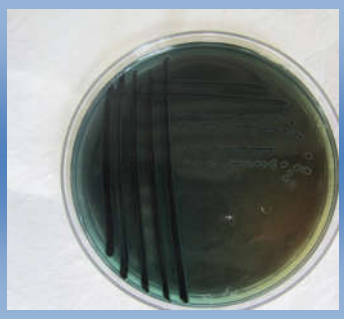
Training Manual

Microbiological methods and HACCP concepts for Seafood Industry



2018

ICAR-Central Institute of fisheries technology (ICAR-CIFT)
Visakhapatnam Research centre

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Microbiological Methods and HACCP concepts for Seafood industry

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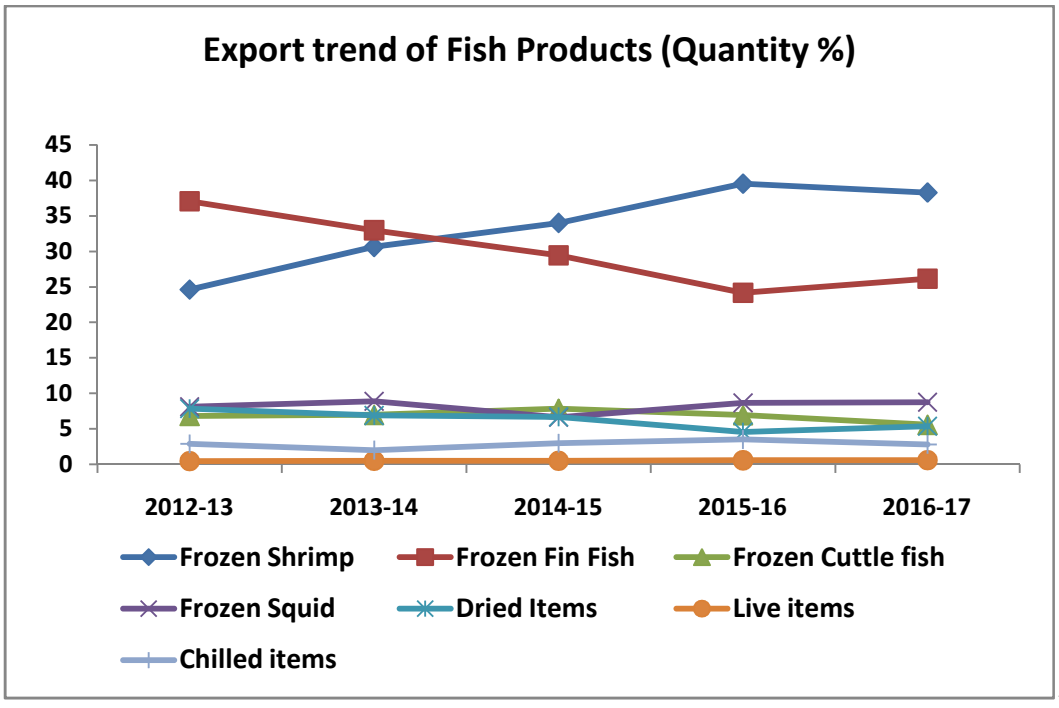
TABLE OF CONTENTS

1. INTRODUCTION

Role of fisheries in Indian economy:

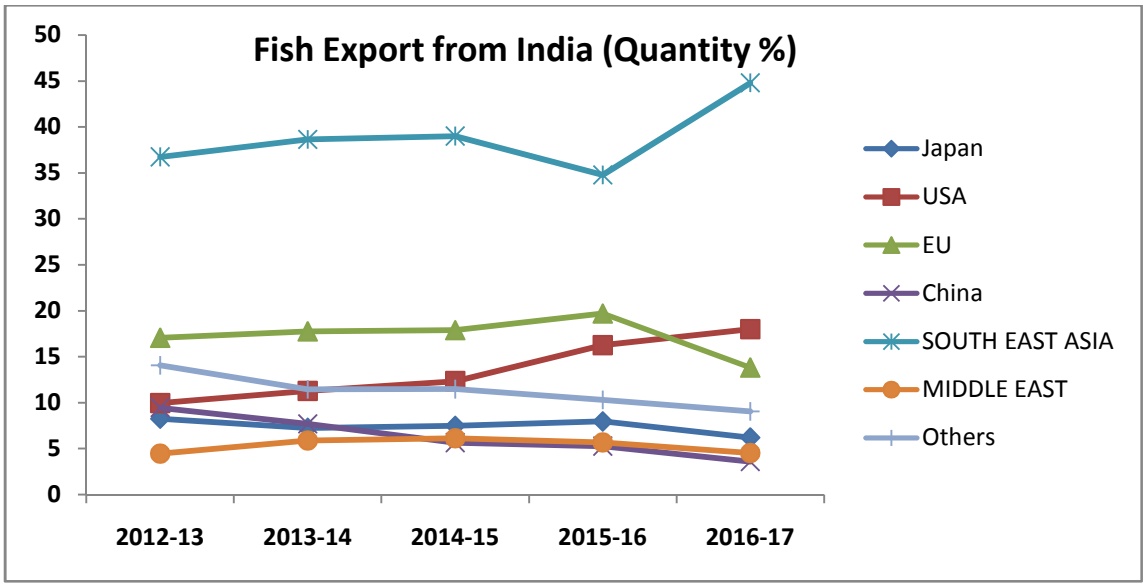
Fisheries sector has been steadily contributing to the Indian economy and is poised to attain greater heights in the recent future. Fisheries contribute 1.0% of GDP and 5.3% of Agricultural GDP. Fisheries forms 18% of agricultural export and has shown an annual growth rate of 4.5% in the last five year plan period. Indian marine exports witnessed impressive growth from 37,175 tonnes in 1970 to 13,77,244 tonnes in 2017-18. In terms of value the increase was from Rs 35.54 crores in 1970 to Rs. 45,107 crores in 2017-18. These exports have generated valuable foreign exchange which increased from US $ 47.38 million (1970) to US $ 7,082 million (2017-18).

In 2017-18, frozen shrimp is the major export value item accounting for a share of 41% in quantity and 68.4% of the total US $ earnings in 2017-18. The quantity of frozen shrimp exported was 5,65,980 tonnes which earned Rs. 30,868 crores (US $ 4,848 million).Frozen fin fish was the second major item in terms of quantity (25.6%; 3,53,192 tonnes) and Value (10.4%; 4,674 crores). Other major seafood products exported were, frozen cuttle fish, frozen squid, dried fish, live fish and chilled fish.



Quantity wise, in 2016-17, South East Asia was the major market for India fish exports (6,16,707 tonnes) followed by USA (2,47,780 tonnes) and European Union (1,90,314 tonnes). Other export markets for India fish exports were Japan, Middle East and China.

	Quantity (Tonnes)	Value (Rs Crores)
Japan	85,651	2,846.3
USA	**2,47,780**	**14,769.83**
EU	1,90,314	7,115.96
China	49,701	1,448.03
SOUTH EAST ASIA	**6,16,707**	**14,250.26**
MIDDLE EAST	62,220	1,849.1
Others	1,24,871	2,827.4
Total	**13,77,244**	**45,106.89**



The export of *L. vannamei* shrimp has shown tremendous growth from 91,171 MT in 2012-13 and reached 3,29,766 tonnes in 2016-17. Andhra Pradesh was the leading state in shrimp production followed by Tamil Nadu and Gujarat.

Implications of quality in domestic and international seafood trade:

Consumers' prime concern is safe food. Microorganisms are one of the most clearly identified acute risks to fish consumers. Infections due to pathogenic bacteria, enteroviruses, fungi and biogenic amine poisoning are the most significant microorganism associated illnesses. Fish consumers succumb to illness due to ingestion of preformed microbial toxins (*Clostridium botulinum*, *Bacillus cereus*, *Staphylococcus aureus*) or by ingestion of sufficient number of viable pathogenic bacteria (*Listeria*, *Salmonella*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Shigella*) or by microbial decarboxylation of histidine to the allergic histamine. Microbial hazards enter the fish either due to human/animal faecal pollution, from microorganisms autochthonous to the aquatic environment or as a result of growth of microorganisms due to improper post-harvest handling. Quality is perceived differently by consumers, processors, regulatory officials and scientists. All over the world, consumers insist on fresh, nutritious and health food. The quality of a product may be defined as its measurement against a standard regarded as excellent at a particular price which is satisfactory both to the producer and the consumer. Quality can be measured in terms of the senses, chemical composition, physical properties and microbial flora. Strict quality guidelines have been put in place for sale of fish and fishery products in the domestic (Food Safety Standards Authority of India; Bureau of Indian Standards) and international markets (Export Inspection Council of India, European Union Directives, specific requirements of each importing country). The quality requirements set are either the complete absence of the particular bacteria or a maximum allowed number per gram. Only those fish and fishery products that meet the quality requirements of the importing country are allowed to enter that particular country. Rejections have a negative effect on exports both in terms of monetary loss and tarnish the brand image of the country's fishery products.

Rapid Alert System for Food and Feed (RASFF) of the European Commission:

RASFF of the EU is a key tool that is used to avert food safety risks in food and feed before they can harm consumers.. The RASFF was put in place by the European Commission to provide food and feed control authorities with an effective tool to exchange information about measures taken responding to serious risks detected in relation to food or feed. An 'alert notification' or 'alert' is sent when a food or a feed presenting a serious risk is on the market or when rapid

action is required. Alerts are triggered by the member of the network that detects the problem and has initiated the relevant measures, such as withdrawal/recall. The notification aims at giving all the members of the network the information to verify whether the concerned product is on their market, so that they can take the necessary measures. Products subject to an alert notification have been withdrawn or are in the process of being withdrawn from the market.

Quality issues raised by the European Union in fish exported from India:

Over the last fifteen years (2001-2015), a total of 362 RASFF notifications related to fishery exports from India to the EU were notified. Higher incidence of quality issues were associated with crustaceans (71%) compared to cephalopods (15%) and fin fish (14%). The quality issues that were notified in fish exported from EU include physical, chemical and biological hazards (Table 2). The major quality issue responsible for RASFF notifications was veterinary medicinal products (52%), followed by heavy metals (14.4%) and the presence of pathogenic microorganisms (12.4%) indicating the need for focused action on these three quality issues. The residues of veterinary medicinal products (antibiotics) detected in fish exported from India were Furazolidone (AOZ); Nitrofurazone (SEM), Oxytetracycline and Chloramphenicol. The heavy metals, cadmium and mercury and the pathogenic bacteria Vibrios sp (*Vibrio cholerae*/ *Vibrio cholerae*non-O1/non-O139, *Vibrio parahaemolyticus*, *Vibrio vulnificus*) and Salmonella (*Salmonella paratyphi* B; *Salmonella Weltevreden*) were also reported in fish exported from India. However, there was a dominance of specific quality issue depending on the fish species. The major quality concerns associated with crustaceans was attributed to residues of veterinary medicinal products (72%) and pathogenic microorganisms (14%) whereas with cephalopods it was heavy metals (80%). However, in the case of fin fish, the quality issues were diverse viz., biocontaminants (33%), heavy metals (18%), pathogenic microorganisms (12%), food additive & flavourings (12%), non-pathogenic microorganisms (6%) and organoleptic aspects (6%).

Quality issues responsible for RASFF alerts of fish exported from India to the EU

	Hazard Category	Specific cause for RASFF alert
1.	Adulteration Fraud	improper health certificate
2.	Biocontaminants	histamine
3.	Biotoxins (other)	ciguatera poisoning
4.	Food additive & flavourings	Sodium carbonate, sulphite, allura red, sorbic acid, E160b annato/bixin/norbixin; E122 Azorubine; sulphite
5.	Foreign bodies	Faeces; defective packaging and infested with insects, foreign body (scraps of paper, cardboard, wire wool, hair and insects)
6.	Heavy metals	Cadmium, mercury
7.	Labeling	Insufficient/ incorrect labeling
8.	Non pathogenic microorganisms	high aerobic plate count, thermotolerant Coliforms, Enterococci, *Enterobacteriaceae*
9.	Organoleptic aspects	Altered organoleptic characteristics and abnormal smell; spoilage and poor temperature control
10.	Packaging defects	Defective packaging and infested with insects; bulging
11.	Pathogenic microorganisms	Vibrios (*Vibrio cholerae*/ *Vibrio cholerae* NON O:1/NON O:139), *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *V. alginolyticus*).
		Salmonella (*Salmonella paratyphi* B; *Salmonella Weltevreden*)
12.	Residues of Veterinary medicinal products (antibiotics)	Furazolidone (AOZ); Nitrofurazone (SEM), Oxytetracycline, Chloramphenicol, Leucomalachite green

Quality issues raised by the United States in fish imported from India:

The United States has charged that the fish imported from India is subject to refusal of admission pursuant to Section 801(a)(3) in that such fish appears to contain Salmonella, a biological hazard which may render it harmful to health (OASIS charge code: Salmonella); or it appears to be adulterated in that it or contains a new animal drug (its metaabolites) that is unsafe (OASIS charge code – Vetdrugres) or it appears to consist in whole or in part of a filthy, putrid, or decomposed substance, (insect, rodent, and/or other animal filth), or to be otherwise unfit for food (OASIS charge code – Filthy). A total of 43 import refusals were reported in fish exported from India and were mainly due to presence of Salmonella, veterinary drugs and filth. Import refusals were reported in finfish, shrimp, crab, squid and octopus. Maximum quality issues were reported in fin fish (42%) followed by shrimp (30%). The antibiotics responsible for import alerts in shrimps were nitrofurans and chloramphenicol.

The pursuit of safe food is the prime objective of food microbiologists and they try to achieve it by applying a series of control measures (hurdles) along the food chain aimed to eliminate, prevent or reduce the microbial hazards to an acceptable level based on HACCP program. HACCP (Hazard Analysis and Critical Control Point) is a system to prevent hazards so that the food processed will be safe for human consumption. A concerted and coordinated approach by the food microbiologists and food technologists is needed to develop safe and healthy fishery products that meet consumer expectations. Moreover, consumers need for convenience products, lightly preserved products and ready-to-products pose new challenges to both the microbiologist and the food technologist.

The future of fisheries depends on the production of safe and wholesome products, and this goal can be achieved by the strict enforcement of HACCP-based management practices, during primary production /harvest stage, processing stage in fish processing units, distribution to domestic and international markets and storage during retail sale. The aim of quality assurance is to ensure that a product conforms as closely as possible and consistently to that standard at all times. The training material on ‘Microbiological methods for analysis of fish and shrimp in seafood processing plants’ is prepared with an aim to provide a simple understanding of the routine microbiological methods so as to equip the technologists of the seafood processing establishment to conduct own checks related to microbiological hazards.

2. AEROBIC PLATE COUNT (APC)

Aerobic plate count (APC) is intended to indicate the level of bacteria in solid food product (fish, shrimp etc) or in liquids (water, ice) or on food contact surfaces (worker's palm, processing tables, trays, crates, floor etc). The terms Total Plate Count (TPC), Total bacterial count (TBC), heterotrophic bacterial count (HBC), total viable count (TVC), Standard Plate Count (SPC) are used synonymous to APC to indicate total bacteria in a sample. Pour plate method or spread plate methods are used to determine APC.

Requirements:

- Sterile media [Plate count Agar (PCA) or Tryptone Glucose Agar (TGA)]
- Sterile diluent (Butterfields phosphate-buffered dilution water (450ml in 1000ml flask; 90ml in 150ml flask)
- Sterile petri plates (15 x 90 mm)
- Sterile pipettes (10ml, 1ml) or sterile disposable tips
- Sterile stainless steel scissors, sterile stainless steel forceps
- Sterile bent (L-shaped) applicator
- Blender or Stomacher
- Incubators maintained at 35°C and 22°C.

A. Determining APC in Solid Food Samples (Fish, Shrimp, crab, squid, cuttle fish etc):

1. Preparing dilutions of the shrimp/fish sample:

Aseptically cut the shrimp/fish sample and weigh 50 grams in a sterile sample dish. (Note that adding or removing food pieces from the sample dish to obtain 50 grams should be performed aseptically near the flame in a laminar flow chamber). Transfer the 50g to a stomacher bag and homogenize with 450 ml of diluent (Butterfields phosphate-buffered dilution water) using a stomacher blender. The resultant homogenized material is 1 : 10 dilution or 10^{-1} dilution which means that 1 gram of food sample is present in 10ml of the homogenized material. 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.

Understanding Dilutions:

DILUTION			QUANTITY OF MEAT AT THE SPECIFIC DILUTION	
1 : 10	or	10^{-1}	1g in 10ml	0.1g per 1ml
1 : 100	or	10^{-2}	1g in 100ml	0.01g per 1ml
1 : 1,000	or	10^{-3}	1g in 1000ml	0.001g per 1ml
1: 10,000	or	10^{-4}	1g in 10,000ml	0.0001g per 1ml

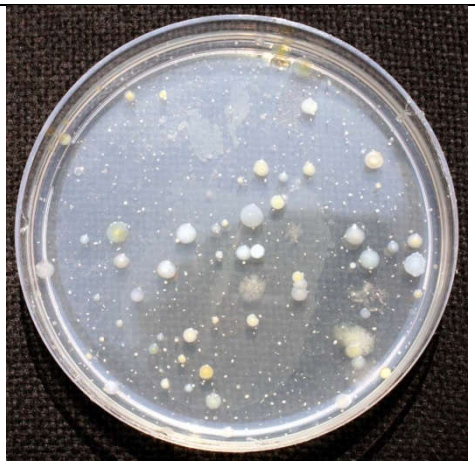
2. Pour plate method for determining APC:

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 15 to 18ml of molten and cooled (40 to 45°C) PCA/TGA 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°C for 48 hours.

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 15 to 18ml of molten and cooled PCA/TGA in a sterile petri plate. For diluent control, transfer 1ml of sterile Butterfields phosphate-buffered dilution water to a sterile petri plate and add 15 to 18ml of molten and cooled PCA/TGA. For air control, leave an empty sterile petri plate open on the work table during the period of analysis for 15 minutes and add 15 to 18ml of molten and cooled PCA/TGA. Allow the agar to set (leave the plates undisturbed for 15 minutes), invert the solidified agar plates and incubate at 35°C for 48 hours.

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.



Petri plate with bacterial colonies of different sizes

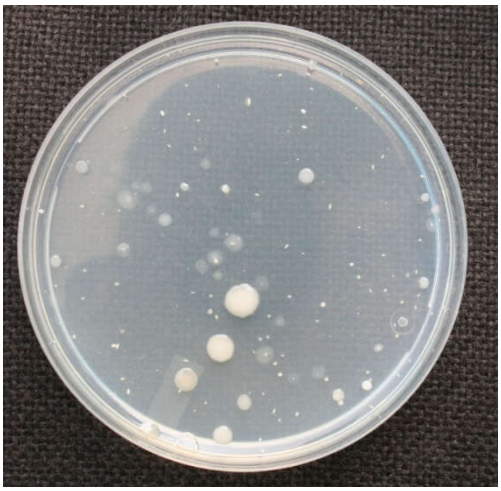
The colony counts of duplicate plates within the same dilution should agree within 10% limit and counts between different dilutions should agree decimally. Record the results dilution wise as shown below.

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

4. **Calculating APC**:

Select 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.



Petri plate with colonies within countable range (25 to 250)

APC is calculated using the formula

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

Where,

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

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.

See the worked out example given below for ease of understanding the calculating APC.

(***Note***: APC is expressed as colonies forming units (cfu) as we count the colonies formed but not individual bacteria. The number of colonies formed indicate the number of bacteria in the

original sample because each colony is formed from a single bacteria that is trapped in the agar medium.)

Example for calculating APC

Dilution	Petri Plate 1	Petri Plate 2
10^{-1} or 0.1	2200	2100
10^{-2} or 0.01	240	230
10^{-3} or 0.001	26	28
10^{-4} or 0.0001	2	3

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

$$N = \frac{524}{[(1 \times 2) + (0.1 \times 2)] \times 0.01}$$

$$N = \frac{524}{[2.2] \times 0.01}$$

$$N = \frac{524}{0.022}$$

APC = 23,818 cfu/g*

***Round off the value and report APC:** Report only the first two significant digits. Round off to two significant figures only at the time of final calculation. Round by raising the second digit to the next highest number when the third digit is 6, 7, 8, or 9 and use zeros for each successive digit toward the right from the second digit. Round down when the third digit is 1, 2, 3, or 4. When the third digit is 5, round up when the second digit is odd and round down when the second digit is even.

For the above example, after rounding off the APC is

APC = 24,000 cfu/g or 2.4 x 10^4 cfu/g

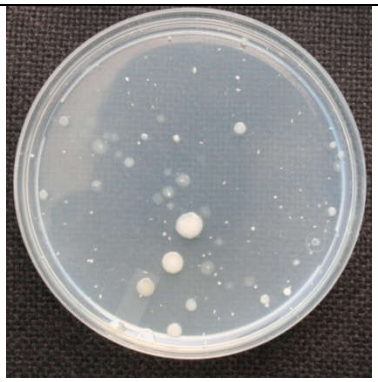
Exceptions:

i) 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.

ii) When number of colonies per plate exceeds 250, for all dilutions, record the counts as too numerous to count (TNTC) for all plates except the plate closest to 250. Count the colonies in the last plate. Calculated APC but record the result as EAPC (estimated APC) to denote that it was estimated from counts outside 25-250 per plate range.

Petri plate with more than 250 colonies

iii) When number of colonies per plate is less than 25, for all dilutions, count the colonies in the first plate. Calculated APC but record the result as EAPC (estimated APC) to denote that it was estimated from counts outside 25-250 per plate range.



Petri plate with less than 25 colonies

iv) If all the plates from all dilutions have no colonies, report APC as less than 1 times the corresponding lowest dilution used. (If 1:10 was the lowest dilution used and there were no colonies on that plate then give the result as APC = <10 cfu/g).

v) When plates from a sample are known to be contaminated or otherwise unsatisfactory, record the result as laboratory accident (LA).

5. **Spread Plate method for determining APC**:

For spread plate method (surface plate method) plate count agar (PCA) plates have to be prepared in advance. For this, melt a flask containing 200 ml of PCA in boiling water bath, cool the agar to 45-50°C and pour it into 10 petri plates (approximately 15 to 18 ml) per petri plate. Allow the PCA to set (solidify). Dry the surface of the agar by placing the petri plate in laminar flow chamber (under low speed air flow) for 45 minutes. The dried plates are ready for use to determine APC.

Prepare 10^{-2}, 10^{-3} and 10^{-4} dilutions of the shrimp/fish sample as described previously (point 1). Arrange six petri plates in 3 rows in duplicate. Label the plates appropriately (indicating the product name/code, media, date and dilution). Pipette 0.5 ml from the respective dilution and inoculate on the surface of two petri plates (0.5ml each). Using a sterile bent glass rod (or sterile disposable L shaped plastic rod) spread the inoculums uniformly on the surface of each plate. Leave the plates undisturbed for 30 minutes to allow the inoculums to be absorbed. Invert the plates and incubate at 35°C for 48 hours. Count and calculate as mentioned previously but remember that the **APC count has to be multiplied by 2** as 0.5ml was used in spread plate method instead of 1 ml that was used in pour plate method.

B. **<u>Determining APC of Liquid samples (water / ice):</u>**

Collect water in a sterile container. Transfer 1ml of water to four petri plates (one ml in each plate). Add approximately 15 to 18ml of molten and cooled (40 to 45°C) 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 two plates at 37°C for 48 hours and the other two plates at 22°C for 96 hours. Count the colonies and calculate APC.

APC (cfu/ml) = Average count

C. Determining APC of surface samples (swab samples of Workers hands, tables, floors, trays etc):

Sterilize swabs (swab should be made of absorbent cotton) and metal template having 25cm^2 of inner open area. Moisten the sterile swab by dipping it in a flask containing 100ml of sterile diluent (Butterfields phosphate-buffered dilution water). Drain excess diluent from the swab by pressing the swab to the inner walls of the flask. Using the moist swab, thoroughly swab 25cm^2 of the test surface (worker's hand or processing table or freezing tray or floor) so that the bacteria present on the test surface are transferred to the swab. Replace the swab into the Butterfields phosphate-buffered dilution flask and shake well so that the bacteria present on the swab will be transferred to the diluent. Transfer 1ml of from the dilution flask to two petri plates (one ml in each plate). Add approximately 15 to 18ml of molten and cooled (40 to 45°C) 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 the plates at 37°C for 48 hours. Count the colonies and calculate APC.

$$\text{APC (cfu/cm}^2\text{)} = \text{Average count} \times \frac{100}{25}$$

Interpreting the APC result:

Interpret the APC result obtained by comparing the standard values specified for the particular product. Food Safety Standards Authority of India (FSSAI) has specified limits for fish and fishery products for the domestic market. Export Council of India (EIC) has listed the specifications for fish and fishery products export. Moreover, importing countries have laid

down certain specifications for import of fish and shrimp into their countries (European Union Council Directives, National standards of other importing countries).

Total Plate count at 37°C per gram, Maximum allowed TPC values for different fishery products meant for export (EIC of India)

S.O. 729 (E) dated 21st August 1995; subsequently amended vide No. Orders S.O. 792 (E) dated 17th August 2001, S.O. 722 (E) dated 10th July 2002, S.O. 464 (E) dated 24th April 2003, S.O. 1227 (E) dated 23rd October 2003 & S.O. 1227 (E) dated 31st July 2006.

	Fresh/ Chilled/ Frozen	**Cooked / boiled**
Shrimps / Prawns	5,00, 000 cfu/g	1,00,000 cfu/g
Fish: whole, dressed, fillets or any other type (pomfrets, seer, pearl spot, sardine, mackerel, hilsa etc.)	5,00, 000 cfu/g	1,00,000 cfu/g
Crab and crab meat	10,00, 000 cfu/g	1,00,000 cfu/g
Cephalopods (cuttle fish, squid, octopus.) and their body parts including roes	5,00, 000 cfu/g 2,00, 000 cfu/g for products meant for raw Consumption	1,00,000 cfu/g
Clam/ Mussel meat	10,00, 000 cfu/g	1,00,000 cfu/g

Total Plate count requirements for sea foods for domestic markets
Food Safety and Standards Authority of India (FSSAI).
***FSS (Food product standards and food additives) regulation 2011*, Part II, 496 pp.**

Frozen shrimps or prawns (Raw)	Not more than five lakhs per gram
Frozen shrimps or prawns (Cooked)	Not more than one lakh per gram
Frozen fish	Not more than five lakhs per gram
Frozen fish fillets, or minced fish flesh, or mixtures thereof	Not more than five lakhs per gram
Salted fish / dried salted fish	Not more than five lakhs per gram
Canned fish, canned shrimp, canned crab meat	Nil

Total Plate count (maximum permissible) for water intended for human consumption
(includes water used in shrimp/ fish processing)
(EU Council directive, 98/83/EC; Part A, Microbiological parameters)

Colony count at 22°C	100/ml
Colony count at 37°C	20/ml

Maximum Permissible limit of Total Plate count for Swab Samples
Document No EIC/F & FP/Ex.Inst./March/2012/Issue 4
Export Inspection Council

1	Tables, Utensils, Trays etc	100 per cm^2
2	Workers hand	100 per cm^2

3. PALM IMPRESSION TECHNIQUE FOR POPULARIZING HYGIENE LITERACY AMONG FISH HANDLERS

B. Madhusudana Rao, J Charles Jeeva and Imam Khasim (2005) Palm impression technique for popularizing hygiene literacy among fish handlers. ICAR NEWS, Volume 11 (2): 18-19

Safe food means lower incidence of food borne diseases, low public health costs, fewer barriers to international trade, lower production losses and better competitiveness. To achieve safe food and ensure the safety of the fish consumer, it is of utmost importance to popularize good sanitation and hygiene practices among primary fish handlers viz. crew of mechanized trawlers, traditional boat operators, loading and unloading workers at fishing harbor, fish vendors and pre-processing and processing workers in seafood processing plants so that they apply the same during harvesting, handling, processing and sale of fish. Most of them are unaware that they are potential carriers of pathogenic microorganisms and that poor person at hygiene makes the fish unsafe for human consumption. Moreover, physically it is almost impossible to distinguish between unsafe fish and safe fish, as both look normal, smell normal and taste normal.

In spite of repeated efforts through lectures, discussions, exhibitions etc., the hygiene literacy gained little momentum amongst the fish handlers as they had little knowledge of microorganisms. The conventional bacteriological techniques to demonstrate the total bacterial load on worker's palm by swabbing method, was beyond their level of comprehension. Most people retain 10 to 15% of what they had heard, 30 to 55% of what they had seen, 50% or more of what they had seen and heard and up to 90% by participating with the involvement of all the senses. A simple participatory technique whose results can be easily read by illiterate or less educated people was needed to create awareness on need for personal hygiene among fish handlers. To address that issue, the Palm Impression Technique (PIT), was developed and its usefulness as an effective participatory tool for popularizing the need for good hygiene practices was tested among fish handlers.

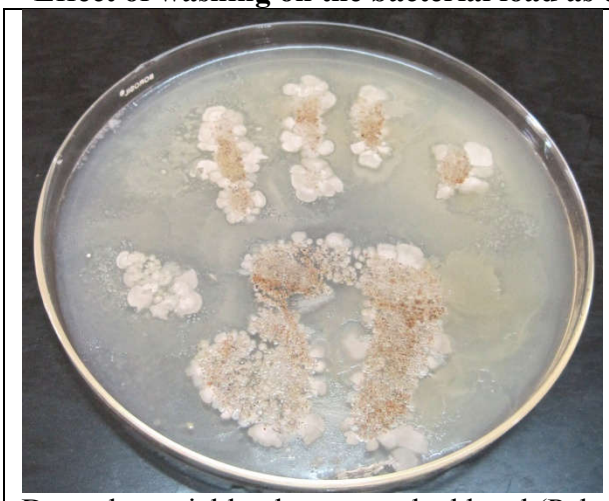
PIT was done by placing the palm of fish handler on a large-sized petri dish preset with nutrient medium. Briefly, 50 ml of molten Tryptone Glucose Agar (tryptone 0.5%, beef extract 0.3%, sodium chloride 0.5%, D-glucose 0.1%, agar-agar 1.5%, pH 7.1±0.1) was poured in large sized petri dishes (195 mm bottom plate diameter), allowed to set and dry. The handlers were

asked to place their soiled palm on pre-set agar plate and allowed to remain in contact for 30 seconds. They were then directed to wash their hands thoroughly with soap and water and after drying their palm, they were then directed to wash their hands thoroughly with soap and water and after drying their palm, they were once again asked to place their palm on a different agar plate. The plates were then incubated at 37°C and observed after 24 hours for visible bacterial growth.

The result was very striking to the fish handlers. The plate on which unclean palm was placed showed very dense bacterial growth, the shape of the fish handler's palm, whereas the plate on which clean palm was placed showed negligible bacterial growth. The result was easily read by all the fish handlers. This can have an everlasting impression in their minds, which in turn makes them follow good personal hygiene.

The seafood technologists can employ the Palm Impression Technique to popularize good sanitation and hygiene practices among pre-processing and processing workers.

Effect of washing on the bacterial load as demonstrated using Palm impression technique



Dense bacterial load on unwashed hand (Palm) of a fish handler

Bacteria load reduced to a large extent by washing the hands (palm)

3. FAECAL INDICATOR BACTERIA

The term 'indicator bacteria' can be applied to any taxonomic, physiological or ecological group of bacteria whose presence or absence provides indirect evidence concerning a particular feature in the past history of the water or fish/shrimp sample. Microbial indicators are more often employed to assess food safety and hygiene than quality.

Ideally a food safety indicator should meet certain important criteria. It should

i) Be easily and rapidly detectable
ii) Be easily distinguishable from other members of the food flora
iii) Have a history of constant association with the pathogen whose presence it is to indicate
iv) Always be present when the pathogen of concern is present
v) Possessing growth requirements equaling those of the pathogen
vi) Have a die off rate that at least parallels that of the pathogen and ideally persists slightly longer than the pathogen of concern.

Faecal indicator bacteria, as the name suggests are employed to detect and measure faecal contamination in the assessment of food and water safety. Faecal indicator bacteria are almost exclusively of faecal origin, and are transmitted through faecal contamination of foods and water, as well as cross-contamination, or by direct human contact during fish / shrimp processing. The commonly employed faecal indicator bacteria are Faecal Coliforms, *Escherichia coli (E.coli)*, *Enterococci* and *Clostridium perfringens* (sulphite reducing Clostridia). Faecal indicator bacteria, in most cases are not harmful but their presence in food or water may indicate the possible presence of enteric pathogens such as *Salmonella* and *Vibrio cholerae*. Face

4. MOST PROBABLE NUMBER (MPN) METHOD FOR TOTAL COLIFORMS, FAECAL COLIFORMS AND *E.COLI* IN SHRIMP/FISH SAMPLES

3 TUBE MPN METHOD

MPN methods are used to detect low numbers of bacteria in large volumes of sample. MPN is only a statistical approximation of the test bacteria in the given sample and not the actual number. MPN is 'that bacterial density, which if it is present in the sample, would more frequently than any other, have given the observed analytical result'. In the MPN method, measured volumes of food sample homogenate are added to a series of tubes containing a liquid indicator growth media. The media receiving test bacteria show growth and characteristic colour change, which is, absent in those tubes receiving an inoculums of homogenate without test bacteria. From the number and distribution of positive and negative reactions, the MPN of the test bacteria in the fish/shrimp sample can be estimated by reference to statistical tables (MPN tables).

MPN method can be used to detect Coliforms and *E.coli* in fish/shrimp samples provided the expected numbers of bacteria are less than 100 per gram; otherwise the results may not be meaningful. Coliforms are Gram-negative, facultatively anaerobic bacteria. They produce gas from glucose and ferment lactose to acid and gas within 48 hours at 35°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 45.5°C (except shellfish isolates, 44.5°C).

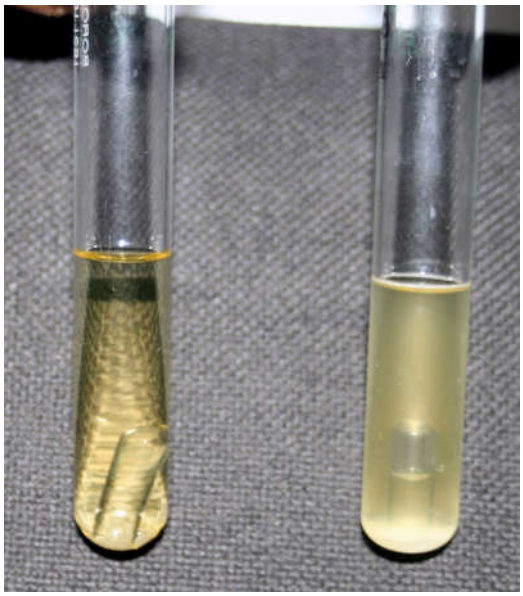
a) **Preparation of dilutions of the shrimp/fish sample**:

Aseptically cut the shrimp/fish sample and weigh 50 grams in a sterile sample dish. Note that adding or removing food pieces from the sample dish to obtain 50 grams should be performed aseptically near the flame in a laminar flow chamber). Transfer the 50g to a stomacher bag and homogenize with 450 ml of diluent (Butterfields phosphate-buffered dilution water) using a stomacher blender. The resultant homogenized material is 1 : 10 dilution (0.1 g per ml). Pipette 10 ml from the above 10^{-1} dilution to 90ml of sterile diluent and mix well. This gives 1 : 100 (0.01g per ml). Pipette 10 ml from the above 10^{-2} dilution to 90ml of sterile diluent and mix well. This gives 1 : 1000 (0.001g per ml).

b) Step I: Presumptive test for Total Coliforms:

Media: Lauryl tryptose broth (LST broth), 10ml each in 9 test tubes; place inverted Durham's tube in each tube

Method: Arrange LST tubes in triplicate sets in a test tube stand. Inoculate 1 ml aliquots (0.1g) from the fish/shrimp sample homogenate from 10^{-1} dilution into 3 LST tubes for a 3 tube MPN analysis. Use a 1 ml pipette for inoculation. Hold pipette at angle so that its lower edge rests against the tube. 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 (0.001g) into three LST tubes and label them as 0.001g. Incubate all the LST tubes at 35°C± 0.5°C. Examine tubes after 24 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 0.1g, 0.01g and 0.001g tubes. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 hours. Perform confirmed test on all presumptive positive (gas) tubes.



Negative **Positive**

c) Step II: Confirmed Test for Coliforms:

Media: Brilliant Green Lactose Bile Broth (BGLB 2%), 5ml each in test tubes; place inverted Durham's tube in each tube

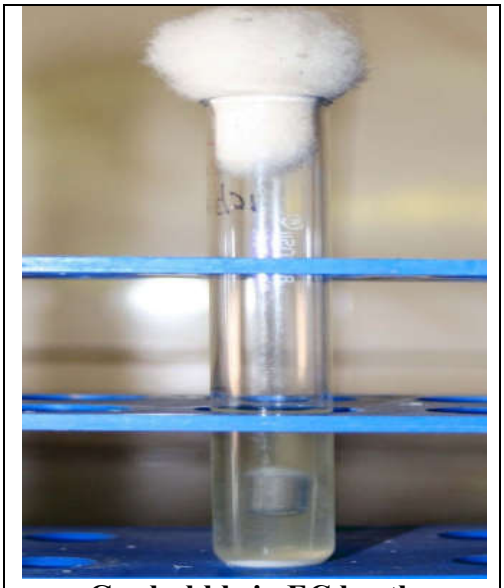
Method: The number of BGLB tubes required depends on the number of positive LST tubes in Step I. Inoculate loopful of culture from the positive LST tubes to BGLB broth and mark the corresponding label (either 0.1g or 0.01g or 0.001g). Incubate BGLB tubes at 35°C± 0.5°C. Examine for gas production after 48 hours of incubation. Note the result as number of positives in each set of 0.1g, 0.01g and 0.001g tubes. 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 Coliforms MPN per gram.

d) Step III: Test for Faecal Coliforms:

Media: EC Broth, 5ml each in test tubes; place inverted Durham's tube in each tube.

Method: 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 0.1g or 0.01g or 0.001g). Incubate EC tubes at 45.5°C. Examine tubes after 24 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 0.1g, 0.01g and 0.001g tubes. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 hours. Note the result as number of positives in each set of 0.1g, 0.01g and 0.001g tubes. 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 Faecal Coliforms MPN per gram.

Note: For shellfish and shellfish harvest water analysis, the incubation temperature is 44.5± 0.2°C.



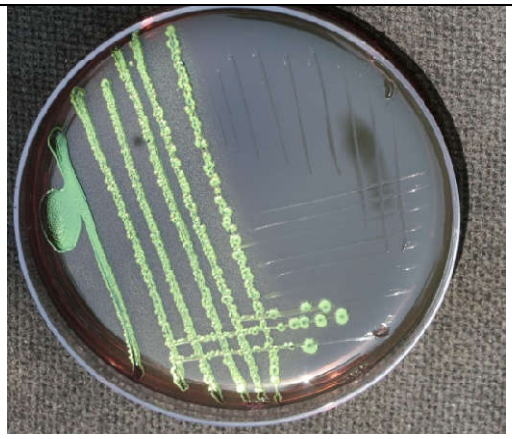
Gas bubble in EC broth

e) Step IV: Test for *E.coli*:

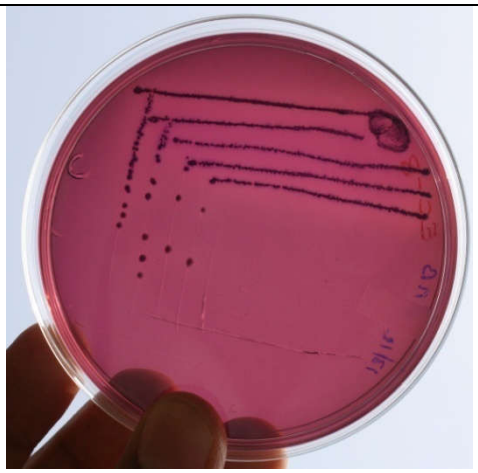
Media: Levines Eosin Methylene Blue (EMB) agar, Nutrient agar slants, Tryptone broth (5ml in test tubes), MR-VP broth (5ml in test tubes), Koser's citrate (5ml in test tubes) or Simmons citrate agar slants.

Method:

Melt EMB agar, cool to 50°C, pour 15 to 18ml 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 0.1g or 0.01g or 0.001g). Incubate EMB agar plates tubes at 35°C± 0.5°C for 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 Nutrient agar slants and incubate at 35C for 24 hours. Label the nutrient agar slants appropriately (0.1g, 0.01g and 0.001g).



E.coli colonies- greenish metallic sheen by reflected light



E.coli colonies - dark centered flat colonies by transmitted light.

Confirm the suspected *E.coli* colonies by performing IMVC tests (Indole test, Methyl red test, Voges-Proskauer test and Citrate test). All cultures appearing as Gram-negative, short rods should be tested for the IMVC reactions mentioned below and also re-inoculated back into LST to confirm gas production.

i) Indole test: Inoculate the suspected *E.coli* culture from nutrient agar slant into tryptone broth and incubate at 35°C for 24 hours. Test for the presence of indole by adding 0.2 to 0.3ml of Kovac's indole reagent. Appearance of pink colour in the upper layer (pink ring) is positive. Yellow colour is negative.

ii) Methyl red test: Inoculate the suspected *E.coli* culture from nutrient agar slant into MRVP broth and incubate at 35°C for 48 hours. Add 5 drops of

methyl red reagent indicator to each tube. Appearance of distinct red colour is positive. Yellow colour is negative.

iii) Voges-Proskauer test: Inoculate the suspected *E.coli* culture from nutrient agar slant into MRVP broth and incubate at 35°C for 48 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.

iv) Citrate test: Inoculate the suspected *E.coli* culture from nutrient agar slant into Koser's citrate media tubes (inoculate lightly to avoid detectable turbidity) or streak on Simmon's citrate agar slants and 35°C for 48-96 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 IMVC pattern ++-- are confirmed as E.coli (biotype 1). E.coli biotype 2 give -+-- IMVC pattern.

Note the result as number of positives in each set of 0.1g, 0.01g and 0.001g 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.

Maximum allowed *E.coli* count in different fishery products meant for export (EIC of India)

S.O. 729 (E) dated 21st August 1995; subsequently amended vide No. Orders S.O. 792 (E) dated 17th August 2001, S.O. 722 (E) dated 10th July 2002, S.O. 464 (E) dated 24th April 2003, S.O. 1227 (E) dated 23rd October 2003 & S.O. 1227 (E) dated 31st July 2006.

	Fresh/ Chilled/ Frozen	**Cooked / boiled**
Shrimps / Prawns	20 per gram	Nil per gram
Fish: whole, dressed, fillets or any other type (pomfrets, seer, pearl spot, sardine, mackerel, hilsa etc.)	20 per gram	Nil per gram
Crab and crab meat	20 per gram	Nil per gram
Cephalopods (cuttle fish, squid, octopus.) and their body parts including roes	20 per gram	Nil per gram
Clam/ Mussel meat	20 per gram	Nil per gram

3 Tube MPN Table for food samples

For 3 tubes each at 0.1g, 0.01g and 0.001g inocula

Number of Tubes giving a Positive Reaction			MPN per gram
3 x 0.1g	3 x 0.01g	3 x 0.001g	
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**

5. MOST PROBABLE NUMBER (MPN) METHOD FOR TOTAL COLIFORMS, FAECAL COLIFORMS AND *E.COLI* IN WATER / ICE SAMPLES

5 TUBE MPN METHOD

Step I: Presumptive test for Total Coliforms:

Media:

Double strength MacConkey purple broth (1 flask with 50ml double strength broth and 5 large sized test tubes with 10ml of double strength broth), place inverted Durham's tube in each tube

Single strength MacConkey purple broth; place inverted Durham's tube in each tube

Method: Collect water aseptically. Transfer 50 ml of water sample to the flask containing 50 ml double strength MacConkey purple broth. Transfer 10ml of water sample to each of the 5 large test tubes containing double strength MacConkey purple broth. Transfer 1 ml of water sample to each of the 5 test tubes containing single strength MacConkey purple broth. While transferring, hold the pipette at angle so that its lower edge rests against the tube to avoid gas bubble formation. Label the flask as 50ml, large test tubes as 10ml and test tubes with single strength media as 1ml. Incubate the flask and tubes 35°C± 0.5°C for 48 hours. Examine the flask and tubes for acid (yellow colour) and gas production (bubbles in Durham's tube). If acid and gas production are noticed, then, note the reaction as positive. Note the result as number of positives in each set of 50ml, 10ml and 1ml tubes. Perform confirmed test on all presumptive positive (gas) tubes.

Step II: Confirmed Test for Coliforms:

Media: Brilliant Green Lactose Bile Broth (BGLB 2%), 5ml each in test tubes; place inverted Durham's tube in each tube

Method: The number of BGLB tubes required depends on the number of positive MacConkey purple broth flask and tubes in Step I. Inoculate loopful of culture from the

positive MacConkey purple broth flask and tubes to BGLB broth and mark the corresponding label (either 50ml, 10ml or 1ml). Incubate BGLB tubes at 35°C± 0.5°C. Examine for gas production after 48 hours of incubation. Note the result as number of positives in each set of 50ml, 10ml and 1ml tubes. Calculate MPN per gram from the 5 tube MPN table (MPN table for 50 ml, 10ml or 1ml. Give the result as Coliforms MPN per 100 ml.

Step III: Test for Faecal Coliforms:

Media: EC Broth, 5ml each in test tubes; place inverted Durham's tube in each tube.

Method: 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 (50 ml, 10ml or 1ml). Incubate EC tubes at 45.5°C. Examine tubes after 24 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 50ml, 10ml and 1ml tubes. Calculate MPN per gram from the 5 tube MPN table (MPN table for 50 ml, 10ml or 1ml). Give the result as Faecal Coliforms MPN per 100 ml.

Note: For shellfish and shellfish harvest water analysis, the incubation temperature is 44.5± 0.2°C.

Step IV: Test for *E.coli*:

Media: Levines Eosin Methylene Blue (EMB) agar, Nutrient agar slants, Tryptone broth (5ml in test tubes), MR-VP broth (5ml in test tubes), Koser's citrate (5ml in test tubes) or Simmons citrate agar slants.

Method:
Melt EMB agar, cool to 50°C, pour 15 to 18ml 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 50 ml, 10ml or 1ml). Incubate EMB agar plates tubes at 35°C± 0.5°C for 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 centred flat colonies by transmitted light. Pick

suspicious colonies and transfer to Nutrient agar slants and incubate at 35C for 24 hours. Label the nutrient agar slants appropriately (50 ml, 10ml or 1ml).

Confirm the suspected *E.coli* colonies by performing IMVC tests (Indole test, Methyl red test, Voges-Proskauer test and Citrate test). All cultures appearing as Gram-negative, short rods should be tested for the IMVC reactions mentioned below and also re-inoculated back into LST to confirm gas production.

i) Indole test: Inoculate the suspected *E.coli* culture from nutrient agar slant into tryptone broth and incubate at 35°C for 24 hours. Test for the presence of indole by adding 0.2 to 0.3ml of Kovac's indole reagent. Appearance of pink colour in the upper layer (pink ring) is positive. Yellow colour is negative.

ii) Methyl red test: Inoculate the suspected *E.coli* culture from nutrient agar slant into MRVP broth and incubate at 35°C for 48 hours. Add 5 drops of methyl red reagent indicator to each tube. Appearance of distinct red colour is positive. Yellow colour is negative.

iii) Voges-Proskauer test: Inoculate the suspected *E.coli* culture from nutrient agar slant into MRVP broth and incubate at 35°C for 48 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.

iv) Citrate test: Inoculate the suspected *E.coli* culture from nutrient agar slant into Koser's citrate media tubes (inoculate lightly to avoid detectable turbidity) or streak on Simmon's citrate agar slants and 35°C for 48-96 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 IMVC pattern ++-- are confirmed as E.coli (biotype 1). E.coli biotype 2 give -+-- IMVC pattern.

Note the result as number of positives in each set of 50 ml, 10ml or 1ml labeled EMB agar plates. Calculate MPN per gram from the 5 tube MPN table (Table for 50 ml, 10ml or 1ml). Give the result as *E.coli* MPN per 100 ml.

E.coli in water intended for human consumption

(includes water used in shrimp/ fish processing)

(EU Council directive, 98/83/EC; Part A, Microbiological parameters)

Escherichia coli (*E.coli*)	0 per 100/ml

5 Tube MPN Table for water samples

For a set of one 50ml, five 10ml and five 1ml volumes

Number of Tubes giving a Positive Reaction			MPN per 100 ml
1 x 50 ml	5 x 10 ml	5 x 1 ml	
0	0	0	<1
0	0	1	1
0	0	2	2
0	1	0	1
0	1	1	2
0	1	2	3
0	2	0	2
0	2	1	3
0	2	2	4
0	3	0	3
0	3	1	5
0	4	0	5
1	0	0	1
1	0	1	3
1	0	2	4
1	0	3	6
1	1	0	3
1	1	1	5
1	1	2	7
1	1	3	9
1	2	0	5

1	**2**	**1**	**7**
1	**2**	**2**	**10**
1	**2**	**3**	**12**
1	**3**	**0**	**8**
1	**3**	**1**	**11**
1	**3**	**2**	**14**
1	**3**	**3**	**18**
1	**3**	**4**	**21**
1	**4**	**0**	**13**
1	**4**	**1**	**17**
1	**4**	**2**	**22**
1	**4**	**3**	**28**
1	**4**	**4**	**35**
1	**4**	**5**	**43**
1	**5**	**0**	**24**
1	**5**	**1**	**35**
1	**5**	**2**	**54**
1	**5**	**3**	**92**
1	**5**	**4**	**161**
1	**5**	**5**	**>180**

6. DETERMINING COLIFORMS EMPLOYING SOLID MEDIA

Media : Violet Red Bile Agar (VRBA).

Note: VRBA media has LACTOSE. Do not confuse with Violet Red Bile Glucose Agar (VRBGA) which has glucose.

Method: Prepare 10^{-1} and 10^{-2} dilutions of the fish/shrimp sample as described in Aerobic Plate Count method. Transfer 1 ml aliquots from 10^{-1} dilution to two sterile petri plates (1ml to each plate). Similarly, transfer 1 ml aliquots from 10^{-2} dilution to two sterile petri plates (1ml to each plate). Pour 10 ml of VRBA cooled to 45°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. Incubate. Examine plates under magnifying lens and with illumination.

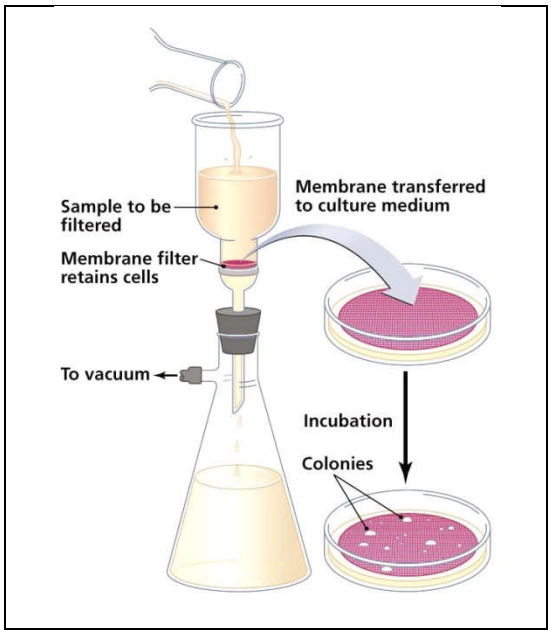
Counting of Coliforms: Count purple-red colonies that are 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids.

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.*

7. MEMBRANE FILTRATION METHOD

Membrane filtration method provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter. A membrane filter is a thin cellulose acetate filter with a pore size of 0.22 µm or 0.45 µm that is capable of retaining bacteria while allowing water to pass through. A known volume of water sample is filtered through the membrane. Bacteria in the sample are concentrated on the surface of the membrane. After filtration, the membrane filter containing the bacterial cells is placed in a petri plate containing a selective medium and incubated at the appropriate temperature for a specific period of time depending upon the bacteria that is targeted. The passage of nutrients through the filter facilitates the growth of target bacteria organisms on the upper surface of the membrane. After the incubation period, the number of specific colonies developed on the filter reveals the number of target bacteria in the water sample.



Membrane Filtration Method

Membrane filtration (MF) method vis-à-vis Most Probable Number (MPN) method:

- The membrane filtration technique can be used to test large volumes of sample.
- The MF Technique offers the advantage of isolating discrete colonies of bacteria, thus providing real numbers whereas the MPN gives only a probable number based on statistical approximation based on MPN tables.

- Membrane filtration cannot be used effectively for turbid waters or food homogenates as they clog the filters but MPN methods can be used for turbid waters and food homogenates.

8. MEMBRANE FILTRATION METHOD FOR DETECTING *ENTEROCOCCI* IN WATER SAMPLES

Enterococci include *Enterococcus faecalis*, *E. faecium*, *E. gallinarum*, and *E. avium*. m-Enterococcus Agar is used for the detection of *Enterococci* employing the membrane filtration technique for testing water.

Method:

1. Aseptically collect the water sample
2. Using forceps aseptically remove the membrane filter from its sterile package and place it into the membrane filter assembly.
3. Pour 100ml of the water sample into the funnel of the filter assembly.
4. Allow the entire 100ml volume of water sample to flow through the filter. (*Note: Vacuum pump can be used to effectively draw the sample completely through the filter*).
5. Flame the forceps and remove the membrane filter from the funnel of the filter assembly.
6. Place the membrane filter into pre-prepared m-Enterococcus agar plate. (*Sterilize m-Enterococcus agar, melt and cool the agar to 45 – 50°C, add required amount of TTC, pour the m-Enterococcus agar into petri plates and finally dry the petri plates*).
7. Incubate the m-Enterococcus plates at 35°C for 24 hours.
8. Count all light and dark red colonies as *Enterococci* (Enterococci reduce Triphenyl tetrazolium chloride (TTC) to formazan (red colour)

$$\textbf{\textit{Enterococci} per 100 ml} = \frac{\textbf{Red colonies counted}}{\textbf{Volume of sample (ml) filtered}} \textbf{ X 100}$$

***Enterococci* in water**
(includes water used in shrimp/ fish processing)
(EU Council directive, 98/83/EC; Part A, Microbiological parameters)

Enterococci	0 per 100/ml

9. MEMBRANE FILTRATION METHOD FOR DETECTING Clostridium perfringens IN WATER SAMPLES

Membrane Clostridium Perfringens (m-CP) Medium is a selective and chromogenic medium for the identification of *Clostridium perfringens* from water samples and has been recommended in European Council Directive 98/83/EC for testing the quality of water intended for human consumption.

Method:

1. Aseptically collect the water sample
2. Using forceps aseptically remove the membrane filter from its sterile package and place it into the membrane filter assembly.
3. Pour 100ml of the water sample into the funnel of the filter assembly.
4. Allow the entire 100ml volume of water sample to flow through the filter. (*Note: Vacuum pump can be used to effectively draw the sample completely through the filter*).
5. Flame the forceps and remove the membrane filter from the funnel of the filter assembly.
6. Place the membrane filter into pre-prepared m-CP agar plate. (*Sterilize m-CP agar, melt and cool the agar to 45 – 50°C, add the required amount of supplements, pour the m-CP agar into petri plates and finally dry the petri plates*).
7. Incubate the m-CP agar plates anaerobically at 44±1C for 21±3 hours.
8. Count all opaque yellow colonies.
9. Expose the yellow colonies to ammonium hydroxide vapours for 20 to 30 seconds.
10. Count all colonies that turn pink or red after exposure to ammonium hydroxide vapours. (*Clostridium perfringens* lack β-D glucosidase activity but ferment sucrose resulting in characteristic opaque yellow *Clostridium perfringens* colonies on m-CP agar. Presumptive positive *Clostridium perfringens* colonies are further tested for acid phosphatase activity. *Clostridium perfringens* colonies turn pink or red as phenolphthalein diphosphate is cleaved by acid phosphatase).

The composition of m-CP agar is:

Basal medium:

Tryptose	30 g
Yeast extract	20 g
Sucrose	5 g
L-cysteine hydrochloride	1 g
MgSO4·7H2O	0,1 g
Bromocresol purple	40 mg
Agar	15 g
Water	1000 ml

Dissolve the ingredients of the basal medium, adjust pH to 7.6 and autoclave at 121 °C for 15 minutes. Allow the medium to cool and add supplements.

Supplements:

D-cycloserine	400 mg
Polymyxine-B sulphate	25 mg
Indoxyl-β-D-glucoside to be dissolved in 8 ml sterile water before addition	60 mg
Filter sterilised 0.5% phenolphthalein diphosphate solution	20 ml
Filter sterilised 4.5% $FeCl_3 \cdot 6H_2O$	2 ml

$$\textbf{\textit{Clostridium perfringens} per 100 ml} = \frac{\textbf{Pink/Red colonies counted}}{\textbf{Volume of sample (ml) filtered}} \textbf{ X 100}$$

***Clostridium perfringens* (including spores) in water intended for human consumption (EU Council directive, 98/83/EC; Part C, Indicator parameters)**

Clostridium perfringens (including spores)	0	per 100/ml

10. Pathogenic Bacteria

Fish consumers may succumb to illness due to ingestion of preformed microbial toxins (*Clostridium botulinum*, *Bacillus cereus*, *Staphylococcus aureus*) or by ingestion of sufficient number of viable pathogenic bacteria (*Listeria*, *Salmonella*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Shigella*) or by microbial decarboxylation of histidine to the allergic histamine. Pathogenic bacteria enter the fish/shrimp either due to human/animal faecal pollution, from microorganisms autochthonous to the aquatic environment or as a result of growth of microorganisms due to improper post-harvest handling.

Infectious dose of enteric pathogens:

Organism	**Estimated minimum infectious dose**
Bacteria	
Campylobacter jejuni	1000
Salmonella	10^4 - 10^{10}
Shigella dysenteriae	10 - 10^4
E. coli	10^6 – >10^7
E. coli $O_{157}H_7$	10 - 100
Staphylococcus aureus	10^5 - > 10^6
Vibrio cholerae	1000
Vibrio parahaemolyticus	10^6 - 10^9
Yersinia enterocolitica	10^7
Bacillus cereus	10^4 - 10^8
Clostridium botulinum	10^3
Clostridium perfringens	10^6 - 10^7
Viruses	
Hepatitis A	<10
Norwalk-like virus	<10

The presence of pathogens make the fish/shrimp product unfit for export. Rejections of Indian fish/shrimp products due to the presence of pathogens such as *Vibrio cholerae*, *Vibrio parahaemolyticus, Salmonella* have been reported from the European Union and USA.

Conventional methods or traditional methods for the detection of pathogenic bacteria refer to those methods which are in commonly use. They involve homogenizing solid food sample using a stomacher blender, pre-enrich to allow sub-lethally injured cells to repair their damaged membranes and metabolic pathways, enrich the target organisms using enrichment media which encourages the growth of the target organisms and suppresses the growth of other microorganisms, and inoculate on specific selective agar. Suspected colonies are confirmed by performing a battery of biochemical tests.

Rapid methods are alternatives to the conventional methods and are designed to obtain the end result in less laboratory time. Major improvements have been in three areas:

- sample preparation – agar slides
- separation and concentration of target cell – immunomagnetic separation, direct epifluorescent filter technique.
- Rapid end detection
    - Better media design
    - Assessment of hygiene using ATP bioluminescence for monitoring (1pg ATP=1000 bacterial cells; 10^{-15} g ATP per cell
    - Antibody based detection systems – ELISA, Latex agglutination
    - Nucleic acid based detection- probes, PCR, multiplex PCR, Real time PCR, gene chips

11. SALMONELLA

Salmonella is a Gram negative, rod shaped, non-spore forming bacterium, causes Typhoid fever in humans. Sources are animals, especially in poultry and swine. Environmental sources of the organism include water, soil, insects, factory surfaces, kitchen surfaces, animal feces,

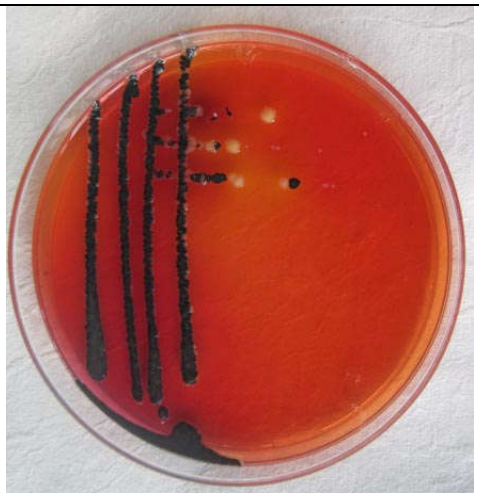
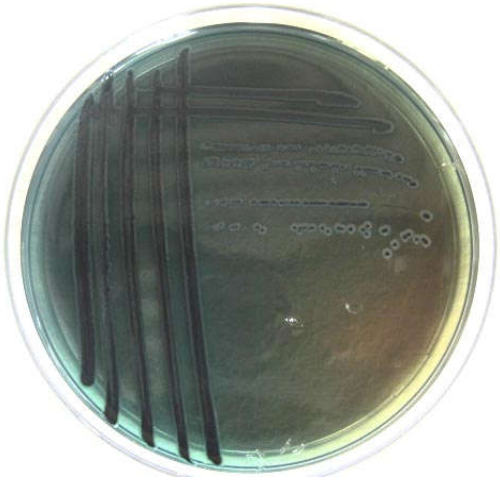
Method:

	Aseptically cut and weigh 25 g of shrimp/fish sample and put it into sterile stomacher bag.		
Primary Enrichment *(**Note**: Sterilize 225 ml of lactose broth in a 500ml flask)*	Homogenize with add 225 ml sterile lactose broth using a stomacher blender.		
	Aseptically transfer homogenized mixture back into the 500 ml flask		
	Incubate the sample mixture at 35°C for 24 h		
Selective enrichment			
	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
Selective plating *(**Note**: Prepare BSA plates one day before streaking and store in dark at room temperature until streaked).*	Mix and streak a loopuf of growth from TTB on Bismuth Sulfite agar (BSA), Hektoen enteric agar (HEA) and Xylose Lysine desoxycholate (XLD) agar.		Mix and streak a loopuf of growth from RV medium on Bismuth Sulfite agar (BSA), Hektoen enteric agar (HEA) and Xylose Lysine desoxycholate (XLD) agar.

	Incubate plates at 35°C for 24 ± 2 h		Incubate plates at 35°C for 24 ± 2 h
Examine all the plates for presence of Salmonella colonies			
Bismuth Sulfite agar BSA	Xylose Lysine desoxycholate XLD agar	HEA Hektoen enteric agar	
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	Pink colonies with or without black centers.	Blue-green to blue colonies with or without black centers.	
.	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 centers on HE* ***XLD agars:*** *yellow colonies with or without black centers* ***BS agar:*** *Green colonies with little or no darkening of the surrounding medium.*	
	Lightly touch the very center 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	***Note****: Store picked selective agar plates at 5-8°C.*	

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

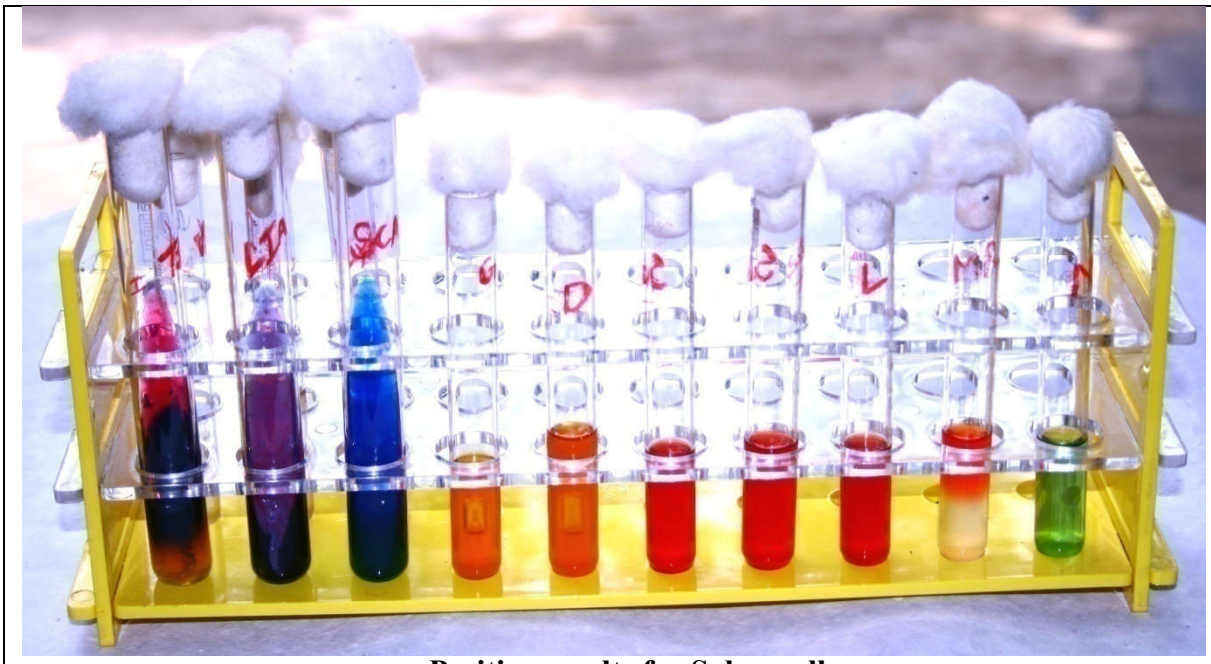
 Xylose Lysine desoxycholate (XLD) agar*:* Pink colonies with or without black centers.	 Hektoen enteric agar (HEA) Blue-green to blue colonies with or without black centers
 Bismuth Sulfite agar (BSA) Brown, gray, or black colonies; sometimes they have a metallic sheen. Surrounding	

medium is usually brown at first, but may turn black in time with increased incubation	

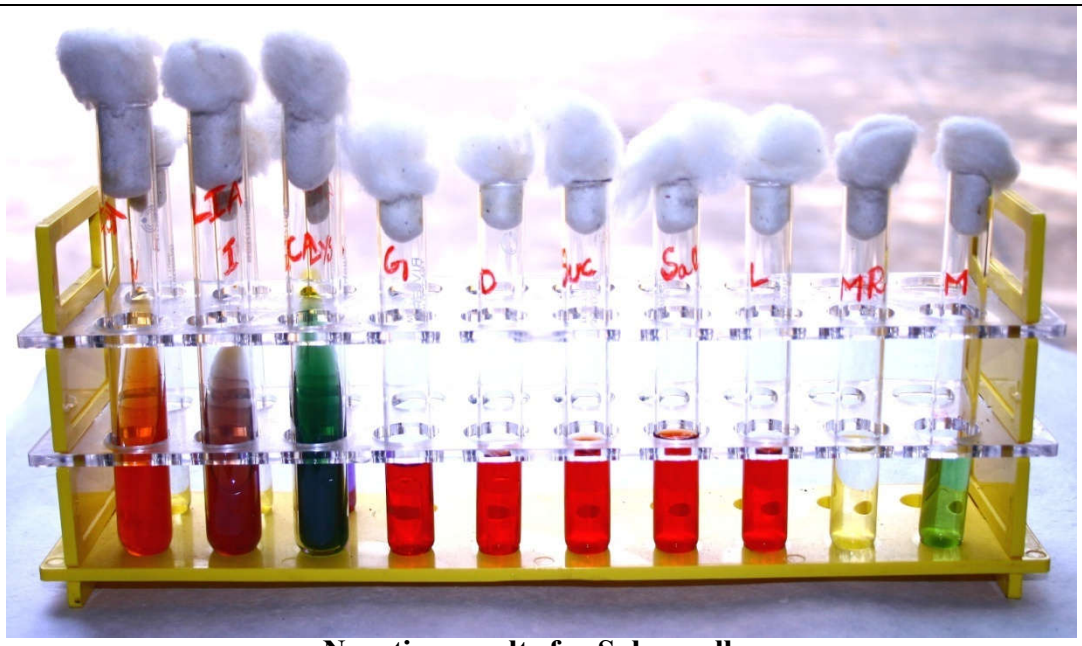
Table : Biochemical and serological reactions of *Salmonella*

Biochemical test	***Test Result***		***Reaction result for Salmonella species***
	Positive test result	***Negative test result***	
TSI	*Yellow Butt*	*Red Butt*	Yellow Butt
LIA	*Purple Butt*	*Yellow 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	growth; blue color
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



Positive results for Salmonellas



Negative results for Salmonella

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 = 25g**; 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 **375g 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**

<u>Sample plan for Raw frozen shrimps</u>:

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

Homogenize the 375g of sample with 3375ml of lactose broth and proceed further for Salmonella testing.

Salmonella in different fishery products meant for export (EIC of India)

S.O. 729 (E) dated 21st August 1995; subsequently amended vide No. Orders S.O. 792 (E) dated 17th August 2001, S.O. 722 (E) dated 10th July 2002, S.O. 464 (E) dated 24th April 2003, S.O. 1227 (E) dated 23rd October 2003 & S.O. 1227 (E) dated 31st July 2006.

	Fresh/ Chilled/ Frozen	**Cooked / boiled**
Shrimps / Prawns	Absent in 25g	Absent in 25g
Fish: whole, dressed, fillets or any other type (pomfrets, seer, pearl spot, sardine, mackerel, hilsa etc.)	Absent in 25g	Absent in 25g
Crab and crab meat	Absent in 25g	Absent in 25g
Cephalopods (cuttle fish, squid, octopus.) and their body parts including roes	Absent in 25g	Absent in 25g
Clam/ Mussel meat	Absent in 25g	Absent in 25g

Salmonella requirements for sea foods
Food Safety and Standards Authority of India (FSSAI).
***FSS (Food product standards and food additives) regulation 2011*, Part II, 496 pp.**

Frozen shrimps or prawns (Raw)	Absent in 25g
Frozen shrimps or prawns (Cooked)	Absent in 25g
Frozen fish	Absent in 25g
Frozen fish fillets, or minced fish flesh, or mixtures thereof	Absent in 25g
Salted fish / dried salted fish	Absent in 25g
Canned fish, canned shrimp, canned crab meat	Absent in 25g

12. *Vibrio cholerae*

Bergey's manual of Systematic Bacteriology (2005) lists 44 species under the genus Vibrio 12 are pathogenic to humans viz., *V.cholerae, V.vulnificus*, *V.parahaemolyticus*, *V.furnissi*, *V.metschnikovii, V.cincinnatiensis*, *V.alginolyticus*, *V.mimicus*, *V.fluvialis*, *V.hollisae*, *V.damsela* and *V.harveyi*.

The Gram negative, non-spore forming, comma shaped bacterium, *V. cholerae* is the etiological agent of the dreaded disease 'cholera'. The World Health Organization (WHO) defines cholera as "an acute intestinal infection caused by ingestion of food or water contaminated with the bacterium *Vibrio cholerae*. *V.cholerae* causes profuse watery diarrhea, vomiting, and muscle cramps. The diarrhea has a characteristic "rice water" appearance. Cholera enterotoxin (CT) is the primary virulence factor of the disease cholera.*V. cholerae* is excreted in great numbers in the feces of cholera patients and convalescents. The disease is transmitted primarily by the fecal-oral route, indirectly through contaminated water supplies.

Serogroups of V. cholerae

The antigenic structure of Vibrio species is defined on the major classes of bacterial antigens viz., somatic (O), capsular (K) and flagellar (H) associated with the bacterial lipopolysaccharide, capsule and flagellum. Strains of *V.cholerae* are divided into 2 serogroups designated as *V.cholerae* O1 and *V.cholerae* non O1. Non O1 *V.cholerae* are morphologically and biochemically similar to *V. cholerae* O1 but do not agglutinate with group O1 specific antiserum. Within *V.cholerae* there are over 200 defined O antigens.In late 1992 epidemics of severe acute watery diarrohea clinically resembling cholera and mainly affecting adults was reported in Madras, India and in Southern Bangladesh (Ramamurthy *et al*., 1993). This bacterium did not belong to any of the 138 serogroups for *V. cholerae.* described until then; the conclusion was it belonged to a new group - O139. Genetic and phenotypic evidence strongly suggests that the O139 strain arose from a *V.cholerae* O1 strain probably El Tor biotype by horizontal gene transfer. Only serogroups O1 and O139 contain all strains have caused all large epidemics and pandemics of cholera. Strains of *V.cholerae* O1 have been further differentiated into 3 serotypes designated Ogawa, Inaba and Hikojima, which have antigenic formulae of AB, AC and ABC types.

Biotypes of *V.cholerae* O1:

Classical biotype: does not haemolyse red blood cells, does not agglutinate chicken red blood cells, Voges-Proskauer test negative, inhibited by polymyxin B (50-unit disc), lysed by Classical IV and FK bacteriophages. The sixth pandemic classical strains are no longer isolated from patients or the environment. El Tor biotype: hemolyses red blood cells, agglutinates chicken red blood cells, Voges-Proskauer test positive, not inhibited by polymyxin B (50-unit disc, lysed by El Tor 5 bacteriophage, continues to cause cholera outbreaks. Atypical El Tor: Recent years have seen the emergence of new variants of *V. cholerae* O1 that have traits of both the classical and El Tor (eg. PB-sensitive El Tor strains)

Method:

Aseptically cut and weigh 25 g of shrimp/fish sample and put it into sterile stomacher bag.

↓

Primary Enrichment *(**Note**: Sterilize 225 ml of APW in a 500ml flask)*	Homogenize with add 225 ml of APW (Alkaline Peptone Water) using a stomacher blender.	
	↓	
	Aseptically transfer homogenized mixture back into the 500 ml flask	
	↓	
	Incubate the sample mixture at 35°C ±2°C	
	↓	
Selective enrichment		
	After 6 to 8 h incubation*	Re-incubate the flask overnight*
	↓	↓
Prepare dried plates of TCBS	Transfer a loopful from the surface pellicle of APW	Transfer a loopful from the surface pellicle of APW culture

agar	culture to the surface of a dried TCBS plate and streak in a manner that will yield isolated colonies	to the surface of a dried TCBS plate and streak in a manner that will yield isolated colonies
	⬇	⬇
	Incubate TCBS plates at 35°±2°C for 18 to 24h	Incubate TCBS plates at 35°±2°C for 18 to 24h
	⬇	⬇

Examine the TCBS plates for presence of V.cholerae colonies

⬇

Large (2 to 3 mm), smooth, yellow and slightly flattened with opaque centers and translucent peripheries.

⬇

Purify colonies by streaking on a non-selective agar (T_1N_1, T_1N_3, or TSA-2% NaCl agar).

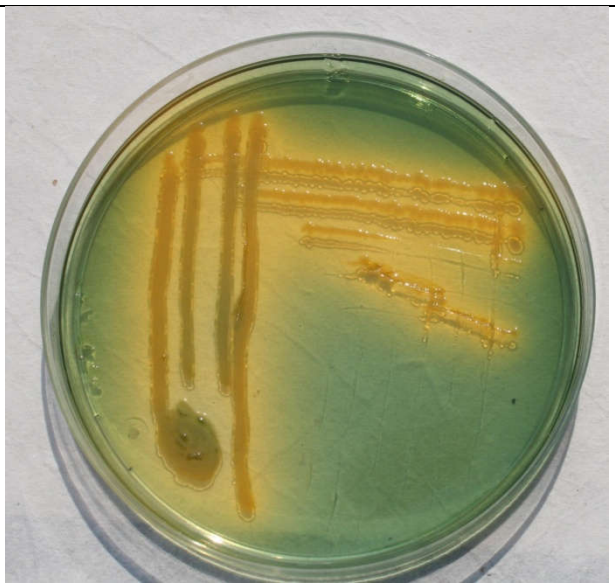
⬇

Incubate overnight at 35 ±2° C

⬇

Proceed with biochemical and serological identification.

**(Note: APW provides suitable enrichment for incubation periods of 6 to 8 h, but other competing microflora may overgrow V. cholerae during longer enrichment periods for certain types of samples. If the product was subjected to a processing step, i.e. heating, freezing, drying, or low densities are expected, incubation overnight is recommended to thoroughly resuscitate injured cells.So it is recommended to streak APW enrichments both after 6 to 8 h and after overnight incubation).*



Vibrio cholerae colonies- yellow and slightly flattened with opaque centers and translucent peripheries

Table: Biochemical characteristics of *Vibrio cholerae*

TCBS Agar		Yellow Colony
Oxides test		+
Arginine dihydrolase		-
Ornithine decarboxylase		+
Lysine decarboxylase		+
Growth in NaCl (w/v)	0%	+
	3%	+
	6%	-
	8%	-
	10%	-
Growth at 42°C		+
Production of acid	Sucrose	+
	D-cellobiose	-

	Lactose	-
	Arabinose	-
	D-Mannose	+
	D-Mannitol	+
ONPG test		+
Voges-Proskauer test		variable
Sensitivity to 0/129	10 μg O/129	S
	150 μg O/129	S
Gelatinase		+
Urease		-

***Vibrio cholerae* requirements for sea foods**
Food Safety and Standards Authority of India (FSSAI).
***FSS (Food product standards and food additives) regulation 2011*, Part II, 496 pp.**

Frozen shrimps or prawns (Raw)	Absent in 25g
Frozen shrimps or prawns (Cooked)	Absent in 25g
Frozen fish	Absent in 25g
Frozen fish fillets, or minced fish flesh, or mixtures thereof	Absent in 25g
Salted fish / dried salted fish	Absent in 25g
Canned fish, canned shrimp, canned crab meat	Absent in 25g

13. *Vibrio parahaemolyticus*

V. parahaemolyticus is a halophilic estuarine organism found in coastal waters. *V. parahaemolyticus* causes gastroenteritis. Most clinical isolates of *V. parahaemolyticus* are differentiable from environmental strains by their ability to produce a thermostable direct hemolysin (TDH), termed the Kanagawa phenomenon.

Quantitative method: MPN method to *analyse the fish/shrimp sample for number of V.parahaemolyticus* present in the fish/shrimp sample

MPN Method

Aseptically cut and weigh 50 g of shrimp/fish sample and put it into sterile stomacher bag.
(For fish -obtain surface tissues, gills, and gut of fish
For crustaceans such as shrimp, use the entire animal if possible;
if it is too large, select the central portion including gill and gut)

↓

Prepare 1:10 (10^{-1}) dilution by homogenizing
with 450 ml of PBS in a stomacher blender
(*Phosphate Buffered Saline*)

↓

Prepare 1:100 (10^{-2}) dilution by transferring
10ml from 10^{-1} to 90 ml of sterile PBS

↓

Inoculate 10 ml of 10^{-1} dilution to 3 tubes (10 ml to each tube)
containing 10 ml double strength APW.
(This represents the 1 g portion)

↓

Inoculate 1 ml of 10^{-1} 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 10^{-2} dilution to 3 tubes (1 ml to each tube)
containing 10 ml of single strength APW.
(This represents the 0.01 g portion)

↓

Incubate APW tubes overnight at 35 ±2°C

↓

Streak a loopful from the top 1 cm of APW tubes onto TCBS agar
(*9 TCBS plates are required*)

↓

Incubate TCBS plates overnight at 35 ±2°C

↓

V. parahaemolyticus colonies appear as round, opaque,
green or bluish colonies, 2 to 3 mm in diameter

↓

Purify colonies by streaking on a non-selective agar
(T_1N_3, or TSA-2% NaCl agar).

↓

Incubate overnight at 35 ±2° C

↓

Proceed with biochemical identification.

↓

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 1g, 0.1g and 0.01g).
Give the result as *V. parahaemolyticus* MPN per gram.

Vibrio parahaemolyticus colonies - **round, opaque, green or bluish colonies, 2 to 3 mm in diameter**

Table: Biochemical characteristics of *Vibrio parahaemolyticus*

TCBS Agar		Green Colony
Oxides test		+
Arginine dihydrolase		-
Ornithine decarboxylase		+
Lysine decarboxylase		+
Growth in NaCl (w/v)	0%	-
	3%	+
	6%	+
	8%	+
	10%	-
Growth at 42°C		+
Production of acid	Sucrose	-
	D-cellobiose	variable
	Lactose	-
	Arabinose	+
	D-Mannose	+
	D-Mannitol	+

ONPG test		-
Voges-Proskauer test		-
Sensitivity to 0/129	10 µg O/129	Resistant
	150 µg O/129	Sensitive
Gelatinase		+
Urease		variable

Qualitative method: to test the sample for *the presence or absence of V. parahaemolyticus in 25 grams* of the sample

Aseptically cut and weigh 25 g of shrimp/fish sample and put it into sterile stomacher bag. ↓
Homogenize with 225 ml of APW containing 3% NaCl ↓
Incubate overnight at 35 ±2°C ↓
Streak a loopful from the top 1 cm of APW growth onto TCBS agar ↓
Incubate TCBS plates overnight at 35 ±2°C ↓
V. parahaemolyticus colonies appear as round, opaque, green or bluish colonies, 2 to 3 mm in diameter ↓
Purify colonies by streaking on a non-selective agar (T_1N_3, or TSA-2% NaCl agar). ↓
Incubate overnight at 35 ±2° C ↓
Proceed with biochemical identification as mentioned above.

***Vibrio parahaemolyticus* requirements for sea foods**
Food Safety and Standards Authority of India (FSSAI).
***FSS (Food product standards and food additives) regulation 2011*, Part II, 496 pp.**

Frozen shrimps or prawns (Raw)	Absent in 25g
Frozen shrimps or prawns (Cooked)	Absent in 25g
Frozen fish	Absent in 25g
Frozen fish fillets, or minced fish flesh, or mixtures thereof	Absent in 25g
Salted fish / dried salted fish	Absent in 25g
Canned fish, canned shrimp, canned crab meat	Absent in 25g

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

1g	0.1g	0.01g	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

14. *Listeria* monocytogenes

Listeriaare Gram positive, motile, non-spore forming bacteria. The genus *Listeria* contains 6 species namely *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi.* Only *L. monocytogenes* is commonly associated with human listeriosis. *L. monocytogenes* is quite hardy and resists the deleterious effects of freezing, drying, and heat. Its ability to grow at temperatures as low as 3°C permits multiplication in refrigerated foods. The manifestations of listeriosis include septicemia, meningitis (or meningoencephalitis), encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (2nd/3rd trimester) or stillbirth.

***Method*:**

Aseptically cut and weigh 25 g of fish / shrimp sample

⇓

Homogenize with 225 ml of buffered Listeria Enrichment Broth (BLEB)

⇓

Incubate at 30°C for 4 hours

⇓

Add the selective agents

(supplements – acriflavin, nalidixic acid, cyclohexamide)

⇓

Continue incubation at 30° C for a total of 48 h

⇓

Streak on selective agar

(either OXA or PALCAM or MOX or LPM fortified with esculin and Fe^{3+}

⇓

Incubate OXA, PALCAM or MOX plates at 35° C for 24-48 h;

fortified LPM plates at 30° C for 24-48 h

⇓

Listeria colonies are black with a black halo

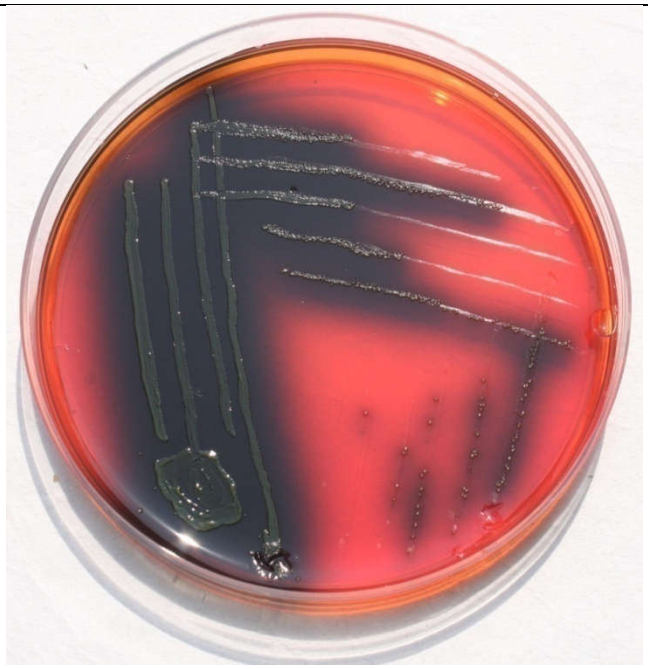
⇓

Transfer typical colonies from OXA and PALCAM or modified LPM or MOX

to Trypticase soy agar streaking for purity and typical isolated colonies

⇓

Proceed with biochemical identification



Listeria **colonies -black with a black halo**

Biochemical tests for Listeria

Gram Staining	Gram positive rods
Motility	Motile
Methyl Red Test	+
Voges-Proskauer	+
Urease test	-
Catalase test	+
	Note: gas evolution is slow, observe under microscope)
Oxidase test	-
Esculin hydrolysis	+
Acid from glucose	+
	(no gas)
Acid from salicin	+

Tests for confirmation of *Listeria monocytogenes*

Nitrate reduction	-
Acid from D-Mannitol	-
Acid from L-Rhamnose	+
Acid from D-Xylose	-
Acid from α-methyl D-mannopyranoside	+
B-haemolysis on blood agar	+

***Test for haemolysis*:** Inoculate the culture from TSA agar on the surface of pre-dried blood agar plates and incubate at 35°C for 18 to 24 hours. 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.

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.

CAMP test haemolytic enhancements for Listeria species

	Staphylococcus aureus	***Rhodococcus equi***
L. monocytogenes	+	-
L. ivanovii	-	+
L. innocua	-	-
L. welshimeri	-	-
L. seeligeri	+	-

15. *Staphylococcus aureus*

S. aureus is a Gram positive, spherical bacterium (coccus). Human intoxication is caused by ingesting enterotoxins produced in food by some strains of *S. aureus*. A toxin dose of less than 1.0 microgram in contaminated food will produce symptoms of staphylococcal intoxication. This toxin level is reached when *S. aureus* populations exceed 1,00,000 per gram. Staphylococci exist in air, dust, sewage, water, milk, and food or on food equipment, environmental surfaces, humans, and animals. Humans and animals are the primary reservoirs. Staphylococci are present in the nasal passages and throats and on the hair and skin. Although food handlers are usually the main source of food contamination in food poisoning outbreaks, equipment and environmental surfaces can also be sources of contamination.

Transfer the 50g to a stomacher bag and homogenize with 450 ml of diluent (Butterfields phosphate-buffered dilution water) using a stomacher blender. The resultant homogenized material is 1 : 10 dilution or 10^{-1} dilution which means that 1 gram of food sample is present in 10ml of the homogenized material.

Method:

Melt sterile Baird-Parker agar, cool to 45-50C 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 fish / shrimp sample

⇩

Homogenize with 450 ml of Butterfields phosphate-buffered dilution water
using a stomacher blender. This is 10^{-1} dilution (0.1 gram per ml)

⇩

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.

Examine the BP plates for typical *S.aureus* colonies

S. aureus colonies are 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

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*/g**

Coagulase test:

Coagulae plasma is used to qualitatively determine the pathogenecity 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°C for 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°C and examine periodically over 6 h period for clot formation.
- Only firm and complete clot that stays in place when tube is tilted or inverted is considered positive for *S. aureus*.

- Partial clotting must be tested further by performing Grams staining, catalase test, anaerobic utilization of glucose, anaerobic utilization of mannitol and thermostable nuclease production.
- Staphylococci aureus appears as Gram +ve cocci, catalase +ve, produce thermonuclease and utilize glucose and mannitol anaerobically.

Catalase test: Use growth from TSA slant for catalase test on glass slide or spot plate, and illuminate properly to observe production of gas bubbles.

Anaerobic utilization of glucose: Inoculate tube of carbohydrate fermentation medium containing glucose (0.5%). Immediately inoculate each tube heavily with wire loop. Make certain inoculum reaches bottom of tube. Cover surface of agar with layer of sterile paraffin oil at least 25 mm thick. Incubate 5 days at 37°C. Acid is produced anaerobically if indicator changes to yellow throughout tube, indicating presence of *S. aureus*.

Anaerobic utilization of mannitol: Repeat 2, above, using mannitol as carbohydrate in medium. *S.aureus* is usually positive but some strains are negative.

Thermostable nuclease production: Prepare microslides by spreading 3 ml toluidine blue-deoxyribonucleic acid agar on the surface of each microscope slide. When agar has solidified, cut 2 mm diameter wells (10-12 per slide) in agar and remove agar plug by aspiration. Add about 0.01 ml of heated sample (15 min in boiling water bath) of broth cultures used for coagulase test to well on prepared slide. Incubate slides in moist chamber 4 h at 35°C. Development of bright pink halo extending at least 1 mm from periphery of well indicates a positive reaction.

16. Colony Characteristics of bacteria

	Bacteria	**Agar**	**Colony Characteristics**
1	*Salmonella*	Xylose Lysine desoxycholate agar (XLD agar)	Pink colonies with or without black centers
2	*Salmonella*	Hektoen enteric agar (HEA)	Blue-green to blue colonies with or without black centers
3	*Salmonella*	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
4	*Stapylococcus aureus* (coagulase positive Staphylococci)	Baird-Parker agar (BP agar)	colonies are 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
5	*Listeria*	PALCAM Listeria Agar (Polymyxin-B-sulfate, **A**criflavine-HCl, **L**ithium chloride, **C**eftazidime, **A**esculin, and **M**annitol)	gray-green colonies surrounded by dark brown to black halos in the medium

6	*Vibrio cholerae*	Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS agar)	Large (2 to 3 mm), smooth, yellow and slightly flattened with opaque centers and translucent peripheries
7	*Vibrio parahaemolyticus*	Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS agar)	round, opaque, green or bluish colonies, 2 to 3 mm in diameter
8	*Coliforms*	Violet Red Bile Agar (VRBA)	purple-red colonies that are 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids
9	*E.coli*.	Eosin Methylene Blue (EMB) agar	colonies appear with a greenish metallic sheen by reflected light and dark centred flat colonies by transmitted light.
10	*Enterococci*	m-Enterococcus agar	light and dark red colonies
11	*Clostridium perfringens*	Membrane Clostridium perfringens agar (m-CP agar)	opaque yellow colonies on m-CP agar. *Clostridium perfringens* colonies turn pink or red as phenolphthalein diphosphate is cleaved by acid phosphatase

EU directives pertaining to Food and Feed

1	Food Law	(EC) 178/2002
2	General Hygiene Criteria Reg.	(EC) 852/2004
3	Specific Hygiene Criteria Reg.	(EC) 853/2004
4	Official control of Feed and Food General Reg.	(EC) 882/2004
5	Official Control of Feed and Food of Animal origin Reg.	(EC) 854/2004
6	Water Quality Directive	98/83/EC
7	Microbial and Chemical criteria Reg.	2073/2006, (EC) 1881/2006, (EC) 2074/2005
8	Food Additives Reg.	(EC) 1333/2008
9	Labelling Reg.	(EC) 1169/2011
10	Requirements to sampling and testing Reg.	(EC) 333/2007 and (EC) 589/2014
11	Minimum required performance limits (MRPLs) for certain residues in food of animal origin (**Antibiotics**)	2003/181/EC
12	sampling methods and the methods of analysis for the official control of the levels of **benzo(a)pyrene** in foodstuffs	2005/10/EC
13	Amending Regulation (EC) No 466/2001 as regards **heavy metals**	(EC) No 78/2005
14	setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin (**malachite green and leucomalachite green)**	2004/25/EC
15	Fixing the total volatile basic nitrogen (TVB-N) limit values for certain categories of fishery products	95/149/EC
16	Food additives other than colours and sweeteners	**95/2/EC**
17	Feed hygiene Reg.	(EC) 183/2005
18	Veterinary Medicines Dir.	82/2001/EC
19	Aquatic Animal Health and disease control Dir.	88/2006/EC
20	Animal by-product rules Reg.	(EC) 1069/2009 and Reg. (EC) 142/2011

Government of India notifications related to fish and fishery products

1	Notifications of Fresh, Frozen and Processed Fish & Fishery Products Export Inspection Council (Ministry of Commerce and Industry, Govt. of India) • S.O. 730 (E) dated 21st August 1995 Subsequently amended vide No. Notifications • S.O. 415 (E) dated 11th April 2002, • S.O. 1029 (E) dated 24th September 2002, • S.O. 1034 (E) dated 9th September 2003, • S.O. 717 dated 25th February 2005, • S.O. 612 dated 15th February 2007, • S.O. 1519 dated 16th June 2008, • S.O. 2714 (E) dated 28th October 2009 & • S.O. 143 (E) dated 21.1.2011 • S.O. 497(E) dated 10.3.2011
2	Orders of Fresh, Frozen and Processed Fish & Fishery Products , Products Export Inspection Council (Ministry of Commerce and Industry, Govt. of India) • S.O. 729 (E) dated 21st August 1995 Subsequently amended vide No. Orders ▪ S.O. 792 (E) dated 17th August 2001, ▪ S.O. 722 (E) dated 10th July 2002, ▪ S.O. 464 (E) dated 24th April 2003, ▪ S.O. 1227 (E) dated 23rd October 2003 & ▪ S.O. 1227 (E) dated 31st July 2006.
3	FOOD SAFETY AND STANDARDS (CONTAMINANTS, TOXINS AND RESIDUES) REGULATIONS, 2011 Ministry of Health and Family Welfare, (Food Safety and Standards Authority of India) NOTIFICATION, New Delhi, dated the 1st August, 2011
4	FOOD SAFETY AND STANDARDS (FOOD PRODUCT STANDARDS AND FOOD ADDITIVES) THIRD AMENDMENT REGULATION, 2017: Ministry of Health and Family Welfare, (Food Safety and Standards Authority of India) NOTIFICATION, New Delhi, the 13th February, 2017. This regulation prescribes revised microbiological requirements for sea foods and covers new varieties of fish and fisheries products, maximum permissible limits of hygiene indicator organisms and safety indicator organisms in vide range of fish and fishery products. These standards have been finalised after consideration of the comments received from stakeholders in this respect and shall come into force on 1st January, 2018. The revised microbiological standards are aimed at ensuring quality and food safety of sea food produce in the domestic market.

Websites related to seafood industry:

1	**CIFT** :Central Institute of Fisheries Technology	http://www.cift.res.in/
2	**ICAR**: Indian Council of Agricultural Research	http://www.icar.org.in/
3	**EIC**: Export Inspection Council of India	http://www.eicindia.gov.in/
4	**MPEDA**: Marine Products Export Development Authority	http://www.mpeda.com/
5	**FSSAI** : Food Safety Standards Authority of India	http://www.fssai.gov.in/
6	**BIS**: Bureau of Indian Standards	http://www.bis.org.in/
7	European Union directives	http://eur-lex.europa.eu/en/index.htm
8	**RASFF**: rapid alert system for food and feed	http://ec.europa.eu/food/food/rapidalert/index_en.htm
9	**USFDA**: United States Food and Drug Administration	http://www.fda.gov/
10	**BAM**: Bacteriological Analytical Manual	http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm
11	Seafood Network Information Centre	http://seafood.ucdavis.edu/
12	The Australia and New Zealand Food Standards Code	http://www.foodstandards.gov.au/
13	Russia- Federal Service for Veterinary and Phytosanitary Surveillance	www.fsvps.ru

SUGGESTED READING/REFERENCES:

1. *Bacteriological Analytical Manual*. USFDA-BAM US Food and Drug Administration. Bacteriological Analytical Manual Online. **http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm2006949.htm**
2. *Bad Bug Book*: Foodborne Pathogenic Microorganisms and Natural Toxins Handbook. U.S. Food and Drug Administration

   http://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf

3. European Union – http://eur-lex.europa.eu/en/index.htm

   Commission regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs
   Council directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official Journal of the European Communities, L 330/32

4. Export Inspection Council. (2012) *Executive instructions for approval and monitoring of fish & fishery products for export*. Document No. EIC/F&FP/Ex.Inst./March /2012/Issue 4. Export Inspection Council, Ministry of Commerce & Industry, Govt. of India, www.eicindia.gov.in

5. *Fish and Fishery Products Hazards and Controls Guidance* - Fourth Edition – APRIL 2011. Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Food Safety. http://www.fda.gov/downloads/food/guidanceregulation/ucm251970.pdf

6. Food safety and standards (contaminants, toxins and residues) regulations, 2011. Food Safety and Standards Authority of India, Ministry of Health and Family welfare, Govt. of India. Notification dated the 1st August, 2011. http://www.fssai.gov.in/
7. *Practical Medical Microbiology* (Collee, J.G., Duguid, J.P., Fraser, A.G. and Marmion, B.P. Eds), Churchill Livingstone, UK
8. Atlas, M.R. (2004). *Handbook of Microbiological Media*. 3rd edn. CRC Press, LLC, USA.
9. Huss, H.H., Ababouch, L. and Gram, L. (2004) Assessment and management of seafood safety and quality. FAO fisheries technical paper. No. 444, Food and Agricultural Organization, Rome

10. Forsythe, S.J. (2000).*The Microbiology of safe food*. Blackwell Science, London.

11. Surendran, P.K., Thampuran, N., Nambiar, V.N., and Lalitha, K.V. (2006). *Laboratory Manual for microbiological examination of seafoods*. CIFT, Cochin, India.

Compendium of Fish and Fishery Products

Export Inspection Council

(Ministry of Commerce and Industry, Govt. of India)

Specification for shrimps/prawns

BACTERIOLOGY

Parameter	**Fresh/ Chilled/ Frozen**	**Cooked**
Total plate count at 37°C per g (max)	5,00,000	1,00,000
E. coli count per g (max)	20	Nil
Coagulase positive staphylococcus per g. (max).	100	100
Salmonella & S. arizona per 25 gm	Nil	Nil.

PESTICIDES

Pesticide	Maximum permissible residual level (ppm)
BHC	0.3
Aldrin	0.3
Dieldrin	0.3
Endrin	0.3
DDT	5.0

ANTIBIOTICS

Antibiotic	Maximum permissible residual level (ppm)
Chloramphenicol	Nil
Furazolidone	Nil
Neomycin	Nil
Tetracycline	0.1
Oxytetracycline	0.1
Trimethoprim	0.05
Oxolinic acid	0.3
Nalidixic acid	Nil
Sulphamethoxazole	Nil

HEAVY METALS

Heavy Metal	Maximum permissible residual level (ppm)
Mercury	1.0
Cadmium	3.0
Arsenic	75
Lead	1.5
Tin	250
Nickel	80
Chromium	12

MINISTRY OF HEALTH AND FAMILY WELFARE
(Food Safety and Standards Authority of India)

Food Safety and Standards (Food Products Standards and Food Additives) Third Amendment Regulations, 2017.

"Table 1A

Microbiological Requirements for Fish and Fishery products -Hygiene Indicator Organisms

Sl. No.	Product Category*	Aerobic Plate Count				Coagulase positive Staphylococci				Yeast &mold count				Stage where criterion applies	Action in case of unsatisfactory results
		Sampling Plan		Limits (cfu/g)		Sampling Plan		Limits (cfu/g)		Sampling Plan		Limits (cfu/g)			
		n	c	m	M	n	c	m	M	n	c	m	M		
1.	Chilled/Frozen Finfish	5	3	$5x10^5$	$1x10^7$	-	-	-	-	-	-	-	-	After Chilling/Freezing	Improvement in hygiene; Time-Temperature Control along value chain
2.	Chilled/Frozen Crustaceans	5	3	$1x10^6$	$1x10^7$	-	-	-	-	-	-	-	-	After Chilling/Freezing	Improvement in hygiene; Time-Temperature Control along value chain
3.	Chilled/Frozen Cephalopods	5	2	$1x10^5$	$1x10^6$	-	-	-	-	-	-	-	-	After Chilling/Freezing	Improvement in hygiene; Time-Temperature Control along value chain
4.	Live Bivalve Molluscs#	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5.	Chilled/Frozen Bivalves	5	2	$1x10^5$	$1x10^6$	-		-	-	-	-	-	-	After Chilling/Freezing	Improvement in hygiene; Time-Temperature Control along value chain
6.	Frozen Cooked Crustaceans/Frozen Heat Shucked Mollusc	5	2	$1x10^5$	$1x10^6$	5	2	$1x10^2$	$1x10^3$	-	-	-	-	End of Manufacturing process	Improvement in hygiene; Selection of raw material; Time-Temperature Control along value chain; process control
7.	Dried/Salted and Dried Fishery Products	5	0	$1x10^5$		-	-	-	-	5	2	100	500	End of Manufacturing process	Improvement in hygiene; Selection of raw material; Adequate drying (water activity ≤ 0.78)
8.	Thermally Processed Fishery Products	Commercially Sterile**				-	-	-	-	-	-	-	-	End of Manufacturing process	Revalidation of thermal process

** Commercial sterility should be established as per APHA (2015). Canned Foods—Tests for Commercial Sterility. Compendium of Methods for the Microbiological Examination of Food.

No hygienic indicators are currently prescribed for the Live Bivalve Molluscs

Table 1B

Microbiological Requirements for Fish and Fishery Products –Safety Indicator Organisms

Sl. No.	Product Category*	*Escherichia coli*				*Salmonella*				*Vibriocholerae* (01 and 0139)				*Listeria monocytogenes*				*Clostridium botulinum*			
		Sampling Plan		Limits (MPN/g)		Sampling Plan		Limits		Sampling Plan		Limits		Sampling Plan		Limits		Sampling Plan		limits (MPN /g)	
		n	c	m	M	n	c	m	M	n	c	m	M	n	c	m	M	n	c	m	M
1.	Chilled/Frozen Finfish	5	3	11	500	5	0	Absent/25g		5	0	Absent/25g		-	-	-	-	-	-	-	-
2.	Chilled/Frozen Crustaceans	5	3	11	500	5	0	Absent/25g		5	0	Absent/25g		-	-	-	-	-	-	-	-
3.	Chilled/frozen Cephalopods	5	0	20		5	0	Absent/25g		5	0	Absent/25g		-	-	-	-	-	-	-	-
4.	Live Bivalve Molluscs	5	1	230/ 100g	700/ 100g	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5.	Chilled/Frozen Bivalves	5	0	46		10	0	Absent/25g		5	0	Absent/25g		-	-	-	-	-	-	-	-
6.	Frozen cooked crustaceans/Frozen heat shucked mollusca	5	2	1	10	5	0	Absent/25g		5	0	Absent/25g		5	0	Absent/25g		-	-	-	-
7.	Dried/ Salted and dried fishery products	5	0	20		5	0	Absent/25g		-	-	-	-	-	-	-	-	-	-	-	-
8.	Thermally processed fishery products	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Absence of viable spores or vegetative cells of *Clostridium botulinum* and absence of botulinum toxin.			

Sampling Plan:

The terms n, c, m and M used in this standard have the following meaning:

n = Number of units comprising a sample.

c = Maximum allowable number of units having microbiological counts above m.

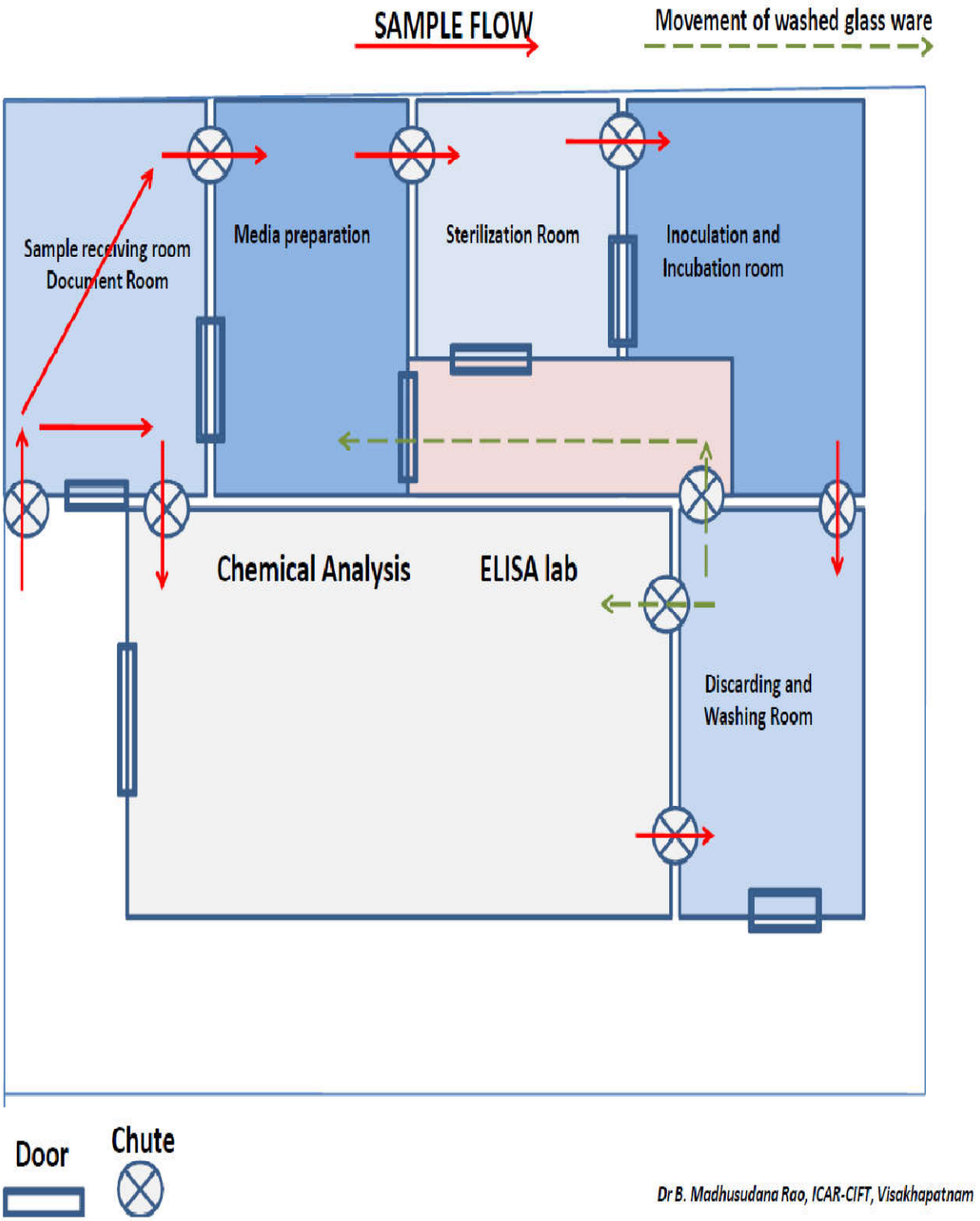
m = Microbiological limit that may be exceeded number of units c.

M = Microbiological limit that no sample unit may exceed.

List of Antibiotics and other pharmacologically active substances banned for using in shrimp aquaculture

Sl. No.	Antibiotics and other Pharmacologically Active Substances
1	Chloramphenicol
2	Nitrofurans including: Furaltadone, Furazolidone, Furylfuramide, Nifuratel, Nifuroxime, Nifurprazine, Nitrofurantoin, Nitrofurazone
3	Neomycin
4	Nalidixic acid
5	Sulphamethoxazole
6	Aristolochia spp and preparations thereof
7	Chloroform
8	Chlorpromazine
9	Colchicine
10	Dapsone
11	Dimetridazole
12	Metronidazole
13	Ronidazole
14	Ipronidazole
15	Other nitroimidazoles
16	Clenbuterol
17	Diethylstilbestrol (DES)
18	Sulfonamide drugs (except approved Sulfadimethoxine, Sulfabromomethazine and Sulfaethoxypyridazine)
19	Fluroquinolones
20	Glycopeptides

Layout of Laboratory (Microbiology and Chemical)



Fumigation of Microbiology Lab

Procedure

- Use safety goggles and hand gloves during fumigation.
- Put 'OFF' the A/C unit .
- Take Potassium Permanganate (about 15 gm.) in a petri dish and keep in the area where fumigation is to be carried out.
- Place the petri plate on a polyethene bag.
- Add about 25 ml of 35 % Formaldehyde solution to Potassium Permanganate . Immediately close the area.
- Label the area as "Area Under Fumigation" so that nobody enters the room.
- Keep the room under fumigation for a minimum of 8 - 12 hrs.
- Defumigate the room by putting on the A/C unit .

Frequency :

Once a week and after completion of any civil work.

Hazards of Formaldehyde:

- It is toxic to inhalation and ingestion and causes irritation to eyes and respiratory tract.
- Skin contact can cause dermatitis. Also cause skin burns.
- Exposure to a concentration of 10 to 20 PPM can cause difficulty in breathing.
- Slightly corrosive in nature, suspected carcinogen.

Biochemical quality indices

Viji P

During spoilage of fish, a number of chemical reactions take place in the fish muscle, producing lot of organic compounds which can be quantitatively measured and used to assess the quality of fish. These compounds are produced in fish muscle by autolytic enzymes, putrefactive microorganisms or by chemical reactions such as lipid oxidation. During the course of spoilage, these compounds gradually get accumulated in the flesh and their quantification is an important means to measure the progress of spoilage. Following is an overview of some of the most widely used chemical indices of seafood quality.

1. Total volatile base nitrogen (TVB-N)

TVBN is a measure of decomposition of proteins. It gives an index of volatile bases formed from solubilised nitrogen derivatives. TVB-N in fish is mainly composed of ammonia and primary, secondary and tertiary amines. Bacterial catabolism of aminoacids in fish muscle results in the accumulation of ammonia and other volatile bases. Ammonia and primary amines are bound by formalin, therefore this fraction is called the formalin bound nitrogen (FBN). The trimethyl amine (TMA) represents the fraction, which is not bound by formalin. The TVB-N value is used as an index of quality for deciding the state of freshness of fish (along with TMA). TVB-N gradually increases as the spoilage progresses. A level of 35-40 mg 1VB-N /100g of fish muscle is usually regarded as the limit of acceptability, beyond which the fish can be regarded as spoiled.

2. Trimethylamine (TMA)

Trimethyl amine nitrogen (TMA) is derived from trimethylamineoxide (TMAO) which is critical for osmo regulation in marine fish. During spoilage, TMAO is reduced by enzymes to TMA. TMAO is a tasteless non-protein nitrogen compound whose content varies with the season, size and age of fish. The concentration of amines in fish tissues is both time and temperature dependent and is related to the deterioration of fish. The determination of TMA as an indicator of freshness (actually of decay) has been a useful criterion for evaluating the quality of fish. TMA-N between 10-15 mg / 100g muscle is considered as the

limit of acceptability for round, whole chilled fish. This index is not suitable for freshwater fish and heat treated fish products.

3. Ammonia

Free amino acids produced by proteolytic enzymes are further broken down to ammonia. The amount of ammonia produced can also give an index of fish spoilage. In elsamobranchs, more ammonia is produced during spoilage as they have larger amounts of urea in muscles. Though ammonia has been identified as a volatile component in a variety of spoiling fish, it is often impossible to determine its relative contribution to the overall increase in total volatile bases. For squid, ammonia has been reported as an excellent quality indicator. However, ammonia can be better used as the quality indicator during advanced spoilage.

4.pH

As the fish spoils, a number of basic compounds are generated and hence, pH increases. Change in fish pH is also used as an index to measure the quality of fish. The postmortem pH limit of acceptability is usually 6.8~7.0.

5.Biogenic amines

Decarboxylation of amino acids by proteolytic bacteria leads to the formation of a wide variety of amine compounds. Most spoilage bacteria possessing decarboxylase activity do so in response to acidic pH, presumably so that the organisms may raise the pH of the growth medium through the production of amines. Biogenic amines are non-volatile compounds, which are found at very low level in fresh fish and their accumulation is related to bacterial spoilage which are thermally stable and can be used as indicator of poor quality of raw material in preserved products.

Histamine, putrescine, cadaverine and tyramine are produced from the decarboxylation of histidine, ornithine, lysine and tyrosine, respectively. Histamine has received most of the attention since it has been associated with incidents of scombroid poisoning in conjunction with the ingestion of tuna, mackerel, mahi-mahi. It is interesting to note that most of the biogenic amines are stable to thermal processing, so their presence in finished canned products is a good indication that the raw material was spoiled prior to

processing. Although the biogenic amines have been associated with fish spoilage legal limit has been established for histamine only. The European Union set a maximum average content of 100 mg/kg fish for canned products and trace for ripened products. The US Food and Drug administration lowered the limit from 100 to 50 mg/kg, recommending that not only histamine level but also other biogenic amine content had to be taken into account. India has also limited maximum permissible level for histamine content in frozen fish 200 mg/kg. Less than 5 mg/kg is considered safe for consumption; 5 – 20 mg/kg is safe; 20-100 mg/kg is probably safe and > 100 mg/kg is toxic and unsafe for consumption. Incidence of histamine poisoning after eating fish are mainly due to poor quality of raw material.

6. Nucleotide Catabolites

After the death of fish, ATP synthesis stops and hydrolysis begins. ATP is broken down to ADP, AMP, IMP and hypoxanthine by nuclease enzymes. The final stages of this process is the formation of a compound called hypoxamthine, which gradually increase with time and can be used as a measure of quality of fish. The nucleotide degradation products especially inosine monophosphate (IMP), hypoxantine (H_x) clearly reflects the quality loss in fish. The presence of higher levels of IMP in the muscle indicates relatively high quality, whereas accumulation of inosine and hypoxathine is an indicator of poor quality. Inosine has a sweet taste where as hypoxanthine tastes bitter. The amount of nucleotide degradation products is measured by the enzymic method or by High Pressure Liquid Chromatography (HPLC) method. K value is proposed as an index for assessing the freshness of fishHPLC method is used to determine the K value. K value as an index of estimating the freshness of fish has become widely used in Japan.

K value can be defined as,

$$K = \frac{H_xR + H_x}{H_xR + H_x + ATP + ADP + AMP + IMP} \times 100$$

Where H_xR = Inosine

H_x = Hypoxathine

ATP = Adenosines triphosphate

ADP = Adenosines diphosphate

AMP = Adenosines monophosphate

IMP = Iosines monophosphate

In a freshly caught fish, K value could be as low as 0, 10-20 in moderate quality fish and 80-90 and above in spoiled fish. K value is found to have good correlation with sensory data for a number of fish.

7. Indole

Estimation of indole gives an indication of decomposition in shrimp. Indole is a degradation product of tryptophan. Indole is measured by spectrophotometric method of AOAC. Indole is extracted with light petroleum from trichloroacetic acid precipitated shrimp muscle. The extracted indole is reextracted with Ehrlich's reagent. The acceptability limit of indole in shrimp is established as 25mg/100 g by USFDA.

8.Peroxide value

The highly unsaturated fatty acids found in fish lipids are very susceptible to oxidation. Fatty acids are oxidized to hydroperoxides, peroxides and then to aldehydes, ketones, alkenals etc. Peroxides value gives an index of primary lipid oxidation as it measures the amount of hydroperoxides generated during the chain reaction of lipid oxidation. The concentration of the hydroperoxides may be determined by titrimetric or by spectrophotometric methods, giving the peroxide value (PV) as milliequivalents (mEq) peroxide per 1 kg of fat extracted from the fish. The most common method is based on iodometric titration which measures the iodine produced from potassium iodide (KI) by the peroxide present in fat. PV is a good guide to assess the quality of fat. Fresh oil should have PV 1 mg.oxygen/kg. On storage it may increase to 10 mg/kg. Values above 20 give rancid smell.

9.Thiobarbituric Acid Value (TBA Value)

TBA index is the most used indicator for advanced/secondary lipid oxidation. The peroxides formed may break down to carbonyls, form polymers, or react with protein, vitamins, pigments etc. The most widely used test for measuring extent of oxidative deterioration of lipids in muscle foods is the 2- thiobarbituric acid test or TBA test, which expresses lipid oxidation in mg of malonaldehyde/Kg of the sample . Malonaldehyde was shown to be a secondary oxidation product of polyunsaturated fatty acids. TBA measures the

malonaldehyde produced during fat oxidation. TBA reacts specifically with malodadehyde to give a red chromogen which can be determined spectrophotometrically.

10.Free fatty acids (FFA)

Free fatty acids gives an index of lipid hydrolysis occurred in fish. Triglycerides are cleaved by lipolyitc enzymes to di and monoglycerides and glyceridesm making free fatty acids. Free fatty acids are suspected of deriving primarily from phospholipids, as the latter disappear with time of storage which can be affected by the action of bacteria, enzymes or non-enzymic catalysis. During spoilage, the amount of free fatty acids increases, which can be measured by reacting with alkali and is expressed as %oleic acid. Free fatty acids are mostly generated from phospholipids and are more prone to oxidation.

Protocol of biochemical quality indices

1.Total Volatile Base Nitrogen

Total volatile bases in the samples are determined as TVB-N by the micro diffusion method (Conway, 1950). For this, extract 5 gm of fish muscle with 10% trichloro acetic acid (TCA) and filter through Whatman No. 1filter paper. The filtrate is made up to 50 ml in a standard flask using distilled water. From the TCA extract, TVB-N is liberated by treating with alkali and absorbed in standard acid in Conway diffusion unit. Take 1 ml of standard N/100 sulphuric acid in the inner chamber of the diffusion unit. To the outer chamber, add 1 ml of TCA extract followed by 1 ml of saturated potassium carbonate. Seal the unit with the glass lid. Gently mix the unit to mix the solution in the outer chamber. Keep the unit undisturbed overnight. The amount of un reacted acid in the inner chamber can be determined by titrating against standard N/100 sodium hydroxide with tashiro's indictor. Similarly, a blank can be run using 1 ml of TCA solution without fish juice in the outer chamber. TVB-N is calculated and expressed as mg % of the sample.

1 ml of N/100 H_2SO_4 = 0.l4 mg of nitrogen

$$\text{TVB-N (mg\%)} = \frac{\text{(Blank-Titre value)x 0.14x 100x 100}}{\text{Wt. of sample x volume pipetted}}$$

2.Tri methyl amine nitrogen (TMA-N)

TMA-N is also determined by the micro diffusion method (Conway, 1950). Take 1 ml of standard N/100 sulphuric acid in the inner chamber of the conway diffusion unit. In the outer chamber, add 1 ml of TCA extract followed by 1 ml neutralized formaldehyde and 1 ml saturated potassium carbonate. TMA is liberated while all the other volatile bases will be held back by formaldehyde. TMA is absorbed by the standard acid and is measured by titration with acid as explained for TVB-N. TMA-N is calculated and expressed as mg/100 g of the sample.

$$\text{TMA-N (mg\%)} = \frac{\text{(Blank-Titre value)x 0.14x 100x 100}}{\text{Wt. of sample x volume pipetted}}$$

3.Determination of TBARS

TBARS of the fish muscle is determined according to the procedure of Tarladgis *et al.* (1960). Weigh 10 gm of the ground muscle accurately and blend with 50 ml of distilled water using a mortar and pestle. Wash out the mixture into a 250 ml flask with 47.5 ml distilled water followed by 2.5 ml of 4 N HCl. Connect the flask to a distillation unit and heat by an electric mantle in such a way that 50 ml of the distillate is collected in 10 min. Transfer 5 ml of the distillate in a stoppered tube and add 5 ml of TBA reagent. Placer the tubes in boiling water for exactly 35 minutes. Prepare the blank using 5ml distilled water and 5ml TBA reagent. After cooling, measure the optical density at 538 nm by a UV spectrophotometer.

TBARS number, expressed as mg malon di aldehyde (MDA) / kg sample = 7.8 x optical density.

4.Free fatty acid

Hydrolytic rancidity is expressed in terms of free fatty acid present, calculated as any of the predominant fatty acid. FFA value was determined as per AOAC (1989) to assess the hydrolytic rancidity. For this, suitable quantity of the fish sample is blend with anhydrous sodium sulphate in a mortar until all the water is removed. The blend is shaken well with chloroform and filtered. Take 20 ml of chloroform extract in 100 ml conical flask and evaporate to dryness by keeping it on a waterbath and calculate the fat weight by difference in weight of the flask. After cooling, add 10 ml of neutral alcohol to dissolve the fat and titrate against 0.01 N NaOH using phenolphthalein as indicator. Free fatty acid is calculated and expressed as % oleic acid

$$\text{FFA (\% oleic acid)} = \frac{0.282 \times V \times N \times 100}{\text{Wt. of fat}}$$

5.Estimation of peroxide value (PV)

Peroxide value of the fish sample is estimated according to the procedure of Yildiz *et al.* (2003). Prepare chloroform extract of fat as described for free fatty acid. For analysis of PV, take 20 ml of the filtrate in an iodine flask. To this, add 30 ml glacial acetic acid and a pinch of potassium iodide, shake well and keep in dark for 30 min. After incubation period, the take out the solution, add 15 ml of distilled water and titrate against 0.01 N $Na_2S_2O_3$ using starch as indicator. The weight of fat in 20 ml filtrate is calculated from the weight difference before and after evaporating 20 ml of chloroform extract in a beaker. The peroxide value is calculated as follows.

$$\text{PV (meq } O_2\text{/kg sample)} = \frac{\text{Titre value x 0.01} \quad \text{X 100}}{\text{Wt. of fat}}$$

Suggested Books for reading:

Principle of total quality On Achonu, V.K. and Ross, J.E. (1994) St. Lucie Press, Florida.

Quality Assurance in Seafood processing (2002) T.S.G. Iyer, M.K. Kandoran, Mary Thomas and P.T. Mathew, Society of Fisheries Technologists (I), Cochin

Quality Assurance in the Fish Industry (1992), H.H. Huss *et al (eds)* Elsvier Science Publishers, BV

Seafood Quality Determination (1986), DE Kramer and J. Liston, Elsiver Science Publishers B.V. Amolerdam.

HACCP

Jesmi Debbarma, Viji P and B. Madhusudhana Rao

HACCP is merely an acronym that stands for Hazard Analysis and Critical Control Point. HACCP is a total quality management system with stress on safety based on a systematic approach to hazard identification, assessment and control which make it different from traditional inspection and quality control procedures. In HACCP system, control is transferred from end product testing to on-line checking, that is a change from 'testing for failure' to 'preventing failure'. HACCP is a preventive system of hazard control rather than a reactive one. Food processors can use it as a management tool to ensure safer food products for consumers. The HACCP system is designed to identify hazards and establish controls. HACCP is product specific and plant specific; therefore, a unique plan has to be chalked out for each product and/or process. In Principle, HACCP can be applied throughout the food chain, starting from the primary producer to final consumer. However, HACCP is not a zero-risk system, but it is designed to minimize the risk of food-safety hazards to acceptable levels.

HACCP is a preventive system for ensuring food safety, but it is not a stand-alone system. HACCP must be built upon current food-safety programs such as Good Manufacturing Practices (GMPs) (e.g., sanitation and personal hygiene programs) to make it work. GMP and SSOP are fundamental for a sound HACCP plan.

Principles of HACCP:

The HACCP system consists of 7 principles,

1. Conduct a hazard analysis
2. Identify the critical control points (CCP's)
3. Establish critical limits
4. Establish a monitoring system
5. Establish the corrective actions
6. Establish verification procedures
7. Establish documentation and record keeping

Preliminary steps to developing a HACCP plan:

The HACCP is implemented in 12 steps which include five preliminary steps and the seven principles. Failure to properly address the preliminary steps may lead to ineffective design, implementation and management of the HACCP plan. The five preliminary step includes,

1. Assembly of HACCP team,
2. Description of the products
3. Identify intended use and consumers of food
4. Construct a flow diagram of process steps
5. On-site verification of flow diagram

1. Assembly HACCP team:

Assembling a HACCP team is an important step in building a HACCP program. To ensure that all likely hazards and critical controls points (CCPs) are identified, a multidisciplinary team of experts must be assembled to develop, implement and maintain the HACCP system. The HACCP team should include members who have operational experience, product specific knowledge and a good understanding of the production process. The team may include personnel from maintenance, production, sanitation, quality control and laboratory. The team develops the HACCP plan, writes SSOPs, and verifies and implements the HACCP system. The team should be knowledgeable about food-safety hazards and HACCP principles. When issues arise that cannot be resolved internally, it may be necessary to enlist outside expertise.

2. Description of Products:

Once a HACCP team is established, the members first describe the product and the method of distribution. A full description of the product needs to be prepared which help to determine the food safety hazards associated with its production. Product descriptions must describe relevant food safety information. Example of description of products are given below,

PRODUCT DESCRIPTION OF FROZEN RAW SHRIMP	
Species	*Litopenaeus vennamei*
Type	Head on; Head Less (HL); Peeled and Deveined (PD); PD tail on; Peeled and Undeveined (PUD); and PUD tail on; Butterfly
Count	13-16 pieces/pound
Freezing method	IQF/Block Frozen
Packaging	Packed in LDPE/laminated duplex cartons which are packed in 5 or 7 PL paper board master carton
Storage	Stored at -18°C or below
Instruction for use	To be fully cooked before use
Shipment/Transport	Refrigerated containers at -18°C or below

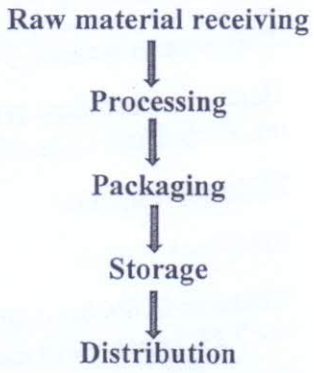
3. Identify intended use and consumers of food

The HACCP team also has to state the intended use for the product which describes target consumers, anticipated preparation and use of the products by consumers. Intended use information also needs to identify whether the end user will be the general public or a specific public or a specific consumer group such as infants, the elderly, pregnant women or immune compromised persons. Example of intended use of products are given below,

INTENDED USE OF THE PRODUCT	
Name of the product	Frozen raw shrimp
Consumer	General public
Anticipated use	To be consume only after cooking
Special consideration	Shrimp may be allergic for some persons

4. Construct a flow diagram of process steps

A flow diagram provides an important visual tool that the HACCP team can use t complete the remaining steps of the HACCP plan development. It is important to include all th steps within the facility's control, including receiving and storage steps for all raw materials. Th flow diagram should be clear and complete enough so that people unfamiliar with the process ca quickly comprehend your processing stages. The flowing is an example of a basic flow diagram,



5. On-site verification of flow diagram

Since the accuracy of the flow diagram is critical to conduct a hazard analysis, the steps outlined in the diagram must be verified at the plant. If a step is missed, a significant safety issue may not be addressed. The HACCP team should walk through the facility and make any changes required in the flow chart. The walk-through allows each team member to gain an overall picture of how the product is made. It may be helpful to invite additional plant personnel to review the diagram during the walk-through.

Principles of HACCP:

1. Conduct a hazard analysis:

The hazard analysis step is fundamental to the HACCP system. Hazard Analysis is a system used to analyses the significance of a hazard for consumer safety. To establish a plan that effectively prevents food safety hazards, it is crucial that all significant safety hazards and the measures to control them be identified.

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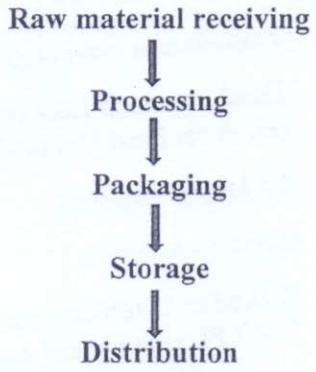

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Principles of HACCP:

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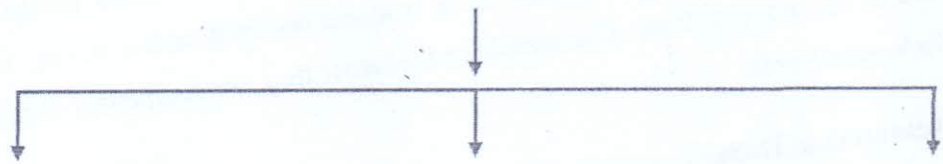
Hazard refers to the conditions or contaminants in food that can cause illness or injury to the consumers. Hazard is a biological, chemical or physical agent that is reasonably likely to cause illness or injury in the absence of its control (NACMCF, 1997). Hazards in HACCP system are calcified to Biological, chemical and physical Hazards.

Hazard



Biological Hazard

1. Pathogenic Bacteria

- *Clostridium botulinum*
- *Clostridium perfringens*
- *Bacillus cereus*
- *Escherichia coli* O157:H7
- *Listeria monocytogenes*
- *Salmonella spp.*
- *Staphylococcus aureus*
- *Vibrio cholerae,*
- *V. parahaemolyticus,*
- *Yersinia enterocolitica*

2. Pathogenic Viruses

- Hepatitis A and E
- Norwalk virus group
- Rotavirus

3. Parasitic Protozoa and Worms

- *Anasakis simplex*
- *Ascaris lumbricoides*
- *Cryptosporidium parvum*
- *Diphyllobothrium latum*
- *Entamoeba histolytica*
- *Giardia lamblia*
- *Pseudoterranova decipiens*
- *Taenia solium,*
- *Trichinella spiralis*

Chemical Hazard

1. **Naturally Occuring Chemicals**
   - Mycotoxins (e.g., aflatoxin)
   - Scombrotoxin (histamine)
   - Ciguatoxin
   - Shellfish toxins
     - Paralytic shellfish poisoning (PSP)
     - Diarrheic shellfish poisoning (DSP)
     - Neurotoxic shellfish poisoning (NSP)
     - Amnesic shellfish poisoning (ASP)/Domoic acid

2. Intentionally Added Chemicals

- Food additives
  - Preservatives (e.g., nitrite and sulfiting agents)
  - Nutritional additives (e.g., niacin)
  - Color additives

3. **Unintentionally or Incidentially Added Chemicals**
   - Agricultural chemicals (e.g., pesticides, fungicides, herbicides, fertilizers, antibiotics and growth hormones)
   - Prohibited substances
   - Toxic elements and compounds (e.g., lead, zinc, arsenic, mercury and cyanide)
   - Polychlorinated biphenyls (PCBs)
   - Plant chemicals (e.g., lubricants, cleaning compounds, sanitizers and paints)

Physical Hazard

- Metal pieces
- Glass

Hazard analysis are conducted in the following steps,

1. Identification of Hazards
2. Evaluation of hazards
3. Identification of control measures

1. **Identification of Hazard:** the HACCP team should list all hazards that may be reasonabl expected to occur at each step from production to consumption. After identifying the type an nature of contamination, the stage at which it might occurred, either during or after processing should be ascertained. It must be established whether they are completely eliminated or brought down to safe levels.

2. Evaluation of Hazard: All the potential hazards identified are evaluated based on severity and likelihood of occurrence. Severity is defined as seriousness of the consequences of exposure to hazard. Likelihood of occurrence of hazard is based on a combination of experience, epidemiological data and information in technical literature.

3. Identification of control measures: Control measures are actions and activities that can be used to prevent or eliminate a food safety hazard or reduce it to an acceptable level. The following are examples of control measures that could be used to control the three types of hazards.

A. Control Measure for Biological Hazards:

Bacteria and other pathogenic microorganism:

1. Time/temperature control (e.g., proper control of refrigeration and storage time minimizes the growth of pathogens).
2. Heating and cooking processes (e.g., thermal processing).
3. Cooling and freezing (e.g., cooling and freezing retard the growth of pathogenic bacteria).
4. Fermentation and/or pH control (e.g., lactic acid-producing bacteria in yogurt inhibit the growth of some pathogenic bacteria that do not grow well in acidic conditions).
5. Addition of salt or other preservatives (e.g., salt and other preservatives inhibit growth of some pathogenic bacteria).

6. Drying (the drying process may use enough heat to kill pathogenic bacteria, but even when drying is conducted at lower temperatures, it may remove enough water from the food to prevent some pathogens from growing).
7. Source control (e.g., the presence or amount of pathogens in raw materials may be controlled by obtaining them from noncontaminated sources).

B. Control measures of Chemical Hazards

1. Source control (e.g., vendor certification and raw-material testing).
2. Production control (e.g., proper use and application of food additives).
3. Labeling control (e.g., finished product properly labeled with ingredients and known allergens).

C. Control measure of Physical Hazards

1. Source control (e.g., vendor certification and raw-material testing).
2. Production control (e.g., use of magnets, metal detectors, sifter screens, destoners, clarifiers, air tumblers, x-ray equipment, and visual inspection).

Hazard Analysis Worksheet

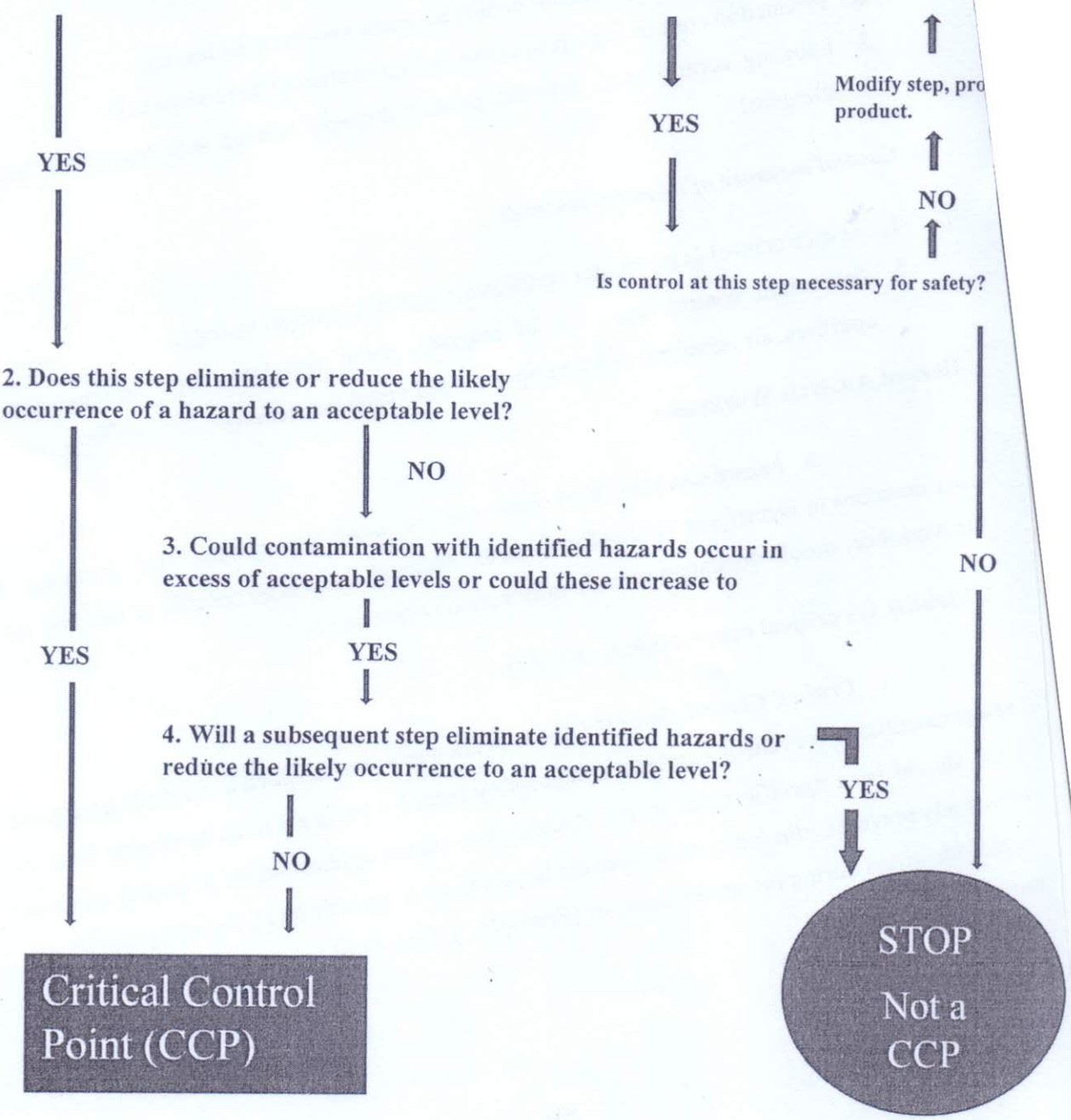
A hazard-analysis worksheet can be used to organize and document the considerations in identifying food-safety hazards. Although there is no specific or required form, the worksheet should document specific information (Annexure I).

2. Identify the critical control points (CCP's)

Critical Control Point (CCPs) is defined as a step at which control can be applied and is essential to prevent or eliminate a food-safety hazard or reduce it to an acceptable level. A CCP should be a specific point in the process flow where application of a control measure effectively prevents, eliminates or reduces the hazard to an acceptable level. For every significant hazard identified during the hazard analysis (Principle 1), there must be one or more CCPs where the hazard is controlled.

The determination of CCP is facilitated by applying the decision tree develo
Codex Alimentarius Commission. The decision tree consists of series of questior
processor/manufacturer has to answer. Based on the answer, processor will determine wheth
activity is CCP.

CCP DECISION TREE



Explanation of CCP decision Tree:

Question 1. Does a control measure(s) exist at this step or subsequent steps in the process flow for the identified hazard?

If your answer is yes, ask Question 2.

If you cannot identify a control measure in the process for the hazard, answer no. If the answer is no, then ask: Is control at this step necessary for safety? If this answer is no too, the step is not a CCP for that hazard. Move to the next hazard at that step or to the next step with a food-safety hazard. If the answer is yes, then you have identified a significant hazard that is not being controlled. In this case, the step, process or product must be redesigned to include a control measure. Sometimes there is no reasonable control measure available. In such cases, HACCP does not provide assurance that food products are safe.

Question 2. Does this step eliminate or reduce the likely occurrence of a significant hazard to an acceptable level?

To answer this question, consider if this is the best step at which to control the hazard? If the answer is yes, then the step is a CCP; move to the next food-safety hazard. If the answer is no, ask Question 3.

Question 3. Could contamination with an identified hazard or hazards occur in excess of acceptable levels, or could these increase to unacceptable levels?

The question refers to contamination that exists, occurs or increases at this step. If the answer is no, then the step is not a CCP for that hazard. Move to the next hazard at that step or the next step with a food-safety hazard.

If the answer is yes, then ask the 4th question.

Question 4. Will a subsequent step eliminate the identified hazard or hazards or reduce the likely occurrence to an acceptable level? If you answer no, then this step is a CCP. If you answer yes, then this step is not a CCP for this hazard. In this case, be sure the hazard is controlled by a subsequent processing step.

HACCP plan form:

HACCP plan form is a tool which helps to manage each CCPs. The plan form w address the last five principles of HACCP. A typical HACCP plan form is given in Annexure II

3. Establish critical limits

Critical limits must be established for each CCP identified in the hazard analysi Critical Limit is a maximum and/or minimum value to which a biological, chemical or physica parameter must be controlled at a CCP to prevent, eliminate or reduce to an acceptable level th occurrence of a food-safety hazard.

A critical limit represents the boundaries that are used to ensure that an operatior produces safe products. Each CCP must have one or more critical limits for each food-safety hazard. When the process deviates from the critical limit, a corrective action must be taken tc ensure food safety.

The appropriate critical limit for the operation may not be readily apparent or available. They must be established through the tests conducted or information gathered from sources such as scientific publications, regulatory guidelines, experts or experimental studies.

Source of information on critical limits

General Information	Examples
Scientific publications	Journal articles, food science texts, microbiology texts
Regulatory guidelines	National and International guidelines, BIS, EIA/MPEDA publications; USFDA/EU guidelines, tolerances and action levels
Experts	CIFT, NACMCF, thermal process authorities; consultants, food scientists/microbiologists, equipment manufacturers, sanitarians, university extension, trade associations
Experimental studies	In-house experiments; contract labs

Examples of critical limits,

Hazard	CCP	Critical Limit
Microbial Pathogen	Cooking	85°C for 3 mins for elimination of pathogens in shrimp.
	Drying	aw<0.07

4. Establish a monitoring system:

Monitoring is important to ensure that the critical limits are consistently met. Monitoring is the process to conduct a planned sequence of observations or measurements to assess whether a CCP is under control and to produce an accurate record for future use in verification. Purpose of Monitoring,

- To track the operation of the process and enable the identification of trends toward a critical limit that may trigger process adjustments,
- To identify when there is loss of control (a deviation occurs at a CCP).
- To provide written documentation of the process control system.

The monitoring procedures are used to determine if the control measures are being enacted and the critical limits are being met. Monitoring procedures must identify the following parameters,

- ✓ What will be monitored?
- ✓ How the critical limits and control measures will be monitored?
- ✓ How frequently monitoring will be performed?
- ✓ Who will perform the monitoring?

What will be monitored?

Monitoring may mean measuring a characteristic of the product or of the process to determine compliance with a critical limit.

Examples:

- Measurement of cold storage compartment temperature
- Measurement of the pH of an acidified food such as pickle.
- Measurement of line speed when critical to adequate cooking or chilling processes.

Monitoring may also involve observing if a control measure at a CCP is being performed. For examples: Checking that a vendor's certificate accompanies a lot of raw material.

How Critical Limits and Control Measures will be Monitored?

Monitoring must be designed to provide rapid (real-time) results. There is no time for lengthy analytical testing because critical limit failures must be detected quickly and an appropriate corrective action instituted before distribution.

Example:

- ✓ Time and temperature using a calibrated thermometer and stopwatch
- ✓ Water activity (aw) using a calibrated RH meter
- ✓ Acidity using a calibrated pH meter

How frequently monitoring will be performed?

Monitoring can be continuous or noncontinuous. Where possible, continuous monitoring should be used. Continuous monitoring is possible for many types of physical and chemical parameters. Examples of continuous monitoring:

- The time and temperature of a batch cooker process for IQF shrimp may be continuously monitored and recorded on a temperature recording chart.
- Each package of frozen, mechanically-cut fish blocks may be passed under a metal detector.

Examples of nonconscious monitoring:

- Routine, daily checks for properly iced fish.
- Periodic sensory examination for decomposition in histamine forming seafood.

Who will Monitor?

Assignment of the responsibility for monitoring is an important consideration when developing a HACCP plan. Individuals assigned to CCP monitoring can be:

- Line personnel,
- Equipment operators,
- Supervisors,
- Maintenance personnel or
- Quality-assurance personnel.

5. Establish the corrective actions:

Corrective Action can be defined as procedures to be followed when a deviation occurs. Corrective actions must be taken when critical limits at a CCP have been compromised. When possible, these actions should be predetermined when developing the HACCP plan.

Effective corrective action plans must:

- Correct and eliminate the cause of the noncompliance to assure that the CCP is brought back under control.

- Segregate, assess and determine the disposition of the noncompliant product.

All corrective actions taken must be documented. Documentation will assist the firm in identifying recurring problems so that the HACCP plan can be modified. Additionally, corrective action records provide proof of product disposition. There are two components of corrective actions:

1) to correct and eliminate the cause of the deviation and restore process control.

2) to identify the product that was produced during the process deviation and determine its disposition.

Example of corrective action:

IF deviation	Product (e.g., hot smoked fish) does not reach required internal temperature for the required time.
THEN corrective action	Recook or destroy product.

6. Establish verification procedures:

Verification are those activities, other than monitoring, that determine the validi of the HACCP plan and that verify the system is operating according to the plan. The prop development and implementation of the verification principle is fundamental to the successf execution of the HACCP plan. The purpose of the HACCP plan is to prevent food safety hazard and the purpose of verification is to provide a level of confidence that the plan is based on soli scientific principles, is adequate to control the hazards associated with the product and proces: and is being followed.

Elements of Verification:

- Validation
- CCP verification activities
    - ✓ Calibration of monitoring devices
    - ✓ Calibration record review
    - ✓ Targeted sampling and testing
    - ✓ CCP record review
- HACCP system verification
    - ✓ Observations and reviews
    - ✓ Microbiological
- Regulatory agencies

7. Establish documentation and record keeping:

Accurate record keeping is an essential part of a successful HACCP program. Records provide documentation that the critical limits have been met or that appropriate corrective actions were taken when the limits were exceeded. They also provide a means of monitoring so that process adjustments can be made to prevent a loss of control.

Four kinds of categories are kept as part of the HACCP system.

1. HACCP plan and support documentation used in developing the plan
2. Records of CCP monitoring
3. Records of corrective action
4. Records of verification activities

Annexure I: Hazard Analysis worksheet

(1) Ingredient/processing step	(2) Identify potential hazards introduced, controlled or enhanced at this step.	(3) Are any potential food-safety hazards significant? (Yes/No)	(4) Justify your decision for column 3.	(5) What control measure(s) can be applied to prevent significant hazards?	(6) Is this step a critical control point? (Yes/No
Receiving Fresh Shrimp	BIOLOGICAL Bacterial pathogens	YES	Raw seafoods can be natural reservoirs for marine vibrios and depending on the quality of the harvest, can harbor terrestrial pathogens such as Salmonella.	A cook step follows that assumes a high bacterial load.	
	CHEMICAL Sulfiting agent	Yes	Sulfiting agents may cause an allergic-type reaction.	Labeling control based on product screening.	
	PHYSICAL None				
Cold Storage	BIOLOGICAL Bacterial pathogen growth	Yes	Without controlled temperatures, bacterial pathogens may increase in numbers	A cook step followas that assumes a high bacterial load	
	CHEMICAL None				
	PHYSICAL None				

Name of the firm: .....

Address of the firm: .....

.....

Signature: .....

Date: .....

Products Description: .....

.....

Method of storage and distribution: .....

.....

Intended use and consumer:.....

.....

Annexure II: Hazard Plan Form

COOKED SHRIMP

1	2	3	4	5	6	7	8	9	10
Critical Control Point (CCP)	**Significant Hazards**	**Critical Limits for each Control Measures**	**Monitoring**				**Corrective Action(s)**	**Verification**	**Records**
			What	**How**	**Frequency**	**Who**			
Cooker	Survival of bacterial pathogen	Cook at 100°C for 15 min	Cook temperature	Monitor temperature with a continuous temperature recorder	Temperature monitored continuously with hourly visual checks.	Quality control supervisor	If the temperature or time parameters are not met, then processing line will be stopped and required adjustments made. All the product produced during the deviation will be recorded or destroyed.	• Daily record review. • Quarterly calibration of thermometer • Semi-annual finished product microbial testing • Process validation study of time and temp. of cook and its effect on the final internal temp. of various sizes of shrimp and initial temp. • Cooking equipment validation study	
			Cook time	Monitor cook time by timing the movement of a block placed on belt through cooker.	Cook time monitored hourly	Quality control supervisor			

Name of the firm: .....

Address of the firm: .....

.....

Signature: .....

Date: .....

Products Description: .....

.....

Method of storage and distribution: .....

.....

Intended use and consumer:.....

.....

GOOD FOOD LABORATORY PRACTICES (GFLPs)

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1. Introduction

In the early 70's FDA (United States Food and Drug administration) have realized cases of poor laboratory practice throughout the United States. These include poor equipment calibration, incorrect or inaccurate accounts of the actual lab study and incompetent test systems. Although the term "good laboratory practice" might have been used unofficial already for some time in many laboratories, across the world GLP was originated in the United States and it had a significant effect over worldwide. In 1978, GLP as an official regulation was created by the FDA. The OECD (Organisation for Economic Co-operation and Development) Principles of Good Laboratory Practice were first formed by an Expert Group on GLP set up in 1978 under the Special Programme on the Control of Chemicals. GFLPs in this chapter is as per the FSSAI, India.

2. Scope of testing applicable for a food laboratory

GFLPs guidelines state the general requirements to perform systematic sampling of food samples, conduct chemical, microbiological tests and testing of packaging materials to ascertain the quality of food. These Guidelines are applicable to all organizations performing tests to ascertain the quality of food material including packaging material. These Guidelines are for use by laboratories in developing their management system for quality, administrative and technical operations. Laboratory customers, regulatory authorities and accreditation bodies may also use it in confirming or recognizing the competence of laboratories.

3. Structure of Food Lab:

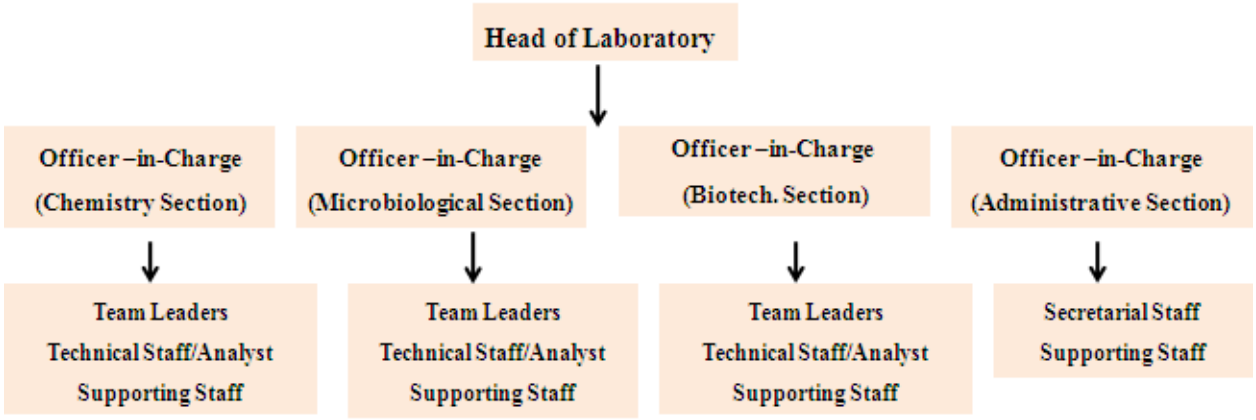
a. Personnel

Personnel must obviously understand the nature of the foods they are testing and reasons for testing when undertaking contract review and method selection.

b. The Management Structure:

A chart with continuous updating on the organizational structure and lines of responsibility of the laboratory is an important feature of the quality assurance programme and must appear in the Quality Assurance Manual. The common organization of staff is shown in Figure A

Fig. The structure of a staff for a typical food testing laboratory is mentioned below:



The office of Head of Laboratory (other titles such as "Chief" or "Director" is often used) may include a Deputy if the laboratory staff is sufficiently large. The laboratory Head is usually the spokesman for the laboratory in many instances. The supervisor is the on-site manager of the laboratory. A reasonable maximum number of analysts for one person to supervise is 10 to 12. The core responsibilities of a Supervisor are to assist the head in overall laboratory work planning and planning the work of the group and receiving and assigning samples for analysis, within the group. Reviewing the reports of completed work, making appropriate recommendations and ensuring proper laboratory safety is the duty of a supervisor. A team leader is a senior analyst who has been assigned a small group, usually not more than 4, to do a specific task or type of analysis. The leader has no supervisory functions as such, but is the coordinator of the group's activities and is the contact point for the supervisor. The basic job of the analytical staff is to analyze the samples received and to issue a report. They may also be called onto offer advice to industry and trade, to assist in improvement of food quality, or advice on conformity with standards or other legal requirements. The duties of supporting staff include for example

glassware washing, cleaning and housekeeping maintenance, disposal of sample reserves (when no longer required) and pest control.

4. Infrastructure and accommodation and related requirements:

Although final design of the laboratory is made by architects and engineers, the analytical staff should be involved in some of the decisions that will ultimately affect their working environment and conditions. There is often a 5 year period from the decision in principle to build a new laboratory to when it is accepted and operational. Also there will usually be an expectation that it will not require major alteration for a further 10 years. The Laboratory requires generic activities which will serve extensive provision of fixed benches with water, power, sinks, fume cupboards, reagent shelves, glassware cleaning and storage. Specialized rooms may be required for "clean air" work (e.g. on some environmental contaminants) or for work with substances which need to be handled with special care either for safety or for cross-contamination reasons. To provide for a prompt exit in the event of fire or other emergency, at least two entrances/exits must be provided for each laboratory whenever possible.

4.1 The Chemical Laboratory, Equipment and Instruments:

Laboratory design must provide complete segregation of trace analyses from highly concentrated formulations and from pure substances used in preparing analytical standards is virtually essential. The segregation must apply to all facilities for washing/cleaning equipment, washing and storage of glassware, use of protective clothing and even transfer of notebooks and records. The complexity of equipping a laboratory and the consequent delay in production of useful results should not be underestimated. In the early stages, the requirements for equipment may seem large and complex but once the laboratory is established, the running costs are relatively low. The logistical problems of maintenance, repair and replacement of equipment are also considerable. The extensive use of solvents, ashing and noxious chemicals in food analysis, requires more fume hoods than other types of laboratory work.

4.2 Environment Conditions, Safety and related requirements

Adequate control of temperature, humidity and dust is important to staff comfort, instrumental performance and safe working (e.g. with flammable solvents). All the needs such as Electronic equipment with operating ranges, requirements of cooling water, storing of test materials, reagents, standards under controlled conditions etc, have to be identified and documented so that proper procedures for monitoring them and taking necessary action can be included in the quality assurance system. Records will be needed which show that: samples are received, stored, handled and analyzed under environmental conditions that will not adversely affect analyses.

4.3 Housekeeping Control:

The responsibility of cleaning staff and laboratory staff must each have clear instructions as to their respective duties in relation to cleaning of floors, vertical surfaces (e.g. cupboards, walls, windows and doors), horizontal surfaces (e.g. work surfaces, shelves), equipment, interiors of refrigerators, freezers, fume cupboards, controlled environment stores, control of the contents of refrigerators, freezers, fume cupboards, controlled environment stores, checking the performance of air-conditioning and dust extraction equipment and fume-cupboards and pest control.

5 Safety Features:

The building and laboratory design should include a number of safety features including:

i. The fire areas of corridors should be formed of concrete blocks.
ii. Services should include a shower sprinkler system near each doorway so that a worker can take an immediate shower, clothes and all, in the case of accidental general contact with corrosive or poisonous liquids or fire.
iii. It must always be possible to leave the laboratory safely irrespective of the initial site of a fire. Serious thought must be given to the number and location of fire extinguishers and stand pipe systems, and to the availability of sprinkler systems.
iv. Flooring needs to be of a non – slip material, resistant to acids and solvents, but not so hard as to be tiring to stand on for a few hours at a time. Well-laid linoleum and a filled epoxy resin on top of concrete are among the best available.

v. Pollutants generated within the laboratory must be removed safely, quickly and efficiently. In particular, toxic or noxious gases must be removed expeditiously through a duct system that does not exhaust near the building air conditioning intake.

vi. The building must be planned for security. Restriction of access is of considerable importance because of the extremely valuable and sensitive equipment used in the laboratory work as well as to protect the integrity of official samples.

vii. It is very advisable to have an efficient fire and smoke detection system with appropriate alarms.

6. Personnel related requirements

i. The personnel should be technically competent to perform their duties as allotted to them whether operating on specific equipments/ performing tests /evaluating results/signing the reports.

ii. Qualification for doing specific tasks shall be judged on the basis of their education, training, specific experience and demonstrated skill.

iii. Regular and refresher training should be organized to keep the personnel update in their domain of activity.

iv. Specific job description for each personnel should be defined with their role and responsibility.

v. Personnel should wear proper uniform and protective clothing's, etc as required depending upon the test method.

vi. Normally blank determination along with the known-standards must be carried out in duplicate/ replicate to check the accuracy of the results obtained and human error etc.

vii. All the analysis records must be documented either through hardcopy or through soft copy to demonstrate that the tests are really been carried out.

viii. Random checking of the result should be done inter-laboratory and intralaboratory to check the proficiency of the personnel.

ix. First Aid box should be available in the lab along with emergency Telephone no. of hospital/doctors/contact person.

x. Personnel should be medically fit depending upon the test method he is deployed to avoid any hazards.

7. Test methods:

i. The laboratory shall use only official methods depending on the requirement of the test, its sensitivity and nature of the commodity which is being tested and quality/safety factors to be determined.

ii. In case of non-official method, validation of the methods as per set norms is a must and their range of detection/quantification, L.O.D./L.O.Q. limitations etc. must be established.

iii. Selection of method is very important depending upon the requirement of the test and customer requirement.

iv. External calibration of the equipment is a must annually or depending upon its use. However in case of any equipment being used very frequently, internal calibration facility should be available and done regularly with a record thereof.

v. SOP as far as possible should be available for test method along with the protocol.

vi. In case of micro biological analysis standard culture must be available to establish the confirmation of the microbes. SWAB testing must be done for inoculation room and media preparation room regularly to ensure that it is not contaminated.

vii. All the apparatuses specially glass should be contamination free and should be cleaned and rinsed thoroughly before use. No chemicals should be used after its expiry or otherwise if it looks like deteriorated or decomposed.

8. Equipments:

i. The equipment must be calibrated depending upon the requirements by an outside accredited lab and/or internally as the case may be.

ii. In case the sophisticated instruments are shifted from one place to another the same should be re-calibrated.

iii. Depending upon the uses, the equipments should be internally calibrated either daily or at a periodically interval as the case may be.

iv. Instruction manual, operation manual and other details of the equipments like calibration, due date of calibration, safety precaution, etc must be available at the side of the equipment.

v. Each sophisticated equipment should have IQ, OQ and PQ Certificates from the manufacturer.

vi. LOD/LOQ/ Range of detection/ range of quantification must be established for each equipment in context of the test method, nature of the food commodity, constituent to be determined.

vii. The SOP for safe handling, transportation, storage, use and plant maintenance of the equipments must be available to ensure proper functioning and to prevent deterioration /contamination.

9. Certified Reference Materials / Standards and Reference Cultures:

Testing, validation/calibration, standardization & reference materials are inter-related due to dependent on each other. Without proper reference materials, it is not possible to make up any idealized and reliable measurement system. As per the lab quality assurance procedure reference materials are required for all types of testing and validation/calibration. These are widely used for validation/calibration of an apparatus and testing procedure, assessing the true value. The laboratory shall ensure to maintain the reference standards, which are certified by the competent body having traceability to a national/international system like NIST etc. The certificate provided by the supplier/manufacturer shall be maintained in the laboratory for records. All reference standards shall be kept under responsible person to maintain proper storage, transport, security, integrity, mishandling etc and the relevant records are also to be maintained. The utmost care & protection shall be taken during handling & preparation of standards to avoid cross contamination & health hazard. The reference culture/microbial pure cultures are used establishing acceptable performance of media, performance of the kits, validation of methods and assessing/evaluating the laboratory performance. The reference microbial strains are directly collected by laboratory from recognized national or international collection (ATCC, MTCC, NCIM etc) with traceability. Generally the reference strains are received in lyophilized stage or deep-frozen stage. If the reference strain has been thawed they shall not be refrozen.

10. Calibration and performance assessment related requirements:

For accurate test results, lab shall be ensured that the equipments which are suitable for intended purpose and capable of providing valid results, such instruments would be regularly inspected,

checked & calibrated accordingly. So laboratory should establish a schedule for the calibration and performance verification of equipments/instruments, which will be direct influence on the test results. It is the laboratory responsibility to verify / check the calibration certificate in terms of the lab requirements, traceability to the primary standard, ensure the capability / calibration range, uncertainty, due date of next calibration (if require) etc and laboratory has to evaluate the services. All records are to be maintained. The equipments like thermometer, pressure gauge, humidity meter, laboratory may calibrate through external calibration agencies with proper traceability in regular intervals / as per the lab protocol and the laboratory may use the same equipments as standards for verification.

11.Sampling & sample handling:

Sampling for testing or analysis is a process of taking a representative portion from a material or product to test (e.g. by physical measurements, chemical analysis, microbiological examination), typically for the purposes of identification, quality control, or regulatory assessment. The sampling is a significant role in testing activities as it reflects the ultimate test results**.** All incoming samples shall receive through the receiving section maintained and supervised by laboratory responsible person. On receiving section the laboratory responsible personnel initially must check the relevant overall criteria like sample identity/labelling, mode of transportation, condition of the sample including packaging, sample quantity, verification of fees (whenever necessary), parameter to be tested etc against the customer declaration / requirements. Any abnormalities / deviation / doubts from the normal condition, suitability of the sample for tests etc , the same shall be clarified from the customer / laboratory responsible personnel before accepting the samples for registration / testing. In case microbiological test samples, the same shall be received in the sterilized container/sample box etc. The laboratory documentation system shall includes all relevant information such as customer details, date of receipt, condition of the sample on receipt, sample quantity, transportation , parameters to be tested ,observation/remark (if any) etc.

12. Laboratory reports: It must contain all the information normally necessary for the customer to utilize the result which include

- Name, Address of the Laboratory

- Name, Address of the customer
- Certificate/Report Number
- Page Identification (Page X of Y)
- Sample received details (Dates, Names of deliverable, receiver)
- Unambiguous identification of sample / test material (Description, Laboratory Number etc.)
- Analysis conducted, Methods, Procedures any deviation from standard practices.
- Preparation of test material, taking of test portions
- Results
- Uncertainty of measurements
- Comments on significant of findings (if expected by the customer)
- Date of report
- Authorizing signature

Documentation for QA programme:

- Analyst worksheet
- Laboratory report
- Procedures for checking of results
- Procedures for authorization for report
- Period for retention of documents

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