



ENVIRONMENT AGENCY

**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**

*Methods for the Examination of Waters and Associated Materials*



**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**

**Methods for the Examination of Waters and Associated Materials**

This booklet contains three membrane filtration and most probable number methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment. This booklet supercedes “The Microbiology of Drinking Water (2002) - Part 9 - Methods for the isolation and enumeration of *Salmonella* and *Shigella* by selective enrichment, membrane filtration and multiple tube most probable number techniques”.

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* by membrane filtration
  - Part 10 - Methods for the isolation and enumeration of *Yersinia*, *Vibrio* and *Campylobacter* by selective enrichment and membrane filtration

- The Microbiology of Drinking Water (2004) -
  - Part 6 - Methods for the isolation and enumeration of sulphite-reducing clostridia and *Clostridium perfringens* 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. An index of methods and details on how to obtain copies are available from the Agency's webpage ([www.environment-agency.gov.uk/nls](http://www.environment-agency.gov.uk/nls)) or from the Secretary.

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

Dr D Westwood  
*Secretary*

May 2004

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## 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<sup>(1)</sup> 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<sup>(2)</sup>.

### **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<sup>(3)</sup> 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  $\beta$ -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.

### **A4 Principle**

Isolation is based on concentration from water by membrane filtration, or the use of a filter aid, followed by pre-enrichment involving incubation in a non-selective medium (to recover environmentally-stressed organisms) and selective enrichment with subculture to a selective agar containing lactose and an indicator of acidity. 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 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. This method is not suitable for the recovery of *Salmonella typhi* 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<sup>(4)</sup> and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere<sup>(2)</sup> 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<sup>(2)</sup> 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  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  per 100 ml of sample, or equivalent).
- A7.2 Incubators capable of maintaining temperatures of  $37 \pm 1$  °C and  $41.5 \pm 0.5$  °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.



Unless otherwise stated chemical constituents should be added as anhydrous salts.

A8.1 *Buffered peptone water*<sup>(5)</sup>

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Distilled, deionised or similar grade 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  $7.2 \pm 0.2$ . Autoclaved media may be stored in the dark at room temperature, protected from dehydration, and used within one month.

A8.2 *Rappaport Vassiliadis enrichment broth*<sup>(6, 7)</sup>

*Solution A*

Soya peptone	4.5 g
Sodium chloride	7.2 g
Potassium dihydrogen phosphate	1.26 g
Dipotassium hydrogen phosphate	180 mg
Distilled, deionised or similar grade water	800 ml

*Solution B*

Magnesium chloride anhydrous	13.6 g
Distilled, deionised or similar grade water	100 ml

*Solution C*

Malachite green	36 mg
Distilled, deionised or similar grade 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  $5.2 \pm 0.2$ . Autoclaved media may be stored between 2 - 8 °C, protected from dehydration, and used within one month.

A8.3 *Brilliant green agar*<sup>(8)</sup>

Yeast extract	3.0 g
Proteose peptone or polypeptone	10.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Phenol red (0.2 % m/v aqueous solution)	40 ml

Brilliant green (0.5 % m/v aqueous solution)	2.5 ml
Agar	20.0 g
Distilled, deionised or similar grade 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  $6.9 \pm 0.2$ . Cool the molten medium to approximately 50 °C and pour into sterile Petri dishes. Allow the medium to solidify, store at between 2 - 8 °C, protected from dehydration, and use within one month. Alternatively, the bottled medium may be stored in the dark at room temperature and use within one month

The medium may be made more selective by the addition of a sulphonamide, for example, sulphapyridine at 1000 mg/l, sulphadiazine at 800 mg/l, or sulphamandelate supplement<sup>(9)</sup>. The latter comprises an aqueous filter-sterilised solution of sodium sulphacetamide at 1000 mg/l and sodium mandelate at 250 mg/l. Sodium desoxycholate at 2500 mg/l has also been used to prevent swarming of species of *Proteus*.

#### A8.4 *Xylose lysine desoxycholate agar*<sup>(10)</sup>

##### *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
Distilled, deionised or similar grade water	1 litre

##### *Solution A*

Sodium thiosulphate pentahydrate	34.0 g
Ammonium iron(III) citrate	4.0 g
Distilled, deionised or similar grade water	100 ml

##### *Solution B*

Sodium desoxycholate	10.0 g
Distilled, deionised or similar grade 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 and 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 of  $7.4 \pm 0.2$ . Pour the complete medium into sterile Petri dishes and allow the medium to solidify.

#### A8.5 *Lysine iron agar*<sup>(11)</sup>

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
Distilled, deionised or similar grade 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  $6.7 \pm 0.2$ . Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored at between 2 - 8 °C, protected from dehydration, and used within one month.

#### A8.6 *Triple sugar iron agar*<sup>(12)</sup>

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
Distilled, deionised or similar grade 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  $7.4 \pm 0.2$ . Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored at between 2 - 8 °C, protected from dehydration, and used within one month.

### A8.7 *Urea broth*

#### *Broth base*

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
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH to  $6.8 \pm 0.2$ . 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  $6.8 \pm 0.2$ . Prepared base medium may be stored in the dark at room temperature and 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 broth base and aseptically dispense in 2 - 3 ml volumes in sterile capped containers.

### A8.8 *Filter-aid*<sup>(13)</sup>

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. Store in the dark at room temperature and use within 12 months.

### A8.9 *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<sup>(2)</sup>.

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of

the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter, grid-side upwards, onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. 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 capped container containing 90 ml of buffered peptone water. If more than one membrane filter is required, all filters are transferred to the 90 ml of buffered peptone water. Mix well. 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 water for at least one minute. During the filtration of a series of samples, the filter base need not be sterilised unless it becomes contaminated or a membrane filter becomes damaged. Known polluted and non-polluted samples should be filtered using separate filtration equipment. Alternatively, polluted samples should only be processed after non-polluted samples. When funnels are not in use they should be covered with a sterile lid or a sterile Petri dish lid.

The time between the end of the filtration step and the beginning of the incubation stage should be as short as possible and no longer than 2 hours.

#### *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) is filtered to form an initial layer on the absorbent pad. A second aliquot (typically, 15 ml) of filter-aid is 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, is mixed thoroughly and incubated at 37 °C for 24 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 24 hours. After this time, plate out loopfuls of the Rappaport Vassiliadis enrichment broth onto xylose lysine desoxycholate agar and brilliant green agar. Incubate the selective agars at 37 °C for 24 hours. After plating, return the Rappaport Vassiliadis enrichment broth to the incubator at 41.5 °C for a further 24 hours. After this time, again plate out loopfuls of the Rappaport Vassiliadis enrichment broth onto xylose lysine desoxycholate agar and brilliant green agar. Incubate the selective agars at 37 °C for 24 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
<i>Salmonella</i>	Smooth red colonies 2 - 3 mm in diameter, typically, with black centres or wholly black colonies
Xylose-fermenting coliform bacteria	Yellow colonies
<i>Pseudomonas</i> species	Red or yellow colonies with grey-black centres
<i>Shigella</i> species	Small pink-red colonies
<i>Proteus</i> species	Red colonies that are irregular and may have small black centres

#### *Colonial appearance on brilliant green agar*

Organism	Characteristic appearance
<i>Salmonella</i>	Smooth red colonies, approximately 2 mm in diameter
Lactose- and sucrose-fermenting coliform bacteria	Yellow/green colonies
<i>Pseudomonas</i> species	Small crenated colonies
<i>Proteus</i> species	Small red colonies

Where isolates are overgrown, then subculture 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.

### A9.4 Confirmation tests

#### A9.4.1 Biochemical confirmation

Using a straight wire, subculture characteristic colonies from each Petri dish 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, ie 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 <sup>1</sup>	Butt <sup>1</sup>	H <sub>2</sub> S production <sup>2</sup>
<i>Salmonella</i>	Alkaline	Alkaline	+ve
<i>Arizona</i>	Alkaline	Alkaline	+ve
<i>Proteus</i>	Red	Acid	+ve or -ve
<i>Providencia</i>	Red	Acid	-ve
<i>Citrobacter</i>	Alkaline	Acid	+ve
<i>Escherichia</i>	Alkaline	Acid or no change	-ve
<i>Shigella</i>	Alkaline	Acid	-ve
<i>Klebsiella</i>	Alkaline	Alkaline	-ve
<i>Enterobacter</i>	Alkaline	Acid	-ve

<sup>1</sup> Alkaline reaction is purple, acid reaction is yellow

<sup>2</sup> +ve = blackening of the medium, -ve = no blackening

### Reactions in triple sugar iron agar and urea broth

Genus	Triple sugar iron agar			Urea broth <sup>3</sup>
	Slope <sup>1</sup>	Butt <sup>1</sup>	H <sub>2</sub> S production <sup>2</sup>	
<i>Salmonella typhi</i>	NC or Alk	Acid	+ve (weak)	-ve
Other <i>Salmonella</i>	NC or Alk	Acid + gas	+ve	-ve
<i>Shigella</i>	NC or Alk	Acid	-ve	-ve
<i>Proteus morgani</i>	NC or Alk	Acid ± gas	-ve	+ve
<i>Proteus vulgaris</i>	Acid	Acid + gas	+ve	+ve
<i>Escherichia</i>	Acid	Acid + gas	-ve	-ve
<i>Citrobacter</i>	Acid	Acid + gas	+ve	+ve or -ve
<i>Klebsiella</i>	Acid	Acid + gas	-ve	+ve or -ve
<i>Enterobacter</i>	Acid	Acid + gas	-ve	-ve

<sup>1</sup> Alkaline (Alk) reaction is red, acid reaction is yellow or no change (NC)

<sup>2</sup> +ve = blackening of the medium, -ve = no blackening

<sup>3</sup> +ve = alkaline reaction (red), -ve is no change in colour

#### A9.4.2 Serological confirmation

Subculture characteristic colonies from each Petri dish to moist nutrient agar slopes. For optimum flagellar 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 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) 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 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.

#### **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<sup>(2)</sup> in this series.

#### **A13 References**

1. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 1 - Water Quality and Public Health. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
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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<sup>(1)</sup> 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<sup>(2)</sup>.

### **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<sup>(3)</sup> 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  $\beta$ -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.

### **B4 Principle**

Isolation and enumeration is based on concentration from water by membrane filtration, followed by multiple tube pre-enrichment involving incubation in a non-selective medium (to recover environmentally-stressed organisms) and selective enrichment with subculture to a selective agar containing lactose and an indicator of

acidity. 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 is not suitable for the recovery of *Salmonella typhi* 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<sup>(4)</sup> and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere<sup>(2)</sup> 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<sup>(2)</sup> 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<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O 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.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

### B8.1 *Buffered peptone water*<sup>(5)</sup>

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water. Dispense the resulting solution (typically, 10 - 15 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  $7.2 \pm 0.2$ . Autoclaved media may be stored in the dark at room temperature, protected from dehydration, and used within one month.

### B8.2 *Rappaport Vassiliadis enrichment broth*<sup>(6,7)</sup>

#### *Solution A*

Soya peptone	4.5 g
Sodium chloride	7.2 g
Potassium dihydrogen phosphate	1.26 g
Dipotassium hydrogen phosphate	180 mg
Distilled, deionised or similar grade water	800 ml

#### *Solution B*

Magnesium chloride anhydrous	13.6 g
Distilled, deionised or similar grade water	100 ml

#### *Solution C*

Malachite green	36 mg
Distilled, deionised or similar grade 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  $5.2 \pm 0.2$ . Autoclaved media may be stored between 2 - 8 °C, protected from dehydration, and used within one month.

### B8.3 *Brilliant green agar*<sup>(8)</sup>

Yeast extract	3.0 g
Proteose peptone or polypeptone	10.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Phenol red (0.2 % m/v aqueous solution)	40 ml
Brilliant green (0.5 % m/v aqueous solution)	2.5 ml
Agar	20.0 g
Distilled, deionised or similar grade 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  $6.9 \pm 0.2$ . Cool the molten medium to approximately 50 °C and pour into sterile Petri dishes. Allow the medium to solidify, store at between 2 - 8 °C, protected from dehydration, and use within one month. Alternatively, the bottled medium may be stored in the dark at room temperature and use within one month.

The medium may be made more selective by the addition of a sulphonamide, for example, sulphapyridine at 1000 mg/l, sulphadiazine at 800 mg/l, or sulphamandelate supplement<sup>(9)</sup>. The latter comprises an aqueous filter-sterilised solution of sodium sulphacetamide at 1000 mg/l and sodium mandelate at 250 mg/l. Sodium desoxycholate at 2500 mg/l has also been used to prevent swarming of species of *Proteus*.

### B8.4 *Xylose lysine desoxycholate agar*<sup>(10)</sup>

#### *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
Distilled, deionised or similar grade water	1 litre

#### *Solution A*

Sodium thiosulphate pentahydrate	34.0 g
Ammonium iron(III) citrate	4.0 g
Distilled, deionised or similar grade water	100 ml

#### *Solution B*

Sodium desoxycholate	10.0 g
Distilled, deionised or similar grade 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 and 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 of  $7.4 \pm 0.2$ . Pour the complete medium into sterile Petri dishes and allow the medium to solidify.

#### B8.5 *Lysine iron agar*<sup>(11)</sup>

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
Distilled, deionised or similar grade 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  $6.7 \pm 0.2$ . Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored at between 2 - 8 °C, protected from dehydration, and used within one month.

#### B8.6 *Triple sugar iron agar*<sup>(12)</sup>

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
Distilled, deionised or similar grade 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  $7.4 \pm 0.2$ . Cool in a sloping position to give an agar slope with a deep butt. The prepared medium may be stored at between 2 - 8 °C, protected from dehydration, and used within one month.

#### B8.7 *Urea broth*

##### *Broth base*

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
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH to  $6.8 \pm 0.2$ . 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  $6.8 \pm 0.2$ . Prepared base medium may be stored in the dark at room temperature and 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 broth base and aseptically dispense in 2 - 3 ml volumes in sterile capped containers.

#### B8.8 *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.

#### B9.2 *Sample processing*

##### B9.2.1 *Membrane filtration-multiple tube technique*

Appropriate volumes of sample are filtered through appropriate 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, grid-side upwards, onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the

funnel. Open the stopcock and apply a vacuum not exceeding 65 kPa (500 mm of mercury) and filter the 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. Mix well. Other volumes of sample are 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. Each tube or bottle of buffered peptone water is mixed well.

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 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 is incubated at 37 °C for 24 hours. After incubation, mix well 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 24 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 and brilliant green agar. Incubate the selective agars at 37 °C for 24 hours. After plating, return the Rappaport Vassiliadis enrichment broth to the incubator at 41.5 °C for a further 24 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 and brilliant green agar. Incubate the selective agars at 37 °C for 24 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
<i>Salmonella</i>	Smooth red colonies 2 - 3 mm in diameter, typically, with black centres or wholly black colonies
Xylose-fermenting coliform bacteria	Yellow colonies
<i>Pseudomonas</i> species	Red or yellow colonies with grey-black centres
<i>Shigella</i> species	Small pink-red colonies
<i>Proteus</i> species	Red colonies that are irregular and may have small black centres

*Colonial appearance on brilliant green agar*

Organism	Characteristic appearance
<i>Salmonella</i>	Smooth red colonies, approximately 2 mm in diameter
Lactose- and sucrose-fermenting coliform bacteria	Yellow/green colonies
<i>Pseudomonas</i> species	Small crenated colonies
<i>Proteus</i> species	Small red colonies

Where isolates are overgrown, then subculture 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.

**B9.4** *Confirmation tests*

**B9.4.1** *Biochemical confirmation*

Using a straight wire, subculture characteristic colonies from each Petri dish 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, ie 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 <sup>1</sup>	Butt <sup>1</sup>	H <sub>2</sub> S production <sup>2</sup>
<i>Salmonella</i>	Alkaline	Alkaline	+ve
<i>Arizona</i>	Alkaline	Alkaline	+ve
<i>Proteus</i>	Red	Acid	+ve or -ve
<i>Providencia</i>	Red	Acid	-ve
<i>Citrobacter</i>	Alkaline	Acid	+ve
<i>Escherichia</i>	Alkaline	Acid or no change	-ve
<i>Shigella</i>	Alkaline	Acid	-ve
<i>Klebsiella</i>	Alkaline	Alkaline	-ve
<i>Enterobacter</i>	Alkaline	Acid	-ve

<sup>1</sup> Alkaline reaction is purple, acid reaction is yellow

<sup>2</sup> +ve = blackening of the medium, -ve = no blackening

### Reactions in triple sugar iron agar and urea broth

Genus	Triple sugar iron agar			Urea broth <sup>3</sup>
	Slope <sup>1</sup>	Butt <sup>1</sup>	H <sub>2</sub> S production <sup>2</sup>	
<i>Salmonella typhi</i>	NC or Alk	Acid	+ve (weak)	-ve
Other <i>Salmonella</i>	NC or Alk	Acid + gas	+ve	-ve
<i>Shigella</i>	NC or Alk	Acid	-ve	-ve
<i>Proteus morgani</i>	NC or Alk	Acid ± gas	-ve	+ve
<i>Proteus vulgaris</i>	Acid	Acid + gas	+ve	+ve
<i>Escherichia</i>	Acid	Acid + gas	-ve	-ve
<i>Citrobacter</i>	Acid	Acid + gas	+ve	+ve or -ve
<i>Klebsiella</i>	Acid	Acid + gas	-ve	+ve or -ve
<i>Enterobacter</i>	Acid	Acid + gas	-ve	-ve

<sup>1</sup> Alkaline (Alk) reaction is red, acid reaction is yellow or no change (NC)

<sup>2</sup> +ve = blackening of the medium, -ve = no blackening

<sup>3</sup> +ve = alkaline reaction (red), -ve is no change in colour

#### B9.4.2 Serological confirmation

Subculture characteristic colonies from each Petri dish to moist nutrient agar slopes. For optimum flagellar 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 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) 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 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.

## 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 multiply the result by 10, however, if no organisms are found then this would equate as 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 and 5 x 100 ml volumes of sample

Number of tubes giving a positive reaction		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, these are numbers that are at least 95% as probable as the 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<sup>(2)</sup> in this series.

## B13 References

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## **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<sup>(1)</sup> 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<sup>(2)</sup>.

### **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<sup>(3)</sup> and appropriate risk assessments should be made before adopting these methods. Standard laboratory microbiology safety procedures should be followed and guidance is given elsewhere<sup>(2)</sup> 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<sup>(2)</sup> 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<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.5H<sub>2</sub>O 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.

Unless otherwise stated chemical constituents should be added as anhydrous salts.

### **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
Distilled, deionised or similar grade water	1 litre

Dissolve the ingredients in the water and adjust the pH to  $7.2 \pm 0.2$ . Dispense the resulting solution (typically, 90 ml) into suitable capped containers and sterilise by steaming at  $100\text{ }^{\circ}\text{C}$  for 30 minutes. After steaming, the pH of the medium should be checked to confirm a pH of  $7.2 \pm 0.2$ . The medium should be stored at between  $2 - 8\text{ }^{\circ}\text{C}$ , protected from dehydration, and used 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% <i>m/v</i> aqueous solution)	3 ml
Sodium desoxycholate	500 mg
(DL) Serine	1.0 g
Tetracycline hydrochloride	32 mg
Agar	14.0 g
Distilled, deionised or similar grade 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  $50\text{ }^{\circ}\text{C}$  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. Store at between  $2 - 8\text{ }^{\circ}\text{C}$ , protected against dehydration, and use within one month. Prepared dishes should be dried before use.

#### C8.3 *Modified Hektoen agar*<sup>(4)</sup>

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 % <i>m/v</i> 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
Distilled, deionised or similar grade 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. Prepared dishes should be dried before use.

#### C8.4 *Filter-aid*<sup>(5)</sup>

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. 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<sup>(2)</sup>.

Place the sterile or disinfected filtration apparatus in position and connect to a source of vacuum, with the stopcock turned off. Remove the funnel and, holding the edge of the membrane filter with sterile smooth-tipped forceps, place a sterile membrane filter, grid-side upwards, onto the porous disc of the filter base. Replace the sterile funnel securely on the filter base. Pour or pipette the required volume of sample into the funnel. 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 90 ml of modified Hajna GN enrichment broth. If more than one membrane filter is required, all filters are transferred to the 90 ml of modified Hajna GN enrichment broth. Mix well.



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 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 a membrane filter to act as a supporting base for the filter-aid. An aliquot of filter-aid (typically, 15 ml) is filtered to form an initial layer on the absorbent pad. A second aliquot (typically, 15 ml) of filter-aid is 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, is 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:

*Colonial appearance on modified desoxycholate citrate agar*

Organism	Characteristic appearance
<i>Shigella</i>	Small raised cream coloured colonies
<i>Salmonella</i>	Large black coloured colonies with a thin white periphery
<i>Pseudomonas</i>	Very small flat cream coloured colonies
<i>Proteus</i>	Cream coloured colonies with a small black centre
<i>Escherichia</i>	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
<i>Shigella</i>	Moist green coloured colonies 2-4 mm in diameter, <i>Shigella sonnei</i> may produce larger irregular colonies
<i>Salmonella</i>	Blue-green colonies, with or without black centres
<i>Pseudomonas</i>	Large rough textured green coloured colonies
<i>Proteus</i>	Pale green or ochre yellow coloured colonies
Coliform bacteria	Yellow coloured colonies. The medium around the colonies often turns salmon pink

**C9.4** *Confirmation tests*

Subculture typical colonies to a non-selective medium such as nutrient agar and incubate at 37 °C for 24 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<sup>(2)</sup> 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.

2. Standing Committee of Analysts, The Microbiology of Drinking Water (2002) - Part 3 - Practices and Procedures for Laboratories. *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
3. The Control of Substances Hazardous to Health Regulations 1999, Statutory Instrument 1999 No. 437.
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*, King, S. & Metzger W.I., 1968, **16**, 577-581
5. Concentration technique for demonstrating small amounts of bacteria in tap water. *Acta Pathologica et Microbiologica Scandinavia*, Hammarstrom, E. & Ljutov, V., 1954, **35**, 365-369.

## **Address for correspondence**

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below.

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