

STUDIES ON VIBRIO PARAHAEMOLYTICUS IN BOMBAY

**THESIS SUBMITTED
UNIVERSITY OF BOMBAY**

**FOR THE DEGREE OF
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IN
MICROBIOLOGY**

**SUBMITTED BY
A. K. PATIL**

**UNDER THE GUIDANCE OF
DR. M. V. SANT**

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HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
BOMBAY**

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by

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CHAPTER I

CHAPTERI

GENERAL INTRODUCTION

Food poisoning is a prehistoric malady and innumerable outbreaks have occurred down the centuries, but despite advanced technology of recent times, the causative organisms of food poisoning generally elude Microbiologists.

The danger arising from sewage pollution of coastal waters were shown as early as 1883 when Robinson, the Medical Officer of Health to the East Kent Combined Sanitary Districts recorded two cases of enteric fever due to eating raw shellfish gathered near the Ramsgate sewage outfall. Similar cases were reported by Piggot of Teignmouth in 1894. Since 1872, Ireland, the town of Belfast, had recorded an unaccountably high death rate from this condition. The commission, appointed by the local Government Board for Ireland to enquire into the matter laid the blame on contaminated shellfish gathered from the shores of Belfast Lough.

Newsholm, Medical Officer of Health for Brighton, reported cases in 1894, 1895 and 1896 caused by shellfish. Other outbreaks occurred in Southampton and Winchester in 1902, in Workington in 1914 and New York and Chicago in 1925.

In 1908 George F. Buther produced a report incriminating shellfish in the city of Birmingham and Hamer (1911) investigated certain localised prevalences in London in 1910, blamed imported flat fish caught near the mouth of the River Elbe.

The Chief importance of shell fish from Public health point of view is their liability to give rise to enteric fever. In France alone it was estimated that within a period of 15 years more than 100,000 cases of typhoid fever due to the consumption of shell fish occurred of which 25,000 proved fatal (Belin 1934).

Oysters and shell fish derive their nourishment by filtering water through their gills by ciliary action. In this connection Sherwood and Thomson (1953) state that a single shell fish will remove the suspended matter from over 10 gallons of sea-water in a single day. According to Eyre (1924) about 48 litres of water enter and leave the shell of an oyster a day. Any bacteria contained in the water are thus concentrated and Knott (1951) estimates that E.coli content of an oyster is at least ten times as high as the concentration in the surrounding polluted water.

Food poisoning/outbreaks occur generally in the summer season with the symptoms of acute gastroenteritis. Of late, much attention has been focussed on V.parahaemolyticus which is found to give rise to outbreaks of food-poisoning. Reports pouring out of Japan have amply established that V.parahaemolyticus causes acute gastroenteritis.

Sakazaki (1969) emphasized that 70% of the cases of bacterial gastroenteritis implicating sea fish in Japan are caused by the facultatively halophilic, enteropathogenic V.parahaemolyticus. In Japan it is a custom for the people to eat raw sea-fish which is held responsible for the outbreaks of gastroenteritis due to V.parahaemolyticus.

The organism in question was first reported by Fujino and his co-workers (1951, 1953) who found the organism in autopsy materials collected from an outbreaks of gastroenteritis involving 272 persons and including 20 fetal cases. In this

'Shirasuboshi', or boiled and semi-dried young sardines, was considered as the causative foodstuff of the outbreaks.

Takikawa (1958) studied 34 strains, all of which, except one, had been isolated on ordinary agar plate containing 4% sodium chloride from the faeces of patients in several outbreaks of gastroenteritis.

After Takikawa many Japanese investigators concentrated on this organism, which has been isolated repeatedly from further outbreaks of gastroenteritis implicating sea-fish and its products. Now it is said that over 50% of the diagnosed outbreaks of bacterial gastroenteritis in Japan may have been caused by this organism (Sakasaki et al. 1963).

The organism was studied ecologically by Miyamoto and his co-workers (1960, 1961a, 1961b), Horie and his co-workers (1963), and Wagatsuma (1962). These investigators described that the organism is found frequently in coastal sea-water and sea-fish but not in pelagic. It has been reported by many workers that organism with the same biochemical and serological properties as the organism originated from patients were distributed widely in sea-water and sea-fish.

Miyamoto et al. (1960, 1961 b,c), Sekine et al. (1962), Horie et al. (1963), Wagatsuma (1961), and Noguchi (1964) demonstrated that the vibrios are widely distributed in the coastal sea of Japan.

Tubosaki and Sakasaki (1962 unpublished data) Takahira (1965), and Yasunara (1964) found the vibrios in the coastal sea waters of the United States, the Philippines, Taiwan, HongKong, and Singapore. It may well be that V. parahaemolyticus is an inhabitant of the coastal sea.

No attention was paid to Fujino et al's report for several years until the second outbreak of food-poisoning, involving

120 persons consisting of 73 in-patients and 47 hospital employees of the Yokohama National Hospital, was described by Takikawa and Fujisawa (1956). The causative foodstuff was salted cucumber, which is called "asazuke" in Japan. Later, Takikawa (1958) encountered several endemic and sporadic cases of similar food-poisoning. In 1960, an explosive epidemic of food-poisoning due to horse mackerel, affecting several thousands of persons, broke out along the Pacific Coast of Japan. It was confirmed by many workers that the causative agent of the epidemic was V. parahaemolyticus. Since then, the isolation of vibrios from the cases of gastroenteritis has been carried out in almost all bacteriological laboratories in Japan as a routine procedure. Consequently, it has been clarified that vibrio gastroenteritis is one of the diagnoses encountered in bacterial food-poisoning. According to statistics prepared by the Ministry of Welfare of Japan, over 70% of food poisoning cases in 1963 were caused by the vibrios. Incidentally, most of the larger outbreaks were recorded by the Ministry of Welfare, but the smaller outbreaks and sporadic cases were probably overlooked. Therefore, there is a general impression, that the real incidence of food poisoning is much higher than the number reported in the statistics.

The causative foodstuff in vibrio food-poisoning is associated directly or indirectly with sea fish or sea water; raw sea fish meat is the most important foodstuff. Sometimes salted vegetables are the source of food poisoning. In this case, the vegetables become contaminated with the vibrios from a chopping board or a kitchen knife with which the sea fish has been prepared for cooking.

Food poisoning due to the vibrios may exist in other countries, especially in tropical and subtropical zones. Sakazaki and Gomez (unpublished data in 1965) have found several cultures of vibrios among the so-called NAG (Nonagglutinable) vibrios isolated from Cholera cases in the Philippines.

The first isolation of V. parahaemolyticus in U.S.A. was reported from the sediments and marine animals of the West Coast (Baross J. et al. 1968). Organisms purporting to resemble

V.parahaemolyticus were isolated from the frozen sediments and waters of the Gulf and South Atlantic coasts (Ward B.W. 1968). V.parahaemolyticus was isolated from Chesapeake Bay crabs retained in commercial shedding tanks (Krantz et al. 1969), Fishbein et al. (1970) reported the recovery of 56 strains of V.parahaemolyticus from the cooked, picked, packed and refrigerated meat of Chesapeake Bay blue crabs offered for the retail market.

V.parahaemolyticus has been isolated from Gulf coast shrimp in U.S.A. and shown to be able to survive in shrimp at temperatures ranging from -18°C to 80°C (Vandersant et al. 1970 and 1972).

J.Howard Beard et al. (1972) reported V.parahaemolyticus gastroenteritis in Maryland U.S.A.. Steamed crabs were found to be causative agents served at the picnic.

Mary Charleson et al. (1972) isolated V.parahaemolyticus from gastroenteritis cases who had eaten the crabs. V.parahaemolyticus as a cause of food borne outbreaks have been responsible for the increasing number of reports of this type of food-poisoning in two states in U.S.A. Since 1969, Maryland has reported five outbreaks, Washington has reported four.

Ray L.Gregory et al. (1972) reported such large outbreaks in U.S.A. and shown for the first time that shrimps have been implicated as the vehicle for V.parahaemolyticus gastroenteritis.

Barros et al. (1968) confirmed the occurrence of V.parahaemolyticus in sea water, sediments and shell-fish. According to them V.parahaemolyticus was found in a relatively large numbers during the summer from all Puget Sound water, sediment and oyster samples in U.S.A.

The isolation of V.parahaemolyticus from in-shore water and it's apparent association with oysters is of considerable significance, particularly in view of the outbreaks of food poisoning caused by this organism (Dack G.M. 1966).

At present V. parahaemolyticus gastroenteritis has become an international problem and besides Japan and U.S.A. it is being studied in Canada, Viet-Nam, Thailand, Indonesia, Denmark, Germany, Spain, Yugoslavia, Italy, Australia, Korea, England, and India on different aspects.

In Calcutta, gastroenteritis due to V. parahaemolyticus was first recorded in 1970 (Chatterjee et al. 1970). Sanyal et al. (1972) reported the isolation of this organism in Varanasi during the study of enteric infections in Sunderpur Slum area.

R. Bradley Sack (1973) examined 500 dogs in Calcutta in search of canine reservoir of V. cholerae and found no evidence for this although 14% of dogs harboured non-cholera vibrios in their intestines. Identical serotypes of these organisms were isolated from dogs, crows and humans in the same geographical areas. Their results indicate that non-human reservoirs exist for noncholera vibrios in contrast to V. cholerae and that these reservoirs may serve as sources of infections in human.

The organism is causing a global concern in the wake of its world wide distribution in sea water and sea fish for which it has been acclaimed as a marine bacterium (Sakazaki 1971).

Chatterjee et al. (1971) reported first observations on non-marine habitat of V. parahaemolyticus in pondfishes in Calcutta and in 1972 reported the isolation of this particular enteric pathogen from a non-marine water source in Calcutta.

V. parahaemolyticus was first reported as a cause of food-poisoning in Japan (Sakazaki 1965) and has since been encountered in cases of gastroenteritis in various parts of the world, including Thailand, Philippines, (Lamba, quoted by Sakazaki, 1971), Australia (Battey et al. 1970), Malaysia (Prescott, quoted by Sanyal S.C. et al. 1973), Calcutta (Chatterjee et al. 1970) and England (Hobbs and Barrow, quoted by Sanyal S.C. et al. 1973).

BACTERIOLOGY

Cultural Characteristics

The vibrios are facultatively anaerobic. They can be cultured readily on ordinary media if 1 to 3% sodium chloride is added, but they grow very poorly or do not grow at all in media containing no salt. When the vibrios are inoculated into 1% peptone water (pH 7.0), they grow if 0.5 to 0.8% salt is added; the most abundant growth is obtained from media containing 2 to 4% salt. However, the optimum concentration of salt necessary to induce growth of the vibrios may be influenced by the kinds of medium employed and the temperature of incubation. For example, the vibrios grow well on blood agar or brain heart infusion agar plates without additional salt.

The vibrios can be cultured readily in a pH range from 5.0 to 9.6, but they grow best at pH 7.5 to 8.5. They grow at temperatures between 15°C and 40°C, but the maximum growth is obtained at 37.5°C.

Colonies of the vibrios freshly isolated on ordinary agar containing 3% sodium chloride are moist, smooth, circular, and opaque in appearance, and they attain a size of 2 to 3 mm after 24 hours. Following several subcultures, however, various colonies which are translucent, mucoid, or wrinkled dissociate from the original colonies mentioned above. On blood agar plate a hemolytic zone is produced around the colonies. In a broth medium, the vibrios produce clouding and, sometimes, a pellicle on the surface of the broth.

The vibrios grow relatively well on MacConkey and Aronson agars containing 3% sodium chloride, but they fail to grow on SS agar (Sakazaki 1969).

Inorganic requirements for growth of the halophilic organisms were studied by using a chemically defined medium according to MacLeod and Onofry (1956). It was confirmed that

none of the organisms tested grew unless Na^+ , K^+ , Mg^{++} and Po_4^{---} were added to the medium, and that Cl^- , Ca^{++} , and So_4^{--} were not essential for their growth (Sakazaki et al. 1963).

According to the observations with a scanning binocular microscope, the colonies of the organisms were divided roughly into 6 groups of phases I to VI. Phase I was a dull, moist, circular, slightly yellow, dome-shaped colony with a surface slightly textured at the edge. Phase II was a moist, circular, bluish, slightly raised colony with an entire margin and a finely textured surface. Phase III was a dark slightly dried, circular, flat colony with no defined surface. Phase IV was a translucent colony with a surface more coarsely textured than Phase II. Phase V was a very dull, pink-yellowish glistening, dome-shaped colony with a surface not well-defined. In addition, to these 5 colonial phases, Phase VI, or a wrinkled colony, was observed in some cultures. (Sakazaki et al. 1963).

On NTB-Teepol agar and TCBS agar the colonies of V. parahae-molyticus after 18-24 hours incubation, are round and 2-3 mm in diameter with a green or blue coloured center which is stained by alkaline bromothymol blue (Sakazaki, 1965b).

Chatterjee and Neogy (1971) studied the colonial features of V. parahae-molyticus by using nutrient agar, 10% sheep blood agar, bile salt agar, MacConkey's agar, SS agar, TCBS agar, and cholera medium after overnight incubation at 37°C.

Colonies freshly isolated on nutrient agar were slightly mucoid and butyrous in consistency, they appeared circular and moderately elevated with shiny surface and entire margin. Cultures on 3% NaCl nutrient agar assumed yellowish grey colouration. In old cultures, the central part of the colony was convex with minute daughter colonies, the periphery was relatively flat and the margin looked undulated.

On TCBS agar the colonies were coloured blue (Being a non-sucrose fermenter) having an opaque raised centre and relatively

flat translucent periphery. On cholera medium the colonies revealed fairly large size having blackish centre and light grey periphery. No non-lactose fermenting colonies were visible on MacConkey's agar. Growth on SS agar could be seen after heavy inoculation. Colonies after fresh isolation were saline and acriflavine (1:1000) stable; but on repeated subculture or even on keeping in nutrient agar for few months they showed evidence of roughness in saline and/or acriflavine. Old cultures on nutrient agar slants (prepared from meat extract) showed formation of crystals underneath the colonies.

Growths of all strains particularly with those of rugose variants, were hastened as well as improved on nutrient agar containing 3% NaCl, in the presence of 10% CO₂ and on blood agar. On ordinary nutrient agar the rugose colonies showed delayed and scanty growth, i.e. appearing 48 to 72 hours after inoculation. In ordinary peptone water medium freshly isolated strains yielded turbid growth with no pellicle and this was improved on adding 3% NaCl to the medium. In nutrient broth containing 3% NaCl diffuse turbidity, granular deposit and slimy surface pellicle was observed. The organism was grown at temperatures 18°C to 21°C, 37°C, 42°C at pH 5 to 11 and in NaCl concentrations of 0.5 to 8%.

Morphology

V. parahaemolyticus is a gram-negative straight, and occasionally slightly curved rod which varies from 1 to 3 μ in length and from 0.4 to 0.6 μ in width. The vibrios exhibit pleomorphism, and undulating filament and spheroplast forms which show single polar flagellation. (Sakazaki 1969).

Sakazaki et al. (1963) tested morphologically 100 cultures of halophilic organism. All the cultures tested were composed of Gram-negative, straight and occasionally slightly curved rods which exhibited pleomorphism. Undulating filaments and spheroplast forms were seen in almost all of them. All of them showed single polar flagellation. They found that the variety in cell-form of the organism was very similar to that of vibrio cultures. Spheroplast form was regularly observed in almost all vibrio cultures. Although somatic curvature was shown, as a rule, in some organisms of vibrio comm, it was relatively rare to occur. Pleomorphism of cell-form was seen in all vibrio cultures except two. The cultures did not exhibit the same degree of pleomorphism as the other cultures of the genus, consisting only of straight and occasionally curved rods and spheroplasts.

Chatterjee and Neogy (1971) studied the cultural characteristics of this organism and reported that a large proportion of V. parahaemolyticus cells examined from fresh cultures were highly motile and presented in general coccoid to avoid Gram-negative bacillary forms with evidence of pleomorphism. Pleomorphic forms were exaggerated in old strains showing mucoid and rough colonies. The cells from nutrient agar culture were arranged singly, in pairs, short chains and groups. Bipolar staining was evident after treatment of the smears with dilute carbol fuchsin or Wayson's stain. Besides the variable depth of staining, appearances like long thick filaments, clubs, short rods with pointed ends and other irregular forms were enhanced in old cultures, particularly in those grown on 3% NaCl nutrient agar media. Presence of well-formed capsule was noted by following Benian's technique. Overnight culture presented mostly monotrichous-looking cells but typical peritrichous forms with indiscriminate flagellar distribution were chiefly ascertained in short undivided cells from a study of 5 hour culture.

Biochemical Characteristics

All the vibrios reduce nitrate to nitrite, fail to produce hydrogen sulfide in the butt of Kligler's iron agar, liquefy gelatin, produce indole, and give a positive cytochrome oxidase test of Gaby and Hadley (1957) and negative Voges-Proskauer and phenylalanine deaminase tests. Generally, citrate is utilised as a sole carbon source, and urea and malonate are not decomposed.

The vibrios ferment glucose, maltose, trehalose and mannitol without forming gas in the modified Hugh-Leifson medium (MOP medium according to Leifson, 1963), and they fail to acidify lactose, rhamnose, sucrose, xylose, adonitol, dulcitol, inositol, and salicin. Starch is not hydrolyzed, and cellobiose is not fermented within 24 hours of incubation. Fermentation of arabinose is variable with biotypes. Very rarely does, a strain acidify sucrose.

A majority of the vibrios give a positive reaction in the cholera red test. No luminescence is observed. (Okazaki 1969).

Neogy et al. (1971) reported the observations that a string, test, positivity of V. parahaemolyticus may be lost on storage but reappears when grown in presence of additional nutrient and phosphate.

Chatterjee et al. (1970) reported growth of halophilic V. parahaemolyticus in 0.5%, 3%, 6% and 8% sodium chloride peptone water media, no turbidity was, however, observed at 0% and 10% and strains were found to be ampicillin resistant, polymyxin-B sensitive and were negative to lactose, V-P, MR, ONPG, Simmons' citrate, K-P organic acids, and haemolysis test. All strains studied were positive for gelatin degradation, nitrate reduction and indole production. They produced acid in mannitol, and showed growth at 42°C. None of them produced acid in inositol, dulcitol, adonitol, rhamnose, raffinose, salicin, cellobiose and sorbitol.

Fishbein et al. (1970) isolated strains of V. parahaemolyticus which were positive for TSI (ALK/Acid), Cytochrome oxidase, indole, methyl red, citrate utilisation, gelatin liquefaction, nitrate reduction, catalase, motility, haemolysis, starch hydrolysis, halophilism 3% NaCl in peptone, 7% NaCl, glucose, maltose, mannitol and trehalose. They were negative for urease, Voges-Proskauer, malonate-utilisation, phenylalanine deaminase, cholera red, Gram stain, halophilism 0.5 NaCl, 10% NaCl, adonitol cellobiose, inositol, lactose, rhamnose, salicin, sorbitol, sucrose, and xylose.

Tentative method of Food and Drug Laboratories Ottawa (August 1970) gives positive tests for mannitol, 3% NaCl, 7% NaCl, glucose, maltose, arabinose, gelatin, motility, tartate, starch, indole, haemolysis of human RBCs, catalase, nitrite production and negative tests to lactose, sucrose, 10% NaCl, Salicin, Cellobiose, adonitol, inositol, H_2S , haemolysis of sheep RBCs and Voges-Proskauer.

Some variation has been reported for V. parahaemolyticus in these reactions, depending on the source of the strains examined.

Pathogenicity

Since 1960, many Japanese investigators have studied the vibrios. Judging from the results of feeding experiments with human volunteers performed by Takikawa (1958) and Aiso and Fujiwara (1963), there is no doubt that V. parahaemolyticus produces gastroenteritis.

Zen-Yoji (1964) and Sakazaki and his co-workers (1963) demonstrated that, when the broth cultures of the vibrios were inoculated into the ligated rabbit gut, by the method of De and Chatterjee (1953), severe enteritis was produced in the loop of the gut. When 3-week-old mice of CFW strain received intraperitoneally 0.5 ml of an overnight broth culture of the vibrios, almost all of the animals died within 24 hours, but majority

of the mice inoculated with 0.5 ml of a 10 fold dilution of the broth cultures survived.

Under natural conditions V. parahaemolyticus may be pathogenic only to man. Aiso (1961) stated that V. parahaemolyticus could produce experimental gastroenteritis in dogs and monkeys, but other workers (Zen-Yogi, 1964) reported that the vibrios produced no illness in any animals.

It is uncertain whether all members of the species are enteropathogenic to human beings. Sakazaki (1965a) observed that most of K antigen types were found not only in sea water and sea fish but also in patients, and that K antigen types frequently isolated from sea water and sea fish were also found predominantly in cultures from patients. From these results, he considered that all members of the V. parahaemolyticus may be able to produce human gastroenteritis.

Fujino et al. (1951), described a hemolytic activity of the vibrios. Kato et al. (1965) found that the vibrio cultures isolated from human patients affected with gastroenteritis were hemolytic and those from sea water and sea fish were nonhemolytic when the vibrios were grown on brain heart infusion agar containing human blood.

Using blood agar plates which contained 0.5% Thiotone (BBL), 1% NaCl, 0.2% $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 1.5% agar, 0.2% glucose, and 1% washed human red cells, Sakazaki et al. (1968) demonstrated that 9% of 2720 cultures which were isolated from diarrheal stools were hemolytic, whereas only 1% of 650 cultures from sea fish showed such activity. The same results were obtained by Kato et al. (1966) and Asakawa et al. (1966). No relationships were found between biochemical and serological properties and the hemolytic activity of the vibrios.

Sakazaki et al. (1968) carried out feeding experiments with the hemolytic and nonhemolytic vibrios on human adult volunteers. When a volunteer received 10 million organisms of the hemolytic

vibrios isolated from diarrheal stools, he revealed clinical signs of abdominal pain and diarrhoea after 5 hours of incubation. However, 14 volunteers who received nonhemolytic vibrios originating from sea fish revealed no signs of illness, although over a thousand million organisms were consumed.

The hemolytic activity of the vibrios is demonstrable in supernatant from a centrifuged broth cultures of the organisms. The hemolytic substance in the supernatant was studied by Kato et al. (1966, 1967). A hemolytic substance toxic for mice and Hela cells was precipitated in a sugar-free and nucleic acid free protein fraction by 30 to 60% saturation of ammonium sulphate at pH4.5 and zone chromatography on a sephadex G-200. The hemolytic activity of the fraction remained after heating at 100°C for 30 minutes at pH6.0, but was destroyed by trypsin digestion. The fraction revealed no activity of lecithinases A and B or gelatinase.

It is clear, from the results described above that only the hemolytic culture is enteropathogenic. However, the role of the enteritis producing power of the toxic substance obtained from the hemolytic vibrios is uncertain. Kato et al. (1967), demonstrated with intraduodenal dog inoculation tests that the enteritis producing power of the toxic substance was much weaker than that of live organisms. They also found that a variant of the vibrios which lacked like the X antigen could not produce enteritis in inoculated dogs, although the vibrios were strongly hemolytic. From results of epidemiological and clinical investigations of vibrios food poisoning, there is no doubt that the gastroenteritis caused by the vibrios is of the infectious and not the toxic type. It is probable, therefore, that the enteritis producing power of the hemolytic vibrios may be in the living bacterial cell itself. The antigen and toxic substance may have a supplemental effect in producing enteritis.

According to recent studies on the hemolytic activity of the vibrios, a controversy has arisen about the epidemiology of vibrio food poisoning. As stated previously, it is evident from the epidemiological investigations carried out by many workers that sea fish and their products are the main causative vibrios originating from sea fish and are pathogenic for man. On the other hand, it has been demonstrated by many workers that the vibrio infection is not transmitted from person to person.

Chatterjee et al. (1972) reported that the epidemiology of V.parahaemolyticus infection in Calcutta appears different from that already experienced in Japan.

Aims and Objects of Study

V.parahaemolyticus has attracted a lot of attention lately as cases of gastroenteritis/food poisoning are attributed to it in different parts of the world though it was very rampant in Japan earlier. Sea fish and fish products are proved to be vehicles that communicate V.parahaemolyticus and give rise to outbreaks of bacterial food poisoning.

In Bombay cases of gastroenteritis are quite common round the year, but generally the causative organism goes undetected and so far, no attention has been focussed on the isolation of V.parahaemolyticus.

Taking into consideration the world wide distribution of V.parahaemolyticus as a cause of food poisoning the present investigation was undertaken to study V.parahaemolyticus in fish particularly shell fish, oysters, crabs and prawns/shrimps in the coastal region of Bombay. The study on stool samples of gastroenteritis cases was also carried out to find out the corelationship between the consumption of fish and food poisoning due to V.parahaemolyticus.

The organism is causing a global concern in the wake of its world wide distribution in sea water and sea fish for which it has been acclaimed as a marine bacterium but it has been isolated from non-marine sources in Calcutta and from stool samples in Varanasi, very far from the coastal region. These reports clearly indicate that V.parahaemolyticus has not only a marine habitat but also has a non-marine habitat. The vibrios have been also isolated from animal excreta in Calcutta and Varanasi.

As regards the habitat of V.parahaemolyticus the study was undertaken to isolate it from animal excreta and stool samples of gastroenteritis cases in the city of Farbhani far away from the sea coast.

CHAPTER II

PRESENT PROBLEM COVERING THE WORLD WIDE

DISTRIBUTION AND EPIDEMIOLOGY

V.parahaemolyticus is a new marine bacterium among the enteropathogens. Large number of food poisoning or gastroenteritis cases take place in Japan generally in summer season due to V.parahaemolyticus (Sakazaki et al. 1963). Many Japanese microbiologists were attracted by this organism since the first report of Fujino et al. (1951) who could isolate it from autopsy material. It is a custom in Japan to eat raw sea fish and fish products which are held responsible for these outbreaks. In 1965 and 1966 it was responsible for more than 45% of all the cases of bacterial food poisoning occurring in that country (Fishbein et al. 1970). Now it is reported that over 70% of the cases of bacterial gastroenteritis in Japan may be caused by this organism (Sakazaki et al. 1963). Thus, it became clear beyond doubt that V.parahaemolyticus was the causative agent for about 70% of all reported gastroenteritis cases in Japan particularly in summer season due to consumption of raw sea fish and fish products.

Japanese bacteriologists Miyamoto et al. (1960, 1961b,c) Sekine et al. (1962), Horie et al. (1963), Wagatsuma (1961), and Naguchi (1964), proved the wide distribution of the organisms in the sea coast of Japan. Tubosaki and Sakazaki (1962, unpublished data), Takahira (1965), and Yasunara (1964) reported the occurrence of the Vibrios in the sea coast of the United States, the Philippines, Taiwan, HongKong and Singapore and opined

that V.parahaemolyticus is an inhabitant of the coastal sea.

Taking into consideration, the peuring reports out of Japan the study on V.parahaemolyticus was undertaken in many countries. Baross et al. (1968) reported the first isolation of the organism from the sediments and marine animals of the West coast in United States. After that the isolation was reported by Ward (1968) from the frozen sediments and water of the Gulf and South Atlantic coasts, Krantz et al. (1969) from Chesapeake Bay crabs, Fishbein et al. (1970) from cooked, picked, packed and refrigerated meat of Chesapeake Bay blue crabs and Vanderzant et al. (1970, 1972) from Gulf coast shrimp.

Beard et al. (1972) reported V.parahaemolyticus gastroenteritis in Maryland due to eating crabs. Mary Charleson et al. (1972) isolated the organisms from gastroenteritis cases with history of having consumed the crabs. Gregory et al.(1972) reported such large outbreak due to shrimps.

V.parahaemolyticus as a cause of foodborne outbreaks has been responsible for increasing number of reports of this type of food poisoning in two states in U.S.A. . Since 1969, Maryland has reported five outbreaks, Washington has reported four. Thus within a few years, this organism became recognised as an important cause of foodborne disease in the United States and other countries.

Chatterjee et al. (1970) were the first to isolate and report this V.parahaemolyticus gastroenteritis in the city of Calcutta, India. After that it was isolated repeatedly by them in Calcutta.

Chatterjee et al. (1971, 1972) reported first observation of non-marine habitat of V.parahaemolyticus in pond-fishes and from a non-marine water source in Calcutta. Sanyal et al. (1972) also reported the isolation of this organism in Varanasi, a hinterland. These reports clearly indicate that the organism is not only the habitat of sea coasts and sea animals but also the habitat of the non-marine sources. Therefore, Chatterjee et al. (1972) opined that the epidemiology of V.parahaemolyticus infection in Calcutta appears different from that already experienced in Japan.

In U.S.A., V.parahaemolyticus has been isolated not only from gastroenteritis cases but also from other sources. Roland (1971) reported a case of gasgangrene which necessitated the amputation of a leg. The pathogenicity of this organism was indicated also by it's isolation from infection of the ear and eye as well as from localized skin infections. Weaver isolated this organism in U.S.A. from human blood infection, lesions and abscesses (Quoted by Fishbein et al. 1970) and opined that V.parahaemolyticus solely as a foodborne enteric agent may have to be modified to include the ability of this organism to infect man by a route other than food.

Besides Japan, U.S.A. and India the sporadic cases and outbreaks due to V.parahaemolyticus have been reported in Australia, the Philippines, South-Vietnam, Thailand, Malaysia, Togo, Mexico and Panama. In England it has been reported twice in persons arriving by plane from Asian countries.

According to Sakazaki (1969) food poisoning due to V.parahaemolyticus is of infectious type and symptoms of gastroenteritis appear 12 hours after ingestion of the infected food, but the incubation period can be as short as 2 hours or delayed to 40 hours. The outstanding symptoms are abdominal pain and diarrhoea, associated with nausea and vomiting. Recovery from this V.parahaemolyticus takes about 2 to 5 days. The fatality rate is very low.

Dysenteric stools having almost fresh blood mixed with mucous and no normal content were investigated by Chatterjee et al. (1970) and reported that besides the acute attack of bloody diarrhoea the patients suffered from abdominal pain, vomiting, pyrexia and dehydration. Further more cases were not all mild in nature. Accordingly, Chatterjee and Neogy suggested the name of V.dysenteriae in view of such acute dysenteric syndrome, resembling bacillary dysentery.

The spectrum of signs and symptoms observed in a series of well reported outbreaks in the U.S.A. are as follows (William H. Barker et al. 1974).

In most outbreaks, explosive watery diarrhoea was the cardinal manifestation in over 90% of cases. Nausea and cramping abdominal pain were the other most common manifestations, with vomiting in approximately 50% of cases. Fever, rarely exceeding 102°F, chills and headache were reported less frequently. Duration of illness ranged from 2 hour to more than 10 days with a median of 3 days. Median incubation periods for the series of outbreaks ranged from 13 to 23 hours with incubation periods for individual cases ranging from 4 to 96 hours. No age or sex predilection has been noted. Several cases with profuse rice water stools have been reported from India and Togo but the profound fluid loss seen in cholera gravis has not occurred.

Death has occurred in fewer than 1 per 1000 cases reported from Japan and in none of the approximately 1000 cases reported in the United States.

CHAPTER III

CHAPTERIII

OCCURRENCE OF V. PARAHAEVOLYTICUS IN

SEA-ANIMALS AROUND THE COAST

OF BOMBAY

(Oysters)

Miyamoto et al. (1960, 1961 a, 1961 b) reported that V. parahaemolyticus is found frequently in coastal sea water and sea fish. Tubosaki and Sakazaki (1962 unpublished data) Takahira (1965) and Yasunara (1964) reported the occurrence of the Vibrios in the coastal sea waters of the United States, Philippines, Taiwan, HongKong and Singapore and opined that V. parahaemolyticus might be an inhabitant of the sea coast.

Barosa et al. (1968) were first to isolate it from the sediments and marine animals of the West Coast in U.S.A. and according to Duck (1966) the isolation of V. parahaemolyticus from in-shore water and it's apparent association with oysters is of considerable significance, particularly in view of the outbreaks of food poisoning caused by this organism.

Kampelmacher et al. (1972) reported the occurrence of V. parahaemolyticus in mussels, oysters and estuarine waters in the Netherlands.

Oysters derive their nourishment by filtering water through their gills by ciliary action and according to Eyre (1924) about 48 litres of water enter and leave the

shell of an oyster a day. Any bacteria contained in the water are thus concentrated and Knott (1931) estimated that bacterial content of a oyster is at least ten times higher than the concentration in the surrounding polluted water.

Thus, this necessitated us to undertake the study on oysters in the coast of Bombay as there is possibility of this giving rise to food poisoning due to eating raw or improper cooking and handling.

Materials and Methods

Oyster is a marine animal sedentary in habits. It has a shell consisting of two unequal valves. The right valve is attached to the left by ligaments forming a sort of lid over it. The foot is absent and the animal is attached to the stones or rocks by the left valve.

Stones or rocks between low and high tide marks are generally covered by these animals. At high tide when submerged, animals open the right valve and obtain their food by ciliary currents set in the water. Stones or rocks at the mouth of the creek on the coast of the sea are most densely covered by these animals as they can easily draw the microscopic animals and plants that are suspended in the sea water. Fisherfolk staying on the coastal region pick up densely covered stones and bring them on the shore for removing the animals from the shell valves. In case of rocks this is not possible and therefore animals are removed from shell valves at low tide by means of a sickle like pointed sharp instrument specially designed for the purpose.

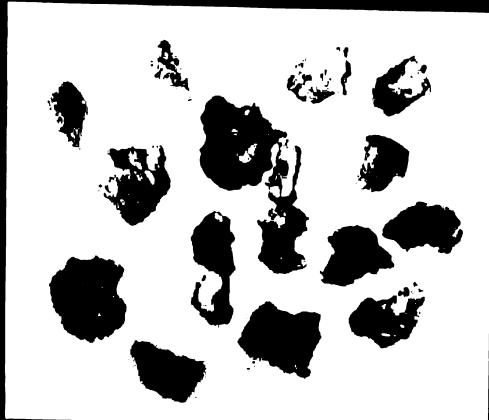


Photo No. 1

Oysters, entire and opened



Photo No. 3

Crabs



Photo No. 2

Seeds, etc.



Photo No. 4

Seeds, etc.

We collected the oysters by former method from Mahim and Colaba coastal regions. Stones densely covered, were brought on the sea shore where complete shells of oysters were removed. Shells were brought to the Laboratory soon after their removal.

Ten shells of average size were selected. They were washed in sterile distilled water separately. A small portion of the shell at one end was nibbled away with sterile nibbling forceps and through the opening the blade of a sterile scalpel was inserted. With this muscles and other attachments of oysters were cut and, holding them with the paper interposed between them and the hands the two valves of the shell were separated and upper one removed. All the fluid, inside the shell was poured off and complete body of the oysters was transferred to a sterile preweighed Petridish by means of a scalpel. In this way ten oysters were removed from the shells and pooled together. A 10 per cent suspension of the pooled sample was prepared by means of a mixer at top speed in 3 per cent peptone water under aseptic conditions. (Tentative Method of Food and Drug Laboratories, Ottawa, 1970).

i) A loopful of the blended material was streaked on TCBS agar (Thiosulphate Citrate Bile Salt agar) and BTB agar (Bromothymol Blue-Teebol agar) and incubated overnight.

ii) 5ml of the material was inoculated into 3% NaCl-peptone water and incubated at 37°C for 6 hours and then subcultured on TCBS and BTB agar.

After overnight incubation the suspected colonies were picked up (as given under Materials and Methods) and subcultured on nutrient agar slants containing 3 per cent sodium chloride and incubated overnight at 37°C. The isolates were

subjected to bacteriological detailed study following the methods of Sakazaki Riichi (1965 b, publication of NIHON EIYO KAGAKU Co., Ltd., Tokyo, Japan) and Thatcher and Clark (1968). Accordingly, bacteriological tests, carried out for identification of the isolates were with typical characteristics as follows:

Primary Test

1) Lactose	-	Negative (No gas)
2) Sucrose	-	Negative (No gas)
3) Mannitol	-	Acid (No gas)
4) 0% NaCl-Peptone water	-	No growth
5) 3% NaCl-Peptone water	-	Profuse growth
6) 8% NaCl-Peptone water	-	Relatively profuse growth
7) 10% NaCl-Peptone water	-	No growth

Secondary Test

8) Glucose	-	Acid (No gas)
9) Maltose	-	Acid (No gas)
10) Arabinose	-	Variable
11) Salicin	-	Variable
12) Cellobiose	-	Negative (within 24 hours)
13) Adonitol	-	Negative
14) Inositol	-	Negative
15) Gram staining	-	Negative
16) Motility	-	Positive
17) Oxidase	-	Positive
18) H ₂ S production	-	Negative
19) Voges-Proskauer	-	Negative
20) Methyl red	-	Positive
21) Indole production	-	Positive
22) Catalase	-	Positive
23) Nitrate reduction	-	Positive
24) Gelatin liquefaction	-	Positive

However, very small, translucent colonies of few strains of coliform bacteria, *Proteus* and enterococci may grow on these agar but can be differentiated from those of *V. parahaemolyticus*.

Composition of media

BIB-Teepol Agar (Akiyama et al. 1963 modified)

Yeast extract	-	3 g
Peptone	-	10 g
Sodium Chloride	-	40 g
Sucrose	-	20 g
Bromothymol blue	-	0.04 g
Thymol blue	-	0.04 g
Teepol	-	2.0 ml
Agar	-	15.0 g

All the ingredients were added to one litre of distilled water, dissolved by boiling and pH adjusted to 9.0. It was dispensed in MacKartney bottles and sterilised at 121°C for 15 minutes.

TCBS agar (Kobayashi et al. 1963)

Yeast extract	-	5 g
Peptone	-	10 g
Sucrose	-	20 g
Sodium thiosulphate (2H ₂ O)	-	10 g
Sodium Citrate (2H ₂ O)	-	10 g
Sodium Cholate	-	3 g
Cx - gall	-	5 g
Sodium Chloride	-	10 g
Ferric citrate	-	1 g
Bromothymol blue	-	0.04 g
Thymol blue	-	0.04 g
Agar	-	15 g

All the ingredients except Ox gall were added to 1 litre of distilled water. Proportionate Ox-bile was added as a substitute for Ox-gall powder as it was not available locally. pH was adjusted to 8.6 dissolving all the ingredients and dispensed in MacKartney bottles. Bottles were sterilised by boiling in a water bath as it should not be autoclaved (Sakazaki, 1965 b).

Enrichment medium

Sakazaki emphasized that enrichment procedure may be necessary for the isolation of V.parahaemolyticus from sea water, sea fish and faeces of convalescent patients., and reported that the addition of 2 per cent sodium chloride and Colistin into the ordinary broth not only greatly enhances the growth of V.parahaemolyticus but also inhibits the growth of the majority of coliform bacteria and many strains of Proteus, Pseudomonads and Gram positive organisms.

Chatterjee et al. (1970) isolated 13 strains of V.parahaemolyticus from acute diarrhoeal cases when preliminary enrichment for 6 hours in alkaline peptone water was performed with all the faecal samples for the isolation of Vibrios.

Food and Drug Laboratories, Ottawa, recommended bismuth sulphite salt broth for preliminary enrichment for the isolation of V.parahaemolyticus.

However, before using the TCBS, BTB agar and enrichment medium, a comparative study was made for observing the growth of the V.parahaemolyticus. For this study standard strains of V.parahaemolyticus were obtained from Canada, and U.S.A.. Both TCBS agar plates, one prepared from local medium and other from Japanese one were inoculated with V.parahaemolyticus simultaneously and incubated at 37°C overnight, under identical conditions. After several pilot experiments it was found that

locally prepared TCBS agar was equally good and was used throughout our study.

At the same time enrichment media, bismuth sulphite slat broth and 3 per cent sodium chloride peptone water; were simultaneously inoculated and incubated and growth was compared by turbidity method. Peptone water containing 3 per cent NaCl was found equally good as a preliminary enrichment medium and was used throughout our study. Suspected colonies -round, mucoid, translucent with a greenish or bluish opaque centre were picked up. While picking up the colonies they were first looked at through the medium by holding it up against the light. The selected colonies were marked by glass marking pencil on the bottom of the dish. The plate was held vertically with the left hand, then the holder of the wire was grasped like a pen with fingers quite close to the wire. Care was taken in picking up single colonies, particularly when they were very close to one another, that the point of the wire did not touch any of the neighbouring colonies. In this way colonies, were easily removed without touching the others.

Whenever plates showed heavy growth e.g. crowding of the colonies; and picked up colonies were suspected to contain a mixture of bacteria, they were cultured on selective medium (TCBS agar) by inoculating them very thinly on the surface of the TCBS agar. The suspected colonies were picked up in the pure form after overnight incubation at 37°C.

The suspected pure colonies were streaked on Bacto-peptones agar slants containing 3% NaCl. After overnight incubation at 37°C the slants were removed from the incubator and checked for the purity of the culture by examining them visually. Selected slants were sealed with paraffin wax, labelled properly and stored at room temperature in a close container. Refrigeration or freezing was reported and

found lethal to the strains of V. parahaemolyticus (Thomson, Personal communication).

These isolates were subjected to bacteriological detailed study for identification.

The procedure followed throughout this study for isolation and identification of V. parahaemolyticus is based on that described by Thatcher and Clark (1968) in 'Microorganisms in Food'. Their significance and method of enumeration' (University of Toronto Press, Canada P. 107-114) and Miichi Sakasaki 'Vibrio parahaemolyticus, Isolation and Identification', Department of Bacteriology, National Institute of Health, Kamosaki, Shinagawa-Ku, Tokyo, Japan.

Non-sucrose fermenters on TCBS agar were isolated from 6 pooled samples of oysters out of 30 pooled samples processed.

Isolates were subjected to motility test by cultivation in semi-solid nutrient agar. 0.4% Japanese agar was used for this purpose and exact optimal concentration was determined by trial. After dissolving it in peptone water containing 1% NaCl. 10 ml of it was dispensed in test tubes and sterile, clear and transparent agar tubes were inoculated with a straight wire making a single stab down the centre of the tube to about half the depth of the medium. Inoculated tubes were incubated at 37°C and observed at different intervals for diffuse, hazy growth that spread throughout the medium rendering it slightly opaque. (Cruickshank, 1972).

General morphology of the isolates was studied by Gram staining method.

Oxidase test

The isolates were tested for oxidase test as follows:

Wet filter paper method:- A strip of filter paper was soaked with a little freshly prepared 1 per cent solution of tetramethyl-p-phenylene-diamine dihydrochloride and then at once used by rubbing a speck of fresh culture on it with a platinum loop. A positive reaction was indicated by an intense deep-purple hue, appearing within 5-10 seconds. (Cruickshank, 1972).

Catalase test

The isolates were tested for catalase by following method.

A small amount of the culture to be tested was picked from a nutrient agar slope, using a clean sealed capillary tube and this was inserted into hydrogen peroxide solution held in a small clean tube. The production of gas bubbles from the surface of the solid culture material indicated a positive reaction which occurred almost immediately. (Cruickshank, 1972).

Gelatin liquefaction test

The test was performed by stabbing the nutrient gelatin containing 3 per cent sodium chloride, using inoculum from a 24 hours agar culture, and incubated at 37°C.

Medium - Nutrient gelatin

Fifteen gram of gelatin (Oxoid) was added to 100 ml of nutrient broth and held overnight at 4°C.

Next day the gelatin was dissolved at low temperature and pH was adjusted to 8.4, and steamed for 10 minutes. 5 ml of serum was added at low temperature and steamed for 30 minutes, stirring occasionally. Medium was filtered and pH was readjusted to 7.6. Twelve ml of the medium was dispensed in test tubes which were sterilised in the autoclave for 10 minutes at 115°C. The resulting medium was transparent when solid yet not so stiff that it could split when inoculated. Liquefaction was tested at intervals by removing the nutrient gelatin cultures from the incubator and holding them at 4°C for 30 minutes before reading the results. (Cruickshank, 1972).

Indole test

Indole test was carried out by inoculating indole broth containing 3 per cent sodium chloride with a fresh culture of the organisms. After 48 hours incubation at 37°C indole reaction was examined by adding Kovac's reagent which produced red colour indicating a positive reaction after shaking gently.

Medium - Two gram peptone powder (Difco) was added to 100 ml of distilled water containing 3 per cent sodium chloride. The pH was adjusted to 7.4 and sterilised by autoclaving at 121°C for 15 minutes.

Kovac's reagent - Amyl or isoamyl alcohol - 15 ml
p-Dimethyl-aminobenzaldehyde - 1 g
Conc. hydrochloric acid, HCl - 5 ml

Fresh solution was prepared by dissolving aldehyde in alcohol and then adding the acid slowly. (Cruickshank, 1972).

Nitrate reduction test

One per cent peptone water containing 0.1 per cent potassium nitrate and 3 per cent sodium chloride was inoculated with a fresh culture of the organisms. After 24 hours incubation, the sulfanilic acid and the alpha-naphthylamine solutions were added drop by drop which developed the red colour within a few minutes indicating the presence of nitrite. (Cruickshank, 1972).

Hydrogen Sulphide Production Test

For H_2S production test, medium used contained meat extract 7.5 g., peptone 25 g., sodium chloride, 30 g., and gelatin 120 g. in one litre of distilled water. pH was adjusted to 7.6 and filtered after steaming. Then sterilised in the autoclave for 10 minutes in free steam and again at $115^\circ C$ for 10 minutes. Five ml of a freshly prepared 10 per cent solution of ferrous chloride, presterilised, by filtration was added after cooling the medium at $55^\circ C$. The medium was tubed and sealed with corks impregnated with paraffin wax.

Tubes were inoculated with a straight wire and incubated at least 7 days and inspected daily for blackening due to the production of H_2S . (Cruickshank, 1972).

Voges-Proskauer test

V.P. test was carried out by inoculating lightly glucose phosphate peptone water medium with a young agar slope culture and incubated at $37^\circ C$ for 48 hours and then 1 ml of Barritt's reagent was added containing 40 per cent, potassium hydroxide and 3 ml of a 5 per cent solution of α -naphthol in absolute ethanol. Tube was shaken at intervals to ensure maximum

aeration. A positive reaction was indicated by the development of a pink colour in 2-5 minutes becoming crimson in 30 minutes.

Medium (Glucose phosphate peptone water)

Peptone	5 g
Dipotassium hydrogen phosphate K_2HPO_4	5 g
Water	1 l

3 per cent sodium chloride was added.

pH of the medium was adjusted to 7.6 after dissolving the ingredients. Glucose solution in a final concentration of 0.5 per cent was added in each tube containing about 5 ml medium. (Cruickshank, 1972).

Methyl red test

MH test was carried out by inoculating "Glucose phosphate peptone water" medium with a young agar slope culture and incubating it at 37°C for 48 hours. Five drops of the methyl red reagent were added to the incubated medium, mixed and results read immediately. Positive tests were bright red and negative were yellow.

Methyl red indicator solution was prepared as follows:

Methyl Red	-	0.1 g
Ethanol	-	360 ml

Made up to a volume of 500 ml with distilled water.
(Cruickshank, 1972).

Haemolysis

The haemolytic activity was studied on ordinary blood agar plates containing 10% defibrinated human as well as sheep blood. (Cruickshank, 1972).

Nutrient agar was melted in a water bath. When the temperature of the melted nutrient agar was about 45-50°C 10% defibrinated blood was added aseptically. Mixed it uniformly by shaking the bottles gently and about 15 ml of blood agar was poured in a pre-sterilised Petridish aseptically. In case of a bubble in the blood agar it was gently flamed when blood agar was completely solidified. All the plates were transferred to an incubator where they were incubated for 48 hours at 37°C. Contaminated plates were discarded and the sterile ones were stored in the refrigerator.

At the time of studying the haemolytic activity of the isolation the plates were warmed up in the incubator at 37°C and then inoculated by streaking a fresh culture in a single line on the agar plates. After 24 hours incubation at 37°C haemolysis under and round the growth was examined.

The isolates were also studied for haemolysis by Feeley and Pittman method (1963). For this purpose human and sheep RBCs were used. Half (0.5) ml of a 24 hour broth culture was mixed with 0.5 ml of one per cent washed human and sheep blood cells. The mixture was incubated for two hours at 35-37°C and held at 4°C overnight and then it was read for the presence of haemolysis.

Kanagawa phenomenon

The isolates were subjected to Kanagawa phenomenon on special medium which determines whether the isolate is pathogenic or not (Sakazaki et al. 1968). For this phenomenon Wagatsuma agar was used which consisted of -

Bacto-yeast extract	-	0.3	%
Bacto peptone	-	1.0	%
Sodium Chloride	-	7.0	%
Dipotassium phosphate	-	0.5	%
Maunitol	-	1.0	%
Crystal Violet	-	0.001	%
Bacto agar	-	1.5	%
Rabbit blood	-	5	%

The above ingredients except blood were added to water and steamed until agar was melted. The pH of the basal medium was adjusted to 8.0. Without autoclaving the melted basal agar medium was cooled to 50°C, blood was added and poured into petridishes.

A loopful of overnight broth cultures of the Vibrios to be tested was inoculated by streaking in a single line on the agar plates. After 24 hours incubation at 37°C, plates were examined for haemolysis under and round the growth.

Each isolate was individually studied for Kanagawa phenomenon on locally prepared medium as Japanese medium was not available in the market.

Susceptibility to Antibiotics

Isolates were tested also for their sensitivity to common antibiotics by disc diffusion technique (Bauer et al. 1966).

Nutrient agar containing 3% NaCl was melted and 15 ml of it was poured in each presterilised Petridish. Agar was allowed to solidify and then they were dried in the incubator before use.

A loopful of the original fresh culture was introduced into a test tube containing 4 ml of 3% NaCl peptone water. Tube was then incubated for 2 to 5 hours at 37°C to produce a bacterial suspension of moderate cloudiness. If necessary suspension was diluted with saline solution to a density visually equivalent to that of a standard (0.5 ml of 1% Bact₂ to 99.5 ml of 1% H₂So4 (0.36N).

Bacterial broth suspension was streaked on the surface of the medium evenly with a cotton swab from which surplus bacterial suspension was removed by rotating it against the side of the tube before the plates were seeded. After five minutes the discs were placed on the agar with flamed forceps and gently pressed down to ensure contact.

After overnight incubation at 37°C the zone diameters were measured with a ruler on the under surface of the petridish.

Isolates were tested for their sensitivity to chloramycetin, tetracycline, streptomycin, kanamycin, polymyxin B and ampicillin.

Pathogenicity

Pathogenicity in adult and infant rabbits by a loop technique -

Pathogenicity of the strains was studied by injecting a dose of culture into the lumen of a loop of an adult rabbit following De et al. (1953) technique.

Rabbits weighing about 1.5 kilogram, not fed for twenty-four hours, were used in the experiment. Under anaesthesia and sterile conditions, midline incision was made through which small intestine was taken out. A segments of it were isolated with ligatures by avoiding the blood vessels carefully. Three segments were inoculated with 1 ml of 24 hour culture of V.parahaemolyticus in peptone water, 1 ml of normal saline, and 1 ml of 24 hour culture of V.cholerae in peptone water in serial order. The abdomen was closed in two layers with thread. The animal was not fed for further 24 hours and then killed by intravenous injection of air.

The inoculated loops of the small intestine were examined carefully and the fluid contents of the distended loops were aspirated aseptically for detection of Vibrios bacteriologically.

Infant rabbits: Pathogenicity in infant rabbits was carried out following Dutta and Habbu technique (1955):

Infant rabbits, ten days old, of either sex, weighing about 200 gram were utilised for the experiments.

Under light anaesthesia and aseptic conditions incision was made through which the small intestine was pulled out. One ml of peptone water culture was inoculated into it and abdomen closed in two layers with thread. Inoculated animals were observed carefully for 24 hours.

Fluid content of the small intestine of the dead animals was aspirated for detection of *Vibrios* bacteriologically.

Pathogenicity in adult mice and Guinea pigs

Kasauli strain, of mice weighing 18-20 gms of either sex were used for the experiment.

Mice were inoculated intraperitoneally with 0.5 ml of overnight incubated broth culture and observed for 48 hours. Mice were opened after the death and heart blood collected aseptically and inoculated in peptone water containing 3 per cent sodium chloride for detection of organisms. (Thatcher and Clark, 1968).

Guinea pigs: Guinea pigs weighing about 300 grams of either sex bred at Haffkine Institute were used for the experiment.

Guinea pigs were inoculated intraperitoneally with 5.0 ml of overnight incubated broth culture and observed for 48 hours. Heart blood was collected after the death of the Guinea pigs and cultured in a peptone water containing 3 per cent sodium chloride for detection of the *Vibrios*.

Results

One hundred and fifty oysters were collected from coastal region of Mahim and same number was collected from the coastal region of Colaba, Bombay. Ten oysters were peeled together and 15 samples each from Mahim and Colaba coastal regions were prepared and tested (Table I).

TABLE IStrains of V.parahaemolyticus isolated fromOysters

Coastal Region	No. of pooled samples tested	Strains of V.parahaemolyticus isolated	Percentage of isolation
Mahim	15 (150 sub-samples)	2	13.33
Colaba	15 (150 sub-samples)	4	26.66

TABLE IISensitivity to various antibiotics

No.of strains tested - 6 (Oysters)

Antibacterial agents	Concentration per disc	Inhibitory Zone in mm	Results
Chloromycetin	30 mcg	16 or more	Sensitive
Tetracycline	30 mcg	18 or more	Sensitive
Streptomycin	10 mcg	14 or more	Sensitive
Kanamycin	30 mcg	17 or more	Sensitive
Polymyxin B	50 IU	12 or more	Sensitive
Ampicillin	10 mcg	10 or less	Resistant

TABLE III

Pathogenicity of strains isolated from Oysters

Culture No.	Mice	Guinea Pigs	Ligated rabbit gut	Infant rabbit	Haemolysis		Kanagawa Phenomenon
					Human Blood	Sheep Blood	
1	2/2	2/2	0+	1/2	+	& ±	+
2	2/2	2/2	+	2/2	+	-	+
3	2/2	2/2	+	1/2	+	-	±
4	1/2	2/2	+	1/2	+	-	-
5	2/2	2/2	0±	1/2	±	-	+
6	2/2	1/2	+	2/2	+	-	+

Mice, Kasauli strain, weighing 18-20 g. received intraperitoneally 0.5 ml of the overnight incubated broth culture.

Guinea pigs bred at Haffkine, weighing 250-350 g. received intraperitoneally 0.5 ml of the overnight incubated broth culture.

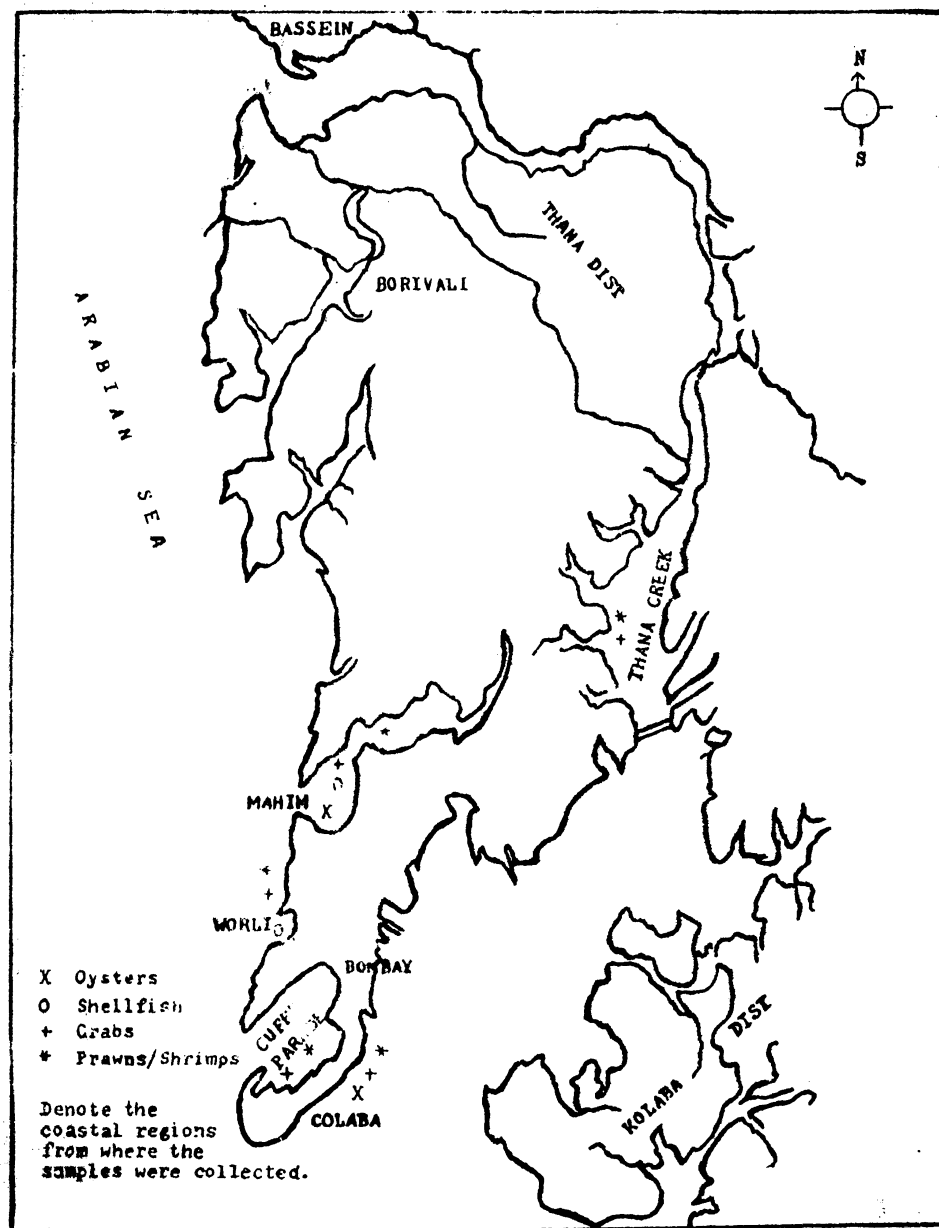
The ligated rabbit gut test was performed by using a method of De and Chatterjee.

The infant rabbit technique was performed by using a method of Datta and Habbu.

The denominators indicate the number of animals used and the numerators indicate the number of animals that died.

0 Indicates the positive reaction and 0 Indicates very weak reaction.

& Indicates the negative reaction.



GREATER BOMBAY

Two and four strains of V.parahaemolyticus could be isolated from coastal region of Mahim and Colaba respectively. These six strains did not grow in peptone water containing 0 per cent and 10 per cent sodium chloride but showed abundant growth in peptone water containing 3 per cent sodium chloride and comparatively less growth in peptone water containing 8% NaCl. Haemolysis was observed on human blood agar but not on sheep blood agar. Results were confirmed by Feeley and Pittman method (1963). Pathogenicity was tested in mice, guinea pigs, adult and infant rabbits. The mice and guinea pigs died within 24 hours. Rabbits showed rather weak positive reaction. The isolates were found to be positive for Kanagawa phenomenon (Table III) and sensitive to chloramphenicol, tetracycline, streptomycin, kanamycin and polymyxin B but not to ampicillin (Table II). When studied biochemically (as given in this Chapter under Materials and Methods) they showed all typical characteristic reactions of V.parahaemolyticus (Sakazaki, 1965 b).

Discussion

From thirty pooled samples six strains of V.parahaemolyticus were isolated from coastal region of Mahim and Colaba, Bombay (Table I).

Most of the Bombay's sewage outfalls and defecations by slum dwellers are on West side from where these samples were gathered. Due to this sewage outfalls, sewage pollution of coastal waters cannot be ruled out as Robinson had shown it as early as 1883.

Oysters derive their nourishment by filtering water through their gills by ciliary action, and according to Eyre (1924) about 48 litres of water enter and leave the shell of an oyster a day, and bacteria contained in the water are thus concentrated. Knott (1951) reported that bacterial content of a oyster is at least ten times as high as the concentration in the surrounding polluted water. Hence the isolation of the *Vibrios* from samples of oysters indicates the possibility of sewage pollution of coastal waters, and liability of oysters to give rise to gastroenteritis due to eating raw or improperly cooked and handled. However fish is generally properly cooked (boiled, fried, roasted) in Bombay. Bombay being a cosmopolitan city fish is also consumed less as compared to Calcutta.

Though, we could not isolate *V. parahaemolyticus* from stool samples of gastroenteritis cases, there is evidence to it's presence in oysters in the coast of Bombay. This is in agreement with findings of Barros et al. (1968) U.S.A., Duck (1966) U.S.A., Kampelmacher (1972), Netherlands, who could isolate *V. parahaemolyticus* from oysters. This probably is the first report on isolation of *Vibrios* from oysters in the coast of Bombay which opens a new field of research in view of epidemiology of gastroenteritis.

Shellfish

Robinson (1883) reported the cases of enteric fever due to eating raw shellfish gathered near the Ramsgate sewage outfall. Similar cases were reported by Piggot of Teignmouth in 1894 and Ireland, the town of Belfast had recorded high death rate due to contaminated shellfish.

Newsholm reported similar cases in 1894, 1895 and 1896 caused by shellfish and other outbreaks took place in Southampton and Winchester in 1902, in Workington in 1914 and New York and Chicago in 1925. Bucher incriminated shellfish in the city of Birmingham in 1908.

Belin (1934) reported several cases of enteric fever due to the consumption of shellfish in France alone.

Shellfish derive their nourishment by filtering water through their gills by ciliary action and in this connection Sherwood and Thomson (1953) stated that a single shell-fish will remove the suspended matter from over 10 gallons of sea water in a single day. Thus any bacteria contained in the water are concentrated by shellfish.

Baross et al. (1968) were first to isolate V. parahaemolyticus in U.S.A. from the sediments and marine animals of the west coast and Ward (1968) reported the isolation of it from the frozen sediments and waters of the Gulf and South Atlantic coasts.

Thomson (1973, unpublished data) reported the isolation of the V. parahaemolyticus from Canadian shellfish while Kampelmacher et al. (1972) studied the occurrence of V. parahaemolyticus in shell-fish in the Netherlands.

In Bombay, and other coastal regions of the country shellfish are consumed due to low cost and availability throughout the year. This made us to investigate the shellfish in the coastal region of Bombay.

Materials and Methods

The shellfish is a sluggish animal more or less sedentary in habits and incapable of actively capturing its prey. Due to this it obtains its food by ciliary action set in the water. Sea water shellfish are found at the bottom along the coastal region and in large numbers near the opening of the creek where they are partly buried in the sand or the mud.

During the period of low tide the shellfish were collected from coastal regions of Mahim-Worli and Worli-Colaba. Among them shellfish of average size were selected and brought to the laboratory in living condition for processing.

Each shellfish was opened aseptically after washing in sterile distilled water as follows: each shellfish was placed on a piece of sterile parchment paper by grasping it with the left hand. A small portion of the shellfish at the posterior end was nibbled away with sterile nibbling forceps through which a sterile scalpel was inserted and the posterior adductor muscle and other attachments were cut. Thus two halves of the shell were separated and left one removed. All the fluid in the shell was poured off and with the help of the scalpel the body was transferred to a sterile Petridish.

Ten bodies of an average size shellfish were pooled together and 10% suspension was prepared in 3% NaCl peptone water by means of a mixer at top speed.

A loopful of the blended material was streaked on TCBS agar (Thiosulphate Citrate Bile Salt agar) and BTB agar (Bromothymol Citrate Bile Salt agar; Methods and preparation and ingredient given in this chapter under Oysters) and incubated overnight.

5.0 ml of the blended material was inoculated into 3% NaCl peptone water (about 100 ml) and subcultured on TCBS and BTB agar after 6 hours incubation at 37°C.

The suspected colonies were picked up after overnight incubation which were studied bacteriologically after checking for purity as given under oysters.

Results

Eight hundred shellfish were collected from coastal region of Worli-Mahim. Ten shell-fish of an average size were pooled together and thus eighty pooled samples were studied bacteriologically for identification of the V.parahaemolyticus. Twelve pooled samples were found to be positive for V.parahaemolyticus. (Table IV). Five from Mahim and seven from Worli. All isolates were subjected to biochemical, pathogenicity and anti antibiotic sensitivity tests. They showed all the characteristics of V.parahaemolyticus as reported in this chapter under oysters. (Table V and VI).

Discussion

Twelve pooled samples of shellfish five and seven from Mahim and Worli respectively were found to be positive for V.parahaemolyticus (Table IV).

TABLE IV

Strains of V. parahaemolyticus isolated from
Shellfish

<u>Coastal</u>	<u>No. of pooled samples tested</u>	<u>Strains of V. parahaemo- lyticus isolated</u>	<u>Percentage of isolation</u>
Mahin	40 (400 sub-samples)	5	12.5
World	40 (400 sub-samples)	7	17.5

TABLE V

Sensitivity to various antibiotics

No. of strains tested - 12 (Shellfish)

<u>Antibacterial agents</u>	<u>Concentration per disc</u>	<u>Inhibitory Zone in mm</u>	<u>Results</u>
Chloromycetin	30 mcg	18 or more	Sensitive
Tetracycline	30 mcg	18 or more	Sensitive
Streptomycin	10 mcg	15 or more	Sensitive
Kanamycin	30 mcg	17 or more	Sensitive
Polymyxin B	50 IU	11.5 or more	Sensitive
Ampicillin	10 mcg	11 or less	Resistant

TABLE VI
Pathogenicity of strains isolated from Shellfish

Culture No.	Mice	Guinea Pigs	Ligated rabbit gut	Infant rabbit	Hemolysis		Kanagawa Phenomenon
					Human Blood	Sheep Blood	
1	2/2	2/2	0+	2/2	±	± -	+
2	2/2	2/2	+	2/2	+	-	-
3	1/2	2/2	+	1/2	±	-	±
4	2/2	1/2	0±	2/2	+	-	-
5	2/2	2/2	+	1/2	+	±	+
6	1/2	2/2	+	2/2	+	-	±
7	2/2	2/2	+	1/2	+	-	-
8	2/2	2/2	+	1/2	±	-	+
9	2/2	2/2	±	1/2	+	-	±
10	2/2	1/2	+	2/2	+	-	+
11	2/2	2/2	+	1/2	+	-	+
12	2/2	1/2	+	2/2	+	±	+

Mice, Kasauli strain, weighing 18-20 g. received intraperitoneally

0.5 ml of the overnight incubated broth culture.

Guinea pigs bred at Haffkine, weighing 250-350 g. received intraperitoneally

5.0 ml of the overnight incubated broth culture.

The ligated rabbit gut test was performed by using a method of De and Chatterjee.

The infant rabbit technique was performed by using a method of Dutta and Habbu.

The denominators indicate the number of animals used and the numerators indicate the number of animals that died.

⊕ Indicates the positive reaction and 0 indicates very weak reaction.

⊖ Indicates the negative reaction.

Sherwood and Thomson (1953) reported the capability of shellfish of filtering the water through the gills by ciliary action for their nourishment and accordingly a single shellfish will remove the suspended matter from over 10 gallons of sea water in a single day. Thus any bacteria in the polluted water are concentrated by shellfish. As stated before the samples of shellfish were collected from Mahim and Worli coastal region where the water pollution can be suspected due to sewage outfalls.

Shellfish is consumed deliciously in Bombay and all over coastal area throughout the season due to which there is very possibility of shellfish giving rise to food poisoning if not properly cooked and handled.

Both oysters and shellfish showed the presence of the V.parahaemolyticus though stool samples of gastroenteritis cases were found to be negative. This might be due to proper cooking and secondly less consumption of fish in the Bombay, the city being a cosmopolitan one. This was also reported by Hakanishi et al. (1963) who could not isolate V.parahaemolyticus from stool samples of gastroenteritis cases but could isolate the organism from seafish in Germany. Fish is held responsible for V.parahaemolyticus gastroenteritis in Japan where it is a custom for the people to eat raw fish but in Bombay fish is consumed completely boiled, fried or roasted. The consumption is moderate in amount.

Our finding on isolation of V.parahaemolyticus from shellfish in the coast of Bombay, clearly indicates a possibility of food poisoning arising due to eating raw shellfish or improper handling.

However, this is a first report on isolation of V.parahaemolyticus from shellfish opening a new line of research.

Crabs

Crab is one of the most highly organised Crustaceans. The carapace is fused with all the thoracic segments to form the cephalothorax. The eyes are stalked. The first three pairs of thoracic appendages are turned forward and modified as maxillipeds, and the rest as the legs, suited for walking or swimming.

Baross et al. (1968) reported the isolation of V. parahae-molyticus from the sediments and marine animals of the west coast. Krantz et al. (1969) isolated this organism from Chesapeake Bay crabs retained in commercial shedding tanks in U.S.A. Fishbein et al. (1970) reported the recovery of 56 strains of V. parahae-molyticus from the cooked, picked, packed, and refrigerated meat of Chesapeake Bay blue crabs offered for the retail market.

V. parahae-molyticus gastroenteritis was reported in Maryland U.S.A. in 1972 by Beard et al. in which steamed crabs were found to be causative agents served at the picnic. In second incidence, Charleson et al. (1972) isolated V. parahae-molyticus from gastroenteritis cases arising out of crabs. Since 1969, in U.S.A., Maryland has reported five outbreaks of gastroenteritis, and Washington has reported four. V. parahae-molyticus as a cause of food borne outbreaks has been responsible for the increasing number of reports of this type of food poisoning in U.S.A. due to eating crabs. Taking into consideration the involvement of the crabs as a vehicle the study was undertaken to survey the coast of Bombay.

Materials and Methods

Crabs are generally found in the coastal rocky and muddy places of the sea. They usually come out in search of food during the period of high tide. At this particular time they were caught by means of a circular bag like net. A net was

fixed to a circular iron^{ring} to which pieces of fish as a bait were tied diametrically. The net was left at the bottom and blindly pulled periodically. They remained in the net due to impact of the water if they happened to be in it.

Crabs are very furious sea animals and when touched do bite any touching object by means of the maxillae. So, in order to avoid the bite their maxillae were tied with a strong thread and then brought to the laboratory.

Crab was opened by removing the carapace with scissors under aseptic conditions. Small pieces of each of the internal organs were separated out and poked together in a preweighed Petridish.

Ten per cent suspension of pooled sample each of the crab was prepared in peptone water containing 3 per cent sodium chloride by means of a mixer at top speed.

A loopful of the ground material was streaked on TCBS agar and BTB agar (Methods of preparation and ingredient given under the Oysters).

5.0 ml of the material was inoculated into 3% NaCl peptone water (about 100 ml) and subcultured on TCBS and BTB agar after 6 hours incubation at 37°C.

The suspected colonies were picked up after overnight incubation which were studied bacteriologically after checking for purity as given under Oysters.

Results

Fifty crabs were collected each from the coastal regions of Mahim, Worli, Colaba, Cuffeparade and Thana Creek. Each pooled sample contained ten crabs and thus 25 pooled samples

TABLE VII

Strains of V.parahaemolyticus isolated from

Crabs

Coastal	No.of pooled samples tested	Strains of V.parahaemolyticus isolated	Percentage of isolation
Colaba	5 (50 sub-samples)	2	40
Cuffeparade	5 (50 sub-samples)	1	20
Worli	5 (50 sub-samples)	3	60
Mahim	5 (50 sub-samples)	2	40
Thana	5 (50 sub-samples)	1	20

TABLE VIII

Sensitivity to various antibiotics

No.of strains tested - 9 (Crabs)

Antibacterial agents	Concentration per disc	Inhibitory Zone in mm	Results
Chlormycetin	30 mcg	13.5 or more	Sensitive
Tetracycline	30 mcg	14 or more	Sensitive
Streptomycin	10 mcg	12 or more	Sensitive
Kanamycin	30 mcg	15 or more	Sensitive
Polymyxin B	50 IU	12 or more	Sensitive
Ampicillin	10 mcg	9 or less	Resistant

TABLE IX

Pathogenicity of strains isolated from Crabs

Culture No.	Mice	Guinea Pigs	Ligated rabbit gut	Infant rabbit	Haemolysis		Kanagawa Phenomenon
					Human Blood	Sheep Blood	
1	2/2	2/2	0 +	2/2	±	± -	±
2	1/2	2/2	0 ±	1/2	+	-	-
3	2/2	1/2	+	1/2	+	±	+
4	2/2	2/2	+	2/2	+	-	±
5	1/2	2/2	+	2/2	+	-	+
6	2/2	2/2	+	1/2	±	-	+
7	2/2	2/2	±	2/2	+	-	±
8	2/2	2/2	+	1/2	±	-	+
9	1/2	2/2	+	1/2	+	-	±

Mice, Kasauli strain, weighing 18-20 g. received intraperitoneally 0.5 ml of the overnight incubated broth culture.

Guinea pigs bred at Haffkine, weighing 250-350 g. received intraperitoneally 5.0 ml of the overnight incubated broth culture.

The ligated rabbit gut test was performed by using a method of De and Chatterjee.

The infant rabbit technique was performed by using a method of Dutta and Habbu.

The denominators indicate the number of animals used and the numerators indicate the number of animals that died.

0 Indicates the positive reaction and ± indicates very weak reaction

± Indicates the negative reaction.

were processed, five each from above mentioned coastal region. Of twentyfive samples tested nine were found to be positive for V.parahaemolyticus 2, 1, 3, 2 and 1 from Colaba, Cuffparade, Worli, Mahim and Thana Creek, respectively. (Table VII).

These isolates were studied for biochemical reactions, pathogenicity and antibiotic sensitivity tests. They showed all the characteristics of V.parahaemolyticus as reported under oysters. (Table VII and IX).

Discussion

Twentyfive samples of crabs were collected from coastal regions of Colaba, Cuffparade, Worli, Mahim and Thana Creek. The strains of V.parahaemolyticus could be isolated from each area mentioned above which indicated ample presence of Vibrios in the crabs around the coast of Bombay. (Table VII).

V.parahaemolyticus gastroenteritis due to eating steamed crabs in U.S.A. (Maryland) was reported by Beard et al. (1972). Charleson et al. (1972) isolated this organism from gastroenteritis cases arising out of eating crabs. Krantz et al. (1969) reported the isolation of V.parahaemolyticus during studies of mortality in population of the blue crab callinectes sapidus from lethargic and moribund crabs being retained in commercial tanks during shedding of soft crabs.

Bombay, mostly surrounded by sea coast is densely populated with poor sanitary facilities due to large number of slum dwelling units. In addition to this sewage outfalls are on coastal regions, resulting in pollution of the sea water. The samples collected from these areas show the ample presence of Vibrios in the crabs around the coast of Bombay which is likely to give rise to outbreaks of food poisoning if crabs are improperly consumed.

Our results confirm the findings of Baross et al. (1968), Krantz et al. (1969) Fishbein et al. (1970), Beard et al. (1972) who isolated V.parahaemolyticus from crabs.

Probably this is the first report on crabs in Bombay.

Prawns and Shrimps

Baross et al. (1968) were first to isolate V.parahaemolyticus in U.S.A. from the sediments and marine animals of the west coast and Ward (1968) reported the isolation of it from the frozen sediments and waters of the Gulf and South Atlantic coasts.

Vandersant et al. (1970, 1972) reported the isolation of V.parahaemolyticus from Gulf coast shrimps in U.S.A. and showed it to be capable of surviving in shrimps at temperatures ranging from -18°C to 80°C.

Gregory et al. (1972) reported such large outbreaks in U.S.A. and showed for the first time that shrimps have been implicated as the vehicle for V.parahaemolyticus gastroenteritis.

Chatterjee et al. (1971) were first to isolate V.parahaemolyticus from small pond-fishes including 59 shrimps and prawns collected periodically from different markets of Calcutta, during a peak season of Cholera in Calcutta.

Prawns/shrimps contain high proteins and generally are very favourite with the people all over the world. They are consumed in large numbers in India particularly all over the coastal regions. Taking into consideration it's consumption we had undertaken this study to find out whether these animals also are liable to act as a vehicle for V.parahaemolyticus.

Materials and Methods

Prawns and shrimps are found both in the sea and in fresh water. The body of the prawn or shrimp is covered with a chitin known as an exoskeleton and segmented like that of a earthworm. The appendages are used in swimming and collecting

the food paraticles. As they are moved, a current is set up which brings in the particles serving as food.

The prawns and shrimps were caught by means of a square type net dragged manually on the muddy areas of the coastal regions near the mouth of the creek. The prawns and shrimps were brought to the laboratory in living condition as far as possible. About 50 prawns and shrimps of an average size were selected for making the pool of sample. Each of them was washed thoroughly in a physiological salt solution and placed in a preweighed Petridish. Ten per cent suspension in peptone water containing 3 per cent sodium chloride was prepared by means of a mixer at top speed.

A loopful of the blended material was streaked on TCBS agar and BTB agar and incubated at 37°C overnight.

5.0 ml of the blended material was inoculated into peptone water containing 3 per cent sodium chloride and subcultured on TCBS and BTB agar after 6 hours incubation at 37°C.

The suspected colonies were picked up after overnight incubation which were subjected to biochemical and other tests for identification. (Details given under oysters in this Chapter).

Results

One thousand two hundred and fifty prawns and shrimps were caught from coastal region of Colaba, Cuffeparade, Worli, Mahim and Thana Creek. Fifty prawns and shrimps were pooled together for making a sample. Thus twenty five samples five each from above mentioned areas were processed for the isolation of V. parahaemolyticus. Eight samples were found to be positive for V. parahaemolyticus, 1, 2, 2, 2, and 1 from Colaba, Cuffeparade, Worli, Mahim and Thana respectively. (Table X).

TABLE X**Strains of V.parahaemolyticus isolated from****Prawns/Shrimps**

Coastal Region	No.of pooled samples tested	Strains of <u>V.parahaemolyticus</u> isolated	Percentage of isolation
Colaba	5 (250 sub-samples)	1	20
Cuffeparade	5 (250 sub-samples)	2	40
Worli	5 (250 sub-samples)	2	40
Mahim	5 (250 sub-samples)	2	40
Thana	5 (250 sub-samples)	1	20

TABLE XI**Sensitivity to various antibiotics****No.of strains tested - 8 (Prawns/Shrimps)**

Antibacterial agents	Concentration per disc	Inhibitory Zone in mm	Results
Chloromycetin	30 mcg	17 or more	Sensitive
Tetracycline	30 mcg	19 or more	Sensitive
Streptomycin	10 mcg	14 or more	Sensitive
Kanamycin	30 mcg	17 or more	Sensitive
Polymyxin B	50 IU	11 or more	Sensitive
Ampicillin	10 mcg	10 or less	Resistant.

TABLE XII

Pathogenicity of strains isolated from Prawns/Shrimps

Culture No.	Mice	Guinea Pigs gut	Ligated rabbit	Infant rabbit	Haemolysis		Kanagawa Phenomenon
					Human Blood	Sheep Blood	
1	2/2	1/2	O +	1/2	±	& -	-
2	2/2	2/2	O ±	1/2	+	±	+
3	2/2	2/2	+	2/2	+	-	±
4	1/2	2/2	+	1/2	±	-	-
5	2/2	2/2	+	1/2	±	-	+
6	2/2	1/2	+	1/2	+	-	±
7	2/2	2/2	+	1/2	+	-	+
8	2/2	2/2	±	2/2	+	&	+

Mice, Kasauli strain, weighing 18-20 g. received intraperitoneally

0.5 ml of the overnight incubated broth culture.

Guinea pigs bred at Heffkine, weighing 250-350 g. received intraperitoneally 5.0 ml of the overnight incubated broth culture.

The ligated rabbit gut test was performed by using a method of De and Chatterjee.

The infant rabbit technique was performed by using a method of Dutta and Habbu.

The denominators indicate the number of animals used and the numerators indicate the number of animals that died.

O Indicates the positive reaction and & indicates very weak reaction

& Indicates the negative reaction.

These isolates were tested for biochemical reactions, pathogenicity and sensitivity to antibiotics. They showed all the characteristics of V.parahaemolyticus as reported under Oysters (Table XI and XII).

Discussion

Twenty five samples five each from coastal region of Colaba, Cuffeepara, Worli, Mahim and Thana Creek, were examined. V.parahaemolyticus could be isolated from each region. (Table X).

Prawns and shrimps usually are found at bottom in muddy regions. The respiration is effected by gills. As mentioned under oyster, shellfish and crabs sewage outfalls of Bombay are on coastal region due to which water pollution takes place. As samples collected were from these areas, the contamination of sea animals cannot be denied which was indicated by the presence of V.parahaemolyticus in them.

Ray L.Gregory et al. (1972) reported large outbreaks in U.S.A. and showed for the first time that shrimps have been a vehicle for V.parahaemolyticus gastroenteritis. Chatterjee et al. (1972) reported typical V.parahaemolyticus from local pond shrimps and prawns in Calcutta which is in complete agreement with our findings.

For several years there are no cholera cases in Bombay (Health Bulletin of Bombay Municipal Corporation) and according to Sanyal et al.(1972) in Calcutta and in the Philippines the frequency of V.parahaemolyticus infections followed that of V.cholerae indicating the less chances gastroenteritis due to this organisms in the greater Bombay. Though stool samples of gastroenteritis cases were examined for isolation of

V.parahaemolyticus, it could not be isolated. However, danger of food poisoning cannot be ruled out taking into consideration it's ample presence in the sea animals if improperly handled or cooked. Same observations were also reported by Nakanishi et al. (1968) in Germany where they could isolate V.parahaemolyticus from sea fish and not from stool samples of gastroenteritis cases.

CHAPTER IV

CHAPTERIV (A)

STUDY ON STOOL SAMPLES OF

GASTROENTERITIS

CASES

Japanese people are in the habit of eating the raw sea fish and consume oysters and shellfish etc. with a dash of pepper and salt. According to Sakazaki et al. (1965) this custom of eating raw is held responsible for outbreaks of gastroenteritis due to V.parahaemolyticus.

Fujino (1951) was first to isolate V.parahaemolyticus from autopsy material investigated during the outbreaks of gastroenteritis in Japan. Fujino and co-workers (1951, 1953) after a thorough study found that "Shirasuboshi" or boiled and semidried young sardine is the foodstuff causing the outbreaks in Japan. In this outbreak about 276 people were affected out of which 26 died.

After the reports of Takikawa many Japanese investigators were attracted by this organism which was isolated repeatedly during further outbreaks of gastroenteritis. Sakazaki et al. (1963) reported that over 50% of gastroenteritis cases may have been caused by this organism.

Takikawa and Fujisawa (1956) reported the food poisoning involving employees of the Yokohama National Hospital along with the other people. Later, Takikawa (1958) encountered several endemic and sporadic cases of similar food poisoning.

In 1960 thousands of persons were affected due to food poisoning along the Pacific Coast of Japan. Many workers reported after confirmation that the V.parahaemolyticus was the causative organism of the epidemic. Since then, isolation of Vibrios is routinely followed in the bacteriological laboratories in Japan.

V.parahaemolyticus as a cause of foodborne outbreaks of gastroenteritis has been responsible for a number of outbreaks of this type of food poisoning in two states in U.S.A.. Since 1969, Maryland has reported five outbreaks, Washington has reported four. Gregory et al. (1972) reported largest such outbreak due to V.parahaemolyticus in the United States.

Chatterjee et al. (1970) were first to isolate V.parahaemolyticus in India from persons suffering from acute diarrhoea who were admitted in the Infectious Diseases Hospital, Calcutta without prior therapy.

Sanyal et al. (1972) isolated this organism in Varanasi, India, during the study of enteric infections in Sunderpur alum area.

Though V.parahaemolyticus was first reported as a cause of food poisoning in Japan (Sakazaki, 1965) it has been also encountered in cases of gastroenteritis in various parts of the World including Thailand, Philippines (H.Inaba, quoted by Sakazaki 1971), Australia (Battey et al. 1970), Malaysia (Prescott, quoted by Sanyal, 1973) and England (Hobbs and Barrow, quoted by Sanyal 1972).

In Greater Bombay, cases of diarrhoea and gastritis occur round the year due to which about 300 persons die per month, in the city. (Public Health Department of the Bombay Municipal Corporation).

This provoked us to undertake this study on gastro-enteritis cases in Bombay as there were no reports on work on V.parahaemolyticus.

Materials and Methods

Kasturba Hospital is situated centrally in the city of Bombay, in which generally all cases of communicable diseases are admitted.

It has a special division for admitting the gastro-enteritis cases whose rectal swabs are collected soon after the admission for routine isolation of V.Cholerae as one of the causative agents.

Alkaline peptone water was used for the collection of the specimen (Chatterjee et al. 1970). About 10 ml alkaline peptone water was dispensed in each test tube and sterilised at 15 pounds pressure for 15 minutes. After sterilisation tubes were incubated at 37°C for 48 hours and only sterile tubes were used for the collection of the rectal swabs of the gastroenteritis cases by the medical staff.

The swabs were immediately put in sterile alkaline peptone water (Chatterjee et al. 1970) and incubated at 37°C for 6 hours. After the incubation a loopful of the material was streaked onto the MacConkey's Agar plates for isolation of V.cholerae.

Duplicate samples of rectal swabs of gastroenteritis cases were brought to the Haffkine Institute within $\frac{1}{2}$ hour and incubated at 37°C for short time (3 hours). A loopful of the incubated sample was plated on TCBS agar and BTB agar plates (Preparation and composition given under materials and methods in Chapter III) incubated at 37°C overnight.

Next morning suspected colonies - round, mucoid, translucent with a greenish or bluish opaque centre - were subcultured on nutrient agar containing 3% NaCl and incubated at 37°C overnight. After incubation they were checked for purity and then subjected to detail study for identification.

Stool samples of gastroenteritis cases from Civil Hospital, Parbhani (in Marathwada region of Maharashtra State) about 450 kilometer to east of Bombay and away from the sea were also collected and studied culturally for the isolation of V.parahaemolyticus.

A laboratory was set up in the Civil Hospital, Parbhani for collection and processing of these samples. Public Health Department had helped in collection of the specimens from the selected areas of the city and isolation ward of the Hospital which was close to the laboratory. After collection samples were immediately processed by inoculating a loopful of the stool sample in 3% NaCl peptone water. After 5-6 hours incubation at 37°C a loopful of the culture was streaked onto the TCBS and BTB agar plates which were incubated at 37°C overnight and suspected colonies subcultured on nutrient agar slants containing 3% NaCl and incubated overnight at 37°C which were studied further for isolation of V.parahaemolyticus.

Results

Six hundred and fifty two rectal swabs of gastroenteritis cases from Kasturba Hospital, Bombay were collected during the period from May 1972 to June 1973 particularly in summer months and processed for the isolation of V.parahaemolyticus but no V.parahaemolyticus could be isolated from these samples (Table XIII).

About five hundred stool samples of gastroenteritis cases from Civil Hospital, Parbhani, were collected mostly during the rainy season during the period from July 1972 to September 1973 and processed for the isolation of V.parahaemolyticus but not a single strain could be isolated from these samples. (Table XIII).

TABLE XIII

Strains of V.parahaemolyticus isolated from gastroenteritis cases

Place	No. of samples of rectal swabs/stool collected	No. of strains isolated
Kasturba Hospital Bombay	652	Nil
Civil Hospital, Parbhani	500	Nil

Discussion

In this study we could not isolate a single strain of V. parahaemolyticus though six hundred and fifty two stool samples of gastroenteritis cases from Kasturba Hospital, Bombay and five hundred stool samples of gastroenteritis cases from Civil Hospital, Parkbani were bacteriologically examined. In Japan, United States, Canada and other countries shellfish and oysters are consumed raw with a dash of pepper and salt which is generally held responsible for outbreaks of food poisoning but in India fish is usually cooked properly (boiled, roasted or fried) and then consumed. This might be one of the reasons why we could not isolate this organism from gastroenteritis cases.

We have isolated V. parahaemolyticus from the varieties of fish from the coast of Bombay but it could not be isolated from stool samples of gastroenteritis cases from Bombay. Some observations have been reported by Nakanishi, Leistner, Hechelmann and Baumgart (1968). According to them V. parahaemolyticus could not be isolated from seven hundred and thirteen human diarrhoeal stools collected in Southern Germany although the Vibrios were found in 31% of sea fish at fish markets along the Baltic Sea.

According to Chatterjee et al. (1970) the fishes are seldom eaten raw or half cooked in Calcutta. The consumption of true marine fishes in comparison to those of fresh water origin (i.e. those living in the ponds) is infrequent and outbreaks of gastroenteritis in Calcutta due to V. parahaemolyticus seems to be quite different from that already reported in Japan and other countries. They state that consumption of pond water for drinking and handling of infected fishes may be indirectly responsible for the cases of human gastroenteritis. Secondly, in Calcutta most of the people consume a lot of fish where as in Bombay a cosmopolitan city fish is consumed less as compared to Calcutta.

and in Bombay there are no reports of indigenous cholera cases for last several years (Health Bulletin of Greater Bombay).

In Bombay, cases of gastroenteritis are quite common round the year and according to Public Health Department of the Bombay Municipal Corporation about 300 deaths take place per month. Generally the causative organism goes undetected and so far, no attention has been focussed on the isolation of V.parahaemolyticus.

Though we have not detected this organism from stool samples of the gastroenteritis cases it is quite possible that V.parahaemolyticus might be a cause of some bacterial food poisoning cases, taking into consideration it's rampance in the coast of Bombay. More work is necessary on this aspect.

(B)

STUDY ON ANIMAL FAECES

Sanyal et al. (1973) carried out the study on animal excreta for the presence of V.cholerae in Varanasi and reported that from 1,287 specimens of animal excreta examined, fifty one strains of V.cholerae ogawa and other serotypes were isolated. Some of the animals excreted V.cholerae repeatedly.

Sharanjit Singh et al. (1973) reported the isolation of V.cholerae from different sources like human faeces, excreta of animals, potable water and sewage in a community in Varanasi during the period, June 1972 to May 1973. The examination of 823 samples of animal excreta yielded eight strains of serotype, ogawa and twenty seven strains belonging to other serotypes.

R.Bradley Sack (1973) carried out elaborate study on dogs in Calcutta in search of canine reservoir of V.cholerae and found no evidence of it but seventy dogs out of 500 investigated, showed presence of non-cholera vibrios in their intestines.

Identical serotypes of these organisms were isolated from dogs, crows and human beings in the same geographical area. These results indicated that non-human reservoirs exist for non-cholera vibrios in contrast to V.cholerae and that these reservoirs may serve as sources of infections in humans.

Materials and Methods

Parbhani, a city in the Marathwada region of the Maharashtra State is situated 450 kilometer to East of Bombay. Population of the city is about 60,000 and consists

of Hindus and Muslims with poor hygienic conditions. The sanitary facilities in the city are also not satisfactory and the cases of gastroenteritis start occurring at the beginning of the monsoon every year.

A laboratory was set up in the Civil Hospital, Parbhani for processing the stool samples of gastroenteritis cases and animal excreta.

Plastic containers were supplied to the staff for collection of the animal excreta from different localities of the Parbhani city. The samples of animal excreta were received in the morning and pools of five samples of same species were made soon after they were received. During the study on animal excreta one thousand and seventy samples were collected. Of these two hundred and fourteen pools of each of 5 samples were made species-wise. It included dog, cow/bull, sheep, fowl, buffalo, donkey, horse, and some pooled samples of fresh water fish.

A loopful of material of each sample was inoculated into peptone water containing 3% NaCl. After six hours incubation at 37°C a loopful of this was streaked onto a TCBS agar plate which was incubated overnight at 37°C. (Methods of preparation of TCBS agar is given under Materials and Methods in Chapter III).

The suspected colonies - round, mucoid, translucent with a greenish or bluish opaque centre were picked up. (More details in Chapter III).

These isolates were studied culturally, biochemically and for pathogenicity and Kanagawa phenomenon (Methods given in Chapter III). They were subjected to antibiotic sensitivity tests against different antibiotics such as

chloromycetin, tetracycline, streptomycin, kanamycin, ampicillin and polymyxin B (Methods given in Chapter III). They were studied for most of the characteristics of V.parahaemolyticus so far reported as given in Chapter III under Materials and Methods.

Results

One thousand and seventy samples of animal excreta of dogs, fowls, sheep, cattle, donkey, horses and some samples of fresh water fish were collected from different areas of Parbhani city during the period from January 1973 to September 1973 and bacteriologically investigated. Five excreta of the same animal species were pooled together for convenience of the processing. (Table XIV).

Eleven pooled samples of dog excreta out of 86 examined, were found to be positive for V.parahaemolyticus and the rest were negative. Twelve pooled samples of fresh water fish did not show presence of V.parahaemolyticus. - These eleven strains showed halophilism when tested for NaCl tolerance test. They did not grow in peptone water containing 0% NaCl and 10% NaCl but showed abundant growth in 3% NaCl peptone water and comparatively less growth in peptone water containing 8% NaCl. Haemolysis was observed on human blood agar but not on sheep blood agar. Same results were observed when tested according to Feeley and Pittman method (1963) for haemolysis. When tested for pathogenicity in guineapigs and mice, the animals died within 24 hours. In the case of ligated-loop in adult rabbits, it gave rather weak positive reaction. Same trend was observed in the infant rabbits also. (Table XVI). (Methods are given in Chapter III under Materials and Methods). The isolates were tested for Kanagawa phenomenon and were

Strains of V.parahaemolyticus isolated from
Animal faeces from Parbhani

Place	Name of the Animal	No.of pooled samples processed	No.of strains isolated	Percentage
Parbhani	Dog	86	11	12.8
Parbhani	Powl	41	Nil	-
Parbhani	Sheep	21	Nil	-
Parbhani	Cow/Bull	16	Nil	-
Parbhani	Buffalow	21	Nil	-
Parbhani	Donkey	13	Nil	-
Parbhani	Fish	10	Nil	-
Parbhani	Horse	6	Nil	-

TABLE XV

Sensitivity to various antibiotics
No.of strains tested - 11 (Dog excreta)

Antibacterial agents	Concentration per disc	Inhibitory Zone in mm	Results
Chloromycetin	30 mcg	17 or more	Sensitive
Tetracycline	30 mcg	16 or more	Sensitive
Streptomycin	10 mcg	14.5 or more	Sensitive
Kanamycin	30 mcg	16.5 or more	Sensitive
Polymyxin B	50 IU	12 or more	Sensitive
Ampicillin	10 mcg	8 or less	Resistant.

TABLE XVI

Pathogenicity of strains isolated from Dogs excreta

Culture No.	Mice	Guinea Pigs	Ligated rabbit gut	Infant rabbit	Haemolysis		Kanagawa Phenomenon
					Human Blood	Sheep Blood	
1	2/2	1/2	±	1/2	±	± -	±
2	2/2	2/2	0 +	2/2	+	-	±
3	2/2	2/2	+	2/2	+	-	+
4	2/2	2/2	0 ±	1/2	+	-	+
5	2/2	2/2	+	1/2	+	±	-
6	2/2	2/2	+	1/2	+	-	-
7	2/2	2/2	+	2/2	+	-	+
8	2/2	1/2	+	2/2	+	-	-
9	1/2	2/2	+	2/2	±	-	±
10	2/2	2/2	+	2/2	+	-	+
11	2/2	2/2	±	1/2	+	-	+

Mice, Kasauli strain, weighing 18-20 g. received intraperitoneally

0.5 ml of the overnight incubated broth culture.

Guinea pigs bred at Heffkine, weighing 250-350 g. received intraperitoneally

5.0 ml of the overnight incubated broth culture.

The ligated rabbit gut test was performed by using a method of De and Chatterjee.

The infant rabbit technique was performed by using a method of Dutta and Habbu.

The denominators indicate the number of animals used and the numerators indicate the number of animals that died.

0 Indicates the positive reaction and 0 indicates very weak reaction

± Indicates the negative reaction.

found to be positive (Method in Chapter III). They were also found sensitive to chloramycetin, tetracycline, streptomycin, kanamycin and polymyxin B except ampicillin. (Table IV). The isolates showed all typical characteristics when studied biochemically as given in Chapter III under Materials and Methods.

Discussion

It is wellknown that animals play an important role in transmission of the diseases from animal to animal and from animal to human beings. Small Gram negative bacteria of genus *Brucella* are essentially pathogens of animals, notably goats, sheep, cattle and pigs, but are causative organisms of brucellosis in man through his accidental contact with the discharges of infected animals. Some of the organisms of the genus *Leptospira* cause leptospirosis in man and animals and most types of pathogenic strains of leptospiras are carried by various species of wild rodents. Pathogenic species of *Mycobacterium* affect man, animals, birds and reptiles. Horse dung contain *Clostridium tetani*.

Sanyal et al. (1973) reported the isolation of *V.cholerae* from excreta of cow and chicken. Sharanjit Singh et al. (1973) reported also the isolation of the eight strains of *V.cholerae* serotype-ogawa and twenty seven strains belonging to other serotypes, from samples of animal excreta.

R.Bradley Sack (1973) reported the isolation of non-cholera *Vibrios* from the intestines of 70 dogs out of 500 examined in Calcutta. We have screened 86 pooled samples of dogs excreta which included 430 sub-samples. Eleven pooled samples were found to be positive for *V.parahaemolyticus*.

V.parahaemolyticus has also been isolated from non-marine sources. J.Rapara (Unpublished data) reported the presence of halophilic Vibrios in waters of Navarra (Spain) far away from the sea. Sanyal et al. (1972) isolated V.parahaemolyticus from stool samples in Varanasi which is also far off from the sea. In the place like Parbhani about 450 kilometer away from the sea, we could isolate this organism from dogs excreta. It is an interesting finding which opens a new field for further study. This is also in agreement with Chatterjee's findings (Isolation of V.parahaemolyticus from non-marine sources) Chatterjee and Neogy 1972 b).

Though V.parahaemolyticus is a marine bacterium it could be detected at a distant place like Parbhani and it is apparent that this organism does not confine its habitat to the coastal region but can survive in hinterland too.

CHAPTER V

SUMMARY AND CONCLUSIONS

- 1) The investigation was carried out to study V.parahaemolyticus in fish in the coast of Bombay particularly from shellfish, oysters, crabs, prawns and shrimps.
- 2) Stool samples of gastroenteritis cases from Kasturba Hospital, Bombay were investigated to find out the corelationship between the contaminated sea animals and the gastroenteritis cases.
- 3) Stool samples of gastroenteritis cases from Civil Hospital, Parbhani and animal faeces from different areas of the same city were bacteriologically processed for detection of V.parahaemolyticus.
- 4) As Japanese TCBS agar was not available in the market, TCBS medium prepared at this Institute, was utilised throughout this study for isolation of V.parahaemolyticus after comparative study between Japanese TCBS agar and locally prepared one, which gave equally good results.
- 5) Forty pooled samples of shellfish (400 sub-samples) each from Mahim and Worli coastal regions were examined and five and seven strains of V.parahaemolyticus were.

isolated from the above mentioned coastal regions respectively.

- 6) Fifteen pooled samples of oysters (150 sub-samples) each from Mahim and Colaba coastal regions were processed and two and four strains were isolated respectively from the above mentioned coastal regions.
- 7) Five pooled samples of clams (50 sub-samples) each from Colaba, Cuffeparade, Worli, Mahim and Thana Creek, coastal regions were processed and two, one, three, two and one strains were isolated respectively from the above mentioned coastal regions.
- 8) Five pooled samples of Prawns/Shrimps, (250 sub-samples) each from Colaba, Cuffeparade, Worli, Mahim and Thana Creek coastal regions were studied and one, two, two, two and one strains were isolated respectively from the above mentioned coastal regions.
- 9) Two hundred and fourteen pooled samples of animal excreta (1070 sub-samples) of dog, fowl, sheep, cow/bull, buffalo, donkey, horse including some samples of fish from different areas of Parbhani were investigated and eleven strains were isolated from eightysix pooled samples of dogs excreta and rest were found to be negative including river fish samples.
- 10) Six hundred and fifty two rectal swabs of gastroenteritis cases and about five hundred stool samples of gastroenteritis cases from Kasturba Hospital, Bombay and Civil Hospital, Parbhani, respectively were investigated but V.parahaemolyticus could not be detected in a single stool sample.

- 11) The isolated strains were studied for biochemical reactions; pathogenicity in animals, antibiotics sensitivity, Kanagawa phenomenon and most of them showed positive results.

Conclusions

The results of the present investigations warrant inference that V.parahaemolyticus is definitely present in a variety of sea animals particularly shellfish, oysters, prawns/shrimps and crabs, in the coastal regions of Bombay mainly Colaba, Cuffeparade, Werli, Mahim and Thana Creek.

Most of the isolated strains were found pathogenic in mice, guinea pigs, adult and infant rabbits. Kanagawa phenomenon was also studied and most of the strains gave positive phenomenon. From these results it is apparant that V.parahaemolyticus may/can be the causative organism of some outbreaks of food poisoning or gastroenteritis in Bombay or other coastal territories of the country.

No V.parahaemolyticus could be isolated from stool samples of gastroenteritis in Bombay and Parbhani.

Strains of V.parahaemolyticus could be isolated from dog excreta in the different areas of Parbhani. These strains showed most of the characteristics of V.parahaemolyticus. These results clearly show that this organism does not confine it's habitat to the coastal region but can survive in hinterland too.

Probably this is the first attempt to study Y. parahae-
molyticus in Bombay which opens a new field of research in
view of gastroenteritis epidemiology for further investiga-
tion particularly regarding it's habitat, characteristics
and pathogenicity.

CHAPTER VI

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"Studies on Vibrio parahaemolyticus in Bombay"

A

SYNOPSIS

submitted to

THE UNIVERSITY OF BOMBAY

for

the degree of

MASTER OF SCIENCE

in

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by

A.K.Patil

under the guidance of

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Food poisoning and/or gastroenteritis very often takes place generally in the summer season with the symptoms of acute gastroenteritis. Of late much attention has been focussed on V.parahaemolyticus which is found responsible to give rise to outbreaks of food poisoning and/or gastroenteritis. Reports from Japan have amply established that V.parahaemolyticus causes acute gastroenteritis.

Sakazaki (1969) emphasized that 70% of the cases of bacterial gastroenteritis in Japan are caused by the facultatively halophilic, enteropathogenic V.parahaemolyticus. In Japan it is a custom of the people to eat raw sea fish which is responsible for outbreaks of gastroenteritis due to V.parahaemolyticus.

Baross J. et al. (1968) first reported the isolation of V.parahaemolyticus in U.S.A. from the sediments and marine animals of the West Coast. Fishbein et al. (1970) reported the recovery of 56 strains of V.parahaemolyticus from the cooked, pickled, packed and refrigerated meat of Chesapeake Bay blue crabs offered for the retail market. J. Howard Beard et al. (1972) reported V.parahaemolyticus in Maryland, U.S.A. and steamed crabs were found to be the causative agents served at the picnic. Mary Charleson et al. (1972) isolated V.parahaemolyticus from gastroenteritis case who had eaten the crabs. V.parahaemolyticus as a cause of foodborne outbreaks have been responsible for the increasing number of reports of this type of food poisoning in two states in U.S.A. Since 1969, Maryland has reported five outbreaks, Washington has reported four. Roy L. Gregory et al. (1972) reported largest number of such outbreaks and for the first time shrimps have been implicated as the vehicle for V.parahaemolyticus gastroenteritis in the U.S.A. Barross et al. (1968) confirmed the occurrence of V.parahaemolyticus in sea water, sediments, shellfish and oysters etc.

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At present V.parahaemolyticus has become an international problem and besides Japan and U.S.A. it has been isolated from marine animals, sea water and sediments etc. and elaborately studied in different aspects in Canada, Viet-Nam, Thailand, Indonesia, Denmark, Germany, Spain, Yugoslavia, Italy, Australia, Korea, England and India. In Calcutta, gastroenteritis due to V.parahaemolyticus was first recorded in 1970 (Chatterjee et al. 1970). Sanyal et al. (1972) reported the isolation of this organism in Varanasi during the study of enteric infections in Sunderpur alum area. Chatterjee et al. (1971, 1972) reported the isolation of V.parahaemolyticus from pond fishes and pond waters during the preliminary studies in Calcutta. M.Bradley (1973) reported the presence of non-cholera vibrios in the intestine of 14% of dogs. The results indicate that non-human reservoirs exist for non-cholera vibrios in contrast to V.cholerae and that these reservoirs may serve as sources of infections in humans.

The present investigation was undertaken to study V.parahaemolyticus in varieties of fish particularly shellfish, oysters, crabs and prawns/shrimps in the coast of Bombay. The rectal swabs/stool samples of gastroenteritis cases from Kasturba Hospital, Bombay and Civil Hospital, Parbhani were also studied to isolate this organism in order to correlate with the sea animal food. Animal faeces from Parbhani were also included in the study.

The present study covers almost whole coast of Bombay. Isolation of V.parahaemolyticus from varieties of fish. Samples of shellfish were collected and processed from Mahim, Worli area throughout the year and strains of V.parahaemolyticus were isolated. In the same way samples of oysters were collected and processed from Mahim-Colaba coastal region and strains of V.parahaemolyticus were isolated.

Samples of crabs and prawns/shrimps were collected from coastal region of Colaba, Cuffeparade, Worli, Mahim and Thana Creek and processed for isolation of V.parahaemolyticus. About 35 strains of V.parahaemolyticus were isolated from these marine animals.

Stool/rectal swabs, samples of gastroenteritis cases from Kasturba Hospital, Bombay and Civil Hospital, Parbhani were collected and processed for isolation of V.parahaemolyticus covering all the seasons of the year. Surprisingly no V.parahaemolyticus could be isolated from these samples.

Animal faeces of different animals in different localities at Parbhani were collected and processed and about 11 strains of V.parahaemolyticus were isolated from dog faeces only.

All the isolated strains were studied individually for their growth in varying concentrations of NaCl in peptone water from 0% to 10% in addition to other many biochemical characteristics. Haemolytic activity of individual strain was studied by using the human RBCs and sheep RBCs on blood agar plates. These results were confirmed by testing individual strains for haemolysis following Feeley and Pittman (1963) technique using the human and sheep RBCs. These strains were further studied for their haemolytic activity on a special blood agar, (Wagatsuma agar) known as Kanagawa phenomenon following Sakazaki et al. (1968) technique which determined whether the strains isolated were pathogenic or not.

Pathogenicity of all the strains of V.parahaemolyticus was investigated in

- 1) Mice
- 2) Guinea pigs
- 3) Infant rabbits and
- 4) Adult rabbits.

Mice, guinea pigs were injected intraperitoneally with 0.5 ml and 5.0 ml of overnight incubated culture respectively. Infant rabbits were injected intraintraintestinally with each strain isolated individually following Dutta and Habbu (1955) technique. In addition to this the reaction was observed in the test with the ligated rabbit intestine by using the method of De and Chatterjee (1953). Sensitivity to different antibiotics, chloromycetin, tetracycline, streptomycin, kanamycin, ampicillin and polymyxin B. was studied using individual strain following agar-diffusion technique using drug impregnated discs. (Bauer, et al. 1966).

The thesis is presented in four main Chapters:-

- Chapter I : Gives a general introduction including the history of the V.parahaemolyticus, of the experimental investigations and the review of literature.
- Chapter II : Dwells upon present problem covering the worldwide distribution and epidemiology caused by the V.parahaemolyticus.
- Chapter III : Presents the actual experimental work carried out : (a) Materials and Methods (b) Results and discussion. Each subsection will cover details on shellfish, oysters, crabs and prawns/shrimps subchapterwise. This includes also the detail studies on morphology biochemical reactions, haemolytic activity, sensitivity to antibiotics and pathogenicity in animals etc.
- Chapter IV : Deals with the isolation of V.parahaemolyticus from : (a) Stools/rectal swabs samples of gastroenteritis cases from Hospitals. (b) It gives also the findings on the animals faeces.

A glimpse into the work - General summary and conclusion.

V. parahaemolyticus has become a global problem as cases of gastroenteritis/food poisoning are attributed to it in different parts of the world. This is very rampant in Japan. Sea fish and fish products are proved to be vehicles that communicate V. parahaemolyticus and give rise to outbreaks of bacterial food poisoning.

The results of the present investigation into varieties of fish e.g. shellfish, oysters and prawns/shrimps show the presence of V. parahaemolyticus in the coast of Bombay. Crabs from this region also show ample presence of V. parahaemolyticus.

Surprisingly V. parahaemolyticus could not be isolated from human stool samples from cases of gastroenteritis despite meticulous investigations. This situation has also been observed in Germany by other workers though V. parahaemolyticus was detected in fish and sea water in the same region (Nakanishi H. et al. 1968).

Out of 200 faecal pooled samples tested from different animals from Parbhani 11 strains of V. parahaemolyticus could be isolated from dog faeces. Though it is a marine bacterium, it could be detected at a distant place like Parbhani and it is apparent that this organism can survive in hinterland too.

The strains isolated have manifested typical characteristics. It shows haemolysis on human blood agar and not on sheep blood agar. In animal pathogenicity tests it shows positive results in mice and guinea pigs both these animals die within 24 hours. The organism could be isolated from the site of injection. In the case of ligated loop in adult rabbits it gave rather weak positive as the loop was not

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fully distended with the fluid accumulation. So also, same results were observed in the infant rabbits injected intra-intestinally with V.parahaemolyticus.

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