *Bacillus*, *Enterococcus* and *Staphylococcus* are Gram-positive, whereas coliform bacteria are Gram-negative bacilli.

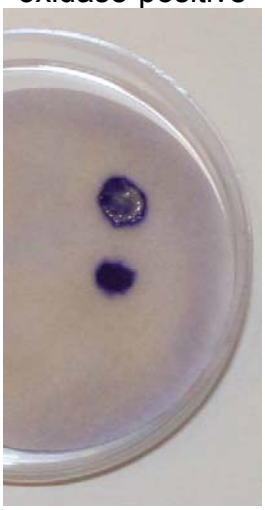
The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA. Place 2 - 3 drops, typically 0.1 - 0.2 ml (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth from the NA incubated at 37 °C onto the treated filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction. See Figure A3.

Figure A3 Oxidase test

Escherichia coli
oxidase-negative



Pseudomonas aeruginosa
oxidase-positive



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*).

Examine the LPW incubated at 44 °C for 21 ± 3 hours for the production of acid, see Figure A2. Examine the TW incubated at 44 °C for 21 ± 3 hours for the production of indole by adding 2 - 3 drops, typically 0.1 - 0.2 ml of Kovàcs' reagent. Shake well and allow to settle. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer (see Figure A4).

Figure A4 Demonstration of indole production in tryptone water using Kovàcs' reagent

No colour developed
i.e. regard as negative

Red colour developed
i.e. regard as positive

Typically, coliform bacteria are oxidase-negative and produce acid from lactose at 37 °C and may produce acid from lactose at 44 °C. Typically, *E. coli* 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] and may differentiate other species of coliform bacteria which exhibit the same confirmation profile as *E. coli* (for example *Klebsiella* spp). Thermotolerant (i.e. "faecal") coliform bacteria are oxidase-negative and produce acid from lactose at 44 °C, and are indole-negative.

Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests[10]. Commercial test kits may be used following appropriate performance verification at the laboratory.

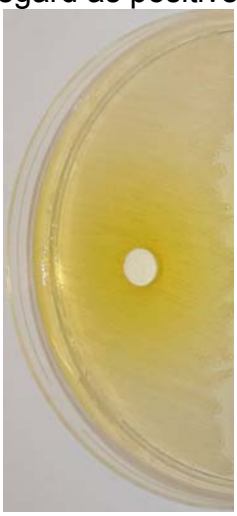
A9.4.2 *Confirmation of coliform bacteria and E. coli using TNA*

Coliform bacteria and *E. coli* can also be confirmed by demonstration of production of β-galactosidase, indole production (for *E. coli*) and lack of oxidase production, following sub-culture to Petri dishes containing TNA. Data on the verification of the performance of these confirmation procedures are given in Appendix 1. Thermotolerant coliform bacteria are oxidase-negative and produce β-galactosidase at 44 °C, and are indole-negative.

From the MLSB or MLSA membrane filters incubated at 37 °C and 44 °C, sub-culture each yellow colony to be tested to two Petri dishes containing TNA. Aseptically place an ONPG disc onto each area of sub-culture. Incubate one TNA Petri dish at 37 °C for 21 ± 3 hours and the other TNA Petri dish at 44 °C for 21 ± 3 hours. After incubation, examine the TNA dish incubated at 37 °C for the presence of yellow colouration around the ONPG disc. Confirmation of expression of β-galactosidase is demonstrated by the production of yellow colouration (see Figure A5).

Figure A5 Demonstration of β-galactosidase production on tryptone nutrient agar with ONPG discs

Yellow colour developed
i.e. regard as positive



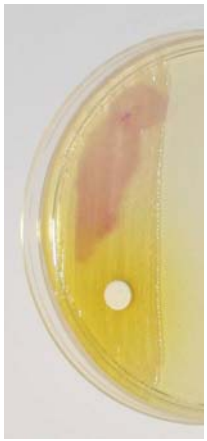
No colour developed
i.e. regard as negative

Growth on the TNA dish incubated at 37 °C may also be used for the oxidase test, provided it is pure. Pure cultures are essential for the oxidase test (see A9.4.1 and Figure A3) and if mixed colonies develop, it may be necessary to sub-culture representative colonies to Petri dishes containing MA and incubating at 37 °C for up to 24 hours, with subsequent sub-culture of representative lactose fermenting colonies to TNA for confirmation.

Examine the TNA dish incubated at 44 °C for the expression of β-galactosidase (see Figure A5). Test for indole production by adding 2 - 3 drops, typically 0.1 - 0.2 ml of either Kovàcs' reagent (A8.5) or modified Kovàcs' reagent (A8.6) to an area of growth. Use of the modified Kovàcs' reagent avoids exposure to amyl alcohol fumes. Indole production is demonstrated by the rapid appearance of a red colour (see Figures A6 and A7).

Figure A6 Demonstration of indole production with Kovàcs' reagent on tryptone nutrient agar

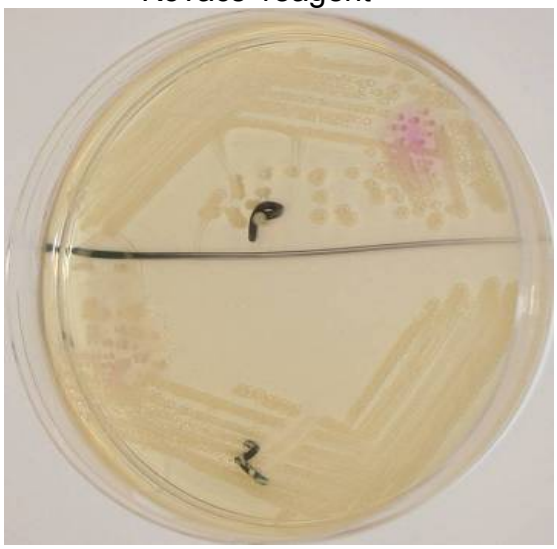
Escherichia coli
ONPG-positive and indole-positive



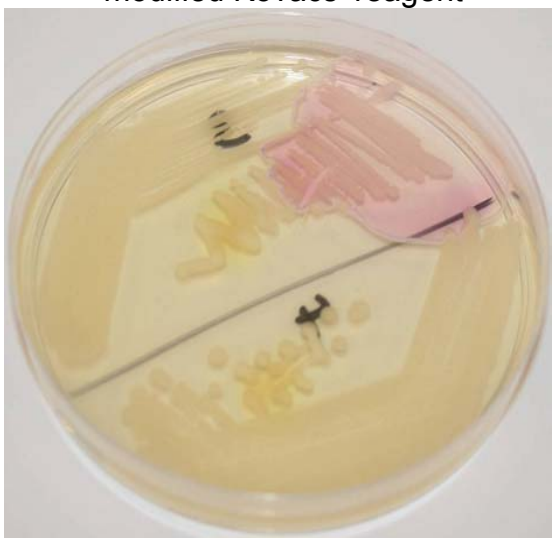
Pseudomonas aeruginosa
ONPG-negative and indole-negative

Figure A7 Demonstration of indole production on tryptone nutrient agar

Red colour developed with Kovàcs' reagent



Red colour developed with modified Kovàcs' reagent



Further identification may be carried out using characteristic colonies on TNA or MA by means of appropriate biochemical and other tests[10]. Commercial test kits may be used following appropriate performance verification at the laboratory.

A10 Calculations

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

The count from the MLSB or MLSA membrane filter for the 37 °C incubation is regarded as presumptive coliform bacteria, and that from the MLSB or MLSA membrane filter for the 44 °C incubation is regarded as presumptive *E. coli*. The number of presumptive coliform bacteria and *E. coli* is generally expressed as the number of colonies per 100 ml of sample. Taking into account any dilution (as necessary) the presumptive count is:

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

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 lactose-positive (in LPW) or β-galactosidase-positive, 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) or β-galactosidase-positive, produce indole at 44 °C, and are oxidase-negative.

On rare occasions, a significant number of isolates from the MLSB or MLSA membrane filter incubated at 37 °C may confirm as *E. coli* and the count calculated may be higher than that calculated from the MLSB or MLSA membrane filter incubated at 44 °C. For these samples, 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 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.

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4. Membrane filtration media for the enumeration of coliform bacteria and *Escherichia coli* in water: comparison of Tergitol 7 and lauryl sulphate with Teepol 610, by a Joint Committee of the Public Health Laboratory Service and Standing Committee of Analysts. *Journal of Hygiene*, 1980, **85**, 181-191.

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6. Eine vereinfachte Methode zum Nachweis der Indolbildung durch Bakterien. *Zeitschrift fur Immunitatsforschung und experimentelle Therapie*, N Kovàcs, 1928, **55**, 311-315.

7. Membrane filter procedure for enumerating the component genera of the coliform group in seawater. *Applied Microbiology,* A P Dufour & V J Cabelli, 1975, **29**, 826.

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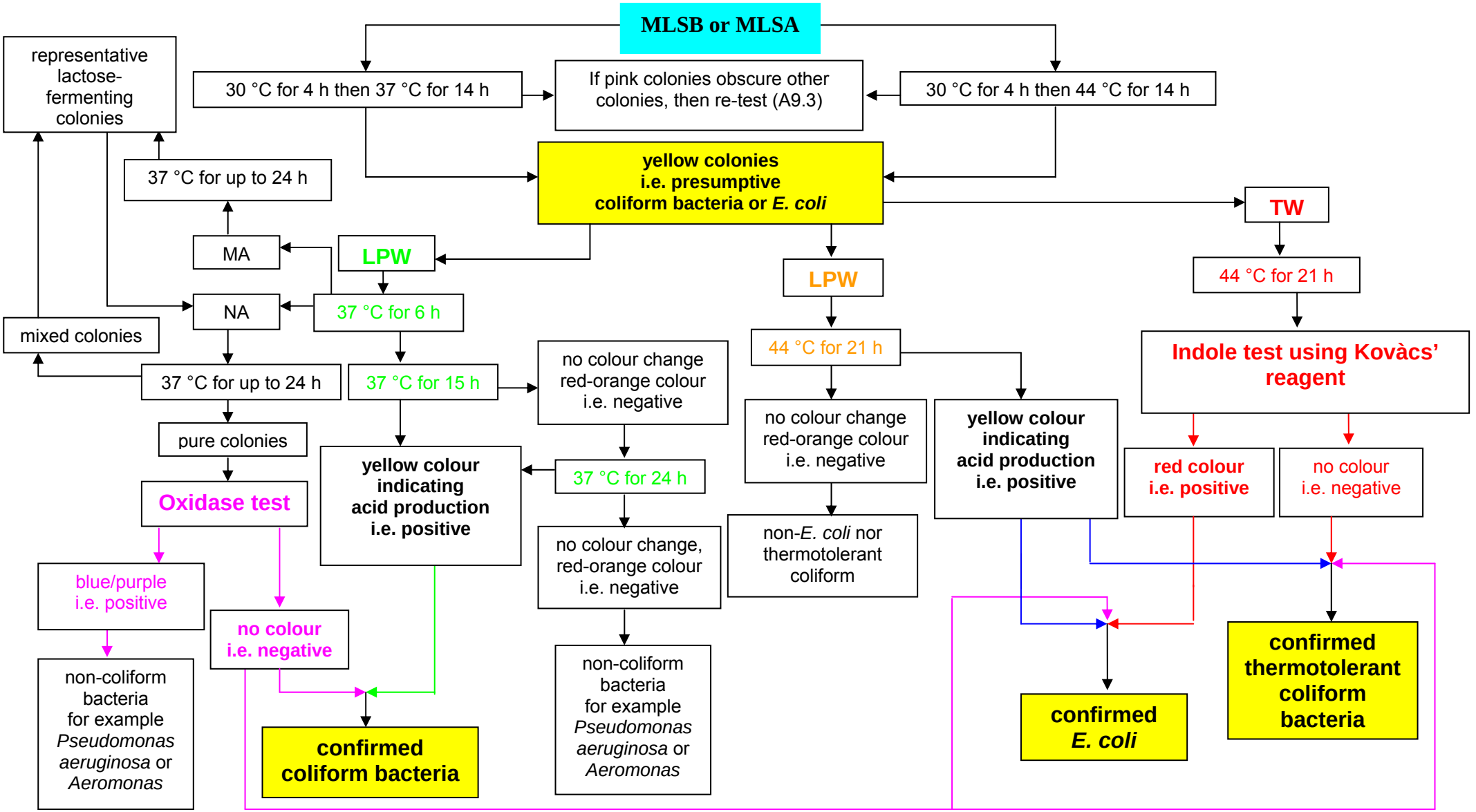
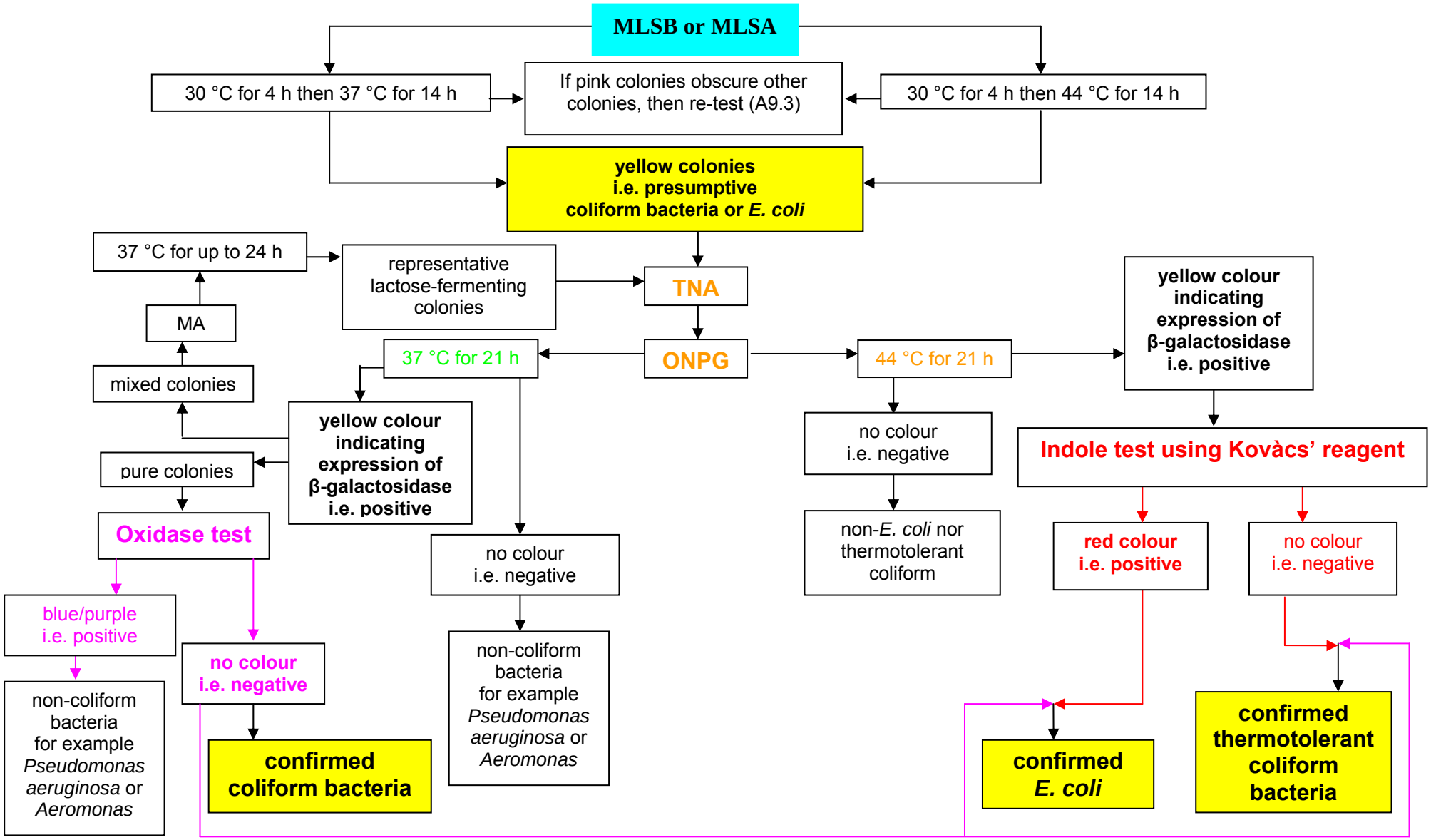
9. Glycosidase profiles of members of the family Enterobacteriaceae. *Journal of Clinical Microbiology*, P Kampfer, O Rauhoff & W Dott, 1991, **29**, 2877-2879.

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

Flow chart A1 **Confirmation of yellow colonies of presumptive coliform bacteria and *E. coli* using LPW and TW on colonies obtained on MLSB or MLSA incubated at 37 °C and 44 °C** (see section A9.4.1)



Flow chart A2 **Confirmation of yellow colonies of presumptive coliform bacteria and *E. coli* using TNA on colonies obtained on MLSB or MLSA incubated at 37 °C and 44 °C** (see section A9.4.2)



B The enumeration of coliform bacteria and *Escherichia coli* by a single membrane filtration technique using membrane lactose glucuronide agar incubated at 37 °C.

B1 Introduction

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. These tests provide a sensitive means for detecting faecal contamination, for assessing raw water quality, 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 or express β-galactosidase, and form all shades and sizes of yellow colonies on membrane filters (after incubation at 30 °C for 4 hours followed by incubation at 37 °C for 14 hours) are regarded as coliform bacteria. 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 (i.e. non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia, Klebsiella*, *Leclercia*, *Pantoea*, *Raoultella*, *Serratia*, and *Yersinia.*

Isolates which produce acid from lactose and produce β-glucuronidase forming green colonies after incubation at 30 °C for 4 hours followed by incubation at 37 °C for 14 hours 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 confirmatory tests for acid production from lactose, oxidase-negative reactions 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 sodium thiosulphate pentahydrate per 100 ml of sample, or equivalent).

B7.2 Incubators capable of maintaining temperatures of 30.0 ± 1.0 °C, 37.0 ± 1.0 °C and/or 44.0 ± 0.5 °C, or cycling incubators, fitted with timers, capable of attaining these temperatures.

B7.3 Filtration apparatus, sterile filter funnels or filter funnels that can be sterilised, and vacuum source.

B7.4 Sterile membrane filters, for example, white, 47 mm diameter, cellulose-based 0.45 µm nominal pore size. Gridded membrane filters may facilitate the counting of organisms.

B7.5 Smooth-tipped forceps.

B7.6 Water baths (or incubators) set at 30.0 ± 1.0 °C, 37.0 ± 1.0 °C and/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 formulation. Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the 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 the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

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
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 1M 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 the medium at 121 °C for 15 minutes. Allow the medium to cool, distribute it in Petri dishes and allow it to solidify. Petri dishes containing the agar medium may be stored at temperatures in the range 5 ± 3 °C for up to one week, protected against dehydration. Storage beyond this time may result in a deterioration of the performance of the medium. The pH of the medium 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
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, mix well and distribute the solution in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored in the range 5 ± 3 °C for up to one month.

B8.3 *Tryptone water*

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[6]. Care should, therefore, be taken in the appropriate selection of the tryptone used.

Tryptone	20 g
Sodium chloride	5 g
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 the medium in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored in the range 5 ± 3 °C for up to one month.

B8.4 *Tryptone nutrient agar*

Beef extract powder	1 g
Yeast extract	2 g
Peptone	5 g
Tryptone	20 g
Sodium chloride	5 g
Agar	15 g
Water	1 litre

Dissolve the ingredients in the water. The final pH of the sterile medium should be 7.4 ± 0.2. Sterilise by autoclaving at 121 °C for 15 minutes. Bulk sterile medium may be stored in the dark at room temperature for up to one month. Alternatively, allow the medium to cool to about 50 °C, distribute it in Petri dishes and allow the medium to solidify. Petri dishes containing tryptone nutrient agar (TNA) medium may be stored in the range 5 ± 3 °C for up to one month, protected against dehydration. If bulk medium is used, heat the medium to melt the agar, distribute it into Petri dishes and allow the medium to solidify.

Alternative nutrient media may be used, for example yeast extract agar or blood agar base, and supplemented with tryptone (to a concentration of 20 g/l) but nutrient media containing fermentable carbohydrates should not be used. Alternative media would need to be validated accordingly.

B8.5 *Kovàcs' reagent*[7]

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. Mix well. The reagent should be pale yellow or straw-coloured when freshly prepared and may be stored in the dark in the range 5 ± 3 °C for up to six months. Depending on the grade, some batches of amyl alcohol may be unsatisfactory and produce a dark colour with the p-dimethylaminobenzaldehyde.

B8.6 *Modified Kovàcs' reagent*[8]

p-Dimethylaminobenzaldehyde	5.0 g
Ethyl alcohol	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the p-dimethylaminobenzaldehyde in the ethyl alcohol and slowly add the hydrochloric acid. Mix well. The reagent should be a pale yellow colour when freshly prepared and may be stored in the dark in the range 5 ± 3 °C for up to six months.

B8.7 *ONPG discs*

These are small discs impregnated with ortho-nitrophenol-β-D-galactopyranoside (ONPG) Not all commercially available ONPG discs are suitable for this method and discs should be validated before use. See Appendix 1.

B8.8 *Other media*

Standard and commercial formulations of other media and reagents used in this method include Gram stain reagents, nutrient agar (NA), MacConkey agar (MA), oxidase reagent, Ringer's solution, quarter strength 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 MLGA 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. The minimum volume filtered should not be less than 1 ml. This is to ensure that colonies are evenly spread across the membrane filter and the volume filtered is still representative of the sample submitted. If smaller volumes are required to ensure the count of colonies lies within the above range, then appropriate dilutions should be made.

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, 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 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 membrane lactose glucuronide agar, which should be well-dried (for example, the Petri dish should be 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 4.00 ± 0.25 hours then transferred to an incubator at 37 °C for a minimum of 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, for any reason, an early indication of a result is required, the membrane filter at 37 °C may be examined after an incubation time of approximately 8 hours but the membrane filter must be returned to the incubator 37 °C for the full minimum incubation period of 14 hours prior to counting.

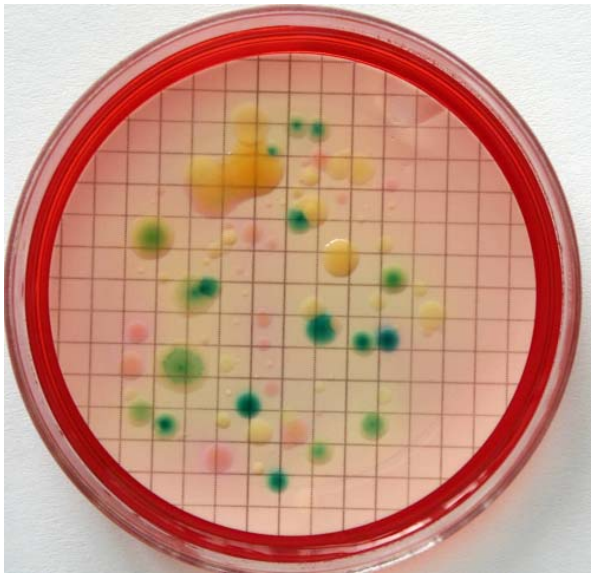
B9.3 *Reading of results*

After the total incubation period of 18 hours, examine the MLGA 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 colouration may change on cooling and standing. All yellow colonies are presumptive non-*E. coli* coliform bacteria and green colonies are *E. coli* (see Figure B1). The total combined count of yellow and green colonies, and blue colonies if present, is regarded as the number of presumptive 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 obscure lactose-fermenting colonies, a further sample should be taken and re-submitted for examination and this fact recorded. Alternatively, any portion of sample retained in a refrigerator may be re-examined using an appropriate dilution of the sample, to enable isolated colonies to develop. However, whilst a count of the number of colonies (of the re-examined sample) on the MLGA membrane filter may be made, this value should not be reported and used to assess water quality, as this may not reflect the

number of colonies in the original sample when first examined. In addition to appropriate dilution of the sample, MPN or presence-absence methods should also be considered.

Occasionally, blue colonies may be noted and recorded. On rare occasions 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.

Figure B1 Yellow and blue colonies of presumptive coliform bacteria and green colonies of *E. coli* on membrane lactose glucuronide agar



B9.4 *Confirmation tests*

Depending on the intended purpose of the analysis and the required accuracy, sub-culture a suitable number of yellow, blue (if present) 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.

If the aim of the examination 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 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. Where one type of colonial species greatly exceeds another type, random choosing of colonies may result in the failure to culture the less frequently occurring species. In these cases, additional considerations should be given to choosing all colonial species. 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 confirmatory testing, 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 confirmatory testing 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. Petri dishes should be stored in the appropriate incubator prior to sub-culturing, if confirmation is not undertaken immediately after counting.

Coliform bacteria can be confirmed by testing for lactose fermentation in lactose peptone water (LPW) at 37 °C and at 44 °C as being indicative of the possession of β-galactosidase enzyme, and for the absence of the oxidase enzyme. Also, *E. coli* can be similarly confirmed but with the inclusion of a test for indole production in tryptone water (TW) at 44 °C. See flow chart B1. Alternatively, these bacteria can be confirmed by testing directly for β-galactosidase and indole production from growth on nutrient agar supplemented with tryptone (i.e. TNA) with a disc containing ortho-nitrophenyl-β-D-galactopyranoside (ONPG) on the agar surface. See flow chart B2.

B9.4.1 *Confirmation of coliform bacteria and E. coli using LPW and TW*

From the MLGA membrane filters incubated at 37 °C, sub-culture each colony to be tested

(i) LPW and incubate at 44 °C for 21 ± 3 hours, and
(ii) TW and incubate at 44 °C for 21 ± 3 hours.
(iii) If colonies on MLGA membrane filters are small, sub-culture to LPW and incubate at 37 °C for approximately 6 hours. After approximately 6 hours of incubation, from the LPW tube or bottle incubated at 37 °C, sub-culture to MA and NA. Incubate the MA and NA at 37 °C for up to 24 hours. This is to check for purity and colonial appearance. After sub-culturing, the LPW is returned to the incubator at 37 °C for a further incubation period of 15 hours. Alternatively, if colonies on MLGA membrane filters are large, sub-culture directly to MA and NA, and to LPW. Incubate the MA and NA at 37 °C for up to 24 hours. This is to check for purity and colonial appearance. Incubate the LPW at 37 °C for 21 hours.

After 21 hours of incubation at 37 °C, the LPW is examined for acid production. If no colour change is exhibited, i.e. the results are negative, the LPW is returned to the incubator at 37 °C and incubated for a further 24 hour period, and then re-examined for acid production. Confirmation of acid production is demonstrated by a change of colour from red to yellow (see Figure B2).

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, exhibit 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.

Following incubation of MA and NA Petri dishes at 37 °C for up to 24 hours, examine the MA and NA to ascertain whether pure isolates develop. If the culture is pure, carry out an oxidase test on colonies only from NA. Pure cultures are essential for the oxidase test and it may be necessary to make further sub-cultures. If MA or MA exhibit mixed colonies, sub-culture a representative number of each lactose-fermenting colony from MA to NA. Incubate the NA at 37 °C for up to 24 hours.

Figure B2 Demonstration of lactose fermentation in lactose peptone water

No fermentation exhibited, i.e. no colour change, regard as negative

Fermentation exhibited, i.e. colour change from red to yellow, regard as positive

In situ oxidase tests carried out directly on colonies on the MLGA 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.

Typically, coliform bacteria produce large pink to red, mucoid or non-mucoid, colonies on MA, often with a halo of precipitation of bile salts. Some species of *Bacillus*, *Enterococcus* and *Staphylococcus* may grow on MLGA membrane filters producing very small opaque yellow colonies. *Bacillus*, *Enterococcus* and *Staphylococcus* can be readily recognised by colony characteristics on MA and by Gram staining. If *Bacillus*, *Enterococcus* and *Staphylococcus* grow on MA, they produce very small opaque red or colour-less colonies. *Bacillus*, *Enterococcus* and *Staphylococcus* are Gram-positive, whereas coliform bacteria are Gram-negative bacilli.

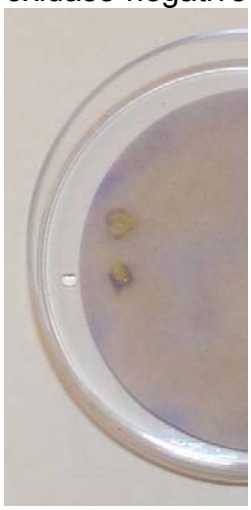
The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA. Place 2 - 3 drops, typically 0.1 - 0.2 ml (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, 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. See Figure B3.

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.

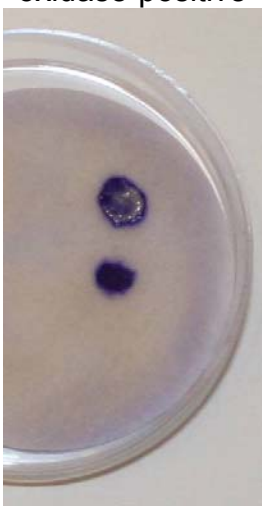
On each occasion where oxidase reagent is used, conduct control tests with organisms, of which one species is known to give a positive reaction (for example *Pseudomonas aeruginosa*) and one species is known to give a negative reaction (for example *E. coli*).

Figure B3 Oxidase test

Escherichia coli
oxidase-negative



Pseudomonas aeruginosa
oxidase-positive



Examine the LPW incubated at 44 °C for 21 ± 3 hours for the production of acid, see Figure B2. Examine the TW incubated at 44 °C for 21 ± 3 hours for the production of indole by adding 2 - 3 drops, typically 0.1 - 0.2 ml of Kovàcs' reagent. Shake well and allow to settle. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer (see Figure B4).

Figure B4 Demonstration of indole production in tryptone water using Kovàcs' reagent

No colour developed
i.e. regard as negative

Red colour developed
i.e. regard as positive

Typically, coliform bacteria are oxidase-negative and produce acid from lactose at 37 °C and may produce acid from lactose at 44 °C. Typically, *E. coli* 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*[9, 10] and may differentiate other species of coliform bacteria which exhibit the same confirmation profile as *E. coli* (for example *Klebsiella* spp). Thermotolerant coliform bacteria are oxidase-negative and produce acid from lactose at 44 °C, and are indole-negative.

Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests[11]. Commercial test kits may be used following appropriate performance verification at the laboratory.

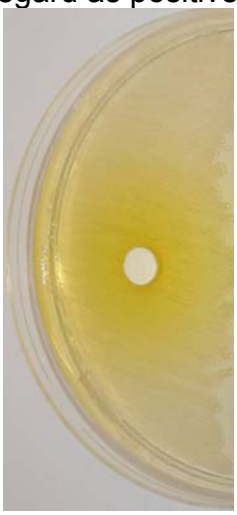
B9.4.2 *Confirmation of coliform bacteria and E. coli using TNA*

Coliform bacteria and *E. coli* can also be confirmed by demonstration of production of β-galactosidase, indole production (for *E. coli*) and lack of oxidase production, following sub-culture to Petri dishes containing TNA. Data on the verification of the performance of these confirmation procedures are given in Appendix 1.

From the MLGA membrane filters incubated at 37 °C, sub-culture each yellow colony to be tested to two Petri dishes containing TNA. Aseptically place an ONPG disc onto each area of sub-culture. Incubate one TNA Petri dish at 37 °C for 21 ± 3 hours and the other TNA Petri dish at 44 °C for 21 ± 3 hours. After incubation, examine the TNA dish incubated at 37 °C for the presence of yellow colouration around the ONPG disc. Confirmation of expression of β-galactosidase is demonstrated by the production of yellow colouration (see Figure B5).

Figure B5 Demonstration of β-galactosidase production on tryptone nutrient agar with ONPG discs

Yellow colour developed
i.e. regard as positive



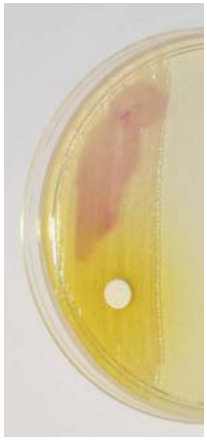
No colour developed
i.e. regard as negative

Growth on the TNA dish incubated at 37 °C may also be used for the oxidase test, provided it is pure. Pure cultures are essential for the oxidase test (see B9.4.1 and Figure B3) and if mixed colonies develop, it may be necessary to sub-culture representative colonies to Petri dishes containing MA and incubating at 37 °C for up to 24 hours, with subsequent sub-culture of representative lactose fermenting colonies to TNA for confirmation.

Examine the TNA dish incubated at 44 °C for the expression of β-galactosidase (see Figure B5). Test for indole production by adding 2 - 3 drops, typically 0.1 - 0.2 ml of either Kovàcs' reagent (B8.5) or modified Kovàcs' reagent (B8.6) to an area of growth. Use of the modified Kovàcs' reagent avoids exposure to amyl alcohol fumes. Indole production is demonstrated by the rapid appearance of a red colour (see Figures B6 and B7).

Figure B6 Demonstration of indole production with Kovàcs' reagent on tryptone nutrient agar

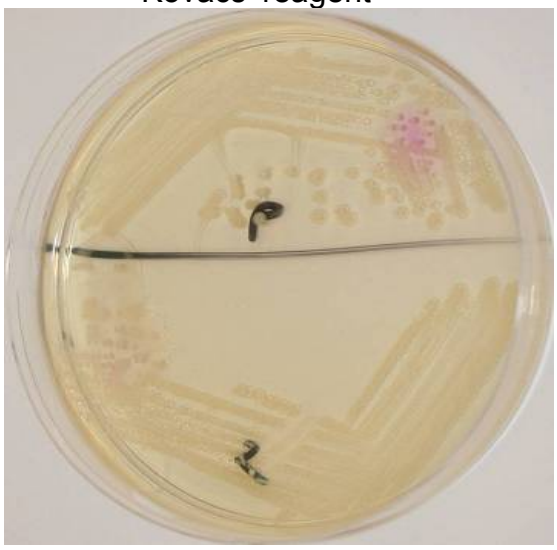
Escherichia coli
ONPG-positive and indole-positive



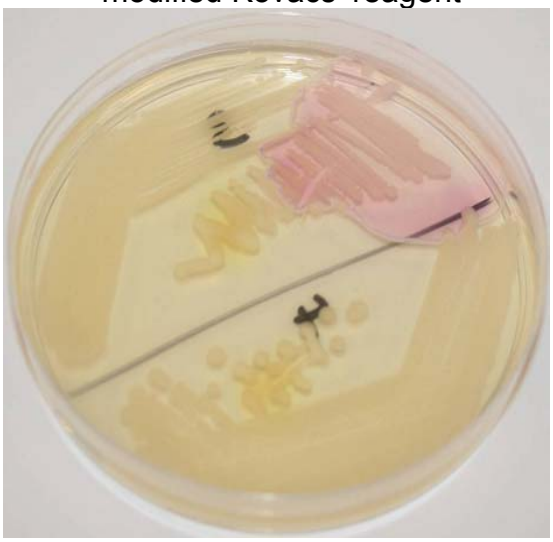
Pseudomonas aeruginosa
ONPG-negative and indole-negative

Figure B7 Demonstration of indole production on tryptone nutrient agar

Red colour developed with Kovàcs' reagent



Red colour developed with modified Kovàcs' reagent



Thermotolerant coliform bacteria are oxidase-negative and produce β-galactosidase at 44 °C, and are indole-negative.

Further identification may be carried out using characteristic colonies on TNA or MA by means of appropriate biochemical and other tests[11]. Commercial test kits may be used following appropriate performance verification at the laboratory.

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:

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

Where N is the number of yellow, green and blue colonies counted on the MLGA membrane filter, and DF is the appropriate dilution, if required.

The count of the total number of yellow, green and blue colonies on the MLGA membrane filter is regarded as the presumptive coliform bacteria count and the number of green colonies is regarded as the presumptive *E. coli* count.

B10.2 *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) or β-galactosidase-positive, 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) or β-galactosidase-positive, produce indole at 44 °C and are oxidase-negative, combined with any proportion of yellow and blue colony isolates that subsequently confirm as *E. coli*.

B11 Expression of results

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

B12 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 as appropriate. Further details are given elsewhere[2] in this series.

B13 References

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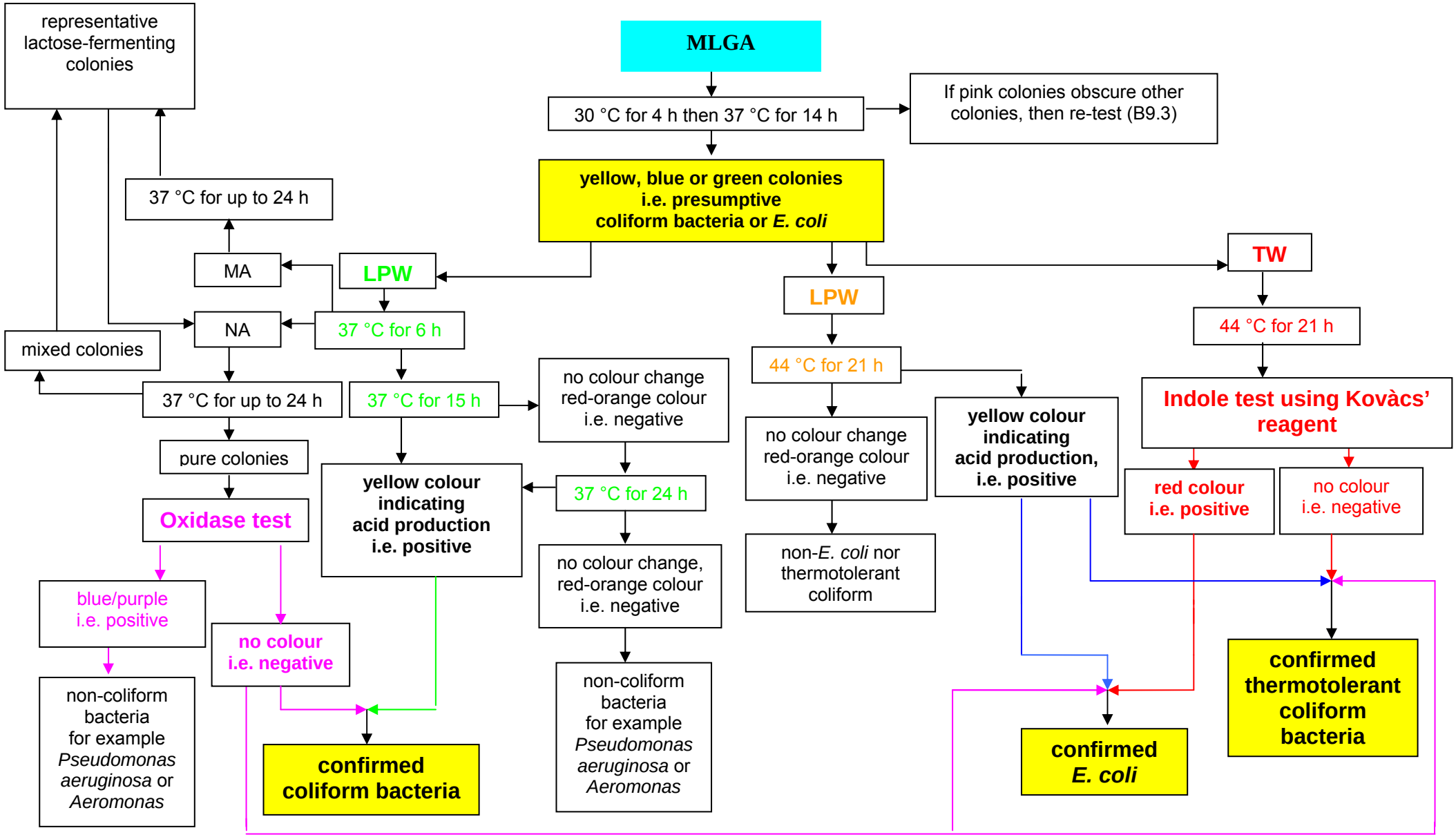
8. Membrane filter procedure for enumerating the component genera of the coliform group in seawater. *Applied Microbiology,* A P Dufour & V J Cabelli, 1975, **29**, 826.

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

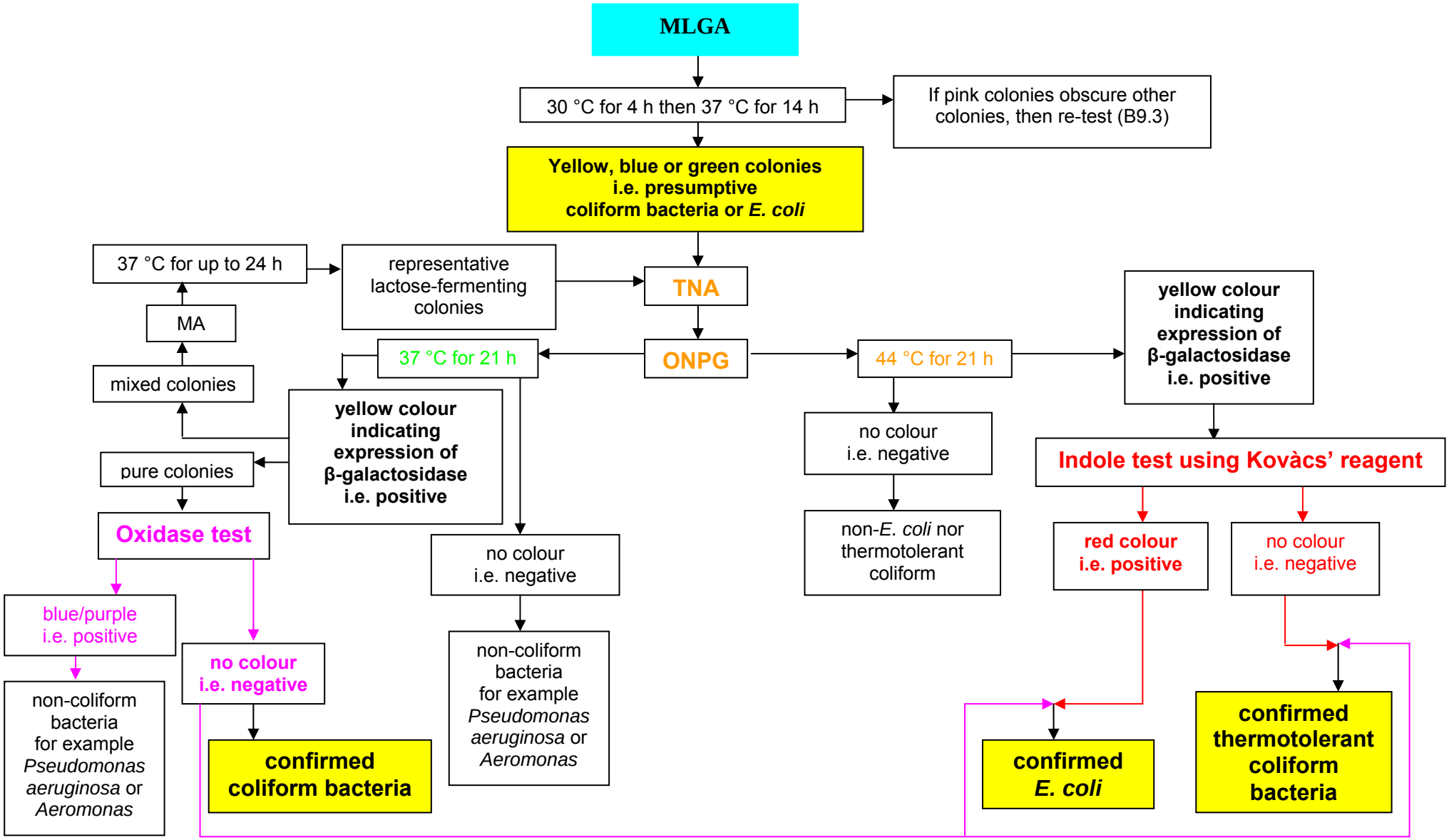
10. Glycosidase profiles of members of the family Enterobacteriaceae. *Journal of Clinical Microbiology*, P Kampfer, O Rauhoff & W Dott, 1991, **29**, 2877-2879.

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

Flow chart B1 **Confirmation of yellow colonies of presumptive coliform bacteria and *E. coli* using LPW and TW on colonies obtained on MLGA incubated at 37 °C** (see section B9.4.1)



Flow chart B2 **Confirmation of yellow colonies of presumptive coliform bacteria and *E. coli* using TNA on colonies obtained on MLGA incubated at 37 °C** (see section B9.4.2)



C The enumeration of coliform bacteria and *Escherichia coli* by a multiple tube most probable number technique using minerals modified glutamate medium incubated at 37 °C

C1 Introduction

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. These tests provide a sensitive means for detecting faecal contamination, for assessing raw water quality, 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.

C2 Scope

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

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

C3 Definitions

In the context of the method, organisms which are oxidase-negative and produce acid from lactose within 48 hours at 37 °C in a chemically defined medium 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 (i.e. non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia, Klebsiella*, *Leclercia*, *Pantoea*, *Raoultella*, *Serratia*, and *Yersinia.*

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 48 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.

C4 Principle

Bacteria are grown in a liquid medium containing lactose and bromocresol purple as an indicator of acidity. This is followed by confirmation tests for acid production from lactose (or expression of β-galactosidase), negative oxidase reaction and, where necessary, indole formation.

In this method (which is a variation of the presence/absence test described in method E) measured volumes of sample, or dilution of sample, are added to a series of tubes or bottles containing liquid differential medium. If, within the series, some of the tubes or bottles exhibit no characteristic growth in the medium following incubation, and other tubes or bottles exhibit some characteristic growth in the medium following incubation, then the

most probable number of organisms in 100 ml of sample can be estimated from appropriate probability tables, see Appendix C1. Confirmation that positive reactions (i.e. those tubes or bottles showing characteristic growth) are due to a particular organism can be obtained by sub-culture to tubes of confirmation media. This may involve incubation at higher temperatures, depending on the particular organism.

C5 Limitations

This method is suitable for all types of water and related samples and is particularly suitable for the examination of sludges and waters containing sediment.

C6 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.

C7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [2] in this series. Principally, fan assisted incubators are required. Other items include:

C7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate per 100 ml of sample, or equivalent).

C7.2 Incubators (or water baths) capable of maintaining temperatures of 37.0 ± 1.0 °C and 44.0 ± 0.5 °C.

C7.3 Suitable bottle or test tube racks.

C8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the 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 the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

Minerals modified glutamate medium[4, 5] is used for the isolation of coliform bacteria from waters. The use of MacConkey broth may not be suitable because of variations in the inhibitory properties of different batches of bile salts.

C8.1 *Minerals modified glutamate medium*

Lactose	20.0 g
L (+) Glutamic acid sodium salt	12.7 g
L (+) Arginine monohydrochloride	40 mg
L (-) Aspartic acid	48 mg
L (-) Cystine	40 mg
Sodium formate	500 mg
Dipotassium hydrogen phosphate	1.8 g
Ammonium chloride	5.0 g
Magnesium sulphate heptahydrate	200 mg
Calcium chloride dihydrate	20 mg
Iron(III) citrate	20 mg
Thiamine (Aneurin hydrochloride)	2 mg
Nicotinic acid	2 mg
Pantothenic acid	2 mg
Bromocresol purple (1 % m/v ethanolic solution)	2 ml
Water to	1 litre

This formulation enables double-strength medium to be prepared. This is conveniently prepared in quantities of 10 (or more) litres. If the medium is not to be distributed in tubes immediately, the lactose and thiamine should be omitted and added before dispensing.

Several of the ingredients are more conveniently added as separate solutions and these may be prepared as follows:

SOLUTION 1

L (+) Arginine monohydrochloride	400 mg
L (-) Aspartic acid	480 mg
Water	50 ml

Heat the water to about 50 °C and dissolve the ingredients.

SOLUTION 2

L (-) Cystine	400 mg
5M Sodium hydroxide	10 ml
Water	90 ml

Heat the water to about 50 °C and dissolve the ingredients.

SOLUTION 3

Nicotinic acid	20 mg
Pantothenic acid	20 mg
Water	5 ml

Dissolve the ingredients in the water without heating the solution.

SOLUTION 4

Iron(III) citrate	200 mg
Water	10 ml

Heat the water to about 50 °C and dissolve the ingredients.

SOLUTION 5

Calcium chloride dihydrate	5 g
Water	100 ml
Concentrated hydrochloric acid	0.1 ml

Dissolve the ingredients in the water without heating the solution and sterilise the solution at 121°C for 20 minutes. Store as a stock solution.

SOLUTION 6

Thiamine	100 mg
Water	99 ml

Prepare a sterile 0.1 % m/v solution of thiamine in the water. This can be carried out by adding the contents of an ampoule of thiamine (100 mg) to 99 ml of sterile water.

The above solutions may be stored at temperatures in the range 5 ± 3 °C and any remaining unused solution should be discarded after 6 weeks.

To prepare 10 litres of double-strength medium, dissolve the appropriate quantities of L (+) glutamic acid sodium salt, sodium formate, dipotassium hydrogen phosphate, ammonium chloride and magnesium sulphate heptahydrate in 9 litres of hot distilled water about 50 °C. Add the whole of solutions 1, 2, 3 and 4, and 4 ml of solution 5. Adjust the pH of the medium to 6.9 ± 0.2 or higher if necessary, so that the final pH (when completely prepared and after sterilisation is 6.7 ± 0.2). After adjustment of the pH, add 20 ml of a 1 % m/v ethanolic solution of bromocresol purple. Dilute to a final volume of 10 litres.

If the medium is not required for immediate use, dispense the mixed solution (medium without lactose and thiamine) into suitable containers in 500 ml volumes. Autoclave the solutions at 115 °C for 10 minutes and store in the dark at room temperature for up to one month.

For use, add the necessary amounts of lactose and solution 6 (i.e. 10 g and 1 ml respectively). Allow the added lactose to dissolve and distribute the medium into suitable tubes or bottles in 10 ml and 50 ml volumes. Cap the containers and sterilise at 115 °C for 10 minutes.

Prepare single-strength medium by diluting the double-strength medium with an equal volume of water and distribute the medium in 5 ml volumes in tubes. Sterilise the medium at 115 °C for 10 minutes.

The sterile media can be stored in the range 5 ± 3 °C for up to one month.

C8.2 *Lactose peptone water*

Peptone	10 g
Sodium chloride	5 g
Lactose	10 g
Phenol red (0.4 % m/v aqueous solution)	2.5 ml
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, mix well and distribute it in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored at temperatures in the range 5 ± 3 °C for up to one month.

C8.3 *Tryptone water*

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[6]. Care should, therefore, be taken in the appropriate selection of the tryptone used.

Tryptone	20 g
Sodium chloride	5 g
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 the medium in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored at temperatures in the range 5 ± 3 °C for up to one month.

C8.4 *Tryptone nutrient agar*

Beef extract powder	1 g
Yeast extract	2 g
Peptone	5 g
Tryptone	20 g
Sodium chloride	5 g
Agar	15 g
Water	1 litre

Dissolve the ingredients in the water. The final pH of the sterile medium should be 7.4 ± 0.2. Sterilise by autoclaving at 121 °C for 15 minutes. Bulk sterile medium may be stored in the dark at room temperature for up to one month. Alternatively, allow the medium to cool to about 50 °C, distribute it in Petri dishes and allow the medium to solidify. Petri dishes containing tryptone nutrient agar (TNA) medium may be stored in the range 5 ± 3 °C for up to one month, protected against dehydration. If bulk medium is used, heat the medium to melt the agar, distribute it into Petri dishes and allow the medium to solidify.

Alternative nutrient media may be used, for example yeast extract agar or blood agar base, and supplemented with tryptone (to a concentration of 20 g/l) but nutrient media containing fermentable carbohydrates should not be used. Alternative media would need to be validated accordingly.

C8.5 *Kovàcs' reagent*[7]

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. Mix well. The reagent should be pale yellow or straw-coloured when freshly prepared and may be stored in the dark in the range 5 ± 3 °C for up to six months. Depending on the grade, some batches of amyl alcohol may be unsatisfactory and produce a dark colour with the p-dimethylaminobenzaldehyde.

C8.6 *Modified Kovàcs' reagent*[8]

p-Dimethylaminobenzaldehyde	5.0 g
Ethyl alcohol	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the p-dimethylaminobenzaldehyde in the ethyl alcohol and slowly add the hydrochloric acid. Mix well. The reagent should be a pale yellow colour when freshly prepared and may be stored in the dark in the range 5 ± 3 °C for up to six months.

C8.7 *ONPG discs*

These are discs of filter paper impregnated with ortho-nitrophenol-β-D-galactopyranoside (ONPG). Not all commercially available ONPG discs are suitable for this method and discs should be validated before use. See Appendix 1.

C8.8 *Other media*

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

C9 Analytical procedure

C9.1 *Volumes of sample for inoculation*

A series of different volumes of sample is inoculated into tubes or bottles of minerals modified glutamate medium.

For waters expected to be of good quality, use 1 x 50 ml and 5 x 10 ml volumes of sample. Add the 50 ml and 10 ml volumes of sample to equal volumes of double-strength medium.

For waters expected to be of doubtful or unknown quality, use 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample. Add the 50 ml and 10 ml volumes of sample to equal volumes of double-strength medium and the 1 ml volumes of sample to 5 ml of single-strength medium.

For waters expected to be of a more polluted nature, use 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample. Add the 10 ml volumes of sample to equal volumes of double-strength medium, and the 1 ml and 0.1 ml volumes of sample to 5 ml of single-strength medium.

For waters expected to be heavily polluted, dilutions (in a suitable diluent, for example, quarter-strength Ringer's solution or maximum recovery diluent) of a hundred-fold, or a thousand-fold or higher, may need to be prepared. Suitable volumes of these diluted samples should then be used and added to 5 ml of single-strength medium.

For all types of samples and in all cases, sufficient volumes of sample should be added to the medium so that, after incubation, some of the tubes or bottles exhibit characteristic growth within the medium and some of the tubes or bottles exhibit no growth within the medium. Sterile pipettes are used to transfer the sample to the tube or bottle containing the minerals modified glutamate medium.

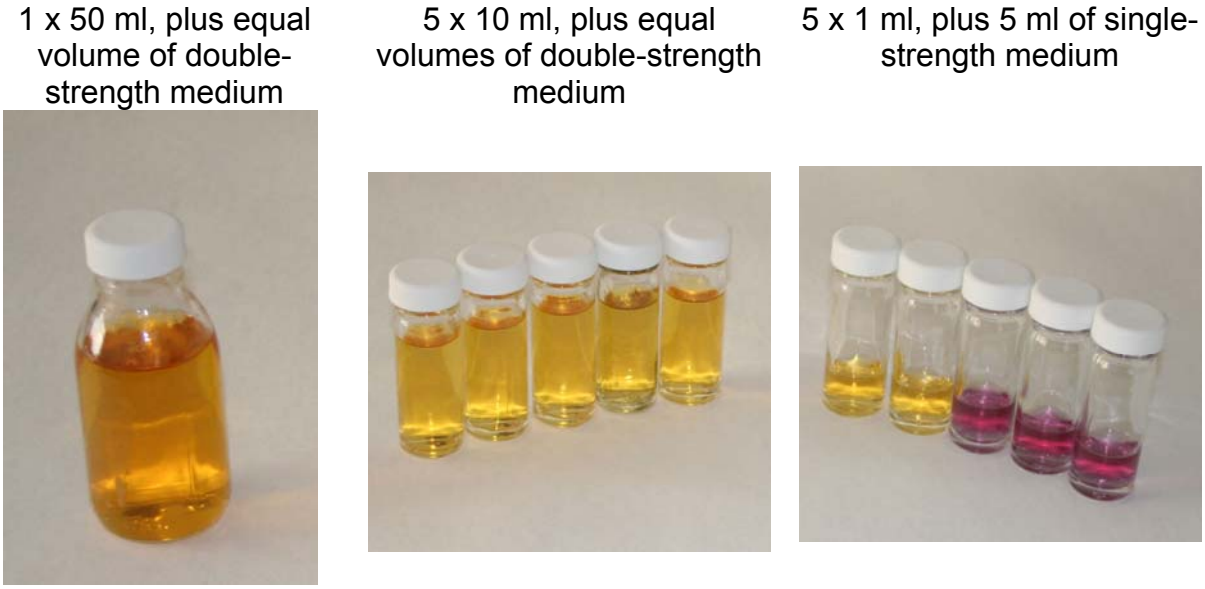
C9.2 *Sample processing*

After the tubes or bottles of minerals modified glutamate medium have been inoculated with the appropriate volume of sample, or diluted sample, each tube or bottle is capped or sealed and placed in an incubator at 37 °C. After 24 hours, the tubes or bottles are examined for acid production (as demonstrated by the presence of yellow colouration). After a further 24 hours, the tubes or bottles are re-examined and results recorded. All tubes or bottles that exhibit positive (characteristic) growth within the medium are retained for confirmatory testing. Some tubes or bottles may exhibit growth without a colour change. In these cases, these tubes are regarded as negative.

C9.3 *Reading of results*

The number of tubes or bottles for each series of volume of sample is recorded where a positive reaction is given, as demonstrated by characteristic growth within the medium and the production of a yellow colouration (see Figure C1). After this, confirmation tests may be carried out as required.

Figure C1 A typical 11 tube MPN test with minerals modified glutamate broth



bottles that exhibit growth within the medium are indicated by yellow colouration, regard these as positive
bottles that exhibit no growth within the medium are indicated by purple colouration, regard these as negative

When dilutions of sample have been used, a consecutive series of volumes should be chosen whereby some of the tubes or bottles exhibit growth within the medium, i.e. are positive, and some tubes or bottles exhibit no growth within the medium, i.e. are negative. From the results, the MPN of bacteria in the sample is determined from probability tables, see Appendix C1.

C9.4 *Confirmation Tests*

Whenever tubes or bottles showing a positive reaction are used in confirmatory tests, they should be tested for confirmation both as coliform bacteria and as *E. coli*.

Coliform bacteria can be confirmed by testing for lactose fermentation in lactose peptone water (LPW) at 37 °C and at 44 °C as being indicative of the possession of β-galactosidase enzyme, and for the absence of the oxidase enzyme. Also, *E. coli* can be similarly confirmed but with the inclusion of a test for indole production in tryptone water (TW) at 44 °C. See flow chart C1. Alternatively, these bacteria can be confirmed by testing directly for β-galactosidase and indole production from growth on nutrient agar supplemented with tryptone (i.e. TNA) with a disc containing ortho-nitrophenyl-β-D-galactopyranoside (ONPG) on the agar surface. See flow chart C2.

C9.4.1 *Confirmation of coliform bacteria and E. coli using LPW and TW*

For each tube or bottle showing characteristic growth within the MMGM, sub-culture to

(i) MA and NA and incubate at 37 °C for up to 24 hours, and
(ii) MA and incubate at 44 °C for up to 24 hours.

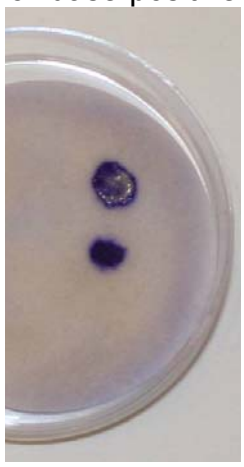
If pure cultures are obtained on NA at 37 °C then perform the oxidase test. The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA at 37 °C.

Place 2 - 3 drops, typically 0.1 - 0.2 ml (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 prepared filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction. See Figure C2.

Figure C2 Oxidase test

E. coli
oxidase-negative

Pseudomonas aeruginosa
oxidase-positive



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

If there is any doubt about the purity of the cultures then sub-culture typical coliform colonies from MA to NA, incubate the NA at 37 °C for up to 24 hours and carry out the oxidase test on pure colonies from NA.

Typically, coliform bacteria produce large pink to red, mucoid or non-mucoid, colonies on MA, often with a halo of precipitation of bile salts. Some species of *Bacillus*, *Enterococcus* and *Staphylococcus* may grow in MMGM producing turbid growth. These can be readily recognised by colony characteristics on MA and by Gram staining. If *Bacillus*, *Enterococcus* and *Staphylococcus* grow on MA, they produce very small opaque red or colour-less colonies. *Bacillus*, *Enterococcus* and *Staphylococcus* are Gram-positive, whereas coliform bacteria are Gram-negative bacilli.

If the isolates are oxidase-negative, then perform the LPW test for lactose fermentation.

For each isolate to be tested, sub-culture (from NA at 37 °C) to LPW and incubate at 37 °C for 21 ± 3 hours. Following incubation, examine for acid production and if no colour change is exhibited, i.e. the results are negative, re-examine after a further 21 ± 3 hours. Confirmation of acid production is demonstrated by a change of colour from red to yellow (see Figure C3).

Figure C3 Demonstration of lactose fermentation in lactose peptone water

No fermentation exhibited, i.e. no colour change, regard as negative

Fermentation exhibited, i.e. colour change from red to yellow, regard as positive

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, exhibit 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. Thermotolerant coliform bacteria are oxidase-negative and produce acid from lactose at 44 °C, and are indole-negative.

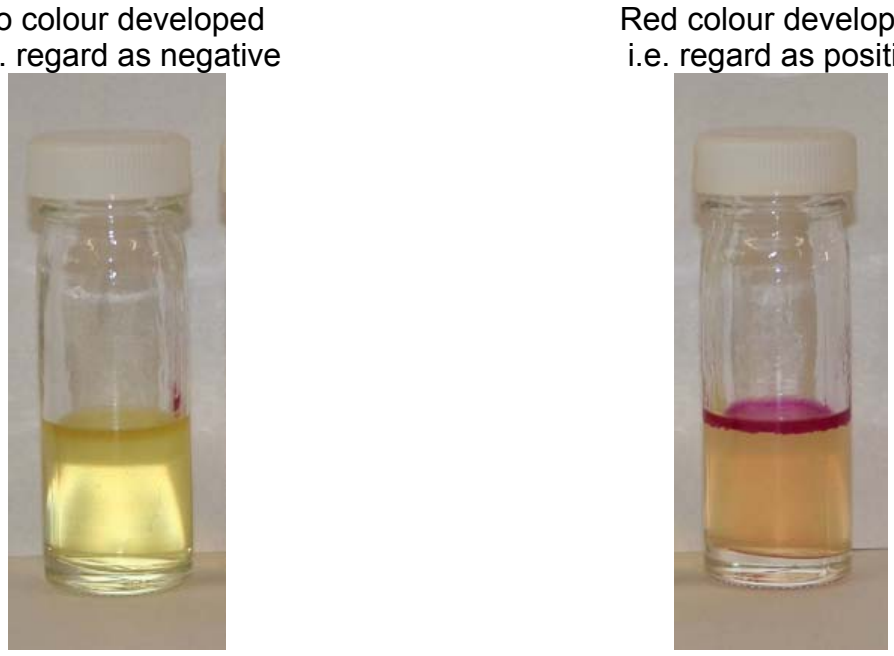
Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests[9]. Commercial test kits may be used following appropriate performance verification at the laboratory.

Inoculate typical coliform colonies (from MA at 44 °C) into tubes of TW and incubate at 44 °C for 21 ± 3 hours. After incubation of the TW tubes at 44 °C, add 0.1 - 0.2 ml of Kovàcs' reagent. Shake well and allow to settle. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer (see Figure C4) confirming the presence of *E. coli*.

It may be necessary to inoculate typical coliform colonies (from MA at 44 °C) into tubes of LPW and incubate at 44 °C for 21 ± 3 hours. Following incubation, examine for acid production. Confirmation of acid production is demonstrated by a change of colour from red to yellow (see Figure C3).

Tests for β-glucuronidase may assist in the early confirmation of *E. coli*[10, 11]. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

Figure C4 Demonstration of indole production in tryptone water using Kovàcs' reagent



C9.4.2 *Confirmation for coliform bacteria* and *E. coli using TNA*

Coliform bacteria and *E. coli* can also be confirmed by demonstration of production of β-galactosidase, indole production (for *E. coli*) and lack of oxidase production, following sub-culture to Petri dishes containing TNA. See Appendix 1 for additional information.

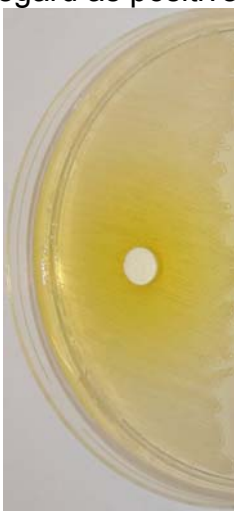
For each tube or bottle showing characteristic growth within the MMGM incubated at 37 °C, sub-culture to

(i) MA and NA and incubate at 37 °C for up to 24 hours, and
(ii) MA and incubate at 44 °C for up to 24 hours.

If a pure culture is obtained on NA at 37 °C then perform the oxidase test as described in section C9.4.1. If the isolate is oxidase-negative, sub-culture (from NA at 37 °C) to a Petri dish containing TNA and aseptically place an ONPG disc onto the area of sub-culture. Incubate the TNA Petri dish at 37 °C for 21 ± 3 hours. After incubation, examine the TNA Petri dish for the presence of yellow colouration around the ONPG disc. Confirmation of expression of β-galactosidase is demonstrated by the production of yellow colouration (see Figure C5).

Figure C5 Demonstration of β-galactosidase production on tryptone nutrient agar with ONPG discs

Yellow colour developed
i.e. regard as positive



No colour developed
i.e. regard as negative

If there is any doubt about the purity of the NA culture at 37 °C then sub-culture typical coliform colonies from MA to NA, incubate at 37 °C for up to 24 hours and carry out the oxidase test.

Thermotolerant coliform bacteria are oxidase-negative and produce β-galactosidase at 44 °C, and are indole-negative.

Further identification may be carried out using characteristic colonies on TNA or MA by means of appropriate biochemical and other tests[9]. Commercial test kits may be used following appropriate performance verification at the laboratory.

Sub-culture typical coliform colonies (from MA at 44 °C) onto Petri dishes containing TNA, aseptically place an ONPG disc onto the area of sub-culture, and incubate the TNA Petri dish at 44 °C for 21 ± 3 hours. After incubation, examine the TNA Petri dish for the presence of yellow colouration around the ONPG disc. Confirmation of expression of β-galactosidase is demonstrated by the production of yellow colouration (see Figure C5). After reading the ONPG reactions, conduct the indole test on the growth on the TNA Petri dish. After incubation of the TNA plates at 44 °C, add 2-3 drops, typically 0.1-0.2 ml of Kovàcs'

reagent (C8.5) or modified Kovàcs' reagent (C8.6) to an area of growth. Use of the modified Kovàcs' reagent avoids exposure to amyl alcohol fumes. Indole production is demonstrated by the rapid appearance of a red colour (see Figures C6 and C7).

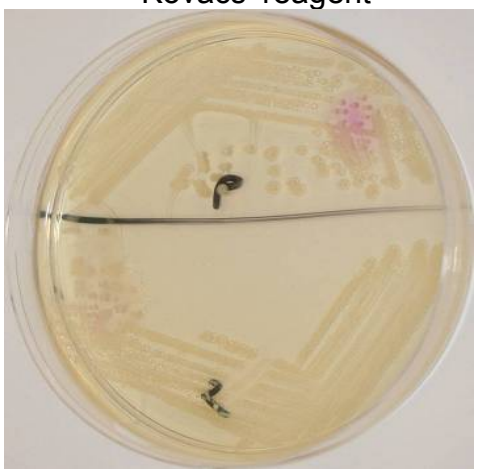
Figure C6 Demonstration of indole production with Kovàcs' reagent on tryptone nutrient agar

Escherichia coli
ONPG-positive and indole-positive

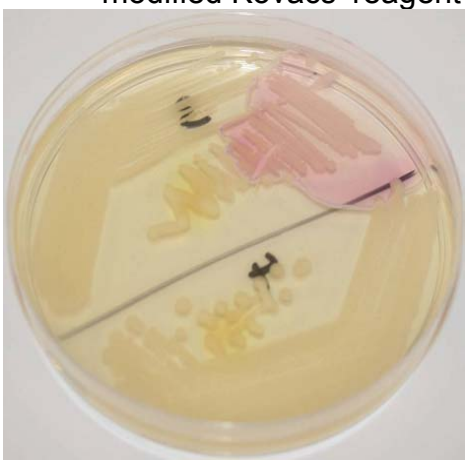
Pseudomonas aeruginosa
ONPG-negative and indole-negative

Figure C7 Demonstration of indole production on tryptone nutrient agar

Red colour developed with Kovàcs' reagent



Red colour developed with modified Kovàcs' reagent



The presence of *E. coli* is demonstrated by the production of indole on TNA.

Tests for β-glucuronidase may assist in the early confirmation of *E. coli*[10, 11]. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

Typically, *E. coli* colonies are oxidase-negative, express β-galactosidase at 37 °C and at 44 °C, and produce indole on TNA at 44 °C.

C10 Calculations

C10.1 *Presumptive coliform bacteria*

The number of minerals modified glutamate medium tubes or bottles of each volume of sample showing a positive reaction is counted, and then by reference to the appropriate tables in appendix C1, the MPN of presumptive coliform bacteria and *E. coli* present in 100 ml of sample is determined. For example, if in a 15-tube test comprising 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample, the number of tubes showing positive reactions in each consecutive series is 3, 2 and 0 respectively, then, from Table C3, the MPN is 13 organisms per 100 ml.

C10.2 *Confirmed coliform bacteria and E. coli*

Confirmed coliform bacteria are calculated by reference to the appropriate table in appendix C1 for the number of tubes or bottles that yield isolates that produce typical coliform colonies on MA, produce acid from LPW or β-galactosidase on TNA at 37 °C and are oxidase-negative.

Confirmed *E. coli* are calculated by reference to the appropriate table in appendix C1 for the number of tubes or bottles that yield isolates that produce typical coliform colonies on MA, produce acid from LPW or β-galactosidase on TNA at 44 °C, produce indole at 44 °C and are oxidase-negative.

C11 Expression of results

Presumptive coliform bacteria and confirmed coliform bacteria and *E. coli* counts are expressed as MPN per volume of sample. For drinking water, the volume is typically, 100 ml.

C12 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*). Tubes or bottles should be incubated as appropriate. Further details are given elsewhere[(2)] in this series.

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Appendix C1 Tables of most probable numbers

From the various combinations of positive and negative reactions for the different volumes examined, the following tables indicate the MPN of bacteria in 100 ml of sample. It is important to realise that the MPN is only an estimate, based on statistical probabilities and that the actual number may lie within a range of values. Approximate 95 % confidence intervals (the MPR) which demonstrate the range of possible numbers which could yield the number of positive reactions, have been published[12]. A procedure for estimating these confidence intervals for other dilution series has also been published[13]. These confidence intervals are seldom of practical use when reporting results because they apply to the accuracy of the method and not the likely variability of organisms at the sampling source[14]. The MPR in Tables C1 - C3 illustrates those situations where the method becomes relatively imprecise, particularly when nearly all the tubes show growth within the medium. In these situations, further dilutions should have been prepared and added to tubes of medium.

Table C1 gives the MPN (and where applicable the MPR) for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample. Similarly Table C2 gives the MPN (and where applicable the MPR) for an 11-tube series comprising 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample. Table C3 shows data for a 15-tube series of 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of samples but gives only those values of the more likely combinations of positive and negative reactions. For example, positive reactions in the 0.1 ml tubes would not be expected if all of the 10 ml and 1 ml tubes were negative. Hence, MPN and MPR values for a combination of results like for instance 0, 0, 2 etc are not tabulated. If these unlikely combinations are observed in practice with greater than expected frequencies, then this might indicate that the statistical assumptions underlying the MPN estimation are not correct[12, 15, 16]. For example, the organisms may not have been uniformly distributed throughout the sample, or toxic substances may have been present.

Calculation of MPN

The number of positive reactions for each set of tubes is recorded and, from the relevant table, the MPN of organisms present in 100 ml of the sample is determined.

Where a series of dilutions of the sample is used, then the following rules should be applied, as illustrated by the numbers in bold, underlined, italic type in table C4.

(i) Use only three consecutive sets of dilutions for calculating the MPN.

(ii) Wherever possible, select three consecutive dilutions where the results are neither all positive nor all negative. The most efficient statistical estimate will result when about half the tubes are positive (see examples (a), (b) and (c) in Table C4).

(iii) If less than three sets of dilutions give positive results, begin with the set containing the largest volume of sample (see example (d) in Table C4).

(iv) If only one set of tubes gives a positive reaction, use this dilution and the one higher and one lower (see example (e) in Table C4).

Table C1 MPN and MPR per 100 ml of sample for a 6-tube series containing 1 x 50 ml and 5 x 10 ml volumes of sample

Number of tubes giving a positive reaction		MPN per 100 ml	MPR* per 100 ml
1 x 50 ml	5 x 10 ml		
0	0	None found	
0	1	1	
0	2	2	
0	3	3	
0	4	4	4-5
0	5	6	
1	0	1	
1	1	2	
1	2	5	4-5
1	3	9	8-10
1	4	15	13-18
1	5	>18**	

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 18.

Table C2 MPN and MPR per 100 ml of sample for an 11-tube series of 1 x 50 ml, 5 x 10 ml and 5 x 1 ml volumes of sample

Number of tubes giving a positive reaction			MPN per 100 ml	MPR* per 100 ml
1 x 50 ml	5 x 10 ml	5 x 1 ml		
0	0	0	None found	
0	0	1	1	
0	1	0	1	
0	1	1	2	
0	2	0	2	
0	2	1	3	
0	3	0	3	
1	0	0	1	
1	0	1	2	
1	1	0	2	
1	1	1	4	
1	1	2	6	
1	2	0	4	4-5
1	2	1	7	6-7
1	2	2	9	9-10
1	3	0	8	7-9
1	3	1	10	10-11
1	3	2	13	12-13
1	3	3	17	15-18
1	4	0	12	11-14
1	4	1	16	15-19
1	4	2	21	19-24
1	4	3	27	24-30
1	4	4	33	30-38
1	5	0	23	20-27
1	5	1	33	29-40
1	5	2	53	44-65
1	5	3	91	75-110
1	5	4	160	134-190
1	5	5	>180**	

* These numbers are at least 95 % as probable as the MPN.

** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 180.

Table C3 MPN and MPR per 100 ml of sample for a 15-tube series containing 5 x 10 ml, 5 x 1 ml and 5 x 0.1 ml volumes of sample

Number of tubes giving a positive reaction			MPN per 100 ml	MPR* per 100 ml
5 x 10 ml	5 x 1 ml	5 x 0.1 ml		
0	0	0	None found	
0	0	1	2	
0	1	0	2	
1	0	0	2	
1	0	1	4	
1	1	0	4	
2	2	0	4	
2	0	1	5	
2	1	0	5	
2	1	1	7	
2	2	0	7	7-9
2	3	0	11	
3	0	0	7	
3	0	1	9	
3	1	0	9	
3	1	1	13	
3	2	0	13	
3	2	1	16	14-16
3	3	0	16	14-16
4	0	0	11	11-13
4	0	1	14	14-16
4	1	0	16	14-16
4	1	1	20	18-20
4	2	0	20	18-22
4	2	1	25	23-27
4	3	0	25	23-27
4	3	1	31	29-34
4	4	0	32	29-34
4	4	1	38	34-41
5	0	0	22	20-23
5	0	1	29	25-34
5	0	2	41	36-50
5	1	0	31	27-36
5	1	1	43	36-50
5	1	2	60	50-70
5	1	3	85	70-95
5	2	0	50	40-55
5	2	1	70	60-80
5	2	2	95	80-110
5	2	3	120	105-135
5	3	0	75	65-90
5	3	1	110	90-125
5	3	2	140	120-160
5	3	3	175	155-200
5	3	4	210	185-240
5	4	0	130	110-150
5	4	1	170	150-200
5	4	2	220	190-250
5	4	3	280	240-320
5	4	4	345	300-390
5	5	0	240	200-280
5	5	1	350	290-420
5	5	2	540	450-600
5	5	3	910	750-1100
5	5	4	1600	1350-1900
5	5	>1800**		

* These numbers are at least 95 % as probable as the MPN.

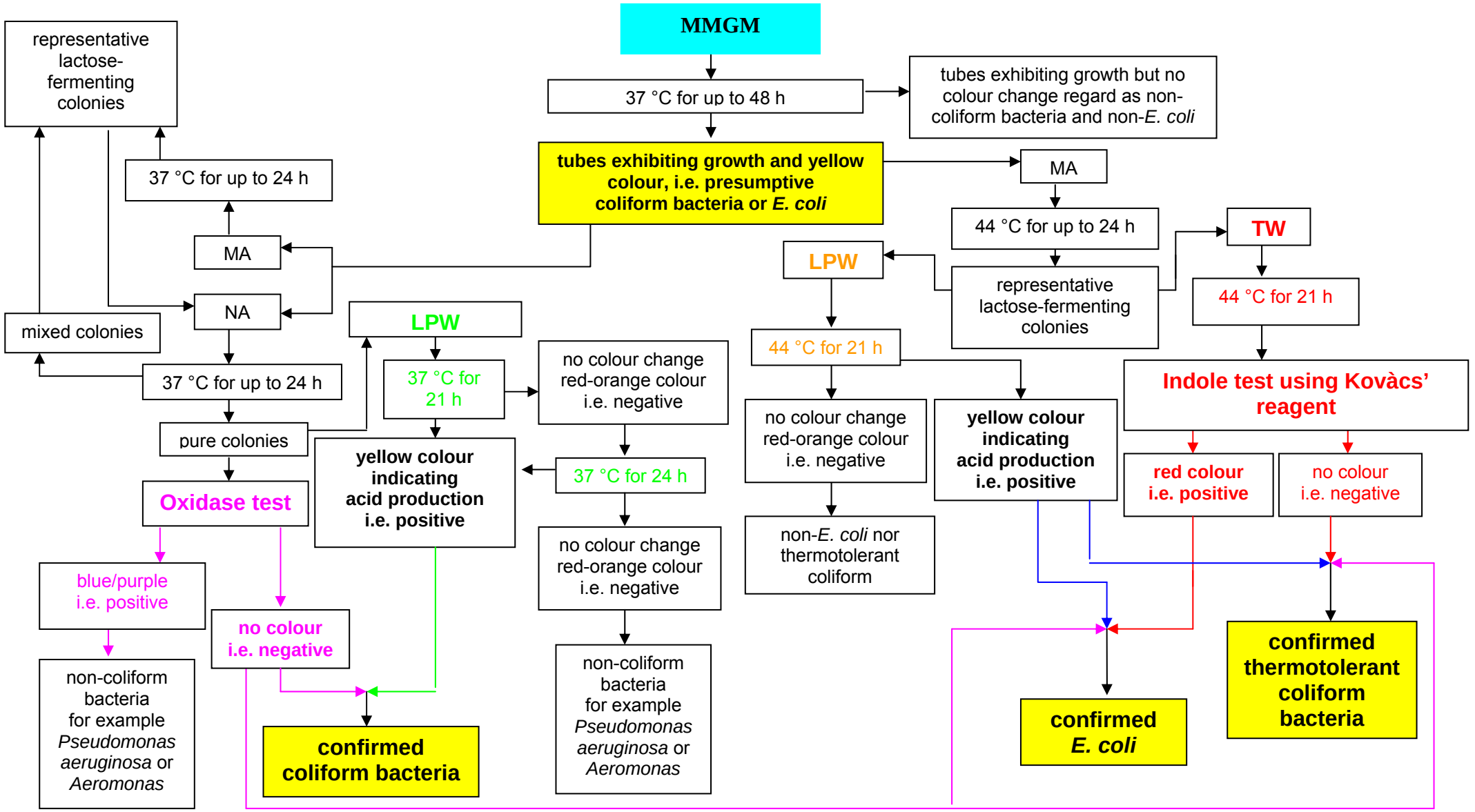
** There is no discrimination when all tubes are positive; the theoretical MPN is infinity. The true count is likely to exceed 1800.

Table C4 Examples of the derivation of the MPN from the numbers of positive reactions in a series of dilutions*

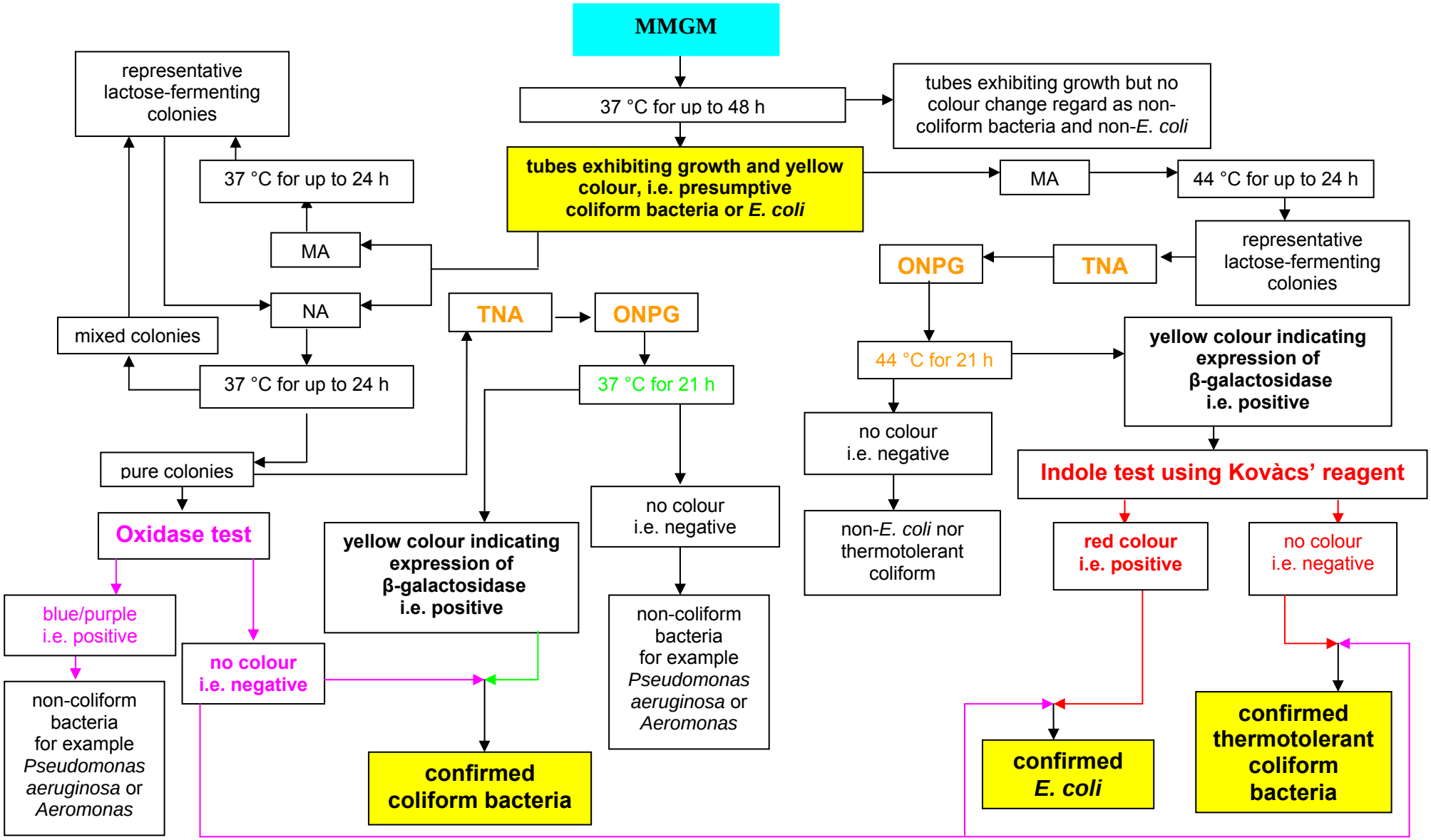
Example in text	Volume of sample (ml)					MPN per 100 ml
	10	1	0.1	0.01	0.001	
(a)	***5***	***3***	***2***	0		140
(b)	5	***5***	***3***	***2***	0	1400
(c)	5	***5***	***2***	***0***	0	500
(d)	***3***	***1***	***0***	0		9
(e)	***0***	***1***	***0***	0		2

* Numbers in bold, underlined, italic type indicate which results should be used in determining the MPN.

Flow chart C1 **Confirmation of yellow colonies of presumptive coliform bacteria and *E. coli* using LPW and TW obtained from MMGM tubes exhibiting growth when incubated at 37 °C for 48 hours** (see section C9.4.1)



Flow chart C2 **Confirmation of yellow colonies of presumptive coliform bacteria and *E. coli* using TNA obtained from MMGM tubes exhibiting growth when incubated at 37 °C for 48 hours** (see section C9.4.2)



D The enumeration of coliform bacteria and *Escherichia coli* by a defined substrate most probable number technique incubated at 37 °C

D1 Introduction

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. These tests provide a sensitive means for detecting faecal contamination, for assessing raw water quality, 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.

D2 Scope

This method comprises a most probable number (MPN) technique and is suitable for the examination of drinking waters, including samples from all stages of treatment and distribution, and those source waters of moderate to high turbidity. Whilst details of a specific commercial product are described in this document, this does not constitute an endorsement of this product but serves only as an illustrative example of the type of products available. Equivalent products may be available.

Users wishing to employ this method, or similar methods from other manufacturers, should verify the performance under their own laboratory conditions[2]. Details of evaluation trials are reported elsewhere[3].

D3 Definitions

Defined substrate media are chemically defined formulations containing substrates for the specific detection of diagnostic enzymes associated with a particular group of organisms.

In the context of this method, organisms which produce β-galactosidase, as demonstrated by the production of a yellow colour through the enzymatic cleavage of ortho-nitrophenyl-β-D-galactopyranoside in a defined substrate medium, are regarded as coliform bacteria. In addition, organisms which produce β-glucuronidase, as demonstrated by the production of a yellow colour and blue-white fluorescence (under long wavelength ultra-violet illumination) through the enzymatic cleavage of 4-methylumbelliferyl-β-D-glucuronide in a defined substrate medium, are regarded as *E. coli*. This method is reported to be highly specific for coliform bacteria and *E. coli*, and confirmation tests including a negative oxidase test are not usually required.

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 (i.e. non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia, Klebsiella*, *Leclercia*, *Pantoea*, *Raoultella*, *Serratia*, and *Yersinia.*

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.

D4 Principle

Organisms are grown in a defined liquid medium containing substrates for the specific detection of the enzymes β-galactosidase and β-glucuronidase. The dehydrated medium is dissolved in 100 ml of sample, or dilution of sample, which is then added to a 51-well reaction pouch. This is then sealed and incubated at 37 °C for between 18 and 22 hours. If, within the pouch, some of the wells exhibit no growth in the medium after incubation, while other wells exhibit some growth in the medium after incubation, then the most probable number of organisms in 100 ml of sample can be estimated from appropriate probability tables, see Appendix D1.

D5 Limitations

This method is suitable for most types of aqueous samples. Those with high turbidities, however, may mask or inhibit colour development. The presence of very high numbers of *Aeromonas* may result in false positive reactions.

D6 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] in this series.

When ultra-violet lamps are used gloves and either goggles or a face shield suitable for use with appropriate ultra-violet emitting sources should be worn.

D7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [2] in this series. Principally, fan assisted incubators are required. An example of the methodology for this type of method is presented and is based upon a commercially available system. Some of the equipment listed is specific to this system and alternative systems may be available for which other equipment may be required. Other items include:

D7.1 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 per 100 ml of sample, or equivalent).

D7.2 Incubator capable of maintaining a temperature of 37.0 ± 1.0 °C.

D7.3 Sterile 100 ml plastic bottles containing anti-foaming agent as supplied by the manufacturer of the test system or suitable equivalent.

D7.4 MPN reaction pouches as supplied by the manufacturer (for example, a 51-well system) and associated heat-sealing equipment.

D7.5 Ultra-violet long wavelength (365 - 366 nm) lamp, and viewer.

D7.6 Colour and fluorescence comparator as supplied by the manufacturer.

D8 Media and reagents

Different commercial formulations of these media and reagents may be available. Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the method[2].

D8.1 *Colilert® 18 medium*[5].

The medium is a commercially available formulation provided in sachets and is suitable for single samples. The medium is a chemically defined formulation with minimal nutrients and substrates for the specific detection of the enzymes β-galactosidase and β-glucuronidase. For MPN counts the medium can be used in conjunction with Quanti-Tray® reaction pouches.

D9 Analytical procedure

D9.1 *Sample preparation*

The volume, or dilution, of samples should be chosen so that not all the wells show a positive response. For treated waters, 100 ml of sample will generally be appropriate, whilst for contaminated waters, appropriate dilutions should be prepared, and 100 ml of diluted sample used. When preparing dilutions use sterile distilled, deionised or similar grade water. Buffered solutions should not be used as they may adversely affect the performance.

D9.2 *Sample processing*

The sample, or appropriate dilution, (usually 100 ml) is decanted into a sterile bottle containing anti-foaming agent. Following the manufacturer's instructions, the contents of one sachet of medium is then aseptically added. After capping the bottle, the contents are gently agitated to ensure dissolution of the medium and then the bottle is left to stand, typically, for a few minutes to allow completion of dissolution and dispersal of any air bubbles. The contents of the bottle are then added to the MPN reaction pouch, which is then sealed in the apparatus provided by the manufacturer to produce a 51-well reaction pouch. Prolonged exposure of the inoculated reaction pouch to direct sunlight should be avoided as this may result in hydrolysis of the specific substrates causing false-positive reactions. The time between the inoculation of the reaction pouch and the beginning of the incubation stage should be as short as possible and no longer than 2 hours.

Sealed MPN reaction pouches are then incubated, 'well-side' down, at 37 °C for not less than 18 hours and not more than 22 hours.

D9.3 *Reading of results*

After incubation, the pouch is examined and the number of wells that have a sufficient yellow colour (see Figure D1) compared against the manufacturer's comparator, is recorded. The pouch is then re-examined under an ultra-violet long wavelength lamp and the number of wells, that produce a blue-white fluorescence (see Figure D2) of sufficient intensity compared against the manufacturer's comparator, is recorded. If the pouch is examined before the completion of 22 hours incubation and this examination reveals

borderline responses, then it should be returned to the incubator for the remaining incubation period. After 22 hours incubation, the pouch is removed and re-examined as before.

Figure D1 Example of MPN reaction pouch with a defined substrate medium with 19 wells showing presence of coliform bacteria

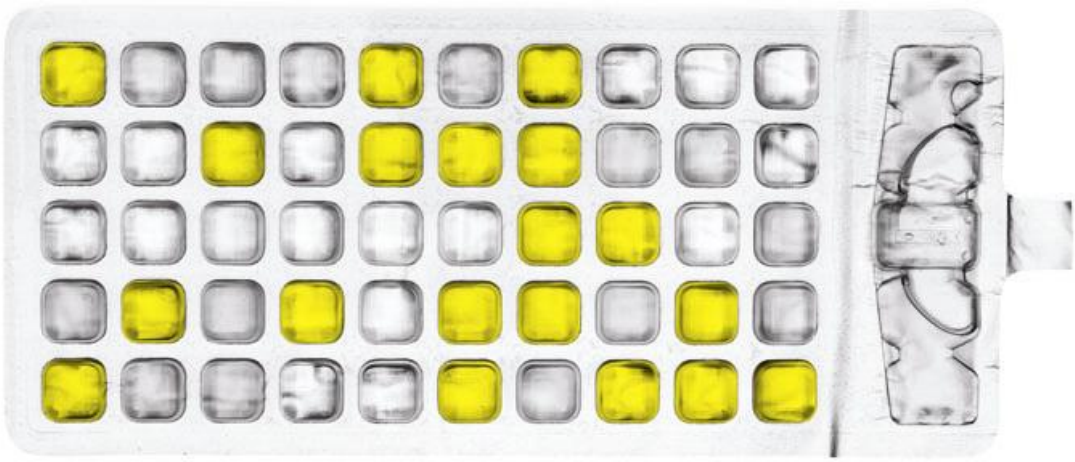
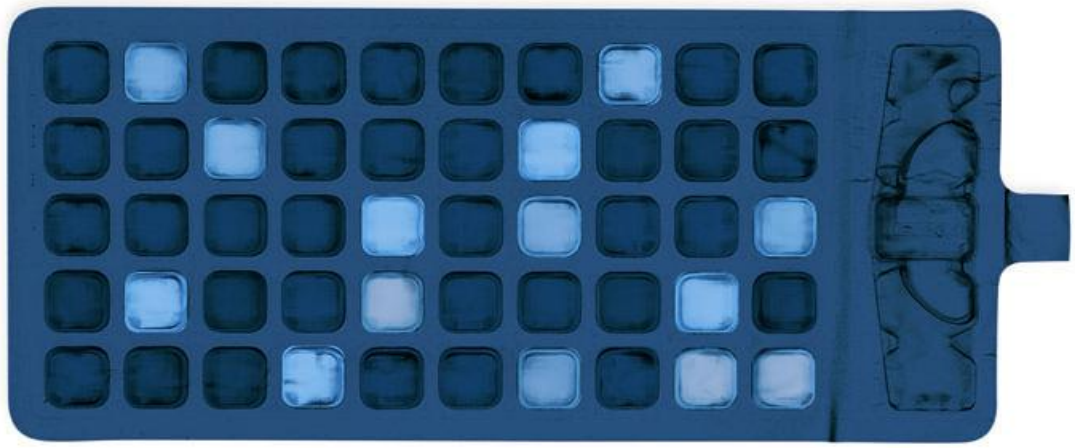


Figure D2 Example of MPN reaction pouch with a defined substrate medium with 14 fluorescing wells showing presence of *E. coli*



D9.4 *Confirmation tests*

This method is reported to be highly specific for coliform bacteria (yellow colouration in the wells) and *E. coli* (blue-white fluorescence in the wells). Hence, confirmation tests may not usually be required. Should there be any doubt as to the type of organism and response detected, then wells showing a characteristic response should be sub-cultured and confirmatory tests undertaken. See methods A, B, C or E.

D10 Calculations

D10.1 *Confirmed coliform bacteria and E. coli*

The MPN of coliform bacteria is determined by reference to appropriate probability tables, see for example Appendix D1. This is derived from the number of wells showing a yellow colouration. For example, if there are 31 wells showing a yellow colouration in the reaction pouch, then from Appendix D1, the MPN of coliform bacteria is 48 per 100 ml of sample, or diluted sample, examined. Any dilution needs to be taken into account.

The MPN of *E. coli* is determined by reference to the same probability table. This is derived from the number of wells showing a blue-white fluorescence. For example, if there are 12 wells showing a blue-white fluorescing in the reaction pouch then, from Appendix D1, the MPN of *E. coli* is 14 per 100 ml of sample, or diluted sample examined. Any dilution needs to be taken into account.

D11 Expression of results

Confirmed coliform bacteria and *E. coli* counts are expressed as MPN counts per volume of sample. For drinking water, the volume is typically 100 ml.

D12 Quality assurance

New batches of media should be tested with appropriate reference strains of target bacteria (for example, *E. coli* and *Enterobacter aerogenes*) and non-target bacteria (for example, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*). Pouches should be incubated for 18 - 22 hours at 37 °C. Further details are given elsewhere[(2)] in this series.

D13 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. Standing Committee of Analysts, Evaluation trials for two media for the simultaneous detection and enumeration of *Escherichia coli* and coliform organisms 1998, *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

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

5. IDEXX Laboratories, Milton Court, Churchfield Road, Chalfont St Peter, Buckinghamshire, SL9 9EW.

Appendix D1 MPN (and 95 % confidence intervals) per 100 ml for a 51-well defined substrate medium reaction pouch

Number of wells showing a positive reaction	MPN per 100 ml	95 % confidence limits	Number of wells showing a positive reaction	MPN per 100 ml	95 % confidence limits
0	0	0-4	26	36	25-54
1	1	0-6	27	38	26-57
2	2	1-7	28	41	28-60
3	3	1-9	29	43	30-63
4	4	2-11	30	45	32-66
5	5	2-12	31	48	33-69
6	6	3-14	32	50	35-73
7	8	4-16	33	53	38-76
8	9	5-17	34	56	40-80
9	10	5-19	35	59	42-84
10	11	6-21	36	62	45-89
11	12	7-22	37	66	47-94
12	14	8-24	38	70	50-99
13	15	9-26	39	74	53-105
14	16	10-28	40	78	56-111
15	18	11-29	41	83	60-118
16	19	12-31	42	89	64-126
17	21	13-33	43	95	68-135
18	22	14-35	44	101	73-146
19	24	15-37	45	109	79-159
20	25	17-39	46	118	85-175
21	27	18-42	47	130	93-195
22	29	19-44	48	145	102-224
23	31	20-46	49	165	115-272
24	32	22-49	50	201	136-388
25	34	23-51	51	>201	

E The detection of coliform bacteria and *Escherichia coli* by a presence-absence technique using minerals modified glutamate medium incubated at 37 °C

E1 Introduction

Tests for coliform bacteria and *Escherichia coli* (*E. coli*) are the most important routine microbiological examinations carried out on drinking water. These tests provide a sensitive means for detecting faecal contamination, for assessing raw water quality, 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.

E2 Scope

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

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

E3 Definitions

In the context of the method, organisms which are oxidase-negative and produce acid from lactose within 48 hours at 37 °C in a chemically defined medium 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 (i.e. non-gas producing) strains. The following genera have been commonly isolated in routine practice: *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia, Klebsiella*, *Leclercia*, *Pantoea*, *Raoultella*, *Serratia*, and *Yersinia.*

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 48 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.

E4 Principle

The presence-absence test is a modification of the multiple tube technique described in method C. The test incorporates a single volume of medium (usually 100 ml) instead of a series of tubes or bottles of different volumes. Several media have been evaluated[3] and procedures are based on the principle that coliform bacteria and *E. coli* should be absent in 100 ml of drinking water. A positive result (as indicated by characteristic growth in the medium) indicates that presumptive coliform bacteria or *E. coli* may be present.

E5 Limitations

Presence-absence tests are not quantitative. They only provide an indication of the presence or absence of presumptive coliform bacteria or *E. coli*. Where a positive result (indicated by characteristic growth in the medium) is recorded, an immediate operational response should be made on the assumption that any positive growth may contain *E. coli*. Operational decisions should, therefore, not be delayed until confirmation tests for coliform bacteria and *E. coli* have been completed.

E6 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] in this series.

Whilst the first stage of this test (i.e. the filling and incubation of bottles) can be conducted at non-laboratory sites, the method involves the growth of cultures of potentially pathogenic bacteria. Therefore, if used at such sites the tests should be conducted in suitably equipped facilities.

E7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [2] in this series. Principally, fan assisted incubators are required. Other items include:

E7.1 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 per 100 ml of sample, or equivalent).

E7.2 Incubators (or water baths) capable of maintaining temperatures of 37.0 ± 1.0 °C and 44.0 ± 0.5 °C.

E8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the 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 the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

E8.1 *Minerals modified glutamate medium*[5]

Lactose	20.0 g
L (+) Glutamic acid sodium salt	12.7 g
L (+) Arginine monohydrochloride	40 mg
L (-) Aspartic acid	48 mg
L (-) Cystine	40 mg
Sodium formate	500 mg
Dipotassium hydrogen phosphate	1.8 g
Ammonium chloride	5.0 g
Magnesium sulphate heptahydrate	200 mg
Calcium chloride dihydrate	20 mg
Iron(III) citrate	20 mg
Thiamine (Aneurin hydrochloride)	2 mg
Nicotinic acid	2 mg
Pantothenic acid	2 mg
Bromocresol purple (1 % m/v ethanolic solution)	2 ml
Water to	1 litre

This formulation enables double-strength medium to be prepared. This is conveniently prepared in quantities of 10 (or more) litres. If the medium is not to be distributed in tubes immediately, the lactose and thiamine should be omitted and added before dispensing.

Several of the ingredients are more conveniently added as separate solutions and these may be prepared as follows:

SOLUTION 1

L (+) Arginine monohydrochloride	400 mg
L (-) Aspartic acid	480 mg
Water	50 ml

Heat the water to about 50 °C and dissolve the ingredients.

SOLUTION 2

L (-) Cystine	400 mg
5M Sodium hydroxide	10 ml
Water	90 ml

Heat the water to about 50 °C and dissolve the ingredients.

SOLUTION 3

Nicotinic acid	20 mg
Pantothenic acid	20 mg
Water	5 ml

Dissolve the ingredients in the water without heating the solution.

SOLUTION 4

Iron(III) citrate	200 mg
Water	10 ml

Heat the water to about 50 °C and dissolve the ingredients.

SOLUTION 5

Calcium chloride dihydrate	5 g
Water	100 ml
Concentrated hydrochloric acid	0.1 ml

Dissolve the ingredients in the water without heating the solution and sterilise the solution at 121°C for 20 minutes. Store as a stock solution.

SOLUTION 6

Thiamine	100 mg
Water	99 ml

Prepare a sterile 0.1 % m/v solution of thiamine in the water. This can be carried out by adding the contents of an ampoule of thiamine (100 mg) to 99 ml of sterile water.

The above solutions may be stored in the range 5 ± 3 °C and any remaining unused solution should be discarded after 6 weeks.

To prepare 10 litres of double-strength medium, dissolve the appropriate quantities of L (+) glutamic acid sodium salt, sodium formate, dipotassium hydrogen phosphate, ammonium chloride and magnesium sulphate heptahydrate in 9 litres of hot distilled water at about 50 °C. Add the whole of solutions 1, 2, 3 and 4, and 4 ml of solution 5. Adjust the pH of the medium to 6.9 ± 0.2 or higher if necessary, so that the final pH (when completely prepared and after sterilisation is 6.7 ± 0.2). After adjustment of the pH, add 20 ml of a 1 % m/v ethanolic solution of bromocresol purple. Dilute to a final volume of 10 litres.

If the medium is not required for immediate use, dispense the mixed solution (medium without lactose and thiamine) into suitable containers in 500 ml volumes. Autoclave the solution at 115 °C for 10 minutes. The medium may be stored in the dark at room temperature for up to one month.

For use, add the necessary amounts of lactose (10 g) and solution 6 (1 ml). Allow the added lactose to dissolve and distribute the solution in 100 ml volumes into suitable bottles, usually of 200 ml capacity. Cap the containers and sterilise the medium at 115 °C for 10 minutes.

The sterile media can be stored at room temperature for up to one month.

E8.2 *Lactose peptone water*

Peptone	10 g
Sodium chloride	5 g
Lactose	10 g
Phenol red (0.4 % m/v aqueous solution)	2.5 ml
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, mix well and distribute medium in 5 ml volumes into tubes. Cap the tubes. Autoclave the tubes at 110 °C for 10 minutes. Sterile media can be stored in the range 5 ± 3 °C for up to one month.

E8.3 *Tryptone water*

The use of certain peptones which give satisfactory results in tests carried out 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 the tryptone used.

Tryptone	20 g
Sodium chloride	5 g
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 the medium in 5 ml volumes into suitable containers and cap and autoclave at 115 °C for 10 minutes. Sterile media can be stored in the range 5 ± 3 °C for up to one month.

E8.4 *Tryptone nutrient agar*

Beef extract powder	1 g
Yeast extract	2 g
Peptone	5 g
Tryptone	20 g
Sodium chloride	5 g
Agar	15 g
Water	1 litre

Dissolve the ingredients in the water. The final pH of the sterile medium should be 7.4 ± 0.2. Sterilise by autoclaving at 121 °C for 15 minutes. Bulk sterile medium may be stored in the dark at room temperature for up to one month. Alternatively, allow the medium to cool to about 50 °C, distribute it in Petri dishes and allow the medium to solidify. Petri dishes containing tryptone nutrient agar (TNA) medium may be stored in the range 5 ± 3 °C for up to one month, protected against dehydration. If bulk medium is used, heat the medium to melt the agar, distribute it into Petri dishes and allow the medium to solidify.

Alternative nutrient media may be used, for example yeast extract agar or blood agar base, and supplemented with tryptone (to a concentration of 20 g/l) but nutrient media containing fermentable carbohydrates should not be used. Alternative media would need to be validated accordingly.

E8.5 *Kovàcs' reagent*[7]

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. Mix well. The reagent should be pale yellow or straw-coloured when freshly prepared and may be stored in the dark in the range 5 ± 3 °C for up to six months. Depending on the grade, some batches of amyl alcohol may be unsatisfactory and produce a dark colour with the p-dimethylaminobenzaldehyde.

E8.6 *Modified Kovàcs' reagent*[8]

p-Dimethylaminobenzaldehyde	5.0 g
Ethyl alcohol	75 ml
Hydrochloric acid (concentrated)	25 ml

Dissolve the p-dimethylaminobenzaldehyde in the ethyl alcohol and slowly add the hydrochloric acid. Mix well. The reagent should be a pale yellow colour when freshly prepared and may be stored in the dark in the range 5 ± 3 °C in the dark for up to six months.

E8.7 *ONPG discs*

These are discs of filter paper impregnated with ortho-nitrophenol-β-D-galactopyranoside (ONPG). Not all commercially available ONPG discs are suitable for this method and should be validated before use. See Appendix 1.

E8.8 *Other media*

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

E9 Analytical procedure

E9.1 *Sample preparation*

A volume of sample (typically 100 ml) is added directly to the same volume of double strength MMGM.

E9.2 *Sample processing*

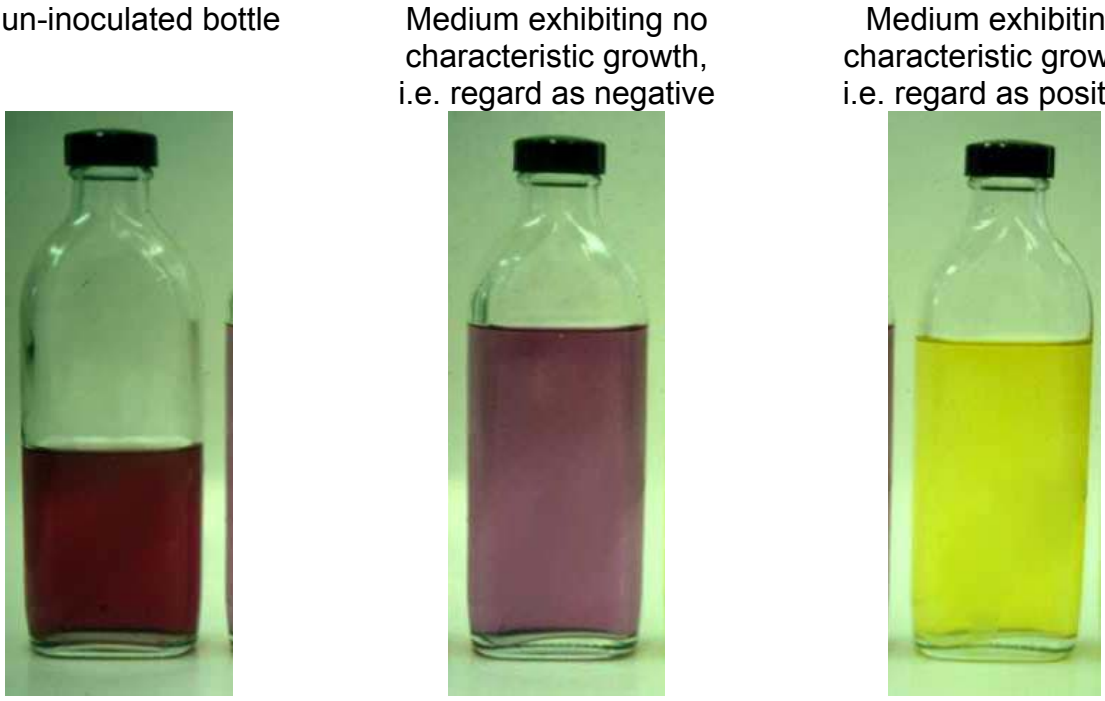
A volume of sample (typically, 100 ml) is added to each bottle containing 100 ml of double strength MMGM. For convenience, the bottle can be marked at the 200 ml level. The sample can be poured directly into the bottle from a sample bottle, or run from a sampling tap provided that care is taken to avoid contamination of the tap, sample and medium. The addition of chlorine-neutralising agents is unnecessary in this case.

The bottle is loosely capped and then incubated at 37 °C and examined after 24 hours. The production of acid (demonstrated by the production of a yellow colouration) should be regarded as a presumptive positive result for coliform bacteria. If a negative result is obtained (where no acid or yellow colouration is produced) after 24 hours, the bottle should be incubated for a further 24 hours before a final result is reported.

E9.3 *Reading of results*

After incubation, examine the bottles for the production of acid (demonstrated by a yellow colouration) which indicates a positive result (see Figure E1). Confirmatory testing should then be carried out to establish the presence of coliform bacteria and *E. coli*.

Figure E1 A presence/absence test with MMGM incubated at 37 °C



E9.4 *Confirmation tests*

Bottles showing characteristic growth in the medium, i.e. positive results are subjected to confirmatory testing for coliform bacteria and *E. coli*.

Coliform bacteria can be confirmed by testing for lactose fermentation in lactose peptone water (LPW) at 37 °C and 44 °C, as being indicative of the possession of the β-galactosidase enzyme, and for the absence of the oxidase enzyme. Also, *E. coli* can be similarly confirmed but with the inclusion of a test for indole production in tryptone water (TW) at 44 °C. See flow chart E1. Alternatively, these bacteria can be confirmed by testing directly for β-galactosidase and indole production from growth on nutrient agar supplemented with tryptone (i.e. TNA) with a disc containing ortho-nitrophenyl-β-D-galactopyranoside (ONPG) on the agar surface. See flow chart E2.

E9.4.1 *Confirmation of coliform bacteria and E. coli using LPW and TW*

For each tube or bottle showing characteristic growth within the MMGM, sub-culture to

(i) MA and NA and incubate at 37 °C for up to 24 hours, and
(ii) MA and incubate at 44 °C for up to 24 hours.

If pure cultures are obtained on NA at 37 °C then perform the oxidase test. The oxidase test is carried out with pure cultures of lactose-fermenting organisms grown on NA at 37 °C.

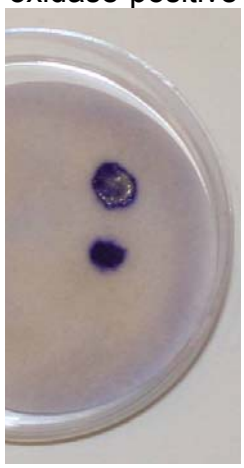
Place 2 - 3 drops, typically 0.1 - 0.2 ml (sufficient to moisten the filter paper) of freshly prepared oxidase reagent on to a filter paper contained in a Petri dish. With a platinum (not nichrome) wire loop, plastic loop, wooden stick or glass rod, smear some of the growth

from the NA onto the prepared filter paper. Regard the appearance of a deep blue purple colour within approximately 10 seconds as a positive reaction. See Figure E2.

Figure E2 Oxidase test

E. coli
oxidase-negative

Pseudomonas aeruginosa
oxidase-positive



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

If there is any doubt about the purity of the cultures then sub-culture typical coliform colonies from MA to NA, incubate the NA at 37 °C for up to 24 hours and carry out the oxidase test on pure colonies from NA.

Typically, coliform bacteria produce large pink to red, mucoid or non-mucoid, colonies on MA, often with a halo of precipitation of bile salts. Some species of *Bacillus*, *Enterococcus* and *Staphylococcus* may grow in MMGM producing turbid growth. These can be readily recognised by colony characteristics on MA and by Gram staining. If *Bacillus*, *Enterococcus* and *Staphylococcus* grow on MA, they produce very small opaque red or colour-less colonies. *Bacillus*, *Enterococcus* and *Staphylococcus* are Gram-positive, whereas coliform bacteria are Gram-negative bacilli.

If the isolates are oxidase-negative, then perform the LPW test for lactose fermentation.

For each isolate to be tested, sub-culture (from NA at 37 °C) to LPW and incubate at 37 °C for 21 ± 3 hours. Following incubation, examine for acid production and if no colour change is exhibited, i.e. the results are negative, re-examine after a further 21 ± 3 hours. Confirmation of acid production is demonstrated by a change of colour from red to yellow (see Figure E3).

Figure E3 Demonstration of lactose fermentation in lactose peptone water

No fermentation exhibited, i.e. no colour change, regard as negative

Fermentation exhibited, i.e. colour change from red to yellow, regard as positive

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, exhibit 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.

Further identification may be carried out using characteristic colonies on MA by means of appropriate biochemical and other tests[9]. Commercial test kits may be used following appropriate performance verification at the laboratory.

Inoculate typical coliform colonies (from MA at 44 °C) into tubes of TW and incubate at 44 °C for 21 ± 3 hours. After incubation of the TW tubes at 44 °C, add 2 - 3 drops, typically 0.1 - 0.2 ml of Kovàcs' reagent. Shake well and allow to settle. Indole production is demonstrated by the rapid appearance of a deep red colour in the upper non-aqueous layer (see Figure E4) confirming the presence of *E. coli*.

It may be necessary to inoculate typical coliform colonies (from MA at 44 °C) into tubes of LPW and incubate at 44 °C for 21 ± 3 hours. Following incubation, examine for acid production. Confirmation of acid production is demonstrated by a change of colour from red to yellow (see Figure E3).

Thermotolerant (i.e. "faecal") coliform bacteria are oxidase-negative and produce acid from lactose at 44 °C, and are indole-negative.

Tests for β-glucuronidase may assist in the early confirmation of *E. coli*[10, 11]. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

Figure E4 Demonstration of indole production in tryptone water using Kovàcs' reagent

No colour developed
i.e. regard as negative

Red colour developed
i.e. regard as positive

E9.4.2 *Confirmation for coliform bacteria* and *E. coli using TNA*

Coliform bacteria and *E. coli* can also be confirmed by demonstration of production of β-galactosidase, indole production (for *E. coli*) and lack of oxidase production, following sub-culture to Petri dishes containing TNA. See Appendix 1 for additional information.

For each tube or bottle showing characteristic growth within the MMGM, sub-culture to

(i) MA and NA and incubate at 37 °C for up to 24 hours, and
(ii) MA and incubate at 44 °C for up to 24 hours.

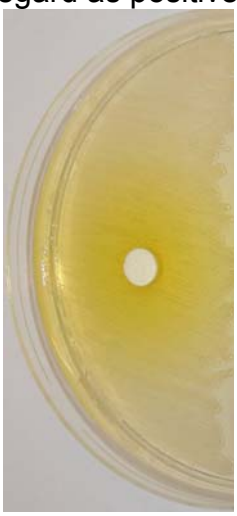
If a pure culture is obtained on NA at 37 °C then perform the oxidase test as described in section E9.4.1. If the isolate is oxidase-negative, sub-culture (from NA at 37 °C) to a Petri dish containing TNA and aseptically place an ONPG disc onto the area of sub-culture. Incubate the TNA Petri dish at 37 °C for 21 ± 3 hours. After incubation, examine the TNA Petri dish for the presence of yellow colouration around the ONPG disc. Confirmation of expression of β-galactosidase is demonstrated by the production of yellow colouration (see Figure E5).

If there is any doubt about the purity of the NA culture at 37 °C then sub-culture typical coliform colonies from MA to NA, incubate at 37 °C for up to 24 hours and carry out the oxidase test.

Thermotolerant coliform bacteria are oxidase-negative and produce β-galactosidase at 44 °C, and are indole-negative.

Figure E5 Demonstration of β-galactosidase production on tryptone nutrient agar with ONPG discs

Yellow colour developed
i.e. regard as positive



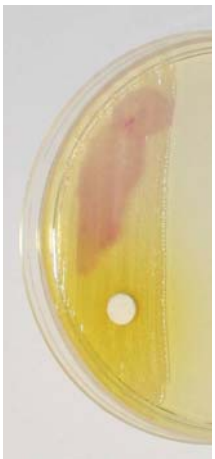
No colour developed
i.e. regard as negative

Further identification may be carried out using characteristic colonies on TNA or MA by means of appropriate biochemical and other tests[9]. Commercial test kits may be used following appropriate performance verification at the laboratory.

Sub-culture typical coliform colonies (from MA at 44 °C) onto Petri dishes containing TNA, aseptically place an ONPG disc onto the area of sub-culture, and incubate the TNA Petri dish at 44 °C for 21 ± 3 hours. After incubation, examine the TNA Petri dish for the presence of yellow colouration around the ONPG disc. Confirmation of expression of β-galactosidase is demonstrated by the production of yellow colouration (see Figure E5). After reading the ONPG reactions, conduct the indole test on the growth on the TNA Petri dish. After incubation of the TNA plates at 44 °C, add 2 - 3 drops, typically 0.1 - 0.2 ml of either Kovàcs' reagent (E8.5) or modified Kovàcs' reagent (E8.6) to an area of growth. Use of the modified Kovàcs' reagent avoids exposure to amyl alcohol fumes. Indole production is demonstrated by the rapid appearance of a red colour (see Figures E6 and E7).

Figure E6 Demonstration of indole production with Kovàcs' reagent on tryptone nutrient agar

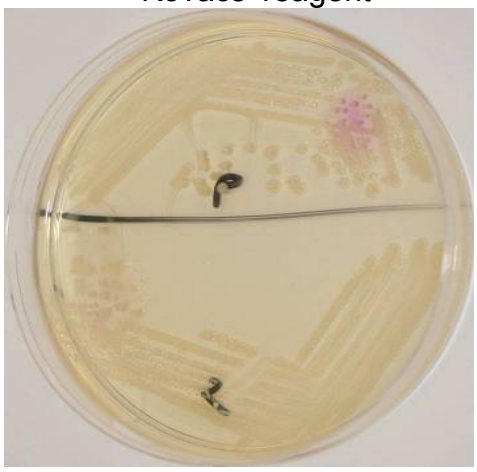
Escherichia coli
ONPG-positive and indole-positive



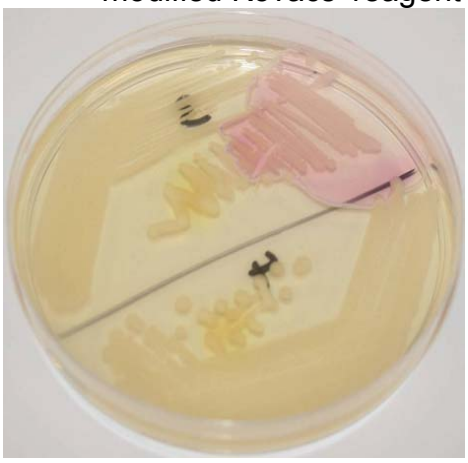
Pseudomonas aeruginosa
ONPG-negative and indole-negative

Figure E7 Demonstration of indole production on tryptone nutrient agar

Red colour developed with Kovàcs' reagent



Red colour developed with modified Kovàcs' reagent



The presence of *E. coli* is demonstrated by the production of indole on TNA.

Tests for β-glucuronidase may assist in the early confirmation of *E. coli*[10, 11]. Suitable commercial test kits may be used following appropriate performance verification at the laboratory.

Typically, *E. coli* colonies are oxidase-negative, express β-galactosidase at 37 °C and at 44 °C, and produce indole on TNA at 44 °C.

E10 Calculations

This test indicates the presence or absence of presumptive coliform bacteria and *E. coli*.

E11 Expression of results

Presumptive and confirmed coliform bacteria and *E. coli* are expressed as being present or absent. How these results are handled electronically will depend on the computer systems used.

E12 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*). Bottles should be incubated for 24 hours at 37 °C or 44 °C as appropriate. Further details are given elsewhere[2] in this series.

E13 References

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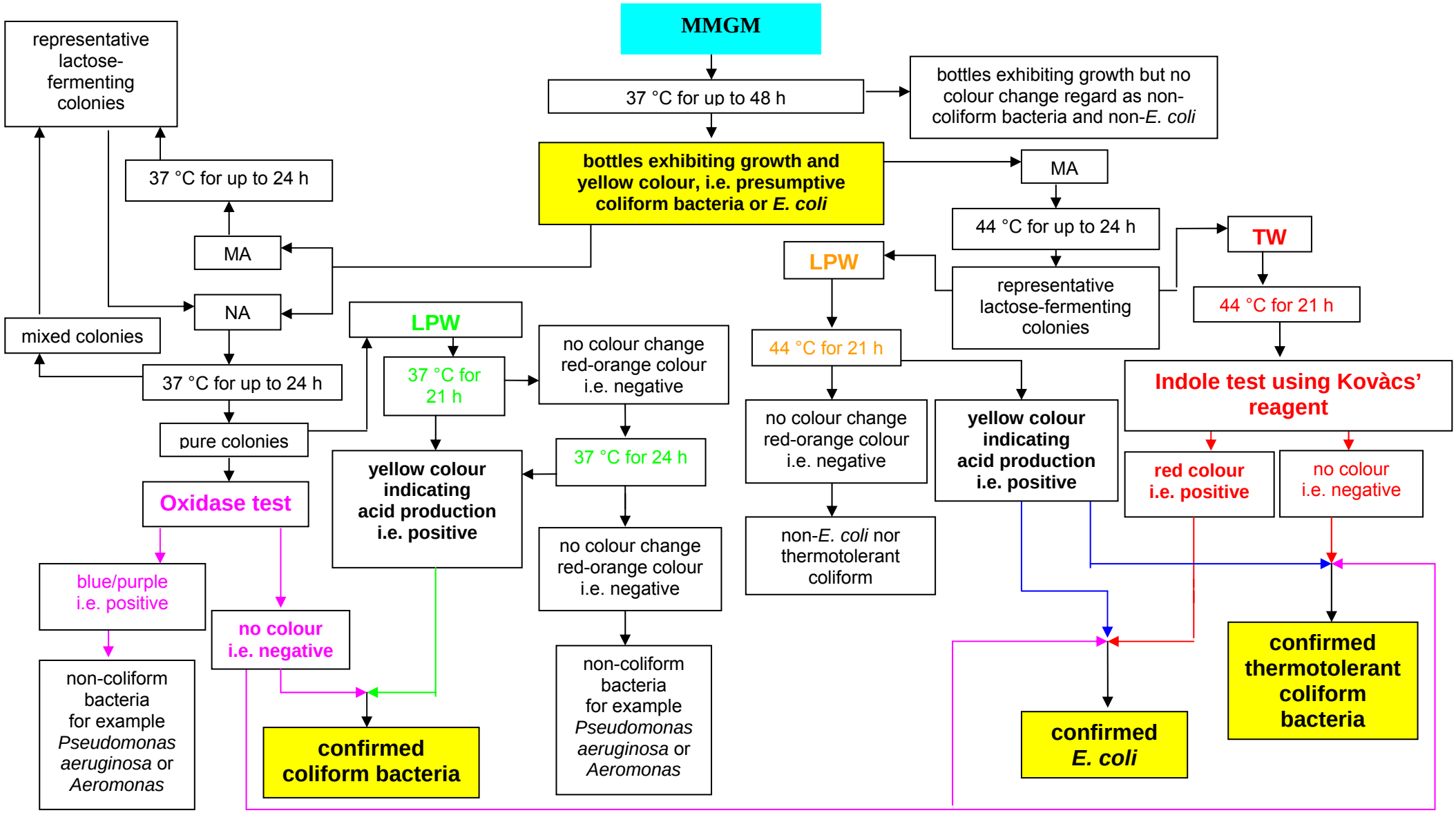
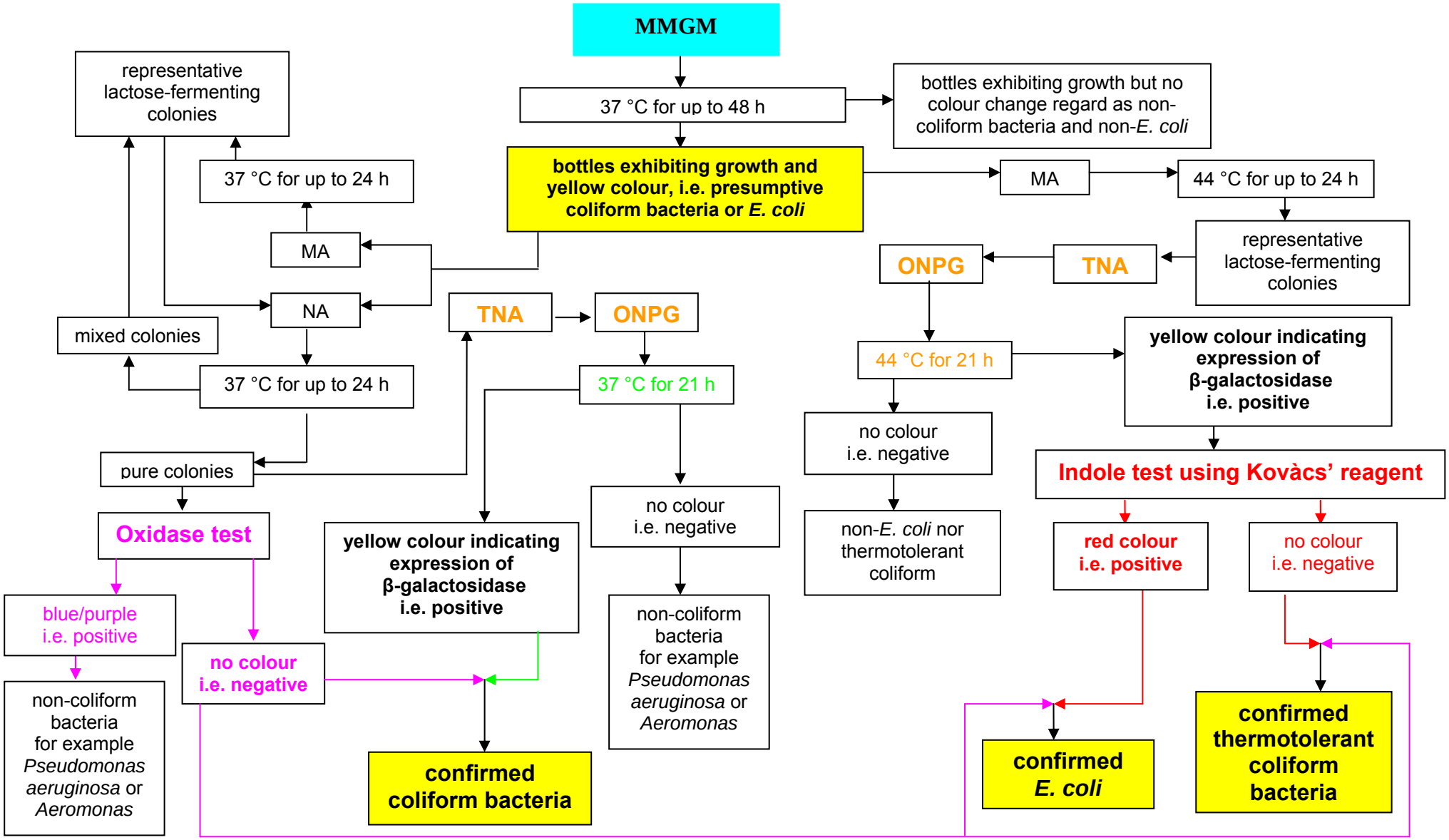
Flow chart E1 **Confirmation of yellow colonies of presumptive coliform bacteria and *E. coli* using LPW and TW obtained from MMGM tubes exhibiting growth when incubated at 37 °C for 48 hours** (see section E9.4.1)



Flow chart E2 **Confirmation of yellow colonies of presumptive coliform bacteria and *E. coli* using TNA obtained from MMGM tubes exhibiting growth when incubated at 37 °C for 48 hours** (see section E9.4.2)



F The detection of *Escherichia coli* O157:H7 by selective enrichment and immuno-magnetic separation

F1 Introduction

The recovery of *Escherichia coli* O157:H7 (*E. coli* O157:H7) from environmental samples is often difficult because of the altered physiological state that certain bacteria develop in order to survive hostile environments. Infections involving *E. coli* O157:H7 have occasionally been implicated with contaminated water, but food-borne infections are more common. The significance of *E. coli* and other coliform bacteria in water treatment and supply are described elsewhere[1] in this series.

E. coli O157:H7 is a recognised cause of haemorrhagic colitis, an illness characterised by bloody diarrhoea and severe abdominal pain but little or no fever. It is also one of the causes of haemolytic uraemic syndrome. Outbreaks involving *E. coli* O157:H7 have been associated with the consumption of food and contaminated water, and person-to-person contact also occurs. Symptoms can persist for up to 7 days. Strains of *E. coli* O157:H7 produce a toxin which is similar to that produced by *Shigella dysenteriae* Type 1 which is cytotoxic to Vero cells in cell culture.

This method may be subject to cross-contamination and aerosol production during the immuno-magnetic separation (IMS) procedure. Any laboratory that does not have access to automated IMS beads retrieval, for example a “Dynal Beadretriever” or equivalent equipment, should consider whether to engage in the examination for *E. coli* O157:H7.

E. coli O157:H7 has been classified as a hazard group 3 organism. Presumptive isolates and waters for which epidemiology strongly suggests *E. coli* O157 may be present should be handled under containment level 3 conditions (as for *Salmonella Typhi*).

The automated IMS procedure should be performed in a class 1 safety cabinet. Access to the room during this procedure should be restricted.

Presumptive colonies on agar plates should be transferred to Category 3 facilities. All confirmatory tests should be performed in a containment level 3 laboratory.

F2 Scope

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

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

F3 Definitions

In the context of this method, *E. coli* O157:H7 are strains of *E. coli* which do not ferment sorbitol, produce colourless to pale orange colonies on cefixime tellurite sorbitol MacConkey agar, and which subsequently confirm by biochemical and serological tests.

F4 Principle

Organisms are isolated by membrane filtration or entrapment with filter-aid, and then selective enrichment followed by immuno-magnetic separation (IMS) and inoculation onto a selective agar medium containing sorbitol as a fermentable carbohydrate and neutral red as an indicator of acidity. Isolation of colonies is followed by selection of typical non-sorbitol-fermenting colonies for identification by biochemical and serological tests.

F5 Limitations

This method does not identify atypical sorbitol-fermenting strains of *E. coli* O157 or other serotypes of *E. coli* that produce verocytotoxins.

This method is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter. This will limit the volume of sample that can be filtered. In these instances, the use of several membrane filters or filter aid may be more appropriate.

When low numbers of *E. coli* O157 are present, detection is improved when larger volumes of sample are examined. However, the presence of high numbers of competing organisms may inhibit the growth or detection of *E. coli* O157.

F6 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.

Strains of *E. coli* O157:H7 which produce verocytotoxin have been reclassified from "Hazard Group 2" to "Hazard Group 3"[4]. However, where samples are not expected to contain *E. coli* O157:H7, routine examination may be undertaken in "Hazard Group 2" containment facilities. Where substantial sub-culture work is required, this should be undertaken in "Hazard Group 3" containment facilities. In addition, those strains used as positive control strains should not produce verocytotoxin. Suitable strains are available commercially (for example, National Collection of Type Cultures 12900). Caution should be exercised in the disposal of contaminated materials, especially those containing *E. coli* O157:H7.

Disposable gloves and safety glasses should be worn throughout the IMS procedure.

F7 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [2] in this series. Principally, appropriate membrane filtration apparatus, equipment for IMS and fan-assisted incubators are required. Other items include:

F7.1 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 per 100 ml of sample, or equivalent).

F7.2 Incubators (or water baths) capable of maintaining temperatures of 37 ± 1 °C and 42 ± 1 °C.

F7.3 Filtration apparatus, sterile or sterilisable filter funnels, and vacuum source.

F7.4 Sterile, membrane filters, for example, white, 47 mm diameter, cellulose-based, 0.45 µm nominal pore size. Gridded membrane filters may facilitate the counting of organisms.

F7.5 Smooth-tipped forceps.

F7.6 Vortex mixer.

F7.7 Rotary sample mixer for IMS mixing (for example, Dynal or equivalent) suitable for use with Eppendorf tubes or screw-capped tubes.

F7.8 Magnetic particle concentrator (for example, Dynal MPC–m or equivalent) suitable for use with Eppendorf tubes or screw-capped tubes.

F7.9 Eppendorf tubes and tube opener or suitable screw-capped tubes.

F8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulation. Commercial formulations should be used and stored according to manufacturer's instructions. The performance of all media and reagents should be verified prior to their use in the 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 the medium is not within its stated range, then, before heating, it should be adjusted accordingly. Where media are stored in a refrigerator they should be allowed to reach room temperature before use.

F8.1 *Modified tryptone soya broth*[5]

Tryptone soya broth	30 g
Bile salts number 3	1.5 g
Dipotassium hydrogen phosphate	1.5 g
Novobiocin	20 mg
Water	1 litre

Dissolve the ingredients in the water and adjust the pH to 7.4 ± 0.2. Dispense the resulting solution in 90 ml volumes into suitable screw-capped containers and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. The sterilised medium may be stored in the dark, protected from dehydration, at room temperature and used within one month.

F8.2 *Buffered peptone water*[6]

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Water	1 litre

Dissolve the ingredients in the water. Dispense the resulting solution in 90 ml volumes into suitable screw-capped tubes or bottles and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. Autoclaved media may be stored in the dark at room temperature, protected from dehydration, and used within one month.

F8.3 *Cefixime tellurite sorbitol MacConkey agar*[7]

Peptone	20.0 g
Sorbitol	10.0 g
Bile salts number 3	1.5 g
Sodium chloride	5.0 g
Neutral red	30.0 mg
Crystal violet	1 mg
Potassium tellurite	2.5 mg
Cefixime	0.05 mg
Agar	15.0 g
Water	1 litre

Dissolve the ingredients, except cefixime and potassium tellurite, in the water. To achieve this, it will be necessary to heat to boiling. Dispense in appropriate volumes into suitable screw-capped bottles and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.1 ± 0.2. Allow the medium to cool. This basal medium can be stored in the dark at room temperature, protected from dehydration, and used within one month. Prior to use melt the basal medium if taken from store. Allow the molten medium to cool to approximately 50 °C and add the following selective supplements which should be filter-sterilised.

(i) Cefixime solution: Dissolve 500 mg of cefixime in 100 ml of ethanol. This may be stored at between 2 - 8 °C and used within one month. Add 1 ml of this solution to 100 ml of ethanol and add 1 ml of the resulting solution to 1 litre of the basal medium to give a final concentration of 0.05 mg/l.

(ii) Potassium tellurite solution: Dissolve 25 mg of potassium tellurite in 10 ml of water. The filter-sterilised solution may be stored at approximately -20 °C and used within one month. Add 1 ml of this solution to 1 litre of the basal medium to give a final concentration of 2.5 mg/l.

Mix the complete medium thoroughly and pour into sterile Petri dishes and allow the agar to solidify. Petri dishes may be stored in the range 5 ± 3 °C, protected against dehydration, and used within one month. Dishes should be dried in a suitable oven at 45 - 50 °C for 30 minutes before use.

F8.4 *Modified phosphate buffered solution*

Sodium chloride	8 g
Potassium chloride	20 mg
Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Polyoxyethylene-sorbitan monolaurate (for example, Tween 20)	0.5 ml
Water	1 litre

Dissolve the ingredients in the water and check that the pH is 7.4 ± 0.2. Sterilise the resulting solution by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the solution should be checked to confirm a pH of 7.4 ± 0.2. Allow the solution to cool. This solution can be stored in the dark at room temperature and used within one month.

F8.5 *Filter-aid*[8]

Diatomaceous earth	1 g (approximately)
Water	15 ml

Weigh out appropriate amounts of filter-aid into suitable bottles and add the water. Sterilise by autoclaving at 121 °C for 15 minutes. Store in the dark at room temperature and use within 12 months.

F8.6 *Magnetic beads*

Para-magnetic beads coated with antibodies to *E. coli* O157 antigen[9]
(for example, Dynabeads or equivalent).

F8.7 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar (NA), MacConkey agar (MA) and *E. coli* antisera and latex agglutination kits.

F9 Analytical procedure

F9.1 *Sample preparation.*

If present in drinking water, *E. coli* O157 are likely to be found in low numbers. Hence, a sample volume of at least 1000 ml should be examined. Smaller volumes may be more appropriate for polluted source waters.

F9.2 *Sample processing*

The sample is filtered using either a membrane filter or with filter aid and any residue added to medium. After incubation, a portion of the medium is then used for the detection of the organism.

F9.2.1 *Membrane filtration*

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

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter, 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 into the funnel. 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 90 ml of modified tryptone soya broth or buffered peptone water. Whereas modified tryptone soya broth is suitable for polluted waters, buffered peptone water may be more appropriate for the recovery of stressed *E. coli* O157 from drinking waters and relatively unpolluted waters[10, 11].

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.

F9.2.2 *Filter-aid*

The usual membrane filtration apparatus may be used but with a sterile absorbent pad in place of a membrane filter to act as a supporting base for the filter-aid. An aliquot of filter-aid (15 ml) is added to the filter funnel and filtered to form an initial layer on the absorbent pad. The contents of a second aliquot of filter aid are then mixed with the sample, which is then filtered. For heavily polluted waters, additional aliquots of filter-aid may be required. When filtration is complete, remove the funnel carefully and transfer the absorbent pad and filter-aid to modified tryptone soya broth. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 90 ml.

F9.2.3 *Enrichment, immuno-magnetic separation and sub-culture to selective agar*

Thoroughly mix the modified tryptone soya broth or buffered peptone water from sections F9.2.1 or F9.2.2. Incubate the modified tryptone soya broth at 42 °C for 21 hours and the

buffered peptone water at 37 °C for 21 hours[10, 11]. Enrichment broths should be subjected to IMS, firstly, after incubation for 6 - 7 hours, and then again at 21 hours.

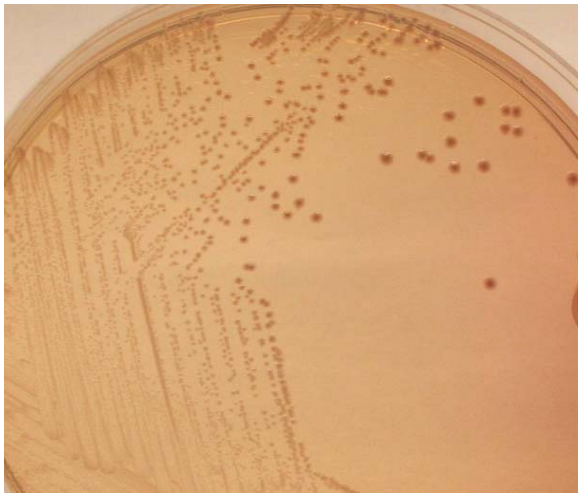
Thoroughly mix the antibody-coated para-magnetic beads and transfer 20 µl of the suspension to a 1.5 ml Eppendorf tube, or suitable screw-capped tube. Add 1 ml of the thoroughly mixed incubated enrichment broth to the tube and mix again, gently, by inversion. Ensure that no air bubbles are trapped at the bottom of the tube. Place the tube onto a rotating mixer set at 30 revolutions per minute and gently mix for approximately 30 minutes. After mixing, place the tube into the magnetic particle concentrator with the associated magnetic strip in position. To concentrate the beads into a small pellet onto the side of the tube, gently invert the magnetic particle concentrator repeatedly for about 1 minute. With the magnetic strip in position, carefully open the tube and aspirate the liquid from the tube and any remaining liquid that might be inside the cap. Remove the magnetic strip from the magnetic particle concentrator and add 1 ml of modified phosphate buffered solution (F8.4) to the tube. Close the cap and gently invert to re-suspend the beads. Re-position the magnetic strip in the magnetic particle concentrator and concentrate the beads into a small pellet as before. Repeat the rinsing step with more modified phosphate buffered solution (F8.4). Re-suspend the beads in 50 µl of modified phosphate buffered solution (F8.4) and inoculate the beads onto cefixime tellurite sorbitol MacConkey agar, following manufacturer's instructions where provided, and incubate at 37 °C for 21 hours.

F9.3 *Reading of results*

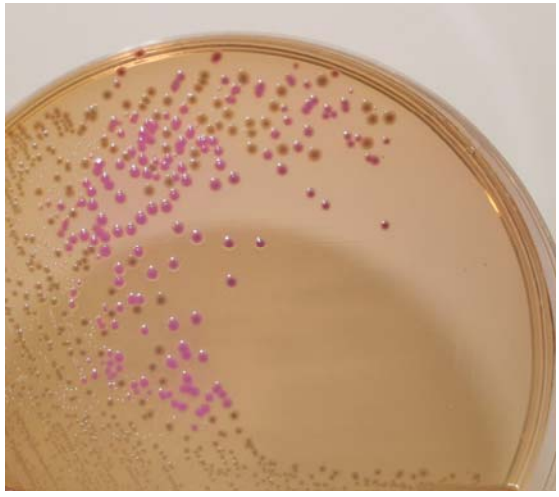
After incubation, examine the cefixime tellurite sorbitol MacConkey agar Petri dishes for typical non-sorbitol-fermenting colonies that are smooth and circular, 1 - 3 mm in diameter and colourless to pale orange in colour, usually with dark centres, see Figure F1. Strains of *E. coli* which ferment sorbitol are pink in colour.

Figure F1 Colonies on cefixime tellurite sorbitol MacConkey agar

E. coli O157



Pink colonies of sorbitol-fermenting *E. coli* and pale orange non-sorbitol-fermenting colonies of *E. coli* O157

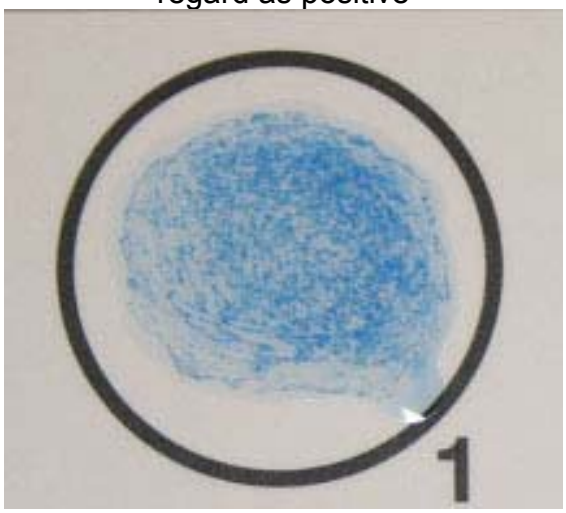


F9.4 *Confirmation tests*

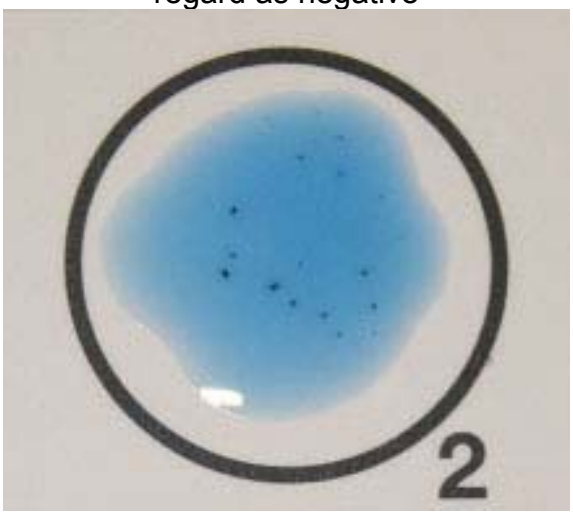
Inoculate typical colonies onto NA (and MA if isolate purity needs to be checked) and incubate at 37 °C for 21 hours. Isolates can then be subjected to serological identification using commercially available antisera or latex agglutination kits. Examine the slides for evidence of agglutination and carry out the tests with appropriate positive (non-verocytotoxin-producing strain of *E. coli* O157) and negative (non-O157 strain of *E. coli*) controls, see Figure F2. Some isolates may require further identification by biochemical testing as some non-sorbitol-fermenting coliform bacteria (for example, *Escherichia hermanii*) can cross react in the latex agglutination test. While chromogenic media can be used to demonstrate the lack of β-glucuronidase, some strains of *E. coli* O157:H7 may produce atypical biochemical profiles and results should be interpreted with caution.

Figure F2 Agglutination test

reaction with *E. coli* O157
regard as positive



reaction with another serotype of *E. coli*
regard as negative



F10 Calculations

The test indicates the presence or absence of *E. coli* O157.

F11 Expression of results

E. coli O157 are reported as being detected or not detected in the volume of sample examined.

F12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example, *E. coli* O157) and non-target bacteria (for example, other *E. coli*). Further details are given elsewhere[(2)] in this series.

F13 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.

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Appendix 1 Comparison of confirmation techniques for coliform bacteria and *Escherichia coli* using a tryptone nutrient agar plate method versus sub-culture to lactose peptone water and tryptone water

1 Introduction

The current conventional tests for confirming presumptive coliform bacteria and *Escherichia coli* (*E. coli*) from water samples, described previously in this series[1, 2] involves sub-culture to tubes or bottles of lactose peptone water (LPW) for incubation at 37 °C and 44 °C, and to tryptone water (TW) for incubation at 44 °C. In addition, coliform bacteria and *E. coli* are oxidase-negative, i.e. do not possess the enzyme cytochrome oxidase. Oxidase-negative isolates that ferment lactose at 37 °C are considered to be confirmed as coliform bacteria. Oxidase-negative isolates that ferment lactose at 44 °C are considered to be confirmed as thermotolerant (or 'faecal') coliform bacteria. Oxidase-negative isolates that ferment lactose at 44 °C and produce indole in tryptone water at 44 °C are considered to be confirmed as *E. coli*. These tests require at least 18 hours incubation and, for some strains of coliform bacteria, may require up to two days for the demonstration of acid production from lactose at 37 °C. In addition, these tests do not detect
β-galactosidase-positive coliform bacteria that may lack other enzymes involved in lactose fermentation, for example lactose permease.

An alternative method for confirming coliform bacteria and *E. coli* based upon the demonstration of β-galactosidase and production of indole on a solid medium (tryptone nutrient agar, TNA) has been proposed by members of the Standing Committee of Analysts (SCA) WG2 committee. The definition of coliform bacteria, faecal coliform bacteria and *E. coli* includes possession of the enzyme β-galactosidase which is involved in the first step of lactose fermentation, and can be demonstrated by the chromogenic substrate ortho-nitrophenol-β-D-galactopyranoside (ONPG) test. A colony is streaked onto nutrient agar supplemented with 2 % tryptone contained in Petri dish and an ONPG-impregnated disc is then placed on the surface of the agar where the colony has been inoculated. Expression of β-galactosidase during incubation results in the disc and the surrounding medium turning yellow. Indole production can be demonstrated in the same Petri dish by the addition of Kovàcs' reagent to colony growth after incubation. The same dish can also be used to perform the oxidase test by the addition of oxidase reagent on another part of the colony growth. By testing for the presence of β-galactosidase the TNA method is more in keeping with the current definitions of coliform bacteria and *E. coli*. The TNA method enables a more rapid, cost effective result to be obtained compared to the current conventional LPW and TW tube tests.

This study was conducted under the auspices of the SCA WG2 to assess the TNA plate technique for the confirmation of coliform bacteria and *E. coli* and establish whether the technique can be regarded as equivalent to the conventional tests involving LPW and TW[1, 2]. In addition, an alternative (modified) formulation of Kovàcs' reagent, originally designed for testing indole production by colonies on membrane filters, was evaluated against the traditional formulation for colonies on TNA. Modified Kovàcs' reagent is prepared using ethyl alcohol instead of amyl alcohol.

2 Materials and methods

Samples of a range of drinking waters and environmental waters were analysed by procedures previously described[1, 2] involving membrane filtration and enumeration on

membrane lauryl sulphate broth (MLSB) or membrane lactose glucuronide agar (MLGA). Following incubation, colonies were counted in accordance with previously published procedures[1, 2], presumptive counts recorded and colonies selected for confirmation.

2.1 *Confirmation of isolates from drinking water samples*

Where possible all presumptive coliform and *E. coli* colonies on each membrane filter exhibiting growth on MLSB at 37 °C or 44 °C, or MLGA at 37 °C were selected for confirmation. Colonies were inoculated into three tubes - two tubes containing LPW and one tube containing TW. One tube of LPW was incubated at 37 °C, the other tube of LPW incubated at 44 °C and the tube containing TW incubated at 44 °C. After 6.0 hours incubation the LPW tube incubated at 37 °C was inoculated onto nutrient agar (for the oxidase test) and onto each of two Petri dishes of TNA. The LPW tube was returned to the incubator at 37 °C to complete incubation for a further 15 hours. Into each TNA Petri dish an ONPG disc was placed at one end of the inoculum. The nutrient agar was incubated at 37 °C for 21 hours. One of the TNA dishes was incubated at 37 °C and the other TNA dish incubated at 44 °C, both dishes being incubated for 21 hours.

A range of drinking waters was examined varying from customer tap and service reservoir samples, hydrant samples, treatment works samples and samples from vending machines.

2.2 *Confirmation of isolates from environmental water samples*

Where necessary, sample dilutions were prepared in sterile maximum recovery diluent or quarter-strength Ringer's solution such that membranes containing between 10 and 30 colonies are obtained.

With environmental waters membrane filters may well contain a mixture of different types of lactose and non-lactose fermenting colonies together with *Aeromonas* spp. In addition, it may not be essential for confirmation results to be obtained within the same time frame as that for drinking waters. Given these two scenarios, yellow colonies, irrespective of size, from MLSB at 37°C and 44 °C, or yellow or green colonies (and blue colonies if present) from MLGA at 37 °C, were sub-cultured to nutrient agar at 37 °C for 21 hours to obtain pure cultures and to perform the oxidase test before being tested for confirmation. Where sub-cultures were pure, the oxidase test was performed and any oxidase-positive isolates recorded before being discarded.

Once oxidase-negative pure cultures were obtained, representative colonies were inoculated into three tubes - two tubes containing LPW and one tube containing TW. One tube of LPW was incubated at 37 °C, the other tube of LPW incubated at 44 °C and the tube containing TW incubated at 44 °C. In addition, the colonies were inoculated onto each of two Petri dishes of TNA. For each TNA dish an ONPG disc was placed at one end of the inoculum. One of the TNA dishes was incubated at 37 °C. The other TNA dish was incubated at 44 °C. Both dishes were incubated for 21 hours.

The environmental waters examined included surface freshwaters (for example river, canal, lake and reservoir waters), ground waters and springs, and sewage effluents.

2.3 Reading of test reactions

After a total incubation time of 21 hours the reactions in tubes containing LPW and TW were recorded. Indole production from TW was tested using Kovàcs' reagent. Development of a red colouration was considered to be positive. Lactose fermentation in LPW was indicated by a colour change from red to yellow. Any tubes containing LPW incubated at 37 °C which did not exhibit lactose fermentation after 21 hours incubation were returned to the incubator and incubated for a further 21 hours and then re-examined.

For the TNA method, the ONPG discs in the TNA dishes at 37 °C and 44 °C were examined for any colour development. A bright yellow colouration on the disc or in a zone in the agar around the disc was recorded as a positive reaction for the presence of β-galactosidase. No development of a yellow colour on the disc or the surrounding agar was regarded as a negative reaction. After recording the ONPG result from the dishes incubated at 37 °C and 44 °C, two or three drops (typically 100 - 200 µl) of Kovàcs' reagent were placed on any colony growth observed in the TNA dish incubated at 44 °C. The development of a red colour in and around the colonies within a few seconds is indicative of a positive indole reaction. No change in the colour of the colonies or the medium is indicative of a negative indole reaction. Following this, two or three drops (typically 100 - 200 µl) of the modified Kovàcs' reagent were placed on a different area of the colony growth. A similar development of a red colour within a few seconds is indicative of a positive indole reaction. No change in the colour of colonies or the medium is indicative of a negative indole reaction.

All data were entered onto a spreadsheet provided by the organisers.

Where possible, laboratories were requested to identify a selection of isolates that confirmed by both methods and a selection of isolates that produced discrepant outcomes by an appropriate identification system (for example API 20E, VITEK etc.). Identifications and their probability scores of good identification were recorded on the spreadsheet.

2.4 *Source of ONPG discs for TNA plate method*

All participating laboratories used Rosco Diagnostica Diatab ONPG discs (BioConnections, Wetherby, UK). Preliminary work had demonstrated poor colouration with some commercially available discs and that colouration tended to fade during the 24-hour incubation.

2.5 *Quality control*

All the media used for the trial were prepared and quality controlled in accordance with previously published guidance in this series[1, 3]. A known strain of *E. coli*, for example NCTC 9001, was included as a positive control for the whole procedure. A suitable negative control, for example *Pseudomonas aeruginosa*, was also used.

3 Results and discussion

Whilst sixteen laboratories participated in the study, data from only 15 laboratories were suitable for analysis. Data were generated for 3203 isolates from a range of samples, including drinking waters, surface freshwaters, ground waters and sewage effluents.

The data for the reactions of these isolates obtained from the LPW and TW tube tests and TNA method are summarised by laboratory in Table 1. Of the 3203 isolates,

i) 144 isolates were oxidase-positive (these were discarded and not tested further), and

ii) 50 isolates were oxidase-negative but failed to ferment lactose in LPW or produce β-galactosidase on TNA.

The resulting 3009 isolates were confirmed as coliform bacteria or thermotolerant faecal coliform bacteria, or *E. coli* by both or one of the confirmation techniques. Including oxidase-positive and non-coliform isolates the overall agreement between the two confirmation methods was 83.6 % with rates for individual laboratories ranging from 63.2 % to 100 %.

For the analysis of the data, it has been assumed that any isolate confirmed by either method is a true confirmed isolate. Typically, more isolates confirmed by the TNA method than by the LPW and TW tube tests (see Table 2). This is particularly noticeable for those isolates that confirmed as coliform bacteria at 37 °C only. Of 813 such isolates, 696 confirmed by both methods, 16 by the LPW and TW tube tests only and 101 by the TNA method only, giving overall confirmation rates of 87.6 % for the LPW and TW tube tests and 98.0 % for the TNA method (Tables 1 and 2). This result is to be expected as the LPW and TW tube tests confirm only isolates that are able to complete the fermentation of lactose, whilst the TNA method confirms β-galactosidase-positive isolates regardless of their ability to complete the process of fermentation of lactose, which is more in keeping with current definitions of coliform bacteria. Similar results are seen in the data for the non-*E. coli* faecal coliform bacteria with overall confirmation rates of 78.0 % for the LPW and TW tube tests and 87.0 % for the TNA method (Table 2). For *E. coli*, the confirmation rates for both techniques were higher, being 93.3 % for the LPW and TW tube tests and 96.3 % for the TNA method (Table 2). The greatest discrepancy between the two techniques for *E. coli* was with indole production. More isolates were indole-positive by the TNA method using either Kovàcs' or modified Kovàcs' reagents (1315 + 95 = 1410) compared to the LPW and TW tube tests (1315 + 47 = 1362) (Table 1). The reasons for this discrepancy are not clear, but may be due to poorer growth in TW when presented with a minimal inoculum. Failure to produce indole on TNA was often associated with no or very limited growth of the isolates. Similarly, failure to ferment lactose or produce β-galactosidase at 44 °C may be due to insufficient inoculum or poor growth at the higher temperature. It is known that some strains of *E. coli* (for example, *E. coli* O157) grow poorly at 44 °C.

Combining all the data for those isolates that confirmed as coliform bacteria (including *E. coli*) leads to overall confirmation rates of 88.1 % for the LPW and TW tube tests and 94.5 % for the TNA method (Table 2). Higher rates were obtained for non-thermotolerant coliform bacteria and *E. coli* than for the thermotolerant non-*E. coli* faecal coliform bacteria. Based on the assumption that all confirmed isolates (by both or either technique) are truly coliform bacteria or *E. coli*, the data indicate that the TNA plate method is superior to the LPW and TW tube tests for the confirmation of these bacteria. However, both techniques appear to be capable of giving false-negative results, most likely due to poor inoculation or poor growth, especially at the elevated temperature of 44 °C.

Table 1 Number of isolates confirming as non-*E. coli* coliform bacteria, non-*E. coli* faecal coliform bacteria or *E. coli* by either or both of the LPW and TW tube tests and TNA methods for the confirmation of coliform bacteria and *E. coli*

Lab	n	Oxidase +ve	Oxidase -ve, not confirm by any technique	Isolates confirmed as non-*E. coli* coliform bacteria by either or both techniques							Isolates confirmed as *E. coli* by either or both techniques							% agree
				by both		by one only					by both	by one only						
							at 37 °C only		at 44 °C			lactose test			indole test			
				at 37 °C only	at 37 °C and 44 °C	LPW TNA	+ve -ve	-ve +ve	+ve -ve	-ve +ve		LPW TNA	+ve -ve	-ve +ve	TW TNA	+ve -ve	-ve +ve	
1	369	28	0	84	182						75							100
2	122	3	0	44	14				4	4	50						3	91.0
3	178	2	5	87	2		1	17		5	56			2*		1	2	85.4
4	242	53	20	55	53		6	26	1	16	11						1	79.3
5	173	4	11	35	11		1	21	3	9	73			3*			5	77.5
6	114	2	0	4	8						99						1	99.1
7	173	6	3	58	2		1	21	2	4	66			7*		2	8	78.0
8	139	1	0	24	13		2	4	7	11	67		3†	3*		4	6	75.5
9	119	2	0	9	3		1			13	73			7*		8	10	73.1
10	166	0	2	43	24			9		20	52		5			1	10	72.9
11	696	0	0	134	62		1	2	17	16	425		10††	2*		22	14	89.2
12	144	1	0	18	27		2		10	35	45		1†			1	5	63.2
13	121	0	0	2	27				20	8	64							76.9
14	347	42	9	69	38		1	1	30	15	105		6†	8**		8	26	75.8
15	100	0	0	30	7				1	4	54			1*			4	91.0
Total	**3203**	**144**	**50**	**696**	**473**		**16**	**101**	**95**	**160**	**1315**		**25**	**33**		**47**	**95**	**83.6**

† These isolates were all also indole-negative on TNA and indole-positive in TW.

†† Seven of these isolates were all also indole-negative on TNA and indole-positive in TW (4 due to no growth on TNA).

* These isolates were all also indole-negative in TW and indole-positive on TNA.

** Five of these isolates were also indole-negative in TW and indole-positive on TNA.

Table 2: Sensitivities of two techniques for the confirmation of non-thermotolerant coliform bacteria, thermotolerant coliform bacteria and *E. coli*

Non-thermotolerant non-*E. coli* coliform bacteria		
	LPW at 37 °C	TNA at 37 °C
Number of isolates	813	
Number confirmed	712	797
Percent confirmed	87.6 %	98.0 %
Thermotolerant non-*E. coli* coliform bacteria		
	LPW at 44 °C	TNA at 44 °C
Number of isolates	728	
Number confirmed	568	633
Percent confirmed	78.0 %	87.0 %
E. coli		
	LPW + TW at 44 °C	TNA at 44 °C
Number of isolates	1468	
Number confirmed	1370	1413
Percent confirmed	93.3 %	96.3 %
All coliform bacteria		
	Total	Total
Number of isolates	3009	
Number confirmed	2650	2843
Percent confirmed	88.1 %	94.5 %

3.1 *Identification of confirmed isolates*

A total of 283 isolates that confirmed by both methods were identified, comprising 87 non-thermotolerant non-*E. coli* coliform bacteria, 54 thermotolerant non-*E. coli* and 142 isolates that confirmed as *E. coli* (see Table 3). All isolates were identified as genera or species of coliform bacteria. The non-thermotolerant coliform bacteria contain several typical environmental coliform bacteria, whilst the thermotolerant coliform bacteria are dominated by those genera typically regarded as faecal coliforms. In the latter group there were three strains identified as *E. coli* that failed to confirm as *E. coli*, due to failure to produce indole from tryptone. In contrast, 135 isolates confirmed as *E. coli* were identified as *E. coli*. These data indicate that 2.2 % of *E. coli* were indole-negative, which is in agreement with other published data[4].

Four strains of *Klebsiella oxytoca*, two strains of *Citrobacter freundii* and one strain of *Escherichia hermannii* confirmed as *E. coli* by both techniques (see Table 3), representing a 4.9 % false-confirmation rate. *Klebsiella oxytoca* and *Escherichia hermannii* are indole-positive coliform bacteria, whilst between 5 % and 20 % of strains of *Citrobacter freundii* are indole-positive. All three species contain strains capable of growth at 44 °C. These results demonstrate the shortcomings of relying on the indole test for the confirmation of *E. coli*.

A further 152 isolates that yielded discrepant results with the two confirmation techniques were also identified (see Table 4). Again all isolates were identified as genera or species of coliform bacteria, representing a wide range of species. For the non-*E. coli* isolates, similar species producing discrepant results were identified for both techniques. The

greater range of identified coliform bacteria from the TNA method is probably reflective of this technique being based on the detection of β-galactosidase compared to the LPW tests being based upon lactose fermentation. The reasons for the disparity in results from the two techniques are unknown, but may be related to failure of the isolate to grow on sub-culture and for the thermotolerant coliform bacteria to produce little or no growth at the higher temperature on sub-culture. Both techniques are dependent upon sufficient inoculum being used for sub-culture to the test media and the response of the isolates in subsequent growth. The disparity of some of the results for the thermotolerant coliform bacteria may also be related to the limited ability of weakly thermotolerant organisms to grow in liquid or on solid media at 44 °C.

Table 3: Identifications (number) of isolates confirmed as either non-thermotolerant coliform bacteria, thermotolerant coliform bacteria or *E. coli* by both confirmation techniques

Confirmed as non-thermotolerant non-*E. coli* coliform bacteria	**Confirmed as thermotolerant non-*E. coli* coliform bacteria**	**Confirmed as *E. coli***
Buttiauxella agrestis (1) *Citrobacter farmeri* (1) *Citrobacter freundii* (7) *Citrobacter youngae* (14) *Citrobacter* spp. (24) *Enterobacter amnigenus* (2) *Enterobacter asburiae* (2) *Enterobacter cloacae* (7) *Enterobacter intermedius* (3) *Enterobacter sakazakii* (2) *Escherichia coli* (1) *Klebsiella oxytoca* (5) *Klebsiella pneumoniae* (3) *Kluyvera ascorbata* (1) *Kluyvera* spp. (2) *Pantoea agglomerans* (3) *Pantoea* spp. (3) *Raoutella terrigena* (2) *Serratia fonticola* (1) *Serratia liquefaciens* (1) *Serratia* spp. (1) *Yersinia* spp. (1)	*Citrobacter freundii* (3) *Citrobacter* spp. (1) *Enterobacter amnigenus* (1) *Enterobacter cloacae* (7) *Enterobacter* spp. (1) *Escherichia coli* (3) *Escherichia fergusonii* (1) *Klebsiella oxytoca* (2) *Klebsiella pneumoniae* (11) *Klebsiella rhinoscleromatis* (1) *Klebsiella* spp. (21) *Serratia liquefaciens* (1) *Serratia odorifera* (1)	*Citrobacter freundii* (2) *Escherichia coli* (135) *Escherichia hermannii* (1) *Klebsiella oxytoca* (4)

The most notable aspect regarding the disparity in confirmed *E. coli* isolates is the range of non-*E. coli* species identified, especially for the TNA plate method (see Table 4). Again the principal species resulting in false confirmations are *Klebsiella oxytoca* and species of *Citrobacter* (the identifications of species of *Enterobacter* and *Serratia* should be considered suspect as these species are indole-negative and do not normally grow at 44 °C). The high number of occurrences of *Klebsiella oxytoca* confirming as *E.* coli from the TNA method compared to the TW tube test may be indicative of a greater sensitivity of detecting indole production on the solid medium.

Table 4 Identifications of isolates confirmed as either non-thermotolerant coliform bacteria, thermotolerant coliform bacteria or *E. coli* by one confirmation technique only

Identification	Lactose or β-galactosidase at 37 °C		Lactose or β-galactosidase at 44 °C		Indole production at 44 °C	
	LPW +ve TNA -ve	LPW -ve TNA +ve	LPW +ve TNA -ve	LPW -ve TNA +ve	TW +ve TNA -ve	TW -ve TNA +ve
Buttiauxella agrestis		3				
Citrobacter braakii						2
Citrobacter farmeri				2		
Citrobacter freundii			7	6	1	4
Citrobacter koseri				1		
Citrobacter youngae	1			2		
Citrobacter spp.		1		5		1
Enterobacter amnigenus		3	1			
Enterobacter asburiae	1			1		
Enterobacter cloacae			7	4	2‡	2‡
Enterobacter sakazakii			1			1‡
Enterobacter spp.				1		
Escherichia coli				3*	7	14
Escherichia hermannii	1					
Escherichia vulneris			1	1		
Hafnia alvei		4				
Klebsiella ornithinolytica			4	2		
Klebsiella oxytoca	1			9†		14
Klebsiella pneumoniae				5		
Klebsiella spp.			5			
Kluyvera cryocrescens			1			
Kluyvera spp.			3			
Pantoea agglomerans		1				
Pantoea spp.	1	3	1	1		
Raoutella terrigena		1				
Serratia fonticola	1	5				
Serratia liquefaciens	1				1‡	
Serratia odorifera			3	1		
Serratia spp.				1		
Yersinia enterocolitica			1			
Yersinia spp.				1		
Total (n = 152 isolates)	**7**	**21**	**35**	**46**	**11**	**38**

* Two of these isolates were also indole-negative in TW and indole-positive on TNA
† Four of these isolates were also indole-negative in TW and indole-positive on TNA
‡ These identifications should be treated with caution as these bacteria are indole-negative and do not normally grow at 44 °C.

In addition to the identified isolates listed in Table 4, three further isolates were identified as *E. coli* with unusual confirmation test reactions. Two of these isolates were *E. coli* which were indole-positive by both techniques but LPW-negative and β-galactosidase-negative at 44 °C. The laboratory that isolated these two bacteria sub-cultured them to MacConkey agar at 37 °C where they grew as non-lactose fermenting colonies. However, they both produced β-galactosidase at 37 °C (as demonstrated by the TNA ONPG test) and, thus, can be regarded as *E. coli* lacking the ability to complete the fermentation of lactose despite expressing β-galactosidase. The third *E. coli* isolate was also indole-positive at 44 °C, but LPW-negative and β-galactosidase-negative at both 37 °C and 44 °C.

3.2 Evaluation of modified Kovàcs' reagent

For 14 of the 15 laboratories indole reactions using the conventional formulation of Kovàcs' reagent (prepared in amyl alcohol) and modified Kovàcs' reagent (prepared in ethyl alcohol) were predominantly the same. Twelve laboratories reported 100 % agreement between the two formulations and the remaining two laboratories reported 99.3 % and 99.4 % agreement respectively. For these 14 laboratories, 2044 isolates were tested, of which 2038 (99.7 %) were either indole-positive or indole-negative with both formulations. Of the remaining six isolates, four were indole-positive with the conventional formulation only and the other two isolates were indole-positive with the modified formulation only. In contrast to these data, the results from laboratory 15 showed considerable variation between the two formulations of Kovàcs' reagent. Of 70 isolates tested, 13 were indole-negative with both formulations, 18 isolates were indole-positive with both formulations, 16 isolates were indole-positive with the conventional formulation only and 23 isolates were indole-positive with the modified formulation only. The reasons for this much greater discrepancy in results for this laboratory compared to the other laboratories is not known, but may be indicative of problems within the laboratory conducting the indole test using the TNA method.

4 Conclusions

This study compared the efficacy of a solid medium method, the TNA method, with the traditional broth-based LPW and TW tube tests for the confirmation of coliform bacteria and *E. coli* from water samples. The principal conclusions of the study are:-

(i) There is a confirmation agreement rate of 83.6 % between the TNA method and the LPW and TW tube tests.

(ii) Based on the assumption that all confirmed isolates (by both or either technique) are true coliform bacteria or *E. coli*, the data indicate that the TNA method is superior (94.5 % confirmation rate) to the LPW and TW tube tests (88.1 % confirmation rate) for the confirmation of these bacteria.

(iii) Both confirmation techniques are reliable for the confirmation of coliform bacteria. The confirmation rates for non-thermotolerant coliform bacteria are higher with the TNA method compared to the LPW and TW tube tests (98.0 % versus 87.6 %) The confirmation rates for thermotolerant faecal coliform bacteria are also higher with the TNA method compared to the LPW and TW tube tests (87.0 % versus 78.0 %). This is probably reflective of a greater number of coliform bacteria being confirmed by the β-galactosidase test compared to the demonstration of lactose fermentation.

(iv) The confirmation rate for *E. coli* was higher by the TNA method (96.3 %) compared to the LPW and TW tube tests (93.3 %). However, both confirmation techniques are prone to false confirmed *E. coli* results due to thermotolerant indole-positive coliform bacteria (especially *Klebsiella oxytoca* and species of *Citrobacter*). Owing to the apparent greater sensitivity of the TNA method for the testing of indole-production there is a higher probability of detecting a falsely-confirmed *E. coli* using this method.

(v) The TNA method has been shown to produce similar or higher confirmation rates of coliform bacteria and *E. coli* from a range of water samples compared to the traditional LPW and TW tube tests.

(vi) Modified Kovàcs' reagent (using ethyl alcohol) has been shown to produce similar results to the traditional formulation (using amyl alcohol) for the testing of indole production using the TNA method.

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However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advance notice of forthcoming publications, please contact the Secretary.

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ENVIRONMENT AGENCY
EMERGENCY HOTLINE
0800 80 70 60

ENVIRONMENT AGENCY