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# *VIBRIO PARAHAEMOLYTICUS* IN MARINE PRODUCTS AND ITS ISOLATION AND IDENTIFICATION

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*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 an 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. *V.parahaemolyticus* food poisoning is due to ingestion of the organisms and not due to any toxin.

*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) is prepared by the organism and it grows between temperature of15°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 (Wagatsuma 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 hour 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 or APS Broth on to the surface of Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar plates and incubate the plates for 18 hours at 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) butt with no production of gas and $H_2S$.

4. The TSI positive cultures are confirmed by the following biochemical and other tests:Table 1

Table 1 Characoristics 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) | : | Nega tive |
| 8. | Mannitol (Acid) | : | Positive |
| 9. | Arabinose (Acid) | : | Positive |
| 10. | Inositol (Acid) | : | Negative |
| 11. | Lysine Decarboxylase | : | Positive |
| 12. | Ornithine Decarboxylase | : | Positive |
| 13. | Arginine Dehydrolase | : | 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 Wagatsuma 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 Lugols 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 vasaline 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 paraphenylene 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

See chapter on *E. coli*

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. Halophilism Test

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

8. Kanagawa Test

Spot several loopfuls of the culture on a single well-dried Wagatsuma 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.Dispense 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 chloride | 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 hours 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 sterlize at 121° C for for 12 *minutes. Cool tubes* in slanted position to obtain butts 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 e alcohol and the ammonium oxalate in distilled water. Mix two solutions and store the mixture for 24 hrs before use.

4.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 | 0.25g |
| Ethyl alcohol | 10 ml |
| Distilled water | 100 ml |

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

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 g L-lyzine 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 mts. Final pH 7.5.

8. *Wagatsuma Agar*

| | |
|---|---|
| Yeast extract | 5.0 g |
| Peptone | 10.0 g |
| Sodium chloride | 70.0g |
| 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 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.