Standing Committee of Analysts

The Microbiology of Recreational and Environmental Waters (2016) – Part 8 – Methods for the isolation and enumeration of *Salmonella* and *Shigella*

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 methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, presence-absence and most probable number methods.

A The isolation and enumeration of *Salmonella* species by selective enrichment, presence-absence and multiple tube most probable number technique

B The isolation and enumeration of *Shigella* species by selective enrichment, presence-absence and multiple tube most probable number technique

This bluebook updates and replaces sections 7.7 and 7.14 of the earlier version of The Microbiology of Recreational and Environmental Waters published in 2000.

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

Contents

About this series

Introduction

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

Performance of methods

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

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

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

Standing Committee of Analysts

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

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

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

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

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

Robert Carter
Secretary
June 2015

Warning to users

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

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

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

A The isolation and enumeration of *Salmonella* species by selective enrichment, presence-absence and multiple tube most probable number technique

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 wastewater, reclaimed wastewater effluents, and effluents associated with agriculture. The numbers of salmonellas present in water are, generally, much lower than those of other micro-organisms.

Most *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 that contain eggs. The significance of *Salmonella* bacteria in surface water, wastewater and sea water is described elsewhere[(1)] in this series.

A2 Scope

The method is suitable for the examination of fresh and saline surface waters, swimming pools, spa pools and hydrotherapy pools, primary and secondary wastewater effluents and sediments including sand. Clean water samples may be filtered directly using membrane filters or a suitable filter-aid used. Sediments may be suspended in a suitable diluent or inoculated directly into pre-enrichment broths.

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 broth 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. Salmonellas of subgenus I (the largest group) are considered pathogenic towards humans and are β-galactosidase-negative. Salmonellas are further sub-divided into serovars on the basis of genus-specific combinations of somatic and flagellar antigens, and 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 belongs to subgenus 1 and 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 termed *Salmonella enterica* subspecies *enterica* serovar Typhi which is conventionally shortened to *Salmonella* Typhi. Similarly for *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Montevideo, etc.

A4 Principle

Samples are incubated first in a pre-enrichment broth, followed by inoculation into one or both of two enrichment broths (Rappaport Vassiliadis broth and selenite enrichment broth). These are inoculated onto two selective agars (xylose lysine desoxycholate agar and brilliant green agar) for the diagnostic detection of *Salmonella* species by a presence-absence determination or a multiple tube most probable number technique.

A5 Limitations

This method is labour intensive and may require the preparation of large numbers of tubes or bottles of media and appropriate sub-cultures.

In certain cases, only one enrichment broth and one selective medium may need to be used, but some species of *Salmonella* may not be detected. For investigations involving possible 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 A9.5.1) 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 sub-culture 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.

Sodium biselenite is toxic and should be handled with care. Ingestion by pregnant laboratory workers may cause miscarriages or teratogenic effects. To minimise any possible risk, sodium biselenite should not be added to the other ingredients as a dry powder but should be prepared separately as a solution and the remaining ingredients added to it.

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

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

A7.3 Filtration apparatus, filter funnels, (either sterilised or capable of being sterilised) 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[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salt. If the pH of the media are not within the stated range, then, before heating, they should be adjusted accordingly. Media with a pH outside the required range should be discarded. Where media are stored in a refrigerator, they should be allowed to warm to room temperature before use.

A8.1 *Single-strength buffered peptone water*[6]

Peptone	10 g
Sodium chloride	5 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 of 7.2 ± 0.2. Autoclaved media may be stored in the dark at room temperature, for up to one month, if protected from dehydration.

Double-strength buffered peptone water can be prepared using double the amounts of ingredients in the 1000 ml of water.

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 capped containers and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 5.2 ± 0.2. The autoclaved media may be stored between 5 ± 3 °C, for up to one month, if protected from dehydration.

A8.3 *Selenite cysteine broth*[9, 10]

Tryptone	5 g
Lactose	4 g
Disodium hydrogen phosphate (Na_2HPO_4)	10 g
L-cysteine	10 mg
Sodium biselenite	4 g
Water	1 litre

Dissolve the sodium biselenite in the water with gentle heating and then add the remaining ingredients. Mix well to dissolve. Dispense the resulting solution into suitable screw-capped bottles or tubes to a minimum depth of 60 mm. Sterilise by placing in free-flowing steam for 15 minutes. The medium should not be autoclaved. After steaming, the pH of the medium should be checked to confirm a pH of 7.0 ± 0.2. The autoclaved media may be stored between 5 ± 3 °C, for up to one month, if protected from dehydration.

A8.4 *Brilliant green agar*[11]

Yeast extract	3 g
Proteose peptone or polypeptone	10 g
Sodium chloride	5 g

Lactose	10 g
Sucrose	10 g
Phenol red (0.2 % m/v aqueous solution)	40 ml
Brilliant green (0.5 % m/v aqueous solution)	2.5 ml
Agar	20 g
Water	960 ml

Dissolve the ingredients in the water. To achieve this, it will be necessary to heat to boiling. Dispense the resulting solution in appropriate volumes into suitable 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 6.9 ± 0.2. Cool the molten medium to approximately 50 °C and pour into sterile Petri dishes. Allow the medium to solidify. The medium may be stored at between 5 ± 3 °C, for up to one month, if protected from dehydration.

The medium may be made more selective by the addition of a sulphonamide, for example, sulphapyridine at a concentration of 1000 mg/l, or sulphadiazine at a concentration of 800 mg/l. Alternatively, a sulphamandelate supplement[9] added as an aqueous filter-sterilised solution of sodium sulphacetamide at a concentration of 1000 mg/l and sodium mandelate at a concentration of 250 mg/l may be used.

Most bacteria produce single colonies on media, but *Proteus* colonies tend to spread or swarm over the medium. Sodium desoxycholate at a concentration of 2500 mg/l may be added to the medium to prevent swarming of species of *Proteus*.

Prepared dishes should be dried before use, by placing for example the open Petri dish in an incubator at 37 °C for 30 minutes.

A8.5 *Xylose lysine desoxycholate agar*[12]

Basal medium

Lactose	7.5 g
Sucrose	7.5 g
Xylose	3.75 g
L(-) Lysine hydrochloride	5 g
Sodium chloride	5 g
Yeast extract	3 g
Phenol red (0.4 % m/v aqueous solution)	20 ml
Agar	12 g
Water	1 litre

Solution A

Sodium thiosulphate pentahydrate	34 g
Ammonium iron(III) citrate	4 g
Water	100 ml

Solution B

Sodium desoxycholate	10 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 screw-capped bottles and sterilise by autoclaving at 115 °C for 10 minutes. The basal medium may be stored in the dark at room temperature for up to 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 melted basal medium and mix well. The pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Some commercial formulations do not require formal sterilisation and should be prepared to their instructions. Pour the complete medium into sterile Petri dishes and allow the medium to solidify. The complete medium may be stored at between 5 ± 3 °C, for up to one month, if protected against dehydration.

Prepared dishes should be dried before use, by placing, for example the open Petri dish in an incubator at 37 °C for 30 minutes.

A8.6 *Lysine iron agar*[13]

Peptone	5 g
Yeast extract	3 g
Glucose	1 g
L (-) Lysine	10 g
Ammonium iron(III) citrate	500 mg
Sodium thiosulphate pentahydrate	40 mg
Bromocresol purple (1 % m/v ethanolic solution)	2 ml
Agar	15 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 capped containers. Sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.7 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored at between 5 ± 3 °C, for up to one month, if protected against dehydration.

A8.7 *Triple sugar iron agar*[14]

Beef extract	3 g
Yeast extract	3 g
Peptone	20 g
Sodium chloride	5 g
Lactose	10 g
Sucrose	10 g

Glucose	1 g
Iron(III) citrate	300 mg
Sodium thiosulphate pentahydrate	300 mg
Phenol red (0.4 % m/v aqueous solution)	6 ml
Agar	15 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 and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 7.4 ± 0.2. Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored at between 5 ± 3 °C, for up to one month, if protected against dehydration.

A8.8 *Urea broth*

Broth base

Peptone	1 g
Glucose	1 g
Disodium hydrogen phosphate	1 g
Potassium dihydrogen phosphate	800 mg
Sodium chloride	5 g
Phenol red (0.4 % m/v aqueous solution)	1 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 screw-capped bottles and sterilise by autoclaving at 115 °C for 10 minutes. After autoclaving, the pH of the medium should be checked to confirm a pH of 6.8 ± 0.2. The prepared broth base may be stored in the dark at room temperature, for up to one month. Prior to use, add 5 ml of an aqueous 40 % m/v filter-sterilised solution of urea to each 95 ml of broth base and aseptically dispense in 2 - 3 ml volumes in sterile containers and cap. The complete medium should be prepared on the day of use.

A8.9 *Filter-aid*[15]

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. The sterilised filter-aid may be stored in the dark at room temperature for up to 12 months.

A8.10 *Other media*

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

Note: Chromogenic agar media are now available commercially. They should be stored, prepared and used according to the manufacturer's instructions following verification of their performance in the laboratory[2].

A9 Analytical procedure

A9.1 *Sample preparation*

A9.1.1 *Surface waters and sea waters*

Due to the likelihood that, if present, the numbers of *Salmonellas* in some surface waters and sea waters are likely to be low, for presence-absence determinations a sample volume of at least 1000 ml should be examined.

For the membrane filtration multiple tube technique, typically, an 11-tube series can be used, i.e. the membrane filtration of 1 x 500 ml, 5 x 100 ml and 5 x 10 ml of sample. Alternatively, volumes of 1 x 500 ml and 5 x 100 ml can be filtered and the 10 ml volumes can be added directly to 10 ml volumes of double-strength buffered peptone water. For a different series, smaller volumes of sample, for example 1 ml, may be appropriate and these can be added directly to 9 ml of single-strength buffered peptone water. Turbid waters, unsuitable for direct membrane filtration, may be filtered using filter aid.

A9.1.2 *Treated wastewater*

Treated wastewater may be analysed as described in A9.1.1 although several membrane filters may be required for presence-absence determinations. A sample volume of at least 100 ml may need to be examined.

The volumes may be reduced and volumes of 1 x 50 ml, 5 x 10 ml and 5 x 1 ml may be used. The 1 x 50 ml and 5 x 10 ml volumes can be membrane filtered or added to equal volumes of double-strength buffered peptone water. The 1 ml volumes can be added directly to 9 ml of single-strength buffered peptone water. To represent smaller volumes of samples, a 1:10 dilution of the sample, for example 1 ml of sample diluted with quarter strength Ringer's solution or maximum recovery diluent, may be appropriate. Typically, 1 ml of these diluted samples can be added directly to 9 ml of single-strength buffered peptone water.

A9.1.3 *Untreated wastewater*

For presence-absence determinations, 100 ml of untreated wastewater sample may be required, as it may not be possible (owing to turbidity) to process larger volumes by membrane filtration. For an 11-tube most probable number series, the volumes of untreated wastewater are usually 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 x 50 ml and 5 x 10 ml volumes can be filtered or added to equal volumes of double-strength buffered peptone water. The 1 ml volumes can be added to 9 ml of single-strength buffered peptone water. To represent smaller volumes, for example 0.1 ml and 0.01 ml volumes of sample, a 1:10 and 1:100 dilution of the sample, may be appropriate. Typically, 1 ml of these diluted samples can be added directly to 9 ml of single-strength buffered peptone water.

A9.1.4 *Sediment and sand*

Solid material can be dispensed as a single weight for presence-absence determinations by weighing, for example 10 g of sample into an appropriate volume (typically 100 ml) of single-strength buffered peptone water. For the multiple tube technique, weigh 1 x 50 g, 5 x 10 g and 5 x 1 g quantities of sample into appropriate volumes (typically 450 ml, 5 x 100 ml and 5 x 10 ml respectively) of single-strength buffered peptone water. For smaller quantities, for example 100 mg, these may be added directly to 10 ml of single-strength buffered peptone water.

A9.2 Sample processing

A9.2.1 *Membrane filtration*

For membrane presence-absence or membrane multiple tube techniques 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 gridded 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, typically, 10 - 15 ml of single-strength buffered peptone water, ensuring that the membrane filter is fully submerged. Record the volume filtered. Other volumes of sample should be similarly treated until all the filters are transferred to the corresponding tubes or bottles of single-strength buffered peptone water. The largest 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 single-strength buffered peptone water. Ensure that all 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 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 a 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 single-strength buffered peptone water. With the same medium, rinse any filter-aid adhering to the funnel into the culture vessel and make up to 100 ml.

For presence-absence determinations, place the culture vessel in an incubator and incubate at 37 °C for 21 ± 3 hours. For a most probable number test, re-suspend the filter aid in the single-strength buffered peptone water and dispense in a multiple tube most probable number series using 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 ml volumes can be inoculated into 9 ml of sterile single-strength buffered peptone water.

A9.2.3 *Direct inoculation*

Where the numbers of salmonellas in the sample are likely to be high, smaller volumes of sample, for example 1 x 50 ml and 5 x 10 ml can be inoculated directly into equal volumes of double-strength buffered peptone water. Volumes of 1 ml and subsequent dilutions of the sample can be inoculated directly into 9 ml of single-strength buffered peptone water.

A9.2.4 *Sediment and sand*

Samples of sediment and sand may be analysed by weighing appropriate amounts, for example, a single aliquot of 10 g for presence-absence determinations or 1 x 5 g, into 90 ml, and 5 x 1 g and 5 x 0.1 g into 9 ml of single-strength buffered peptone water for a most probable number series. Larger weights of sample should be weighed into appropriately larger volumes of single-strength buffered peptone water.

A9.3 *Enrichment and sub-culture to selective agar*

The buffered peptone water and membrane filters, and if appropriate filter-aid or solid sample material, or direct inoculation is mixed thoroughly and incubated at 37 °C for 21 ± 3 hours.

After incubation, gently mix each tube or bottle and sub-culture to either or both of the enrichment broths:-

i) 0.1 ml of the buffered peptone water from each tube or bottle into separate containers of 10 ml of Rappaport Vassiliadis enrichment broth. Incubate these containers at 41.5 °C for 21 ± 3 hours.

ii) 1 ml of the buffered peptone water from each tube or bottle into separate containers of selenite cysteine enrichment broth at a depth of at least 60 mm. Incubate these

containers at 37 °C for 21 ± 3 hours.

After the appropriate enrichment broth incubation, transfer a loopfull of each enrichment broth to xylose lysine desoxycholate agar and to brilliant green agar. Return the Rappaport Vassiliadis enrichment broth to the incubator at 41.5 °C for a further 21 ± 3 hours. Discard the selenite cysteine enrichment broth, as selectivity is reduced after 24 hours of incubation.

Incubate both selective agars at 37 °C for 21 ± 3 hours.

After the agar incubation, examine each Petri dish for typical *Salmonella* colonies. Where no growth is observed and samples are negative, transfer a loopful of the Rappaport Vassiliadis enrichment broth from each tube or bottle incubated for a total of 48 hours onto both xylose lysine desoxycholate agar and brilliant green agar. Incubate the selective agars at 37 °C for 21 ± 3 hours.

A9.4 *Reading of results*

After each respective incubation period, examine the Petri dishes under good light, if necessary using a hand lens. Colonies on the selective agars may be 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 A1)
Xylose- or lactose- or sucrose-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

Colonial appearance on brilliant green agar

Organism	Characteristic appearance
Salmonella	Smooth red colonies, approximately 2 mm in diameter (see Figure A2)
Lactose- or sucrose-fermenting coliform bacteria	Yellow/green colonies
Pseudomonas species	Small crenated colonies
Proteus species	Small red colonies

Where isolates are overgrown on the xylose lysine desoxycholate agar or brilliant green agar, then sub-culture respectively to fresh xylose lysine desoxycholate agar and brilliant green agar. This facilitates the production of pure cultures and enables typical colonial morphology to be observed.

Figure A1 *Salmonella* isolated from wastewater growing on xylose lysine desoxycholate agar

Figure A2 *Salmonella* isolated from wastewater growing on brilliant green agar

A9.5 *Confirmation tests*

A9.5.1 *Biochemical confirmation*

Select at least one typical *Salmonella* colony, or, if the intention is to isolate different species of *Salmonella* or for epidemiological investigations, select a minimum of five colonies from each selective agar and sub-culture to separate Petri dishes of nutrient agar. Incubate the Petri dishes at 37 °C for 21 ± 3 hours. Spreading colonies on nutrient agar are *Proteus* and these can be discarded at this stage. Providing that cultures are pure, using a straight wire, sub-culture colonies from each Petri dish of nutrient agar to lysine iron agar (see Figure A3), triple sugar iron agar (see Figures A4 and A5) and urea broth (see Figure A6). 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 go no further than 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 salmonellas, depending on serological tests. Alternatively, a commercially available identification system or direct confirmation by serological tests (see A9.5.2) 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, no change is red

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) in colour

[2] +ve = alkaline reaction (red), -ve is no change in colour

Figure A3 Biochemical reactions on lysine iron agar

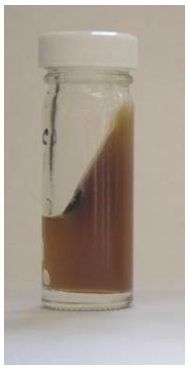
Citrobacter	*E. coli*	*Salmonella*
		
yellow slope and butt with sulphide production	neutral	black butt (sulphide production) and alkaline slope

Figure A4 *Citrobacter*, *E. coli* and *Salmonella* on triple sugar iron agar

Citrobacter	*E. coli*	*Salmonella*
		
acid slope and butt, with a hint of sulphide production	acid slope and butt	acid butt, alkaline slope with blackening i.e. sulphide production

Figure A5 As Figure A4 above but viewed from the front

Citrobacter	*E. coli*	*Salmonella*
		
acid slope and butt, with a hint of sulphide production	acid slope and butt	acid butt, alkaline slope with blackening i.e. sulphide production

Figure A6 Growth of *Salmonella* and *Proteus* in urea broth

Salmonella

yellow colour - negative

Proteus

red colour - positive

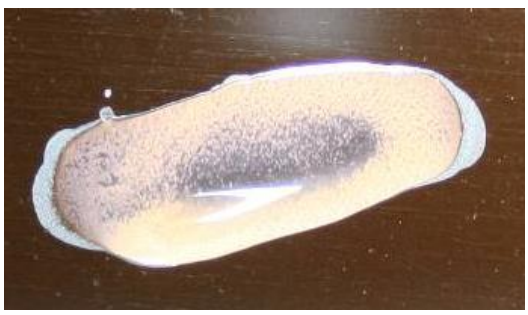
A9.5.2 *Serological confirmation*

Sub-culture any confirmed *Salmonella* isolates (as shown by characteristic growth on lysine iron agar, triple sugar iron agar and urea broth) from the nutrient agar dishes to moist nutrient agar slopes. For optimum flagella formation, it is essential that 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 of 0.02 ml) of saline solution onto a clean microscope slide. Emulsify growth from the slope in the first drop to produce homogeneous suspensions and growth from the moist butt in the second drop. Moisture encourages flagella formation and should enhance the agglutination in the second drop. 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, see Figure A7. 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 or brilliant green agar and dry smooth colonies treated as previously described. Organisms that agglutinate with PSO and PSH anti-sera, or strains that agglutinate with PSH serum only, can be regarded as members of the *Salmonella* group.

Figure A7 Positive slide agglutinations for *Salmonella*

Poly O slide agglutination

Poly H slide agglutination



Auto-agglutination control, no clumping

A10 Calculations

For presence-absence determinations, the tests indicate the presence or absence of *Salmonella* species in the volume or weight examined.

The number of buffered peptone water tubes or bottles of each volume of sample showing a positive reaction is counted, and then by reference to the appropriate tables in Appendix 1, the MPN of presumptive *Salmonella* species 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 3 in Appendix 1, the MPN is 13 organisms per 100 ml.

A11 Expression of Results

The result is expressed as the presumptive and confirmed *Salmonella* species being detected, or not detected, in the volume or weight of sample examined for presence or absence.

For the most probable number, the count is expressed as the number of *Salmonella* per volume or weight 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 of required quality control are given elsewhere[(2)] in this series.

A13 References

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15. Concentration technique for demonstrating small amounts of bacteria in tap water, *Acta Pathologica et Microbiologica Scandinavia*, E Hammarstrom and V Ljutov, 1954, **35**, 365-369.

B The isolation and enumeration of *Shigella* species by selective enrichment, presence-absence and multiple tube most probable number technique

This method has not been subjected to widespread use within the UK or verification of performance. Users of this method are encouraged to contact the Secretary of the Standing Committee of Analysts at the address given at the end of this booklet with their experiences and any relevant data on its performance. Information on the routine use of this method and similar methods, would be welcomed to assess their full capabilities.

B1 Introduction

Members of the genus *Shigella* normally inhabit the intestinal tract of humans but do not infect animals. The presence of *Shigella* 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 surface water, wastewater and sea water is described elsewhere[(1)] in this series.

B2 Scope

The method is suitable for the examination of fresh and saline surface water, swimming pools, spa pools and hydrotherapy pools, primary and secondary wastewater effluents and sediments including sand. Clean water samples may be filtered directly using membrane filters or a suitable filter-aid used. Sediments may be suspended in a suitable diluent or inoculated directly into pre-enrichment broths.

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 *Shigella* species.

Bacteria in the genus *Shigella* are facultative anaerobes, Gram-negative, non-motile rods. Four species are recognised; *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 a few exceptions, carbohydrates are fermented without gas production.

B4 Principle

This method uses one enrichment broth and two selective agars for the diagnostic detection of *Shigella* species by a presence-absence determination or a multiple tube most probable number technique.

B5 Limitations

This method is labour intensive and may require the preparation of large numbers of tubes or bottles of media and appropriate sub-cultures.

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 used. 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.

B6 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.

Strains of *Shigella* are “Hazard Group 3”[4]. However, where samples are not expected to contain *Shigella* 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. Caution should be exercised in the disposal of contaminated 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 (at least 1 litre) made of suitable material. For swimming pools, spa pools and hydrotherapy pools the containers should not be made of glass and should contain sufficient sodium thiosulphate pentahydrate to give a final concentration in the sample of not less than 18 mg/l. For example, 0.1 ml of a 1.8 % m/v solution of sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) per 100 ml of sample, or equivalent may be suitable.

B7.2 Incubator capable of maintaining a temperature of 37 ± 1 °C.

B7.3 Filtration apparatus, filter funnels, (either sterilised or capable of being sterilised) and vacuum source.

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

B7.5 Smooth-tipped forceps.

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[2]. Variations in the preparation and storage of media should also be verified. Water should be distilled, deionised or of similar grade quality. Unless otherwise stated chemical constituents should be added as the anhydrous salt. If the pH of the media is not within the stated range, then, before heating, it should be adjusted accordingly. Media with a pH outside the required range should be discarded. Where media are stored in a refrigerator, they should be allowed to attain room temperature before use.

B8.1 *Single-strength modified Hajna GN enrichment broth*

Tryptone	20 g
Glucose	1 g
Mannitol	2 g
Sodium citrate	5 g
Sodium desoxycholate	500 mg
Dipotassium hydrogen phosphate	4 g
Potassium dihydrogen phosphate	1.5 g
Sodium chloride	5 g
(DL) Serine	1 g
Water	1 litre

Dissolve the ingredients in the water and adjust the pH of the medium to 7.2 ± 0.2. Dispense the resulting solution (typically, 90 ml) into suitable capped containers and sterilise by steaming at 100 °C for 30 minutes. After steaming, the pH of the medium should be checked to confirm a pH of 7.2 ± 0.2. The medium may be stored at between 5 ± 3 °C for up to one month, if protected from dehydration.

Double-strength medium may be prepared using double the amount of ingredients in the 1000 ml of water.

B8.2 *Modified desoxycholate citrate agar*

Tryptone	20 g
Lactose	10 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 g
Tetracycline hydrochloride	32 mg

Agar	14 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 and allow the agar to solidify. The complete medium may be stored at between 5 ± 3 °C for up to one month, if protected against dehydration. Prepared dishes should be dried before use, by placing, for example the open Petri dish in an incubator at 37 °C for 30 minutes.

B8.3 *Modified Hektoen agar*[5]

Yeast extract	3 g
Proteose peptone	12 g
Lactose	12 g
Sucrose	12 g
Salicin	2 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 g
Sodium chloride	5 g
Sodium thiosulphate pentahydrate	5 g
Agar	14 g
Novobiocin	15 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. The medium may be stored at between 5 ± 3 °C for up to one month, if protected from dehydration. Prepared dishes should be dried before use, by placing, for example the open Petri dish in an incubator at 37 °C for 30 minutes

B8.4 *Filter-aid*[6]

Diatomaceous earth	1 g (approximately)
Distilled, deionised or similar grade 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. The sterilised aid may be stored in the dark at room temperature for up to 12 months.

B8.5 *Other media*

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

B9 Analytical procedure

B9.1 *Sample preparation*

B9.1.1 *Surface waters and sea water*

Due to the likelihood that, if present, the numbers of *Shigella* in some surface waters and sea waters are likely to be low, for presence-absence determinations, a sample volume of at least 1000 ml should be examined.

For the membrane filtration multiple tube technique, typically, an 11-tube series can be used, i.e. the membrane filtration of 1 x 500 ml, 5 x 100 ml and 5 x 10 ml of sample. Alternatively, volumes of 1 x 500 ml and 5 x 100 ml can be filtered and the 10 ml volumes can be added directly to 10 ml volumes of double-strength modified Hajna GN enrichment broth. For a different series, smaller volumes of sample, for example 1 ml, may be appropriate and these can be added directly to 9 ml of single-strength modified Hajna GN enrichment broth. Turbid waters, unsuitable for direct membrane filtration, may be filtered using filter aid.

B9.1.2 *Treated wastewater*

Treated wastewater may be analysed as described in B9.1.1 although several membrane filters may be required for presence-absence determinations. A sample volume of at least 100 ml may need to be examined.

The volumes may be reduced and volumes of 1 x 50 ml, 5 x 10 ml and 5 x 1 ml may be used. The 1 x 50 ml and 5 x 10 ml volumes can be membrane filtered or added to equal volumes of double-strength modified Hajna GN enrichment broth. The 1 ml volumes can be added to 9 ml of single-strength modified Hajna GN enrichment broth.Typically, 1 ml of these diluted samples can be added directly to 9 ml of single-strength modified Hajna GN enrichment broth.

B9.1.3 *Untreated wastewater*

For presence-absence determinations, 100 ml of untreated wastewater sample may be required, as it may not be possible (owing to turbidity) to process larger volumes by membrane filtration. For an 11-tube most probable number series, the volumes of untreated wastewater are usually 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 x 50 ml and 5 x 10 ml volumes can be filtered or added to equal volumes of double-strength modified Hajna GN enrichment broth. The 1 ml volumes can be added to 9 ml of single-strength modified Hajna GN enrichment broth. To represent smaller volumes, for example 0.1 ml and 0.01 ml volumes of sample, a 1:10 and 1:100 dilution of the sample, may be appropriate. Typically, 1 ml of these diluted samples can be added directly to 9 ml of single-strength modified Hajna GN enrichment broth.

B9.1.4 *Sediment and sand*

Solid material can be dispensed as a single weight for presence-absence determinations by weighing, for example 10 g of sample into an appropriate volume (typical 100 ml) of single-strength modified Hajna GN enrichment broth. For the multiple tube technique,

weigh 1 x 50 g, 5 x 10 g and 5 x 1 g quantities of sample into appropriate volumes (typical 450 ml, 5 x 100 ml and 5 x 10 ml respectively) of single-strength modified Hajna GN enrichment broth. For smaller quantities, for example 100 mg, these may be added directly to 10 ml of single-strength modified Hajna GN enrichment broth.

B9.2 Sample processing

B9.2.1 *Membrane filtration*

For membrane presence-absence or membrane multiple tube techniques 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 gridded 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, typically, 10 - 15 ml of single-strength modified Hajna GN enrichment broth, ensuring that the membrane filter is fully submerged. Record the volume filtered. Other volumes of sample should be similarly treated until all the filters are transferred to the corresponding tubes or bottles of single-strength modified Hajna GN enrichment broth. The largest 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 single-strength modified Hajna GN enrichment broth. Ensure that all 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 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 *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 (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 single-strength 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 100 ml.

For presence-absence determinations, place the culture vessel in an incubator and incubate at 37 °C for 6 - 8 hours. For a most probable number test, re-suspend the filter aid in the single-strength modified Hajna GN enrichment broth and dispense in a multiple tube most probable number series using 1 x 50 ml, 5 x 10 ml and 5 x 1 ml. The 1 ml volumes can be inoculated into 9 ml of sterile single-strength modified Hajna GN enrichment broth, and incubated as above.

B9.2.3 *Direct inoculation*

Where the numbers of *Shigella* in the sample are likely to be high, smaller volumes of sample, for example 50 ml and 10 ml can be inoculated directly into equal volumes of double strength modified Hajna GN enrichment broth. Volumes of 1 ml and subsequent dilutions of the sample can be inoculated directly into 9 ml of single-strength modified Hajna GN enrichment broth.

B9.2.4 *Sediment and sand*

Samples of sediment and sand may be analysed by weighing appropriate amounts, for example, a single aliquot of 10 g for presence-absence determinations or 1 x 5 g, into 90 ml, and 5 x 1 g and 5 x 0.1 g into 9 ml of single-strength modified Hajna GN enrichment broth for a most probable number series. Larger weights of sample should be weighed into appropriately larger volumes of single-strength modified Hajna GN enrichment broth.

B9.3 *Enrichment and sub-culture to selective agar*

The modified Hajna GN enrichment broth and membrane filters, and if appropriate filter-aid or solid sample material, is mixed thoroughly and incubated at 37 °C for 6 - 8 hours. After incubation, the tubes or bottles are examined for growth. After incubation, gently mix each tube or bottle and plate out loopfuls of modified Hajna GN enrichment broth onto both modified desoxycholate citrate agar and modified Hektoen agar. Incubate the selective agars at 37 °C for 18 - 24 hours.

B9.4 *Reading of results*

After incubation, examine the Petri dishes of modified desoxycholate citrate agar (see Figure B1) and modified Hektoen agar (see Figure B2) under good light, and if necessary use a hand lens. Colonies may be differentiated as follows

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

Colonial appearance on modified Hektoen agar

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 B1 Colonies of *Shigella* on modified desoxycholate citrate agar

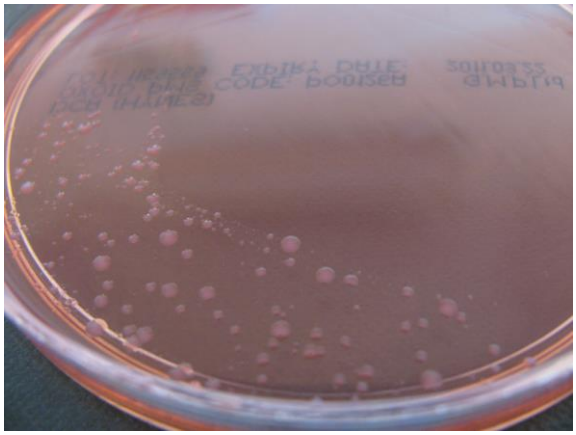

Figure B2 Colonies of *Shigella* and *Salmonella* on modified Hektoen agar

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

B9.5 *Confirmation tests*

Sub-culture typical colonies from the selective agars 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. Slide agglutination can be conducted direct from initial colonies if considered pure.

B10 Calculations

For presence-absence determinations, the tests indicate the presence or absence of *Shigella* species in the volume or weight examined.

The number of modified Hajna GN enrichment broth tubes or bottles of each volume of sample showing a positive reaction is counted, and then by reference to the appropriate tables in Appendix 1, the MPN of presumptive *Shigella* 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 3 in Appendix 1, the MPN is 13 organisms per 100 ml.

B11 Expression of results

For presence-absence determinations, the result is expressed as the presumptive and confirmed *Shigella* species being detected, or not detected, in the volume or weight of sample examined.

For multiple tube most probable number techniques, the count is expressed as the number of *Shigella* per volume or weight of sample examined.

B12 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 of required quality control are given elsewhere[(2)] in this series.

B13 References

1. Standing Committee of Analysts, The Microbiology of Recreational and Environmental Waters (2014) - Part 1 - Water quality epidemiology and public health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.

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Appendix 1 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, which demonstrate the range of possible numbers (the MPR) which could yield the number of positive reactions, have been published[1]. A procedure for estimating these confidence intervals for other dilution series has also been published[2]. 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[3]. The MPR in tables 1 - 3 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 1 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 2 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 3 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[1, 4, 5]. 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 4.

(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 4).

(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 4).

(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 4).

Table 1 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 2 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 3 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	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 4 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.

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