Global Salm-Surv

A global *Salmonella* surveillance and laboratory support project of the World Health Organization

Laboratory Protocols

Level 1 Training Course

Identification of *Salmonella*

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Foreword

Despite the controls that have already been put into place, *Salmonella* infection arising from contaminated food continues to be an immense problem with millions of cases occurring annually throughout the world. In addition to the misery caused, financial loss is enormous.

Detection of *Salmonella* before contaminated foods can be consumed is therefore an essential feature of safeguarding public health and incidentally preserving the reputations and fortunes of food manufacturers and processors.

Surveillance of *Salmonella* in all the different stages of feed-food chain constitutes an important element in the exploration of epidemiology of foodborne salmonellosis, and in the development and implementation of efficient *Salmonella* control strategies.

Efficient laboratory methods for isolation, identification and typing of *Salmonella* are essential elements in *Salmonella* monitoring and control programmes. This protocol describes methods that have been extensively documented in the scientific literature, are accepted by international standardisation bodies and can be applied under most conditions and circumstances in laboratories worldwide.

It is, however, important to note the following:

There are many different procedures for isolation of *Salmonella*. The ideal method has a high sensitivity and specificity, and at the same time is simple, rapid and inexpensive. No single method fulfils all these criteria, and no single method is optimal for all purposes. Therefore, it is advisable to consult the literature before choosing a method for a particular purpose. Frequently comparing your existing methods to newer methods is also highly advisable.

In this manual we present the methods for isolation of *Salmonella* according to the ISO-6579 standard but other methods may be used. The criteria for biochemical identification of *Salmonella* are relatively standard, however the format varies. Molecular methods are increasingly being introduced as an alternative. These methods often lead to a faster diagnosis and may be simpler to conduct, but they have the disadvantage that they may be expensive.

The *Salmonella* serotyping system is probably the best phenotypic bacterial typing system ever developed. It has a high discriminatory power and provides information that has great epidemiological significance. Molecular typing methods such as PFGE can yield supplementary information, but are so far not a substitute for serotyping or micro tray agglutination, etc. Availability and cost of high quality antisera can be a problem in some countries and regions.

In a survey of national *Salmonella* reference laboratories, conducted before the launch of Global Salm-Surv, laboratories were asked about which guidelines/standards they used for antimicrobial susceptibility testing. Nearly 3 out of 4 laboratories used the NCCLS guidelines. In this light, GSS has decided to promote the use of the NCCLS guidelines, but GSS laboratories are not obligated to follow these guidelines.
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1. Isolation, identification and serotyping of *Salmonella* from faeces and food

Introduction
The following procedures will guide you through the steps necessary to isolate *Salmonella* confidently from faeces and from food, carry out a biochemical identification of *Salmonella* and serotype the strains using slide agglutination tests:

1. Isolation of *Salmonella* from food and faeces.
2. Biochemical confirmation of *Salmonella* suspect colonies (after isolation from faeces or food).
3. Serotyping of *Salmonella*.

The genus consists of two species: (1) *S. enterica* which is divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*; and (2) *S. bongori* (formerly called *S. enterica* subsp. *bongori*). The subspecies name does not need to be indicated, as only serovars of subspecies *enterica* has a name. Therefore we write e.g. *Salmonella Typhimurium*.

*Salmonella* is generally identified as being a non-lactose fermenting, (NLFs) Gram negative rod shaped organism, ranging 0.7 to 1.5 x 2 to 5 µm in size. With the exception of *S. Pullorum* and *S. Gallinarum*, they are motile with peritrichous flagellae. D-glucose is fermented with the production of acid and usually gas. Other carbohydrates usually fermented are L-arabinose, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol (except ssp VI), trehalose, D-xylose and dulcitol.

*Salmonella* is oxidase negative, catalase positive, indole and Voges Proskauer (VP) negative, methyl red and Simmons citrate positive, H₂S producing and urea negative. Some of these characteristics are used for biochemical confirmation of *Salmonella*.

Isolation of *Salmonella* from animal faeces and food

Some farm animals are infected with *Salmonella* without showing signs of the illness, i.e. they are subclinically infected. Faeces from these herds may contain *Salmonella* in low numbers. In food, *Salmonella* may also be present in low numbers in addition to a lot of other micro-organisms, and they may be injured. To diminish the risk of obtaining false negative results, a non-selective pre-enrichment of a large faeces or food sample, a combination of two selective enrichments and plating on two selective media are performed:

- Pre-enrichment in non-selective medium (buffered peptone water).
- Selective enrichment in Tetrathionate broth (Müller-Kauffmann) and Rappaport Vassiliadis soy peptone (RVS) broth.
- Subcultivation on Xylose Lysine Desoxycholate (XLD) agar and on Brilliant Green agar (BGA) (or another selective agar media).

The ISO-6579 standard is applicable to products intended for human consumption or feeding of animals. The procedures for isolation of *Salmonella* from food and animal faeces given in this protocol follow the ISO-6579: 2002 standard (ref. 1). An overview of the procedure of isolating *Salmonella* according to other standards is given in Appendix 1.
It is advisable to include an extra selective pre-enrichment medium other than RVS as this is a very selective media and may not propagate some Salmonella spp. like Salmonella Typhi and Salmonella Paratyphi. In addition, RVS broth and XLD plates are already included in the Nordic standard for detection of Salmonella in food (ref. 2). Instead of BGA plates you may use other selective agar plates, e.g. Bismuth sulphite agar, Hektoen agar, Mannitol Lysine Crystal Violet Brilliant Green agar, Desoxycholate-citrate agar or Salmonella Shigella agar (ref. 3).

The manual does not contain a method for the isolation of Salmonella from human faeces, as it is not included in the training course.

**Biochemical confirmation and serotyping of Salmonella**

Subsequently it is confirmed with biochemical tests whether the colonies resembling Salmonella on XLD and BGA are Salmonella. The ISO-6579 standard (ref. 1) recommends using the TSI agar, Urea agar (Christensen), L-lysine decarboxylase, β-galactosidase (ONPG), Voges Proskauer and Indole tests in this order. These tests are described in this manual except that the urease test is performed with urea broth instead of urea agar. (According to ref. 2 Triple Sugar Iron (TSI) agar, mannitol, urea, ornithine decarboxylase and lysine decarboxylase could be suitable to confirm Salmonella suspect colonies).

In addition, the Salmonella colonies are serotyped and classified on subspecies level. The biochemical confirmation of Salmonella and the serotyping may be performed at the same time.

**Safety**

Several countries follow the CDC/NIH biosafety recommendations indicated in "Biosafety in Microbiological and Biomedical Laboratories", 4th Edition, 1999 (ref. 4) that recommend Biosafety Level 2 practices for all the Salmonella, except S. Typhi. For S. Typhi they recommend Biosafety Level 2 practices for activities with clinical materials and cultures; and Biosafety Level 3 practices for activities that can generate aerosols or for activities involving big volumes of organisms.

Carry out all procedures in accordance with local safety codes of practice.

**References**

1.2 Identification of *Salmonella* spp.

**Materials**

**Equipment**
- Disposable inoculation loops (1 µl)
- Straight wire
- Bunsen burner
- Incubators at 37°C

**Media and reagents**
- TSI agar
- Urea broth
- L-lysine decarboxylation medium for LDC test
- LDC control medium (amino acid decarboxylation medium without lysine) for LDC test
- Sterile paraffin oil
- ONPG medium or ONPG disks for β-galactosidase test
- Tryptone/tryptophane medium for indole test
- VP medium for Voges Proskauer test
- Kovacs reagent for indole test
- Creatine solution for Voges Proskauer test
- KOH solution for Voges Proskauer test
- 1-naphthol, ethanolic solution for Voges Proskauer test

**Bacterial strains**
- *Salmonella* suspect strains on non-selective agar plates (e.g. nutrient agar)
- Reference strains for quality control of the media.

**Safety**

Carry out all procedures in accordance with the local codes of safe practice.
**Procedure**

**Day 5**
From a pure culture on nutrient agar plates, inoculate the media:

- TSI (use a straight wire to stab the butt and streak the agar surface).
- Urea broth (use a 1 µl loop full).
- L-lysine decarboxylation medium and the LDC control medium (use a 1 µl loop full) pour a layer of sterile paraffin oil above.
- ONPG medium (use a 1 µl loop full).
- VP medium (use a 1 µl loop full).
- Tryptone/tryptophane medium for indole, (use a 1 µl loop full).

Pour a layer of sterile paraffin oil above.

Incubate all biochemical tests at 37°C for 18 to 24 hours (overnight). Except for VP, which need 48h of incubation.

Perform the β-galactosidase test:
Suspend a loop full of the suspected colony in a tube containing 0.5 ml of ONPG medium and incubate at 37°C. A positive reaction might be seen already after 20 minutes, but usually the test is read after 3-4 hours or more. Or use ONPG disks as described by the manufacturer.

**Day 6: Read biochemical tests**
Add reagents to the following tests:

- **VP:** Add four drops of the creatine solution, six drops of the ethanolic solution of 1-naphthol and four drops of the potassium hydroxide solution. Shake well after addition of each reagent.

- **Indole:** Add 1 ml of the Kovacs reagent to the medium.

Read the results of the biochemical tests according to table 1 and table 2 and write the results in the record sheets to identify *Salmonella*. *Salmonella* should then be serotyped according to the following procedure.

**Theory / comments**

The creatine solution may be omitted, as it only facilitates the colour development.
Table 1. Interpretation table.
Refer to appendix 2 for a colour presentation of the reactions.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Reactions/enzymes</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>TSI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Acid production (if the butt is yellow, and the slope is red, acid production is only from glucose)</td>
<td>Butt red</td>
</tr>
<tr>
<td>TSI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Acid production from lactose and/or sucrose</td>
<td>Surface red</td>
</tr>
<tr>
<td>TSI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Gas production</td>
<td>No air bubbles in butt</td>
</tr>
<tr>
<td>TSI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;S production</td>
<td>No black colour</td>
</tr>
<tr>
<td>Urea broth</td>
<td>Urease</td>
<td>Yellow</td>
</tr>
<tr>
<td>LDC test</td>
<td>Lysine decarboxylase</td>
<td>A yellow/brown colour</td>
</tr>
<tr>
<td>ONPG</td>
<td>β-Galactosidase</td>
<td>Remain colourless</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>Acetoin production</td>
<td>Remain colourless</td>
</tr>
<tr>
<td>Indole</td>
<td>Indole production</td>
<td>Yellow ring</td>
</tr>
</tbody>
</table>

<sup>a</sup> Regarding TSI:
Read the colour of the butt and of the surface of the medium.
ALK: A red colour corresponding to no acid production
NC: No change in the colour of the medium
A: A yellow colour corresponding to acid production
G: Gas production in the butt
H<sub>2</sub>S production
+: Black colour
-: No black colour
### Table 2. Biochemical results for *Salmonella* (ref. 1).

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive or negative reaction</th>
<th>Percentage of <em>Salmonella</em> inoculations showing the reaction&lt;sup&gt;2)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSI glucose (acid formation)</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>TSI glucose (gas formation)</td>
<td>+</td>
<td>91.9&lt;sup&gt;3)&lt;/sup&gt;</td>
</tr>
<tr>
<td>TSI lactose</td>
<td>-</td>
<td>99.2&lt;sup&gt;4)&lt;/sup&gt;</td>
</tr>
<tr>
<td>TSI sucrose</td>
<td>-</td>
<td>99.5</td>
</tr>
<tr>
<td>TSI hydrogen sulfide</td>
<td>+</td>
<td>99.2</td>
</tr>
<tr>
<td>Urea splitting</td>
<td>-</td>
<td>99</td>
</tr>
<tr>
<td>Lysine decarboxylation</td>
<td>+</td>
<td>94.6&lt;sup&gt;5)&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-Galactosidase reaction</td>
<td>-</td>
<td>98.4&lt;sup&gt;5)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Voges-Proskauer reaction</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Indole reaction</td>
<td>-</td>
<td>98.9</td>
</tr>
</tbody>
</table>


2) These percentages indicate only that not all strains of *Salmonella* show the reactions marked + or -. These percentages may vary from country to country and from food product to food product.

3) *Salmonella* Typhi is anaerogenic.

4) The *Salmonella* subspecies III (Arizona) gives positive or negative lactose reactions but is always β-galactosidase positive. The *Salmonella* subspecies II gives a negative lactose reaction, but gives a positive β-galactosidase reaction. For the study of strains, it may be useful to carry out complementary biochemical tests.

5) *S*. Paratyphi A is negative.

### References

1. ISO 6579 :2002(E) 4<sup>th</sup> ed. Microbiology - General guidance on methods for the detection of *Salmonella*. International Organization for Standardization, Geneve, Switzerland

4. Composition and preparation of culture media and reagents

If no reference is given, it is the procedure used at DVL. The media and reagents are available from several companies including Oxoid, Merck and Difco. The composition of the dehydrated media given below is an example and may vary a little among the different manufacturers. Also, the media should be prepared according to the manufacturers description if it differs from the description given here. Refer to Appendix 2 for a colour presentation of growth of *Salmonella* on selective agar media and positive and negative reactions of biochemical tests.

**Creatine solution for VP test (ref. 2)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine monohydrate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Dissolve the creatine monohydrate in the water.

**Kovacs reagent for indole reaction (ref. 2)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Dimethylaminobenzaldehyde</td>
<td>5 g</td>
</tr>
<tr>
<td>Hydrochloric acid, ( \rho = 1.18 - 1.19 \text{ g/ml} )</td>
<td>25 ml</td>
</tr>
<tr>
<td>2-Methylbutan-2-ol</td>
<td>75 ml</td>
</tr>
</tbody>
</table>

**Preparation:**

Mix the components.

**LDC control medium for the LDC test**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>D(+)-glucose-Monohydrate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Lab-Lemco Powder</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Peptone &quot;Orthana&quot;</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Cresol red 0.2%</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Bromocresol purple 1.6% in 96% ethanol</td>
<td>0.63 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Preparation:**

Dissolve the chemicals in water (boiling for 2 min may be necessary). Adjust pH to 6.0, transfer into 2 ml tubes and autoclave at 121°C for 10 min.

**L-Lysine decarboxylation medium for the LDC test**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-lysine dihydrochloride</td>
<td>10.0 g</td>
</tr>
</tbody>
</table>
D(+)-glucose-Monohydrate 0.5 g  
Lab-Lemco Powder 3.0 g  
Peptone "Orthana" 5.0 g  
Cresol red 0.2% 2.5 ml  
Bromocresol purple 1.6% in 96% ethanol 0.63 ml  
Distilled water 1000 ml

Preparation:
Dissolve the chemicals in water (boiling 2 min may be necessary). Adjust pH to 6.0, transfer into 2 ml tubes and autoclave at 121°C for 10 min.

Description:
The LDC broth is used for the test of production of lysine decarboxylase. This enzyme decarboxylates lysine to yield the alkaline compound cadaverin and CO₂. A paraffin oil layer is added after inoculation to keep the pH alkaline. Often glucose is metabolised in the beginning of the incubation period and a yellow colour develops in the media after some hours of incubation, but later the media turns purple if the lysin decarboxylase is present because of formation of the alkaline compound cadaverin.

As other compounds in the media could be broken down to alkaline compounds, the LDC control media without lysine is also inoculated, a layer of paraffin oil added and it is incubated at the same time. If both the LDC media and the LDC control media turn purple, it can not be shown that lysine decarboxylase is present and the test is evaluated as negative.

1-Naphthol, ethanolic solution for VP test (ref. 2)

| 1-Naphthol | 6 g |
| Ethanol, 96 % (V/V) | 100 ml |

Dissolve the 1-naphtol in the ethanol.

ONPG medium

| Sodium dihydrogen phosphate (NaH₂PO₄) | 6.9 g |
| o-Nitrophenyl β-D-galactopyranoside (ONPG) | 2.0 g |
| Water | 1000 ml |

Adjust pH to 7.0. Dispense 0.5 ml into tubes.

Description:
This medium shows the presence of β-galactosidase producing bacteria. β-galactosidase liberates o-nitrophenol, which is yellow at alkaline pH, from ONPG. The reaction is positive if a yellow colour develops.
**Potassium hydroxide solution for VP test (ref. 2)**

Potassium hydroxide  40 g  
Water  100 ml  

Dissolve the potassium hydroxide in the water.

**Triple sugar/iron agar (TSI agar) (ref. 2)**

Meat 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  0.3 g  
Sodium thiosulfate  0.3 g  
Phenol red  0.024 g  
Agar  12 g to 18 g  
Water  1000 ml  

1) Depending on the gel strength of the agar.

**Preparation**

Dissolve the dehydrated medium in the water by heating if necessary. Adjust pH to ~ 7.4 after sterilisation, dispense into 10 ml tubes and autoclave at 121°C for 10 min. Place tubes in a sloping position to obtain a butt depth of 2.5 cm.

**Description:**

Triple-sugar iron agar is used for differentiation of *Enterobactericeae* according to their ability to ferment lactose, sucrose and glucose. The colour of the slope and the butt and gas production are noted. Acid production from fermentation of one or more of the sugars results in a yellow colour because the phenol red indicator turns yellow at low pH. Very little glucose is present in the medium, so if a bacteria, like *Salmonella*, only ferments glucose then only a little acid will be formed. On the slope, the acid will be oxidised by the air and by the breakdown of protein in the medium and the colour will remain red while the butt is yellow. H₂S production from thiosulphate will be seen as black areas in the medium due to FeS production. Air production from fermentation of sugars will be seen as air bubbles in the medium. The medium is only lightly inoculated.

**Tryptone/tryptophane medium for indole reaction (ref. 2)**

Tryptone  10 g  
Sodium chloride  5 g  
DL-Tryptophane  1 g  
Water  1000 ml
Preparation:
Dissolve tryptone and chemicals in the water at 100°C. Adjust pH to ~ 7.5 after sterilisation. Dispense 5 ml of medium into tubes and autoclave at 121°C for 15 min.

Description
The media is used for testing the liberation of indole from tryptophane. When Kovacs reagent containing amyl alcohol and p-dimethylaminobenzaldehyde is added, indole can be extracted into the amyl alcohol layer by shaking a little. Indole and p-dimethylaminobenzaldehyde produces a red/pink colour.

**Urea broth**

**Base**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1.0 g</td>
</tr>
<tr>
<td>D(+) Glucose-Monohydrat</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate (KH₂PO₄)</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Phenol red 0.2%</td>
<td>6 ml</td>
</tr>
<tr>
<td>Water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Preparation**
Dissolve the dehydrated base in the water, by heating if necessary. Adjust the pH to ~ 6.8 after sterilisation and sterilise in the autoclave at 121°C for 20 min.

**Urea solution**

Urea (e.g. no. 8486 from Merck) 20 g
Water, to a final volume of 100 ml

**Preparation**
Dissolve the urea in the water. Sterilise by filtration and check the sterility.

**Complete medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>950 ml</td>
</tr>
<tr>
<td>Urea solution</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

Prior to use, melt the base and cool it to 45°C ± 1°C, add the sterile filtered urea solution. Dispense 100 ml of medium in sterile tubes and allow it to set in a sloping position.

**Description**
Urea medium tests for high urea activity. Urea hydrolyses urea to NH₃ and CO₂

\[(\text{NH}_2)\text{CO} + \text{H}_2\text{O} \rightarrow 2 \text{NH}_3 + \text{CO}_2\]
The phenol red turns red at alkaline pH so a positive reaction is shown as the development of a red-pink colour.

**VP medium (ref. 2)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>7.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate (K$_2$HPO$_4$)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Preparation:**
Dissolve peptone and the chemicals in the water by heating if necessary. Adjust the pH to $\sim 6.9$ after sterilisation. Dispense 0.4 ml of medium into tubes and autoclave at 115°C for 20 min.

**Description:**
This is a test for acetoin production from glucose. The acetoin produced is oxidised to diacetyl, which produces a red colour with $\alpha$-naphtol at alkaline pH. A positive reaction is seen as a very pale red colour.

**References**
APPENDIX 1

Photographs of positive and negative reactions in biochemical tests of *Salmonella*.

The positive and negative control strains for the biochemical tests are indicated in brackets.

**Indol Test.**

1. Uninoculated medium.
2. Positive reaction: *Escherichia coli* (*Escherichia coli* ATCC 25922)
3. Negative reaction: *Salmonella* (*Ps. Aeruginosa* ATCC 27853)

This test is used to determine whether an organism can split indole from tryptophan. After incubation in tryptophan or peptone broth, Kovac's reagent is added.

**ONPG Test.**

The purpose of this test is to determine whether an organism has the enzyme beta-galactosidase by using the compound o-nitrophenyl-beta-D-galactopyranoside (ONPG). This test is used to differentiate between lactose-negative and lactose-delayed organisms.

ONPG is hydrolysed by the enzyme to o-nitrophenol, which is yellow, and galactose.

1. Uninoculated medium.
2. Positive reaction: *Citrobacter* (*Citrobacter freudii* ATCC 8090)
3. Negative reaction: *Salmonella* (*Salmonella enteritidis* ATCC 13076)
Lysine decarboxylase test.

Used to determine whether an organism can decarboxylate an amino acid leading to formation of an amine. Two tubes are used, one containing Møller's decarboxylase medium (control tube), and the other containing Møller's decarboxylase medium and an amino acid (lysine, ornithine or arginine). After inoculation both tubes are overlayed with sterile paraffin oil. Here lysine is used.

Glucose-fermenting organisms produce acid in the media and cause the control tube to turn yellow after incubation. Organisms that utilise the amino acid cause alkalinity due to formation of an amine. The indicators are bromcresol purple and cresol red.

1. Uninoculated medium.
2. The control tube is more yellow after incubation than an uninoculated medium
3. Positive reaction: *Salmonella. (Edwardsiella tarda ATCC 15947)*
5. Negative reaction: *Citrobacter. Here the medium is more yellow than an uninoculated medium.* *(Citrobacter freudii ATCC 8090)*

Urease test.

The purpose of this test is to determine whether an organism can split urea into ammonia and carbon dioxide by the action of the enzyme urease. Christensen's urea medium without agar is used. The production of ammonia leads to alkalinity in the medium and will cause the medium to turn red (the indicator used is phenol red).

1. Uninoculated medium.
2. Positive reaction: *Proteus. (Proteus vulgaris ATCC 8427)*
3. Negative reaction: *Salmonella. The medium is more yellow than the uninoculated medium due to acid produced during fermentation of glucose.* *(E.coli ATCC 25922)*

Voges-Proskauer test. (VP-test).

The purpose of this test is to determine whether an organism can produce acetyl-methylcarbinol (acetoin) from fermentation of glucose. The culture is incubated in Clark and Lubb's medium. Half of this medium is then used for the methyl red test and the other half for a Voges-Proskauer test. Alpha-napthol (5%) and potassium hydroxide (40%) are added to the medium. If acetoin is present the medium will turn pink-reddish in colour.

1. Uninoculated medium.
2. Positive reaction: *Klebsiella. (Klebsiella pneominae ATCC 10031)*
3. Negative reaction: *Escherichia coli. (Citrobacter freudii ATCC 8090)*
Triple Sugar Iron Agar (TSI)

The purpose of this test is to determine whether organisms can ferment glucose, sucrose and/or lactose with or without production of gas. The ability of the organism to produce hydrogen sulphide from thiosulphate in an acid environment is also tested. This test is typically used to help differentiate between groups, genera and species among *Enterobacteriaceae*. Fermentation of glucose alone will show as a yellow colour in the butt of the medium, fermentation of sucrose and/or lactose will cause both butt and slant to be yellow.

Production of hydrogen sulphide leads to blackening. Results are given as slant/butt/gas production/hydrogen sulphide production.

Bacteria that attack glucose by oxidation or not at all are often obligate aerobes and will only grow on the slant of the agar and often cause an alkaline reaction there due to the utilisation of peptones.

1. Uninoculated medium.
2. Alkaline/acid/gas/hydrogen sulphide: *Salmonella* (18 hours). The gas can be seen at the bottom of the tube. (*Salmonella enteritidis* ATCC 13076)
3. Acid/acid/gas: *Klebsiella*. (*E.coli* ATCC 25922)
4. Acid/acid: *Yersinia enterocolitica*. After 48 hours the slant of the medium appears slightly red because the bacterium produces only a small amount of acid which is oxidised from the surface of the agar. (*Ps. Aeruginosa* ATCC 9027)
5. Acid/acid/gas/hydrogen sulphide: *Citrobacter*. (*Proteus vulgaris* ATCC 13315). *Citrobacter* may also produce an Alkaline/acid/gas/hydrogen sulphide reaction which is like that of *Salmonella*, but the degree of blackening is less. The gas produced is not visible in this picture.
6. Alkaline/neutral: *Pseudomonas fluorescens*, which attacks sugars oxidatively.
Flow diagram for detection of *Salmonella*

**Non-selective enrichment**
25 g food / faeces in 10% phosphate buffer 37°C, 24 h.

**Selective enrichment**
0.1 ml in 10 ml Rappaport-Vassiliadis Soy Broth 37°C, 24 h.
1 ml in 10 ml Tetrathionate broth (Müller-Kauffman) 41.5°C, 24 h.

**Isolation**
XLD with an inoculation loop 37°C, 24 h.
BLA with an inoculation loop

**Streaking on nutrient agar** 37°C, 24 h.

**Biochemical confirmation** 37°C, 24 h.
TSI
Urea broth
LDC
ONPG
VP
Indole

**Serotyping** 37°C, overnight
O-antigens
H-antigens
Phase I 37°C, overnight
Phase II
Date: __________ Record sheet:
Initials: __________ Isolation of *Salmonella* from faeces and food.
Biochemical tests

Sample: __________

<table>
<thead>
<tr>
<th>Colour</th>
<th>Results</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>TSI Butt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSI Slope</td>
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<tr>
<td>TSI Gas</td>
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<td></td>
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<tr>
<td>TSI $H_2S$</td>
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<tr>
<td>Urea splitting</td>
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<tr>
<td>Lysine decarboxylation</td>
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<tr>
<td>$\beta$-galactosidase reaction</td>
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<tr>
<td>Voges-Proskauer reaction</td>
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<tr>
<td>Indole reaction</td>
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Overall result: _______________________________________

Sample: __________

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<tr>
<th>Colour</th>
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<td>Indole reaction</td>
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Overall result: _______________________________________
**Biochemical tests**

**Triple Sugar Iron Agar (TSI):**

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<thead>
<tr>
<th>QC-strains</th>
<th>Colour / Butt:</th>
<th>Colour / Slope:</th>
<th>Gas prod.:</th>
<th>H₂S prod.:</th>
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<tbody>
<tr>
<td><em>E. coli</em></td>
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<tr>
<td>ATCC 25922</td>
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<td><em>Proteus hauseri</em></td>
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<td>ATCC 13315</td>
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<td><em>Ps. aeruginosa</em></td>
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<td>ATCC 9027</td>
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<tr>
<td><em>Salmonella enteritidis</em></td>
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Result of QC of the medium:

**Urease test:**

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<tbody>
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<td><em>Proteus vulgaris</em></td>
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Result of QC of the medium:

**Lysine decarboxylase test:**

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<th>Colour / test-tube:</th>
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<tbody>
<tr>
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Result of QC of the medium:
**ONPG test:**

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<tbody>
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<td>ATCC 13076</td>
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<tr>
<td><em>Citrobacter freundii</em></td>
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<td>ATCC 8090</td>
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</table>

Result of QC of the medium:

**Voges-Proskauer test (VP):**

<table>
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<tbody>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
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<td>ATCC 10031</td>
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<td><em>Citrobacter freundii</em></td>
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<td>ATCC 8090</td>
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Result of QC of the medium:

**Indole test:**

<table>
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<tbody>
<tr>
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<td>ATCC 27853</td>
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Result of QC of the medium