Environment Agency

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

Methods for the Examination of Waters and Associated Materials

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

Methods for the Examination of Waters and Associated Materials

This booklet contains three methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment. This booklet supercedes "The Microbiology of Drinking Water (2004) - Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube-most probable number techniques". This document includes an explanation on *Salmonella* taxonomy, removes brilliant green agar as isolation media, provides greater detail and makes minor editorial changes contained in the previous document.

Within this series there are separate booklets dealing with different topics concerning the microbiology of drinking water. Other 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 4 - Methods for the isolation and enumeration of coliform bacteria and *Escherichia coli* (including *E. coli* O157:H7)
Part 5 - A method for the isolation and enumeration of enterococci by membrane filtration
Part 7 - Methods for the enumeration of heterotrophic bacteria by pour and spread plate techniques
Part 8 - Methods for the isolation and enumeration of *Aeromonas* and *Pseudomonas aeruginosa* by membrane filtration
Part 10 - Methods for the isolation of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment

The Microbiology of Drinking Water (2004) -
Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* by membrane filtration
Part 11 - Taste, odour and related aesthetic problems
Part 12 - Methods for the isolation and enumeration of 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

Whilst specific commercial products may be referred to in this document this does not constitute an endorsement of these particular materials. Other similar materials may be suitable and all should be confirmed as such by validation of the method.

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About this series

Introduction

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

Performance of methods

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

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

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

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the 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. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the Agency's web-page (www.environment-agency.gov.uk/nls) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary
July 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 isolation of *Salmonella* species by selective enrichment

A1 Introduction

Many different serotypes of *Salmonella* species are present, to varying extents, in humans, animals and birds. All members of the genus are potentially pathogenic. The low numbers of *Salmonella* species found in waters mainly originate from sewage and sewage effluents. The numbers of salmonellas present in water are, generally, much lower than those of other micro-organisms.

Salmonella infections give rise to symptoms of diarrhoea and vomiting. The incubation period varies between 12 - 72 hours and symptoms usually persist for 2 - 3 days. Most cases of infection occur from the consumption of raw, or undercooked, food, particularly poultry and food containing eggs. The significance of *Salmonella* 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.

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 that form characteristic colonies on selective agar media after culture in enrichment media and which produce the serological and biochemical reactions described are regarded as *Salmonella* species.

Salmonella species normally conform to the general definition of the family Enterobacteriaceae(3) and can be further differentiated, biochemically, into 4 subgroups, subgenus I to IV. Those bacteria of subgenus I (the largest group) are considered pathogenic towards humans and are β-galactosidase-negative. Salmonellas are sub-divided into serovars on the basis of genus-specific combinations of somatic and flagellar antigens. Salmonellas may be further differentiated into groups by bacteriophage and plasmid typing.

The usual biochemical reactions include production of hydrogen sulphide; indole and urease are not produced; citrate is utilised as a carbon source; and lysine and ornithine are decarboxylated. Phenylalanine and tryptophan are not oxidatively de-aminated, and sucrose, salicin, inositol and amygdalin are not fermented.

There are currently only three recognised species of *Salmonella* – *Salmonella bongori*, *Salmonella enterica* (which also has six subspecies) and the recently described *Salmonella subterranea.* Most *Salmonella* encountered in the environment are serovars of *Salmonella enterica* or one of its subspecies. To avoid confusion with species' names the names of these serovars are not italicised when written. Thus, the strain formerly written as *Salmonella typhi* is now *Salmonella enterica* subspecies *enterica* serovar Typhi which is conventionally shortened to *Salmonella* Typhi. Similarly for *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Montevideo etc.

A4 Principle

Isolation is based on concentration from water by membrane filtration (with or without the use of filter aid). This is followed by pre-enrichment in a non-selective medium (to recover environmentally-stressed organisms) with further enrichment in a selective medium, after which aliquots are subcultured to a selective agar containing lactose, an indicator of acidity, and an indicator of hydrogen sulphide production. Characteristic colonies are confirmed by biochemical tests and by slide agglutination.

A5 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. In such instances the sample volume can be increased by the use of several membrane filters or filter aid. When low numbers of organisms are present, detection is dependent only on the volume of sample that can be filtered and tested. High numbers of competing organisms may inhibit the growth, or detection, of target organisms. This method uses only one selective agar for the diagnostic detection of *Salmonella* species. Although some species of *Salmonella* may not be detected by the medium used, it may be adequate for routine water monitoring. However, for investigations into possible water-borne outbreaks of salmonellosis a range of selective media should be used(4). This method is not suitable for the recovery of *Salmonella* Typhi (but see A9.4.1) and *Salmonella* Paratyphi.

A6 Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations(5) and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere(2) in this series.

Extra care should be taken in the isolation and identification of salmonellas due to the pathogenic nature of the organisms. Staff should be adequately trained and supervised and work involving subculture and handling of cultures should be performed in a designated area of a properly equipped laboratory. Adequate facilities should be in place for disposal and sterilisation of test materials.

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 are required. Other items include:

A7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

A7.2 Incubators capable of maintaining temperatures of 37 ± 1 °C and 41.5 ± 0.5 °C.

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

A7.4 Sterile membrane filters, for example, white 47 mm diameter, cellulose-based 0.45 µm nominal pore size.

A7.5 Smooth-tipped forceps.

A8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in the method. 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 *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 (typically, 90 ml) 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 value of 7.2 ± 0.2. Autoclaved media may be stored in the dark at room temperature, protected from dehydration, and should be used within one month.

A8.2 *Rappaport Vassiliadis enrichment broth*[7, 8]

Solution A	
Soya peptone	4.5 g
Sodium chloride	7.2 g
Potassium dihydrogen phosphate	1.26 g
Dipotassium hydrogen phosphate	180 mg
Water	800 ml
Solution B	
Magnesium chloride anhydrous	13.6 g
Water	100 ml
Solution C	
Malachite green	36 mg
Water	100 ml

Dissolve the ingredients of solution A in the 800 ml of water. To achieve this, it may be necessary to heat to boiling. Prepare this solution on the day of use. To 800 ml of solution A, add 100 ml of solution B and 100 ml of solution C and mix well. Dispense the resulting solution (typically, 10 ml) into suitable containers, cap and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to

confirm a pH value of 5.2 ± 0.2. Store autoclaved media at between 2 - 8 °C, protected against dehydration, and use within one month.

A8.3 *Xylose lysine desoxycholate agar*[9]

Basal medium	
Lactose	7.5 g
Sucrose	7.5 g
Xylose	3.75 g
L(-) Lysine hydrochloride	5.0 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red (0.4 % m/v aqueous solution)	20 ml
Agar	12.0 g
Water	1 litre

Solution A	
Sodium thiosulphate pentahydrate	34.0 g
Ammonium iron(III) citrate	4.0 g
Water	100 ml

Solution B	
Sodium desoxycholate	10.0 g
Water	100 ml

Dissolve the ingredients of the basal medium in the water. This will require gentle heating. Dispense the resulting solution in appropriate volumes into suitable bottles, cap and sterilise by autoclaving at 115 °C for 10 minutes. The basal medium may be stored in the dark at room temperature and should be used within one month. Dissolve the ingredients of solution A and solution B in the respective amounts of water and separately pasteurise the individual solutions by heating at approximately 60 °C for 1 hour. To prepare the complete medium, melt the basal medium and cool to approximately 50 °C. Aseptically, add 2.0 ml of solution A and 2.5 ml of solution B to 100 ml of basal medium and mix well. The pH of the medium should be checked to confirm a pH value of 7.4 ± 0.2. Pour the complete medium into sterile Petri dishes and allow the medium to solidify. Store the poured plates at between 2 - 8 °C, protected against dehydration, and use within two weeks. The agar surface of prepared dishes should be dried before use.

A8.4 *Lysine iron agar*[10]

Peptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
L (-) Lysine	10.0 g
Ammonium iron(III) citrate	500 mg
Sodium thiosulphate pentahydrate	40 mg
Bromocresol purple (1 % m/v ethanolic solution)	2 ml
Agar	15.0 g
Water	1 litre

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solutions in small volumes (typically, 5 -10 ml) into suitable

containers and cap. Sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH value of 6.7 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. Store the prepared medium at between 2 - 8 °C, protected against dehydration, and use within one month.

A8.5 *Triple sugar iron agar*(11)

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Iron(III) citrate	300 mg
Sodium thiosulphate pentahydrate	300 mg
Phenol red (0.4 % m/v aqueous solution)	6 ml
Agar	15.0 g
Water	1 litre

Dissolve the ingredients (except phenol red) in the water. To achieve this, it will be necessary to heat to boiling. Add the phenol red solution and mix well. Dispense the resulting solution in small volumes (typically, 5 -10 ml) into suitable containers, cap and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH value of 7.4 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. Store the prepared medium at between 2 - 8 °C, protected against dehydration, and use within one month.

A8.6 *Urea broth*

Base medium	
Peptone	1.0 g
Glucose	1.0 g
Disodium hydrogen phosphate	1.0 g
Potassium dihydrogen phosphate	800 mg
Sodium chloride	5.0 g
Phenol red (0.4 % m/v aqueous solution)	1.0 ml
Water	1 litre

Dissolve the ingredients in the water and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution (typically, 95 ml) into suitable bottles, cap and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH value of 6.8 ± 0.2. Prepared base medium may be stored in the dark at room temperature and should be used within one month. Prior to use, add 5 ml of an aqueous 40 % m/v filter-sterilised solution of urea to each 95 ml of base medium and aseptically dispense in 2 - 3 ml volumes in sterile containers and cap.

A8.7 *Filter-aid*(12)

Diatomaceous earth	1 g (approximately)
Water	15 ml

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

A8.8 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar, saline solution and anti-sera.

A9 Analytical procedure

A9.1 *Sample preparation*

Due to the likelihood that, if present, numbers of salmonellas in drinking water are likely to be low, a sample volume of at least 1000 ml should be examined. Smaller volumes may be appropriate for polluted source waters.

A9.2 *Sample processing*

A9.2.1 *Membrane filtration*

Filter an appropriate volume of sample. If the sample is turbid, several membrane filters may be required. Alternatively, a large volume filtration system can be used(2).

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 onto the porous disc of the filter base. If a grided membrane filter is used, place grid-side upwards. 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 water slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and carefully transfer the membrane filter to a container containing, typically, 90 ml of buffered peptone water. The volume of buffered peptone water can be adjusted according to the nature of the sample and the number of membranes that may be needed. A minimum volume of 50 ml should be taken. Cap the container. If more than one membrane filter is required, all filters should be transferred to the buffered peptone water, ensuring that all the membrane filters are fully submerged.

The funnel 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 of sample is filtered first. For different samples, a fresh pre-sterilised funnel should be taken or remove a funnel from the boiling water bath. Allow the funnel to cool and repeat the filtration process. If funnels are to be re-used, then after filtration of each sample, disinfect the funnel by immersing it in boiling distilled 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 disinfected 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.

A9.2.2 *Filter-aid*

The usual membrane filtration apparatus may be used but with a sterile absorbent pad in place of the membrane filter to act as a supporting base for the filter-aid. An aliquot of filter-aid (typically, 15 ml) should be filtered to form an initial layer on the absorbent pad. A second aliquot (typically, 15 ml) of filter-aid should be mixed with the volume of sample and then filtered. For turbid or dirty 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 buffered peptone water. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 90 ml.

A9.2.3 *Pre-enrichment, enrichment and subculture to selective agar*

The buffered peptone water and membrane filters, and if appropriate filter-aid, should be gently mixed and incubated at 37 °C for 21 ± 3 hours. After incubation, mix well and subculture 0.1 ml of the buffered peptone water into 10 ml of Rappaport Vassiliadis enrichment broth and incubate at 41.5 °C for 21 ± 3 hours. After this time, plate out a loopful of the Rappaport Vassiliadis enrichment broth onto xylose lysine desoxycholate agar. Incubate the selective agar at 37 °C for 21 ± 3 hours. After plating, return the Rappaport Vassiliadis enrichment broth to the incubator at 41.5 °C for a further 21 ± 3 hours. After this time, again plate out a loopful of the Rappaport Vassiliadis enrichment broth onto xylose lysine desoxycholate agar. Incubate the selective agar at 37 °C for 21 ± 3 hours.

A9.3 *Reading of results*

After each respective incubation period, examine the Petri dishes under good light, if necessary using a hand lens. Colonies are differentiated as follows:

Colonial appearance on xylose lysine desoxycholate agar

Organism	Characteristic appearance
Salmonella	smooth red colonies 2 - 3 mm in diameter, typically, with black centres or wholly black colonies (see Figure 1)
Xylose-fermenting coliform bacteria	yellow colonies
Pseudomonas species	red or yellow colonies with grey-black centres
Shigella species	small pink-red colonies
Proteus species	red colonies that are irregular and may have small black centres

Where isolates are overgrown on the xylose lysine desoxycholate agar, subculture to fresh xylose lysine desoxycholate agar. This facilitates the production of pure cultures and enables typical colonial morphology to be observed. See flowchart and Figure 1.

Flowchart for method A procedures

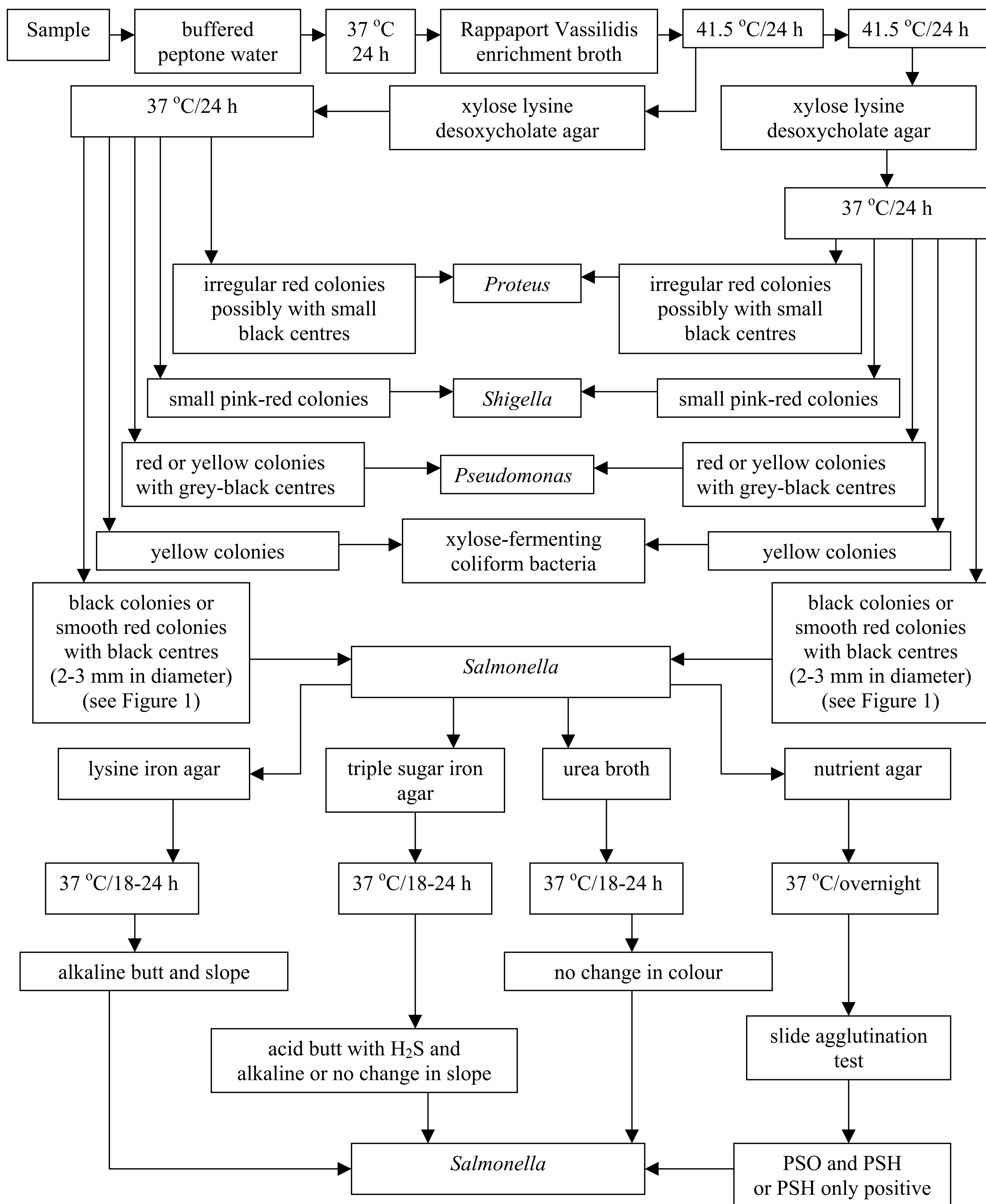
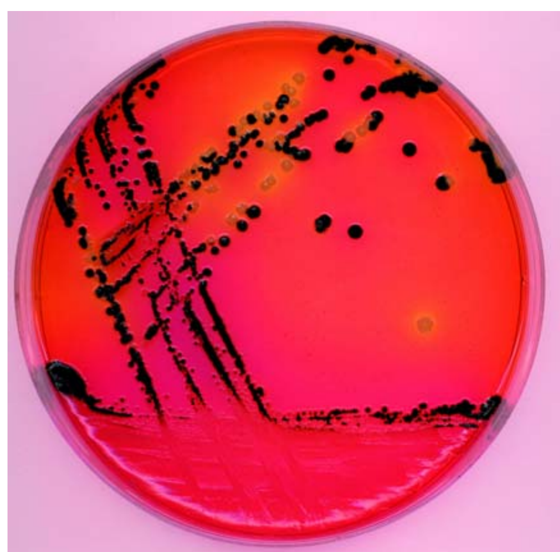


Figure 1 Black *Salmonella* colonies on xylose lysine desoxycholate agar



A9.4 *Confirmation tests*

A9.4.1 *Biochemical confirmation*

Using a straight wire, subculture characteristic colonies from xylose lysine desoxycholate agar to lysine iron agar, triple sugar iron agar, urea broth and nutrient agar as a check for purity. For lysine iron agar and triple sugar iron agar, the wire should be stabbed into the butt and streaked along the slant as it is withdrawn. Avoid stabbing through the butt to the bottom of the tube. The end of the wire should remain approximately 3 mm from the bottom of the tube as gas production may cause the medium to be blown out of the tube. Incubate the inoculated media at 37 °C for 18 - 24 hours. Regard cultures that give characteristic reactions in these confirmatory media, *i.e.* lysine iron agar and triple sugar iron agar, as presumptive salmonellas. Alternatively, a commercially available identification system may be used, following appropriate performance verification at the laboratory.

Reactions in lysine iron agar

Genus	Slope[1]	Butt[1]	H_2S production
Salmonella	alkaline	alkaline	blackening
Arizona	alkaline	alkaline	blackening
Proteus	red	acid	blackening or no blackening
Providencia	red	acid	no blackening
Citrobacter	alkaline	acid	blackening
Escherichia	alkaline	acid or no change	no blackening
Shigella	alkaline	acid	no blackening
Klebsiella	alkaline	alkaline	no blackening
Enterobacter	alkaline	acid	no blackening

[1] Alkaline reaction is purple, acid reaction is yellow

Reactions in triple sugar iron agar and urea broth

Genus	Triple sugar iron agar			Urea broth[2]
	Slope[1]	Butt[1]	H_2S production	
Salmonella Typhi	nc or alk	acid	blackening (weak)	-ve
Other *Salmonella*	nc or alk	acid + gas	blackening	-ve
Shigella	nc or alk	acid	no blackening	-ve
Proteus morganii	nc or alk	acid ± gas	no blackening	+ve
Proteus vulgaris	acid	acid + gas	blackening	+ve
Escherichia	acid	acid + gas	no blackening	-ve
Citrobacter	acid	acid + gas	blackening	+ve or -ve
Klebsiella	acid	acid + gas	no blackening	+ve or -ve
Enterobacter	acid	acid + gas	no blackening	-ve

[1] Alkaline (alk) reaction is red, acid reaction is yellow or no change (nc)
[2] +ve = alkaline reaction (red), -ve is no change in colour

A9.4.2 *Serological confirmation*

Subculture characteristic colonies from xylose lysine desoxycholate agar to moist nutrient agar slopes. For optimum flagellar formation, it is essential that some fluid is present in the tube and sterile broth should be added if required. Incubate overnight at 37 °C. Carry out a slide agglutination test. For example, using a wire loop or pipette, place 3 separate drops (each 0.02 ml) of saline solution onto a clean microscope slide. Emulsify growth from the moist butt of the slope in each separate drop to produce homogeneous suspensions. Mix a loopful of *Salmonella* polyvalent 'O' (PSO) anti-serum with the first drop of suspension and a loopful of *Salmonella* polyvalent 'H' (PSH) anti-serum with the second drop. Gently rock the slide back and forth and examine for agglutination against a black background. The third drop containing no anti-serum indicates whether or not the culture auto-agglutinates. Auto-agglutinating strains should be re-plated on xylose lysine desoxycholate and dry smooth colonies treated as previously described.

Organisms that agglutinate with both PSO and PSH anti-sera, or strains that agglutinate with the PSH anti-serum only, can be regarded as members of the Salmonella group.

A10 Calculations

The tests indicate the presence or absence of *Salmonella* species in the volume examined.

A11 Expression of Results

Presumptive and confirmed *Salmonella* species are reported as being detected, or not detected, in the volume of sample examined.

A12 Quality Assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example *Salmonella* Poona) and non-target bacteria (*Escherichia coli*).

Organisms should be incubated under the appropriate conditions. Further details are given elsewhere[2] in this series.

A13 References

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B Enumeration of *Salmonella* species by a membrane filtration-multiple tube-most probable number technique

B1 Introduction

Many different serotypes of *Salmonella* species are present to varying extents in humans, animals and birds. All members of the genus are potentially pathogenic. The low numbers of *Salmonella* species found in waters mainly originate from sewage and sewage effluents. The numbers of salmonellas present in water are, generally, much lower than those of other micro-organisms.

Salmonella infections give rise to symptoms of diarrhoea and vomiting. The incubation period varies between 12 - 72 hours and symptoms usually persist for 2 - 3 days. Most cases occur from the consumption of raw, or undercooked, food, particularly poultry and food containing eggs. The significance of *Salmonella* 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.

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 that form characteristic colonies on selective agar media after culture in enrichment media and which produce the serological and biochemical reactions described are regarded as *Salmonella* species.

Salmonella species normally conform to the general definition of the family Enterobacteriaceae(3) and can be further differentiated, biochemically, into 4 subgroups, subgenus I to IV. Those bacteria of subgenus I (the largest group) are considered pathogenic towards humans and are β-galactosidase-negative. Salmonellas are sub-divided into serovars on the basis of genus-specific combinations of somatic and flagellar antigens. Salmonellas may be further differentiated into groups by bacteriophage and plasmid typing.

The usual biochemical reactions include production of hydrogen sulphide; indole and urease are not produced; citrate is utilised as a carbon source; and lysine and ornithine are decarboxylated. Phenylalanine and tryptophan are not oxidatively de-aminated, and sucrose, salicin, inositol and amygdalin are not fermented.

There are currently only three recognised species of *Salmonella* – *Salmonella bongori*, *Salmonella enterica* (which also has six subspecies) and the recently described *Salmonella subterranea.* Most *Salmonella* encountered in the environment are serovars of *Salmonella enterica* or one of its subspecies. To avoid confusion with species' names the names of these serovars are not italicised when written. Thus, the strain formerly written as *Salmonella typhi* is now written as *Salmonella enterica* subspecies *enterica* serovar Typhi which is conventionally shortened to *Salmonella* Typhi. Similarly for *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Montevideo, etc.

B4 Principle

Isolation is based on concentration from water by membrane filtration (with or without the use of a filter aid. This is followed by pre-enrichment in a non-selective medium (to recover environmentally-stressed organisms) with further enrichment in a selective medium, after which aliquots are subcultured to a selective agar containing lactose, an indicator of acidity, and an indicator of hydrogen sulphide production. Characteristic colonies are confirmed by biochemical tests and by slide agglutination. The most probable number of organisms in the sample is estimated from appropriate probability tables.

B5 Limitations

This method is suitable for most types of aqueous samples except those with high turbidities, which tend to block the membrane filter. However, since the volume of sample is filtered through a number of membrane filters followed by incubation in a multiple tube situation, this limitation is reduced. When low numbers of organisms are present, detection is dependent only on the volume of sample that can be filtered and tested. High numbers of competing organisms may inhibit the growth, or detection, of target organisms. This method uses only one selective agar for the diagnostic detection of *Salmonella* species. Although some species of *Salmonella* may not be detected by the medium used, it may be adequate for routine water monitoring. However, for investigations into possible water-borne outbreaks of salmonellosis a range of selective media should be used[4]. This method is not suitable for the recovery of *Salmonella* Typhi (but see B9.4.1) and *Salmonella* Paratyphi.

B6 Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations[5] and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

Extra care should be taken in the isolation and identification of salmonellas due to the pathogenic nature of the organisms. Staff should be adequately trained and supervised and work involving subculture and handling of cultures should be performed in a designated area of a properly equipped laboratory. Adequate facilities should be in place for disposal and sterilisation of test materials.

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 are required. Other items include:

B7.1 Sterile sample bottles of appropriate volume, made of suitable material, containing sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l (for example, 0.1 ml of a 1.8 % m/v solution of $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

B7.2 Incubators capable of maintaining temperatures of 37 ± 1 °C and 41.5 ± 0.5 °C.

B7.3 Filtration apparatus, sterile or sterilisable filter funnels, and sources of vacuum.

B7.4 Sterile membrane filters, for example white 47 mm diameter, cellulose-based, 0.45 µm nominal pore size.

B7.5 Smooth-tipped forceps.

B8 Media and reagents

Commercial formulations of these media and reagents may be available, but may possess minor variations to their formulations. The performance of all media and reagents should be verified prior to their use in the method. 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 *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 (typically, 10 - 15 ml) into suitable tubes or bottles, cap and sterilise by autoclaving at 121 °C for 15 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH value of 7.2 ± 0.2. Autoclaved media may be stored in the dark at room temperature, protected from dehydration, and should be used within one month.

B8.2 *Rappaport Vassiliadis enrichment broth*[7, 8]

Solution A	
Soya peptone	4.5 g
Sodium chloride	7.2 g
Potassium dihydrogen phosphate	1.26 g
Dipotassium hydrogen phosphate	180 mg
Water	800 ml
Solution B	
Magnesium chloride anhydrous	13.6 g
Water	100 ml
Solution C	
Malachite green	36 mg
Water	100 ml

Dissolve the ingredients of solution A in the 800 ml of water. To achieve this, it may be necessary to heat to boiling. Prepare this solution on the day of use. To 800 ml of solution A, add 100 ml of solution B and 100 ml of solution C and mix well. Dispense the resulting solution (typically, 10 ml) into suitable containers, cap and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to

confirm a pH value of 5.2 ± 0.2. Store autoclaved media at between 2 - 8 °C, protected against dehydration, and use within one month.

B8.3 *Xylose lysine desoxycholate agar*[9]

Basal medium	
Lactose	7.5 g
Sucrose	7.5 g
Xylose	3.75 g
L(-) Lysine hydrochloride	5.0 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red (0.4 % m/v aqueous solution)	20 ml
Agar	12.0 g
Water	1 litre

Solution A	
Sodium thiosulphate pentahydrate	34.0 g
Ammonium iron(III) citrate	4.0 g
Water	100 ml

Solution B	
Sodium desoxycholate	10.0 g
Water	100 ml

Dissolve the ingredients of the basal medium in the water. This will require gentle heating. Dispense the resulting solution in appropriate volumes into suitable bottles, cap and sterilise by autoclaving at 115 °C for 10 minutes. The basal medium may be stored in the dark at room temperature and should be used within one month. Dissolve the ingredients of solution A and solution B in the respective amounts of water and separately pasteurise the individual solutions by heating at approximately 60 °C for 1 hour. To prepare the complete medium, melt the basal medium and cool to approximately 50 °C. Aseptically, add 2.0 ml of solution A and 2.5 ml of solution B to 100 ml of basal medium and mix well. The pH of the medium should be checked to confirm a pH value of 7.4 ± 0.2. Pour the complete medium into sterile Petri dishes and allow the medium to solidify. Store the poured plates at between 2 - 8 °C, protected against dehydration, and use within two weeks. The agar surface of prepared dishes should be dried before use.

B8.4 *Lysine iron agar*[10]

Peptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
L (-) Lysine	10.0 g
Ammonium iron(III) citrate	500 mg
Sodium thiosulphate pentahydrate	40 mg
Bromocresol purple (1 % m/v ethanolic solution)	2 ml
Agar	15.0 g
Water	1 litre

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solutions in small volumes (typically, 5 -10 ml) into suitable

containers and cap. Sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH value of 6.7 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. Store at between 2 - 8 °C, protected against dehydration, and use within one month.

B8.5 *Triple sugar iron agar*[11]

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Iron(III) citrate	300 mg
Sodium thiosulphate pentahydrate	300 mg
Phenol red (0.4 % m/v aqueous solution)	6 ml
Agar	15.0 g
Water	1 litre

Dissolve the ingredients (except phenol red) in the water. To achieve this, it will be necessary to heat to boiling. Add the phenol red solution and mix well. Dispense the resulting solution in small volumes (typically, 5 -10 ml) into suitable containers, cap and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH value of 7.4 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. Store at between 2 - 8 °C, protected against dehydration, and use within one month.

B8.6 *Urea broth*

Base medium	
Peptone	1.0 g
Glucose	1.0 g
Disodium hydrogen phosphate	1.0 g
Potassium dihydrogen phosphate	800 mg
Sodium chloride	5.0 g
Phenol red (0.4 % m/v aqueous solution)	1.0 ml
Water	1 litre

Dissolve the ingredients in the water and adjust the pH to 6.8 ± 0.2. Dispense the resulting solution (typically, 95 ml) into suitable bottles, cap and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH value of 6.8 ± 0.2. Prepared base medium may be stored in the dark at room temperature and should be used within one month. Prior to use, add 5 ml of an aqueous 40 % m/v filter-sterilised solution of urea to each 95 ml of base medium and aseptically dispense in 2 - 3 ml volumes in sterile containers and cap.

B8.7 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar, saline solution and anti-sera.

B9 Analytical procedure

B9.1 *Sample preparation*

Due to the likelihood that, if present, numbers of salmonellas in drinking water are likely to be low, a sample volume of at least 1000 ml should be examined. For the membrane filtration-multiple tube technique this is, typically, for a 6-tube series, analysed as 1 x 500 ml and 5 x 100 ml aliquots. Smaller volumes and aliquots may be appropriate for polluted waters. Record the volumes of sample filtered so that the MPN can be calculated.

B9.2 *Sample processing*

B9.2.1 *Membrane filtration-multiple tube technique*

Appropriate volumes of sample are filtered through membrane filters.

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 onto the porous disc of the filter base. If a grided membrane filter is used, place grid-side upwards. 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 water slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and carefully transfer the membrane filter to a tube or bottle containing 10 - 15 ml of buffered peptone water, ensuring the membrane filter is fully submerged. Record the volume filtered. Other volumes of sample should be similarly treated until all filters are transferred to the corresponding tubes or bottles of buffered peptone water. The larger single volume of sample may require more than one membrane filter, and if so, all filters used for this volume should be transferred to the bottle or tube of buffered peptone water. Ensure that all the membranes are fully submerged.

The funnel 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 of sample is filtered first. For different samples, a fresh pre-sterilised funnel should be taken or remove a funnel from the boiling water bath. Allow the funnel to cool and repeat the filtration process. If funnels are to be re-used, then 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 disinfected 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.

B9.2.2 *Pre-enrichment, enrichment and subculture to selective agar*

Each tube or bottle of buffered peptone water should be incubated at 37 °C for 21 ± 3 hours. After incubation, gently mix each tube or bottle, and subculture 0.1 ml of buffered peptone water from each tube or bottle into separate containers of Rappaport Vassiliadis enrichment broth (10 ml) and incubate at 41.5 °C for 21 ± 3 hours. After this time, plate out loopfuls of the Rappaport Vassiliadis enrichment broth from each container onto separate Petri dishes of xylose lysine desoxycholate agar. Incubate the selective agar at 37 °C for 21 ± 3 hours. After plating, return the Rappaport Vassiliadis enrichment broth to the incubator at 41.5 °C for a further 21 ± 3 hours. After this time, again plate out loopfuls of the Rappaport Vassiliadis enrichment broth from each container onto separate Petri dishes of xylose lysine desoxycholate agar. Incubate the selective agars at 37 °C for 21 ± 3 hours.

B9.3 *Reading of results*

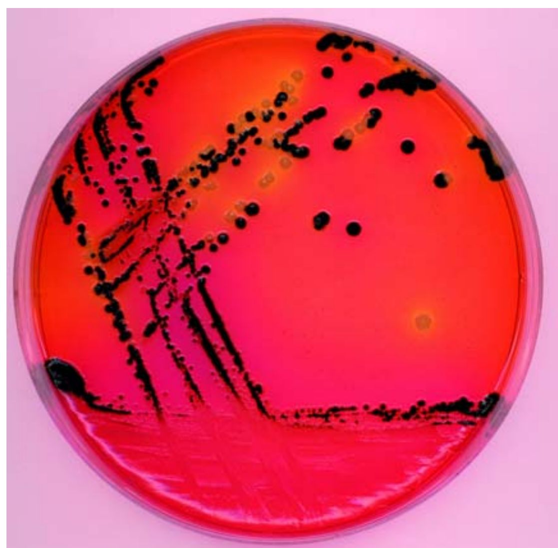
After each respective incubation period, examine the Petri dishes under good light, if necessary using a hand lens. Colonies are differentiated as follows:

Colonial appearance on xylose lysine desoxycholate agar

Organism	Characteristic appearance
Salmonella	smooth red colonies 2 - 3 mm in diameter, typically, with black centres or wholly black colonies (see Figure 1)
Xylose-fermenting coliform bacteria	yellow colonies
Pseudomonas species	red or yellow colonies with grey-black centres
Shigella species	small pink-red colonies
Proteus species	red colonies that are irregular and may have small black centres

Where isolates are overgrown on the xylose lysine desoxycholate agar, then subculture to fresh xylose lysine desoxycholate agar. This facilitates the production of pure cultures and enables typical colonial morphology to be observed. See Figure 1.

Figure 1 Black *Salmonella* colonies on xylose lysine desoxycholate agar



B9.4 *Confirmation tests*

B9.4.1 *Biochemical confirmation*

Using a straight wire, subculture characteristic colonies from each Petri dish of xylose lysine desoxycholate agar to lysine iron agar and triple sugar iron agar, and urea broth and nutrient agar as a check for purity. For lysine iron agar and triple sugar iron agar, the wire should be stabbed into the butt and streaked along the slant as it is withdrawn. Avoid stabbing through the butt to the bottom of the tube. The end of the wire should remain approximately 3 mm from the bottom of the tube as gas production may cause the medium to be blown out of the tube. Incubate the inoculated media at 37 °C for 18 - 24 hours. Regard cultures that give characteristic reactions in these confirmatory media, *i.e.* lysine iron agar and triple sugar iron agar, as presumptive salmonellas. Alternatively, a commercially available identification system may be used, following appropriate performance verification at the laboratory.

Reactions in lysine iron agar

Genus	Slope[1]	Butt[1]	H_2S production
Salmonella	alkaline	alkaline	blackening
Arizona	alkaline	alkaline	blackening
Proteus	red	acid	blackening or no blackening
Providencia	red	acid	no blackening
Citrobacter	alkaline	acid	blackening
Escherichia	alkaline	acid or no change	no blackening
Shigella	alkaline	acid	no blackening
Klebsiella	alkaline	alkaline	no blackening
Enterobacter	alkaline	acid	no blackening

[1] Alkaline reaction is purple, acid reaction is yellow

Reactions in triple sugar iron agar and urea broth

Genus	Triple sugar iron agar			Urea broth[2]
	Slope[1]	Butt[1]	H_2S production	
Salmonella Typhi	nc or alk	acid	blackening (weak)	-ve
Other *Salmonella*	nc or alk	acid + gas	blackening	-ve
Shigella	nc or alk	acid	no blackening	-ve
Proteus morganii	nc or alk	acid ± gas	no blackening	+ve
Proteus vulgaris	acid	acid + gas	blackening	+ve
Escherichia	acid	acid + gas	no blackening	-ve
Citrobacter	acid	acid + gas	blackening	+ve or -ve
Klebsiella	acid	acid + gas	no blackening	+ve or -ve
Enterobacter	acid	acid + gas	no blackening	-ve

[1] Alkaline (alk) reaction is red, acid reaction is yellow or no change (nc)
[2] +ve = alkaline reaction (red), -ve is no change in colour

B9.4.2 *Serological confirmation*

Subculture characteristic colonies from each Petri dish of xylose lysine desoxycholate agar to moist nutrient agar slopes. For optimum flagellar formation, it is essential that some fluid

is present in the tube and sterile broth should be added if required. Incubate overnight at 37 °C. Carry out a slide agglutination test. For example, using a wire loop or pipette, place 3 separate drops (each 0.02 ml) of saline solution onto a clean microscope slide. Emulsify growth from the moist butt of the slope in each separate drop to produce homogeneous suspensions. Mix a loopful of *Salmonella* polyvalent 'O' (PSO) anti-serum with the first drop of suspension and a loopful of *Salmonella* polyvalent 'H' (PSH) anti-serum with the second drop. Gently rock the slide back and forth and examine for agglutination against a black background. The third drop containing no anti-serum indicates whether or not the culture auto-agglutinates. Auto-agglutinating strains should be re-plated on xylose lysine desoxycholate agar and dry smooth colonies treated as previously described.

Organisms that agglutinate with both PSO and PSH anti-sera, or strains that agglutinate with the PSH anti-serum only, can be regarded as members of the Salmonella group.

B10 Calculations

Record the number of tubes or bottles resulting in positive *Salmonella* isolates and calculate the most probable number (MPN) of organisms in the volume of sample using, for example if the 6-tube series was followed, Table 1 below. If smaller volumes and aliquots have been filtered, multiply the result by the appropriate factor. (For example, if 1 x 50 ml and 5 x 10 ml aliquots have been filtered then the value shown in Table 1 would need to be multiplied by 10; however, in the case where no organisms are found then this would equate to a value of less than 10 organisms per litre of sample).

Table 1 MPN and MPR per litre of sample for a 6-tube series containing 1 x 500 ml and 5 x 100 ml volumes of sample

Number of tubes showing positive *Salmonella* isolates		MPN per 1000 ml	MPR* per 1000 ml
1 x 500 ml	5 x 100 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**	

* Most probable range (MPR): these are numbers that are at least 95 % as probable as the most probable number (MPN).
** There is no discrimination when all tubes show growth; the theoretical MPN is infinity. The true count is likely to exceed 18.

B11 Expression of Results

The count is expressed as the number of *Salmonella* per litre of sample.

B12 Quality Assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example *Salmonella* Poona) and non-target bacteria (*Escherichia coli*). Organisms should be incubated under the appropriate conditions. Further details are given elsewhere[2] in this series.

B13 References

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4. Health Protection Agency (2006) Detection of *Salmonella* species. National Standard Method W7. http://www.hpa-standardmethods.org.uk/pdf_sops.asp.

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8. The effect of incubation temperature and magnesium chloride concentration on the growth of salmonella in home-made and in commercially available Rappaport-Vassiliadis broths. *Journal of Applied Bacteriology*, M Peterz, C Wilberg and P Norberg, 1989, **66**, pp523-528.

9. Isolation of Shigellae I. Xylose lysine agars. New media for isolation of enteric pathogens. *American Journal of Clinical Pathology*, W I Taylor and B Harris, 1965, **44**, pp471-475.

10. Lysine Iron Agar in the detection of *Arizona* cultures. *Applied Microbiology*, P R Edwards and M A Fife, 1961, **9**, pp478-480.

11. Report of Enterobacteriaceae Subcommittee and the Nomenclature Committee of the International Association of Microbiological Societies. *International Bulletin of Bacterial Nomenclature and Taxonomy*, 1958, **8**, pp25-70.

C The isolation of *Shigella* species by selective enrichment

C1 Introduction

Members of the genus *Shigella* normally inhabit the intestinal tract of humans but do not infect animals. Their presence in water is, therefore, an indication of human faecal contamination. Infection is commonly by person-to-person contact, or by the consumption of contaminated food or water.

Gastro-intestinal disease is commonly a symptom of infection of which dysentery is the most severe. The disease is typical of conditions of poor hygiene and sanitation. In the UK, *Shigella sonnei* is commonly isolated, although the most severe disease is caused by *Shigella dysenteriae,* type 1, which produces a potent exotoxin (Shiga toxin). The significance of *Shigella* bacteria in water treatment and supply are described elsewhere(1) in this series.

C2 Scope

The method is suitable for the examination of drinking water, 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).

C3 Definitions

In the context of this method, organisms that form characteristic colonies on selective agar media after culture in enrichment media and which produce the serological and biochemical reactions described are regarded as *Shigella* species.

Bacteria in the genus *Shigella* are facultative anaerobes, Gram-negative, non-motile rods. Four species are commonly found; *Shigella dysenteriae*, *Shigella sonnei*, *Shigella flexneri* and *Shigella boydii*. The organisms are oxidase-negative and catalase-positive (with the exception of *Shigella dysenteriae* type 1 which is catalase-negative). Citrate is not used as a sole source of carbon, and with few exceptions, carbohydrates are fermented without gas production.

C4 Principle

Isolation is based on concentration from water by membrane filtration, or the use of a filter aid, followed by selective enrichment and subculture to a selective agar medium with examination for typical colonies. Characteristic colonies may be confirmed by subculturing for further biochemical testing or by slide agglutination for speciation.

C5 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. In such instances, the use of several membrane filters or filter aid is recommended. When low numbers of organisms are present, detection is dependent only on the volume of sample that can be filtered and tested. High numbers of competing organisms may inhibit the growth, or detection, of target organisms.

C6 Health and safety

Media, reagents and bacteria used in these methods are covered by the Control of Substances Hazardous to Health Regulations[3] and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere[2] in this series.

Extra care should be taken in the isolation and identification of *Shigella* species due to the pathogenic nature of the organisms. Staff should be adequately trained and supervised and work involving subculture and handling of cultures should be performed in a designated area of a properly equipped laboratory. Adequate facilities should be in place for disposal and sterilisation of test materials.

C7 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 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 $Na_2S_2O_3.5H_2O$ per 100 ml of sample, or equivalent).

C7.2 Incubator capable of maintaining a temperature of 37 ± 1 °C.

C7.3 Filtration apparatus, sterile or sterilisable filter funnels, and sources of vacuum.

C7.4 Sterile membrane filters, for example, white 47 mm diameter, cellulose-based 0.45 μm nominal pore size.

C7.5 Smooth-tipped forceps.

C8 Media and reagents

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

C8.1 *Modified Hajna GN enrichment broth*

Tryptone	20.0 g
Glucose	1.0 g
Mannitol	2.0 g
Sodium citrate	5.0 g
Sodium desoxycholate	500 mg

Dipotassium hydrogen phosphate	4.0 g
Potassium dihydrogen phosphate	1.5 g
Sodium chloride	5.0 g
(DL) Serine	1.0 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH to 7.2 ± 0.2. Dispense the resulting solution (typically, 90 ml) into suitable containers, cap and sterilise by steaming at 100 °C for 30 minutes. After steaming, the pH of the medium should be checked to confirm a pH value of 7.2 ± 0.2. Store at between 2 - 8 °C, protected against dehydration, and use within one month.

C8.2 *Modified desoxycholate citrate agar*

Tryptone	20.0 g
Lactose	10.0 g
Sodium thiosulphate pentahydrate	6.8 g
Ammonium iron(III) citrate	800 mg
Neutral red (1%m/v aqueous solution)	3 ml
Sodium desoxycholate	500 mg
(DL) Serine	1.0 g
Tetracycline hydrochloride	32 mg
Agar	14.0 g
Water	1 litre

Dissolve the ingredients (except tetracycline hydrochloride) in water. To achieve this, it will be necessary to heat to boiling. Cool the resulting solution to approximately 50 °C and add the tetracycline as an aqueous filter-sterilised solution to give a final concentration of 32 mg/l. Thoroughly mix the complete medium, pour into sterile Petri dishes, replace the lid and allow the agar to solidify. Store at between 2 - 8 °C, protected against dehydration, and use within one month. The agar surface of prepared dishes should be dried before use.

C8.3 *Modified Hektoen agar*[4]

Yeast extract	3.0 g
Proteose peptone	12.0 g
Lactose	12.0 g
Sucrose	12.0 g
Salicin	2.0 g
Ammonium iron(III) citrate	1.5 g
Acid fuchsin	100 mg
Bromothymol blue (1 % m/v aqueous solution)	6.5 ml
Bile salts number 3	9.0 g
Sodium chloride	5.0 g
Sodium thiosulphate pentahydrate	5.0 g
Agar	14.0 g
Novobiocin	15.0 mg
Water	1 litre

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Cool the resulting solution to approximately 50 °C and pour into sterile Petri

dishes. Allow the medium to solidify and store at between 2 - 8 °C, protected from dehydration, and use within one month. The agar surface of prepared dishes should be dried before use.

C8.4 *Filter-aid*[5]

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.

C8.5 *Other media*

Standard and commercial formulations of other media and reagents used in this method include nutrient agar and saline solution.

C9 Analytical procedure

C9.1 *Sample preparation*

Due to the likelihood that, if present, numbers of *Shigella* species in drinking water are likely to be low, a sample volume of at least 1000 ml should be examined. Smaller volumes may be appropriate for polluted source waters.

C9.2 *Sample processing*

C9.2.*1* *Membrane filtration*

Filter an appropriate volume of sample. If the sample is turbid, several membrane filters may be required. Alternatively, a large volume filtration system can be used[2].

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 onto the porous disc of the filter base. If a grided membrane filter is used, place grid-side upwards. 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 water slowly through the membrane filter. Close the stopcock as soon as the sample has been filtered.

Remove the funnel and carefully transfer the membrane filter to, typically, 90 ml of modified Hajna GN enrichment broth. The volume modified Hajna GN enrichment broth can be adjusted according to the nature of the sample and the number of membranes that may be needed. A minimum volume of 50 ml should be used. If more than one membrane filter is required, all filters should be transferred to the modified Hajna GN enrichment broth, ensuring that all the membranes are fully submerged.

The funnel 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 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 to be re-used, then 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 disinfected 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.

C9.2.2 *Filter-aid*

The usual membrane filtration apparatus may be used but with a sterile absorbent pad in place of the membrane filter to act as a supporting base for the filter-aid. An aliquot of filter-aid (typically, 15 ml) should be filtered to form an initial layer on the absorbent pad. A second aliquot (typically, 15 ml) of filter-aid should be mixed with the volume of sample and then filtered. For turbid or dirty 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 Hajna GN enrichment broth. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 90 ml.

C9.2.3 *Enrichment and subculture to selective agar*

The modified Hajna GN enrichment broth and membrane filters, and filter-aid if appropriate, should be mixed well and incubated at 37 °C for 6 - 8 hours. After incubation, gently shake the enrichment broth and plate out loopfuls onto modified desoxycholate citrate agar and modified Hektoen agar. Incubate the desoxycholate citrate and Hektoen agars at 37 °C for 18 - 24 hours.

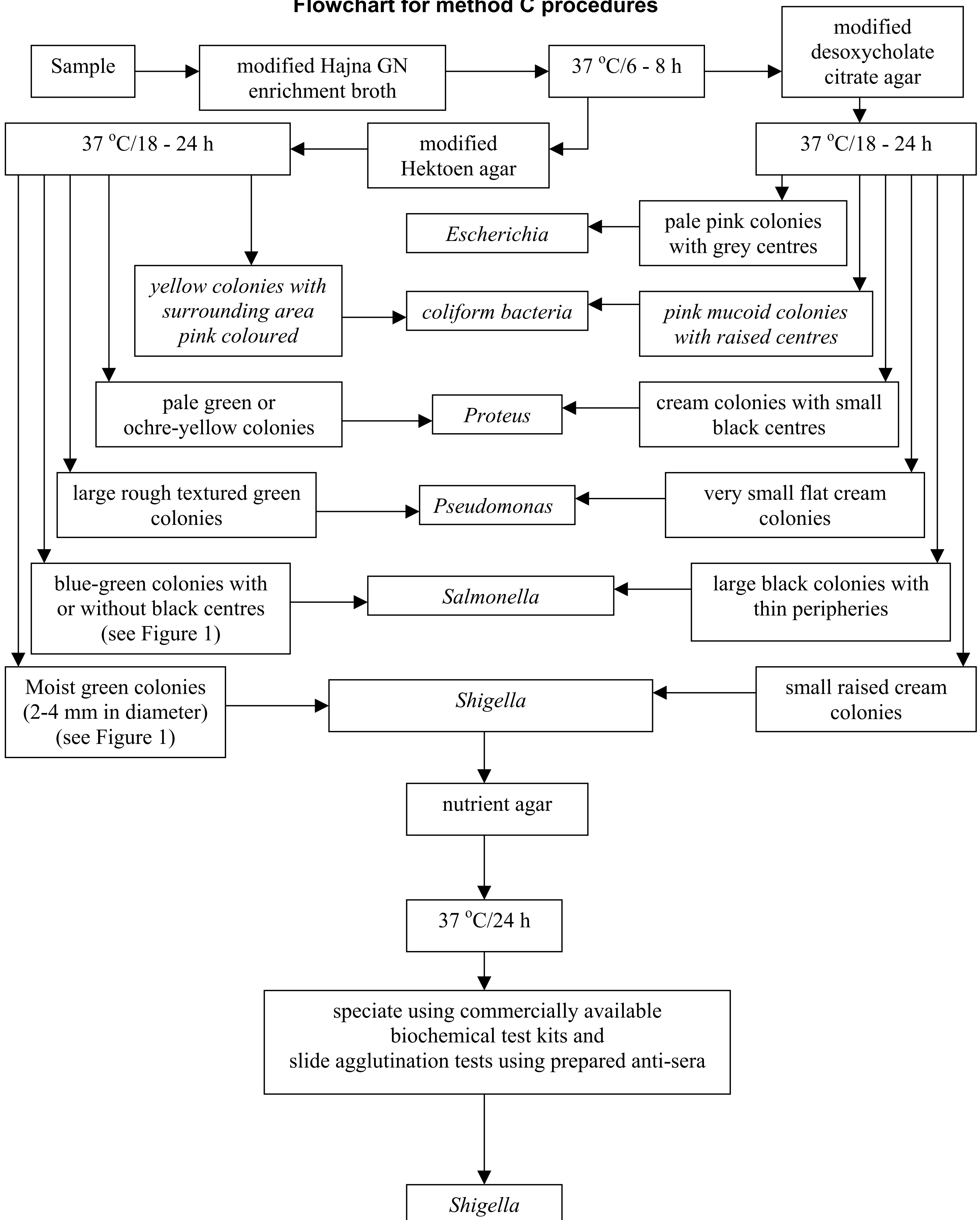
C9.3 *Reading of results*

After incubation, examine the Petri dishes under good light, and if necessary use a hand lens. Colonies are differentiated as follows (see flowchart):

Colonial appearance on modified desoxycholate citrate agar

Organism	Characteristic appearance
Shigella	small raised cream coloured colonies
Salmonella	large black coloured colonies with a thin white periphery
Pseudomonas	very small flat cream coloured colonies
Proteus	cream coloured colonies with a small black centre
Escherichia	pale pink coloured colonies with grey centres
Other coliform bacteria	pink coloured mucoid colonies with raised centres

Flowchart for method C procedures



Colonial appearance on modified Hektoen agar (see Figure 1)

Organism	Characteristic appearance
Shigella	moist green coloured colonies 2-4 mm in diameter (*Shigella sonnei* may produce larger irregular colonies)
Salmonella	blue-green colonies, with or without black centres
Pseudomonas	large rough textured green coloured colonies
Proteus	pale green or ochre yellow coloured colonies
Coliform bacteria	yellow coloured colonies. The medium around the colonies often turns salmon pink

Figure 1 Colonies on modified Hektoen agar

Shigella species (dark green colonies) with *Salmonella* species (light blue-green colonies)

C9.4 *Confirmation tests*

Subculture typical colonies to a non-selective medium such as nutrient agar and incubate at 37 °C for 21 ± 3 hours. Isolates may be speciated using commercially available biochemical test kits and by slide agglutination using prepared anti-sera, following appropriate performance verification in the laboratory.

C10 Calculations

This test indicates the presence or absence of *Shigella* species.

C11 Expression of results

Shigella species are reported as being detected, or not detected, in the volume of sample examined.

C12 Quality assurance

New batches of media and reagents should be tested with appropriate reference strains of target bacteria (for example *Shigella sonnei, Shigella flexneri*) and non-target bacteria

(*Escherichia coli*). Organisms should be incubated under the appropriate conditions. Further details are given elsewhere[2] in this series.

C13 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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3. The Control of Substances Hazardous to Health Regulations 2002, Statutory Instrument 2002 No. 2677.

4. A new plating medium for the isolation of enteric pathogens. I Hektoen enteric agar. II. Comparison of Hektoen enteric agar with SS and EMB agar. *Applied Microbiology*, S King and W I Metzger, 1968, **16**, pp577-581.

5. Concentration technique for demonstrating small amounts of bacteria in tap water. *Acta Pathologica et Microbiologica Scandinavia*, E Hammarstrom and V Ljutov, 1954, **35**, pp365-369.

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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 would like to receive advanced notice of forthcoming publications please contact the Secretary on the Agency's web-page.

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