CHAPTER 6

Isolation and Identification of *Salmonella* spp.

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Foodborne illnesses are among the most common worldwide health issues, and because of the financial costs associated with morbidity and mortality, their implications for public health are widely acknowledged. One of the most prevalent foodborne illnesses with a high zoonotic potential that can cause serious and even deadly infections in both people and animals is Salmonellosis. *Salmonella* is regarded as the primary cause of gastroenteritis and is responsible for the global outbreak of human salmonellosis. Salmonellosis, or infection with *Salmonella*, can result in enteritis, nausea, stomachaches, digestive issues, and even potentially fatal conditions like typhoid and paratyphoid fever. Human adapted *Salmonella* serotypes like Typhi and Paratyphi

result in grave systematic diseases like typhoid fever, whereas, infections with non-typhoid *Salmonella* serotypes most often lead to self-limited acute gastroenteritis.

Salmonella belongs to the Enterobacteriaceae family, characterized as a nonsporulated gram-negative bacillus. It is routinely classified by serotype, based on the expression of three types of antigens: (O) somatic, (H) flagellar and (vi) capsular, according to the Kauffmann-White scheme. This current classification scheme is based on two main Salmonella species: *S. enterica* and *S. bongori*. ***S. enterica*** *subspecies enterica*- 1435 serovars; *S. enterica subspecies salamae*- 485 serovars; *S.enterica subspecies arizonae*- 94 serovars *; S.enterica subspecies diarizonae*- 321 serovars; *S.enterica subspecies houtenae*- 96 serovars and *S.enterica subspecies indica*- 11 serovars. ***S. bongori*- 17** serovars. Based on biochemical characteristics, Salmonella is grouped into three species. ***S. choleraesuis***: Have only one serovar, and affects swine. ***S. typhi***: Have only one serovar, and affects mainly human. ***S. enteritidis***: Contain about 2000 serovars, each of which is given a species name and includes all the serovars infecting animals and human nowadays.

Salmonella is a facultative anaerobic, and oxidase-negative, usually mobile, that produces gas from glucose. Its growth temperature ranges from 7°C to 46°C, with temperature optimum ranging from 35°C to 43°C and, growth pH ranging from 3.8 to 9.5, with optimum pH between 7.0 and 7.5. *Salmonella* is catalase and methyl red positive, and indole, vogues proskauer, malonate and

urea negative. It produces hydrogen sulphide gas (H_2S) from the reduction of sulfur through cysteine desulphydrase and displays as metabolic characteristics decarboxylation capacity regarding the amino acids lysine and ornithine, nitrate to nitrite reduction and the use of citrate as the only carbon.

Isolation and identification of Salmonella

Isolation of Salmonella was performed as per as per ISO 6579-1: 2017 for seafood and soil. For water samples isolation of Salmonella was carried out as per as per ISO 19250: 2010.

Pre-enrichment in non-selective liquid medium

Each 25 g of sample (edible muscle and soil) was aseptically collected in a sterile filter bag and 225 mL of buffered peptone water was added (one to nine ratio) and homogenized using a stomacher blender for 2 min. The pre-enriched samples were incubated for 18 to 24 h at 37 ℃. For water sample, 50 ml sample is added to the same volume of double strength BPW. The samples were mixed well and incubated at 37 ℃ for 18 to 24 h.

Selective enrichment

The pre-enrichment broth (1 mL and 0.1 mL) were transferred aseptically into 10 mL of MKTTn broth and 10 mL of RVS broth, mixed, and then were incubated for 18 to 24 h at 37 ℃ and 41.5℃, respectively.

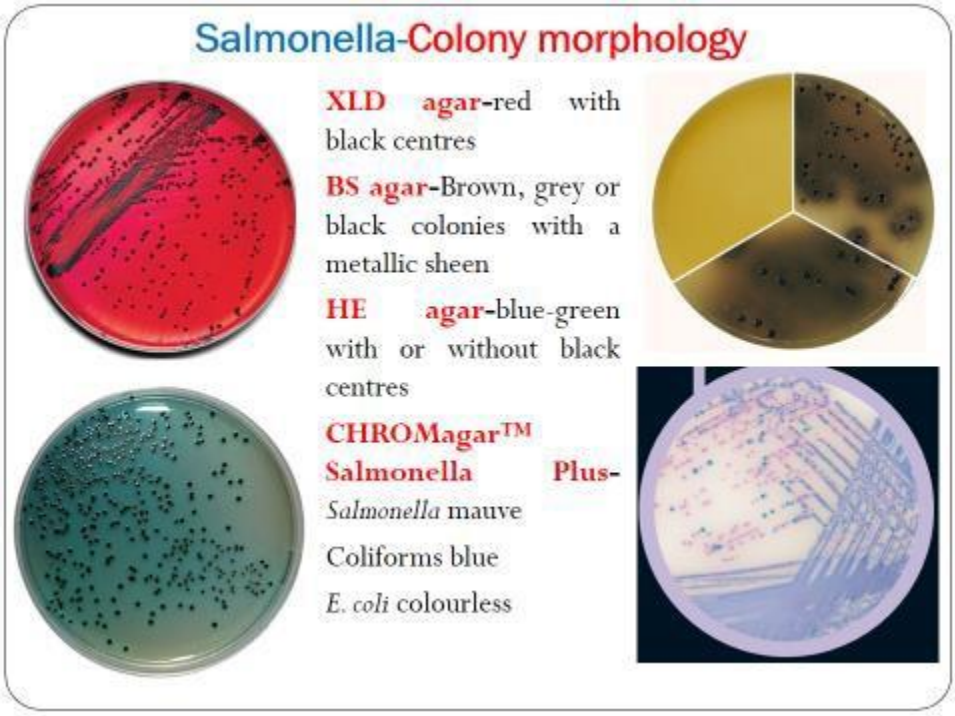
Plating out

Following incubation, a loop-full of each culture was streaked onto the surface of XLD and BSA medium and incubated at 37 ℃ for 18 to 24 h. The XLD and BSA plates were examined for the presence of Salmonella colonies. If growth is slight or if typical colonies of Salmonella were not present, the plates were re-incubated for a further 18 to 24 h and reexamined for the presence of typical Salmonella colonies. The formation of red colonies with black centers and of black colonies with a brown hallow was inspected on XLD and BSA plates, respectively.

The characteristic individual colonies were selected for subculture and confirmation. If there is no individual colony, the suspected colonies were purified by plating on to a non-selective medium. Select up to four more suspect colonies ensuring that these colonies are sub-cultured from different selective enrichment/isolation medium combinations showing suspect growth.

Streak the selected colonies onto the surface of a pre-dried non-selective agar medium in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates at 37°C for 18 to 24 h. Alternatively, if well-isolated colonies (of a pure culture) are available on the selective

plating media, the biochemical confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.



Identification of the Bacterial cultures

Culture characteristics such as colony appearance of each strain were tested according to the standard methods.

Gram's staining

Gram staining is a method that differentiates bacteria in two large group Gram positive and Gram negative. This method differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, in gram positive it is present as a thick layer. A Gram positive results in a purple/blue color while a Gram negative results in a pink/red color. It was also used to find out the morphology of bacteria (rod, cocci, spiral etc).

Catalase test

Catalase is essential for the breakdown of H_2O_2 produced during respiration. In anaerobes, H_2O_2 inhibit their growth in the presence of oxygen because catalase is absent. If few drops of 3% H_2O_2 are added to a drop of broth culture or colony, oxygen gas so released can be seen as white effervescence.

Oxidase test

During aerobic respiration, oxidase enzyme plays a vital role in the operation of electron transport system. Cytochrome oxidase catalyses the oxidation of a reduced cytochrome by molecular oxygen and results in the formation of water and hydrogen peroxide

Biochemical testing

The sub-cultured colonies were picked and inoculated into the following biochemical test tubes for confirmation:

1. **TSI (Triple Sugar Iron) test**

Streak the agar slant surface and stab the butt. Incubate at 37°C for 18 to 24 h. The majority of the typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and (in about 90 % of the cases) formation of hydrogen sulfide (blackening of the agar).

2. **Urea Test**

Streak the agar slant surface. Incubate at 37 °C for up to 24 h. If the reaction is positive, urea is hydrolyzed, liberating ammonia. This changes the colour of phenol red to rose-pink and later to deep cerise. The reaction is often apparent after 2 h to 4 h. Typical *Salmonella* cultures do not hydrolyze urea so that the colour of the urea agar will remain unchanged.

3. **L-Lysine decarboxylation (LDC) test**

Inoculate just below the surface of the liquid medium. Incubate at 37 °C for 18 to 24 h. Turbidity and a purple colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction. The majority of the typical *Salmonella* cultures show a positive reaction in LDC.

4. **Indole test**

Inoculate a tube containing tryptone/tryptophan medium with the suspected colony. Incubate at 37 °C for 24 h ± 3 h. After incubation, add 1 ml of the Kovacs reagent. The formation of a red ring (surface layer) indicates a positive reaction. A yellow-brown ring (surface layer) indicates a negative reaction.

Salmonella serovar Typhi:

A) TSI: Alkaline slant / Acid Butt / Trace H_2S / No Gas
B) Urea: Negative
C) LDM: Lysine Decarboxylase Positive
D) Indole reagent: Indole negative

Salmonella serovar Paratyphi A:

A) TSI: Alkaline slant / Acid Butt / No H2S /Gas
B) Urea: Negative
C) LDM: Lysine Decarboxylase Negative
D) Indole reagent: Indole negative

Most non-typhoidal serovars of *S. enterica*:

A) TSI: Alkaline slant / Acid Butt / H_2S Positive /Gas
B) Urea: Negative
C) LDM: Lysine Decarboxylase Positive
D) Indole reagent: Indole negative

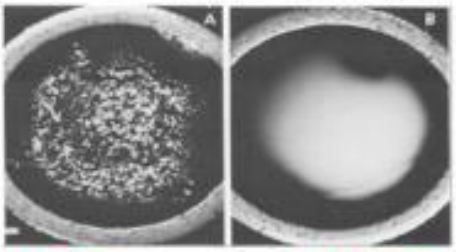
Serological testing

The pure colonies showing typical biochemical reactions for *Salmonella* are also tested for the presence of *Salmonella* O- and H-antigens by slide agglutination using polyvalent antisera. The pure colonies are cultured on a non-selective agar medium and tested for auto-agglutination. Strains that are auto-agglutinable cannot be tested for the presence of *Salmonella* antigens.

Place one drop of saline solution on a clean glass slide. Using a loop, disperse part of the colony to be tested in the saline to obtain a homogeneous and turbid suspension. Rock the slide gently for 5 s to 60 s. Observe the suspension, preferably against a dark background. If the bacteria have formed granules in the suspension, this indicates auto-agglutination and serological confirmation will become complicated. If it is non-auto-agglutinating pure colony continue the test by adding one drop of polyvalent anti-O sera in to the colony in the saline solution for examination of O-antigens. Similarly, an anti-H serum is used for the examination of H antigens. If agglutination occurs, this is considered a positive reaction in both cases.

Salmonella-Serological testing

- Salmonella O- and H- antigen; S. typhi- O- , H- and Vi- antigen by slide agglutination using polyvalent antisera
- Strains are tested for autoagglutination.



Positive Negative

Biochemical reactions	Auto-agglutination	Serological reactions	Interpretation
Typical	No	O- and H-antigens positive (and Vi positive if tested)	Strains considered to be *Salmonella*
Typical	No	O- and/or H-antigens negative	Presumptive *Salmonella*
Typical	Yes	Not tested because of auto-agglutination (see 9.5.4.2)	
No typical reactions	—	—	Not considered to be *Salmonella*

Diagram of procedure for detection of *Salmonella* in seafood and soil

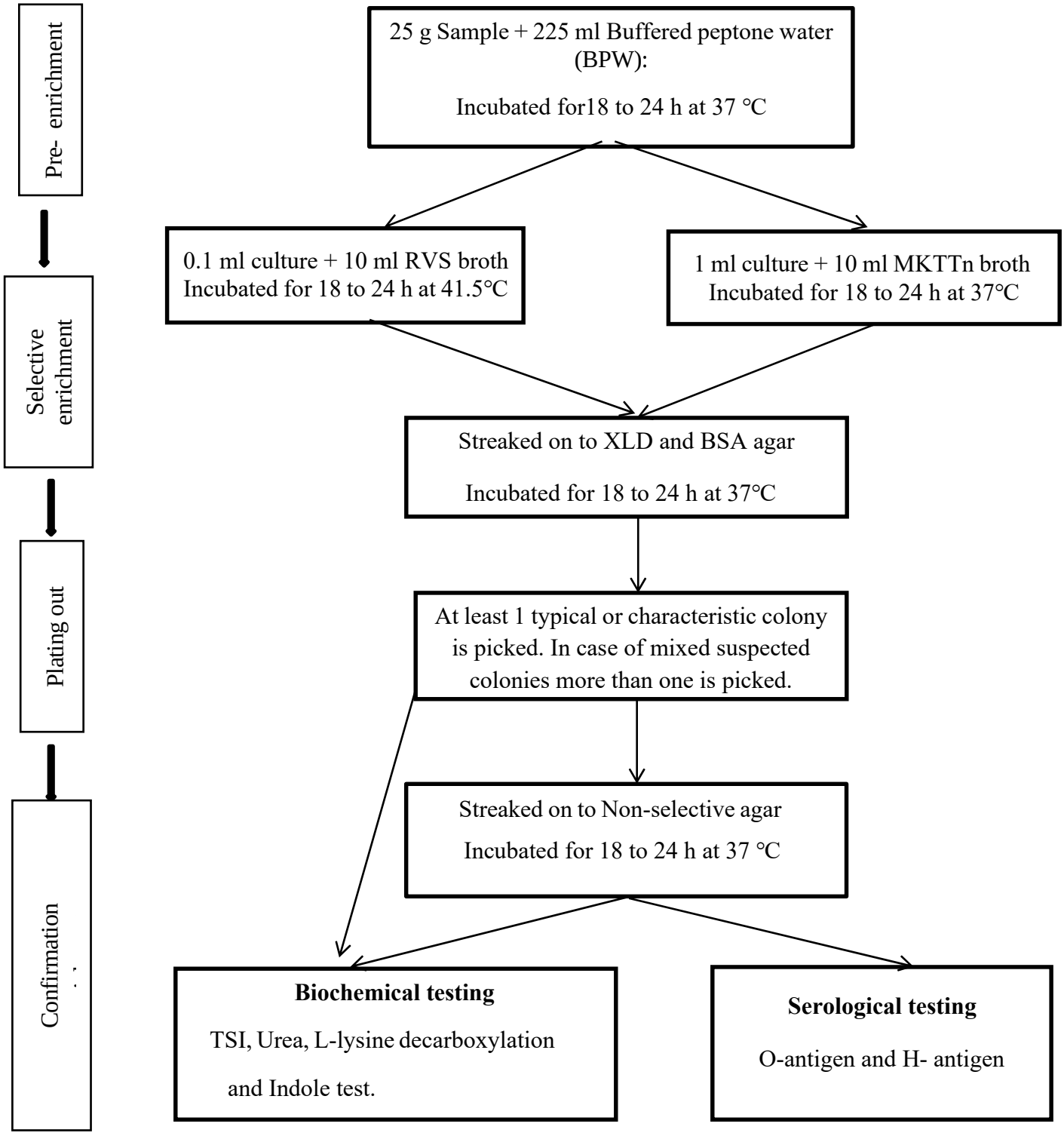


Diagram of procedure for detection of *Salmonella* in water