VIBRIO CHOLERAE IN SEAFOODS AND ITS ISOLATION

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Cholera has been endemic in Eastern India and in East Pakistan (present Bangladesh). The fact that this disease is caused by an organism, called Cholera vibrio was first reported by Robert Koch in 1884, who observed that a wide variety of vibrios commonly found in nature were characteristically haemolytic, whereas true Cholera vibrios were not haemolytic. But subsequently in 1906 haemolytic strains of *Cholera* vibrios were isolated from dead bodies of pilgrims seen at the Eltor Quarantine station in Egypt. The cholera outbreak in Sulawesi (Indonesia) in 1939 was found to be due to the haemolytic biotypes (El Tor biotype) of *Vibrio cholerae*. The marked epidemiological difference between El Tor and classical cholera are that the infection-to-case ratio is higher with El Tor cholera and the El Tor vibrio can survive longer in the environment making it more easily detectable.

Morphology

Cholera vibrios are shaped as a coma or a curved rod measuring 1 to 5 micron in length and 0.3 to 0.6 micron in breadth. They are gram negative, non-spore forming and are actively motile by a single polar flagellum. They are aerobic. The optimum temperature for their growth is 37°C. They are one of the most rapidly multiplying bacteria outgrowing other organisms in the early hours of incubation. They are unusually tolerant of alkali, growing in media as alkaline as pH 9.2, a property utilized for primary isolation of the organisms. On prolonged cultivation, vibrios may become straight rods resembling other gram-negative enteric bacteria.

Natural habitat

The only known natural reservoir of *V. cholerae* is man and it is transmitted from man to man through the environment. Usually food, water, flies and contaminated hands play prominent role in the transmission of this organism. The infected individual will usually excrete vibrios for only a few days. But a few chronic carriers (persons harbouring the organism for more than three months) have been reported and one individual has been reported to have been infected for seven years.

Pathogenesis of *V. cholerae*

In the cholera victim the organisms gain entry through mouth into the small intestine where the alkaline medium and an abundance of products of protein metabolism furnish favourable conditions for their multiplication. The organism on multiplication in the small bowel produces an exotoxin, which acts upon the mucosal cells of the small bowel stimulating the secretion of large quantities of isotonic fluid. The small bowel produces isotonic fluid faster than the colon can absorb, resulting in watery isotonic diarrhoea. This rapid gastrointestinal loss of isotonic fluid is responsible for all the clinical manifestations of the disease.

Clinical symptoms of disease

After an incubation period of 1-4 days, there is a sudden onset of nausea and vomiting and profuse diarrhoea with abdominal cramps. The stools resemble rice water and contain mucus, epithelial cells and a large numbers of vibrios. There is rapid loss of fluids and electrolytes, which leads to profound dehydration, circulatory collapse and anuria. The mortality rate without treatment is between 25 and 50%.

Different serotypes of *V. cholarae*

The cholera vibrio is set apart serologically from other vibrios by its specific O (heat stable) antigen. Three major O antigens can be differentiated between O- group 1 and have been designated A, B and C. Of these, the antigen A is considered to be O - group 1 specific antigen. It occurs in combination with the other O antigens to give vibrio serotypes. The antigen combination AB is the Ogawa serotype, the combination AC the Inaba serotype and the combination ABC the rare Hikojima serotype.

NAG Vibrios

NAG (non-agglutinable) vibrios possess biochemical and morphological characteristics very similar to those of the cholera vibrio, but are non-agglutinable with polyvalent O serum of the cholera vibrio. Such vibrios may produce cholera-such as or mild diarrhoea.

Seafood-related outbreak of cholera

An explosive outbreak of *V. cholerae* El Tor was reported in Philippines during 1961 and 1962. The initial infection was mainly from shrimps that were consumed raw. In 1969, another outbreak occurred in Malaysia. In this, El Tor and non-agglutinating (NAG) strains were isolated from water and shellfish. A cholera epidimic caused by El Tor biotype began in Naples in 1973 and resulted in 25 deaths among 278 bacteriologically confirmed cases. The suspected vehicle of transmission was raw seafoods particularly mussels washed with dockside sea water contaminated with *V. cholerae*. One year later, 48 deaths, on consumption of shellfish, occurred in Portugal and the causative organism was *V. cholerae* biotype El Tor. Forty two per cent of the shellfish samples were found to be infected with *V. cholerae*. During the same period, six cases of cholera occurred in Guam were associated with home preserved fish. In the United States, the first case of cholera since 1911 was reported in Texas in 1973. Although the source of infection was undetermined, the individuals were found to have consumed raw oysters. In 1977, a similar case occurred in Albama in an individual who had eaten large quantities of raw oysters. A cholera outbreak transmitted through boiled crab meat was reported in Lousiana in 1978. In a Food and Drug Administration (FDA) investigation, samples of blue crab from suspected areas revealed numerous non-agglutinable strains of *V. cholerae*.

Thus, it is clear that mostly shellfish and crustaceans are involved in cholera outbreaks but a finfish was incriminated in the Guam outbreak. In the Eastern Hemisphere cholera has usually infected impoverished people who lived in unsanitary conditions. However, in the West, cholera often occurs among the middle class people and is usually more severe in individuals with gastric disorders.

Survival of *V. cholerae* in seafoods and related environment

The survival of *V. cholerae* under various environmental conditions has been investigated by many workers. As early as 1959, the survival of *V. cholerae* in sea water has been demonstrated. In general, survival was enhanced by intermediate salinities, lower temperature, high organic content, neutral pH, dark storage and absence of competing microflora in the substrate. Survival time of the organism has been reported to be 47 days in unsterilized bay water compared to seven days for ocean water. But, survival for more than 285 days has been observed both in ocean and bay water sterilized before being inoculating with *V cholerae*.

Survival of *V. cholerae* in seafoods has also been investigated. Japanese workers have demonstrated the uptake of *V. cholerae* by oysters and clams kept in cholera-polluted sea water. The vibrios entered the gastro-intestinal tract of the shellfish and survived for 1.5 months at 0 to 5°C and 15-20 days at 22°C. In oysters smeared with *V. cholerae* and stored at 20°C, the number of organisms first decreased, then increased with the maximum number occurring after 68 hours. A gradual decrease followed and most of the vibrios disappeared within 171 hours. The survival period of the vibrio increased to about 20 days in oysters and clams which were sterilized or boiled before contamination.

V. cholerae survived only for a few days in fish stored at room temperature but persisted for more than three weeks when refrigerated. Survival of the vibrio for 198 days in sea salt solution at room temperature has been reported and at lower temperature it can be survive for more time. El Tor vibrios survived for more than a month in various foodstuffs frozen at -20°C and much longer in foods frozen at -72°C.

There are also reports that, in summer months *V. cholerae* is also associated with zooplankton and that chitinase activity in vibrios plays a role in this association. The chitinase activity may increase the affinity to curstacea, thus explaining why *V. cholerae*, a chitin digester, is found more frequently in crabs and shrimps than in other seafood.

It is thus clear that the sea water and seafoods are sometimes contaminated with *V. cholerae* and that organisms can survive in these substrates for a pretty long time.

Control measures

It is clear that environmental hygiene and sanitation play a significant role in the contamination of seafoods with *V. cholerae*. Therefore considerable stress has to be given for environmental sanitation and hygiene. The surroundings of the processing plant should be kept clean and disinfected. Only potable water supply should be used and the water used for different purposes should be properly chlorinated. As flies, cockroaches etc. have been implicated in the transmission of various elementary infections including cholera, measures should be taken to combat these insects in the processing premises. Further, more stress should be given to personal hygiene of the fish handlers by providing sufficient number of urinals and facilities for hand and feet washing and disinfection . It is also advised to have periodical medical check-up of the workers engaged in the processing and other operations.

Method for isolation and identification of *V. cholerae*

1. Transfer 25 g of sample to 225ml alkaline peptone water and incubate for 18 hours at 37°C.
2. Streak one loopfull from the surface growth of above to TCBS Agar plates. Also Transfer 1 - 9 ml alkaline peptone water and incubate for 6 hours at 37°C and then streak one loopfull from the second enrichment to another TCBS plate. Incubate both the TCBS plates for 18-24 hours at 37°C.
3. Transfer the suspected colonies (yellow flat smooth colonies with opaque centres and transparent peripheries, 2-3 mm diameter) from both the TCBS plates to Kligler Iron Agar (KIA) and incubate at 37°C for 18 hours. *Vibrio cholerae* will give Acid (yellow) butt and alkaline (pink) slant with no gas and no production of hydrogen sulphide (no black colour).

4. Strains giving the typical positive reaction in KIA are confirmed by the following biochemical tests and by slide agglutination test (Table 1) with *V. cholerae* polyvalent antisera:

Table 1 Characteristics of *Vibrio cholerae*

S. No.	Test	Result
1.	Motility test	Motile
2.	Oxidase test	Positive
3.	Fermentation of glucose	Acid, no gas
4.	Fermentation of sucrose	Acid, no gas
5.	Fermentation of mannitol	Acid, no gas
6.	Fermentation of Inositol	No acid, no gas
7.	Fermentation of Arabinose	No acid, no gas
8.	Lysine decarboxylation	Positive
9.	Ornithine decarboxylation	Positive
10	Arginine dehydrolation	Negative

The cultures with characteristic biochemical reactions of *V. cholerae* and agglutination with *V. cholerae* polyvalent antisera are classified as *V. cholerae* 01 and the cultures with similar biochemical reactions but without agglutination with *V. cholerae* polyvalent antisera are classified as Non-01 *V. cholerae* (NAG).

1. Motility Test

Semi solid medium

Inoculate Motility Medium by stabbing into the top of a tube of the semi-solid medium to a depth of about 5mm. Incubate at 35-37°C for 48 hours and observe for spreading of the growth through the medium.

2. Oxidase Test

See chapter on *V. parahaemolyticus*

3. Carbohydrate Fermentation

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

4. Amino Acid Decarboxylation Test

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

5. Serological Test

a Wash the growth from the Nutrient Agar Slant (4-18 hours at 37°C)
b Mark two sections on a glass slide
c Put a small amount of the suspension of the culture into the upper part of the two marked areas
d Add a drop of polyvalent *V. cholerae* O antiserum to one section only and mix it with the suspension using a sterile loop of the needle.

e Tilt the slide back and forth for one minute and look at it against a dark background. A rapid strong agglutination is a positive reaction

f Test the positive cultures with Ogawa and Inaba antisera. Most serotypes of *V. cholerae* will react with either Ogawa and Inaba antisera but a few serotypes react with both (Hikojima) .

Media and reagents

1. *Alkaline Peptone Water (APW)*

Peptone 10.0 g	
Sodium chloride	10.0 g
Distilled water	1 litre

Adjust pH to 8.5 ± 0.2. Dispense in cornical flasks (225 ml) and in test tubes (10 ml) Autoclave at 15 lbs for 10 minutes.

2. *Nutrient Agar*

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g

Add ingredients to 1 litre of distilled water, heat to boiling until solution is complete, cool to 50-60°C and adjust pH to 6.8-7.0. Distribute in conical flasks and test tubes (for slants) and sterilize at 15 lbs for 15 minutes.

3. *Kilgler Iron Agar (KIA)*

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	15.0 g
Proteose peptone	5.0 g
Lactose	10.0 g
Dextrose	1.0 g
Ferrous sulfate	0.2 g
Sodium chloride	5.0 g
Sodium thiosulfate	0.3 g
Agar	12.0 g
Phenol red	0.024 g

Add all ingredients to 1 litre 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 and sterilize at 15 lbs for 15 minutes. Cool tubes in slanted position to obtain butts 2.5 cm long and slants approximately 5 cm long.

For all other media and reagents sea chapter on *V. parahaemolyticus* (reduce sodium chloride to 0.5% in all media)

4. *Motility Medium*

Beef extract	3.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	3.0 g

Add ingredients to one litre of distilled water, mix well and heat to boiling to obtain complete solution. Cool to 50-60°C and adjust pH to 7.0. Dispense in test tubes and sterilize at 15 lbs for 15 minutes.