# Methods for the Isolation and Identification of Salmonellae (other than Salmonella Typhi) from Water and Associated Materials 1982

Methods for the Examination of Waters and Associated Materials

London Her Majesty's Stationery Office £2.75 net

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## Contents

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# Warning to users

The analytical procedures given in this booklet should only be carried out by competent trained persons, with adequate supervision when necessary. Local safety Regulations must be observed. Laboratory procedures should be carried out only in properly equipped laboratories. Field operations should be conducted with due regard to possible local hazards, and portable safety equipment should be carried. Care should be taken against creating hazards. Lone working, whether in the laboratory or field, should be discouraged. Reagents of adequate purity must be used, along with properly maintained apparatus and equipment of correct specification. Specifications for reagents, apparatus and equipment are given in manufacturers' catalogues and various published standards. If contamination is suspected, reagent purity should be checked before use.

There are numerous handbooks on first aid and laboratory safety. Among such publications are: 'Code of Practice for Chemical Laboratories' and 'Hazards in the Chemical Laboratory' issued by the Royal Society of Chemistry, London; 'Safety in Biological Laboratories' (Editors Hartree and Booth), Biochemical Society Special Publication No 5, The Biochemical Society, London, which includes biological hazards; and 'The Prevention of Laboratory Acquired Infection' Public Health Laboratory Service Monograph, HMSO, London.

Where the Committee have considered that a special unusual hazard exists, attention has been drawn to this in the text so that additional care might be taken beyond that which should be exercised at all times when carrying out analytical procedures. It cannot be too strongly emphasized that prompt first aid, decontamination, or administration of the correct antidote can save life; but that incorrect treatment can make matters worse. It is suggested that both supervisors and operators be familiar with emergency procedures before starting even a slightly hazardous operation, and that doctors consulted after any accident involving chemical contamination, ingestion, or inhalation, be made familiar with the chemical nature of the injury, as some chemical injuries require specialist treatment not normally encountered by most doctors. Similar warning should be given if a biological or radio chemical injury is suspected. Some very unusual parasites, viruses and other micro-organisms are occasionally encountered in samples and when sampling in the field. In the latter case, all equipment including footwear should be disinfected by appropriate methods if contamination is suspected.

The best safeguard is a thorough consideration of hazards and the consequent safety precautions and remedies well in advance. Without intending to give a complete checklist, points that experience has shown are often forgotten include: laboratory tidiness, stray radiation leaks (including ultra violet), use of correct protective clothing and goggles, removal of toxic fumes and wastes, containment in the event of breakage, access to taps, escape routes, and the accessibility of the correct and properly maintained first-aid, fire-fighting, and rescue equipment. If in doubt, it is safer to assume that the hazard may exist and take reasonable precautions, rather than to assume that no hazard exists until proved otherwise.


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# About this series

This booklet is one of a series intended to provide recommended methods for the determination of water quality. In the past, the Department of the Environment and its predecessors, in collaboration with various learned societies, has issued volumes of methods for the analysis of water and sewage culminating in 'Analysis of Raw, Potable and Waste Waters'. These volumes inevitably took some years to prepare, so that they were often partially out of date before they appeared in print. The present series will be published as individual methods, thus allowing for the replacement or addition of methods as quickly as possible without need of waiting for the next edition. The rate of publication will also be related to the urgency of requirement for that particular method, tentative methods being issued when necessary. The aim is to provide as complete and up to date a collection of methods and reviews as is practicable, which will, as far as possible, take into account the analytical facilities available in different parts of the Kingdom, and the quality criteria of interest to those responsible for the various aspects of the water cycle. Because both needs and equipment vary widely, where necessary, a selection of methods may be recommended for a single determinand. It will be the responsibility of the users — the senior analytical chemist, biologist, bacteriologist etc, to decide which of these methods to use for the determination in hand. Whilst the attention of the user is drawn to any special known hazards which may occur with the use of any particular method, responsibility for proper supervision and the provision of safe working conditions must remain with the user.

The preparation of this series and its continuous revision is the responsibility of the Standing Committee of Analysts (to review Standard Methods for Quality Control of the Water Cycle). The Standing Committee of Analysts is one of the joint technical committees of the Department of the Environment and the National Water Council. It now has seven Working Groups, each responsible for one aspect of water cycle quality analysis. They are as follows:

1.0 General principles of sampling and accuracy of results
3.0 Empirical and physical methods
4.0 Metals and metalloids
5.0 General non-metallic substances
6.0 Organic impurities
7.0 Biological methods
9.0 Radiochemical methods

The actual methods etc are produced by smaller panels of experts in the appropriate field, under the overall supervision of the appropriate working group and the main committee. The names of those associated with this method are listed inside the back cover.

Publication of new or revised methods will be notified to the technical press, whilst a list of Methods in Print is given in the current HMSO Sectional Publication List No 5. Corrections and Additions to already published methods will be issued in special compendia as part of this series.

Whilst an effort is made to prevent errors from occurring in the published text, a few errors have been found in booklets in this series. Correction notes for booklets in this series are given in the Reports of The Standing Committee of Analysts, published by the Department of the Environment but sold by the National Water Council, 1 Queen Anne's Gate, London SW1H 9BT. Should an error be found affecting the operation on a method, the true sense not being obvious, or an error in the printed text be discovered prior to sale, a separate correction note will be issued for inclusion in the booklet.

T A DICK
*Chairman*

L R PITTWELL
*Secretary*

*30 November1982*

# Methods for the Isolation and Identification of Salmonellae (other than Salmonella typhi) from Water and Associated Materials 1982

## 1 Introduction

As a result of the worldwide travel and transportation of human foods, animal feedstuffs and fertilizers many different serotypes of salmonellae are present in the human population, domestic and agricultural livestock and also to a lesser degree the wild animal and bird population. Both carriers and infected humans and animals excrete salmonellae in their dejecta. These organisms may be present in domestic sewage, treated sewage effluents as well as agricultural run off and consequently may be found in streams and rivers downstream of discharge points and in tidal and coastal waters. Because they are also associated with wild life they can be transmitted to aquatic environments remote from sewage, treated effluent and livestock.

In order to manage the water cycle effectively and to comply with legislation it is necessary to isolate and sometimes enumerate any salmonellae present. More than 2,000 salmonellae serotypes are now identifiable and no single method of isolation is equally efficient for all of them. The methods given, which are used throughout the water industry, are known to be generally effective for many of these serotypes.

## 2 Hazards

Salmonellae are pathogenic to a greater or lesser degree to man and animals. All cutlures and stages of cultivation must be handled in specified areas with the utmost care by properly trained personnel. All equipment must be sterilized before and after use. Cultures must not be kept longer than is necessary and must be sterilized prior to discarding. Due regard must be paid to the relevant Codes of Practice for Safety in Microbiology Laboratories [2] [3] [23]. See also sections 5.2.

## 3 Definition of Salmonellae

Salmonellae are gram-negative motile rods which are catalase-positive, oxidase-negative and facultatively anaerobic. The majority of serotypes ferment glucose, mannitol and dulcitol with the production of acid and gas, but not lactose, sucrose or salicin. Citrate can be utilized as the sole source of carbon, $H_2S$ is produced and with few exceptions lysine decarboxylated. Urease is not produced and growth does not occur in KCN medium[1].

It should be borne in mind that *S. typhi* and *S. gallinarum* do not produce gas, and other serotypes may give rise to anaerogenic variants.

### 4.1 Types of Method

Methods are given for both the qualitative and quantitative isolation of salmonellae.

## 4 Scope

### 4.2 Types of sample

Fresh waters, sea water, effluents, sewage, sludges, sediment and soil.

### 4.3 Principle

The isolation of salmonellae from these types of sample necessitates four successive stages and, where the number of these bacteria is very small as in good quality waters, a method of concentration is necessary.

4.3.1 **Concentration:** Either by means of gauze pads when sampling, or filtration of the sample through cotton wool or membranes. For quantitative isolation filter aids should be used.

4.3.2 **Pre-enrichment:** The aquatic environment is hostile to salmonellae and any present will be subjected to a degree of attenuation related to the time lapse since they were shed by the host and to adverse environmental stress. Incubation in a non-selective and non-inhibitory medium is required to overcome this attenuation.

4.3.3 **Enrichment:** Inoculation of an enrichment medium from the incubated pre-enrichment culture followed by incubation at 41°C ± 1°C or 42°C ± 1°C as appropriate (see 6.2.2).

4.3.4 **Selection:** The plating out of the enrichment culture after 18–24 hours and 40–48 hours incubation onto solid diagnostic media to produce individual colonies.

4.3.5 **Confirmation:** Sub culture of characteristic colonies to media for biochemical confirmation and serological identification.

### 4.4 Factors Affecting Isolation

In general, the longer the time between sampling and examination, the lower the rate of recovery. Therefore samples must be examined as soon as possible. Exposure of the sample to daylight will increase attenuation and may be bactericidal. It is essential that chlorinated samples should be dechlorinated at the time of collection. Toxic metals will adversely affect bacterial populations and may subsequently limit the success of cultural techniques. Competition from other organisms, in particular members of the pseudomonas group, may also reduce isolation efficiency.

### 4.5 Time Required for Test

Minimum time for a positive result 48–72 hours.
Minimum time for a confirmed negative result 96 hours.
Maximum time for a positive result normally 120 hours.

Complete serological identification of some serotypes may take several weeks, but cultures must not be kept longer than is absolutely necessary.

## 5 Sampling of Sludges, Soil, Herbage, Effluents and Natural Waters for Subsequent Analysis – Sample Collection and Preservation

### 5.1 General Principles

The sampling for the presence of salmonellae must be carried out with care. It is essential to recognise that these organisms are widespread in the environment and may thus cause chance contamination. Samples should therefore be collected only by personnel who are trained and experienced in this work.

It is essential to obtain a representative sample. Sampling equipment should be pre-sterilized before use. Provided visible contamination can be easily removed, subsequent surface sterilization using alcohol and flaming of the equipment is acceptable for its re-use.

### 5.2 Hazards

It may be necessary to take samples from sewer systems, sewage treatment works and fast flowing rivers where certain risks are hazards to the investigator may be present. These include the risk of physical injury, flooding and possible drowning, presence of pathogenic organisms, radio-activity and dangerous atmospheres such as oxygen deficiency, toxic gases and vapours, flammable and explosive gases and vapours.

Personnel engaged in sampling must make themselves familiar with safety equipment and procedures to avoid or prevent injury or infection arising from these hazards, and in the operation of gas/vapour testing equipment used to assess the quality of the atmosphere in restricted spaces. In certain situations, samplers must be accompanied (e.g. in sewers and when necessary in dangerous isolated areas) and must notify their superior officer where and when they intend to sample.

The provisions of the Health at Work Act[25], and the detailed information given in Safety in Sewers and at Sewage works[15][23] should be carefully studied and put into effect.

### 5.3 Natural Waters and Effluents

Procedures and apparatus used for the bacteriological sampling of aquatic situations as described in the fifth edition of The Bacteriological Examination of Water Supplies (Report 71) are applicable. Wide mouth borosilicate glass bottles have proved satisfactory. If the fluid being tested contains chlorine, the addition of sodium thiosulphate in appropriate concentration will be necessary to neutralize any residual chlorine. Apparatus for depth sampling is adequately described by Collins et al[16].

When water is examined for salmonellae, larger volumes of sample may be necessary compared with those required for other organisms. Where the numbers of salmonellae are low or they are suspected of being only intermittently present, the use of Moore's swabs[13] or McCoy's modification[14] is recommended (see 6.1.1.1./2). Where such swabs need longer exposure, or are placed in fast flowing waters in which they may more readily disintegrate, the use of protective purses made from 7 mm mesh Netlon plastic garden netting has proved successful[17].

## 5.4 Sewage Liquors

A separate sterile container for each sample is essential. Wide-mouth autoclavable plastic or disposable containers are suitable and are available in a range of sizes. A sampling pole and clamp facilitate the sampling operation. The use of dippers or weighted buckets is to be avoided because it is difficult to clean them adequately between samples. It is advisable to make up a composite sample from a number of sub-samples in order to obtain a more representative sample.

## 5.5 Sewage Sludge

For sludges it is especially important to obtain representative samples. The sampling procedure will depend on the type of sludge.

### 5.5.1 **Raw Sludge and Humus Sludge**

Sludge often becomes stratified into layers and efforts must be made either to mix the contents of a tank thoroughly before sampling or to obtain a range of samples from different depths or layers. Where the sludge is being transferred to a tanker or lagoon, samples should represent the material being transferred. Using a sampling pole samples should be taken in sterile containers. When samples have to be taken at greater depth, the use of sampling containers with a removable bung activated at the required depth is recommended.

### 5.5.2 **Digested Sludge**

Mixing is usually a feature of digestion and direct sampling can be undertaken from sampling taps commonly provided on digester inlet and outlet pipelines. The tap should be left running to clear the pipework of any residual material. However, the performance of tanks used for sedimentation and consolidation cannot be gauged reliably from samples taken from the inlet and outlet pipelines and depth samples may also have to be taken to give more accurate analysis.

Sampling taps should be cleaned to remove residual material when samples may be drawn directly into sterile wide mouth containers.

### 5.5.3 **Pressed Sludge Cake**

Samples should be obtained which are representative of the sludge mass. Core samples may be taken as described for soil sampling (5.6.1). A composite sample should be prepared by bulking a number of sub-samples.

## 5.6 Soil and Herbage

### 5.6.1 **Sampling Devices**

A corer of suitable design is preferable to trowels or scoops. A convenient corer readily available in the microbiology laboratory is a metal membrane filter funnel and holder. The funnel diameter of 50 mm, for example, is an appropriate size for obtaining approximately 50 g sub-samples up to 25 mm depth. To remove the core sample a suitable rod is required which can be effectively sterilized. The funnel is easily cleaned and surface-sterilized for re-use. Corers of suitable material and dimensions may be readily made.[18]

### 5.6.2 **Sampling Pattern**

The procedures recommended either in Section 8 of "Sampling and Initial Preparation of Sewage and Waterworks Sludges, Soils, Sediments and Plant Materials Prior to Analysis"[18] or in reference 24 are suitable.

### 5.6.3 Plant Material (Herbage)

For small areas, non-rusting shears may be used to harvest plant growth into sterile plastic bags. The shears can be surface-sterilized before use. For larger areas it is recommended that a composite sample be prepared. The sampling pattern in 5.6.2[18] [24] may be followed.

## 5.7 Miscellaneous Material

Techniques for a variety of materials have been developed, largely by modification of existing procedures. The following may be relevant.

### 5.7.1 Bird Droppings

Representative droppings may be collected using a sterile spatula and placed directly into pre-enrichment broth to counteract the bactericidal effect of the dejecta. The weight of the sample material may be obtained by using a container of known weight. Reweighing the container after addition of the droppings gives the weight of the sample by subtraction.

### 5.7.2 Material Adhering to the Surface of Vehicles, Plant and Tools

Soil-sludge mixtures adhering to vehicles or plant used to transport sludge may be collected using a sterile spatula or scraper and placed in sterile honey jars for examination.

Tools such as spanners may be swabbed with sterile alginate wool. The swabs can then be dissolved in Calgon Ringer's solutions (Quarter-strength Ringer's solution with 1% (w/v) Calgon) prior to examination.

## 5.8 Information to be Supplied with Samples

Source
Sampler's name or identification
Date and time sample taken
Type of material
Exact place from which sample was taken and grid reference if possible.

If from a river, stream, lake or reservoir, the depth at which the samples were taken, whether from the side or middle of the water mass, whether the water level was above or below average and whether after heavy rainfall, flooding or during drought.

If from sewage tank, digesters or lagoon, whether from the inlet or outlet pipe; the depth of sample, and whether a composite or sub-sample.

Examination required and reason why the sample was taken.

# 6 Procedures

## 6.1 Concentration Methods

### 6.1.1 Concentration in situ

In certain situations such as in sewerage systems where the presence of salmonellae may be intermittent due to discharge from a single point source, it is better to use concentration methods *in situ* than to transport large volumes or examine numerous samples. However, whilst these methods are useful in proving the presence of salmonellae they do not yield quantitative information.

#### 6.1.1.1. Moore's Swabs[13]

A Moore's swab (B9.1) is fastened with string and suspended in the flowing water or sewage for a period of 2–3 days. It is then collected in a wide-mouthed screw-capped jar and sufficient pre-enrichment medium is added to cover the swab.

#### 6.1.1.2 McCoy's Modification[14]

McCoy recommended the use of sanitary pads. The ends are tied together to form a pear-shaped swab which is used in an identical manner to Moore's swabs as in 6.1.1.1. However, they are collected in plastic bags. In the laboratory a corner is cut from the plastic bag and the fluid squeezed out into a container of pre-enrichment medium.

6.1.1.3 In investigations of sewerage systems to trace Salmonella excretors it is advisable to put the swabs in place on Fridays and collect them on Mondays as people are more likely to be using their household toilets at the weekend.

6.1.1.4 In periods of low flow in sewers or in emergencies, a gauze swab may be wiped along the sewer and subsequently cultivated. This procedure has yielded positive results.[19].

### 6.1.2 **Laboratory Concentration Methods**

#### 6.1.2.1 McCoy's Funnel Method[14]

The sample is filtered through a plug of sterile absorbent cotton wool inserted in the neck of a large sterile funnel which is covered by a sheet of sterile aluminium foil whilst in use. After filtration the cotton wool plug is placed in a container and covered with pre-enrichment medium (A4).

*NB* Non-absorbent cotton wool plugs float on the enrichment medium and should not be used.

The advantage of this method is that very large volumes, for example 20 to 30 litres, can be readily filtered. Filtration can be carried out on site and the plug transported or sent to the laboratory.

Most Probable Number counts on waters before and after such filtration have shown between 50 and 90% retention of *Eschericia coli* in the cotton wool plug.[20]

#### 6.1.2.2 Membrane Filtration

Due to the retention properties of cellulose acetate membranes it is generally only possible to filter relatively small volumes of water using standard laboratory equipment. Consequently this method is only applicable to clear waters. However, pressurized membrane filtration systems can be used to filter larger volumes of turbid waters.

After filtration, the membranes are placed in a container of pre-enrichment medium (A4).

#### 6.1.2.3 Use of Filter Aids

To overcome clogging, filter aids are used with the normal membrane filtration unit, the membrane being replaced by a Whatman No. 17 absorbent pad which retains the filter aid added to the sample.

Pour the contents of a well shaken 30 ml bottle of sterile filter aid suspension into the funnel and apply vacuum to form the initial filter aid layer.

Pour the contents of a well shaken 30 ml bottle of sterile filter aid supsension into the funnel and apply vacuum to form the initial filter aid layer.

Add the contents of a 30 ml bottle of sterile filter aid per litre to the sample, mix and filter through membrane unit, shaking gently before each addition to the filter funnel.

Place the absorbent pad plus layer of filter aid in suitable container and cover with pre-enrichment medium (A4). The filter aid will retain over 90% of the organisms present in the sample[12].

## 6.2 Qualitative Examination

6.2.1 After inoculation of the pre-enrichment medium with the sample concentrate, incubate at 37°C ± 0.5°C for 18–24 hours.

6.2.2 Inoculate enrichment media with the incubated pre-enrichment culture in the ratio of 1:100 for Rappaport medium and 1:10 for Muller-Kauffmann medium (A5 or A6). Incubate Rappaport's Broth at 41°C ± 1°C and Muller-Kauffmann's broth at 42°C ± 1°C.

6.2.3 Plate out after incubation for 18–24 and 40–48 hours if necessary (see 8.5).

## 6.3 Quantitative Examination

Where the number of salmonellae in the samples may be small, even after concentration , it is necessary to use the most probable number (MPN) method.

Measured volumes of the liquid or suspension to be tested, or of one or more dilutions of them, are added to tubes or containers of a suitable medium. These are incubated and subsequently examined to ascertain whether the organisms sought have grown in tubes inoculated with certain volumes of the sample. Provided some tubes give negative results, the most probable number of specific organisms in the original sample may be estimated from the number and distribution of tubes giving a positive result[8].

### 6.3.1 **Pre-enrichment in Buffered Peptone Water**[4] (5.4)

Re-suspend the filter aid obtained, as in 6.1.2.3, in 100 ml of pre-enrichment medium and distribute as follows: pipette 5 separate 10 ml volumes to suitable test tubes or containers to give 5 × 10 ml and leaving 1 × 50 ml. If higher counts are expected pipette 5 × 10 ml, 5 × 1 ml and 5 × 0.1 ml volumes to separate containers. Add sterile pre-enrichment medium to the small volumes to make them up to 10 ml. Incubate at 37°C ± 0.5°C for 18–24 hours. Transfer measured volumes to enrichment media to give the required inoculation ratios and incubate accordingly (6.2.2).

Plate out on selective media after incubation for 18–24 and 40–48 hours if necessary (6.2.3).

## 6.4 Isolation of Salmonellae on Selective Media

In order to confirm the presence of specific salmonellae it is necessary to isolate the organisms in pure culture for final identification. This is done by plating out the enrichment cultures, after incubation, on solid selective media on which salmonellae form characteristic colonies. However, only plating techniques which produce individual separate colonies are of use. Plating methods which do not produce individual colonies may result in 'false negative' results because characteristic colonies are overgrown.

Several solid media are available and workers should familiarize themselves with the type of growth and colonial morphology that salmonellae produce on the medium of their choice. As the selectivity of these media varies, it is necessary to combine a less selective solid medium with a highly selective enrichment medium or vice versa.

### 6.4.1 **Brilliant Green Agar**[6] (5.8)

This is a more selective medium. Colonial characteristics after 18–24 hours incubation are as follows:–

| Organism | Characteristic Appearance |
|---|---|
| Salmonellae | Red/Pink colonies surrounded by bright red medium. Lactose/sucrose fermenters are inhibited to a certain extent, but produced yellow/green colonies when growth does occur. |
| Proteus | Almost completely inhibited: those organisms that grow produce red colonies without swarming. |
| Pseudomonas | growth inhibited: small, crenated red colonies. |

### 6.4.2 **Xylose Lysine Desoxycholate Agar (XLD)**[7] (A7)

This medium was developed originally for the isolation of shigellae. However, salmonella colonies are easily distinguished from other Enterobacteriaceae by a colour reaction resulting from the decarboxylation of lysine plus blackening due to $H_2S$ production.

Colonial appearances after 18–24 hours incubation at 37°C are as follows:

| Organism | Characteristic Appearance |
|---|---|
| Salmonella, Arizona | Red colonies with a black centre (often they are more like black colonies with a red periphery). |
| Shigella<br>Proteus inconstans and<br>P. rettgeri also<br>Pseudomonas | Red colonies |
| Enterobacter, Citrobacter,<br>Escherichia, Klebsiella | Yellow colonies |
| Other Proteus<br>strains | Yellow, sometimes with black centres |

## 6.5 Biochemical Confirmation

6.5.1 Considerable laboratory experience is required to recognise the characteristic salmonella colonies on the medium of choice.

Suspect colonies are sub cultured to specific media for confirmation and to provide sufficient bacterial growth for confirmation of purity, serological tests, and identification.

6.5.2 Plates should be examined both by reflected and transmitted light using a hand lens (circa ×8 magnification) if necessary. On occasions it may be helpful to use a low magnification microscope.

6.5.3 Characteristic colonies (maximum of 5 if available) should be sub cultured with a straight wire. Tapering the end of the wire by rubbing on a whetstone, is advantageous for picking small colonies. To avoid contamination from neighbouring colonies, selected colonies should be picked by a stabbing motion, perpendicular to the plate surface. If necessary, slide agglutination for preliminary identification can be carried out at this stage (6.6.2).

6.5.4 Characteristic colonies should be inoculated into either Lysine Iron Agar Slopes (A.11) or Triple Sugar Iron Agar Slopes (A.12) and Urea Broth, (5.13) and a MacConkey Agar Plate (A.9). The straight wire carrying the organisms from the colony 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 must stop about 3 mm from the bottom of the tube as gas production may blow the medium out of the tube. Incubate overnight at 37°C.

### 6.5.4.1 Lysine Iron Agar[9]

Organisms which produce lysine decarboxylase give an alkaline reaction (purple colour) in the medium. Production of $H_2S$ results in blackening. This medium is suitable for isolating the Arizona group of organisms which can be missed if Triple Sugar Iron agar is used.

| Organism | Slant | Butt | $H_2S$ |
|---|---|---|---|
| Salmonella | Alkaline | Alkaline | + |
| Arizona | Alkaline | Alkaline | + |
| Proteus | Red | Acid | − |
| Providence | Red | Acid | − |
| Citrobacter | Alkaline | Acid | + |
| Escherichia | Alkaline | Acid or neutral | − |
| Shigella | Alkaline | Acid | − |
| Klebsiella | Alkaline | Alkaline | − |

Alkaline = Purple; Acid = Yellow; $H_2S$+ = Blackening

6.5.4.2 Triple Sugar Iron Agar and Urea Broth[10] [12]

Triple Sugar Iron agar is a composite medium for differentiating members of the Enterobacteriaceae. Unfortunately, some members of the proteus group give similar reactions to salmonellae. Although these can be differentiated by smell, it is advisable to inoculate Urea Broth with the same colony.

| Organism | Butt | Slope | $H_2S$ | Urea Broth |
|---|---|---|---|---|
| *Klebsiella spp* | AG | A | – | – |
| *Escherichia coli* | AG | A | – | – |
| *Proteus vulgaris* | AG | A | + | + |
| *Proteus morganii* | A or AG | NC or ALK | – | + |
| *Shigella spp* | A | NC or ALK | – | – |
| *Typical Salmonella* | AG | NC or ALK | + | – |
| *Salmonella cholerae-suis* | AG | NC or ALK | – | – |
| *Salmonella typhi** | A | NC or ALK | + | – |

Key: AG = Acid (yellow) and gas formation
Key: A = Acid (yellow)
NC = No change
ALK = Alkaline (red)
$H_2S$ = Blackening
Urea Broth + = red colour

* Some strains of *S. typhi* may only produce a small disc of blackening at the top of the butt. Others may not produce any blackening of the medium.

## 6.6 Serological Identification of Salmonella Serotypes

6.6.1 Whilst it is feasible to carry out slide agglutination direct from the surface of a selective plate, the amount of material available is limited to the individual colony. The adjacent colony might not be the same orgnaism. Also, if confirmation is to be limited to Polyvalent 'O' and 'H' antisera, many closely related species can and do give false-positive agglutinations with Polyvalent 'O' sera. In addition, it is often difficult to obtain 'H' agglutination with growth from the dry surface of a plate. Consequently, carry out slide agglutination tests only on cultures which give the characteristics listed in 6.5.4.1 or 6.5.4.2 as being typical of salmonellae.

6.6.2 With a short wire loop, place three evenly-spaced drops of saline on a clean microscope slide. Emulsify growth from the slope (6.5.4) in each separate drop to produce homogenous suspensions. A loopful of salmonella Polyvalent 'O' serum is mixed with the first drop of suspension and a loopful of salmonella Polyvalent 'H' serum with the second. Then gently rock the slide back and forth and examine the drops for agglutination against an illuminated background. The third drop indicates whether or not the culture is auto-agglutinating.

6.6.3 Organisms which satisfy the characteristics outlined in 6.5.4.1 or 6.5.4.2 and which also agglutinate with Polyvalent 'O' and Polyvalent 'H' sera can be regarded provisionally as salmonellae and submitted to a specialized laboratory for complete serological identification. Organisms which possess biochemical characteristics typical of salmonella but which only agglutinate with Polyvalent 'H' serum should not be discarded. They should be subjected to further investigation as they may possess antigens not present in the Polyvalent 'O' serum. Strains which give only Polyvalent 'O' agglutination should be passed through a Craigie tube[21] to enhance motility (see also 6.6.5).

6.6.4 Where the sera listed in A.15.2 are available the procedure is as follows:

6.6.4.1 Screen the isolates agglutinating with Polyvalent 'O' serum against the constituent antisera until a positive result is obtained.

6.6.4.2 Screen the isolates against Polyvalent 'H' Phase 1 and 2, and against Polyvalent 'H' Phase 2 sera.

6.6.4.3 If the culture is not in Phase 2, screen it with the Rapid Salmonella Diagnostic sera 1, 2 and 3, and read result according to the following table.

**Rapid Salmonella Diagnostic H Antisera**

| Agglutination with Antiserum 1 | 2 | 3 | Antigen Present |
|---|---|---|---|
| + | + | − | b |
| + | − | + | d |
| + | + | + | E |
| − | − | + | G |
| − | + | + | k |
| − | + | − | L |
| + | − | − | r |

The antigen E should cover all serotype types having antigen e. If E is indicated, test the culture with non-specific enx, and $enz_{15}$ antisera as E may be found in either phase.

The serotype denoted by the symbol G covers the antigens g, m and p.

Those denoted by L should include all those with the antigen l.

If the organism is in the specific phase and does not react with RSD 1, 2 and 3 sera, try a,c,i,y,z, $z_{16}$, $z_{10}$, $z_4$, $z_{23}$ and $z_{29}$.

6.6.4.4 Occasionally strains are isolated which appear to be salmonellae but fail to give 'H' agglutination. Such organisms should be cultured on moist nutrient agar slopes and further 'H' agglutinations tests carried out using the condensation liquid at the foot of the slope. If this fails, culture the organism in nutrient broth and then centrifuge. Use the deposit for 'H' agglutination and check the biochemical reactions.

NB: Non-motile salmonellae varients occur occasionally.

6.6.5 **'H' Phase Reversal**

Complete identification of a serotype requires that both phases be identified. In laboratories where many strains are isolated, Jameson's filter paper method of phase reversal[21] is recommended.

A ditch approximately 1.5 × 4.5 cm is cut in a moist nutrient agar plate. Across the ditch is placed a sterile strip of filter paper approximately 0.5 × 5 cm. In the middle of the strip is placed a loopful of the 'H' serum which agglutinated the strain. The strain is inoculated on one end of the strip and organisms in the other phase will migrate through the area of the strip containing antisera and grow around the other end. This growth is used for further slide agglutinations. The nutrient agar plate must not be too wet or organisms will migrate around the ditch. However, the medium must be moist enough to dampen the paper strip, prior to adding the loopful of antisera.

When difficulty is encountered in obtaining phase reversal using this routine method the technique described by Craigie[22] may be used although it necessitates the use of more antisera.

6.6.6 **Complete Identification and Confirmation**

This can only be carried out by specialized reference laboratories. Cultures should be submitted on nutrient agar slopes (A10).

6.6.6.1 Plate out on nutrient agar to check the purity of the culture before dispatch.

6.6.6.2 PACKAGES CONTAINING THESE ORGANISMS OR OTHER PATHOLOGICAL MATERIAL MUST COMPLY WITH POST OFFICE REGULATIONS. COPIES OF THESE REGULATIONS MAY BE OBTAINED FROM THE POST OFFICE, POSTAL HEADQUARTERS, ST. MARTIN'S LE GRAND, LONDON, EC1A 1HQ.

## 7 Disposal and Safety

7.1 All cultures, test tubes, slopes and slants should be discarded into stainless steel seamless buckets with lids prior to autoclaving at 121°C for 20 minutes.

N.B. When disposing of molten agar from autoclaved cultures pour into liberal quantities of hot water to prevent blockage of drains, etc.

7.2 The remaining sample material is best disposed of after autoclaving by means of a sluice connected directly to the foul sewer.

7.3 All pipettes should, after use, be discarded into a suitable disinfectant.

# Appendix

## A Media and Reagents

Media of a high quality are essential for the successful isolation of salmonellae; satisfactory products are available in dehydrated form from specialist suppliers. For the preparation of the media listed below the formulation and simple directions are given. When media are prepared from individual ingredients, text books in basic bacteriology should be consulted. It should be borne in mind that materials such as agar, peptones and bile salts vary in chemical content from batch to batch consequently each must be evaluated bacteriologically before use.

### A.1 Water

Glass distilled or de-ionized water is stipulated for all the media listed.

### A.2 Physiological Saline (for slide agglutination)

| | | |
|---|---|---|
| Sodium chloride | 8.5 | g |
| Water | 1000 | ml |

Bottle in convenient volumes and autoclave at 121°C for 15 minutes.

### A.3 Ringer's Solution (Quarter strength)

| | | |
|---|---|---|
| Sodium chloride | 2.25 | g |
| Potassium chloride | 0.105 | g |
| Calcium chloride | 0.12 | g |
| Sodium bicarbonate | 0.05 | g |
| Water | 1000 | ml |

Bottle in convenient volumes and autoclave at 121°C for 15 minutes.

### A.4 Buffered Peptone Water[4]

| | | |
|---|---|---|
| Peptone | 10 | g |
| Sodium chloride | 5 | g |
| Disodium hydrogen phosphate (anhydrous) | 3.5 | g |
| Potassium dihydrogen phospate (anhydrous) | 1.5 | g |
| Water | 1000 | ml |

pH 7.2

Bottle in convenient volumes and autoclave at 121°C for 15 minutes.

### A.5 Rappaport's Broth – R10[5]

#### A5.1 **Solution A**

| | | |
|---|---|---|
| Tryptone | 5 | g |
| Sodium chloride | 8 | g |
| Potassium dihydrogen phosphate (anhydrous) | 1.6 | g |
| Water to | 1000 | ml |

Dissolve the ingredients in the distilled water by boiling on the day of use.

#### A5.2 **Solution B**

| | | |
|---|---|---|
| Magnesium chloride ($MgCl_26H_2O$) | 40 | g |
| Distilled water | 100 | g |

### A5.3 **Solution C**

| | | |
|---|---|---|
| Malachite green | 0.4 | g |
| Water | 100 | ml |

### A5.4 **For use**

Add 100 ml of Solution B and 10 ml of Solution C to 1000 ml of freshly prepared Solution A.

Distribute in suitable volumes in screw capped containers and sterilize by steaming for 30 minutes. The complete medium may be stored at room temperature for at least one month without deterioration.

## A.6 Muller-Kauffman Tetrathionate Broth[6]

### A6.1 **Muller-Kauffmann Tetrathionate Broth – Base**

| | | |
|---|---|---|
| Tryptone | 7 | g |
| Soya paptone | 2.3 | g |
| Sodium chloride | 2.3 | g |
| Calcium carbonate | 25 | g |
| Sodium thiosulphate ($Na_2S_2O_35H_2O$) | 40.7 | g |
| Ox bile | 4.75 | g |
| Water | 1000 | ml |

Dissolve all ingredients except calcium carbonate. Bottle in litre volumes and add the calcium carbonate. Autoclave at 121°C for 15 minutes. Prior to use add 19 ml of iodine solution and 9.5 ml of brilliant green solution, mix and pour into sterile containers.

### A6.2 **Iodine Solution**

| | | |
|---|---|---|
| Iodine | 20 | g |
| Potassium iodide | 25 | g |
| Water to | 100 | ml |

To dissolve mix the iodine and potassium iodide in a flask and add water a little at a time. Shake vigorously. The reaction is endothermic therefore slight heating is helpful. Ensure that all the iodine does dissolve.

### A6.3 **Brilliant Green Solution**

| | | |
|---|---|---|
| Brilliant green | 0.1 | g |
| Water | 100 | |

Add the brilliant green to the distilled water and shake to dissolve the dye. Heat the solution to 100°C for 30 minutes and shake from time to time whilst cooling, to ensure that the dye has completely dissolved. Store in brown glass bottle or away from light.

### A6.4 **Preparation of Complete Medium:**

For use, add to each 100 ml of broth base exactly 1.9 ml of iodine solution and 0.95 ml of brilliant-green solution. Mix and distribute in sterile containers as required.

## A.7 Xylose Lysine Desoxycholate Agar (XLD Agar)[7]

### A7.1 **Basal Medium**

| | | |
|---|---|---|
| Agar | 12 | g |
| Lactose | 7.5 | g |
| Sucrose | 7.5 | g |
| Xylose | 3.75 | g |
| L-Lysine HC1 | 5 | g |
| Sodium chloride | 5 | g |
| Yeast extract | 3 | g |
| 1% (W/V) Phenol red | 8 | g |
| Water | 1000 | ml |

Steam to melt ingredients, bottle in appropriate volumes, autoclave at 115°C for 10 minutes.

A7.2 **Solution A**

| | | |
|---|---|---|
| Sodium thiosulphate | 34 | g |
| Ferric ammonium citrate | 4 | g |
| Water to | 100 | ml |

Dissolve over low heat. Pasteurize by heating at 60°C for 1 hour.

A.7.3 **Solution B**

| | | |
|---|---|---|
| Sodium desoxycholate | 10 | g |
| Water | 100 | ml |

Pasteurize by heating at 60°C for 1 hour.

A.7.4 **For use**

Melt the basal medium, cool to approximately 50°C, and aseptically 2.0 ml of Solution A (A.7.2) per 100 ml of base, mix gently. Using a separate pipette aseptically add 2.5 ml Solution B, (A.7.3) mix, adjust pH to 6.9 and pour plates.

A.8 Brilliant Green Agar[6]

A.8.1 **Basal Medium**

| | | |
|---|---|---|
| Meat extract | 4.0 | g |
| Peptone | 10.4 | g |
| Sodium chloride | 3.1 | g |
| Disodium hydrogen phosphate (anhydrous) | 1.0 | g |
| Sodium dihydrogen phosphate (anhydrous) | 0.6 | g |
| Agar | 12.0 | g |
| Water | 900 | ml |

Steam to dissolve and bottle in 900 ml amounts. Autoclave at 115°C for 10 minutes.

A.8.2 **Sugar and Phenol red solution**

(for 900 ml of basal medium 5.8.1).

| | | |
|---|---|---|
| Lactose | 10.4 | g |
| Sucrose | 10.4 | g |
| 1% (W/V) Phenol red | 9 | g |
| Water to | 100 | ml |

Heat at 70°C for 15 minutes, cool to about 50°C. To be used immediately.

A.8.3 **Brilliant Green Solution**

| | | |
|---|---|---|
| Brilliant green | 0.5 | g |
| Water | 100 | ml |

Prepare and store according to A.6.3.

A.8.4 **For use**

Add 0.9 ml of the brilliant green solution to the freshly prepared 100 ml sugar and phenol red solution cooled to 50°C. Add this mixture to the 900 ml molten basal medium cooled to approximately 50°C. Pour plates approximately 4 mm thick.

A.9 MacConkey Agar[8]

| | | |
|---|---|---|
| A.9.1Bile salts | 5 | g |
| Peptone | 20 | g |
| Lactose | 10 | g |
| Sodium chloride | 15 | g |
| Agar | 12 | g |
| 1% (W/V/ Neutral red | 5 | g |
| Water to | 1000 | ml |

Add peptone, sodium chloride and bile salts to the water, steam to dissolve, store overnight at 4°C, filter cold and adjust pH to 7.5. Add lactose, neutral red and agar, steam to dissolve. Dispense into screw-capped bottles and autoclave at 115°C for 15 minutes.

A.9.2 **For use**

Melt by steaming and pour approximately 15 ml per plate.

## A.10 Nutrient Agar Slopes

| | | |
|---|---|---|
| Beef extract | 10 | g |
| Peptone | 10 | g |
| Sodium Chloride | 15 | g |
| Agar | 12 | g |
| Water | 1000 | ml |

Dissolve beef extract and peptone and adjust to pH 7.3. Add agar and steam to dissolve. Mix well and add approximately 7 ml to a 15 ml bottle or approximately 4 ml to a bijou bottle. Autoclave at 115°C for 15 minutes. Allow bottles to cool in a sloping position to form an agar slope.

NB: The agar sope should not reach the neck of the bottle.

## A.11 Lysine Iron Agar[9]

| | | |
|---|---|---|
| Peptone | 5 | g |
| Yeast extract | 3 | g |
| Dextrose | 1 | g |
| L-Lysine | 10 | g |
| Ferric ammonium citrate | 0.5 | g |
| Sodium thiosulphate ($Na_2S_2O_3H_2O$) | 0.04 | g |
| 1% (W/V) in ethanol Brom-cresol purple | 2 | ml |
| Agar | 14.5 | g |
| Water to | 1000 | ml |

pH should be approximately 6.7

Steam to dissolve and dispense in 150 × 12 mm test tubes. Autoclave at 121°C for 15 minutes. Cool in a sloping position to form slopes with a deep butt.

## A.12 Triple Sugar Iron Agar[10]

| | | |
|---|---|---|
| Lab-Lemco | 3 | g |
| Yeast extract | 3 | g |
| Peptone | 20 | g |
| Sodium chloride | 5 | g |
| Lactose | 10 | g |
| Sucrose | 10 | g |
| Dextrose | 1 | g |
| Ferric citrate | 0.3 | g |
| Sodium thiosulphate ($Na_2S_2O_35H_2O$) | 0.3 | g |
| Agar | 15 | g |
| Water to | 1000 | ml |
| 0.2% (W/V) Phenol red | 12 | ml |

pH should be approximately 7.4

Steam to dissolve. Add indicator and mix. Dispense into 150 × 12 mm test tubes and autoclave at 121°C for 15 minutes. Cool in a sloping position to form a slope with a butt of approximately 30 mm.

## A.13 Urea Broth[11]

A.13.1 **Broth Base**

| | | |
|---|---|---|
| Peptone | 1 | g |
| Dextrose | 1 | g |
| Disodium hydrogen phosphate (anhydrous) | 1 | g |
| Potassium dihydrogen phosphate (anhydrous) | 0.8 | g |
| Sodium chloride | 5 | ml |
| 0.2% (W/V) Phenol red | 2 | g |
| Water to | 1000 | ml |

Dissolve the ingredients and check the pH which should be approximately 6.8.

Bottle in 95 ml volumes

Autoclave at 115°C for 15 minutes.

A.13.2 **For use**

Aseptically add 5 ml of sterile 40% (W/V) urea solution to 95 ml sterile broth base (5.13.1). Dispense aseptically in 2–3 ml volumes in sterile bijou bottles.

A.14 Filter Aid[12]

Hyflo-supercel (Hopkins and Williams) approx 1g.
Distilled water approx 15 ml
Bottle in 30 ml screw capped bottles
Autoclave at 121°C for 15 minutes
Make up in large numbers and store until required.

A.15 Salmonella Agglutinating Sera

A.15.1 **Minimum Requirements**

Polyvalent salmonella 'O' serum, groups A–G
Polyvalent 'H' Phase 1 and Phase 2 serum
Somatic antigen 'Vi' serum

A.15.2 To identify most of the commonly occurring serotypes in the UK the following sera are recommended.

Somatic antisera

Polyvalent salmonella 'O' serum, groups A–G
Sera for the individual somatic antigens 2; 3,10; 4; 6,7; 8; 9; 11; 13,22; 15; 19 and Vi

Flagellar antisera

Polyvalent 'H' serum Phase 1 and 2;
Polyvalent 'H' Phase 2 1–7
Rapid Diagnostic Sera 1, 2 and 3
Phase I a, b, c, d, E, G, i, k, L, r, y, z, $z_4z_{23}$, $z_{10}$, $z_{29}$.
Phase II 2, 5, 6, 7, x, $z_{15}$, $\overline{z_6}$.

## B Apparatus

In addition to the normal complement of glassware and other equipment in every day use in a bacteriology laboratory, the following extra items may be necessary:

B.1 **Glassware**

*Screw-capped jars* (1 lb honey jars) for sampling and culturing
*32 mm × 200 mm test tubes* with suitable caps.

B.2 **Racks** for above tubes.

B.3 **Water bath** maintained at 42°C ± 1° with racks to hold 32 mm × 200 mm tubes.

B.4 **Colworth Stomacher** with extra strong bars to homogenize samples of sludge.

B.5 **Safety type bunsen burners** to minimize aerosol dispersal.

B.6 **Seamless stainless steel buckets with lids** for the discarding of cultures and containrs prior to sterilization.

B.7 **Insulated containers** for large samples (the polystyrene containers used to transport 4 Winchesters are ideal).

B8.8 **Filtration Equipment**

B.8.1 *Large funnels*

B.8.2 *Membrane filtration units* with 500 ml funnels.

B.9 **Surgical gauze or sanitary pads** (not flushable or those that contain deodorant) to make swabs.

B.9.1 *Moore's Swabs*[13]
Made from absorbant gauze approximately 100 × 21 cms. See section C.

B.9.2 *McCoy's Modification*[14]
Sanitary pads; tie the end tapes together to form a pear shaped pad. Use as Moore's swab.

B.10 **String and/or wire and wire cutters.**

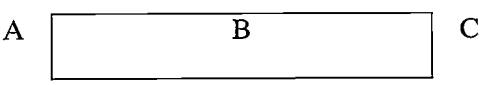
B.11 **Various sizes of bottles and plastic bags** for sampling.

B.12 **Large metal spatula or small trowel** for soil sampling.

B.13 **Blow lamp, matches and spare gas cartridge.**

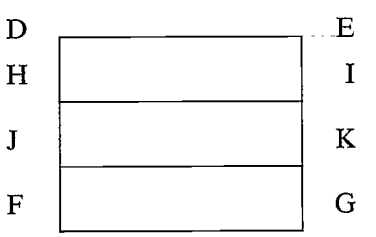
## C Preparation of a Moore's Sewer Swab

1. Take a piece of absorbent gauze approximately 1 metre × 21 cms




Fold ends A and C to centre of line B.

2. Fold in half to give rectangle approximately 25 × 21 cms.
3. Fold edge DE to line JK and edge FG to line HI to form a strip 25 × 7 cms.




4. Roll the strip up to form a tight cylinder approximately 7 × 3 cms.
5. Tie with string. Use about one metre of string and tie the swab with one end. Wrap the rest of the string round the swab.
6. Wrap in Kraft paper. Sterilize in a hot air oven for 2 hours at 180°C.

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## Address for Correspondence

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