

Isolation and identification of *Vibrio cholerae* and *Vibrio Parahaemolyticus* in fish and fishery products

Vibrios are Gram-negative, curved rods that are actively motile by means of polar flagellum. The two most important human pathogenic vibrios associated with fish and fishery products are *Vibrio cholerae* and *V. parahaemolyticus*.

VIBRIO CHOLERAEE

The most important member of the group vibrios is *Vibrio cholerae*, the causative agent of cholera - a food and water borne disease. The clinical symptoms of severe cholera result from the loss of large volumes of diarrhoeal fluid which is a bicarbonate rich, isotonic electrolyte leading to dehydration and death. The incidences of mild asymptomatic infections are more with *eltor* vibrios than with classical cholera vibrios. Non-agglutinable (NAG) vibrios sometimes produce a disease clinically indistinguishable from cholera. The treatment of cholera consists essentially of the prompt and adequate replacement of lost fluid and electrolytes. Fish and fishery products get contaminated with *V. cholera* through domestic sewage contamination of fish growing /harvesting waters, use of contaminated water/ ice for processing and infected food handlers.

As these organisms are expected in very low numbers in fish and fishery products, their detection involves use of selective enrichment broth (alkaline peptone water), selective differential agar medium (thiosulphate citrate bile salt sucrose agar) and confirmation using biochemical tests.

Materials required

- Alkaline Peptone Water (APW)
- Thiosulphate-citrate-Bile salt-Sucrose(TCBS) Agar
- Triple Sugar Iron Agar (TSI)
- Nutrient Agar
- Kligler Iron Agar (KIA)
- 0.85 % Sodium chloride solution
- Polyvalent *V. cholerae* 'O' antiserum
- Ogawa, Inaba, Hikojima antisera (if necessary)
- Dextrose
- Sucrose
- Mannitol
- Arabinose
- Inositol
- Lysine decarboxylase medium
- Ornithine decarboxylase medium
- Arginine dihydrolase medium
- Tryptone broth

Procedure

About 25 g of the composite sample is aseptically weighed, ground or cut into small pieces and transferred into a long necked flat-bottomed flask containing 225 ml of Alkaline Peptone Water (APW) and the suspension is incubated for overnight at $37 \pm 10^\circ\text{C}$ for primary enrichment. A loopful of the incubated peptone water broth is streaked on TCBS plates to obtain isolated colonies and plates are incubated for 18 to 24 hours at $37 \pm 10^\circ\text{C}$. Typical *V. Cholerae* colonies on TCBS agar plates appear as large, smooth, yellow and slightly flattened with opaque center and translucent peripheries. Three or more typical colonies from each of the TCBS plates are subcultured into nutrient agar plates and subjected to following tests to ensure the biochemical identity of *V. Cholerae*.

Characteristics of *V. cholerae*

Sl. No.	Test	Result
1.	Motility test	Motile
2.	Oxidase test	Positive

3.	O/F test	Fermentative (acid & gas Aerobically and anaerobically)
4.	Fermentation of glucose	Positive (Acid, no gas)
5.	Fermentation of sucrose	Positive (Acid, no gas)
6.	Fermentation of mannitol	Positive (Acid, no gas)
7.	Fermentation of inositol	Negative (No acid, no gas)
8.	Fermentation of arabinose	Negative (No acid, no gas)
9.	Lysine decarboxylase	Positive (Purple)
10.	Ornithine decarboxylase	Positive (Purple)
11.	Arginine dihydrolase	Negative (Yellow)
12.	TSI	Negative (No blackening)
13.	Growth in nacl 0 Percent 3 Percent 6 Percent	Positive Positive Negative

Agglutination test

Using a glass marking pencil, two sections of about 1 X 2 cm each are marked in a clean microscope slide. A small amount of the 4-8 h culture in peptone water is placed. One drop of 0.85 % sodium chloride solution is added to both the sections. The culture in the saline is stirred with a clean sterile loop or needle to give an even suspension. A drop of polyvalent *V. Cholerae* '01' antiserum is added to one of the sections and mixed thoroughly with a sterile loop or needle. The slide is tilted back and forth for one minute and the agglutination is observed against a black background. A positive reaction is indicated by a rapid and strong clumping of the organisms in the suspension. Each culture is tested with polyvalent antisera and when positive, test with Ogawa, Inaba and Hikojima antisera, if necessary. Cultures with characteristics biochemical reaction of *V. Cholerae* and agglutination with *V. Cholerae* 01 polyvalent antisera are classified as *V. Cholerae* 01 and the culture with characteristic biochemical reactions but without agglutination with *V. Cholerae* 01 polyvalent antisera is classified as non-01 (i.e) Non-Agglutinable (NAG Vibrios).

Sanitary analysis for *V. Cholerae*

For processing water, 900 ml of water is mixed with 100 ml of ten fold concentrated peptone water at pH 8.6. One or two loopful of the inoculum each after primary and secondary enrichment broths is used for streaking onto previously poured and dried plates of TCBS Agar. The rest of the procedure is same as described for frozen samples. In the case of ice, the ice is allowed to melt into water at room temperature in the laboratory and follow the analytical procedure as described for water and frozen samples. For swab samples also, one or two loopful of inoculum from each shall be taken after primary and secondary enrichments and proceeded in the usual way.

VIBRIO PARAHAEMOLYTICUS

V. parahaemolyticus is a Gram-negative rod shaped, facultatively anaerobic, motile non-spore forming, halophilic bacterium associated with seafoods in their natural environment. It is a causative agent of food poisoning involving consumption of contaminated fish, oyster, crabs, shrimps and lobsters that have been eaten raw/semi-cooked or pre-contaminated cooked food. Therefore, the outbreaks are mostly caused under conditions of improper refrigeration, insufficient cooking, cross-contamination or recontamination. Food poisoning is due to infection and gastro-enteritis develops within 10-15 h. The duration of illness is often short, lasting for 4-6 h, but at times longer for 48 h. The enumeration of *V. Parahaemolyticus* can be done by direct plating method but its presence can be

detected by following enrichment method. Being a halophilic organism, all media used for the isolation of this organism should be supplemented with 3 % sodium chloride.

Materials required

- Alkaline Peptone Salt (APS) broth
- TCBS Agar
- Wagatsuma Agar
- Salt tolerance media
- Buffered Glucose broth
- Lysine decarboxylase medium
- Nutrient agar
- TSI Agar
- Tryptone water
- Saline diluent
- Kovac's reagent
- Alpha-naphthol
- Potassium hydroxide
- Tetramethyl paraphenylene diamine dihydrochloride (O/129)

Procedure

Direct plating method

About 50 g sample is blended thoroughly with 450 ml of saline diluent (sterile 3% NaCl solution), serial dilutions are made using 9 ml saline diluents, 0.1 ml volume of appropriate dilution spread on TCBS Agar plates and incubated at 37±10C for 18 h. The typical colonies of *V. parahaemolyticus* on TCBS agar plates appearing as round blue-green colonies of about 2-3 mm in diameter with dark centre are counted and numbers expressed as CFU/g of sample.

Enrichment method

The same procedure followed for the isolation of *V. cholera* using APW enrichment (containing 3% NaCl) and subculturing on TCBS is followed for isolating *V. parahaemolyticus*. But the typical colonies of *V. parahaemolyticus* on TCBS agar plates appearing as round blue-green colonies of about 2-3 mm in diameter with dark centre are picked, purified and subjected to a battery of biochemical tests for confirmation. These include tests for production of indole, hydrogen sulphide (on TSI slant), fermentation of glucose, lactose and sucrose, oxidase, salt tolerance test, amino acid decarboxylation test and sensitivity to vibriostat substance (O/129).

The typical reaction of *V. parahaemolyticus* are as follows;

Tests	Reactions	Results
Tryptone at 37±10C	Indole	Positive
TSI Agar	H ₂ S	Negative
TSI Agar	Glucose fermentation	Acid, no gas
TSI Agar	Lactose fermentation	Negative
TSI Agar	Sucrose fermentation	Negative
L.D.C.	No change	Positive
Buffered Glucose Broth	Acetoin	Negative
Peptone containing no sodium chloride	Growth	Negative
Peptone containing 8% Sodium chloride	Growth	Negative
Peptone containing 11 % Sodium chloride	Growth	Positive
Tryptone water	Growth at 420C	Positive
Nutrient Agar	Oxidase	Positive

Confirmation

The isolates of *V. parahaemolyticus* identified based on biochemical tests are further confirmed for the production of specific haemolysins by subjecting to Kanagawa reaction on Wagatsuma agar.

The test cultures grown in tryptone broth are spot inoculated onto freshly prepared, dried modified Wagatsuma agar plates, incubated at 370C for 18 h, and observed for clear zone of haemolysis. A Kanagawa-positive reference culture (usually approximately 5 mm) is also used for reference and

comparison. A positive reaction correlating with the pathogenicity of the organisms is characterized by sharply defined, clear, colourless zone of haemolysis of 2-4 mm in diameter surrounding the colony.

Characteristics / reactions of *V. parahaemolyticus*

Tests	Reactions	Results
Growth in 8 % NaCl	Turbid	Positive
Malonate	Green	Negative
Citrate	Turbid	Positive
Nitrate reduction	Purple	Positive
Haemolysis in blood	Clear zone	Positive
Kanagawa reaction	Halo	Positive
Growth at 42°C	Turbid	Positive
Triple sugar iron agar	Alkaline slant and acid butt	Positive
Motility	Motile	Positive
Methyl red	Red	Positive
Beta galactoxidase	No change in colour	Negative
Lysine decarboxylase	Purple	Positive
Ornithine decarboxylase	Purple / yellow	Positive/Negative
Arginine dihydrolase	Yellow	Negative
Catalase	Effervescence	Positive
Urease	No change in colour	Negative
Indole	Red ring	Positive
Oxidase	Purple	Positive
Voges Proskauer	No change in colour	Negative
Hydrogen sulphide	No blackening	Negative Positive

Media composition

Alkaline Peptone water (APW) Broth

Peptone Distilled	: 10.0 g
water	: 1000.0 ml
Ph	: 8.5 ± 0.2

The media is sterilized at 121°C for 15 minutes. For isolating *V. parahaemolyticus*, this media should be supplemented with 3% NaCl.

Thiosulphate Citrate Bile salt Sucrose (TCBS) Agar

Peptone	: 10.0 g
Yeast extract	: 5.0 g
Sucrose	: 20.0 g
Sodium thiosulphate	: 10.0 g
Sodium citrate dehydrate	: 10.0 g
Sodium cholate	: 3.0 g
Oxgall	: 5.0 g
Sodium chloride	: 10.0 g
Ferric citrate	: 1.0 g
Bromothymol blue (BTB)	: 0.04 g
Thymol blue (TB)	: 0.04 g
Agar	: 15.0 g
Distilled water	: 976.0 ml
Ph	: 8.6

Dissolve and boil the ingredients except BTB and TB in sterile distilled water. Add 20 ml of 0.2% BTB and 4 ml of 1% TB. Heat to boil again and cool to 45°C. Do not autoclave.

Wagatsuma Agar

	: 3.0 g
	: 10.0 g
Yeast extract	: 70.0 g
Peptone	: 5.0 g
Sodium chloride	: 10.0 g
Dipotassium hydrogen phosphate	: 0.0001 g
Mannitol	: 15.0 g
Crystal violet	: 1000.0 ml
Agar	: 8.0
Distilled water	
Ph	

Dissolve the above ingredients by gentle heating. Do not autoclave, but steam for 30 minutes and cool to about 50°C. Add 100 ml of 20 % suspension of freshly drawn citrated human red blood cells or rabbit RBCs, which have been washed three times, in sterile physiological saline. Mix well and pour into sterile Petri plates and cool.