Winter School on Product Development and Seafood Safety organized by CIFT Cochin 11 Nov. - 10 Dec. 2003,340-350

Microbiological Quality Evaluation of Seafood

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Since fish is harvested from natural water bodies including farms, it harbours a number of microorganisms found in the environment from where it is caught. These native microorganisms may include fish spoilage bacteria as well as certain pathogens of aquatic origin. In addition to these inherent microorganisms, the fish can get contaminated with other microorganisms during handling, transportation and processing, right from the point of catch to the end product. These microorganisms include both pathogenic and non-pathogenic bacteria. The pathogenic microorganisms can be hazardous to the health of the consumer. The most important pathogens which gain entry into the fish during handling, transportation and processing are Salmonella, *Vibrio cholerae, Staphylococcus aureus* and *Listeria monocytogenes.* In addition, enteropathogenic *Escherichia coli, Clostridium perfringens* and *Bacillus cereus* may also gain entry to the fish.

In order to determine the acceptability of fish as well as shellfish, the assessment of their microbial qualiy is necessary. The microbial parameters that are generally assessed to determine their consumer acceptability and safety are the following.

i) Total plate count (TPC)

ii) Total *Enterobacteriaceae* count (includes all coliforms, Salmonella & Shigella)

iii) *Escherichia coli (E. coli)*

iv) *Staphylococcus aureus*

v) Faecal streptococci

The level of the total bacteria (TPC) will indicate the freshness of the products, as well as their potential shelf life. The type of bacteria in the product can give information regarding how the product has been handled or processed. A high bacterial count indicates the level of contamination of the product, unsuitable conditions of storage, the extent of spoilage, etc. Hence TPC gives an overall picture of the quality of the product. A TPC of 10^6/g or above is considered as a proof of poor quality of the product.

The TPC can be determined by microscopic or cultural method. The cultural method is preferred for determination of TPC because it gives an estimate of viable (live) cells. A suitable culture media, like Tryptone Glucose Agar (TGA) or plate count agar is generally used for determination of TPC. Live bacterial cells are capable of forming colonies on a suitable solid medium. This property of the bacteria is made use of in the determination of TPC.

Total Enterobacteriacea count and total faecal streptococci count gives an indication to whether there was any faecal contamination in the fish at any stage; *E. coli* is a direct faecal indicator organism. *Staphylococcus aureus* can arise mainly from the human handlers; *Salmonella* and *V. cholerae* are pathogenic organisms, implicated in food-borne infections.

Table 1 gives the various microbial parameters to be studied for fish and fish products and the media used for their sampling.

Table1.Microbial parameter and the media for sampling

Parameter	Medium
i) Total plate count (TPC)	Tryptone Glucose Agar (TGA)
ii) Total *Enterobacteriaceae* count (includes all coliforms, Salmonella & Shigella)	Violet Red Bile Glucose Agar (VRBGA)
iii) *Escherichia coli (E. coli)*	Tergitol 7 Agar (T 7)
iv) *Staphylococcus aureus*	Baird Parker Medium (BP)
v) Faecal streptococci	Kenner Faecal Streptococci Agar (KF)
vi) *Salmonella*	A set of media
vii) *Vibrio cholerae*	A set of media

Total Plate Count (TPC)

In the procedure for sampling for TPC, a known quantity of the sample is macerated well with a known volume of a suitable diluent and one ml of the appropriate dilutions are cultured in the plating medium. For this 10 g of the sample is mixed with 90 ml diluent or 25 g sample with 225 ml diluent ie. 1 part sample with nine parts diluent (ie.1 + 9 = 1:10 ratio). This gives 10 times dilution of the sample, ie. 10^{-1} dilution. For further dilution, 1 ml from 10^{-1} dilution is mixed with 9 ml of the diluent (10^{-2} dilution) and so on to get the appropriate dilutions for plating. This type of dilution is called serial decimal dilution. Usually, 3 dilutions are plated in duplicate or triplicate. The selection of the dilutions for sampling depend on the bacterial load in the sample ie., highly contaminated or spoiled samples require higher dilutions than a very fresh one.

Method

10g of the sample is aseptically cut into a sample dish and macerated with 90ml normal saline (NS) in a sterile glass mortar. (Alternatively, 25g sample is blended with 225 ml NS in a stomacher blender)

Sampling scheme

10g sample + 90ml NS: 10^{-1} dilution

1ml + 9ml NS: 10^{-2} dilution (0.5 ml each to BP and T 7; 1 ml each to KF and VRBGA)

1ml + 9ml NS: 10^{-3} dilution (0.5ml each to BP, T 7; 1ml each to KF, VRBGA and TGA)

1ml + 9ml NS: 10^{-4} dilution (1ml to TGA)

1ml + 9ml NS: 10^{-5} dilution (1ml to TGA)

For compositions of media refer any standard manual on Microbiological methods for food analysis.

TPC: Pour plating on TGA, 1 ml of 10^{-3}, 10^{-4} and 10^{-5} dilutions in duplicate is recommended.

For **KF and VRBGA**, pour plating technique is followed. One ml each of 10^{-2} and 10^{-3} dilutions are plated

For **T7 and BP**, pre-set plates have to be prepared

100ml Tergitol-7 agar (T 7) is melted in a water bath, cooled to about 50°C and aseptically added 0.25ml of 1% sterile TTC solution. Poured into sterile Petridishes (15-20 ml each), allowed to set and dried at 56°C for 45min. Cooled to room temperature (RT).

100ml Baird-Parker medium (BP) is melted & cooled to about 50°C; aseptically added 1ml of sterile 1% potassium tellurite solution, followed by 5ml of 50% egg yolk emulsion. Mixed well, poured into sterile Petridishes, allowed to set; dried at 56°C for 45min.cooled to RT.

Arrange 6 Petridishes for TGA & 4 each for KF and VRBGA. Also, arrange 4 plates each of pre-set T 7 and BP agar. Label appropriately, viz: sample name, dilution, medium and date.

For TGA, KF and VRBGA, 1ml each of the appropriate dilutions are pipetted and pour plated with the corresponding medium. Plates are allowed to set, inverted and incubated at 37°C.

For T 7 and BP plates, 0.5ml each of the appropriate dilutions are surface plated using sterile bent glass rod. Plates are inverted and incubated at 37°C for 18-24 h. The plates are examined after the incubation. Observe VRBGA and T 7 plates after 18-24h.

Observations and results

VRBGA plates

Red, small (2-4 mm dia) colonies are counted as *Enterobacteriaceae* colonies. Take average count of duplicate plates.

VRBGA is a medium considered specific for *Enterobacteriaceae*, which includes the Coliform group, Salmonella, Shigella, Klebseilla and Citrobacter. All these bacteria ferment glucose with the production of acid. The medium contains neutral red which turns red in presence of acid. So, all glucose fermenting colonies appear red.

Total *Enterobacteriaceae* count / g = Average count x dilution factor.

T 7 plates

E. coli colonies are lime yellow, occasionally with rust brown centre and an yellow zone around. (Note: Yellow slimy, raised or convex colonies are not to be considered as *E. coli* colonies.) Take average of duplicate plates.

E. coli/g = Average count x 2 x dilution factor.

Average count is multiplied by two because only 0.5ml of the sample dilution was added to the plates.

Tergitol-7 agar containing TTC is a selective and differential medium for *Escherichia coli* and *Enterobacter aerogenes*, which allows their detection in 18-24 hrs. So, this medium has been recommended for routine analysis of water and food. The Tergitol-7, in the medium inhibits the growth of Gram positive bacteria. *E. coli* ferment lactose to produce acid, which changes the colour of bromothymol blue from green to yellow. *E.coli* does not reduce TTC, but other coliforms and *Enterobacter aerogenes* reduce TTC to red dye formazan. Hence, *E.coli* colonies will appear yellow, with deepened yellow centre and with a yellow halo around. Colonies with a red tinged center should not be confused for *E.coli.*

Confirmation of E. coli

The lime yellow colonies, occasionally with rust brown centre and an yellow zone around, on T 7 plates are counted as *E. coli.* However, to confirm them as *E. coli,* the following procedure has to be adopted.

1. Streak on Eosine-Methylene Blue (EMB) agar

EMB agar is melted, cooled to 50°C, poured into Petri dishes and allowed to set. The set plates are dried at 56°C for 45 min and cooled to room temperature. Typical yellow colonies from T 7 plates are picked with a sterile platinum loop and streaked on to EMB plates, by the streak-dilution method, incubated at 37°C for 18-24 h. Well isolated colonies, 2-3mm dia with a greenish metallic sheen by reflected light and dark purple centre by transmitted light is picked and sub cultured on TGA slants and incubated at 37°C for 18-24 h. The green metallic sheen on EMB agar is caused by the precipitation of methylene blue in acid pH due to lactose fermentation by *E. coli*

2. IMViC tests

From the TGA slants above, inoculate to the following media.

a) Tryptone broth (Indole medium)

Inoculate a little of the culture to Tryptone broth and incubate at 37°C for 48 h.

b) MRVP *medium*

Inoculate each culture into 2 tubes of MRVP medium and incubate at 37°C for 48 h.

c) Simmon's Citrate agar

Streak a little of the culture to Simmmon's citrate agar slants and incubate at 37°C for 48 h.

Results

Observe results after 48 h of incubation

1. Tryptone broth

Test for indole production using Kovac's indole reagent (add 0.5 ml and shake, allow to stand). A red or pink colour at top indicates positive test. Indole forms a red dye with p-dimethyl amino benzaldehyde of the Kovac's reagent.

2. MRVP medium

MR Test

Into one tube, add Methyl Red indicator. A red colour indicates positive MR test. *E. coli* ferments glucose to produce acid, which brings down pH of the medium to less than 4.4 indicated by the red colour of methyl red indicator. If the colour is orange, pH is 5.0 – 5.8 and if yellow, pH is more than 6.0

VP Test:

Using 1 ml of the culture from the second tube, do the VP test (To 1 ml culture, add 0.6 ml 5% α-naphthol and 0.2 ml 40% KOH, add a pinch of creatine and mix, allow to stand upto 4 h). Eosine pink colour indicates positive VP test. As an end product of glucose fermentation, some bacterial groups produce acetyl methyl carbinol (acteoin). Addition of potassium hydroxide oxidizes this compound to diacetyl which gives an eosin red colour with guanidine nucleus present in peptone in presence of á- naphthol. The reaction is accelerated by the addition of creatine.

3. Simmon's Citrate agar

Growth indicated by a change in the colour of the medium from green to blue indicates a positive test for citrate utilization by the bacterial culture. A positive growth indicate that citrate is utilized as the sole source of carbon, and colour of the medium turns blue due to the alkaline pH produced by the growth of bacteria. *E. coli* do not utilize citrate and hence give a negative reaction

A culture giving the following results is confirmed as *E. coli*

Indole	Positive
Methyl Red	Positive
VP	Negative
Citrate	Negative

ie., IMViC: + + - -

Eijkman's test

This is used as an optional confirmatory test for *E. coli.*

E. coli cultures will grow and produce gas in EC broth at 44.5± 0.5°C in 24-48h.

BP agar plates

Observe after 36-48 hours. *Staphylococcus aureus* colonies are black with thin white margin and a zone of clearance around.

S. aureus count / g = Average count x 2 x dilution factor.

Average count is multiplied by two since the quantity of sample dilution added is only 0.5 ml.

On BP agar, glycine, lithium chloride and tellurite suppress the growth of bacteria other than

Staphylococcus spp. *S. aureus* reduces tellurite to form grey black or black shiny colonies. A clearance zone around the colonies is produced by the action of lecithinase present in *S.aureus* on the egg yolk. By the action of lipases / phospholipases on lipids or phospolipids *S. aureus* produces a white precipitate as a margin around the black colonies. Typical *S. aureus* gives all the above three characteristics; but the white margin may not be produced by all strains of *S. aureus*.

Confirmation of Staphylococcus aureus

S. aureus is confirmed by Coagulase test. Coagulase is a thrombin-like substance which coagulates blood plasma. Most of the pathogenic *S.aureus* produce coagulase. Difco Bacto-coagulase-EDTA is used for the test. To 0.5 ml. of coagulase reagent in a small sterile test tube, add 2 drops of 24 h old bacterial culture (grown in Brain Heart Infusion broth, BHI; incubate in a serological water bath at 37°C. Observe every 30 min upto 4 h. Coagulation (jell formation) of the contents of the tube indicates a positive reaction for coagulase.

KF Agar plates

Observe after 36-48 h. Count all surface and sub-surface red to pink colonies (some will be with a thin white margin) as faecal streptococci. On KF agar, the sodium azide acts as a selective agent and streptococci reduces TTC to give red coloured formazan dye.

Confirmation of faecal streptococci

Faecal streptococci (Enterococci) group include *Streptococcus faecalis* and *Streptococcus faecium.*

They are confirmed by catalase test.

Pick 5-6 typical colonies from KF agar to BHI broth and incubate at 36 ±1°C for 24-48h. Mix 3ml of the culture with 0.5 ml of dilute Hydrogen peroxide (H_2O_2). (Usually H-$_2O_2$ is available as 30 vol / vol solution, ie 30%. Dilute 2ml to 5ml with distilled water for the test). Note the evolution of gas bubbles (oxygen). No evolution of gas bubbles indicates a negative reaction. Faecal streptococci are catalase negative. A negative result in catalase test confirms faecal streptococci.

MPN method for coliforms

Coliforms, including *E.coli* are determined by Most Probable Number (MPN) method, when present in very low numbers.

The plating methods have limitations to detect low numbers of bacteria in water or food, because the inoculum size is only one ml. per Petri dish. Pathogenic or indicator bacteria may not be present in sufficiently large numbers in water or food to be detected by plating methods. For example, in the case of potable water permitted level of coliforms is less than one per 100ml. In such cases, MPN methods are used, where larger volumes of sample can be used for inoculation. MPN is only a statistical approximation of the test bacteria in the given sample and not the actual number. MPN is defined as *"that bacterial density which, if it had been actually present in the sample would, more frequently than any other, have given the observed analytical results"* (Hoskns & Butterfield). Usually MPN method is used to detect coliform bacteria in water or food provided the expected number of these bacteria in the food products is less than 10^3 per gram. Otherwise the results may not be meaningful. MPN method for coliforms is a three step process and all the media used are liquid media.

Step I: (For presumptive total coliforms)

Requirements

1. Double strength MacConkey broth (DS MC broth) -50 ml - 1 flask
2. Double strength MacConkey broth (DS MC broth) -10 ml - 5 Tubes
3. Single strength MacConkey broth (SS MC broth)- -10 ml - 10 tubes

4. 10ml and 1ml pipettes
5. Serological water bath at 37°C and 44.5°C.
6. Incubator at 37°C.

Arrange the DS and SS MacConkey broths in a test tube rack. 50 ml of the given water sample is inoculated into 50 ml. DS MC broth, 10ml each of the water sample is inoculated into 5 tubes DS MC broth, 1 ml each into 5 tubes of SS MC broth. Make a decimal dilution of the water sample by adding 1 ml of the water to 9 ml of Normal saline and add 1 ml each of the first dilution (equivalent to 0.1 ml of the original sample) to the next five tubes of SS MC broth. Label appropriately. Incubate in the serological water bath at 37°C for 24 h.

After 24 h, observe the tubes for growth and gas production. If the colour of the MC broth has turned towards yellow with gas production in Durhams tubes, the reaction is noted as positive. Note the results as number of positives in each set of 50 ml, 10ml, 1ml and 0.1ml tubes. Compare the results with standard MPN Table (for 5 tube MPN) and note the MPN values for presumptive total coliforms.

Step-II (For confirmed total coliforms).

Inoculate one loopful of culture from the positive tubes of Step-I, to BGLB 2% broth and mark with the corresponding label. Incubate at 37°C in a serological water bath for 24 h. Note growth and gas production. Results are noted as positives if there are growth and gas production. Compare with 5 tube MPN table and obtain the results as MPN confirmed total coliforms.

Step-III (for faecal coliforms and *E. coli*)

From the positive tubes of Step-II, inoculate one loopful each to EC broth and Tryptone broth (indole medium). Label appropriately. Incubate at 44.5° ±0.5°C for 24 h.

EC broth: Note growth and gas production. Note the number of positives in each set. Compare with 5 tube MPN tables. The values obtained gives MPN faecal coliforms.

Tryptone broth: Test for indole production by adding 4 drops of Kovac's indole reagent. A pink or red colour at the top layer indicates a positive test for indole. Note the number of positives in each set. Compare with 5 tube MPN table. Coliform bacteria which produce gas in EC broth and indole in tryptone broth at 44.5 ± 0.5°C are considered as *E. coli*.

Detection and confirmation of pathogens in seafood

Salmonella

The genus Salmonella belongs to the family Enterobacteriaceae. The bacterium is Gram negative. It is motile due to the presence of peritrichous flagella (with a few exceptions).At present more than 2000 serotypes of Salmonella are known to exist

From the epidemiological point of view, Salmonella can be classified into three main groups. The first group is the Typhoid group comprising of *Salmonella typhi* and *S. paratyphi* A and C. They infect only man and are spread either directly or indirectly (via food and water) from person to person. The second group is mainly a veterinary group which includes serovars that are host adapted for particular species of vertebrates, e.g., *S. gallinarum* in poultry, *S. dublin* in cattle, *S. choleraesuis* in swine. Some of these are also pathogenic to man (especially *S. Dublin, S. choleraesuis*). The third group is the food poisoning group (non-Typhoid group) which contains the majority of other salmonella serovars with no particular host preference. They infect both man and animals. This third group includes the principal agents of Salmonellosis, ie.gastroenteritis due to Salmonella.

Fish and fish products are only occasionally associated with salmonellosis although fish meal for animals feed often contain Salmonella as a result of contamination from rodents and birds. Filter-feeding shellfish harvested from polluted waters and frozen pre-cooked prawns have been identified as higher risk products.

Detection and Identification of Salmonella

Pre-enrichment

Macerate 25g of the fish / prawn sample with 225ml of pre-enrichment medium (Lactose broth /Buffered peptone water/Nutrient broth). Incubate at 36±1°C for 18-24h.

Selective enrichment

Pipette 1ml.of culture from the pre-enrichment medium (above) to 10 ml of selective enrichment medium (Selenite cysteine broth and tetrathionate broth). Incubate at 36+1°C for 18-24h.

Selective plating

Streak one loopful from the selective enrichment medium (above) onto pre-dried selective plating medium, viz. (I) Brilliant Green Agar (BGA), (ii) Bismuth Sulphite Agar (BSA) ,(iii) Hekton's Enteric Agar (HEA) , (iv) Xylose Lysine Desoxycholate Agar (XLD) . Incubate at 36 ±1°C for 24 h.

Examine the plates for typical Salmonella colonies.

Salmonella colonies will appear as follows on each of the Selective agar:-

BGA - Smooth, low, convex, moist pink colonies; surrounding medium bright red

All *Salmonella* spp. except *S. typhi* are recovered on BGA. The medium contains lactose and sucrose as sugars and phenol red as acid base indicator. Brilliant green suppresses Gram positive bacteria. *Salmonella* (and other bacteria) which do not ferment lactose and sucrose form 'red-pink-white' opaque colonies surrounded by brilliant red zones. Lactose / Sucrose fermenting organisms like *E. coli* / *Klebsiella* / *Enterobacter* group may form yellow / greenish yellow colonies with intense yellow / green zones (usually, they are inhibited by brilliant green).

BSA - Brown, grey to black colonies with metallic sheen, surrounding medium brown to black.This medium contains glucose as the fermenting sugar. Brilliant green and Bismuth sulphite suppress the growth of Gram positive organisms and coliforms, while permitting growth of *Salmonella*, including *S. typhi*. The metallic ions (Bi^{++} and Fe^{++}) present in the medium stains the *Salmonella* colonies and surrounding medium black or brown in presence of H_2S. *Salmonella* forms brown / black colonies with metallic sheen and surrounding medium turns brown / black. Coliforms and other members of Enterobacteriacea are inhibited on BSA, but occasionally, dull green or brown colonies without metallic sheen may be formed.

HEA - Colonies blue - green with black centre.This medium contains the sugars, lactose, sucrose and salicin. Acid fuchsin and bromothymol blue act as acid / base indicators, and ferric salt as H_2S indicator. *Salmonella* do not ferment these three sugars. Hence *Salmonella* colonies on HEA are blue-green with or without black centre (H_2S production). Shigella appears as green moist raised colonies. Coliforms and other lactose / sucrose / salicin fermenters produce salmon-pink or orange colonies.

Caution: *Proteus* may produce Salmonella – like colonies

XLD - Colonies Red (Pink) with or without black centres.Xylose, lactose, sucrose and lysine are the critical ingredients of XLD. Phenol red is the acid-base indicator and ferric ammonium citrate, the H_2S indicator.

Salmonella do not ferment sucrose and lactose, but rapidly utilize xylose, changing the pH of medium to acidic range, but the active decarboxylation of lysine by *Salmonella* produces the base cadavenine, which neutralizes the acidic pH and changes the reaction to alkaline. Hence Salmonella colonies will appear red (pink) with or without black centre (due to H_2S production by certain *Salmonella* spp.). Other members of the *Enterobacteriaceae* (like *E. coli*) ferment sugars and produce yellow opaque colonies

Caution: *Shigella* form red colonies on XLD.

5. Inoculation to Triple Sugar Iron (TSI) agar, Lysine Iron Agar (LIA) and Urea agar.

Select 2 or 3 typical (or suspected) colonies from each selective agar. Lightly touch the centre of the colony to be picked with sterile needle. Inoculate TSI agar by streaking the slant and stabbing the butt. Without flaming the needle, inoculate LIA by stabbing the butt twice and then streaking the slant. Incubate at 36±1°C, TSI for 24 h and LIA for 48h. Inoculate urea agar slants by streaking and incubate at 36±1°C for 24 h.

Observe the reactions . Salmonella gives the following reactions.

TSI - Alkaline (red) slant and acid (yellow) butt with or without blackening (due to H_2S), sometimes breaking of the agar (gas production).

The TSI agar contains 3 sugars – Glucose (0.1%), sucrose and lactose (1% each), with phenol red (acid base indicator) and ferric citrate (H_2S indicator). Two types of sugar reactions take place – aerobic in the slant (slope) and anaerobic in the butt. Salmonella readily ferments glucose, but lactose and sucrose are not fermented. In the slant, Salmonella gives either alkaline reaction (red) or no change, even though it is glucose fermentative. This is because, in the thin area of slope, the amount of glucose is very low and the acid produced in the beginning gets neutralized by the alkaline products of amino acid metabolism. But in the butt, there is a higher volume of medium, and hence higher quantity of glucose. Hence the acid produced does not get neutralized soon and the butt is acidic (yellow). All the Salmonella give alkaline slant and acid butt. Most of the Salmonella strains (except *S. typhi*) produce gas, indicated by break in medium. H_2S is produced by most of the Salmonella (except *S. typhi* and *S. paratyphi*) indicated by black colour. Sometimes black colour masks the acid production in the butt.

LIA - Alkaline (purple) butt and slant.

A distinct yellow (acid) in butt indicates negative reaction. Other reactions should be considered suspected positives. A black precipitate indicates H_2S production Fermentation of glucose, decarboxylation of lysine and H_2S production are detected in LIA. Bromocresol purpole (BCP) is the acid base indicator and ferric citrate is included for testing H_2S production. Possible reactions in the media are acid production indicated by yellow colour, alkaline reaction indicated by purple colour, no reaction indicated by red colour and H_2S production indicated by black colour. Salmonella ferments glucose and rapidly decarboxylates lysine. So, gives alkaline reaction (purple colour) in both slant and butt (acid from glucose is totally neutralized by bases from lysine decarboxylation).

Urea agar. No colour change

Positive growth with no change of colour indicates a urease negative reaction. Growth with pink colour indicates a urease positive reaction. The phenol red indicator in the media turns red in alkaline pH due to the production of ammonia by hydrolysis of urea by bacteria possessing urease enzyme. Salmonella do not possess urease and hence gives a negative reaction.

6. Retain all cultures giving typical reactions in TSI, LIA and urea agar.

Confirm for Salmonella by biochemical tests.

7. Purification

a) Before biochemical tests, purify the cultures by streaking on MacConkey agar, by streak dilution method. Typical salmonella colonies will be transparent and colourless. (occasionally with dark centre). Pick the typical colonies to NA slants and incubate at 36±1°C.

8. Biochemical tests

Inoculate the following media for biochemical tests:

1) Lysine decarboxylase broth
2) Malonate broth
3) Indole medium (Tryptone broth)
4) Glucose broth
5) Lactose broth
6) Sucrose broth
7) Dulcitol broth
8) Salicin broth
9) MRVP medium (2 tubes; one each for MR and VP tests)
10) Simmon's citrate agar.

Also test for Gram's reaction and motility.

Table 2. Reactions of *Salmonella*

1.	Gram's stain	Gram negative ; short rods
2.	Motility	Motile
3	TSI	Alkaline slant, Acid butt, H_2S positive, Gas positive
4.	LIA	Alkaline slant, alkaline butt, H_2S positive
5.	Urease	Negative
6.	Indole	Negative
7.	Glucose	Acid and Gas
8.	Lactose	Negative
9.	Sucrose	Negative
10.	Dulcitol	Acid and gas
11.	Salicin	Negative
12.	MR test	Positive
13.	VP test	Negative
14.	Lysine decarboxylase	Positive
15.	Malonate utilization	Negative
16.	Citrate	Positive

9. Serological confirmation

All cultures giving typical biochemical reactions are confirmed by agglutination test with Salmonella polyvalent somatic (0) antiserum.

Mark two sections about 1x2 cm each on a glass slide. Emulsify a loopful of the isolated culture with 2ml of 0.85% saline in a test tube. Add one drop of culture suspension to both the marked sections on the slide. Add one drop of saline solution to one section only. Add one drop of the Salmonella Polyvalent O antiserum to the other section only. Using a loop mix culture suspension with saline solution in one section and with the antiserum on the other section. Tilt mixture in back and forth motion for 1 min and observe against dark background in good illumination. Consider any degree of agglutination ie., clumping together of the bacterial cells as a positive reaction.

Positive: agglutination in test mixture; no agglutination in saline control

Negative: no agglutination in test mixture; no agglutination in saline control.

Nonspecific: agglutination in test and in control mixtures.

Vibrio cholerae

V.cholerae comes under the genus *Vibrio* belonging to the family *Vibrionaceae*. *V.cholerae* is divided into 2 biotypes Classical and El Tor based on certain biochemical properties. Each biotype of *V cholerae* is further classified into 2 sub-groups based on the somatic (O) antigenic profile as O1 and non O1 based on agglutination with specific O antisera. *V.cholerae* 'O' group has three known serotypes each possessing distinct antigenic factors. They are Ogawa, Inaba and Hikojima with 'O' antigenic factors AB, AC and ABC respectively.

Detection and Identification.

1. Enrichment

25gm of sample is blended with 225ml Alkaline Peptone Water (APW); transfer aseptically to a sterile 500 ml conical flask and incubate at 36±1°C.

2. Streak on Thiosulphate Citrate Bile salt Sucrose agar (TCBS agar)

After 6-8 h and 16-24 h of incubation (do not shake the flask) streak loopful from the surface growth (pellicle) on to pre-set TCBS agar. Incubate the TCBS plates at 36±1°C for 18-24h. Examine the plates for typical *V.cholerae* colonies.

Typical *V. cholera* colonies are large (2-3 mm dia), smooth, yellow slightly flattened with opaque centres and translucent peripheries (*Vibrio* spp. do not form tiny, creamy yellow colonies on TCBS).

3. Pick typical colonies (2-3) to Nutrient Agar Slants (NA) and incubate at 36±1°C for 24 h.

4. Triple Sugar Iron (TSI) and Kligler Iron Agar (KIA)

Inoculate into TSI and KIA by stabbing butt and streaking slant. Incubate at 36±1C° for 18-24 h. Observe for typical reactions of *V. cholerae*.

TSI - Acidic slant (yellow) and Acidic butt (yellow); No blackening

KIA - Alkaline slant (red) and acidic butt (yellow); No blackening

On TSI, *V. cholerae* ferments both glucose and sucrose, but not lactose and it does not produce gas and H_2S. Hence both slant and butt will turn yellow due to acid production from glucose and sucrose.

KIA, contains glucose (0.1%) and lactose (1%) with phenol red indicator. The level of glucose in the thin slant area is so low that the acid produced by *V. cholerae* by fermentation will be neutralized by the bases produced from peptones during bacterial growth. So, slant will be red (alkaline). But in the butt, the acid produced will be in sufficiently large quantity and will not be completely neutralized by bases at the end of 24h. So, the butt will be yellow (acidic).

Cultures giving typical reactions are confirmed as *V. cholerae* by biochemical tests.

Biochemical tests

Salt tolerance

Inoculate into T_1N_O and T_1N_3 broths and incubate at 36±1°C for 18-24 h. *V. cholerae* will grow in T_1N_O and T_1N_3 broths. (T_1N_0 contains zero salt and T_1N_3 contains 3% salt)

(Note: Some *V. cholerae* Non 01 will grow only at T_1N_3)

H and L glucose O/F test

Incubate by stabbing with a long needle, into H&L glucose O/F medium and incubate at 36±°C for 18-24 h. A yellow colour through out, indicates a fermentative reaction typical of *Vibrio* spp. Vibrios do not produce gas.

Cytochrome oxidase test

Vibrio cholerae gives a positive test for cytochrome oxidase.

d) Gram reaction and motility.

V.cholerae is Gram negative short or curved rods and actively motile.

Fermentation of carbohydrates

Inoculate the following sugar media and incubate at 36±1°C for 48 h.

(i) Glucose, (ii) Sucrose (iii) Arabinose (v) Mannose (v) Mannitol (vi) Inositol

Examine for the production of acid and gas from these medium.

Decarboxylase tests

Inoculate the following three amino acid media, add sterile liquid paraffin (~1cm height) and incubate at 36±1°C for 4 days. Examine for colour change.

A change of colour to purple (alkaline) indicates a positive test for decarboxylation. A yellow colour (acid) indicates negative test.

(i) Lysine decarboxylase medium

(ii) Arginine dihydrolase medium

(iii) Ornithine decarboxylase medium

The medium contains 0.1 % glucose in addition to the test amino acid. In the initial stages glucose is fermented to produce acid (yellow). On further incubation, bases produced by amino acid decarboxylation changes the pH to alkaline, producing purple colour. So, if yellow colour is obtained, continue incubation for 4 days for a conclusion.

Sensitivity to vibriostatic agent 0/129.

Vibrios are sensitive to the vibriostatic agent 0/129 (2,4–Diamino-6,7-diisopropyl pteridine phosphate). Sensitivity of *Vibrio cholerae* to two levels of 0/129, viz. 10 microgram and 150 microgram, are tested. *Vibrio cholerae* is sensitive to both the levels of 0/129.

A heavy inoculum of the test culture is smeared uniformly on the surface of pre-set Nutrient Agar (NA) plate. One disk each containing 10 Mg and 150 Mg. 0/129 compound is placed in each half of the seeded plate and incubated at 36±1°C for 24h. A zone of clearance around the disk indicates sensitivity to that level of 0/129.

Serological confirmation.

The culture identified as *V. cholerae* by cultural and biochemical characteristics as above is confirmed as *V. cholerae* by agglutination test using polyvalent *V. cholerae* O. antiserum. Various reactions for identification of V.*cholerae* are summarized in Table3.

Table 3. Reactions of *Vibrio cholerae* for identification.

Media	Characteristics
TCBS agar	Smooth, yellow, 2-3 mm dia., slightly flattened colonies with opaque centre and translucent periphery.
TSI	Acid slant (yellow) Acid butt (yellow)
KIA	Alkaline slant (red) Acid butt (yellow)
T_1N_0 & T_1N_3	Growth positive
H& L Glucose O / F Test	Fermentative (acid , No Gas)
Cytochrome Oxidase	Positive

Gram stain	Gram negative short rods (Curved rods)
Motility	Motile
Indole	Positive
D-Mannose	Acid only
D-Mannitol	Acid only
L-Inositol	Negative
Glucose	Acid only
Sucrose	Acid only
Arabinose	Negative
L-Lysine decarboxylase	Positive
L-Arginine dihydrolase	Negative
L- Ornithine decarboxylase	Positive
Sensitivity to 0 /129 -10 Mg 150 Mg	Positive; Positive
H_2S Production (Black colour in TSI)	Negative
Agglutination with Polyvalent *V. cholerae* O antisera	Positive for *V. cholerae* O1

The microbiological standards for fish and fish products are given in Table 4.

Table: 4: Microbial standards for fish/fishery products

Products	TPC at 37°C/g.	Coagulase +ve Staphylococci/g.	E. coli/g.	Salmonella	*V. cholerae*
Fresh, chilled or frozen fish, prawn, cuttlefish/ squid	5,00,000	100	20	Absent in 25g.	Absent in 25g.
Cooked/boiled "	1,00,000	100	Nil	Absent in 25g.	Absent in 25g.
Cuttle fish/squid for raw consumption	2,00,000	100	20	Absent in 25g.	Absent in 25g.
Crab, Clam, Mussel, Scallop, Oyster	10,00,000	100	20	Absent in 25g.	Nil