

# *Vibrio parahaemolyticus* in Marine Products and its Isolation and Identification

*Vibrio parahaemolyticus* is an organism causing food poisoning associated with the consumption of raw fish. This organism is widely distributed in the marine environments such as water sediments, fish and shellfish through out the world. This was first isolated in Japan in early 1950. Although the problem of *Vibrio parahaemolyticus* food poisoning has been reported from different parts of the world, it is found more in countries such as Japan where there is a habit of eating raw fish.

## Morphology and other characteristics

*V. parahaemolyticus* is a gram negative, facultatively anaerobic rod, motile and non spore-forming. It grows faster than any other organism for which growth rate has been determined. In many bacteria the generation time is about 20 minutes. But in the case of *Vibrio parahaemolyticus* it is 7 minutes. Hence on initial contamination of fish with this vibrio followed by improper icing will result in the multiplication of this organism and results in the production of food poisoning on consumption of the fish.

*Vibrio parahaemolyticus* grows profusely in ordinary nutrient media with 2-4% NaCl, but grows very poorly in media not containing salt. Although growth is most abundant

in media with 2-4% NaCl, growth also takes place in peptone water containing 8% NaCl. Little or no growth, however, occurs in peptone water with 10% NaCl. It prefers alkaline conditions (pH 7.6-8.6) and it grows between temperature of 15°C to 43°C. The growth is the best at 37°C. Most of the strains do not grow at temperatures below 5°C, although the organism may survive for long periods at these temperatures. It is more sensitive to chilling than to freezing. Therefore, a thorough washing of the fish quickly after catch and immediate icing after washing is the best method to control the incidence of the organism. If seafood is heated to 100°C just before consumption, food poisoning due to this organism would rarely occur. Most of the *V. parahaemolyticus* are destroyed during freezing at -40°C and storage at -20°C for 10 days.

*Vibrio parahaemolyticus* can further be grouped into two types depending on the haemolytic properties in a specified media (Wagatsumo medium). The strains which give the haemolytic reaction are classified as Kanagawa-positive and those which do not give the haemolytic reaction as Kanagawa negative. Studies have shown that about 97% of the cultures isolated from human sources were kanagawa positive, whereas only 1%

of the cultures isolated from marine sources were Kanagawa negative.

## Epidemiology

The food poisoning due to *Vibrio parahaemolyticus* is an infection leading to gastroenteritis in most cases. The symptoms usually appear within 10-15 hours after eating infected food, although the incubation period may be as short as 2 hours or as long as 48 hours. The main symptoms are nausea, vomiting, abdominal pain and diarrhoea, Mild fever, headache and chills may occur. Stools are usually watery, but mucus and blood may be observed.

## Isolation and identification of *V. parahaemolyticus*

1. Transfer 25 g of the sample to 225 ml of Glucose Salt Teepol Broth (GSTB) or Alkaline Peptone Salt Broth (APS) and incubate for 18 hours at 37°C.
2. Streak a 3 mm loopful from the incubated GSTB of APS Broth on to the surface of Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar plates and incubate the plates for 18 hours of 37°C.
3. Transfer two or more suspected *Vibrio parahaemolyticus* colonies from the TCBS plate (on TCBS *Vibrio parahaemolyticus* colonies are round 2-3 mm in diameter, with green or blue centres) into Triple Sugar Iron (TSI) agar and incubate at 37°C for 18 hours. *V. parahaemolyticus* produces an alkaline (red) slant and an acid (yellow) buff with no production of gas and H<sub>2</sub>S.
4. The TSI positive cultures are confirmed by the following biochemical and other tests (table 1).

Table 1 Characteristics of *V. parahaemolyticus*

Test	Result
1. Gramstain	Gram-Negative rods
2. Motility	Motile
3. Oxidase	Positive
4. Indole	Positive
5. V.P	Negative
6. Glucose (Acid)	Positive
7. Sucrose (Acid)	Negative
8. Mannitol (Acid)	Positive
9. Arabinose (Acid)	Positive
10. Inositol (Acid)	Negative
11. Lysine Decarboxylase	Positive
12. Ornithine Decarboxylase	Positive
13. Arginine Dehydrogenase	Negative
14. Growth in NaCl	
0 Percent	Negative
3 Percent	Positive
6 Percent	Positive
8 Percent	Positive
10 Percent	Negative

NB: All media must contain at least 3% NaCl unless different concentrations are specified. Kanagawa phenomenon is also tested using Wagatsumo agar.

1. Procedure for Gram's Stain

- a. Prepare a smear. Put a drop of sterile saline on a slide, aseptically add a little of the colony for staining (18-24 hours old) mix well in the saline, air dry, fix by passing the slide 2-3 times through a Bunsen flame.
- b. Flood the smear with crystal violet for 1 minute
- c. Wash with tap water
- d. Flood the smear with Lugol's iodine solution for 1 minute
- e. Wash with alcohol
- f. Wash with tap water
- g. Flood with Safranin for 30 seconds
- h. Wash with tap water
- i. Air dry

- j. Observe under microscope (oil immersion lens) Gram-positive organisms stain violet or blue, Gram-negative organisms stain red.
2. Motility Test
- Hanging drop
- a. Place vaseline in four corners of a cover slip
  - b. Using a sterile loop, place a drop of an 18 hours nutrient broth culture on to the centre of the cover slip
  - c. Place a clean microscope slide (cavity slide) over the top of the cover slip
  - d. Invert the slide so that the drop is upside down
  - e. Observe under microscope using X40 objectives. The bacterium

can be said to be motile when it is seen to move from one side of the field of view to the other.

3. Oxidase Test

- a. Place a piece of filter paper into an empty petridish and add 3 drops of tetramethyl paraphe-nylene diamine dihydrochloride solution to its center (freshly) prepared 1% aqueous solution)
- b. With a sterile platinum wire or sterile glass rod smear cells thoroughly into the reagent impregnated paper. The oxidase test is positive if transferred cells turn dark purple in 5-10 seconds.

4. Indole Test and V.P. Test

Indole Test

Inoculate tubes of Tryptone Broth. Incubate tubes at 37°C for 24 hours. Add 0.2-0.3 ml of indole Reagent to the tube and shake. Allow 10 minutes and observe the results. A dark red colour in the amyl alcohol surface layer constitutes a positive test.

Voges - proskauer Test

Inoculate tubes of MRVP medium and incubate at 37°C to 48 hours. Pipette 1 ml of each culture to a separate empty culture tube and add 0.6 ml of α naphthol solution and 0.2 ml of potassium hydroxide solution. Shake the tubes and let them stand 2-4 hours. Record the development of pink or crimson color in the mixture as a positive test.

5. Carbohydrate Fermentation

Inoculate one tube each of carbohydrates broth (glucose, sucrose, mannitol, arabinose and inositol) with growth from TSI. Incubate at 37°C for 4 to 5 days. An acid reaction will change the color to yellow.

**6. Amino Acid Decarboxylase Test**

Inoculate tubes of Lysine Decarboxylase, Ornithine Decarboxylase, Arginine Dehydrolase and Basal Medium with loopful of TSI Agar Culture. Incubate at 37°C and examine daily for 4 days. The medium turns yellow because of acid production from glucose. When decarboxylation occurs, the medium becomes alkaline or purple.

**7. Holophilism Test**

Inoculate tubes of Salt Trypticase broth with four salt concentrations (0%, 3%, 6%, 8% and 10%). Incubate at 37°C for 24 hours and observe for growth.

**8. Kanagawa Test**

Spot several loopfuls of the culture on a single well-dried Wagatsumo Agar plate, in a circular pattern. Incubate at 37°C and observe results after 24 hours. Clear transparent zones around the colonies indicate a positive test.

**Media and reagents**

**1. Alkaline Peptone Salt Broth (APS)**

Peptone	10.0 g
Sodium Chloride	30.0 g
Distilled water	1 litre

Dissolve ingredients. Adjust pH to 8.5 ± 0.2. Dispenses in conical flasks. Autoclave 10 minutes at 15 lbs.

**2. Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS)**

Yeast extract	5.0 g
Peptone	10.0 g
Sucrose	20.0 g
Sodium thiosulfate	10.0 g
Sodium citrate	10.0 g
Sodium cholate	3.0 g
Ox-gall	5.0 g
Sodium Chlorate	10.0 g
Ferric citrate	1.0 g

Bromothymol blue (0.2% solution)	20 ml
Thymol blue (1% solution)	4 ml
Agar	15.0 g

Add all ingredients to 980 ml of distilled water and heat to boiling with agitation to obtain complete solution. Do not autoclave. Cool to 45-50°C, adjust pH to 8.6 and pour 15-20 ml volumes into Petri dishes. Dry the surface for 1 hour at 45°C in an inverted position.

Dehydrated TCBS is available commercially. Prepare as directed on the container.

**3. Triple Sugar Iron Agar (TSI)**

Tryptone or polypeptone	20.0 g
Sodium chloride	30.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Ferrous ammonium sulfate	0.2 g
Sodium thiosulfate	0.2 g
Phenol red (0.2% solution)	12 ml
Agar	13.0 g

Add all ingredients to 988 ml of distilled water, mix well and heat to boiling with agitation. Cool to 50-60°C and adjust pH to 7.3 ± 0.1. Fill tubes one-third full and sterilize at 121°C for 12 minutes. Cool tubes in slanted position to obtain buffs of 2.5 cm long and slants of approximately 5 cm long.

**4.a. Crystal violet solution**

Crystal violet (85-90% dye content)	2.0 g
Ethyl alcohol (95%)	20.0 ml
Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Dissolve the crystal violet in alcohol and the ammonium oxalate in distilled water. Mix two solutions and store the mixture for 24 hrs before use.

**b. Lugol's Iodine Solution**

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

Grind the potassium iodide and iodine together in a mortar, adding small increments of water while grinding. Rinse the resulting solution into a volumetric flask and bring the volume to 100 ml.

**c. Counterstain - Safranin**

Safranin O	0.25 g
Ethyl alcohol	10 ml
Distilled water	100 ml

Dissolve the safranin in the ethyl alcohol and mix the resultant solution with the distilled water.

**Indole Reagent (Kovacs)**

Paradimethyl aminobenzaldehyde	5.0 g
Isoamyl (or normal amyl) alcohol	75 ml
Hydrochloric acid (Conc.)	25 ml

Dissolve the benzaldehyde in the isoamyl alcohol and add hydrochloric acid.

**Voges-Proskauer test reagents**

- 5% α naphthol solution. Dissolve 5 g of α naphthol in 100 ml absolute alcohol
- 40% KOH-creatinine solution. Dissolve 40 g of potassium hydroxide and 0.3 g of creatine in 100 ml distilled water.

The α naphthol solution should be prepared fresh each day. The creatine can be omitted from KOH solution.

**Tryptone Broth**

Tryptone	10.00 g
NaCl	30.09 g
Distilled water	1 litre



Dispense in 5 ml portions into culture tubes and sterilize in an autoclave at 15 lbs for 15 minutes.

MRVP Broth

Nacl	30.0 g
Proteose peptone	7.0 g
Potassium monohydrogen phosphate	5.0 g
Glucose	5.0 g
Distilled water	1 litre

Dispense in 5 ml portions into culture tubes and sterilize in an autoclave at 15 lbs for 12-15 minutes.

5. *Carbohydrate Broth (Basal Medium)*

Peptone	10.0 g
Beef extract	3.0 g
Sodium chloride	30.0 g
Bromocresol purple	0.04 g
Distilled water	1 litre
pH 7.0 ± 0.2	

Add Glucose, Sucrose, Mannitol, Arabinose and Inositol (0.5%) to the basal medium separately. Sterilize at 15 lbs for 10 minutes.

6. *Decarboxylase Test Medium*

Peptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Sodium Chloride	30.0 g
Bromocresol purple	0.02 g
Distilled water	1 litre

For arginine broth, add 5 g.L. arginine for 1 litre base, for lysine broth, add 5 gL to 1 litre base and for Ornithine add 5 g L-Ornithine to 1 litre base. Adjust pH to 6.5±0.2. Dispense 5 ml portions into test tubes and sterilize at 15 lbs for 10 minutes.

7. *Salt Trypticase Broth*

Trypticase	10.0 g
Yeast extract	3.0 g

Dissolve ingredients in 1 litre of distilled water. Add 0, 3, 6, 8 and 10 g of NaCl per 100 ml to make, respectively 0, 3, 6, 8 and 10% NaCl trypticase broth. Autoclave at 15 lbs for 15 mnts. 15 Final pH 7.5.

8. *Wagatsuma Agar*

Yeast extract	5.0 g
Peptone	10.0 g
Sodium chloride	70.0 g
Mannitol	5.0 g
Crystal violet (0.1% solution)	1 ml
Agar	15.0 g

Dissolve all ingredients in one litre of distilled water and adjust pH to 7.5. Heat to boiling for several minutes to obtain complete solution. Do not autoclave. Cool to 50°C and add 100 ml of washed 20% suspension of human erythrocytes, mix and solidify as plates. Dry the plates on before use.

To prepare washed human erythrocytes suspension, centrifuge human defibrinated blood, wash erythrocytes with sterilized saline solution (0.85%) three times and suspend, volume of the last sediments of erythrocytes with four volumes of saline solutions.

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