Chapter 5

Isolation of *Salmonella* from seafood

T. Muthulakshmi and Greeshma .S.S

Salmonella is a Gram-negative, catalase positive, oxidase negative, facultative anaerobe food borne pathogen which belongs to *Enterobacteriaceae* family. *Salmonella* was first isolated from animal intestine samples by American bacteriologist named Daniel salmon in 1800s. They normally inhabit the intestine of living organisms and compete for food supply. When the *Salmonella* count increases it cause disease collectively called as Salmonellosis. Nausea, fever, vomiting, diarrhea and abdominal cramps are the common symptoms in Salmonellosis. Salmonellosis can be caused by eating uncooked food contaminated with feces of affected warm-blooded animals.

There are more than 2200 serotypes found to exist. Salmonella serotyping is based on somatic(O) antigen, Capsular (Vi) and flagella(H) antigen. In epidemiological point of view, *Salmonella* can be divided into typhoid group, Animal group and food poisoning group. Animal group consists of the particular species adopted for an animal or bird. Food poisoning group don't have particular host preference. If affects all animals and human-being.

Salmonella are resistant to various environmental factors. They can tolerate and grow in temperature from 8-45^{0}C, water activity above 0.94, pH range of 4-8. *Salmonella* will be completely eliminated in the temperatures above 70^{0}C, but they are resistant to some extend for most of the processing methods like chilling, drying, salting, chlorine and other surface contamination cleaning method.

Isolation from seafood

Salmonella is a fastidious organism, generally the occurrence is less in seafood, so that it requires a pre-enrichment and enrichment prior to plating on selective media. Salmonella should be absent in 25 g of seafood tested

Materials required for isolation

1. Lactose broth
2. Tetrathionate broth
3. Rappaport Vassiliadis enrichment broth
4. Bismuth sulphate agar
5. Hectoen enteric agar
6. Triple sugar iron agar
7. Lysine iron agar
8. Urea agar
9. Simmons citrate agar
10. Brain heart infusion broth
11. Malonate broth
12. Trypticase soy broth
13. Sugars lactose, sucrose, Dulcitol

Pre-enrichment:

Generally, 25g of sample will be pre-enriched in 225ml Lactose broth. But for RTE(Ready to eat) products 225g sample should be pre-enriched in 2.025L of Lactose broth.

Media required: Lactose broth:

Beef extract – 3g

Peptone – 5g

Lactose – 5g

Distilled water – 1L

pH – 6.9 ± 0.1

Blend 25g of sample in 225 ml Lactose broth in stomacher bag with stomacher blender for 30 seconds. Transfer to conical flask and incubate at 37°C for 24 hours. *Salmonella* is lactose negative organism, it cannot ferment lactose to acidic products. Then also we are using lactose broth as Pre-enrichment media because all bacteria other than *Salmonella* can ferment lactose and change media pH to acidic which limits their survival. Only Salmonella can survive in acidic pH.

Selective enrichment

Tetra thionate Broth (TTB)

Tetra thionate Broth (TTB)base: 4.5 g (BD and DifCo)

Distilled water: 100ml

Dissolve properly TTB base in distilled water in boiling water bath. Do not autoclave. Check pH and add 2ml iodine solution (6.5g Iodine crystals and 5g Potassium iodide in 20ml distilled water). Mix properly and transfer 10 ml to sterile tube.

Rappaport Vassiliadis broth (RV Broth)

RV broth base: 26.6g (BD and DifCo)

Distilled water: 1L

pH: 5.1 ± 0.2

Dissolve RV broth base distilled water and transfer 10 ml to tubes. Autoclave at115° C, 10lbs for 20 minutes

USFDA advices the usage of both Tetra thionate Broth (TTB) and Rappaport-Vassiliadis media (RV) for selective enrichment of *Salmonella*. Transfer 1ml of pre-enriched lactose broth to 10 ml TTB and incubate at 37°C for 24 hours. Simultaneously transfer 0.1ml of pre-enriched lactose broth to 10 ml RV media and incubate at 42°C for 24 hours.

Selective plating

Streak one loopful culture from both enrichment media on to pre dried selective plating mediums like Hectoen Enteric Agar (HEA), Xylose Lysine Desoxycholate Agar (XLD) and Bismuth sulphite Agar (BSA). Incubate all plates at 37°C for 24 hours and select typical colonies of *Salmonella* for further confirmation.

Media preparation

Hektoen Enteric Agar (HEA)

Hektoen Enteric Agar (HEA) – 76g (BD and DifCo)

Distilled water – 1L

pH – 7.5 ± 0.2

Do not autoclave the medium

This used to differentiate between *Salmonella* and *Shigella.* HEA contains sugars like lactose, sucrose and salicin and bromo thymol blue and acid fuschin as acid/base indicators. The ferric salt in this media will act as H_2S indicator. *Salmonella* cannot ferment all the above three sugars and will appear as blue to green colour colony with or without black centre in HEA

while all other can ferment sugars and appear as yellow to orange colour colonies.

Xylose Lysine Desoxycholate Agar (XLD)

XLD : 55g(BD and DifCo)

Distilled water: 1L

pH – 7.4 ± 0.2

Do not autoclave the medium

XLD contains sugars like xylose, sucrose, lactose and lysine. Phenol red will act as the indicator and ferric ammonium citrate in the medium will act as H_2S indicator. *Salmonella* cannot ferment lactose and sucrose, but it can ferment the xylose and change the media pH to acidic. But the decarboxylation of lysine by *Salmonella* will produce a basic product called cadaverine which neutralizes the acidic pH and changes the reaction to alkaline. Hence *Salmonella* colonies on XLD will appear a s red to pink colonies with or without black center (due to H_2S production by some *Salmonella spp).* Others will ferment sugars like lactose and sucrose will appear yellow to orange coloured colonies.

Bismuth sulphite Agar (BSA)

BSA : 52g (BD and DifCo)

Distilled water: 1L

pH – 7.0± 0.2

Do not autoclave the medium

BSA contains brilliant green and Bismuth sulphite which suppress the growth of Gram positive organisms and other coliforms. This medium contains glucose as the fermenting sugar. The metallic ions like Bi++ and Fe++, present in the medium will stains salmonella colonies and the surrounding media to black/ brown colour. Typical *Salmonella* forms black to brown colonies with metallic sheen followed by a black to brown background.

Typical colonies from all plates can be picked and confirmed as *Salmonella* by various biochemical reactions, PCR and serotyping.

Table: Biochemical reactions for *Salmonella*

Sl no	Biochemical test	Result	Remarks
1	Gram staining	negative	
2	motility	motile	
3	TSI	Alkaline slant Acid butt	H_2S positive, Gas positive
4	LIA	Alkaline slant, alkaline butt,	
5	Indole	Negative	
6	Urease	Negative	
7	Glucose	Positive	
8	Lactose	Negative	
9	Sucrose	Negative	
10	Dulcitol	Positive	Acid and gas production
11	Salicin	Negative	
12	MR test	Positive	
13	VP test	Negative	
14	Lysine decarboxylase	Positive	
15	Malonate	Negative	
16	Citrate	Positive	

Protocol for the isolation of *Salmonella* from fish

25 g of Sample

(Surface tissue, gills, gut-pooled sample)

⇩

Pre enrichment- Mix 25 g of pooled sample with 225 ml of Lactose broth(pH-6.8), macerate in a stomacher blender

⇩

Incubate overnight at 37^0 C

⇩

Tranfer 1 ml of pre-enrichment to Tetrathionate broth, 0.1 ml of pre-enrichment to Rappaport vassiliadis broth

⇩

Incubate TTB in 37 ^{0} C and RV broth to 42 ^{0} C

⇩

Streak to predried HEA, BSA and XLD plates

⇩

Incubate overnight at 37^0 C

⇩

Pick typical colonies (HEA green with or without black centre, XLD pink with or without black centre, BSA black colonies)on to TSA slants Proceed for biochemical tests

Serological confirmation: *Salmonella* suspected cultures giving typical biochemical reactions are confirmed by agglutination test with *Salmonella* polyvalent antiserum in the slide with one drop of culture and anti-sera

Positive result- agglutination in text mixture, no agglutination in the saline control

Negative result- No agglutination in text mixture, no agglutination in the saline control

Nonspecific- agglutination both in test and control

****