Course Manual on

Shrimp Processing and Quality Assurance for Export

AU-Avanti Aquaculture Skill Development Centre

(AU-Avanti ASDC)

Established by Avanti Foundation

New Building, MLR Department, Andhra University
Visakhapatnam, Andhra Pradesh

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Credits

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Contents

16 Laboratory Methods for Detection of Food Borne Bacteria in Shrimp

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Bacteria are prokaryotic microscopic organisms that not only play a prominent role in causing disease to the shrimp at the hatchery and aquaculture phases but also play active role in spoilage of the shrimp meat stored under improper storage conditions. Moreover, improperly handled shrimp may be contaminated with bacterial pathogens that cause diseases to the humans who consume such contaminated shrimp.

Bacteria and are broadly categorized as Gram-positive (blue/purple colour) and Gram-negative (orange/red colour) based on their colour after being subjected to Gram staining. The important Gram-negative pathogens are *Salmonella, Shigella, Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus*, pathogenic *Escherichia coli* such as Enterotoxigenic *Escherichia coli*(ETEC), Enteropathogenic *Escherichia coli* (EPEC), Enterohemorrhagic *Escherichia coli* (EHEC), Enteroinvasive *Escherichia coli* (EIEC) etc. The important Gram-positive pathogens are *Staphylococcus aureus, Listeria monocytogenes, Clostridium botulinum Bacillus cereus* etc.

Shrimp meat is generally subjected to three main types of bacteriological analysis viz.,

- Aerobic Plate Count (to know the total number of bacteria present in each gram of shrimp meat)
- Faecal indicator bacteria (to estimate the numbers of faecal indicator bacteria such as *Faecal Coliforms, E. coli* in the shrimp meat)
- Pathogens (to detect the presence of human pathogenic bacteria such as *Salmonella, Vibrio cholerae, Listeria monocytogenes, Staphylococcus aureus* in shrimp meat)
- The analyst must aseptically collect representative samples to obtain meaningful results. Established sampling plans / procedures must be strictly adhered to as the results based on these small samples will be used to interpret the condition of large consignments. Always use sterile, leak proof containers to collect the samples. Properly label the sample container. Deliver the samples to the laboratory in iced condition (less than 4 °C) as early as possible. Frozen samples should be kept frozen (-18 °C) at all the times and refrigerated samples (less than 4 °C) should not be frozen.

The methods commonly used for the detection of bacteria are based on

- Morphology and Biochemical methods: based on appearance and presence of enzymes (Gram staining, IMViC tests etc).
- Serological Methods: based on antigen-antibody reaction (Serum agglutination test, Latex agglutination test, Lateral flow immunoassay, ELISA etc)
- Nucleic acid based methods: based on differences in the genome / genes (DNA probes, PCR, RT-PCR etc)

1. Aerobic Plate Count (APC)

Aerobic plate count (APC) is the first analysis that is performed to estimate the number of bacteria in shrimp (solid sample), water and ice (liquid sample) and worker's hand, stainless steel tables, floor (surface samples). Other names for APC are Total Plate Count (TPC), Total Bacterial Count (TBC), Standard Plate Count (SPC). Pour plate method is commonly used to estimate the APC.

1.1. APC of shrimp sample:

- Aseptically collect the shrimp and cut them into small pieces using sterile scissors.

- Weigh 50 grams in a sterile petri dish.
- Transfer the 50 g of meat to a stomacher bag and homogenize with 450 mL of diluent (Butterfields phosphate-buffered dilution water) using a stomacher blender.
- The resultant homogenized mixture is 1:10 dilution or 10^{-1} dilution which means that 1 gram of food sample is present in 10mL of the homogenized mixture.
- Pipette 10 mL from the above 10^{-1} dilution to 90mL of sterile diluent and mix well. This gives 1:100 or 10^{-2} dilution which means that 1 gram of food sample is present in 100mL of the homogenized material.
- Pipette 10 mL from the above 10^{-2} dilution to 90mL of sterile diluent and mix well. This gives 1 : 1000 or 10^{-3} dilution which means that 1 gram of food sample is present in 1000mL of the homogenized material.
- Similarly further dilutions (10^{-4}, 10^{-5} etc) can be made depending upon the anticipated microbial load.
- Arrange six petri plates in 3 rows in duplicate. Label the plates appropriately (indicating the product name/code, media, date and dilution).
- Pipette 1mL from the respective dilution (use 10^{-2}, 10^{-3}, 10^{-4} dilutions) and transfer to two petri plates (1ml each).
- Add approximately 12 to 15 mL of molten and cooled (45 ± 1°C) Plate Count Agar (PCA) to each plate.
- Immediately mix the sample dilution and agar medium thoroughly and uniformly rotating the plate thrice in the clockwise direction and thrice in the anti-clockwise direction on a flat level surface.
- Allow the agar to set (leave the plates undisturbed for 15 minutes).
- Invert the solidified agar plates and incubate at 35 ± 1°C for 48 ± 2 hours.

1.2. Controls:

Include media control, diluent control and air controls to assess the sterilization process and the suitably of the work area for bacteriological analysis.

- For media control, pour 12 to 15 mL of molten and cooled PCA in a sterile petri plate.
- For diluent control, transfer 1 mL of sterile Butterfields phosphate-buffered dilution water to a sterile petri plate and add 12 to 15 mL of molten and cooled PCA.
- For air control, leave an empty sterile petri plate open on the work table during the period of analysis for 15 minutes and add 12 to 15 mL of molten and cooled PCA. Allow the agar to set (leave the plates undisturbed for 15 minutes), invert the solidified agar plates and incubate at 35 ± 1°C for 48 ± 2 hours.

1.3. Counting of colonies and recording result:

- After the desired incubation period, the colonies developed in each plate are counted using a Qubec colony counter.
- Count all colonies including those of pinpoint size.
- The colony counts of duplicate plates within the same dilution should agree within 10% limit and counts between different dilutions should agree decimally.
- Record the results of each plate dilution wise as shown below.

Dilution	Petri Plate 1	Petri Plate 2
10^{-2}		
10^{-3}		
10^{-4}		

1.4. Calculating APC:

- Select only those plates containing 25 to 250 colonies per plate for calculation purpose.
- Plates with colony counts outside the normal 25 to 250 range may give erroneous indication of the actual bacterial population of the shrimp/fish sample.
- In plates with counts of less than 25, the dilution factors may exaggerate low counts giving very high counts.
- In crowded plates with more than 250 colonies, it may be difficult to count or may inhibit the growth of some bacteria due to limitation of nutrients thus giving very low counts.

APC is calculated using the following formula

$$N = \frac{\Sigma C}{[(1 \times n_1) + (0.1 \times n_2)] \times d}$$

Where,

N = Number of colony forming units per gram of the sample (cfu/g)

ΣC = sum of all the colonies on the selected plates (plates with 25 to 250 colonies)

n_1 = number of plates in the first dilution counted

n_2 = number of plates in the second dilution counted

d = dilution from which the first counts were obtained.

2. Most Probable Number (MPN) method for estimation of Coliforms, faecal coliforms and E. coli in shrimp samples

Coliforms, faecal coliforms and *E. coli* are used as faecal indicators and their presence indicates poor sanitary condition in the shrimp processing environment. MPN method can be used to detect coliforms and *E. coli* in shrimp samples provided the expected numbers of bacteria are less than 100 per gram; otherwise, the results may not be meaningful. MPN is only a statistical approximation of the test bacteria in the given sample and not the actual number. From the number and distribution of positive and negative reactions, the MPN of the test bacteria in the shrimp sample can be estimated by reference to statistical tables (MPN tables).

Coliforms are Gram-negative, facultatively anaerobic bacteria. They produce gas from glucose and ferment lactose to acid and gas within 48 hours at 35 ± 0.5°C. The coliform group includes species from the genera *Escherichia, Klebsiella, Enterobacter and Citrobacter*. Faecal coliforms are defined as coliforms that ferment lactose in EC medium with gas production within 48 hours at 44.5 ± 0.2°C, hence they are referred as thermotolerant coliforms.

- Prepare 10^{-1} (0.1 g per ml), 10^{-2} (0.01g per ml) and 10^{-3} dilutions of the shrimp using Butterfields phosphate-buffered dilution water as diluent. The method for preparing dilutions was previously described in the Aerobic Plate Count section (1.1).

2.1. Step I: Test for Total Coliforms:

- Media: Autoclave Lauryl tryptose broth (LST broth), 10ml each in 9 test tubes; place inverted Durham's tube in each tube.
- Arrange LST tubes in triplicate sets in a test tube stand. Inoculate 1 mL aliquots (0.1g) from 10^{-1} dilution into 3 LST tubes. Label the tubes as 0.1g.
- Similarly from 10^{-2} dilution, inoculate 1 mL (0.01g) into three LST tubes and label them as 0.01g.
- Likewise from 10-3 dilution, inoculate 1mL (0.001 g) into three LST tubes and label them as 0.001 g.
- Incubate all the LST tubes at 35± 0.5°C.
- Examine tubes after 24 ± 2 hours for gas production. If gas bubble is noticed in inverted Durham's tube, note the reaction as positive.
- Note the result as number of positives in each set of 0.1 g, 0.01 g and 0.001 g tubes.
- Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 3 hours.
- Perform confirmed test on all presumptive positive (gas) tubes.

2.2. Step II: Confirmed Test for Coliforms:

- Media: Autoclave Brilliant Green Lactose Bile Broth (BGLB 2%), 5 ml each in test tubes; place inverted Durham's tube in each tube
- The number of BGLB tubes required depends on the number of positive LST tubes in Step I.
- Inoculate loopful of culture from the positive LST tubes to BGLB broth and mark the corresponding label (either 0.1 g or 0.01 g or 0.001 g).
- Incubate BGLB tubes at 35°C± 0.5°C.
- Examine for gas production after 48 ± 3 hours of incubation.
- Note the result as number of positives in each set of 0.1 g, 0.01 g and 0.001 g tubes.
- Calculate MPN per gram from the 3 tube MPN table (Table for 3 tubes each at 0.1 g, 0.01 g and 0.001 g).
- Give the result as Coliforms MPN per gram.

2.3. Step III: Test for Faecal Coliforms:

- Media: Autoclave EC Broth, 5 ml each in test tubes; place inverted Durham's tube in each tube.
- The number of EC tubes required depends on the number of positive BGLB tubes in Step II.
- Inoculate loopful of culture from the positive BGLB broth to EC broth and mark the corresponding label (either 0.1 g or 0.01 g or 0.001 g).
- Incubate EC tubes at 44.5 ± 0.2°C.
- Examine tubes after 24 ± 2 hours for gas production. If gas bubble is noticed in inverted Durham's tube, note the reaction as positive.
- Note the result as number of positives in each set of 0.1 g, 0.01 g and 0.001 g tubes.

Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 2 hours.

- Note the result as number of positives in each set of 0.1 g, 0.01 g and 0.001 g tubes.
- Calculate MPN per gram from the 3 tube MPN table (Table for 3 tubes each at 0.1 g, 0.01 g and 0.001 g).
- Give the result as Faecal Coliforms MPN per gram.

2.4. Step IV: Test for E.coli:

- Media: Levine's Eosin Methylene Blue (L-EMB) agar, Nutrient agar slants, Tryptone broth (5 ml in test tubes), MR-VP broth (5 ml in test tubes), Koser's citrate (5 ml in test tubes) or Simmons citrate agar slants.
- Melt EMB agar, cool to 45°C, pour 12 to 15 mL in sterile plates and allow the agar to set.
- Dry the plates in a laminar flow chamber for 45 minutes.
- Streak loopful of culture from the positive EC broth tubes on EMB agar plate and mark the corresponding label (either 0.1 g or 0.01 g or 0.001 g).
- Incubate EMB agar plates tubes at 35°C± 0.5°C for 18-24 hours.
- Observe the EMB agar plates for *E. coli* colonies.
- *E. coli* colonies appear with a greenish metallic sheen by reflected light and dark centered flat colonies by transmitted light.
- Pick suspicious colonies and transfer to PCA slants and incubate at 35 ± 0.5° °C for 18-24 hours.
- Label the nutrient agar slants appropriately (0.1 g, 0.01 g and 0.001 g).
- Perform Gram stain. All the cultures appearing as Gram-negative short rods are further tested by performing IMViC tests (Indole test, Methyl red test, Voges-Proskauer test and Citrate test).
- Indole test: Inoculate the suspected *E. coli* culture from PCA slant into tryptone broth and incubate at 35 ± 0.5°C for 24 ± 2 hours. Test for the presence of indole by adding 0.2 to 0.3 mL of Kovac's indole reagent. Appearance of pink colour in the upper layer (pink ring) is positive. Yellow colour is negative.
- Methyl red test: Inoculate the suspected *E. coli* culture from PCA slant into MRVP broth and incubate at 35 ± 0.5°C for 48± 2 hours. Add 5 drops of methyl red reagent indicator to each tube. Appearance of distinct red colour is positive. Yellow colour is negative.
- Voges-Proskauer test: Inoculate the suspected *E. coli* culture from PCA slant into MR-VP broth and incubate at 35± 0.5°C for 48 ± 2 hours. To 1mL of the incubated culture add 0.6 ml of α-naphthol solution (0.25g α-naphthol in 5ml ethyl alcohol) and 0.2 ml of 40% KOH (2g KOH in 5ml distilled water). Add a few crystals of creatine. Shake and let it stand for 2 hours. Development of Eosin pink colour indicates a positive test.
- Citrate test: Inoculate the suspected *E. coli* culture from PCA slant into Koser's citrate media tubes (inoculate lightly to avoid detectable turbidity) or streak on Simmon's citrate agar slants and 35±0.5°C for 96 hours. Development of distinct turbidity in Koser's citrate medium or development of blue colour in Simmons citrate agar indicates a positive test.
- The cultures that are Indole positive, Methyl red test positive, Voges-Proskauer test negative, and citrate test negative i.e., IMViC pattern ++--are confirmed as *E.coli* (biotype 1). *E. coli* biotype 2 gives -+-- IMViC pattern.
- Note the result as number of positives in each set of 0.1 g, 0.01 g and 0.001 g labeled EMB agar plates.
- Calculate MPN per gram from the 3 tube MPN table (Table for 3 tubes each at 0.1g, 0.01g and 0.001g).
- Give the result as *E.coli* MPN per gram.

2.5. Three Tube MPN Table for food samples (For 3 tubes each at 0.1 g, 0.01 g and 0.001 g)

Number of Tubes giving a Positive Reaction			
3 x 0.1 g	3 x 0.01 g	3 x 0.001 g	MPN per gram
0	0	0	< 3.0
0	0	1	3.0
0	1	0	3.0
0	1	1	6.1
0	2	0	6.2
0	3	0	9.4
1	0	0	3.6
1	0	1	7.2
1	0	2	11
1	1	0	7.4
1	1	1	11
1	2	0	11
1	2	1	15
1	3	0	16
2	0	0	9.2
2	0	1	14
2	0	2	20
2	1	0	15
2	1	1	20
2	1	2	27
2	2	0	21
2	2	1	28
2	2	2	35
2	3	0	29
2	3	1	36
3	0	0	23
3	0	1	38
3	0	2	64
3	1	0	43
3	1	1	75
3	1	2	120
3	1	3	160
3	2	0	93
3	2	1	150
3	2	2	210
3	2	3	290
3	3	0	240
3	3	1	460
3	3	2	1100
3	3	3	>1100

2.6. Determining coliforms employing solid media

- Media : Violet Red Bile Agar (VRBA).
- Prepare 10^{-1} dilution of the shrimp sample as described in Aerobic Plate Count method.
- Transfer 1 mL aliquots from 10^{-1} dilution to two sterile petri plates (1 ml to each plate).
- Pour 10 mL of VRBA cooled to 48°C into plates, swirl plates to mix, and leave them undisturbed for 15 minutes to allow the agar to solidify.
- Overlay with 5 mL of molten and cooled VRBA to prevent surface growth and spreading of colonies.
- Allow the agar to solidify.
- Invert solidified plates and incubate at 35°C for 18-24 h.
- Examine plates under magnifying lens and with illumination.
- Counting of Coliforms: Count purple-red colonies that are 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids.
- Confirmation test for coliforms. To confirm that the colonies are coliforms, pick at least 10 representative colonies and transfer each to a tube of BGLB broth. Incubate tubes at 35°C. Examine at 24 and 48 h for gas production. All cultures that produce gas are confirmed as Coliforms.

Note: If gas-positive BGLB tube shows a pellicle, perform Gram stain to ensure that gas production was not due to Gram-positive, lactose-fermenting bacilli.

3. Pathogens: The presence of these bacteria in shrimp meat causes serious infections to human beings

3.1. Salmonella:

Salmonella is a motile, non-spore forming, Gram negative, rod-shaped bacterium. The genus *Salmonella* is divided into two species that can cause illness in humans viz., *S. enterica* and *S. bongori*. *Salmonella enterica*, which is of the greatest public health concern, is comprised of six subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), *S. enterica* subsp. *indica* (VI). *Salmonella* is further subdivided into serotypes, based on the Kaufmann-White typing scheme which differentiates *Salmonella* strains by their surface (O) and flagellar (H) antigenic properties. S. *enterica* subsp. *enterica* is divided into numerous serotypes. Salmonella causes typhoid disease which is characterized by high fever, diarrhoea, aches, headache, and lethargy (drowsiness or sluggishness) and up to 10% of people who don't get treatment may die. Typhoid fever is caused by serotypes *S. Typhi* and

S. Paratyphi. Salmonella spreads through the fecal-oral route.

Sources: Intestinal tracts of vertebrates, including livestock, wildlife, domestic pets, and humans, and may also live in environments such as pond-water sediment, farm-irrigation water, soil and insects, factory equipment, hands, and kitchen surfaces and utensils. Cross contamination of shrimp when a food handler does not adequately clean utensils, surfaces, equipment, and hands after they have come into contact with contaminated products. Cross contamination may occur at any point in the shrimp processing unit.

Sample plan for Raw frozen shrimps: Raw frozen shrimps fall under Food Category III. Aseptically draw 15 analytical units each weighing 25 grams from 15 different sample units (weighing minimum 100 g). Composite the 25 g x 15 samples as a single 375 g composite sample.

Analysis of shrimp meat for Salmonella

- Aseptically cut and weigh 25 g of shrimp sample and put it into sterile stomacher bag. Homogenize with add 225 mL sterile lactose broth using a stomacher blender. (For US consignments: Homogenize the 375 g of sample with 3375 mL of lactose broth and proceed further for Salmonella testing.
- Aseptically transfer homogenized mixture back into the 500 mL flask (For 375 g sample use 5000 mL flask) and allow it to stand for 60 ± 5 minutes at room temperature.
- Mix well by swirling and determine pH. Adjust pH to 6.8 ± 0.2 if necessary.
- Incubate the sample mixture at 35°C for 24 ± 2 hours.
- Transfer 1 mL to 10 mL of Tetrathionate broth (TTB). Mix well
- Transfer 0.1 mL to 10 mL of Rappaport-Vassiliadis (RV) medium. Mix well.
- Incubate TT broth at 35 ± 2°C for 24 ± 2 h in a water bath
- Incubate RV medium at 42 ± 0.2°C for 24 ± 2 h in a water bath
- Mix and streak a loopful of growth from TTB on Bismuth Sulfite agar (BSA), Hektoen enteric

Table : 1 Sampling plan for Salmonella (USFDA-BAM)

	Food Category I.	Food Category II	Food Category III
Definition	Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption and are intended for consumption by the aged, the infirm, and infants	Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption	Foods that would normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption
Analytical unit	Weight of one **Analytical unit = 25 g**; Take 25 g analytical unit from **100 g sample unit**.		
Number of analytical units to be tested for each food category	60 analytical units	30 analytical units	15 analytical units
Composite unit	To reduce the analytical workload, the analytical units may be composited. Individual 25g Analytical Units may be combined as **375 g Composite Unit**. The maximum size of a composite unit is 375 g or 15 analytical units		
Minimum number of composite units to be tested for each food category	**4 composite units**	**2 composite units**	**1 composite unit**

agar (HEA) and Xylose Lysine desoxycholate (XLD) agar. Incubate plates at 35°C for 24 ± 2 h

- Mix and streak a loopful of growth from RV medium on Bismuth Sulfite agar (BSA), Hektoen enteric agar (HEA) and Xylose Lysine desoxycholate (XLDA). Incubate plates at 35°C for 24 ± 2 h.
- Examine all the plates for presence of Salmonella colonies
- **Salmonella typical colony** appearance on **Bismuth Sulfite agar (BSA):** Brown, gray, or black colonies ; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation
- **Salmonella typical colony** appearance on **Xylose Lysine Desoxycholate (XLDA)**: opaque/ yellow, pink or red with or without black centres
- **Salmonella typical colony** appearance on **Hektoen enteric agar (HEA)**: Blue-green to blue colonies with or without black centres.
- Pick 2 or more colonies of *Salmonella* from each selective agar. Re-incubate BS agar plates for an additional 24 ± 2 h. Pick 2 or more typical colonies, if present, from the BS agar plates.

Note: In the absence of typical or suspicious Salmonella colonies, search for ***Atypical Salmonella colonies*** *as follows:*

- ***HEA:*** *yellow colonies with or without black centres on HE*
- ***XLD agars:*** *yellow colonies with or without black centres.*
- ***BS agar:*** *Green colonies with little or no darkening of the surrounding medium*
- Lightly touch the very centre of the colony to be picked with sterile inoculating needle and inoculate TSI (Triple Sugar Iron) slant by streaking slant and stabbing butt
- Without flaming, inoculate LIA (Lysine Iron Agar) slant by stabbing butt twice and then streaking slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm).
- Incubate TSI and LIA slants at 35°C for 24 ± 2 h.
- **TSI**: Alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar) in TSI.
- **LIA**: alkaline (purple) reaction in butt of tube. Consider only distinct yellow in butt of tube as acidic (negative) reaction. Do not eliminate cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce H_2S in LIA.
- Retain presumed positive cultures
- ***Note***: All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates. Cultures that give an acid butt in LIA and an alkaline slant and acid butt in TSI should also be considered potential *Salmonella* isolates.

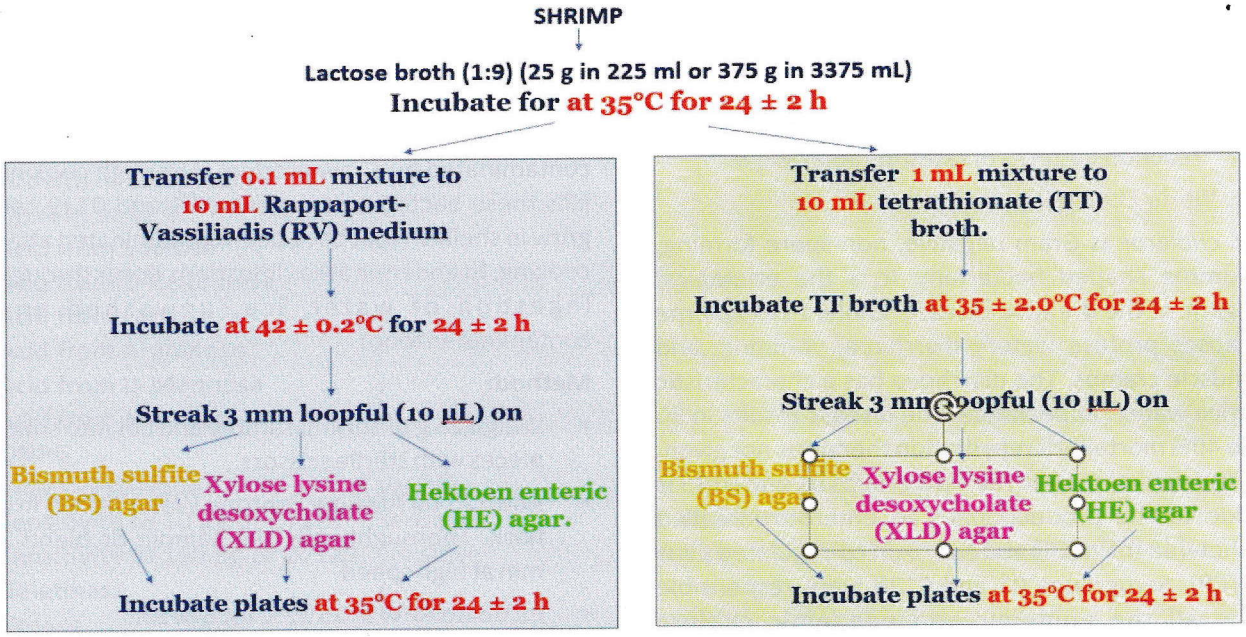

Table : 2: Biochemical and serological reactions of *Salmonella*

Biochemical test	*Test Result*		*Reaction result for Salmonella species*
	Positive test result	***Negative test result***	
TSI	*blackening Butt*	*non blackening*	Yellow Butt
LIA	*blackening Butt*	*blackening Butt*	*Purple Butt*
Urease	*Purple-red colour*	*No colour change*	*No colour change*
Lysine decarboxylase broth	purple color	yellow color	purple color
Phenol red dulcitol broth	yellow color and/or gas	no gas; no color change	yellow color and/or gas (*)
Phenol red lactose broth	yellow color and/or gas	no gas; no color change	no gas; no color change (**)
Phenol red sucrose broth	yellow color and/or gas	no gas; no color change	no gas; no color change
Malonate broth	blue color	no color change	no color change (**)
Indole test	violet color at surface	yellow color at surface	yellow color at surface
Methyl red test	diffuse red color	diffuse yellow color	diffuse red color
Voges-Proskauer test	pink-to-red color	no color change	no color change
Simmons citrate	growth; blue color	no growth; no color change	variable
Polyvalent flagellar test	agglutination	no agglutination	agglutination
Polyvalent somatic test	agglutination	no agglutination	agglutination

* Majority of *S. arizonae* cultures are negative
** Majority of *S. arizonae* cultures are positive

Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella*.

- Streak TSI agar cultures on MacConkey agar or HEA or XLD agar to obtain pure culture. Incubate plates 24 ± 2 h at 35°C. Examine plates for presence of *Salmonella colonies*.
- Transfer single isolated colony to Nutrient Agar slant
- Perform biochemical and serological identification tests
- Classify, as *Salmonella*, cultures that have reaction patterns of listed in Table below.

4. Vibrio cholerae

V. cholerae is Gram negative, non-spore forming, comma shaped bacterium. It is the etiological agent of the dreaded disease 'cholera'. *V.cholerae* causes profuse watery diarrhoea, vomiting, and muscle cramps. The diarrhoea has a characteristic "rice water" appearance. The disease results in 30 to 40% mortality if rehydration therapy is not given. Virulence of *V. cholerae* sero groups 01 and 0139 is due to the production of an enterotoxin called cholerae toxin (CT) and the toxin co-regulated pilus (TCP). Serogroups 01 and 0139 were responsible for all the epidemic and pandemic cholera outbreaks. Non-01 non-0139 serogroups of *Vibrio cholerae* typically do not produce cholera toxin (CT), but can cause diarrhea, stomach cramps, fever, nausea, and/or vomiting, which usually go away by themselves in about a week. *V. cholerae* is excreted in great numbers in the faeces of cholera patients and convalescents. The disease is transmitted primarily by the faecal-oral route, indirectly through contaminated water supplies.

Sources: Cholera infections have been associated with wide variety of seafoods such as oysters, mussels, clams, crab, lobster, shrimp, squid and finfish. Raw, improperly cooked seafood, or cross contaminated by a raw product. Although cooking kills these bacteria, sero groups 01 and 0139 can grow in shellfish that have been contaminated after cooking. In endemic areas infections occur through ingestion of water, ice, unwashed and contaminated food.

Method:

- Weigh 25 g of shrimp sample and cut into small pieces with sterile scissors.
- Add 225 ml Alkaline Peptone Water (APW) in a flask. Thoroughly mix the sample or blend 2 min at high speed.
- Incubate APW at 35 ±2°C for 6 to 8 h.

- Transfer a 3-mm loopful from the surface pellicle of APW culture to the surface of a dried Thiosulfate citrate bile salts sucrose (TCBS) plate and incubate the TCBS plate overnight (18 – 24 hours) at 35°±2°C [Note: May also streak on polymyxin colistin (mCPC) or Cellobiose colistin (CC) agars; Incubate mCPC and CC plates overnight at 35-37°C.
- Re-incubate the flask overnight if the sample had been processed
- Streak on TCBS agar and incubate overnight at 35°±2°C (Also streak on mCPC and CC agar and incubate overnight at 35 – 37 °C).

Table 3: Biochemical and serological reactions of *V. cholerae*

Test	***V. cholerae* reaction**
Arginine Glucose Slant	Alkaline (purple) slant, Acid Butt (Yellow) No gas or H_2S is produced. Arginine is not hydrolyzed
Salt tolerance	Grow without salt (T1N0 medium)
String Test	Positive (DNA strings when a loopful is lifted from the slide)
Oxidase reaction	Positive (Dark purple colour develops within 10 seconds)
Serological Agglutination test	01 serogroups shows strong agglutination using polyvalent *V. cholerae* 01 antiserum. 0139serogroups show agglutination with 0139 antiserum Non 01 and Non 0139 serogroups do not show agglutination with 01 and 0139 antisera.

Table 4 : Biochemical characteristics of human pathogenic Vibrionaceae commonly encountered in seafood

	V. cholerae	***V. parahaemolyticus***	***V. vulnificus***
TCBS agar	Yellow	Green	Green
mCPC agar	Purple	No Growth	Yellow
CC agar	Purple	No Growth	Yellow
AGS	KA (Slant alkaline/ Butt slightly acidic)	KA (Slant alkaline / Butt acidic)	KA (Slant alkaline / Butt acidic)
Oxidase	+	+	+
Arginine dihydolase	-	-	-
Lysine decarboxylase	+	+	+
Ornithine decarboxylase	+	+	+
Growth in 0% NaCl	+	-	-
Growth in 3% NaCl	+	+	+
Growth in 6% NaCl	-	+	+
Growth in 8% NaCl	-	+	-
Growth in 10% NaCl	-	-	-
Growth at 42°C	+	+	+
Acid from Sucrose	+	-	-
Acid from D-Cellobiose	-	Variable	+
Acid from Lactose	-	-	+
Acid from Arabinose	-	+	-
Acid from D-Mannose	+	+	+
Acid from Mannitol	+	+	Variable
ONPG	+	-	+
Voges - Proskauer	Variable	-	-
Sensitivity to 10 µg of O/129	Sensitive	Resistant	Sensitive
Sensitivity to 150 µg of O/129	Sensitive	Sensitive	Sensitive
Gelatinase	+	+	+
Urease	-	Variable	-

- ***V. cholerae* typical colony appearance on TCBS agar**: Large (2 to 3 mm), yellow coloured colonies and slightly flattened with opaque centers and translucent peripheries.
- *V. cholerae* typical colony appearance on mCPC / CC agar: green to purple in colour, with a purple background

5. Vibrio parahaemolyticus

Gram-negative, curve-shaped rod frequently isolated from the estuarine and marine environments. It is a halophilic bacterium and requires at least 0.5% NaCl in all media, and 2% NaCl is optimal. *V. parahaemolyticus* is highly susceptible to low pH, freezing, and cooking. *V. parahaemolyticus* isolates from the environment are usually non-pathogenic. The pathogenic strains of *V. parahaemolyticus* are identified by the presence of one or both of the hemolysins TDH (thermostable direct hemolysin) and TRH (thermostable-related hemolysin). Diarrhea caused by *V. parahaemolyticus* is usually self-limiting.

- **Sources**: Consumption of raw or improperly cooked oysters, fin-fish, squid, octopus, lobster, shrimp, crab and clams.
- Three analytical schemes for enumerating *V. parahaemolyticus* are available. The first is the MPN procedure commonly used by many laboratories. The second is a membrane filtration procedure using hydrophobic grid membrane filter (HGMF). The third is a direct plating method using DNA probes for identification of the total *V. parahaemolyticus* population and pathogenic (TDH containing) strains.
- **MPN Method for enumerating *V. parahaemolyticus***
- Aseptically cut and weigh 50 g of shrimp sample (use the entire animal if possible; if it is too large, select the central portion including gill and gut).
- Add 450 ml Phosphate Buffered Saline (PBS dilution) water and blend for 1 min. This constitutes the 1:10 dilution.
- Similarly prepare 1:100 in PBS by transferring 10 mL from 1:10 dilution to 90 mL of sterile PBS.
- Inoculate 10 mL of 1:10 dilution to 3 tubes (10 ml to each tube) containing 10 mL of double strength APW. (This represents the 1 g portion)
- Inoculate 1 mL of 1:10 dilution to 3 tubes (1 mL to each tube) containing 10 mL of single strength APW. (This represents the 0.1 g portion)
- Inoculate 1 mL of 1:100 dilution to 3 tubes (1 mL to each tube) containing 10 mL of single strength APW. (This represents the 0.01 g portion)
- Incubate all the 9 APW tubes overnight at 35 ±2°C.
- Streak a 3-mm loopful from the top 1 cm of all the APW tubes showing growth onto TCBS agar (and mCPC or CC agars for *V. vulnificus* isolation).
- Incubate TCBS plates overnight at 35 ±2°C (and mCPC or CC plates overnight at 35-37°C).
- *V. parahaemolyticus* typical colony appearance on TCBS agar: Round, opaque, green or bluish colonies of 2 to 3 mm in diameter. Interfering *V. alginolyticus* colonies are large and yellow in colour.
- Most strains of *V. parahaemolyticus* will not grow on mCPC and CC agars.
- Purify colonies by streaking on a non-selective agar (T_1N_3, or TSA-2% NaCl agar), incubate overnight at 35 ±2° C and process for biochemical identification.
- **Biochemical identification of *V. parahaemolyticus* isolates**: The biochemical reactions of *V. parahaemolyticus* and *V. vulnificus* are listed in the *Table:* Biochemical characteristics of human pathogenic *Vibrionaceae* commonly encountered in seafood provided under the subhead *V. cholerae*. Unless otherwise specified, all media used for the detection of *V. parahaemolyticus* and *V. vulnificus* must contain 2% or 3% NaCl. *V. parahaemolyticus* and *V. vulnificus* produce green colour colonies on TCBS agar. They can be differentiated based on ONPG, salt tolerance, cellobiose, arabinose and lactose reactions.
- Based on the biochemical reactions confirm the isolate as *V. parahaemolyticus*. Note the result as number of positives in each set of 1g, 0.1g and 0.01g tubes. Calculate MPN per gram from the 3 tube MPN table (Table for 3 tubes each at 1 g, 0.1 g and 0.01 g). Give the result as *V. parahaemolyticus* MPN per gram.

MPN Index for various combinations of Positive Tubes in a 3-Tube Dilution Series using Inoculum quantities of 1, 0.1 and 0.01 g

1 g	0.1 g	0.01 g	MPN per gram
0	0	0	<0.3
0	0	1	0.3
0	1	0	0.3
0	1	1	0.61
0	2	0	0.62
0	3	0	0.94
1	0	0	0.36
1	0	1	0.72
1	0	2	1.1
1	1	0	0.74
1	1	1	1.1
1	2	0	1.1
1	2	1	1.5
1	3	0	1.6
2	0	0	0.92
2	0	1	1.4
2	0	2	2.0
2	1	0	1.5
2	1	1	2.0
2	1	2	2.7
2	2	0	2.1
2	2	1	2.8
2	2	2	3.5
2	3	0	2.9
2	3	1	3.6
3	0	0	2.3
3	0	1	3.8
3	0	2	6.4
3	1	0	4.3
3	1	1	7.5
3	1	2	12
3	1	3	16
3	2	0	9.3
3	2	1	15
3	2	2	21
3	2	3	29
3	3	0	24
3	3	1	46
3	3	2	110
3	3	3	> 110

6. Staphylococcus aureus

S. aureus is a gram-positive, catalase-positive, cocci (spherical bacterium) which appears as bunch of grape-like clusters under microscopic examination. Human intoxication is caused by enterotoxins produced by some strains of *S. aureus*. *S. aureus* is present everywhere and impossible to eradicate from the environment. Staphylococci exists in air, dust, sewage, water, milk, food, food equipment, environmental surfaces, humans and animals. Staphylococci are present in the nasal passages, throat, hair and skin of humans. *S. aureus* can survive for extended periods in a dry state and can grow at low levels of water activity (as low as 0.83) and are tolerant to salts and sugars. *S. aureus* is predominantly associated with staphylococcal food poisoning. However, *S. aureus* is easily destroyed by heat treatment and most of the sanitizing agents. Therefore, the presence of *S. aureus* in processed shrimp or shrimp processing equipment is considered as an indication of poor sanitation.

Direct plate method for enumeration of *Staphylococcus aureus*:

- Melt sterile Baird-Parker (BP) agar, cool to 45-50 °C and add the required quantity of egg yolk and potassium tellurite. Pour the BP agar into petri plates and allow them to set. Dry the BP plates in laminar flow chamber for 45 minutes.
- Aseptically cut and weigh 50 g of shrimp sample
- Homogenize with 450 ml of Butterfields phosphate-buffered dilution water using a stomacher blender. This is 10^{-1} dilution.
- Aseptically transfer 1 ml from the 10^{-1} dilution to three pre-dried baird-parker agar plates (0.4 ml in first plate, 0.3 ml to the second plate and 0.3 ml to the last plate).
- Spread the inoculums over the surface of the agar using sterile bent glass rod. Keep the plates undisturbed, in up-right position for 30 minutes to allow the inoculums to be absorbed by the agar.
- Invert the plates and incubate at 35 – 37 °C for 45 – 48 hours.
- **Typical appearance of *S. aureus* colonies:** Circular, smooth, convex, moist, 2-3 mm in diameter, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone. Colonies have buttery to gummy consistency when touched with inoculating needle.

- *S. aureus* isolated from frozen foods that have been stored for long periods develop colonies with less black colouration and have a rough appearance.
- Count number of *S. aureus* colonies of all the three plates and record counts
- Test the *S. aureus* colonies for coagulase production
- Add the number of colonies giving positive coagulase test and multiply by the sample dilution factor.
- Report this number as number of *S. aureus*.
- ***Coagulase test:*** Coagulase plasma is used to qualitatively determine the pathogenicity of Staphylococci using the direct tube method. Coagulase plasma, rabbit with ethylenediaminetetraacetate (0.15%) and sodium chloride (0.85%) is used for this test. The coagulase plasma is reconstituted using sterile distilled water as per the manufacturer's instructions.
- Transfer suspect *S. aureus* colonies into BHI broth and incubate at 35 – 37 °C for 18-24 hours.
- Take 0.5 ml of reconstituted coagulase plasma with EDTA into a small test tube.
- Add two drops of the BHI culture and mix thoroughly.
- Incubate at 35 – 37 °C and examine periodically over 6 h period for clot formation.
- Only firm and complete clot (4+) that stays in place when tube is tilted or inverted is considered positive for *S. aureus*.
- Partial clotting (2+, 3+) must be tested further by performing Gram's staining, catalase test, anaerobic utilization of glucose, anaerobic utilization of mannitol and thermostable nuclease production.

Table :5 Typical characteristics of *S. aureus*

Characteristic	Reaction
Catalase test	+
Coagulase production	+
Thermonuclease production	+
Anaerobic utilization of glucose	+
Mannitol	+

- **Most probable number (MPN) method** is recommended for products in which small numbers of *S. aureus* are expected and in foods that contain large numbers of competing species.

7. Listeria monocytogenes

L. monocytogenes is a Gram-positive, motile bacterium that can survive below 1 °C and can grow at low temperatures (less than 3 °C) making it an important organism in seafood industry. *L. monocytogenes* can be found in moist environment and is persistent in food-manufacturing environments. This bacterium causes two forms of disease. The first form manifests as non-invasive gastrointestinal illness and the infected persons suffer from nausea, fever, diarrhoea and is self-limiting. The second form manifests as a serious invasive illness that causes septicaemia, meningitis, encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (2 nd/3 rd trimester) or stillbirth.

Sources: Food workers, food contact surfaces, incoming air, raw materials, and food -processing environment.

Method:

- Aseptically cut and weigh 25 g of shrimp sample
- Homogenize with 225 ml of Buffered Listeria Enrichment Broth (BLEB)
- Incubate at 30°C for 4 hours.
- Aseptically add sterile selective supplements (acriflavine 10 mg / mL, nalidixic acid 40 mg / mL, cycloheximide 50 mg / mL).
- Continue incubation at 30° C for a 24 hours and 48 h.
- At 24 hours and 4 hours streak BLEB enrichments on one esculin based agar (Eg. OXA or PALCAM or MOX or LPM fortified with esculin and Fe^{3+}) and one chromogenic selective agar [CHROMagar *Listeria* or R&F *Listeria monocytogenes* Chromogenic Plating Medium (R&F LMCPM) or RAPID' *L. mono* or Agar *Listeria* according to Ottaviani and Agosti (ALOA) or Oxoid Chromogenic *Listeria* agar (OCLA).
- Incubate OXA, PALCAM or MOX plates at 35° C for 24-48 h; fortified LPM plates at 30° C for 24-48 hours.
- Incubate R&F LMCPM at 35° C; RAPID' *L. mono,* ALOA / OCLA, and CHROM agar at 37 °C.
- **Typical colony appearance of *Listeria* spp., on Oxford agar (OXA):** After 24 hours of incubation *Listeria* species colonies are approximately 1 mm diameter, gray to black colonies surrounded by a black halo. Following

48 hours of incubation *Listeria* species colonies are approximately 2-3 mm diameter, black with a black halo and sunken centre.

- **Typical colony appearance of Listeria on PALCAM agar**: Incubation conditions and appearance of *Listeria* species colonies are the same as for Oxford agar except that the background plate color is red.
- **Typical colony appearance of Listeria on Modified Oxford Agar:** *Listeria* species colonies are the same as for Oxford agar
- **Typical colony appearance of Listeria on LPM agar fortified with Esculin and Fe:** *Listeria* species colony appearance is similar to Oxford agar.
- **Typical colony appearance of Listeria on R&F *Listeria monocytogenes* Chromogenic Plating Medium (R&F LMCPM):** *L. monocytogenes* and *L. ivanovii* produce a 1-3 mm diameter, blue/green colony and small blue/green halo. All other *Listeria* species produce a 1-2 mm, smooth, convex white colony with no halo.
- **Typical colony appearance of Listeria on RAPID' *L. mono*:** *L. monocytogenes* and *L. ivanovii* produce a 1-3 mm diameter, blue/green colony in the red background of RAPID' *L. mono* agar. Additionally, a yellow halo will surround *L. ivanovii* colonies. All other *Listeria* species produce a 1-2 mm, white colony with or without a yellow halo.
- **Typical colony appearance of Listeria on Agar *Listeria* according to Ottaviani and Agosti (ALOA) or Oxoid Chromogenic *Listeria* agar (OCLA):** All *Listeria* species appear as 1-3 mm diameter blue/green colonies. Additionally, *L.monocytogenes* and *L.ivanovii* have an opaque white halo surrounding the colony.
- **Typical colony appearance of Listeria on CHROM agar**: Appearance of *Listeria* colonies are the same as for ALOA except that the background plate color is light blue.
- Select up to 5 typical colonies from each esculin based agar and streak for purity to TSAYE and incubate plates at 30°C for 24 to 48 hours.
- Select up to 2 typical colonies for streaking if using differential chromogenic agars. The plates may be incubated at 35°C.
- If isolated colonies are available use remaining colony growth to stab a 5% sheep blood agar plate. Incubate at 35 °C for 24-48 h. Examine for a clear zone of haemolysis. *L. monocytogenes* is beta haemolytic but yields a very small zone. So examine for haemolysis beneath the colonies. Alternatively inoculate a thick layered blood agar plate so that haemolysis will be visible around the line of the stab.

Biochemical tests for identification of Listeria:

- Gm stain: All *Listeria* spp. are short, Gram-positive rods. Use 16 to 24 h growth
- Motility: *Listeria* is motile, giving a typical umbrella-like growth pattern (Incubate for up to 7 days at 20-25° C).
- Catalase: *Listeria* species are catalase-positive. Gas evolution is slow, observe under microscope.
- Carbohydrate fermentation: All *Listeria* species are positive for dextrose, esculin, and maltose. All *Listeria* spp. except *L. grayi* should be mannitol-negative.
- **CAMP test (Christie-Atkins-Munch-Peterson test)**: A thin layer of sheep blood agar (5% v/v in nutrient agar) is poured on the surface of nutrient agar base plate and dried before use. Streak *Staphylococcus aureus* (NCTC 1803) and *Rhodococcus equi* (NCTC 1621) across the sheep blood agar plate. Test cultures of Listeria are streaked at right angles to *S. aureus* and *R. equi* so that the cultures are at their closest about 1-2 mm apart. Incubate at 37°C overnight. Positive result is indicated by an enhanced zone of haemolysis between the two cultures.

Table : 6 :Identification of different Listeria species:

	Mannitol	Rhamnose	Xylose	Virulence	β-haemolysis	Haemolysis enhancement with *S. aureus*	Haemolysis enhancement with *R. equi*
L.monocytogenes	-	+	-	+	+	+	-
L. ivanovii	-	-	+	+	+	-	+
L. innocua	-	V[a]	-	-	-	-	-
L. welshimeri	-	V	+	-	-	-	-
L. seeligeri	-	-	+	-	+[b]	+	-
L. grayi	+	V	-	-	-		

[a] V, variable biotypes, greater than 10% of strains for this trait

[b] Weakly hemolytic *L. seeligeri* strains may appear non-hemolytic