STUDIES ON INCIDENCE, EFFECT OF PROCESSING AND SURVIVAL OF SELECTED SPECIES OF HALOPHILIC PATHOGENIC VIBRIOS IN SEAFOODS

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in

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by

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Certified that the thesis entitled **"Studies on incidence, effect of processing and survival of selected species of halophilic pathogenic vibrios in seafoods"** is a record of independent bonafide research work carried out by Ms. Smitha N.R. during the period of study from December 1999 to November 2005 under my supervision and guidance for the degree of Doctor of Philosophy and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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DECLARATION

I, Smitha N.R., hereby declare that the thesis entitled **"Studies on incidence, effect of processing and survival of selected species of halophilic pathogenic vibrios in seafoods"** is an authentic record of the research work done by me under the supervision of Dr. Sanjeev S., Principal Scientist, Central Institute of Fisheries Technology, Cochin and has not been previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title of any University or institution.

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DEDICATED TO MY PARENTS

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1. INTRODUCTION

Fish and fishery products have become not only a major source of protein for the ever-increasing human population but also a source of employment for millions and an item to generate immense wealth to nations. In the declaration on World Food Security adopted by the World Food Summit held in Rome in November 1996, fisheries was identified as an important sector to provide food security for the present and future generations.

The world fish production has reached 9,31,90654 mt (Anon, 2002). It is estimated that about 44 % of the world marine stock is heavily to fully exploited leading to the fear that world fisheries is on the brink of disaster (Mace, 1997). Some of the species are exploited to the maximum and some are over exploited indicating the need for resource conservation for sustainable fishery.

India is the eighth largest fish producer contributing a share of 4.0 % to the world fish production (Anon, 2002). The Indian subcontinent with a total land area of 3.3 million $km^2$and a coastline of 8041 km is blessed with marine wealth. India has the strategic advantage to exploit the marine resources compared to other countries bordering the Indian Ocean, and contributes about 45 % of the fish production from the Indian Ocean (Shahul, 1998). Exclusive Economic Zone (EEZ) of India covers an area of 2.02 million sq. km, with an estimated harvesting potential of 3.9 million t per annum (Upare, 2003). Of this, nearly 50 % is reported to be available in inshore areas and the balance in the offshore and the deep sea areas (Upare, 2003).

Export of marine products constitutes a major revenue earning source from agriculture products and it constitutes approximately 3.6 % of the total exports from India and 16 % of the total agricultural products (Upare, 2003). Export of fish and fishery products from India commenced in 1953 with the export of small consignment of frozen shrimp from Cochin. In those days, there were only 3 or 4 exporters in the field and volume of export was too small fetching foreign exchange less than 2 crores. At present there are over 400 exporters in the

country with the total value of exports crossing Rs.7,011 crores (Anon, 2002). Quality control and pre-shipment inspection system was instrumental for this phenomenal growth of the seafood industry. European Union and the United States of America made the quality assurance of food products very stringent and evolved quality assurance programmes based on Hazard Analysis Critical Control Point (HACCP) concept. The Government of India therefore reviewed the existing exporting inspection systems and introduced a new HACCP based quality assurance and monitoring systems (QAMS) for fresh, frozen and processed fish and fishery products with effect from August 21,1995 in line with the requirements of the major importing countries (Shrivastava, 1998).

Safety of seafood and consumer health is of paramount importance. There is a need to improve the quality and safety of seafood products in the international market as well as for domestic trade. Due to the stringent quality control measures adopted by the importing countries, quality of seafood meant for export need prime importance. Developed nations are very much concerned over the safety of food items which may contain pathogenic microorganisms, pesticide residues, harmful chemicals, heavy metals, biotoxins etc., causing health hazards.

Marine occurring vibrios in addition to *V. cholerae* have emerged as significant human pathogen in recent years and 11 species have been documented to cause disease by consumption of raw or improperly cooked seafood or via wounds acquired in the marine environment (Janda *et al.*, 1988). Presence of HPVs (HPVs) in seafood meant for export caused concern during the past two decades. Recently few consignments of frozen fish exported from this country suffered rejection due to the presence of HPVs , viz. *V. parahaemo-lyticus*. To meet the global requirements in seafood trade, it is necessary to produce safe and high quality products. With the implementation of EU directives (No. 91/493/EEC) and the US regulation it has become mandatory to monitor the levels of various hazards in seafoods.

Halophilic vibrios are one of the major groups of bacteria found in marine and estuarine environment. It is therefore to be expected that freshly harvested finfish

and shellfish from these environments might be harboring some of the pathogenic halophilic *Vibrio* spp. Vibrios may cause a variety of diseases including gastroenteritis, wound infections, ear infection and septicemia. A number of halophilic *Vibrio* spp. viz. *V. alginolyticus, V. cincinnatiensis, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii, V. parahaemolyticus and V. vulnificus* are recognized as human pathogens (Dalsgaard, 1998).

HPVs usually encounters different adverse conditions in its native or food-processing environment and the stresses resulting from these conditions may affect the survival of this pathogen. In this regard studies were conducted in which the isolates of HPVs species like *V. cincinnatiensis, V. fluvialis, V. furnissii* and *V. parahaemolyticus* were subjected to different stresses, viz., survival at different processing steps like washing, chilling, freezing, blanching and drying. Viability of the above organisms, when exposed to different concentrations of sodium chloride, chlorine, chlorine dioxide and pH were studied. Antibiotic sensitivity and haemolytic activity of these isolates were also tested.

It is expected that the data generated will be useful for improving the processing strategies and enlarging the vision on the concept for high quality, safe seafood n the domestic as well as international trade.

2. Review of literature

2.1. Incidence of HPVs in fish and fish products

Vibrio species are natural inhabitants of marine aquatic environments of both temperate and tropical regions, with most human infections acquired by exposure to such environments or to foods derived from them (Kelly *et al*, 1991, Colwell and Huq, 1994). Currently, 12 *Vibrio* species viz. *V. alginolyticus, V. carchariae, V. cholerae, V. cincinnatiensis, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii, V. mimicus, V. parahaemolyticus and V. vulnificus* are known to cause or to be associated with human infections. (Kelly *et al.*, 1991; Dalsgaard *et al.*, 1996,a). *Vibrio* spp. mainly associated with intestinal disease may represent health hazards when present in seafood meant for consumption, whereas extra intestinal disease, especially wound infections, can occur after exposure to the aquatic environments and handling of fish. Many *Vibrio* spp are pathogens to humans and have been implicated in food borne disease (Table.1).

Table 1. Association of *Vibrio* spp. with different clinical syndromes

Species	Gastro-enteritis	wound infection	Ear infection	Primary septicemia	Secondary septicemia
V. cholerae O1	+++	+			
V. cholerae non- O1	+++	++	+	+	+
V. mimicus	++		+		
V. fluvialis	++				
V.parahaemolyticus	+++	+	+		+
V. alginolyticus	(+)	++	++	+	
V. cincinnatiensis			+		
V. hollisae	++			+	
V. vulnificus	+	++		++	++
V. furnissii	(+)				
V. damsela		++			
V. metschnikovii	(+)			(+)	
V. carchariae		+			

+++ = frequently reported, ++ = less common (6- !00 reports); + = rare (1-5 reports), and (+) = association is unclear. Pavia *et al.* (1989).

Several authors have reported the incidence of HPVs in seafoods from different parts of the world. In India (Bandekar *et al.*, 1982; Karunasagar *et al.*, 1987; Prasad and Rao, 1994, a; Thampuran *et al.*, 1997; Sanjeev *et al.*, 2000; Deepanjali *et al.*, 2005), Bangladesh (Huq *et al.*, 1980), U.K (Lee *et al.*, 1981), Malaysia (Cann and Taylor, 1981; Elhadi *et al.*, 2004), Indonesia (Lesmana *et al.*, 2002), United states (Blake, 1984; Colwell, 1984; Depaola *et al.*, 1994; Wright *et al.*, 1996; Hlady, 1997; Hackney *et al.*, 1988; Gooch *et al.*, 2002), Japan (Sakazaki, 1983; Alam *et al.*, 2002), Senegal (Schandevyl *et al.*, 1984), Hong Kong (Chan *et al.*, 1989), Srilanka (Foneska and Widarapathirama, 1990), Philippines (Aklani-Rose *et al.*, 1990), Taiwan (Wong *et al.*, 1992, 1995 and 2000), China (Yano *et al.*, 2004), France (Hervio-Heath *et al.*, 2002) Netherlands (Veenstra, 1994), Spain (Sunen *et al.*, 1995; Arias *et al.*, 1999; Castro *et al.*, 2002), Iran (Hosseini *et al.*, 2004), Israel (Bisharat and Raz, 1996), Denmark (Dalsgaard, 1998), Italy (Ripabelli *et al.*, 1999; Baffone *et al.*, 2000; Maugeri *et al.*, 2000; Cavallo and Stabili, 2002; Parisi *et al.*,2004).

Incidence of HPVs from a variety of seafoods collected from fish markets (Sanjeev and Stephen, 1993; Sunen *et al.*, 1995; Elhadi *et al.*, 2004; Parisi *et al.*, 2004) as well as from factory processed raw, cooked, peeled, packed and frozen products (Cann and Taylor, 1981; Sanjeev *et al.*, 2000) have been reported. These include those foods that may be consumed raw (cuttlefish, oysters), partially cooked (steamer clams, mussels) or fully cooked (finfish and shellfish) (Buck, 1991). Occurrence of these species from raw samples collected at sea from commercial vessels (Cann and Taylor, 1981; Jaksic *et al.*, 2002) and also from farmed mussels, shrimps and clams (Maugeri *et al.*, 2000; Bhaskar *et al.*, 1998; Sanjeev, 1999; Castro *et al.*, 2002) has also been reported.

V. parahaemolyticus, V.vulnificus, V. damsela and *V. alginolyticus* may also be fish pathogens (Ruangpan and Kitao, 1991; Liu *et al.*, 2000; Lee *et al.*, 2003). Anderson *et al.* (1988) reported that vibriosis caused 70-95 % reduction in the expected harvests in some farms in Malaysia. *Vibrio spp.* have been isolated from a variety of environmental samples including water, sediment and plankton (Colwell, 1984; Ayres and Barrow, 1978; Aiyamperumal *et al.*, 1994; Montanari *et al.*, 1999; Hervio-Heath *et al.*, 2002). It has been reported that vibrios are the

predominant bacteria in the digestive tracts of oysters, clams, mussels (Sugita *et al.*, 1981; Kueh and Chan, 1985), Prawn (Yasuda and Kitao, 1980; Oxley *et al.*, 2002) and fish (Okuzumi and Horie, 1968, Sera and Ishida, 1972, Depaola *et al.*, 1994).

Vibrio spp. have also been isolated from water showing a broad range of salinities and varying pH values (Dalsgaard, 1998). He reported that a positive correlation exists between water temperature and the number of human pathogenic vibrios isolated as well as the number of reported infection. Such seasonality is particularly noted for *V.parahaemolyticus* and *V. vulnificus* (Oliver and Kaper, 1997; Alam *et al.*, 2002). Due to the halophilic nature and marine source of *Vibrio spp* raw seafood is naturally contaminated and is the main food responsible for infection (Desmarchelier, 2003).

V. alginolyticus, V.cincinnatiensis, V. damsela, V. fluvialis, V. furnissii, V. metschnikovii, V. parahaemolyticus and *V. vulnificus* were the major species isolated during the study. *V.cincinnatiensis V. fluvialis, V. furnissii* and *V. parahaemolyticus* were selected for detailed study for which a detailed review is provided.

2.2. *V. cincinnatiensis*

V.cincinnatiensis, Latin adjective derived from the society of Cincinnati from which the city of Cincinnati, Ohio, was named (Brayton *et al.*, 1986). The organism described was isolated from a 70-year-old male patient with bacteremia and meningitis at the University of Cincinnati hospital (Bode *et al.*, 1986). He had a 24 h. history of lethargy, disorientation, and altered mental status. There was no history of diarrhea, rashes, exposure to seafood or contact with salt water. Although the patient drank alcohol heavily on occasion, he had no liver disease. Physical examination revealed a temperature of 103°F (39.4°C). Laboratory data reported normal hepatic enzymes, leukocytes of 13,200 cells per mm^3, hemoglobin of 14.5 gd l^{-1}, and a platelet count of 194,000 mm^{-3}. Blood and cerebrospinal cultures were inoculated into blood agar plates, and pure cultures of *V. cincinnatiensis* grew from both samples. This was the first reported case of *Vibrio sp* meningitis. Therapy was begun with ampicillin (day 1) and continued with moxalactam for the next 9 days. Recovery was uneventful, representing the

first successful treatment of *Vibrio sp.* meningitis in an adult (Bode *et al.*, 1986). Wuthe *et al.* (1993) have reported the isolation of one strain of *V. cincinnatiensis* from the stool specimen of an immunocompromised elder patient suffering from enteritis and two strains from the rennin stomachs of aborted bovine fetuses.

V.cincinnatiensis is a gram-negative non-sporforming rod, measuring approximately 0.7 by 2.0 μm. Overnight incubation at 25°C and 35°C produces round, smooth, glossy colonies (1 to 2 mm in diameter) that are cream colour on nutrient agar and yellow on thiosulphate citrate bile salts sucrose agar. Single polar flagella are observed attached to cells grown on solid and liquid media (Brayton *et al.*, 1986).

Facultatively anaerobic, sodium chloride is required for growth, ferments glucose, trehalose, sucrose, D-cellobiose, D-manose, m-inositol, salicin, and L-arabinose. Catalase, oxidase, amylase, chitinase and DNase are produced. Gelatinase, urease, alginase, caseinase, lecithinase and elastase are not produced. Positive for lysine decarboxylase, Ortho-nitrophenyl-β-d-galactopyranoside and Voges–Proskauer. Negative for ornithine decarboxylase, arginine dihydrolase and indole production. Sensitive to 150 μg of vibriostatic agent 0/129 (2, 4- diamino 6, 7- di isopropyl pteridine). The DNA base composition is 45 mol % guanine + cytosine (Brayton *et al.*, 1986).

In a study on the phenogram Brayton *et al.* (1986) showed that *V.cincinnatiensis* possessed closest relationships, i.e. > 70 % similarity with *V.diazotrophicus* and *V.nereis.* All three organisms required NaCl for growth, were positive for cytochrome oxidase, reduced nitrate, fermented sucrose, trehalose, and cellobiose, and were sensitive to 150 μ g of 0/129. All were gelatinase negative.

According to MacDonnell and Colwell (1985), the nucleotide base sequence of the 5sr RNA of *V.cincinnatiensis* shares a recent common ancestor with *V. gazogenes* (98.3 % sequence homology), which in turn shares a common ancestory with *V.mimicus, V.fluvialis* and *V.metschnikovii.*

Information on the incidence of *V.cincinnatiensis* in sefood is scanty, although there are reports on the isolation of this species from seafoods, coastal waters and zooplanktons. Ripabelli *et al.* (1999) studied the bacterial pathogens in

mussels (*Mytilus galloprovincialis)*, and showed that 48.4 % of samples contained vibrio pathogens and *V.cincinnatiensis* was isolated from 3.2 % of the samples analyzed. Cavallo and Stabili (2002) observed a selective retention of HPVs viz., *V.cincinnatiensis*, *V. hollisae* and *V.vulnificus* in mussels (*Mytilus galloprovincialis*) from the Mar Piccolo of Taranto (Ionian sea, Italy). *V.cincinnatiensis* was the dominant species isolated from frozen fish products meant for export from Kerala and Tamil Nadu (Sanjeev *et al.*, 2000). Occurrence of *V.cincinnatiensis* in coastal waters of Cochin has been reported by Thampuran *et al.* (1997). Heidelberg *et al.* (2002 & 2002, a) indicated the occurrence of *V.cincinnatiensis* in association with zooplanktons, in the water samples collected from the chop tank river in Chesapeake Bay. Heidelberg *et al.* (2002, a) in another study observed the abundance of *V.cincinnatiensis* during cooler months although the species accounted for less than 0.1 to 3 % in the water samples collected from the chop tank river in Chesapeake Bay. Mao *et al.* (2001) have reported the isolation *V.cincinnatiensis* from diseased mud crabs with different symptoms, cultured in marine ponds of various districts in Ningbo area.

2.3. *V. fluvialis*

Vibrio fluvialis was first identified in 1975 in Bahrain in a patient with diarrhea, and was initially designated as group F vibrios (Furniss *et al.*, 1977). In 1980, the Center for Disease Control renamed the organism as group EF6 (Huq *et al.*, 1980). This organism was responsible for an epidemic involving more than 500 patients in Bangladesh (Huq *et al.*, 1980) and has also caused diarrheal disease especially in Baharin, Bangladesh and Indonesia (Furniss *et al.*, 1977; Joseph *et al.*, 1983). This organism can be misidentified as *Aeromonas* because of similar biochemical reactions in identification scheme and with *V.alginolyticus*, especially because of its tolerance to 8 to 10 % NaCl concentration (Joseph *et al.*, 1978; Furniss *et al.*, 1977; Seidler *et al.*, 1980; Lee *et al.*, 1981)

Lee *et al.* (1981) have done a detailed study on the taxonomy of *V.fluvialis*. Earlier these organisms were frequently isolated from the estuarine environments and were referred to as '*marine-aeromanads*' but later designated them group F (Furniss *et al.*, 1977). A numerical taxonomical study of *Vibrio metschnikovi* and related organisms demonstrated that group F strains formed a distinct phenon

and probably constituted a new species or genus (Lee *et al.*, 1978). Phenotypicaly these organisms appeared to be intermediate between *Aeromonas* and certain species of vibrios, such as *V. anguillarum*. In another study Lee *et al.* (1978) have shown that the minimum inhibitory concentration (MIC) of 2, 4-diamino – 6-7-diisopropyl pteridine (0/129) phosphate for group F organisms was 10-50 μg ml^{-1}. This is similar to that of some species of the genus Vibrio but different from that of strains of the genus Aeromonas which have a MIC ≥ 320 μg 0/129 phosphate $ml^{-1}$and the species *V. anguillarum* which has an MIC of 1-5 μg 0/129 phosphate ml^{-1}(Lee *et al.*, 1978).

Phenon 1, described by Lee *et al.* (1981) corresponds to strains designated group F by Lee *et al.* (1978) and includes strains described as group F strains isolated in 1975 from a patient whose diarrhea was contracted in Bahrain. Mol % (G+C) studies of phenon I strains have shown that it forms two sub clusters (1 a and 1 b) or sub phenons (Lee *et al.*, 1981). The values were found to be in the range 49.3-50.6 mol % (G+C) with a mean of 50.0 and concluded that sub-phenons 1 a and 1 b are biovars of a single species. Jensen *et al.* (1980) have obtained similar values for a number of group F strains in the range of 50.5 - 51.0 mol % (G + C). Group EF6 strains have a mol % G+C of 50 and DNA relatedness tests indicate that all the EF6 strains tested belong to a single species (Brenner *et al.*, 1979). Jensen *et al.* (1980) in another study examined strains of both groups and confirmed that they are synonyms. Lee *et al.* (1981) proposed the inclusion of Phenon 1 in the genus *Vibrio* and given the name *V. fluvials* (belonging to a river) and this would require modification of the genus definition to include aerogenic strains.

Vibrio fluvialis [synonyms group F (Furniss *et al.*, 1977), group EF6 (Huq *et al.*, 1980)] is gram negative short rods, axis straight or curved, sides usually parallel, rods rounded, occurring singly, in pairs and occasionally in short chains of 3 to 4 organisms, may be pleomorphic. Motile by means of single polar sheathed flagellum in liquid media. On solid media lateral, unsheathed flagella of shorter wavelength may be produced. Sodium chloride may be required for growth and the optimum concentration for growth is 1-3 % (w/v). Colonies on TCBS agar are yellow, shiny smooth round, domed and entire may be mucoid and are 2-3 mm in

diameter after 24 h at 37 ± °C. Pigments not produced, facultative anaerobe, metabolism of glucose is positive, gas may be produced. Kovacs oxidase positive, reduce nitrate to nitrite. Grow on simple mineral media on a variety of organic carbon sources.

The species may be divided into biovar I and II. Biovar I strains are widely distributed through out the aquatic environments particularly in brackish and estuarine waters but other sources have included shellfish and sewage. Biovar II strains occur in the same aquatic habitat as biovar I but unlike them they are rarely, if ever, isolated from human faeces. They have, however, been isolated from faeces of cattle, pigs and rabbits (Lee *et al.*, 1981).

V. fluvialis has been reported as the etiologic agent in diarrheal illness in Asia, the Middle East, Africa, Eastern Europe, Great Britain, and United States (Huq *et al.*, 1980; Tacket et *al.*, 1982; Bellet *et al.*, 1989; Hodge *et al.*, 1995). Since 1981, 14 cases of enterocolitis associated with *Vibrio fluvialis* have been reported in the United States (Kolb *et al.*, 1997) two of these cases occurred in infants (Bellet *et al.*, 1989; Hickman *et al.*, 1984) and 11 of 14 occurred in Florida (Klontz and Desenclos, 1990). 10 of these 14 patients reported eaten shellfish 1 to 7 days before onset of symptoms (Hodge *et al.*, 1995; Klontz *et al.*, 1994; Klontz and Desenclos, 1990).

The largest experience with *V. fluvialis* infection was reported by Huq *et al.* (1980) in Bangladesh and involved more than 500 patients, half of whom were young children under 5 years of age, between October 1976 and November 1977. The clinical syndrome described in that study included diarrhea (100 %), vomiting (97 %), abdominal pain (75 %), moderate to severe dehydration (67 %), and fever (35 %), in 75 % patients, blood and leukocytes were found on microscopic examination of stools. According to Kolb *et al.*, 1997, *V. fluvialis* should be included among potential bacterial pathogens causing severe gastroenteritis in infants and known exposures to seafood or coastal waters is not a pre-requisite to *V. fluvialis* infections especially in infants. Hickman *et al.* (1984) reported a similar unremarkable exposure history in a case of *V. fluvialis* in a one-month-old female infant with bloody stools. Bellet *et al.* (1989) isolated *V. fluvialis* from a four-week-old female infant with diarrhea, exposure history was significant

for the mother's consumption of crabs on the day of labor. *V. fluvialis* rarely causes wound infections or primary septicemia (Hlady and Klontz, 1996; Varghese *et al.*, 1996) although the species has been isolated from wound infection in Hawaii (Seidler *et al.*, 1980).

V. fluvialis has been frequently isolated from brackish and marine waters and sediments in the United States (Joseph *et al.*, 1983). From shellfish and water in the Pacific Northwest, Hickman *et al.* (1984), Klontz and Desenclos (1990), Klontz *et al.* (1994), Hodge *et al.* (1995) reported the isolation of *V. fluvialis* in New York Harbor, nearby sewage dumpsites and from shellfish in Louisiana. Thampuran *et al.* (1997) have reported the occurrence of *V. fluvialis* in coastal waters of Cochin (India). Maugeri *et al.* (2000) have observed that *V. fluvialis* was the most frequently recovered species in water and mussel samples collected from two brackish lakes, used as mussel farms in Sicily (Italy).

Sunen *et al.* (1995) have reported the incidence of *V. fluvialis* in 2.04 % of mussels and 13.8 % of clams purchased from retail outlets in the North of Spain. Sanjeev *et al.* (2000) have observed the incidence of *V. fluvialis* in 2.09 % of frozen fish products meant for export. *V. fluvialis I* and *V. fluvialis II* were isolated from seafood and aquacultured food available in Taiwan (Wong *et al.*, 1992). *V fluvialis I* was the major species found in oysters and clams and showed an incidence of 68.8 % and 78.6 % respectively (Wong *et al.*, 1992). Matte *et al.* (1994) reported the incidence of *V. fluvialis* in oysters (*Crassostrea gigas*) (27 %) originating from the southern coast of the state of Sao Paulo-Brazil. Most probable number (MPN 10^{-2}) obtained for the species was 3-150 (Matte *et al.*, 1994).

Venkateswaran *et al.* (1989) have isolated *V. fluvialis* from surface waters and sediment samples of the freshwater Ohta river. Gianelli *et al.* (1984) have reported the occurrence of *V. fluvialis* in shellfish in shores of the Adriatic Sea or purchased from retail shops. This was the first reported incidence of *V. fluvialis* in fishery products of the Mediterranean area.

Incidence of *V. fluvialis* in seawater environment has been reported by Rodriquez and Hofer (1986) in Brazil, Shinoda *et al.* (1985) in Japan and Schandevyl *et al.* (1984) from Senegal. *V. fluvialis* was found positive for chitinase and chitobiase

activities (Osawa and Koga, 1995). *V. fluvialis* was the most frequently isolated *Vibrio* species from shellfish bred in nurseries located in the Ebro river delta (Montilla *et al.*, 1994).

Chang *et al.* (2001) isolated a strain of *V. fluvialis* from the diseased *Paraliethys olivaceies* supplied by fish farm of Xunshan fisheries Group Company of Reng cheng city in July 1988. Six strains of *V. fluvialis* were isolated from diseased black tiger shrimp, *Penaeus monodon* Fabricius (Ruangpan and Kitao, 1991). Engelbrecht *et al.* (1996) found *V.fluvialis* as potential active spoilers on fresh cape Hake and other south Atlantic fish species (Kingklip, Monk, Angel fish and Gurnard). *V. fluvialis* was the predominant microorganisms isolated from the haemolynph of diseased American Lobsters (*Hemarus americanus*) harvested from the Atlantic coastal waters (Tall *et al.*, 1999). It was reported that *V. fluvialis* was responsible for a mysterious disease that has killed 1000 of Maine and New Brunswick lobsters during the past years (Anon, 1999). Guner *et al.* (1997) have reported the isolation of *V. fluvialis* from fresh potatoes .

2.4. *V. furnissii*

The biogroup 2 strains of *V. fluvialis* that produced gas from the fermentation of carbohydrates were named *V. furnissii* by Brenner *et al.* (1983). The separation of *V. furnissii* from *V. fluvialis* was supported by studies of DNA relatedness (Brenner *et al.*, 1983).

Strains of the organism now classified as *V. fluvialis* were first described by Furniss *et al.* in 1977. These organisms, designated group F, were isolated in 1975 from a patient with diarrhea in Bharain, from patients with diarrhea in Bangladesh and from shellfish and estuarine waters in England. Group F required salt and have a number of properties compatible with or mid way between those of vibrios and aeromonads. In a numerical taxonomy study Lee *et al.* (1978) showed that group F strains were a distinct phenon that probably represented a new species, and this group contained two subgroup on the basis of gas production during fermentation of glucose.

Huq *et al.* (1980) studied large number of strains associated with an outbreak of diarrhea in Bangladesh as well as strains isolated from patients with diarrhea in

Indonesia, strains from sewage in Brazil and US strains that had been called group EF-6 in the special bacterial reference activity at the centers for disease control. By both phenotypic tests and DNA relatedness, they found that the organism was closer to the genus *Vibrio* than to the genus *Aeromonas*. All of their strains produced no gas from the fermentation of glucose (were anerogenic) and formed a single DNA relatedness group. Thus, the EF6 group appeared to be identical to group F.

Group F strains isolated from several parts of the world were compared phenotypically and genetically by Seidler *et al.* (1980). They confirmed and extended the observation that group F was more closely related to *Vibrio* than to *Aeromonas*. They further showed that the aerogenic group F strains were in a different DNA relatedness group from the anaerogenic strains, and they recommenced that the two biogroups be considered as two separate species with in the genus *Vibrio*.

The genetic and phenotypic tests indicate that the aerogenic strains formerly included in *V.fluvialis* represent a new species in the genus *Vibrio* and Brenner *et al.* (1983) proposed the name *V.furnissii* for the new species in the genus *Vibrio*, in honour of A.L Furniss, Maidstone Public Health Laboratory, Maidstone, England, for his role in the classification of *V.fluvialis* and for his many contributions to the knowledge of the genus *Vibrio* (Brenner *et al.*, 1983).

V.furnissii is a gram negative, straight to slightly curved rod that is motile by means of polar flagella. It is NaCl requiring, oxidase positive, nitrate positive organism that ferments D – glucose and other carbohydrates with the production of acid and gas, has 50 mol % guanine + cytosine in its DNA (Brenner *et al.*, 1983).

V. furnissii has been isolated from river, estuarine water, marine molluscs and crustacean throughout the world (Oliver and Kaper, 1995). Matte *et al.* (1994) have reported 19 % incidence of *V. furnissii* in oysters (*Crassostrea gigas*) 19 % originating from the southern coast of the state of Sao Paulo-Brazil. Wong *et al.* (1992) found a relatively small percentage (7 to 12 %) of the oysters, clams, shrimps and crabs they examined. Thampuran *et al.* (1997) have reported the incidence of *V.furnissii* in the intestinal contents of fish collected from Cochin and

the percentage varied from 25.9 to 32.9 %. Sanjeev *et al.* (2000) indicated the occurrence of *V.furnissii* in frozen fish and fish products (1.05 %) collected from processing factories situated in Kerala and Tamil Nadu meant for export.

The largest documented outbreaks of *V.furnissii* were reported in 1969, when this species was isolated during an investigation of two outbreaks of acute gastroenteritis in American tourists returning from the Orient (Anon, 1969). In the first outbreak 23 of 42 elderly passengers returning from Tokyo developed gastroenteritis, one woman died and two other persons required hospitalization. Food histories implicated shrimp and crab salad and or the cocktail sauce served with the salads. *V.furnissii* was recovered from seven stool specimens. The second outbreak affected 24 of 59 persons returning from Hong Kong (Anon, 1969). Nine persons were hospitalized. Food vehicle was not identified, but *V. furnissii* was isolated from at least five fecal specimens. In 1994, during a cholera surveillance program in Peru, *V.furnissii* was isolated from 14 patients, 6 with diarrhea and 8 without symptoms (Dalsgaard *et al.*, 1997). Magalhaes *et al.* (1993) isolated sixteen strains of *Vibrio furnissii* from 16 Brazilian patients with diarrhea. Lesmana *et al.* (2002) have reported the isolation of small numbers of *V.furnissii* strains along with *V. parahaemolyticus* and *V.fluvialis* from patients with acute diarrhea in North Jakarta, Indonesia. Although *V.furnissii* had been isolated from dirraheal patients, its role as an enteric pathogen still remains unclear.

Symptoms described by Brenner *et al.* (1983) for the gastroenteritis outbreaks described above included diarrhea (91 to 100 %), abdominal cramps (79 to 100%), nausea (65 to 89 %) and vomiting (39 to 78 %). There were no reports of fever onset of symptoms occurred between 5 and 20 h with the patients recovering within 24 h. Neither the infectious dose of *V.furnissii* nor the susceptible population is known.

Vibrio furnissii is also pathogenic to fishes. Esteve *et al.* (1995) reported for the first time the isolation of *V.furnissii* strains from a European eel culture system, which are pathogenic to eels (*Anguilla anguilla*). Ahsan *et al.* (1992) isolated 14 strains of *V. furnissii* from different ulcerated areas of eel. Their observation clearly establishes the enterotoxicity of these organisms. Sung *et al.* (2001)

isolated *V.furnissii* from cultured tiger shrimp (*Penaeus monodon*) and pond water. Studies of Cantoni *et al.* (2001) have shown the incidence of *V.furnissii* in brined vegetable (*Ocimum basilicum).*

2.5. *V. parahaemolyticus*

Vibrio parahemolyticus as it is now known was first isolated by Fuijino *et al*, in 1951 and designated *Pasteurella parahaemolytica*. The organism caused gastroenteritis in 272 persons resulting in 20 deaths in Osaka, Japan. The fry of sardine boiled in salt water and sold and eaten in the half dried/fried state (Shirasu) was the contaminated food product eaten by all who had acute gastro enteritis. Halophilic nature of this organism was first indicated by Takikawa (1958) and classified the species as *Pseudomonas enteritis*. Miyamoto *et al.* (1961) noted the serological differences between this organism and *Pseudomonas* and proposed the generic name *Oceanomonas*.

Sakazaki *et al.* (1963) were the first to present a detailed description of *V.parahaemolyticus,* based on the differences of growth in peptone water containing 7 and 10 % NaCl, Voges- Proskauer reaction and fermentation of sucrose, arabinose and cellobiose. They recognized three subgroups, subgroup 1 and 2 were designated as *V. parahaemolyticus* and subgroup 3 resembled *V. anguillarum*, which did not grow in 7 or 10 % NaCl. Subgroup 2 grew in 7 and 10 % NaCl, whereas subgroup 1 grew in 7 % NaCl only.

Zen-Yoji *et al.* (1965) confirmed the differences between sub group 1 and 2 and reported the differences in pathogenicity between the two groups. Sub group 1 was isolated frequently from patients with unidentifiable enteritis and subgroup 2 was not pathogenic to man. Sakazaki (1968, a) reexamined 100 cultures of each subgroup and confirmed the results reported by Zen-Yoji *et al.* (1965). Cultures of subgroup 2 grew in 10 % NaCl, fermented sucrose and produced acetoin, whereas those of subgroup 1 did not. Because of these differences, he proposed the specific name *alginolyticus* for subgroup 2 (biotype 2).The organism of subgroup 1(biotype 1) continued to be classified as *V.parahaemolyticus.*

Morphologically *V.parahaemolyticus* is gram-negative rods exhibiting pleomorphism. Slight curved, straight, coccid and swollen forms can be

observed. All strains of *V. parahaemolyticus* are motile by means of a single polar flagellum. In broth cultures, the vibrio has a single polar flagellum but on the surface of nutrient agar young cultures may possess peritichous flagella. On agar plates most cultures appear as smooth, moist, circular, opaque colonies with entire edges. Rough variants have been reported in pure cultures by Tewedt *et al.* (1969). A swarming phenomenon occurs in some instances, when low concentrations of agar are used. This diminishes with increased concentration of agar.

More than five decades have passed since the first report by Fujino *et al.* (1951) that implicated *Vibrio parahaemolyticus* as the cause of an outbreak of seafood poisoning. Since then voluminous literature has accumulated from the investigators worldwide.

V. parahaemolyticus inhabits the marine and brackishwater environments and it is therefore associated with fishes harvested from these environments. Although this halophilic organism was first isolated more than 50 years ago, it has remained practically unknown elsewhere for sometime. At first it was thought to be limited to Japan and Far East. But during the last 30-40years, it has been isolated from different species of fish, shellfish and marine environments such as bottom sediments and plankton. This has been reported from many countries viz. Brazil (Matte *et al.*, 1994), Italy (Maugeri *et al.* (2000), USA (Florida) (Hlady, 1997; Ellison *et al.*, 2001), USA (Liston, 1973), U.K. (Barrow, 1974), Hong Kong (Chan *et al.* (1989), Phillippines, Taiwan, HongKong , Singapore and Japan (Sakazaki, 1969; Wong *et al.*, 2000 and Alam *et al.*, 2002), Panama, West Africa and Indonesia (Beuchat, 1977; Lesmana *et al.*, 2002), Malaysia (Cann and Taylor, 1981; Elhadi *et al.*, 2004) and in India (Chatterjee *et al.*, 1970; Chatterjee and Neogy, 1972; Nair *et al.*, 1975, Victor and Fred. 1976; Natarajan *et al.*, 1979; Lall *et al.*, 1979; Nair *et al.*, 1980; Karunasagar and Mohankumar, 1980; Pradeep and Lakshmanaperumalasamy, 1984; Sanjeev and Iyer, 1986; Sanjeev and Stephen, 1993; Karunasagar *et al.*, 1990; Prasad and Rao, 1994,a; Thampuran *et al.*, 1997; Sanjeev *at el.*, 2000, Deepanjali *et al.*, 2005). The earlier work on *V.parhaemolyticus* has been extensively reviewed by Sakazaki (1969), Lee (1973), Liston (1973), Sakazaki (1973), Barrow (1974) and Joseph *et al.* (1983).

In 1973 an international conference on *V. parahaemolyticus* was held in Tokyo (Anon, 1974).

De *et al.* (1977) showed the incidence of *V.parahaemolyticus* in marine fishes of Calcutta to be 35.2 %. Natarajan *et al.* (1979) reported 36.8 % occurrence in fishes from brackishwater environments. Karunasagar and Mohankumar (1980) found that the incidence varied from 8.33 to 33.3 %. The studies of Nair *et al.* (1980) revealed that 35.6 % of the freshly harvested fishes from the estuarine waters, 40.6 % fishes of mangroves, 37.5 % of freshly caught brackishwater fishes and 44 % fishes from market showed the incidence of *V.parahaemolyticus*. Sanjeev and Iyer (1986) reported the occurrence of *V.parahaemolyticus* in 55.9 % of the market fish samples and 2 out of 15 cooked clam meat samples. Sanjeev and Stephen (1993) showed the incidence of *V.parahaemolyticus* in marine fresh finfish and shellfish varied from 67 to 92 %, whereas in fish products it was less (3.69 to 30.23 %). Sanjeev and Stephen (1993), Karunasagar *et al.* (1990) reported that *V.parahaemolyticus* was the most commonly encountered halophilic pathogenic vibrio in market samples of fish and shellfish and showed 69 % incidence of the organism. Prasad and Rao (1994, a) have reported the incidence of *Vibrio parahaemolyticus* from fresh and frozen prawns and fishes of Kakinada coast. Thampuran *et al.* (1997) were able to isolate *Vibrio parahaemolyticus* in coastal waters and fishes of Cochin. Deepanjali *et al.* (2005) in a study of the oysters along the south west coast of India, detected vibrios in 93.87 % of the samples, and the densities ranged from <10 to 10^4 organisms per gram. They could also detect pathogenic *V.parahaemolyticus* from 10.2 % of the samples. Sanjeev *et al.* (2000) reported 9.42 % occurrence in fish products collected from processing factories situated in Kerala and Tamil Nadu meant for export.

In a study on halophilic vibrios in seafood from Hong Kong markets Chan *et al.* (1989) reported that bivalve shellfish were more frequently and more heavily contaminated with vibrios and in particular with *V.parahaemolyticus* and *V.alginolyticus*. [Mussels ($4.6x10^4g^{-1}$), oysters ($3.4x10^4g^{-1}$) and clams ($6.5x10^3g^{-1}$)]. This differs from the observation of Molitoris *et al.* (1985) who found mackerel, shrimps and squids to be the most frequently contaminated with *V.parahaemolyticus* and *V.alginolyticus* in Indonesia. Elhadi *et al.* (2004) in a

survey of seafood markets and super markets reported the incidence of *V. parahaemolyticus* in 4.7 % of the samples consisting of shrimp, squid, crab, cockles and mussels. Matte *et al.* (1994) analyzed vibrio species in oysters (*Crassortrea gigas*) originating from the southern coast of the state of Sao Paulo Brazil. Most Probable Number (MPN 10^{-2}g) obtained for *V.parahaemolyticus* was <3-1, 200 and showed an incidence of 77 %.

Wong *et al.* (1995) isolated *V.parahaemolyticus* from 36.0 % frozen raw or semi prepared seafoods such as peeled shrimp, fish and shrimp dumplings. Sunen *et al.* (1995) reported 30.23 % incidence of *V.parahaemolyticus* in samples consisting mussels and clams purchased from retail outlets in Spain. Hase *et al.* (1997) have shown the incidence of *V. parahaemolyticus* in 21.1 % of raw seafood samples and 23.3 % of environmental samples collected from Osaka (Japan) seafood market. Baffone *et al.* (2000) have reported 14.8 % incidence of *V. parahaemolyticus* in fresh seafood products in Italy. Jaksic *et al.* (2002) in a study of seafood samples collected along the sea side in Croatia reported 9.40 % incidence of *V. parahaemolyticus*. Wong *et al.* (1992) isolated *V.parahaemolyticus* from 22.8 % freshwater clams in Taiwan. Joseph *et al.* (1983), Sarkar *et al.* (1985), Venkateswaran *et al.* (1989) have also isolated *Vibrio parahaemolyticus* from freshwater samples of India and Japan. Rashid *et al.* (1992) have reported the incidence of *V.parahaemolyticus* in frozen shrimps imported from South East Asia and Mexico.

Aiyamperumal *et al.* (1994) reported the occurrence of *V.parahaemolyticus* in 14.2 % of finfish, 14.5 % of prawns, 23.8 % of crabs and 34.7 % of bivalves of coastal waters of Tuticorin. Sanjeev and Stephen (1993) have shown that densities of *V.parahaemolyticus* in estuarine shellfish were found to be much higher compared with shellfish from the Arabian Sea. This is in agreement with the findings of Nair (1985) with respect to fish from the Bay of Bengal. Similar results were reported by Kaneko and Colwell (1974), Vargu and Heritle (1975).

In man *V.parahaemolyticus* usually causes diarrhea, occasionally dysentery like or gastroenteritis of sudden onset varying from mild to severe. The mortality rate is less than 10 %. There is very little information regarding the dose response in the 5-9 $\log_{10}$ region (FDA CFSAN, 2000). *V.parahaemolyticus* and closely related

organisms have also been isolated occasionally from infected skin or tissue lesions in bathers and fish handlers (Roland, 1970).

In an outbreak in US *V.parahaemolyticus* infections comprisied 59 % with gastroenteritis, 8 % with septicaemia and 34 % with wound infections (Daniels *et al.*, 2000, a). Infections in outbreaks resulted in diarrhea being the most common symptoms, often associated with abdominal cramps, nausea and vomiting (Daniels *et al.*, 2000, a). It is a self-limiting infection generally lasting only for a few days with little evidence of spread of the infection from one person to another (Barrow and Miller, 1976). Tamura *et al.* (1993) have reported an isolated case of reactive arthritis in Japan following *V. parahaemolyticus* infection. A death due to *V. parahaemolyticus* infection following consumption of oysters was reported in New South Wales in 1992 (Kraa, 1995).

There is a link between contamination of seafoods and sea temperature with 89 % of oysters associated with disease coming from waters >22°C (Daniels *et al.*, 2000). Numbers of *V.parahaemolyticus* are higher in seafoods harvested when the water is warmer (DePaola *et al.*, 1990). Cook *et al.* (2002) in a study observed that geometric means for *V. parahaemolyticus* in oysters harvested from the Gulf of Mexico in one study were 7.2 MPN g^{-1} in winter, 1,330 in Spring, 5,150 in Summer and 500 in the Autumn (Cook *et al.*, 2002).

The organism has been isolated frequently from cases of food poisoning accounting for about 40-60 % of all cases of bacterial food poisoning in Japan (Honda and Lida, 1993). In a survey of vibrio infections associated with raw oyster consumption in Florida during the period 1981-1994, *V.parahaemolyticus* was found to be most often identified vibrio species in patients (29 %) with gastroenteritis (Hlady,1997). It is one of the most important causative agents of food poisoning in Japan and was considered a local problem until recently, but it has now been recognized in many countries especially in South East Asia (Chatterjee *et al.*, 1970, Sakazaki *et al.*, 1974). These workers reported the isolation of *V.parahaemolyticus* from up to 15 % patients with diarrhea in Calcutta.

The vibrios have also been isolated from infection of the hands and feets, eyes and ears of the person who have been in contact with marine shore areas. The

disease caused by *V. parahaemolyticus* infection is more prevalent in countries that consume large quantities of seafood and that frequently consume raw seafood (e.g. Japan). Reports on outbreak of infection due to *V.parahaemolyticus* are available from different parts of the world. From USA (Summer *et al.*, 1971; Hlady and Klontz, 1996; Olsen *et al.*, 2000; Daniels *et al.*, 2000, a; CDC, 2001), Japan and Taiwan (Lee *et al.*, 2001), Australia (Batley *et al.*, 1970; Davey,1985; Kraa, 1995), Sweden (Lindqvist *et al.*, 2000); Thailand (Tangkanakul *et al.*, 2000) and UK (Scoging, 1991) Nigeria (Chigbu and Iroegbu, 2000) and Indonesia (Lesmana *et al.* 2002).

V. parahaemolyticus, Kanagawa phenomenon positive, are those mostly associated with disease. This phenomenon represents the production of a thermostable direct haemolysin (TDH). In a study of clinical, marine and shellfish isolates Yam *et al.* (2000) found that all of the 38 clinical isolates were TDH/KP^{+}ve, while only 0.85 of coastal water and 2.5 % of shellfish isolates were TDH/KP^{+}ve. Their suggestion was that it is more important to determine the TDH/KP status of *V.parahaemolyticus* present in foods than it is to enumerate them. Of 115 Japanese clinicial isolates 7 % possessed (TDH and/or the TDH-related haemolysin and urease) all three genes (Lida *et al.*, 1998).

Matsumoto *et al.* (2000) have reported the emergence of a new serotype O3: K6 described as a pandemic clone was first appeared in Bangladesh. This clone posses a high infection frequency and capacity to spread globally (Matsumoto *et al.*, 2000; Daniels *et al.*, 2000, a and Wong *et al.*, 2005). From India Deepanjali *et al.* (2005) have reported the incidence of the pandemic strain from oysters along the south west coast of India. It was also shown that this serotype exhibits increased adherence and cytotoxicity in tissue culture, and this may contribute to the enhanced pathogenic potential of strains of this serotype (Yeung *et al.*, 2002).

2.6. *V. alginolyticus*

V. alginolyticus is a halophilic vibrio first recognized as being pathogenic in humans in 1973 (Zen-Yoji *et al.*, 1973). *V. alginolyticus* was originally classified as biotype 2 of *V. parahaemolyticus* was reclassified as a separate species by Sakazaki (1968, a). The ecological niche occupied by *V. alginolyticus* is similar to that of *V. parahaemolyticus*. Seawater is the normal habitat for *V. alginolyticus*,

and it has been isolated from seawater and seafood in many parts of the world (Baross and Liston, 1970; Kampelmacher *et al.*, 1972; Vasconcelos *et al.*, 1975; Hosseini *et al.*, 2004). *V. alginolyticus* was the dominant species isolated from coastal waters of Cochin (Thampuran *et al.*, 1997). *V. alginolyticus* was isolated from mussels (Matte *et al.*, 1994, a; Mugeri *et al.*, 2000), Clams (Sunen *et al.*, 1995), Oysters (Matte *et al.*, 1994). Reports are available on the incidence of this species in seafood obtained from markets in Hong Kong (Chan *et al.*, 1989) and Malaysia (Elhadi *et al.* 2004). This species was isolated from frozen seafoods mainly from shrimps and crabs (Wong *et al.*, 1995; Sanjeev *et al.*,2000; Jaksic *et al.*, 2002). Wong *et al.* (1992) have reported the incidence of this species in aqua cultured foods comprising oysters and grass shrimps in Taiwan.

Studies by Baross and Liston (1970) in oysters of Washington State showed that *V. alginolyticus* was rarely found in winter, but counts rose rapidly with increasing water temperature, and the organism was abundant in summer, and the minimum growth temperature for *V. alginolyticus* is 8°C.

V. alginolyticus is reported to have been isolated from extra intestinal sites from exposure to seawater, mostly infection of the ear, eye, hand, leg, lung, blood and burns (Rubin and Tilton, 1975; English and Lindberg, 1977; Olsen, 1978; Hansen *et al.*, 1979; Hollis *et al.*, 1976; Pien *et al.*, 1977). Tubiash *et al.* (1970) have reported the association of *V. alginolyticus* with bacillary necrosis of larval and juvenile bivalve molluscs. Resistance to tetracycline and chloramphenicol has been reported in a few isolates of *V. alginolyticus*, but all strains appeared to be sensitive to ciprofloxacin (French, 1990).

2.7. *V. damsela*

Vibrio damsela is a halophilic gram-negative bacillus similar to *V. vulnificus* that strictly causes soft tissue infections following exposure of wounds to brackishwater or injury by saltwater animals (Barber and Swygert, 2000) *V. damsela* infections can be fulminant and are frequently fatal even in immunocompetent hosts. Of the 16 cases of *V. damsela* infection reported between 1982 and 1996, 4 were fatal (Fraser *et al.*, 1997). Thampuran *et al.* (1997) have reported the isolation of *V. damsela* from coastal waters and fishes of Cochin. Sanjeev *et al.* (2000) reported the incidence of this species in iced

prawns. Elhadi *et al.* (2004) have reported the incidence of the species in seafood samples collected from markets and super markets in Malaysia. Hosseini *et al.* (2004) have reported the isolation of the species from shrimp caught off the coast of Iran.

2.8. *V. metschnikovii*

V. metschnikovii, an oxidase-negative and nitrate negative species, of the genus vibrio was first described in 1888 by Gamaleia (1888), redefined in 1978 (Lee *et al.*, 1978), and extensively characterized in 1988 by Farmer *et al.* (1985), is rarely isolated in human infections. Most nonhuman strains of this species were isolated from river water, sewage, cockles, clams, oysters, prawns and lobsters (Lee *et al.*, 1978), crabs and shrimps (Farmer *et al.*, 1985), fish (Hansen *et al.*, 1989) and scallops (Buck, 1991). Thampuran *et al.* (1997) have reported the isolation of *V. metschnikovii* from coastal waters of Cochin. Elhadi *et al.* (2004) have reported the incidence of the species in seafood samples collected from markets and super markets in Malaysia

Although human isolates have been recovered from blood, urine, a foot wound (Farmer *et al.*, 1985; Farmer *et al.*, 1988), gall bladder, and bile duct (Jean-Jacques *et al.*, 1981), as well from feces (Lee *et al.*, 1978). Only one well documented clinical observation concerning a case of bacteremia in a patient with an inflamed gallbladder has been described (Janda *et al.*, 1988). *V metschnikovii* was isolated from 5 infants with diarrhea during a cholera surveillance programme in Peru (Dalsgard *et al.*, 1996). All isolates were identified within a 10-day period. No common source of infection was found and no additional isolates of the organism were identified in the following year. Lesmana *et al.* (2002) have reported the isolation of *V. metschnikovii* from patients with acute diarrhea in Jakarta, Indonesia.

2.9. *V. vulnificus*

This species has been identified as a halophilic "Lactose – positive" marine vibrio (Hollis *et al.*, 1976). *V. vulnificus* is an indigenous bacterium in marine and coastal water environments and is responsible for wound infection and primary septicemia after ingestion of raw seafood, especially oysters or contact with

seawater (Høi *et al.*, 1998; McLaughlin, 1995). The species *V. vulnificus* can be divided into biotypes 1 and 2 on the basis of differences in biochemical and serological properties (Tison *et al.*, 1982). Biotype 1 strains are associated with human disease, whereas biotype 2 strains are pathogenic for fish, especially eels (Amaro and Biosca, 1996).

V. vulnificus is wide spread in the environment and has been isolated from estuarine waters of most U.S. coastal states (Oliver *et al.*, 1982; 1983; Tilton and Ryan, 1987; Pfeffer *et al.*, 2003). Thampuran *et al.* (1997) have reported the incidence of *V. vulnificus* in coastal waters of Cochin. In a survey conducted in Karnataka on the west coast of India, Karunasagar *et al.* (1990) observed the incidence of *V. vulnificus* in fish and shellfish samples collected from the market. *V. vulnificus* was the dominant species isolated from fishes along Kakinada in the eastern coast of India (Prasad and Rao, 1994, a). Sanjeev *et al.* (2000) have reported the occurrence of this species in frozen fish and fish products collected from Kerala and Tamil Nude meant for export. Yamo *et al.* (2004) have reported the isolation of *V. vulnificus* from live seafood samples consisting of razor clams, giant tiger prawns and mantis shrimp samples collected from the markets in coastal cities of China.

Schandevyl *et al.* (1984) have reported the isolation of *V. vulnificus* from marine fish in Senegal, Africa. Vaseeharan and Ramasamy (2003) in a study of the *Penaeus monodon* rearing hatcheries in India, reported the isolation of *V. vulnificus* from, shrimp eggs, post larvae, rearing tank water, source seawater and feed.

The presence of *V. vulnificus* in water and shellfish is seasonal, being most prevalent when the water temperature is high i.e. >20°C (Kelly, 1982). Low salinity (0.5- 1.6 %) also favours the presence of *V. vulnificus* in seawater (Kelly, 1982). Some strains of *V. vulnificus* show bioluminescence, and these strains may also be pathogenic (Oliver *et al.*, 1986).

Food borne infection may result after consuming contaminated raw or under cooked seafood, particularly oysters and clams, with illness usually starting 16-48 h after ingestion. The organism penetrates the intestinal tract and produces primary septicemia. The illness usually begins with malaise, followed by chills,

fever, and prostration. Vomiting and diarrhea are uncommon, but sometimes occur shortly after chills and fever. Hypotension is present in approximately 335 of the cases (Blake *et al.*, 1979; Hollis *et al.*, 1976). Persons with known liver disease, particularly those patients with cirrhosis, are at high risk for *V. vulnificus* primary septicemia. (Vollberg and Herrara, 1997). *V. vulnificus* can also cause skin infection when open wounds are exposed to warm seawater. These skin infections may lead to cellulitis, ulceration, necrotizing fasciitis, and sepsis (Klontz *et al.*, 1988; Howard and Leib, 1988; Oliver, 2005).

2.10. Effect of washing on HPVs

Information regarding the effect of washing on HPVs is scanty. According to Shewan (1977) most of the bacteria adhering to the slime and skin surface could be washed away with water. Several methods have been evaluated for their effectiveness in reducing initial microbial population in seafoods. High-pressure washes have been demonstrated to be effective in reducing bacterial populations on the surface of whole fish by removing the slime layer in seafoods (Mayer and ward, 1991). Karthikeyan *et al.* (1999) reported that washing and chlorine disinfections reduced the total plate counts and faecal coliform counts in cultured shrimp from 6.31 log g^{-1} to the tune of 0.68-1.25 log units. These results corroborate the observations of Vanderzant and Nickelson (1972) and Peranginangin and Suparna (1992). Thampuran and Gopakumar (1990) in a study of the impact of handling practices on the microbial quality of shrimp (*M. dobsoni*) observed that washing resulted in a decrease in the total bacterial load, while the percentage of reduction due to washing was 50.54 to 80.71, on the basis of the count in Sea water agar (SWA). Shucking and washing appear to result in an overall decline in vibrios viz *V.cholerae, V.parahaemolytius* and *Lac+*^{ve} vibrios (Mary and Gregory, 1984). Akalani-Rose *et al.* (1990) in a study of the prawns harvested from prawn farms observed that *V.parahaemolyticus* was always detected in newly harvested prawns where levels varied from 10^2 - 10^3 cfu g^{-1} and washing at the pond site reduced the count slightly. Codex Committee on Food Hygiene (CCFH, 2002) in a review, principally concerning *V. parahaemolyticus* has stressed the need for effective washing of seafood after harvest and during seafood preparation with disinfected seawater or potable water.

2.11. Effect of chilling on HPVs

Reports generally have indicated that vibrios are sensitive to cold. Studies of Muntada-Garriga *et al.* (1995) indicates that high numbers of *V. parahaemolyticus* can be inactivated at chill temperatures; the time of total inactivation depends on the initial number of microorganisms and incubation temperature. Enteropathogenic vibrios grow over the range 5 to 43°C with an optimum at 37°C. There is variation both between species and among strains within species. Both food type and incubation temperature affected survival of vibrios at low temperatures (Corrales *et al.*, 1994).

Cook and Ruple (1992) have shown that *V. vulnificus* held at temperatures of 4°C and 0°C underwent a time dependent decrease in number of recoverable cells. The time required for the bacterium to reach undetectable levels (MPN<$3g^{-1}$) may exceed the usual storage of 14 days for shucked oyster meats and 21 days for shell stock oysters. Gooch *et al.* (2002) have reported a 0.8 $\log_{10}$ decrease in number of *V. parahaemolyticus*, when the oysters were chilled at 3°C after 14 days. Andrews *et al.* (2000) on the contrary have observed that low temperature pasteurization of raw oysters in ice was very effective in reducing *V. vulnificus* and *V. parahaemolyticus* from >100000 to non detectable levels in less than 10 min of processing. Kaysner *et al.* (1989) in a controlled study of *V. vulnificus* in oyster shellstock found that the organism survived upto 2 weeks at 2°C, whereas *V. parahaemolyticus* has been observed to survive storage in shell stock oysters for at least 3 weeks at 4°C (Oliver and Kaper, 1997). To reduce the consumer exposure to *V. vulnificus* the oyster shellstock must be cooled immediately after harvest to eliminate the post harvest growth of the organism (Cook, 1997). Quevedo *et al.* (2005) observed that although rapid chilling by immersion of unwashed whole oysters in ice for 3 h generally declined the *V. vulnifics* numbers, the method cannot be relied upon because of the relatively small decline in *V. vulnificus* number and the possibility of concomitant increases in fecal coliform and total bacterial contamination.

Some workers insisted that inactivation of *V. parahaemolyticus* occurred more rapidly when the organism was chilled (1- 7°C) than when it was frozen at –2 to –30°C (Beuchat, 1977; Johnson and Liston, 1973). Quite the reverse was

observed by Matches *et al.* (1971) who observed that temperatures below 8°C will usually stop growth but it has been observed that the organism can still survive. *V. parahaemolyticus* has been reported to undergo an initial rapid drop in survival (*Ca.*99 %) when incubated on whole shrimp at 3, 7, 10 or −18°C, although survivors remained at the end of the 8th day of study (Oliver and Kaper, 1997). Similar results were observed by Vasudevan *et al.* (2002) when the fish fillets were chilled. Bradshaw *et al.* (1974) found that vibrios grow well at 18°C,in cooked shrimp and crab, but their numbers declined from 0.5 to 1 log at 10°C and below during 48 h holding period.

Wide discrepancies in views may be due to the fact that the organism becoming non-culturable rather than non-viable. Oliver and Wanucha (1989) have observed that while cells rapidly lose culturability at 5°C or 10°C, a significant proportion remained viable and metabolically active.

2.12. Effect of storage at low temperature (4°C and 10°C) on HPVs

Although the vibrios are sensitive to cold; seafoods have also been reported to be protective for vibrios at refrigeration temperatures (Oliver and Kaper, 1997). Wong *et al.* (1994) reported that *V. mimicus*, *V. fluvialis* and V. *parahaemo-lylicus* survived well at low temperature (10°C and 4°C) although it showed 1 to 2 log reduction. In shell stock oysters, *V. parahaemolyticus* was observed to survive for at least 3 weeks with little or no decrease in numbers and it has been shown to grow slowly at 10°C in oyster homogenate (Johnson *et al.*, 1973; Thompson and Thacker,1973). Minimal temperatures reported for *V. parahaemolyticus* multiplication was 5°C (Beuchat, 1973) and 8°C (Baross and Liston, 1970) in artificial media and 10°C in oyster homogenate (Thompson and Thacker, 1973).

Vanderzant and Nickelson (1972) subjected their gulf coast isolates of *V. para-haemolyticus* strain to 0, 3,7,10 and −18°C in whole and homogenized shrimp. The loss of viability in shrimp homogenate was not as great as in the whole shrimp and more than 2 log reduction was observed in 8 days. It is also interesting to note that the strains Vanderzant and Nickleson studied was more readily inactivated at 3°C than at −18°C. Twedt (1989) reported that the initial concentration of *V. parahaemolyticus* suspended for 48 h in peptone broth with

3 % NaCl or raw fish held under refrigeration (4°C) declined from 1 to 4 $\log_{10}$. Similar observations were made in subsequent investigations when *V. parahaemolyticus* was held at low temperature in shrimp (Bradshaw *et al.*, 1974), oysters (Goatcher *et al.*, 1974; Johnson and Liston, 1973; Beuchat, 1977), homogenized and filleted fish (Covert and Woodburn, 1972; Johnson and Liston, 1973; Matches *et al.*, 1971) and crab meat (Beuchat, 1977; Johnson and Liston, 1973).

Oliver (1981) reported rapid and dramatic decrease in cell viability when *V. vulnificus* cells were incubated at 4°C in oyster homogenates. Similar observation was made when the organism was incubated in shrimp homogenate at 4°C (Boutin *et al.*, 1985 and Hopkins and Modlin, 1985). It has been shown that *V. vulnificus* can multiply in post harvest shellfish if they are held at temperatures above 10°C (Oliver, 1989).

At 6 ± 2°C *V. parahaemolyticus* and *V. vulnificus* continued to survive till the end of storage period (90 days) in fish homogenate, although the organism showed a 5 log reduction (Sudha *et al.*, 2003). They also reported that in 3 % salt solution and tryptic soya broth (TSB), *V. vulnificus* could survive only upto 14 days at 6.0± 2°C while *V. parahaemolyticus* remained viable for a longer period of upto 60 days. Vasudevan *et al.* (2002) observed an initial reduction (100- 1000 fold) of *V. parahaemolyticus* in fish fillets when stored at 4 and 8°C although the decline in numbers was less pronounced than when the fillets were frozen. Kaysner *et al.* (1989) indicated the presence of large numbers of cells of endogenous *V. vulnificus* in oysters after 7 days at both 0.5 and 10°C. Covert and Woodburn (1972), Temmyo (1966) have reported that the addition of NaCl to the suspending medium (upto 12 % NaCl) conferred a stabilizing effect on the organism.

2.13. Effect of freezing and frozen storage (–40°C and –20°C) on HPVs

Muntada-Garriga *et al.* (1995) studied the survival of *V. parahaemolyticus* in oyster meat homogenate at various temperatures i.e. –18°C and –24°C with different loads i.e. 10^2, 10^4, 10^5 and 10^7 ml^{-1}. In all cases, the numbers of *V. parahaemolyticus* were a logarithmic function of log time, and the study indicates

that high numbers of *V. parahaemolyticus* can be inactivated at low temperatures.

Covert and Woodburn (1972) studied the interaction of temperature and NaCl concentration in affecting the survival of *V. parahaemolyticus* in trypticase soya broth and shrimp homogenate. Temperature of –5 ± 1°C and –18 ± 1°C reduced the number of viable organism regardless of the NaCl concentration. Fish homogenate was protective as compared with tryptic soya broth.

Vanderzant and Nickelson (1972) reported 2 log reduction in counts of *V. parahaemolyticus* after 8 days storage at –18°C. Thompson and Thacker (1972) observed that oysters held at –20°C for more than 2 weeks seldom contained viable *V. parahaemolyticus* cells. *V. vulnificus* is also sensitive to freezing and rapid die off (6 logs in 40 days) was observed at –20°C (Oliver, 1981; Boutin *et al.*, 1985). Matches *et al.* (1971) inoculated *V. parahaemolyticus* in fish homogenate and observed that the log reduction values of 2.2 to 6.2 at –18°C were attained in 12 to 19 days, and the same reduction values at –34°C were reached before 12^{th} day.

Sudha *et al.* (2003) have reported the complete elimination of *V. vulnificus* from fish muscle homogenate within 3 months of storage at –18°C ± 1 whereas *V. parahaemolyticus* survived the period indicating better survival capacity for this pathogen. Parker *et al.* (1994) showed that oyster samples individually injected with *V. vulnificus* to a level of approximately 1×10^6cfu g^{-1} and vacuum packaged and frozen stored at –20°C had significant effect on decreasing the *V. vulnificus* count to approximately 1×10^1 cfu g^{-1}.

Balasundari *et al.* (1997) observed that vibrios remained viable in edible oysters (*Crassostrea madrasensis*) after 5 months of storage at –18°C in both antioxidants treated and untreated samples. Cook and Ruple (1992) were able to isolate *V. vulnificus* from oysters frozen at –20°C for 12 weeks although freezing and storage of pure cultures of *V. vulnificus* at –20°C reduced the number of culturable cells more quickly than holding the cultures at 0°C. *V. parahaemolyticus* survived freezing at –20°C for 7 weeks in fish fillets (Vasudevan *et al.*, 2002). Wong *et al.* (1994) studied the survival of psychotropic

V. mimicus, *V. fluvialis* and *V. parahaemolyticus* in culture broth at low temperature and found that the strains survived well at 10°C, 4°C and –30°C and could probably enhance the risk of vibrios in seafood. Johnston and Brown (2002) in a study of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* found cells become non culturable over a period of time at 4°C. According to them, these cells in their changed morphological form would not be detected in fish or seafood products by the current vibrio detection methods and freezing at –20°C had no effect in reducing cell numbers.

2.14. Effect of drying on HPVs

The most common method of utilization of fish in India is as fresh fish followed by cured and dried fish (Prasad and Rao, 1994). It is estimated that over 32 % of Indian marine fish catch is consumed as cured/ dried form (Thomas and Balachandran, 1989). Studies on quality of commercial dry fish, both of west and east coasts have been reported (Kalaimani *et al.*, 1988; Basu *et al.*, 1989, Thomas and Balachandran, 1989). But, not much information is available on the effect of drying on HPVs. Sakazaki (1983) have reported that *Vibrio parahaemolyticus* is very sensitive to drying. Temmyo (1966) noticed that *Vibrio parahaemolyticus* exposed to desiccation on inoculated membrane filters or on flat board surfaces died rapidly. Venugopal *et al.* (1984) in a study of dried fishes reported the incidence of *Vibrio parahaemolyticus* in dried white bait to a level of $2 \times 10\ g^{-1}$. In another study, they reported that sun drying for 4 days completely inactivated *V. parahaemolyticus* inoculated into salted and unsalted white bait. Chitu *et al.* (1977) reported isolation of *V. parahaemolyticus* from salted herring and roe in Rumania. Rank *et al.* (1988) have indicated the survival of *V. hollisae* in dried salt fish.

2.15. Effect of blanching on HPVs

Enteropathogenic vibrios are not heat resistant and are readily destroyed by cooking. Resistance depends on several factors, including heating menstrum and physiological condition (Varnam and Evans, 1996). There is considerable variation between species and information is neither complete nor in some cases, is fully reliable. Raw seafoods are mostly implicated in out breaks of food

poisoning, but if they are heated at 100°C shortly before consumption, infection with *V.parahaemolyticus* never occur (Sakazaki, 1983).

The commercial practice of heat shocking oysters in boiling water to facilitate opening reduced counts of *V.parahaemolyticus* and often non-*V. cholera* vibrios to undetectable levels (Hackney *et al.*, 1980). Chang *et al.* (2004) in a study revealed that when *V.parahaemolyticus* were heat shocked at 42°C for 15, 30 or 45 min, it caused an increased demand for NaCl during recovery from heat injury. They also reported that heat shock generally increased the survival of the test organism during subsequent exposure to 47°C. Isolation of *V. hollisae* from fried fish in the absence of evidence of post-process contamination suggests the possibility of a higher level of heat resistance than other species (Lowry *et al.*, 1986).

Delmore and Crisley (1979) observed D values for *V.parahaemolyticus* in clam homogenate of 0.70 min at 49°C, 0.54min at 51°C, 0.31min at 53°C, and 0.24min at 55°C. This study shows that a relatively mild heating process kills the organism. Supporting these findings is the work of Goldmintz *et al.* (1974), who demonstrated that steaming clams, for 5 and 15 min (internal temperature 88 °C and 95 °C respectively) reduced *V.parahaemolyticus* population by 6 log cycles. However, steaming does not provide enough heat to kill vibrios or other pathogens (Hackney and Dicharry, 1988).

In peptone with 3 % NaCl medium 3 - 4 $\log_{10}$ decline of *V.parahaemolyticus* was observed in 5 min at 55° C, at 60°C the decline was 7 $\log_{10}$ (Temmyo 1966). At 65°C 3-4 $\log_{10}$ reduction in count in crabmeat- soybroth-3 % NaCl was reported by Goldmintz (1974). Vanderzant and Nickelson (1972) observed 6 $\log_{10}$ decline at 100°C in 1 min in shrimp homogenate supplemented with 3 % NaCl. The rate of inactivation appeared to be curvilinear in these instances (Goldmintz, 1974).

Heating oysters for 10 min in water at 50°C proved adequate to reduce *V.vulnificus* to a nondetectable level (Cook and Ruple, 1992). This treatment does not impart a noticeable cooked appearance or taste to the oysters and may be employed as a strategy to improve the safety of raw oysters. Hesselman *et al.* (1999) described a technique involving dipping oysters in tanks with water at 67°C for around 5 min followed by spraying with cold water for around one min

to assist in shucking. When combined with market chain procedures such as chilling, packing and cold storage, *V. vulnificus* was reduced by 2 - 4 logs, depending on the original contamination level. The technique has also proved effective in other bivalves.

Ama *et al.* (1994) found *V.vulnificus* cells were more sensitive to heating at 50°C than at 40°C. However the cells were more resistant to heating in oyster or fish homogenate than in buffers at comparable temperature and destruction was rapid at lower pH. Covert and Woodburn (1972) observed that resistance to death is enhanced when cells are heated in substratum containing sodium chloride and at pH near neutrality.

Wong *et al.* (2002) found that logarthimically grown *V. parahaemolyticus* cells heat shocked at 42°C for 30 min were more resistant to thermal inactivation at 47°C than were unshocked cells. They also observed the production of thermostable direct haemolysin, the major virulence factor in *V. parahaemolyticus*, was enhanced in the cells heat shocked at 42°C but not in those heat shocked at 37°C. More recently, Andrews *et al.* (2003) studied the effect of heating on *V. parahaemolyticus* O3:K6, a pathogenic strain with enhanced heat resistance. The researchers found that 6 min heating at 50-52°C reduced a 4 log contamination level to undetectable levels (<0.3 MPN g^{-1}). When the pathogen was present at levels of 5-6 logs, a heating time of 22 min was required to reach non-detectable levels.

2.16. Effect of sodium chloride on HPVs

The vibrios grow very poorly or not at all on or in media lacking salt (Sakazaki, 1983). Most species grow well over the range between the lower and upper growth limits, growth reducing markedly towards the upper limit. The optimum for growth in pure culture is *Ca* 3 %, but may be higher in mixed cultures due to the inhibition of competing microorganisms at higher concentrations (Varnam and Evans, 1996). In practice, the upper limit is of little relevance since all species are able to grow in marine foods with the exception of acid-preserved or heavily salted and dried fish. *V. hollisae* is able to survive in dried salt fish (Rank *et al.*, 1988).

The minimum salt concentration of *V. parahaemolyticus* limiting multiplication in substrates is 0.5 % (Sakazaki and Shinoda, 1986). The organism is readily inactivated in distilled water, with 90 % of the cells inactivated at between 0.9 and 4.4 min (Lee, 1972) good to fair growth occurs at all salt concentrations between 0.5 and 8 %, with luxuriant growth appearing at the optimal concentration of 3 % NaCl (Sakazaki and Shinoda, 1986). Covert and Woodburn (1972) found NaCl appeared to be protective to the cells of *V. parahaemolyticus* in tryptic soya broth and fish homogenate at 48 $\pm$ 1°C. The optimum concentration for growth of *V. parahaemolyticus* may be influenced by the nature of medium employed (Sakazaki, 1983).

The minimum growth temperature reported for *Vibrio parahaemolyticus* is 5°C (Beuchat, 1973; Katoh, 1964), the lower limit is affected by pH and salt concentration (Beuchat, 1973). The minimum pH reported for *V. parahaemolyticus* to allow growth at 5°C in trypticase soy broth with 3 % NaCl was 7.3, when salt concentration was increased to 7 %, the minimum pH rose to 7.6 (Beuchat, 1973).

Kelly (1982) reported that no growth of *V. vulnificus* took place at less than 0.1 % or greater than 5 % NaCl, and optimal growth in 1-2 % NaCl. Oliver and Wanucha (1989) have observed an optimal NaCl concentration between 1 % and 3 % for *V.vulnifucus* although 0.5 % NaCl present in many routine laboratory media provides for very good growth. Marco-Noales (1999) studied the effect of salinity and temperature on long-term survival of the Eel pathogen *V. vulnificus* Biotype 2 (Serovar E). According to them the optimal temperature for survival was dependant on the salinity.

2.17. Effect of pH on HPVs

Vibrios grow over a pH range from 5.6 to 9.6, but it grows best at 7.6 to 8.6 (Anon, 1972). The optimal pH range of *V. parahaemolyticus* varies from 7.5 to 8.5 (Sakazaki *et al.*, 1963). Although *V. parahaemolyticus* has been reported as growing at pH 4.8 (ICMSF, 1980), they are generally sensitive to pH values below 7.0.

All the 79 cultures of *V. parahaemolyticus* tested in a study grew well in media with initial pH values from 5 to 11 (Twedt *et al.*, 1969). Kondo *et al.* (1960) have reported that *V. parahaemolyticus* is more sensitive to acid than *E coli* and growth is completely inhibited at pH 4.5 to 5.0. The vibrios may be killed in vinegar within 1 h and in 0.5 % acetic acid within several minutes (Kondo *et al.*, 1960). However, raw fish or shellfish with vinegar, which is widely used in Japan, has frequently caused food poisoning due to *V. parahaemolyticus*. It is probable that proteins in rich foods interfere with the action of vinegar acid on the vibrio (Kodama, 1967).

Vanderzant and Nickelson (1972) studied the survival of *V. parahaemolyticus* in shrimp homogenates at various pH values. In homogenates adjusted to pH 1, 2, 3 and 4 no survivors could be detected. At pH 5.0, a sharp drop in viable count took place immediately, with no survivors detectable after 15 min. All species of enteropathogenic vibrios grow well at alkaline pH value, the upper limiting values being pH 10-11 (Varnam and Evans, 1996).

Ama *et al.* (1994) observed exponential inactivation rates of *V.vulnificus* at pH 6.5, 6.0, 5.5 during heating and the organisms were inactivated most at pH 5.5 than at any other rates. Yeung and Boor (2004) have reported enhanced survival of acid adapted (pH 5.5) log-phase cells of *V. parahaemolyticus* at pH 3.6, compared to cells not previously exposed to pH 5.5.

2.18. Effect of Chlorine on HPVs

The use of chlorine as a disinfectant has been one of the most important public health practices for the prevention of waterborne diseases over the past 100 years (Anon, 2000). Chlorine is used in fish processing sector as a water disinfectant and is probably the most wide spread disinfectant in use. Its uses include washing fishery products, addition to water for making ice for chilling fish, and in water for thawing frozen products. It is also used in water to cool canned fish after retorting to prevent "leaker" spoilage .The codex fish and fishery products committee recommended upto 10 mg l^{-1} chlorine in water that comes in contact with fishery products and upto 100 mg l^{-1} in water for cleaning equipment and facilities (Anon, 2000).

Venugopal *et al.* (2000) studied the concentration and contact time required by the commonly used sanitizer, hypochlorite for killing/ reducing the cells of *V.parahaemolyticus* in phosphate buffered saline (PBS) and in association with fish. A minimum level of 0.5 ppm of available chlorine was able to reduce the count of both Kanagawa Positive (K^+) and Kanagawa negative (K^-) *V.parahaemolyticus* in PBS by 90 % within 5 min and complete killing of both was achieved in 20 and 30 min, respectively. In fish artificially contaminated with K^+ *V.parahaemolyticus* and exposed to 10 and 20 ppm available chlorine complete destruction of the cells was observed within 10 min, but at 30 ppm, the time required was only 5 min. Venugopal *et al.* (1999) in another study reported the effect of sanitizers on *V. paahaemolyticus* in biofilms on stainless steel surface and hypochlorite at 100 and 200 μg ml^{-1} for 5 min showed a reduction in numbers by only 2-3 log units and failed to completely inactivate biofilm cells.

Studies of Gray and Hsu (1979) have indicated the effectiveness of both chlorine and idophore in killing *V. parahaemolyticus* cells. The inhibitory or lethal activity depends on the amount of free available chlorine in the solution that comes in contact with microbial cells. Free chlorine disinfects by chemically disrupting bacterial cell walls and membrane through oxidation of a chemical group known as the thiol group (WHO, 1998). The exposure of microbial cells to chlorine was also known to cause disruption of cellular enzyme system (Wyss, 1961), protein synthesis (Benarde *et al.*, 1967), oxygen uptake and oxidative phosphorylation (Venkobackar and Rao, 1977) resulting in death or inactivation of cells.

To minimize chlorine waste and optimize its efficient use, chlorine concentration in sanitizing solutions should be monitored (Suslow, 2000). The concentration of the fast acting, antimicrobial hypochlorous acid, the chemical species providing free available chlorine to disinfect solutions, is a function of pH, between pH 6.5 and 7.0, hypochlorite exists as 80-95 % of the free chlorine concentration (Suslow, 2000).The type and form of microorganism will also influence the antimicrobial effectiveness of chlorine disinfectants (Odlang, 1981). Mir *et al.* (1997) have reported that the gram-positive strains were more resistant to chlorine than gram-negative strains and the behaviour of some of them in the presence of chloramphenicol suggests either the synthesis of unique proteins or aggregation of the bacteria as mechanisms of resistance to inactivation.

2.19. Effect of Chlorine dioxide on HPVs

The bactericidal properties of chlorine dioxide (ClO_2) have been known since the beginning of this century, but it has been used in sanitation only since the 1950's (Masschelein, 1979). It has about 2.5 times the oxidation capacity of chlorine (Benarde *et al.*, 1967). Chlorine dioxide has been shown to produce bactericidal effect equivalent to seven times its concentration of chlorine in poultry processing water (Lillard, 1979). Chlorine dioxide maintains its bactericidal activity longer than chlorine. Parts of its disinfection capacity are attributed to the chlorite resulting from the reduction of ClO_2 (Masschelein, 1979). The bactericidal activity of ClO_2 decreases with lower temperatures (Ridenour and Armbruster, 1949) and is not affected by high pH or the presence of ammonia or nitrogenous compounds (White, 1972). Chlorine dioxide is also less reactive than chlorine with organic compounds and its use is preferred, where high organic loads are encountered (White, 1972).

The Food and Drug Administration (FDA) on March 3, 1995, amended the food additive regulations to provide a 3 ppm residual chlorine dioxide for controlling microbial populations in poultry processing water (FDA, 1995). Information is limited regarding the usefulness of ClO_2 in seafood processing. The use of chlorine dioxide is less common in fish processing, probably because of its instability and the hazards involved in handling and transportation (Lin *et al.*, 1996). However, it is used and has been shown to be effective in killing a large number of microorganisms, including some that are resistant to treatment with chlorine and to extend the storage time of many foods, including fishery products. Information regarding effect of ClO_2 on HPVs is scanty.

Shin *et al.* (2004) found initial load of food borne pathogens viz., *E. coli*, *S. typhimurium* and *L. monocytogenes* was reduced by antimicrobial ice containing chlorine dioxide and the lowered microbial level was maintained during treatment. They also reported that the application of antimicrobial ice is a simple and effective method for the safe preservation of fish. Puente *et al.* (1992) observed that sterile seawater treated with lower concentration of chlorine dioxide (less than 47$\mu g\ l^{-1}$ Cl^-) had no effect on the shrimp, but inhibited the growth of *V. parahaemolyticus* and in sewage contaminated seawater chlorine dioxide levels

at 285-2850 μg l^{-1} necessary for the inactivation of *V. parahaemolyticus* and any native bacteria, also destroyed the artemia culture.

2.20. Antibiotic sensitivity of HPVs

Antibiotics and other chemotherapeutic agents are commonly used in fish farm either as feed additives or immersion baths to achieve either prophylaxis or therapy (Li *et al.*, 1999). However, extensive use of these drugs has resulted in an increase drug- resistant bacteria as well as R- plasmids (Son *et al.*, 1997; Saitanu *et al.*, 1994). Furthermore, many species of halophilic vibrios have become recognized as potential human pathogens causing serious gastroenteritis or severe wound infection upon exposure to contaminated seafood and/or seawater (French *et al.*, 1989). In recent years, vibriosis has become one of the most important bacterial diseases in maricultured organisms, affecting large number of species of fish and shellfish (Woo *et al.*, 1995; Wu and Pan, 1997). Elucidation of the antimicrobial susceptibilities of potential pathogenic vibrios will be important for prophylaxis and treatment of vibrio infections in human beings and in cultured marine organisms.

In EU member states, only four or five antimicrobial agents are licensed for use in finfish culture. In the USA, Canada and Norway, regulatory control is equally vigorous. But in many countries there is either no or no effective control on the use of antibiotics in food fish or shellfish species (Alderman and Hastings, 1998).

Li *et al.* (1999) viewed that different vibrio strain had similar antibiotic resistance profiles. He tested the antibiotic sensitivity of seven *Vibrio* species viz *V. alginolyticus, V. vulnificus, V. parahaemolyticus, V. logei, V.pelagius II, V.fluvialis* and *V.medeitterranei* by the agar dilution method. All isolates were sensitive to streptomycin, nalidixic acid, rifampicin and ceftriaxone and almost all were sensitive to chloramphenicol (98 %), sulphamethoxazole (98 %) and ceftazidime (96 %). A large number of strains were found to be resistant to ampicillin, amikacin, kanamycin, trimethoprin and cefuroxime. French *et al.* (1989) reported similar antibiotic susceptibility profiles for *V. alginolyticus, V. parahaemolyticus and V.vulnificus* in a clinical and environmental setting. Ottaviani *et al.* (2001) studied the antimicrobial susceptibility of potentially pathogenic halophilic vibrios isolated from seafood, and confirmed that all isolates were uniformly sensitive to

chloramphenicol, impenem, and meropenem but resistant to lincomycin. Joseph *et al.* (1978) observed that *V.parahaemolyticus* and *V.alginolyticus* produce β-lactamase and are resistant to ampicillin but are inhibited by tetracycline and chloramphenicol. Zanetti *et al.* (2001) have reported similar result and observed the frequency of resistance to β-lactams unexpectadely high among vibrio species.

Susceptibility to antibiotics differs among vibrios species. Bode *et al.* (1986) in a successful treatment of vibrio meningitis caused by *V.cincinnatiensis* have reported the sensitivity of the species towards gentamycin, tobrimycin, chloramphenicol, tetracycline, ticarcillin, ampicillin and moxalactam. Morris and Black (1985) suggested an empiric therapy with tetracycline or chloramphenicol, in combination with aminoglycoside in suspected vibrio sepsis until results of susceptibility testing are available.

Lee *et al.* (1981) while studying the taxonomy of *V. fluvalis* have reported sensitivity towards kanamycin, streptomycin sulphonamide, tetracycline and trimethoprin. Brenner *et al.* (1983) have reported similar antibiotic patterns for *V.furnissii* and *V fluvialis*. They observed sensitivity of the species towards chloramphenicol, nalidixic acid, tetracycline and kanamycin and very much resistant towards penicillin and ampicillin. Sanjeev (1999) has reported the antibiotic sensitivity of *V. parahaemolyticus* from a brackishwater culture pond. All the 250 strains were found sensitive towards chloramphenicol, 68.4 % were sensitive to gentamycin, and 18 % were sensitive to tetracycline and 16.8 % to streptomycin. None of the strains were found sensitive towards penicillin and polymyxin-B. Similar results were reported by Pradeep and Lakshmana-perumalasamy (1985). They observed the antibiotic sensitivity of 120 strains of *V.parahaemolyticus* isolated from water, sediment, plankton, fish and prawns of Cochin backwaters. They also noted higher resistance to ampicillin exhibited by isolates from fish and prawns and none of them were sensitive to kanamycin. Prawns contained more multiple resistant *V.parahaemolyticus* than others samples.

Hollis *et al.* (1976) have reported sensitivity of *V. vulnificus* strains towards ampicillin, chloramphenicol, tetracycline and gentamycin. Similar results were

reported by Sanjeev and Mukundan (2003) while studying the antibiotic sensitivity of *V. vulnificus* strains isolated from iced and frozen fishery products.

2.21. Haemolytic activity of HPVs

Association between heamolysin production and virulence of *V. parahaemolyticus* has been noted by a number of workers. However the role of haemolysin in the virulence is not clear. Lot of information is available on the haemolytic activity of *V. parahaemolyticus*. However information regarding *V. fluvialis*, *V. furnissii* and *V. cincinnatiensis* is scanty.

Kato *et al.* (1965) found that vibrio strains isolated from diarrheal stool gave a haemolytic reaction on autoclaved brain heart infusion agar containing 5 % human blood, 3 % sodium chloride and 0.001 % crystal violet, whereas the strains isolated from marine sources were non haemolytic. This medium was modified by Wagatsuma (1968) to give more clear-cut haemolysis by *V. parahaemolyticus* and the test was named "Kanagawa reaction". Among the virulence factors of *V. parahaemolyticus*, a close correlation between the production of thermostable direct haemolysin (TDH) and human pathogenicity was established by Miyamoto *et al.* (1969). For these authors, 96.5 % of the strains isolated from patients stools produced a thermostable haemolysin, while 99.0 % of those isolated from the marine environment did not. A simple means of revealing this haemolysin is to use the Wagatsuma medium, a blood agar in which strains with β- haemolysis are called KP^{+ve} (Kanagawa phenomenon^{+ve}), and those which are non haemolytic are termed KP^{-ve} (Miyamoto *et al.*, 1969; Blake *et al.*, 1980)

Although *V. parahaemolyticus* has been recognized as an important cause of gastrointestinal disease associated with the consumption of seafood, not all strains of this species are considered to be truly pathogenic (Nichibuchi and Kaper, 1995). TDH is a major virulence determinant of K^{+ve} *V. parahaemolyticus* and that the K^{+ve} phenotype makes a good marker for virulent strains (Nichibuchi and Kaper, 1995). Tdh genes have also been demonstrated in some strains of *V. mimicus*, *V. cholerae non-O1*, *non- O139* and in all strains of *V. hollisae* (Nichibuchi and Kaper, 1995).

However, recently a K^{-ve} *V. parahaemolyticus* strains that produce a toxin TDH related haemolysin (TRH) was found associated with gastroenteritis (Suthienkul *et al.*, 1995), and it appears that both TDH and TRH haemolysin are important virulence factors in the pathogenesis of *V. parahaemolyicus* (Suthienkul *et al.*, 1995). According to Zhang and Austin (2005) there are four haemolysin families in *Vibrio* spp., namely the TDH (Thermostable Direct Haemolysin) family, the HIYA (El Tor Haemolysin) family, the TLH (Thermolabile Haemolysin) family and the δ-VPH (Thermostable Haemolysin) family.

Haemolysins act on erythrocytes membranes thus lysing the cells which lead to the freeing up of iron- binding proteins namely haemoglobin, transferring and lactoferrin (Zhang and Austin 2005). Lang *et al.* (2004) have reported that haemolysin induces cation permeability and activates endogenous gardos K^{+ve} channels, consequences include break down of phosphatidyl serine asymmetry, which depends at least partially on cellular loss of K^{+ve}. The pore -forming activity of haemolysin is not restricted to erythrocytes, but extends to a wide range of other cell types including mast cells, neutrophils, and polymorpho nuclear cells and enhances virulence by causing tissue damage (Zhang and Austin 2005).

Osawa *et al.* (1996) examined the ability of *V. parahaemolyticus* to hydrolyze urea, with specific reference to the presence of the thermostable direct haemolysin gene (tdh) and the gene for thermostable related haemolysin (trh) and suggested that urea hydrolysis is not a reliable marker for identifying tdh- carrying *V. parahaemolyticus* strains but may be a marker for trh- carrying strains. Kelly and Stroh (1989) reported that clinical isolates of *V. parahaemolyticus* obtained from patients with locally acquired gastroenteritis in Canada hydrolyzed urea, but none of the isolates were kanagawa haemolysin positive as determined by the in vitro plate test.

Quadri and Zuberi (1977) were perhaps the first to report a very high percentage of K^{+ve} isolates (52.5 %) from fish and shellfish samples from Karachi, Pakistan. Karunasagar and Mohankumar (1980) observed 25 % incidence of K^{+ve} strains in the environment around Mangalore. Sanjeev (1999) in a study of brackishwater culture pond isolated 12.4 % of K^{+ve} *V. parahaemolyticus* strains. Malathi *et al.* (1988) have reported the isolation of *V. parahaemolyticus* and *V. vulnificus* strains

capable of producing haemolysins from seafoods. Bandekar *et al.* (1982) observed 12 % K^{+ve} strains among isolates from shrimp in Bombay. Hara-kudo *et al.* (2003) have reported the prevalence of pandemic tdh- positive *V. parahaemolyticus* O3:K6 from 10 % of shellfish samples in Japan.

High incidence of K^{+ve} strains among isolates form houseflies led Chaterjee (1980) to speculate that flies might be involved in carrying K^{+ve} strains from human excreta. The high incidence of K^{+ve} strain in the environment in India and Pakistan remain unexplained. Uchimura *et al.* (1993) observed high prevalence of thermostable direct haemolysin like toxin in *V. mimicus* strains isolated from diarrheal patients.

Douet *et al.* (1992) showed that TDH is haemolytic against erythrocytes of various animal species (including human erythrocytes, equine erythrocytes are the most resistant) and cytolytic against cultured mammalian cells.

97 % of *V. fluvialis* and 65.55 % of *V. parahaemolyticus* strain isolated from seafood and aquacultured foods in Taiwan showed haemolytic activity (Wong *et al.*, 1992). In another study Wong *et al.* (1993) have reported the thermostable haemolytic activity of *V. fluvialis* strain after being heated at 100°C but not at 60°C. Chikahira and Hamada (1988) provided an extensive description of the toxic products produced by nine environmental strains of *V. furnissii*. Magalhaes *et al.* (1993) isolated sixteen strains of *V. furnissii* from 16 Brazilian patients with diarrhea and found that most were haemolytic on blood agar.

Zhang and Austin (2005) reported that pathogenic vibrio species were capable of producing various virulence factors consisting of enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and/ or haemagglutinins. Results obtained by Baffone *et al.* (2001) corroborates the above view, they observed vibrio strains consisting of *V. alginolyticus*, *V. para-haemolyticus*, *V. chlorea non-O1*, *V. vulnificus*, *V. fluvialis*, *V. furnissii* and *V. metschnikovii* were in general positive for lipase and gelatinase activity (100 %), haemolytic activity (7.2 %), urease activity (19.2 %), adhesiveness (63 %), cytotoxicity (57.6 %), 23 % of the strains gave positive results in the illeal loop test in rats and 23 % showed the ability to infect the laboratory animals and suggested that pathogenicity of vibrios could be the result of a combination of factors.

3. Materials and Methods

3.1. Incidence of HPVs in fish and fish products

3.1.1. Source of samples

Samples of fish and fishery products meant for internal consumption and export were collected from landing centers, markets and seafood processing plants situated in and around Cochin. One hundred and thirty samples consisting of iced, block frozen and individually quick frozen (IQF) fish and fish products were collected from different sources as mentioned above. Usually the samples were collected on the first working day of each week. Name, type of products and number of samples collected from different sources are given below.

Name	Type of products and numbers			
	Iced	Block frozen	IQF	Total
Shrimp	18	25	3	46
Squid	14	15	10	39
Cuttlefish	19	11	8	38
Others	3	1	3	7
Total	54	52	24	130

3.1.2 . Iced samples

Iced samples consisting of shrimps, squids, cuttlefish, octopus, mussel and mullet were collected from landing centers, markets and processing units situated in and around Cochin. Each sample was immediately transferred into sterile polythene bags. The samples were kept in icebox with sufficient quantity of ice and transported to the laboratory. Analyses were started within 4 h of collection. List of iced samples collected from different sources are given in Table. 2

Table 2. List of iced samples collected from different sources

Iced samples	No. of samples
Shrimp	
Shrimp whole	10
Shrimp PUD	4
Shrimp PD	3
Shrimp headless shell on	1
Squid	
Squid whole	3
Squid whole Cleaned	9
Squid rings	1
Squid tentacles	1
Cuttlefish	
Cuttlefish whole	2
Cuttlefish whole Cleaned	15
Cuttlefish roe	2
Others	
Octopus whole Cleaned	1
Mussel	1
Mullet	1
Total	54

PUD-peeled and undeviened, PD-peeled and deveined

3.1.3. Frozen fish and fishery products

Frozen fishery products consisting of block frozen and IQF (Individually Quick Frozen) fish and products (Table.3) meant for export were collected from nine seafood-processing plants situated in and around Cochin. Frozen samples were selected at random from master cartons. Approximately 100 g was collected aseptically from the slab and transferred to sterile stainless steel sample dishes

using sterile scooper and brought to the laboratory in iced condition in an insulated box and analyzed immediately on arrival.

Table 3. List of frozen fish and fishery products collected from seafood processing plants

Block frozen samples	No. of samples	IQF samples	No of samples
Shrimp			
Shrimp whole	8	Shrimp whole	1
Shrimp PUD	9	Shrimp Peeled and cooked	2
Shrimp PD	6		
Shrimp headless shell on	2		
Squid			
Squid whole	3	Squid whole	2
Squid whole cleaned	11	Squid whole cleaned	5
Squid tentacles	1	Squid rings	2
		Squid tentacles	1
Cuttlefish			
Cuttlefish whole	3	Cuttlefish whole	1
Cuttlefish whole cleaned	7	Cuttlefish whole cleaned	7
Cuttlefish roe	1		
Others			
Crab soft shell	1	Reef cod	2
		Octopus whole cleaned	1
Total	52		24

3.2. Preparation of samples

3.2.1. Iced Samples

Shell and muscle of prawns, muscle and tentacles of cuttlefish, squid and octopus, muscle alone in case of oysters, skin with muscle from both sides of finfish were cut by using sterile scissors and 10 g of the sample was transferred into sterile stomacher bag. Contents in the bags were then homogenized in a

stomacher (Seward) with 90 ml sterile 3 % NaCl solution (C2). The homogenate was kept for few minutes for settling and 1 ml of the supernatant was taken out by using 1 ml sterile pipette. It was then serially diluted by using 9 ml sterile 3 % NaCl solution (C2).

3.2.2. Block frozen and IQF samples

10 g of the samples were transferred to a sterile stomacher bag using sterile scissors. Homogenate was prepared and serial dilutions were made as described in 3.2.1.

3.3. Quantitative studies on HPVs (HPVs)

The incidence of different species of HPVs in the samples was analyzed quantitatively by using 3 tube most probable number (MPN) technique as described in Anon (1995). 10 ml of sample preparation was inoculated into 10 ml of double strength alkaline peptone salt (APS) broth (B1). From the blended samples decimal dilutions were made with 3 % sterile sodium chloride solution. 1 ml of the dilution was inoculated into 10 ml of the APS broth (B1). It was then incubated at 37° C for 18 h. 3 mm loop full of the broth culture was aseptically streaked on to pre-set surface dried thiosulfate citrate bile salts sucrose agar (TCBS) (A4) plates and incubated at 37°C for 18 h in inverted position. The characteristic yellow or green colonies on TCBS plates were regarded as presumptive vibrios.

Isolated colonies from TCBS plates were further purified by streaking on to nutrient agar plates (A2) supplemented with 3 % NaCl. Incubated at 37°C for 18 h in inverted position. Isolated colonies from nutrient agar (A2) plates were streaked on to nutrient agar (A2) slants and Incubated at 37°C for 18 h. These cultures were further subjected to a series of morphological, cultural, and biochemical tests. Isolates were then identified upto the species level as per the key proposed by Alsina and Blanch (1994) counts of each species of HPVs were subsequently derived from the MPN table.

3.4. Studies on morphological cultural and biochemical characteristic of the isolated pathogens

HPVs isolated from iced, frozen fish and fishery products collected from landing centers, markets and processing factories were subjected to a series of morphological, cultural and biochemical tests as mentioned below. 3 % NaCl was added to all media.

3.4.1. Morphological characteristics

Cell form and Gram reaction was studied from growth on nutrient agar (A2) slants. Growth from the edge of an 18 h old culture was taken and a smear was prepared with 3 % NaCl on a clear microscopic slide (Collins and Lyne, 1976). Each smear was stained by using Hucker method (Collins and Lyne, 1976) and observed under the oil immersion objective.

3.4.2. Motility

Motility of the strains was observed by stabbing the culture to a depth of 5-10 mm to a tube containing motility test 3 % NaCl medium (A8). The tubes were incubated at 37°C for 24 h. A circular growth from the line of stab represented a positive test.

3.4.3. Cultural characteristics

Growth on medium subjected to variable range of salt concentration was observed after incubating the cultures at 37°C for 2 days in salt trypticase broth (B8). The sodium chloride concentration in broth media was 3 %, except in the salt range experiments in which concentration of 0, 6, 8 and 10 % were used.

3.4.4. Oxidation and fermentation of glucose

In order to study the oxidation and fermentation of glucose. heated the tubes containing Hugh and Leifson's salt medium (A7) in boiling water bath for 10 min, cooled and inoculated. One set of tube was incubated aerobically and the other set anaerobically by sealing the surface of the medium with 2 cm sterile liquid paraffin and incubated at 37°C for 1 – 2 days. Oxidative metabolism is characterized by acid in aerobic tubes only and fermentative metabolism by the production of acid in both tubes.

3.4.5. Carbohydrate fermentation

Carbohydrate fermentation was observed in bromocresol purple broth (B4) containing 1 % of glucose, sucrose, lactose, manitol and 0.5 % arabinose, salicin and inositol.

3.4.6. Cytochrome oxidase

Placed a 6 cm square piece of filter paper into an empty petridish and added 3 drops freshly prepared 1 % aqueous solution of tetra methyl paraphenylene diamine dihydrochloride solution (C5) to its center. Smeared cells thoroughly on to the reagent impregnated paper in a line of 3 - 6 mm long with an inoculating loop (platinum). Oxidase positive strains turned dark purple in 5 – 10 seconds.

3.4.7. Indole production

The tubes containing 1 % tryptone broth with 3 % NaCl (B10) were inoculated and then incubated at 37°C for 24 h. 0.3 ml of indole reagent (C8) was added to the broth culture, shaken well and allowed the tubes to stand for 10 min and observed the results. A dark red colour in the amyl alcohol surface layer constituted a positive test.

3.4.8. Methyl red test

Inoculated MR-VP broth containing 3 % NaCl (B5) and incubated for 5 days at 37°C. Few drops of methyl red solution (C4) were added to the broth culture. A red colour indicated a positive test.

3.4.9. Voges- Proskauer test

MR-VP broth containing 3 % NaCl (B5) was inoculated, and the tubes were incubated at 37°C for 48 h. Pipetted 1ml of each culture to a separate empty culture tube and added 0.6 ml freshly prepared α naphthol solution (C9.a) and 0.2 ml of potassium hydroxide solution (C9.b). Shaken the tubes and allowed to stand for 2-4 h and observed the changes. A pink or crimson colour in the mixture indicated a positive test.

3.4.10. Nitrate reduction

Motility nitrate medium containing 3 % NaCl (A9) was used to test nitrate reduction. The tubes were inoculated as in 3.4.2. To the positive tubes 2 drops of sulphanilic acid (C6) and naphthylamine acetate (C7) in the ratio 1:1 was added.

The reduction of nitrate to nitrite is indicated by the production of a distinct red coloration.

3.4.11. Gelatinase production

Inoculated the nutrient gelatin tubes containing 3 % NaCl (B7) with the culture and incubated the tubes at 37°C for 5 days. The tubes were placed in ice water for 10 min and then observed the liquefaction.

3.4.12. H_2S and gas production

Streaked the triple sugar iron salt agar containing 3 % NaCl (A5) slants and stabbed the butt. Incubated at 37°C for 24 h. Black colour after incubation indicates H_2S production. Gas production indicates negative test.

3.4.13. Amino acid decarboxylation

Arginine dihydrolase, lysine and ornithine decarboxylase test was performed by inoculating heavily the tubes of arginine dihydrolase, lysine and ornithine decarboxylase broth (B2) and of basal medium containing 3 % NaCl (control) with the culture. A layer of 10 mm thick sterile liquid paraffin was added to each tube. Inoculated the tubes at 37°C for 4-5 days. The medium turned alkaline (Purple colour) when decarboxylation occurred.

3.4.14. Citrate test

Streaked on to Simmon's citrate agar slants containing 3 % NaCl (A3) and incubated the tubes at 37°C for 48 h. A blue colour indicates positive test.

3.4.15. 0/129 pteridine sensitivity

Sensitivity towards 2, 4 – diamino- 6, 7- diisopropyl pteridine was tested by using disc diffusion method as described below:

Sterile cotton swab was inserted into 18 h old nutrient broth containing 3 % NaCl (C2) culture of the organism and rotated it while pressing against the upper sidewall of the tube above the culture fluid level to remove the excess inoculum. The swab was then streaked on to the surface of the pre-set surface dried nutrient agar containing 3 % NaCl (A2) plates and allowed to dry for 10 min at R.T. The pteridine discs having standard strengths i.e. 10 µg/disc and 150 µg/discs were placed apart on the plates using sterile forceps. The plates were then incubated at 37°C for 24 h and observed for zone of inhibition.

3.5. Effect of washing on HPVs

3.5.1. Organism used

V. cincinnatiensis, *V.fluvialis*, *V.furnissii* and *V.parahaemolyticus* isolated from the seafood samples during the study. Strains of *V. fluvialis* and *V. parahaemolyticus* were compared with the type culture obtained from ATCC and NCMB. (*V. fluvialis* ATCC- 33809, *V. parahaemolyticus* NCMB-1902).

3.5.2. Substratum used for inoculation and its preparation

Prawns (*Penaeus indicus*) were purchased from the Cochin market, brought to the laboratory in iced condition, peeled, deveined and washed thoroughly with potable water and boiled for 5 min to destroy the native bacteria present in it. Cooked prawns were then drained and allowed to cool. To study the effect of washing on each species 200 g prawns were used.

3.5.3. Preparation of bacterial suspension

Each species of HPVs were inoculated into tubes containing sterile brain heart infusion broth with 3 % NaCl (B3) and incubated at 37°C for 24 h. The broth culture of each species was then centrifuged for 15 min at 5000 rpm and the supernatent was aseptically decanted off. 10 ml sterile 3 % NaCl solution (C2) was then added to the sediment and the contents of the tube was shaken so as to get a uniform suspension of the cells.

3.5.4. Inoculation of the organisms in to the prawns

2 ml Cell suspension was aseptically transferred to a beaker containing 400 ml sterile 3 % NaCl solution (C2), stirred well with a sterile glass rod. 200 g prawn was then transferred to the cell suspension, stirred well and kept aside for 30 min.

3.5.5. Washing of prawns inoculated with HPVs

Inoculated prawns were transferred to a flask containing sterile tap water (500 ml) with the help of sterile seave and shaken well for 3 min. Prawns were then drained and transferred to another flask containing 500 ml sterile tap water. This procedure was repeated twice, separate sets of sterile beakers and flasks were used for each species.

3.5.6. Enumeration of HPVs after each wash

Load of each species was determined immediately after inoculation and after each washing. Pour plate method was followed for the determination of load of each species.

3.5.7. Determination of the load

Since a particular organism alone had been separately inoculated into sterile prawns and there was no necessity of using a selective medium, nutrient agar (NA) with 3 % NaCl (A2) was used for plating. 1 ml of the homogenate was taken out and plated with nutrient agar supplemented with 3 % NaCl (C2). The plates were incubated at 37°C for 48 h and colony count was taken to find out the load of each species.

3.6. Effect of chilling on HPVs

3.6.1. Organisms used

As mentioned earlier in 3.5.1.

3.6.2. Substratum used for inoculation and its preparation.

Sample was prepared for inoculation as in 3.5.2. To study the effect of chilling on each species of HPVs 250 g prawns were used.

3.6.3. Preparation of bacterial suspension

Cell suspensions were prepared as in 3.5.3.

3.6.4. Inoculation of the organism in to the prawns

2 ml cell suspension was transferred to a flask containing 500 ml sterile 3 % NaCl solution (C2). Cooked prawns were then aseptically transferred to the cell suspension, stirred well with sterile glass rod and kept for 30min. The prawns were then drained.

3.6.5. Chilling of prawns inoculated with HPVs

Inoculated cooked prawns were transferred to sterile polythene bags sealed and placed in an insulated box with flake ice in 1:1 ratio. Inside the icebox polythene bags were kept in such a manner so as to allow maximum contact surface with ice. The icebox was then placed in a chill room (temperature maintained

at < 4°C). Fresh ice was added to the ice box after every 24 h, melted water was removed through the drain pipe of the ice box

3.6.6. Enumeration of HPVs during chilling

Load was analyzed immediately after inoculation. For analyzing the load polythene bags were taken out from the icebox and aseptically cut open. Approximately 10 g of the samples were taken out. Load of each species was analyzed after 24 h, followed by every 48 h for 12 days as mentioned earlier in 3.5.6.

3.6.7. Determination of the load

As mentioned earlier in 3.5.7. After each sampling bag was sealed again and kept inside the icebox.

3.7. Effect of low temperature (4°C and10 °C) storage on HPVs.

3.7.1. Organisms used :

As mentioned earlier in 3.5.1.

3.7.2. Substratum used for inoculation and its preparation

Prawns (*P. indicus*) purchased from the Cochin market was brought to the laboratory in iced condition, peeled and washed with potable water. 20 % prawn homogenate was prepared in three different media i.e. Distilled Water (DW), Normal Saline (NS) (0.85 % NaCl) (C1) and 3 % NaCl solution (C2). 10 ml of the homogenate was transferred to test tubes and sterilized at 121°C for 15 min.

3.7.3. Preparation of bacterial suspension

Cell suspensions were prepared as in 3.5.3.

3.7.4. Inoculation of the organisms in to the prawn homogenate

100 µl of the cell suspension was transferred to each tube containing sterile shrimp homogenate with different media.

3.7.5. Exposure of the HPVs to low temperature

Inoculated tubes were kept at low temperatures i.e. 4°C and 10°C.

4°C was maintained in chill room.10°C was obtained by keeping the tubes in the lower compartment of a refrigerator.

3.7.6. Enumeration of HPVs

Initial load of the homogenate was determined immediately after inoculation as mentioned in 3.5.6. Load during storage at different time intervals was done initially after 24 h followed by every 2 days for the first 2 weeks, weekly till the third month and finally once in a month.

3.8. Effect of freezing (–40°C) and frozen storage (–20°C) on HPVs.

3.8.1. Organisms used

As mentioned earlier in 3.5.1.

3.8.2. Substratum used for inoculation and its preparation

Shrimp homogenate was prepared as mentioned in 3.7.2. 10 ml homogenate was transferred to stainless steel dishes and sterilized at 121°C for 15 min.

3.8.3. Preparation of bacterial suspension

Cell suspension was prepared as mentioned in 3.5.3.

3.8.4. Inoculation of the organism in to the prawn

100 µl of the cell suspension was transferred to each tube containing sterile shrimp homogenate with different media.

3.8.5. Exposure of HPVs to freezing and frozen storage temperatures

Inoculated shrimp homogenates in stainless steel dishes were kept at freezing temperatures i.e.–40°C and –20°C.

3.8.6. Enumeration of HPVs after freezing and frozen storage

Initial load of the homogenate was determined immediately after inoculation as mentioned in 3.5.6. For freezing at –40°C, samples were kept in the freezer only for 2 h. Load of the samples were analyzed at a time interval of 90 min and 120 min.

For frozen storage (–20°C) samples were kept until the complete destruction of the inoculated organism. Initial sampling was done after 24 h followed by every 48 h till the viability was lost.

3.9. Effect of drying on HPVs

3.9.1. Effect of sun drying

3.9.1.1 Organism used

As mentioned earlier in 3.5.1.

3.9.1.2. Substratum used for inoculation and its preparation

Fresh silver belly (*Leognathus* sp.) was purchased from the market and brought to the laboratory in iced condition. Samples were washed with potable water to remove slime and other debris. 500 g of fish were used to study the effect of sun drying on each species.

3.9.1.3. Preparation of bacterial suspension

Bacterial suspensions were prepared as mentioned earlier in 3.5.3.

3.9.1.4. Inoculation of the organism in to the fish

5 ml of the suspension was added to 1000 ml sterile 3 % NaCl solution (C2) taken in a beaker. Stirred well with a sterile glass rod. Fish sample was then dipped in cell suspension for 1 min and drained.

3.9.1.5. Drying the sample

Inoculated fish samples were dried for 3 consecutive days in the sun on cleaned, raised platform covered with net to protect against birds, flies and insects. Air temperature was noted.

3.9.1.6. Enumeration of HPVs

Enumeration of organisms was done immediately after inoculation, after 1 h and 3 h drying. Thereafter load was analyzed every 24 h for 3 consecutive days. Simultaneous with bacterial analysis moisture content and water activity of the inoculated fish samples were determined. Load of each species on the inoculated fish sample was determined by 3 tube MPN method as described in Bacteriological Analytical Manual (Anon, 1995). Alkaline peptone salt (APS) broth (B1) was used as the enrichment medium and incubated at 37°C for 24 h. After streaking from APS broth to thiosulphate citrate bile salts sucrose (TCBS) (A4) agar plates, it was incubated at 37°C for 18 h. Typical colonies appearing on TCBS plates were picked up and subjected to a series of biochemical tests

specific to each species. After biochemical confirmation, MPN table was referred and load was determined (Anon, 1995). Moisture content was analyzed as per AOAC method (AOAC, 1975). Water activity was measured with Aqua Lab Water Activity Meter (AOAC international approved method).

3.9.2. Effect of salt curing and sun drying

3.9.2.1 Organisms used

As mentioned earlier in 3.5.1.

3.9.2.2 Substratum used for inoculation and its preparation

Fresh Barracuda (*Sphyraena sp.*) purchased from the local market was used for the study. Samples were brought to the laboratory in iced condition. Fish was split open by a cut from dorsal side along the vertebral column, gills and intestine were removed. The knife was past deep along the vertebral column and the fish was flattened out. Washed with potable water. 500 g samples were used to study the effect of salt curing and sun drying on each species of HPVs.

3.9.2.3. Preparation of bacterial suspension

Cell suspension was prepared as mentioned earlier in 3.5.3.

3.9.2.4. Inoculation of the organism into the fish

As mentioned earlier in 3.9.1.4.

3.9.2.5. Curing and sun drying

Inoculated samples were arranged in stainless steel basin with sodium chloride in 1:4 ratios. After 48 h the samples were taken out, drained and sun dried for 3 consecutive days. During drying, the fish was turned upside down occasionally to facilitate quicker and even drying.

3.9.2.6. Enumeration of HPVs during drying

Enumeration was done as mentioned earlier in 3.9.1.6. Moisture content and water activity of the inoculated fish samples were determined.

3.10. Effect of blanching on different species of HPVs

3.10.1. Organisms used

As mentioned earlier in 3.5.1.

3.10. 2. Substratum used for inoculation and its preparation

Sample was prepared for inoculation as mentioned earlier in 3.5.2. To study the effect of blanching on each species of HPVs 200 g prawns were used.

3.10.3. Preparation of bacterial suspension

Cell suspension was prepared as mentioned earlier in 3.5.3.

3.10.4. Inoculation of the organism into the prawns

As mentioned earlier in 3.5.4.

3.10.5. Blanching of prawns inoculated with HPVs.

To study the effect of blanching, water was boiled in a stainless steel vessel. Inoculated prawns were transferred to the boiling water. A temperature reduction of 5°C was observed due to the addition and the temperature was maintained at 100°C immediately. Samples were drawn after every 5 seconds for 1 min. A sterile metallic sieve was used for draining the samples from boiling water. Immediately after draining the samples were cooled to protect the surviving cells.

3.10.6. Enumeration of HPVs

Samples were drawn after every 5 seconds for 1 min to analyze the load. Pour plate method was followed as mentioned earlier in 3.5.6.

3.11. Effect of sodium chloride on HPVs

3.11.1 Organisms used

As mentioned earlier in 3.5.1.

3.11.2. Substratum used for inoculation and its preparation

For each species, a set of tubes containing 10 ml salt trypticase broth (B8) supplemented with 1 –10 % NaCl in duplicate were used.

3.11.3. Preparation of bacterial suspensions

Cell suspensions were prepared as mentioned earlier in 3.5.3.

3.11.4. Inoculation of the organism into the broth

25 µl of the cell suspension was aseptically transferred by means of a sterile microtiter pipette.

3.11.5 Incubation of the inoculated tubes

Inoculated tubes were shaken well and incubated at 37°C for 24 h.

3.11.6. Enumeration of HPVs

Optical density of each tube was measured with the help of a spectrophotometer at 666 nm wavelength.

3.12. Effect of pH on HPVs

3.12.1. Organisms used

As mentioned earlier in 3.5.1.

3.12.2. Substratum used for inoculation and its preparation

Substratum used for inoculation was prepared as mentioned earlier in 3.7.2. pH of the homogenate was adjusted (1-14) by using 1N NaOH and 1N HCl with the help of a pH meter. Homogenate was distributed in test tubes (10 ml) and sterilized at 15lbs for 15 min.

3.12.3. Preparation of bacterial suspension

Cell suspension was prepared as mentioned earlier in 3.5.3.

3.12.4. Inoculation of the organism

As mentioned in 3.7.4.

3.12.5. Incubation of the inoculated tubes

Inoculated shrimp homogenates were shaken well and incubated at 37°C for 24 h.

3.12.6. Enumeration of HPVs

Load of each species in the shrimp homogenate (pH 1-14) was determined immediately after inoculation and incubation. Pour plate method was followed as mentioned earlier in 3.5.6.

3.13. Effect of chlorine and chlorine dioxide on HPVs

3.13.1. Effect of chlorine

3.13.1.1. Organisms used

As mentioned earlier in 3.5.1.

3.13.1.2. Substratum used for inoculation and its preparation.

500 ml DW, NS (C1) and 3 % NaCl (C2) solution was prepared in glass stopper bottles and sterilized at 121°C for 15 min.1 bottle of 500 ml 3 % NaCl solution was maintained as control.

3.13.1.3. Preparation of bacterial suspension

Cell suspension was prepared as mentioned earlier in 3.5.3.

3.13.1.4. Inoculation of the organisms

1 ml cell suspension was transferred to each bottle with 500 ml sterile DW, NS and 3 % NaCl solution and control

3.13.1.5. Chlorination of the water samples

Required quantity of sodium hypochlorite was added to the bottle to get residual chlorine level of 1, 2, 5 and 10 ppm and stirred well. Control samples were maintained without chlorine.

3.13.1.6. Enumeration of HPVs subjected to different chlorine levels

Initial load of the sample was noted immediately after inoculation. After chlorination samples were analyzed after 1, 2, 5, 10, 15, 30 and 60 min. 1 ml of the water sample was drawn and load was analyzed as mentioned earlier in 3.5.6.

3.13.2. Effect of chlorine dioxide

3.13.2.1. Organisms used

As mentioned earlier in 3.5.1.

3.13.2.2. Substratum used for inoculation and its preparation

As mentioned earlier in 3.13.1.2.

3.13.2.3. Preparation of bacterial suspension

Cell suspension was prepared as mentioned earlier in as in 3.5.3.

3.13.2.4. Inoculation of the organism

As mentioned in 3.13.1.4.

3.13.2.5. Addition of ClO_2 to the water sample

Chlorine dioxide along with 0.1 % citric acid at calculated level was added to the bottle containing different media and cell suspensions to get 1 ppm, 2 ppm, 5 ppm and 10 ppm available chlorine dioxide, shaken well. Control samples were run without chlorine dioxide.

3.13.2.6. Enumeration of HPVs subjected to different chlorine dioxide levels

As mentioned earlier in 3.13.1.6.

3.14. Antibiotic sensitivity of HPVs

3.14.1. Organism studied

Name and number of different species of HPVs subjected to in vitro sensitivity tests to antibiotics are given below.

Name	Number
V. cincinnatiensis	22
V. fluvialis	3
V. furnissii	2
V. parahaemolyticus	22

3.14.2. Antibiotics used

The antibiotics used in this study, their symbol, concentration per disc and classification of inhibition zones are given in Table 4.

3.14.3. Sensitivity testing

Sterile cotton swab was inserted into 18 h old tryptic soy broth supplemented with 3 % sodium chloride (B9) culture of the organism and rotated it while pressing against the upper sidewall of the tube above the culture fluid level to remove the excess inoculum. The swab was then streaked on to the surface of the preset surface dried Muller Hinton agar (A1) plates supplemented with 3 % NaCl, and allowed to dry for 10 min at room temperature. The antibiotic discs having standard strengths were placed apart on the plates using sterile forceps in such a way that there was no chance of overlapping of zones of inhibition around the

discs. The plates were then incubated at 37°C for 24 h and the zone of inhibition around each disc was measured and interpreted as per Table 4.

Table 4. Concentration of different antibiotics tested, their symbols and classification of inhibition zones

Antibiotics	Symbol	Conc. mcg/disc	Resistant mm or less	Intermediate mm	Sensitive mm or more
Amikacin	Ak	30	14	15 -16	17
Ampicillin	A	10	13	14 -16	17
Ceftriaxone	Ci	30	13	14 -20	21
Chloramphenicol	CH	30	12	13 -17	18
Erythromycin	Er	15	13	14 -22	23
Kanamycin	Ka	30	13	14 -17	18
Nalidixic acid	NA	30	13	14 -18	19
Penicillin G	P	10 units	19	20 -27	28
Streptomycin	S	10	11	12 -14	15
Tetracycline	Te	30	14	15 -18	19
Trimethoprin	Tr	10	10	11 -15	16

3.15. Haemolytic activity of HPVs

3.15.1. Different species of HPVs used for the study

Name	Number
V. cincinnatiensis	22
V. fluvialis	3
V. furnissii	2
V. parahaemolyticus	22

3.15.2. Medium used

Wagatsuma agar, Modified. (A6)

3.15.3 Preparation of washed human erythrocytes suspension.

Fresh human blood was collected, defibrinated by shaking with sterile glass beeds and centrifuged. Washed thrice the erythrocytes with sterile normal saline solution (C1), suspended one volume of the last sedimented erythrocytes with four volumes of saline solution. Washed suspension of the erythrocytes (20 %) was added to the boiled and cooled to 50°C Wagatsuma agar. Mixed and transferred to plates. Surface of the plates was dried by keeping the plates at 45°C for 45 min.

3.15.4. Preparation of broth culture, spotting and incubation

24 h old broth culture of each strain was prepared in typtic soya 3 % NaCl (TS-3) broth (B9). Spotted several loop full of the broth culture on a single surface dried Wagatsuma agar plate in circular pattern. Incubated at 37°C and observed the results after 18-24 h. Clear transparent zones around the colonies indicated a positive test.

3.16. Media formulae and Preparation

A1. Muller-Hinton agar

Beef infusion	300.0 g
Acid hydrolysate of casein	17.5 g
3 % NaCl	30.0 g
Agar	17.0 g
Starch	1.5 g

Added the above to 1 liter of distilled water mixed thoroughly, gently heated to boiling. pH was adjusted to 7.4 ± 0.2 at 25°C. Distributed into flasks and autoclaved at 121°C for 15 min.

A2. Nutrient agar with 3 % NaCl

NaCl	30.0 g
Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g

Added all ingredients to 1 liter distilled water, heated to boiling, cooled to 50-60° C and adjusted pH to 6.8 – 7.0. Distributed in tubes for slants and in flasks for plates, as required, and autoclaved at 121°C for 15 min.

A3. Simmon's citrate agar

Sodium citrate	2.0 g
K_2HPO_4	1.0 g
$(NH_4)H_2PO_4$	1.0 g
Mg $SO_4.7H_2O$	0.2 g
Bromothymol blue	0.08 g
NaCl	30.0 g
Agar	15.0 g

Added all ingredients to 1000 ml distilled water, mixed well and heated to boiling. Cooled to 25°C and adjusted the pH to 6.9 ± 0.2. Filled the tubes 1/3 and plugged. Autoclaved at 121°C for 15 min. Cooled the tubes in slanting position to obtain butts (2.5 cm long) and slants (5 cm long).

A4. Thiosulfate citrate bile salts sucrose agar (TCBS)

Yeast extract	5.0 g
Peptone	10.0 g
Sucrose	20.0 g
Sodium thiosulphate. Pentahydrate	10.0 g
Sodium citrate dihydrate	10.0 g
Sodium cholate	3.0 g
Ox- gall	5.0 g
Sodium chloride	10.0 g
Ferric citrate	1.0 g
Bromothymol blue (0.2 %) solutions	20.0 ml
Thymol blue (1 % solution)	4.0 ml
Agar	15.0 g

Added the ingredients to 980 ml distilled water and heated to boiling with agitation to obtain complete solution, adjusted pH to 8.6. Not autoclaved, cooled to 45-50°C and poured 15-20 ml volume into sterile petridishes.

A5. Triple sugar iron agar

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	15.0 g
Protease peptone	5.0 g
Glucose	1.0 g
Lactose	10.0 g
Sucrose	10.0 g
Ferrous sulfate	0.2g
Sodium chloride	30.0 g
Sodium thiosulphate	0.3 g
Phenol red (0.2 % solution)	12.0 ml
Agar	12.0 g

Added all ingredients to 988 ml distilled water, mixed well and heated to boiling. Cooled to 50-60°C and adjusted the pH to 7.3 to ± 0.1. Filled the tubes 1/3 and plugged. Autoclaved at 121°C for 12 min. Cooled the tubes in slanting position to obtain butts (2.5 cm long) and slants (5 cm long).

A6. Wagatsuma agar, Modified

Yeast extract	5.0 g
Peptone	10.0 g
Sodium chloride	70.0 g
Mannitol	5.0 g
Crystal violet (0.1 % w/v solution in ethyl alcohol)	1.0 ml
Agar	15.0 g

Dissolved all ingredients in 1 liter of distilled water and adjusted pH to 7.5. Heated to boiling for several min. Did not autoclave, cooled to 50°C and added 100 ml of washed 20 % suspension of human erythrocytes, mixed and poured to plates. Dried the plates before use.

A7. Hugh-Leifson salt medium

Peptone	2.0 g
Sodium chloride	30.0 g
Glucose	10.0 g
Potassium monohydrogen Phosphate	0.3 g
Bromthymol blue (0.2 % solution)	15.0 ml
Agar	3.0 g

Added all ingredients to 985 ml distilled water and heated to boiling. Dispensed in 5 ml quantities into small test tubes and autoclaved at 115°C for 15 min. Final pH 7.1

A8. Motility test 3 % NaCl medium

Beef extract	3.0 g
Peptone	10.0 g
Sodium chloride	30.0 g
Agar	4.0 g

Dissolved all ingredients in 1 liter distilled water by heating with occasional agitation. Adjusted pH to 7.4. Dispensed approximately 8 ml portions in to tubes. Autoclaved at 121°C for 15 min.

A9. Motility nitrate medium

Beef extract	3.0 g
Peptone	5.0 g
Potassium nitrate	1.0 g
Agar	3.0 g
NaCl	30.0 g

Dissolved all ingredients in 1 liter distilled water by heating with occasional agitation, adjusted pH to 7.4. Dispensed approximately 8 ml portions into tubes. Autoclaved at 121°C for 15 min.

B1. Alkaline peptone salt (APS) broth

NaCl	30.0 g
Peptone	10.0 g

Added the ingredients to 1 liter distilled water. Heated to boiling to dissolve completely. pH was adjusted to 9 ± 0.2. Distributed 10 ml quantities in tubes and autoclaved at 121°C for 15min. Double strength and single strength were prepared as required.

B2. Amino acid decarboxylase medium

Peptone	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Bromocresol purple (1 % solution)	1.6 ml
NaCl	30.0 g

Dissolved all components in 1 litre distilled water and adjusted the pH to 6.8. Divided the basal broth into 3 portions. Added 0.5 % of L-arginine, L-lysine and L-ornithine to each portion. Dispensed in 3 ml quantities into tubes and autoclaved at 121°C for 10 min.

B3. Brain heart infusion broth

Beef heart infusion	250.0 g
Calf brain infusion	200.0 g
Proteose peptone	10.0 g
NaCl	30.0 g
$Na_2 HPO_4.12 H_2O$	2.5 g
Glucose	2.0 g

Dissolved the ingredients in 1 liter distilled water. Mixed thoroughly and dispensed 10 ml quantities into tubes. Autoclaved at 121°C for 15min.

B4. Bromocresol purple broth

Peptone	10.0 g
Beef extract	3.0 g
Sodium chloride	30.0 g
Bromocresol purple	0.04 g

Dissolved the ingredients in 1 liter distilled water, divided into seven equal parts and added 1 % of glucose, lactose, sucrose, mannitol and 0.5 % of arabinose, salicin and inositol to each portion. Dispensed 5 ml quantities into small tubes and autoclaved at 10 lbs for 15 min.

B5. MRVP Broth

Protease peptone	7.0 g
Glucose	5.0 g
K2 HPO4	5.0 g
NaCl	30.0 g

Added the ingredients to 1 l distilled water, dissolved by heating. Adjusted the pH to 6.9 + 0.1. Dispensed in 5 ml portions into culture tubes, sterilized at 121° C for 15 min.

B6. Nutrient Broth

Beef extract	3.0 g
Peptone	5.0 g
NaCl	30.0 g

Dissolved the ingredients in 1 liter distilled water. Autoclaved at 121°C for 15 min. Final pH 6.9.

B7. Nutrient gelatin

Beef extract	3.0 g
Peptone	5.0 g
Gelatin	120.0 g
NaCl	30.0 g

Suspended the ingredients in 1 liter distilled water, mixed well and heated to boiling. Adjusted the pH to 6.9 ± 0.1. Dispensed 5ml portions into narrow tubes and sterilized at 121°C for 15 min.

B 8. Salt trypticase broth (STB)

Trypticase	10.0 g
Yeast extract	3.0 g
Distilled water	1000 ml

Basal medium was divided into four equal portions, sodium chloride was added at required concentration to get 0 %, 6 %, 8 %, and 10 %. pH of the media was adjusted to 7.5. Autoclaved at 121°C for 15 min.

B9. Tryptic soya 3 % NaCl (TS-3) broth

Trypticase	15.0 g
Phytone	5.0 g
Potassium monohydrogen Phosphate	2.5 g
Glucose	2.5 g
Sodium chloride	30.0 g

Dissolved all ingredients in 1 liter distilled water. Dispensed in 7-10 ml portions in to tubes and autoclaved at 121°C for 15 min.

B10. Tryptone broth

Tryptone	10.0 g
Sodium chloride	30.0 g

Dissolved in 1 litre distilled water, dispensed in 6 ml portions into culture tubes. Adjusted the pH to 7.3 and sterilized in an autoclave at 121°C for 15 min.

C1. Normal saline (physiological saline)

Sodium chloride	8.5 g
Distilled water	1 l

Dissolved sodium chloride in distilled water, sterilized at 121°C for 15 min.

C2. 3 % Sodium chloride solution

Sodium chloride	30.0 g
Distilled water	1 l

Dissolved sodium chloride in distilled water, dispensed 9 ml each into tubes and 90 ml into flasks as required and sterilized at 121°C for 15 min.

C4. Methyl red solution

Methyl red	0.1 g
Ethyl alcohol	300 ml
Distilled water	500 ml

Dissolved methyl red in ethyl alcohol and diluted with water.

C5. 1 % Tetra methyl paraphenylene diamine dihydro chloride

Tetra methyl paraphenylene diamine dihydro chloride	1.0 g
Distilled water	100 ml

C6. Sulfanilic acid reagent

Sulfanilic acid	1.0 g
Acetic acid (5N aqueous soln.)	125 ml

C7. Alpha- naphthol reagent

Alpha- naphthol	1.0 g
Acetic acid 5 N	200 ml

C8. Indole reagent (Kovac)

Paradimetyl amino benzaldehyde	5.0 g
Amyl alcohol	75 ml
HCl (Conc.)	25 ml

Dissolved benzaldehyde in amyl alcohol and added the hydrochloric acid.

C9. Voges- Proskauer test reagents

a) α Naphthol solution

α Naphthol	5.0 g
Absolute alcohol	100 ml

Dissolved α Naphthol in absolute alcohol.

b) 40 % Potassium hydroxide

Potassium hydroxide	40.0 g
Creatine	0.3g
Distilled water	100 ml

Dissolved KOH and creatine in 100 ml distilled water.

4. Results and Discussion

4.1. Incidence of HPVs in fish and fish products

One hundred and thirty samples consisting of iced, block frozen and IQF fish and fish products collected from landing centers and fish processing factories situated in and around Cochin (Table. 5) were examined for the incidence of HPVs. The characteristics colonies of *V. alginolyticus, V. cincinnatiensis, V. fluvialis, V. furnissii, V. metshnikovii, V. parahaemolyticus* and *V. vulnificus* on TCBS are shown in Plate 1 to 7.

HPVs were present in 66.7 % of iced samples, 44.2 % of block frozen fish products and 29.2 % of IQF fish products (Table.5). Among iced samples the incidence was maximum in iced squid 85.7 % followed by iced shrimp (72.2 %) and iced cuttlefish (47.4 %).

Among block frozen fish products the incidence was high in shrimp (54.2 %) followed by squid (40.0 %) and cuttlefish (27.3 %). HPVs were isolated from 33.3 % of IQF shrimp 30.0 % of IQF squid. In general HPVs were isolated from 50.8 % of the sample and all samples of IQF cuttlefish were found free from these organisms.

4.1.1. Incidence of HPVs in iced samples

Incidence (%) and load (MPN g^{-1}) of various species of HPVs isolated from different fish products are given in Table 6. *V. alginolyticus* was isolated from 44.4 % of the shrimp samples, 35.7 % of the squid samples and 10.5 % of the cuttlefish samples. Maximum load was observed with shrimp whole samples and the load varied between $2.3X10^1$ to $3.6x\ 10^2$ MPN g^{-1} (Table.7), followed by shrimp PUD samples (<10 to 2×10^2 MPN g^{-1}) and shrimp PD samples (<10 MPN g^{-1}). Among iced squid samples the maximum load of *V. alginolyticus* was observed with squid rings 1.5×10^2 MPN g^{-1}, followed by squid whole (3×10^1MPN g^{-1}) and squid whole cleaned samples (<10 to 2.1×10^1 MPN g^{-1}). Among iced cuttlefish samples only cuttlefish whole showed the incidence of *V. alginolyticus* and the load varied between 2.7×10^1 to 2×10^2 MPN g^{-1}.

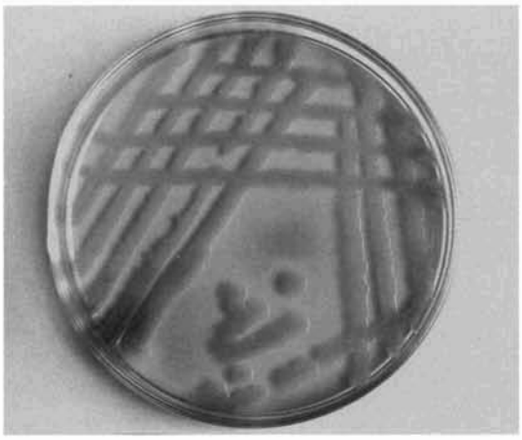


Plate 1. *V. alginolyticus* on TCBS Agar

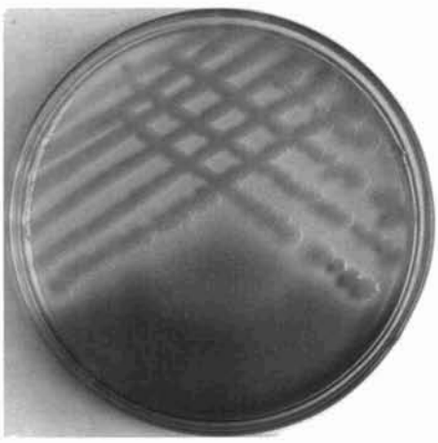


Plate 2. *V. cincinnatiensis* on TCBS Agar

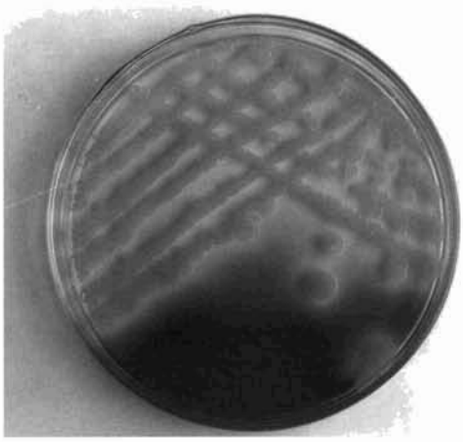


Plate 3. *V.fluvialis* on TCBS Agar

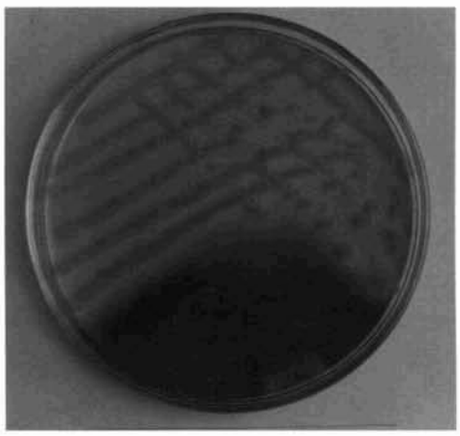


Plate 4. *V. furnissii* on TCBS Agar

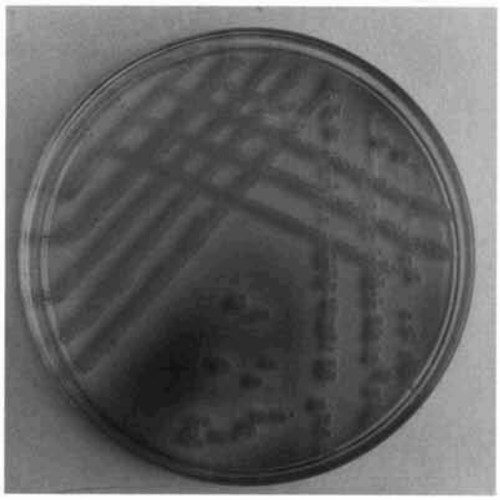


Plate 5. *V. metschnikovii* on TCBS Agar

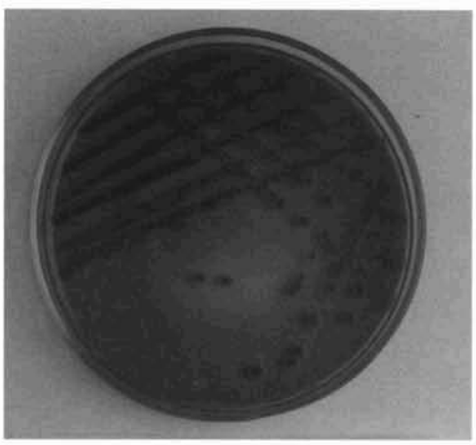


Plate 6. *V. parahaemolyticus* on TCBS Agar

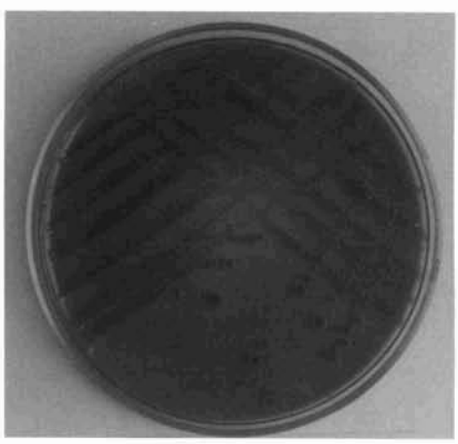


Plate 7. *V. vulnificus* on TCBS Agar

Table 5. Incidence of HPVs in various samples

Name of sample	No. of samples tested	No. of Samples with HPVs	Percentage	Total Percentage
Iced				
Shrimp	18	13	72.2	
Squid	14	12	85.7	
Cuttlefish	19	9	47.4	
Others	3	2	66.7	
				66.7
Block frozen				
Shrimp	24	13	54.2	
Squid	15	6	40.0	
Cuttlefish	11	3	27.3	
Others	2	1	50.0	
				44.2
IQF				
Shrimp	3	1	33.3	
Squid	10	3	30.0	
Cuttlefish	8	0	0.0	
Others	3	3	100.0	
				29.2
Total	130	66		50.8

Table 6. Incidence(%) and load (MPN g^{-1})of various species of HPVs in different fish products

Name of sample		*V. alginolyticus*		*V. cincinnatiensis*		*V. damsela*		*V. fluvialis*		*V. furnissii*		*V. metschnikovii*		*V. parahaemolyticus*		*V. vulnificus*	
		I (%)	L (MPN g^{-1})	I (%)	L (MPN g^{-1})	I (%)	L (MPN g^{-1})	I (%)	L (MPN g^{-1})	I (%)	L (MPN g^{-1})	I (%)	L (MPN g^{-1})	I (%)	L (MPN g^{-1})	I (%)	L(MPN g^{-1})
Iced	Shrimp	44.4	3-360	22.2	30-240	5.3	3.6	-	-	-	-	11.1	3.6-5.6	22.2	6-7.3	33.3	11-530
"	Squid	35.7	1.2-150	50.0	0.3-73	-	-	-	-	7.1	30	7.1	3	7.1	36	57.1	0.6-350
"	Cuttlefish	10.5	27-200	31.6	0.36-930	-	-	5.3	0.3	-	-	-	-	-	-	10.5	30-91
"	Others	-	-	33.3	53	-	-	-	-		-	33.3	0.36	-	-	33.3	0.3
Block frozen	Shrimp	32.0	0.72-3.6	36.0	0.36-9.3	-	-	-	-	-	-	-	-	12.0	0.3-6	20.0	0.3-11
"	Squid	20.0	0.36-9.4	26.7	0.36-2.3	-	-	-	-	6.7	0.3	6.7	0.3	-	-	-	-
"	Cuttlefish	9.1	4.3	27.3	0.36-0.91	-	-	-	-	-	-	-	-	-	-	-	-
"	Others	-	-	-	-	-	-	-	-	-	-	-	-	100.0	3	100.0	11
IQF	Shrimp	33.3	3.6	33.3	9.1	-	-	-	-	-	-	33.3	3.6	-	-	-	-
"	Squid	30.0	0.36-1.5	20.0	0.3-2	-	-	-	-	-	-	10.0	0.36	10.0	0.3	-	-
"	Cuttlefish	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
"	Others	100.0	0.3	100.0	0.73-1.5	-	-	-	-	-	-	-	-	-	-	-	-
Total		26.2	0.3-360	30.8	0.3-930	0.8	3.6	0.8	0.3	1.5	0.3-30	5.4	0.3-5.6	7.7	0.3-36	17.7	0.3-530

I = Incidence
L= Load
- = Nil

Name of iced samples	Total no.of sample	No. of samples with HPVs within the range MPN g^{-1}	*V. algino*	*V. cinci*	*V. dam*	*V. fluvi*	*V. furni*	*V. mets*	*V. para*	*V. vulni*
Shrimp :shrimp whole	10	1-10	0	0	0	0	0	1	0	0
		11-100	2	1	0	0	0	0	0	4
		101-999	2	1	0	0	0	0	0	1
		load range (MPN g^{-1})	23-360	30-110	0	0	0	5.6	0	11-530
shrimp PUD	4	1-10	1	0	1	0	0	1	1	0
		11-101	1	0	0	0	0	0	0	2
		101-999	1	2	0	0	0	0	0	0
		load range (MPN g^{-1})	3-200	110-240	3.6	0	0	3.6	6.2	19
shrimp PD	3	1-10	1	0	0	0	0	0	3	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	7.3	0	0	0	0	0	6-7.3	0
Shrimp headless	1	1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0
Squid : squid whole	3	.1-0.9	0	0	0	0	0	0	0	1
		1-10	0	2	0	0	0	0	0	0
		11-101	1	0	0	0	0	0	2	0
		101-999	0	0	0	0	0	0	0	1
		load range (MPN g^{-1})	30	1.5-3.6	0	0	0	0	36	0.6-350
Squid whole cleaned	9	0.1-0.9	0	1	0	0	0	0	0	0
		1-10	2	1	0	0	0	1	0	4
		11-101	1	2	0	0	1	0	0	1
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	1.2-21	0.3-7.3	0	0	30	3	0	3.6-33
Squid rings	1	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	1	0	0	0	0	0	1
		101-999	1	0	0	0	0	0	0	0
		load range (MPN g^{-1})	150	73	0	0	0	0	0	61

Contd

Squid tentacles	1	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0
Cuttlefish : Cuttlefish whole										
	2	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	1	2	0	0	0	0	0	1
		101-999	1	0	0	0	0	0	0	0
		load range (MPN g^{-1})	27-200	27-71	0	0	0	0	0	91
Cuttlefish whole cleaned	15	0.1-0.9	0	2	0	1	0	0	0	0
		1-10	0	1	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0.36-9.1	0	0.3	0	0	0	0
Cuttlefish roe	2	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	1
		101-999	0	1	0	0	0	0	0	0
		load range (MPN g^{-1})	0	930	0	0	0	0	0	30
Others	3	0.1-0.9)	0	0	0	0	0	1	0	1
		1-10	0	0	0	0	0	0	0	0
		11-101	0	1	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
Total	54	load range (MPN g^{-1})	0	53	0	0	0	0.36	0	0.3

0 = Not detected

Iced squid samples showed the highest incidence of *V. cincinnatiensis* (50.0 %), followed by iced cuttlefish samples (31.6 %) (Table 6) and iced shrimp samples (22.2 %). Maximum load was observed with shrimp PUD (1.1 x 10^2 to 2.4 x 10^2 MPN g^{-1}) and shrimp whole samples (3 x 10^1 to 1.1X 10^2 MPN g^{-1}) (Table.7). Among squid samples,squid rings showed the maximum load (7.3 x 10^1MPN g^{-1}). Load of *V. cincinnatiensis* was found to be < 10 MPN g^{-1} in squid whole and squid whole cleaned samples. Cuttlefish roe samples showed the maximum load (9.3 x 10^2 MPN g^{-1}), followed by cuttlefish whole (2.7 x 10^1 to 7.1 x 10^1 MPN g^{-1}) and cuttlefish whole cleaned samples (<10 MPN g^{-1}). Among others *V. cincinnatiensis* was isolated from an octopus whole cleaned sample and its load was 5.3 x 10^1 MPN g^{-1}.

5.3 % of the iced shrimp PUD sample showed the incidence *V. damsela* and 5.3 % of the iced cuttlefish whole cleaned sample showed the incidence of *V. fluvialis* (Table 6). Load of these organisms were found to be <10 MPN g^{-1}. 7.1 % of the squid whole cleaned samples showed the incidence of *V. furnissii*, load obtained was 3 X10^1 MPN g^{-1} (Table 7). None of the other samples showed the incidence of these species.

V. metschnikovii was isolated from 11.1 % of iced shrimp sample and 7.1 % of iced squid sample (Table 6). This species were isolated from shrimp whole, shrimp PUD and a squid whole cleaned sample (Table 7). Load of the species in the above mentioned samples were found to be <10 MPN g^{-1}. Among others, iced mussel samples also showed the incidence of this species (<10 MPN g^{-1}).

22.2 % of iced shrimp sample and 7.1 % of iced squid samples showed the incidence of *V.parahaemolyticus* (Table 6). Shrimp PUD and shrimp PD samples showed the incidence of the species and load obtained was <10 MPN g^{-1}. Slightly higher load ie. 3.6 X10^1 MPN g^{-1} was observed for this species in the squid whole sample in this study (Table 7).

V.vulnificus was isolated from 57.1 % of iced squid sample, 33.3 % of iced shrimp sample and 10.5 % of iced cuttlefish sample (Table 6). Maximum load was observed in shrimp whole samples (1.1 x 10^1 to 5.3 x 10^2 MPN g^{-1}), followed by shrimp PUD (1.9 x 10^1 MPN g^{-1}). Among iced squid samples maximum load was

observed in squid whole (<1 to 3.5 x 10^2 MPN g^{-1}), followed by squid rings (6.1 x 10^1 MPN g^{-1}) and squid whole cleaned samples (<10 to 3.3 x 10^1 MPN g^{-1}). Among cuttlefish, whole samples showed the maximum load (9.1 x 10^1 MPN g^{-1}) followed by cuttlefish roe sample (3 x 10^1 MPN g^{-1}). Among others mussels (<10 MPN g^{-1}) also showed the incidence of *V.vulnificus* (Table 7).

4.1.2. Incidence of HPVs in block frozen samples

32.0 % of block frozen shrimp samples, 20.0 % of block frozen squid samples and 9.1 % of cuttlefish samples showed the incidence of *V. alginolyticus* (Table 6). Block frozen samples generally showed a load ranging between <1 to 10 MPN g^{-1} (Table 8). Shrimp whole sample showed a load between 1 to 10 MPN g^{-1}, followed by shrimp PD samples (<1 to 10 MPN g^{-1}) and shrimp PUD (<1 MPNg^{-1}). Only squid whole cleaned samples showed the incidence of *V. alginolyticus* and the load varied between <1 to 10 MPN g^{-1}. Among block frozen cuttlefish samples, cuttlefish whole showed the incidence of *V. alginolyticus* and the load observed was <10 MPN g^{-1}.

Block frozen shrimp samples showed the highest incidence of *V. cincinnatiensis* (36.0 %) followed by block frozen cuttlefish (27.3 %) and block frozen squid samples (26.7 %) (Table 6). In shrimp whole samples the load varied between <1 to 10 MPN g^{-1}, while in shrimp PUD and shrimp PD samples load varied between 1 to 10 MPN g^{-1}. A load ranging between <1 to 10 MPN g^{-1} was observed in squid whole cleaned, cuttlefish whole and cuttlefish whole cleaned sample (Table. 8).

V. damsela and *V. fluvialis* couldn't be isolated from block frozen samples. 6.7 % of the block frozen samples showed the incidence of *V. furnissii* and *V. metschnikovii* (Table 6). Both the species were isolated from squid whole cleaned samples (Table 8) and the load was <1 MPN g^{-1}.

12 % of block frozen shrimp sample showed the incidence of *V.parahaemolyticus* (Table 6). In shrimp whole samples the load ranged between <1 to 10MPN g^{-1} and the load observed for shrimp PUD samples was <1MPN g^{-1} (Table 8). Among others crab soft shell showed the incidence of *V.parahaemolyticus* and the load observed was <10 MPN g^{-1}. None of the other block frozen samples showed the incidence of *V.parahaemolyticus*.

Name of block frozen samples	Total no.of sample	No: of samples with HPVs within the range MPN g^{-1}	*V. algino*	*V. cinci*	*V. dam*	*V. fluvi*	*V. furni*	*V. mets*	*V. para*	*V. vulni*
Shrimp : shrimp whole	8	0.1-0.9	0	2	0	0	0	0	1	0
		1-10	4	3	0	0	0	0	1	2
		11-101	0	0	0	0	0	0	0	1
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	1.1-3.6	0.36-9.1	0	0	0	0	0.36-6	1.1-11
shrimp PUD	9	0.1-0.9	2	0	0	0	0	0	1	1
		1-10	0	3	0	0	0	0	0	1
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0.72-0.73	1.5-9.3	0	0	0	0	0.3	0.3-2
Shrimp PD	6	0.1-0.9	1	0	0	0	0	0	0	0
		1-10	1	2	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0.73-1.5	1.5-9.3	0	0	0	0	0	0
shrimp headless shell on	2	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0
Squid : Squid whole	3	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0
Squid whole cleaned	11	0.1-0.9	1	3	0	0	1	1	0	0
		1-10	2	1	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0.36-9.4	0.36-2.3	0	0	0.3	0.3	0	0
squid tentacles	1	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0

Contd.

Cuttlefish: cuttlefish whole										
	3	0.1-0.9	0	2	0	0	0	0	0	0
		1-10	1	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	4.3	0.36-0.62	0	0	0	0	0	0
Cuttlefish Whole cleaned	7	0.1-0.9	0	1	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0.91	0	0	0	0	0	0
cuttlefish roe	1	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0
Others	1	0 1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	1	0
		11-101	0	1	0	0	0	0	0	1
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	3	11
Total	52									

0= Not detected

V.vulnificus was isolated from 20 % of block frozen shrimp samples (Table 6). Load of *V.vulnificus* in shrimp whole samples varied from <10 to 1.1 x 10^1 MPNg^{-1} and shrimp PUD samples showed a load range between <1 to10 MPN g^{-1} . Crab soft shell also showed the incidence of *V.vulnificus* and the observed load was 1.1X10^1 MPN g^{-1}. *V.vulnificus* could not be isolated from any other block frozen samples.

4.1.3. Incidence of HPVs in IQF samples

33.3 % of IQF shrimp samples showed the presence of *V. alginolyticus* (Table 6). Load of this species observed in IQF shrimp whole sample was <10 MPN g^{-1}. 30.0 % of the squid sample showed the presence of *V. alginolyticus*. <1 MPN g^{-1} was observed in IQF squid whole sample and <10 MPN g^{-1} was obtained in IQF squid whole cleaned sample. <1 MPN g^{-1} load was observed in IQF reef cod and IQF octopus whole cleaned sample (Table 9).

V. cincinnatiensis was isolated from 33.3 % (Table 6) of the IQF shrimp whole sample and the load obtained was <10 MPN g^{-1}. 20 % of the squid sample showed the incidence of this species, < 1 MPN g^{-1} load was observed in IQF squid whole sample, whereas <10 MPN g^{-1} was observed in IQF squid whole cleaned sample. IQF reef cod and octopus whole cleaned sample also showed the incidence of this species and the load varied between <1 MPN g^{-1} and <10 MPN g^{-1} (Table 9).

V. damsela, *V. fluvialis* and *V. furnissii* were found to be completely absent in IQF samples.

33.3 % of IQF shrimp sample and 10 % of IQF squid sample showed the incidence of *V. metschnikovii* (Table 6). Load obtained for IQF shrimp whole and squid whole cleaned samples were <10 MPN g^{-1} and <1 MPN g^{-1} respectively (Table 9).

10 % of IQF squid samples showed the incidence of *V.parahaemolyticus* (Table 6). Load observed for this species was <1 MPN g^{-1}. No other samples showed the incidence of this species (Table 9). All IQF samples were found free from *V.vulnificus*.

Name of HPVs isolated from different fish products, their percentage of incidence and load (MPN g^{-1}) are given in Table 10. *V. cincinnatiensis* was found to be the dominant species isolated (30.8 %, <1- 930 MPN g^{-1}) from different fish and fish

products followed by *V. alginolyticus* (26.2 %, <1-360 MPN g^{-1}), *V. vulnificus* (17.7 %, <1-530 MPN g^{-1}), *V. parahaemo-lyticus* (7.7 %, <1-36 MPN g^{-1}), *V. metschnikovii* (5.4 %, 0.3-5.6 MPN g^{-1}), *V. furnissii* (1.5 %, 0.3-30 MPN g^{-1}), *V. fluvialis* (0.8 %, <1 MPN g^{-1}) and *V. damsela* (0.8 %, 3.6 MPN g^{-1}) (Table 10).

HPVs were isolated from 50.8 % of the fish and fish products collected from markets, landing centers and processing plants situated in and around Cochin (Table 5). Not much information regarding the incidence of HPVs in fish and fish products of this country is available except *V. parahaemolyticus*. Prasad and Rao (1994, a) studied the distribution of pathogenic vibrios in fresh, iced and frozen prawns, and fishes of Kakinada coast and reported the incidence of *V. anguillarum, V. metschnikovii, V. parahaemolyticus, V. vulnificus,* and group F vibrios. Thampuran *et al.* (1997) have reported the isolation of *V. alginolyticus, V. cincinnatiensis, V. damsela, V. metschnikovii, V. parahaemolyticus* and *V. vulnificus*, from coastal waters and fishes of Cochin. Sanjeev *et al.* (2000) isolated HPVs i.e., *V. alginolyticus, V. carchariea, V. cincinnatiensis, V. fluvialis, V. furnissii, V. metschnikovii, V. parahaemolyticus and V. vulnificus* from 44.76 % of the samples consisting of iced, frozen fish and fish products collected from different processing factories situated in Kerala and Tamil Nadu.

In this study 66.7 % of the iced samples, 44.2 % of the block frozen samples and 22.9 % of IQF samples showed the incidence of HPVs (Table 5). Dominant species isolated from the samples were *V. cincinnatiensis* (30.8 %) followed by *V. algnolyticus* (26.2 %), *V. vulnificus* (17.7 %), *V. parahaemolyticus* (7.7 %), *V. metschnikovii* (5.4 %), *V. fluvialis* and *V. damsela* (0.8 %) (Table 10). Sanjeev *et al.* (2000) have reported the dominant incidence of *V. cincinnatiensis* (18.06 %) in iced and frozen fish products followed by *V. alginolyticus* (15.18 %), *V. parahaemolyticus* (9.42 %), *V. vulnificus* (5.24 %), *V. metschnikovii* (3.14 %), *V. fluvialis* (2.09 %) and *V. damsela* (0.26 %). Presence of more sucrose positive vibrios than sucrose negative vibrios may be due to the relative abundance of this species in the coastal waters of Cochin. Thampuran *et al.* (1997) have indicated the abundance of these species in the coastal waters of Cochin. Chan *et al.* (1986) have reported similar results.

Name of block frozen samples	Total no.of sample	No. of samples with HPVs within the range MPN g^{-1}	*V. algino*	*V. cinci*	*V. dam*	*V. fluvi*	*V. furni*	*V. mets*	*V. para*	*V. vulni*
Shrimp: shrimp head on	1	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	1	1	0	0	0	1	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	3.6	9.1	0	0	0	3.6	0	0
shrimp peeled cooked	2	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0
Squid: squid whole	2	0.1-0.9	2	1	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0.36	0.3	0	0	0	0	0	0
squid whole cleaned	5	0.1-0.9	0	0	0	0	0	1	1	0
		1-10	1	1	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	1.5	2	0	0	0	0.36	0.3	0
squid rings	2	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0
squid tentacles	1	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0

Contd.

Cuttlefish cuttlefish whole	1	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0
cuttlefish whole cleaned	7	0.1-0.9	0	0	0	0	0	0	0	0
		1-10	0	0	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0	0	0	0	0	0	0
Others	3	0.1-0.9	3	1	0	0	0	0	0	0
		1-10	0	2	0	0	0	0	0	0
		11-101	0	0	0	0	0	0	0	0
		101-999	0.3	0	0	0	0	0	0	0
		load range (MPN g^{-1})	0	0.73-1.5	0	0	0	0	0	0
Total	24									

0 = Not detected

Table 10. Name of HPVs isolated from different fish products, their percentage of incidence and load (MPN g^{-1})

***Vibrio* spp**	**Incidence (%)**	**Load (MPN g^{-1})**
V. alginolyticus	26.2	<1-360
V. cincinnatiensis	30.8	<1-930
V. damsela	0.8	3.6
V. fluvialis	0.8	<1
V. furnissii	1.5	<1-30
V. metschnikovii	5.4	<1-5.6
V. parahaemolyticus	7.7	<1-36
V. vulnificus	17.7	<1-530

Chan *et al.* (1989) studied the summer prevalence of *V. parahaemolyticus* and other HPVs in seafood from Hong Kong markets. They observed that sucrose-positive vibrios were more common than sucrose negative varieties. *V. alginolyticus* was the most frequently isolated species, followed by *V. parahaemolyicus*, *V. fluvialis* and *V. vulnificus*. According to Wong *et al.* (1992) *V. alginolyticus* was the major species isolated from shrimps and crabs. In another study Wong *et al.* (1995) reported *V. alginolyticus* as the dominant species recovered from frozen seafoods consisting of peeled shrimps, fish and shrimp dumplings followed by *V. parahaemolyticus*, *V. cholerae* and *V. fluvialis* at 36.5 %, 15.8 %, 14.9 % and 13.2 %, respectively. Matte *et al.* (1994) have also reported the highest incidence of *V. alginolyticus* (81 %) in oysters followed by *V. parahaemolyticus* (77 %), *V. cholerae non O1* (31 %), *V. fluvialis* (27 %), *V. furnissii* (19 %), *V. mimicus* (12 %), and *V. vulnificus* (12 %). In a study of most widely consumed seafood products Baffone *et al.* (2000) revealed that *V. alginolyticus* was the dominant species isolated (81.48 %).

Schandevyl *et al.* (1984) on the contrary have reported dominant incidence of sucrose negative HPVs over sucrose positive HPVs in seafish from coastal waters in Senegal. *V.parahaemolyticus*, was the most common isolate, followed by *V.alginolyticus*, *V. vulnificus*, *V.damsela*, and *V.fluvialis*. Karunasagar *et al.* (1990) reported that *V. parahaemolyticus* was the commonly encountered HPV (69 %) followed by *V. vulnificus* (25 %) in shrimp, fish and molluscan shellfish collected from shrimp processing factories, fish landing centers and fish markets of Karnataka state.

Information regarding the incidence of *V. cincinnatiensis*, *V.damsela*, *V.fluvialis* and *V. furnissii* in seafood is scanty. Ripabelli *et al.* (1999) in a study on bacterial pathogens in *Mytilus galloprovincialis* mussels isolated *V. cincinnatiensis* from 3.2 % samples. Cavallo and Stabili (2002) observed selective retention of HPVs viz., *V. vulnificus, V. cincinnatiensis, V. orientalis, V. anguillarum, V. marinus* and *V. hollisae* in mussels (*Mytilus galloprovincialis)* collected from 30 sampling sites located in the Mar Piccolo of Taranto (Ionian Sea, Italy).

V.damsela (5.3 %) was isolated only from iced shrimp samples in this study (Table 6). Studies of Sanjeev *et al.* (2000) have also indicated the incidence of *V. damsela* (0.26 %) only in iced shrimp samples. Elhadi *et al.* (2004) in a survey of seafood markets reported the incidence of *V. damsela* in 13 % of the samples consisting of shrimp, squid, crab, cockles and mussels.

V.fluvialis was isolated only from a sample of iced cuttlefish whole cleaned (Table 7) and none of the other samples examined showed the incidence of this HPV. Sanjeev *et al.* (2000) have reported the incidence of this species in block frozen and IQF samples studied (2.09 %). Maugeri *et al.* (2000) revealed that *V.fluvialis* was the most frequently recovered species in water and mussel samples collected from two brackish lakes, used as mussel farms in Sicily (Italy). Elhadi *et al.* (2004) observed the incidence of *V. fluvialis* (7 %) from seafoods collected from markets and supermarkets consisting of shrimp, squid, crab, cockles and mussels. Sunen *et al.* (1995) have indicated the incidence of *V.fluvialis* in 2.04 % of mussels and 13.8 % of clams purchased from retail outlets in the North of Spain. Wong *et al.* (1992); Matte *et al.* (1994) have reported the isolation of this species from oysters and clams. Gianelli *et al.* (1984) have observed the occurrence of *V. fluvialis* in shellfish from shores of the Adriatic Sea or purchased from retail shops. According to Montilla *et al.* (1994), *V. fluvialis* was the most frequently isolated vibrio species from shellfishes bred in nurseries located in the Ebro river delta.

V. furnissii was isolated only from squid samples studied (Table 6). In spite of the relatively low percentage (1.5 %), this species could be isolated from both iced and block frozen samples of squid. Sanjeev *et al.* (2000) have reported the isolation of this species from block frozen and IQF samples (1.05 %). Wong *et al.* (1992) found a relatively small percentage (7 to 12 %) of incidences of *V. furnissii* in oysters, clams, shrimps and crabs samples. Matte *et al.* (1994) have reported 19 % incidence of *V. furnissii* in oysters (*Crassostrea gigas*) originating from the southern coast of the state of Sao Paulo-Brazil. Thampuran *et al.* (1997) have reported the incidence of *V.furnissii* in the intestinal contents of fish collected from the coastal waters of Cochin and the percentage varied from 25.9 to 32.9 %.

Buck (1991) has reported the recovery of *V. metschnikovii* from a variety of finfish and shellfish purchased from fish markets and super markets. This bacterium has also been isolated from food borne disease outbreaks involving shrimp and crab (Farmer *et al.*, 1988). The only report of isolation from finfish (cod, mackerel) was by Lee *et al.* (1978). In the present study *V.metschnikovii* was isolated from iced, blockfrozen and IQF samples and the incidence was 5.4 %. Majority of the isolates were obtained from shrimp and squid samples (Table 6). Sanjeev *et al.* (2000) have reported similar results and maximum number of isolates were obtained from IQF prawns, IQF squids and block frozen prawns.

V. parahaemolyticus was isolated from 7.7 % of the samples examined. Iced samples showed the maximum incidence followed by block frozen samples and IQF samples. In general shrimp samples showed the highest incidence of *V. parahaemolyticus*. Similar results were reported by Karunasagar *et al.* (1990), Sanjeev and Stephen (1993) and Sanjeev *et al.* (2000).

Several authors have reported the incidence of *V.parahaemolyticus* in shellfishes and finfishes collected from marine, estuarine and fresh water environments. Reports are also available on the incidence of *V. parahaemolyticus* in seafood samples obtained from, markets, processing plants and landing centers. De *et al.* (1977) showed the incidence of *V. parahaemolyticus* in marine fishes of Calcutta to be 35.2 %. Natarajan *et al.* (1979) reported 36.8 % occurrence in fishes from brackishwater environments. Karunasagar and Mohankumar (1980) found that the incidence varied from 8.33 to 33.3 %. The studies of Nair *et al.* (1980) revealed that 35.6 % of the freshly harvested fishes from the estuarine waters, 40.6 % fishes of mangroves, 37.5 % of freshly caught brackishwater fishes and 44 % fishes from market showed the incidence of *V.parahaemolyticus*. Sanjeev and Iyer (1986) reported the occurrence of *V.parahaemolyticus* in 55.9 % of the market fish samples and 2 out of 15 cooked clam meat samples. Sanjeev and Stephen (1993) showed the incidence of *V. parahaemolyticus* in marine fresh finfish and shellfish varied from 67 to 92 %, whereas in fish products it was less (3.69 to 30.23 %). Prasad and Rao (1994, a) have reported the incidence of *V. parahaemolyticus* in fresh and frozen prawns and fishes of Kakinada coast. Thampuran *et al.* (1997) were able to isolate *V. parahaemolyticus* from coastal

waters and fishes of Cochin. Aiyamperumal *et al.* (1994) reported the occurrence of *V.parahaemolyticus* in 14.2 % of finfish, 14.5 % of prawns, 23.8 % of crabs and 34.7 % of bivalves from coastal waters of Tuticorin. Sanjeev and Stephen (1993) have shown that the densities of *V.parahaemolyticus* in estuarine shellfish were found to be much higher compared to shellfish from the Arabian Sea.

Wong *et al.* (1995) isolated *V.parahaemolyticus* from frozen raw or semi prepared seafoods such as peeled shrimp, fish and shrimp dumplings (36.0 %). Sunen *et al.* (1995) reported the incidence of *V.parahaemolyticus* in 30.23 % of the samples consisting of mussels and clams purchased from retail outlets in Spain. Hase *et al.* (1997) have shown the incidence of *V.parahaemolyticus* in 21.1 % of raw seafood samples and 23.3 % of environmental samples collected from Osaka (Japan) seafood market. Elhadi *et al.* (2004) in a survey of seafood markets and supermarkets of Malaysia reported the incidence of *V. parahaemolyticus* in 4.7 % of the samples consisting of shrimp, squid, crab, cockles and mussels. Baffone *et al.* (2000) have reported 14.8 % incidence of *V. parahaemolyticus* in fresh seafood products in Italy. Jaksic *et al.* (2002) in a study of seafood samples collected along the sea side in Croatia reported 9.40 % incidence of *V. parahaemolyticus*. Wong *et al.* (1992) isolated *V.parahaemolyticus* from freshwater clams in Taiwan (22.8 %). *V. parahaemolyticus* was isolated also from freshwater samples of India and Japan (Joseph *et al.,* 1983; Sarkar *et al.*, 1985; Venkateswaran *et al.*,1989). Wong *et al.* (1999) in a study of seafood imported from Hong Kong, Indonesia, Thailand and Vietnam recovered *V.parahaemolyticus* from 45.9 % of the samples.

V. vulnificus was isolated from 17.7 % of the samples. Highest incidence of this organism was observed in iced squid samples (57.1 %) (Table 6). IQF samples were found free from this organism (Table 6). Karunasagar *et al.* (1990) have reported the incidence of *V. vulnificus* in 25 % of the samples consisting of shrimp, fish and molluscan shellfish collected from processing factories, fish landing centers and fish markets. Yano *et al.* (2004) have indicated the prevalence of *V. vulnificus* in live seafood available from markets in coastal cities of China, maximum load of *V. vulnificus* obtained was 3.4 log cfu g^{-1} in razor clam samples and 4.9 log cfu g^{-1} in the prawn samples by a direct spreading method.

Sanjeev *et al.* (2000) have reported the incidence of the species in 5.24 % of iced, block frozen and IQF samples collected from different processing plants situated in Kerala and Tamil Nadu meant for export.

Shrimp and squid samples were found more heavily contaminated with HPVs than any other samples in this study (Table 6.). This is in agreement with the observations of Molitoris *et al.* (1985). *V. alginolyticus, V.cincinnatiensis, V. metschnikovii* and *V. parahaemolyticus* were the dominant species isolated from the shrimp, squid and cuttlefish (except for IQF cuttlefish samples) samples (Table 6). *V. furnissii* and *V. vulnificus* were isolated from iced and block frozen samples, *V. damsela* and *V.fluvialis* could be isolated only from iced samples. Iced samples showed greater isolation frequency for vibrios. Incidence of more than five species of HPVs was observed in iced shrimp and squid samples.

HPVs showed their maximum load in iced samples (<1-930 MPN g^{-1}) followed by block frozen samples (<1-11MPN g^{-1}) and IQF samples (<1-9.1MPN g^{-1}) (Table 6). *V.cincinnatiensis* was the dominant species isolated during the study and the load observed was upto 930 MPN g^{-1} followed by *V. vulnificus* 530 MPN g^{-1}, *V. alginolyticus* 360 MPN g^{-1}, *V. parahaemolyticus* 36 MPN g^{-1} etc isolated respectively from iced cuttlefish roe, iced shrimp whole and iced squid whole samples (Table 7). Matte *et al.* (1994) have reported MPN10^{-2}g of HPVs from the oysters originating from the Sao Paulo-Brazil. *V. alginolyticus* showed the maximum load (<3-1,500), *V. parahaemolyticus* (<3-1, 200), *V.fluvialis* (<3-150), *V. furnissii* (<3-40) and *V.vulnificus* (<3-30). *V. fluvialis* showed the lowest load (0.3 MPN g^{-1}) among the species isolated in the present study followed by *V.damsela* (3.6 MPN g^{-1}). They were isolated only from iced samples studied (Table 6).

V. alginolyticus and *V. cincinnatiensis* were the dominant species isolated from block frozen samples and the observed load was <10 MPN g^{-1} (Table 8). *V.parahaemolyticus* was isolated only from shrimp and frozen crab soft shell samples and the load was upto 11 MPN g^{-1}. Recently few consignments of frozen fish products exported from this country were rejected due to the presence of *V. parahaemolyticus* and *V.vulnificus.* Rashid *et al.* (1992) and Wong *et al.* (1999) have reported the isolation of *V. parahaemo- lyticus, V. mimicus, V. alginolyticus,*

V. vulnificus and *V. fluvialis* from imported frozen shrimps, especially those from Southeast Asian countries. This study also has indicated the occurrence of HPVs in frozen fish products and is a matter of serious concern to the seafood industry. However the load of *V.parahaemolyticus* in the samples analyzed in this study was well within the limits of ICMSF specification of 10^2 *V. parahaemolyticus* per gram of the sample (ICMSF, 1974). This is in agreement with the report of Karunasagar *et al.* (1990). They found that the Most Probable Number (MPN) of *V. parahaemolyticus* in frozen shrimp collected along the coast of Karnataka (India) was always less than 10 g^{-1}. No such standards are available to compare the load of other species of HPVs.

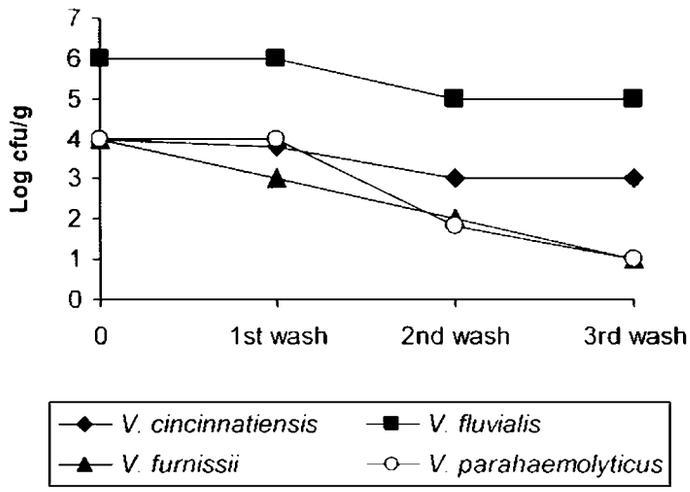
IQF samples showed the lowest load of HPVs and the same was completely absent in IQF cuttlefish samples analyzed. *V. damsela*, *V.fluvialis*, and *V.furnissii* could not be isolated from IQF samples. Like iced and block frozen samples *V.cincinnatiensis* and *V. alginolyticus* were the major species isolated from the IQF samples although the load was less (Table 9). Other species isolated from IQF samples include *V. metschnikovii* (3.6 MPN g^{-1}) and *V. parahaemolyticus* (0.3 MPN g^{-1}). Apparently no information is available for comparison.

4. 2. Effect of washing on HPVs

To study the effect of washing on HPVs four species viz., *V. cincinnatiensis, V. fluvialis, V. furnissii* and *V. parahaemolyticus* were selected. Isolates obtained from the samples were used for the study.

Cooked shrimps inoculated with each species of HPVs were washed in sterile tap water thrice. One to three log reductions in counts were observed in different species of HPVs (Fig 1). After third wash *V.furnissii* and *V. parahaemolyticus* showed the maximum reduction (3 log) followed by *V. fluvialis* and *V. cincinnatiensis* (1 log). *V. cincinnatiensis* and *V. fluvialis* showed one log reduction after second wash, and only a slight decrease in count was observed after third wash. Whereas *V. furnissii* and *V. parahaemolyticus* showed two log reduction in count after second wash. After third wash three log reduction in count was observed with respect to *V. furnissii* and *V. parahaemolyticus* (Fig 1).

Fig.1. Effect of washing on HPVs



In general washing with tap water was found to be very effective against HPVs although complete destruction could not be achieved. One to three log reductions in counts were observed with different species. After third wash *V.furnissii* and *V. parahaemolyticus* showed the maximum reduction (3 log) followed by *V. fluvialis* and *V. cincinnatiensis* (1 log) (Fig.1).

Less reduction in count of *V. cincinnatiensis* and *V. fluvialis* may be due to the deeper penetration and attachment of these organisms. High populations of microorganisms on carcass surfaces may allow for attachment of larger numbers of bacteria (Chung *et al.*, 1989). Shrimp die soon after harvesting. Decomposition begins soon after death and involves bacteria on the shrimp surface, which originate from the marine environment or from contamination during handling and washing (Feiger and Novak, 1961). Molluscan shellfish are sessile and filter feeders, and thus their microflora varies, reflecting the quality of water in which they reside (Jay, 1992), the quality of wash water and other factors.

HPVs are wide spread in the marine and estuarine environments and occur naturally in shellfish and finishes. *V. cininnatiensis* was the dominant species isolated in this study in different fish and fish products. This may be due to the abundance of this species in the aquatic environment. Thampuran *et al.* (1997) have reported the occurrence of this species in the coastal waters of Cochin. In fact this study supports the findings of Chung *et al.* (1989) for the attachment of *V. cincinnatiensis* on fish and fish products.

Timothy *et al.* (1995) have reported that atttached bacteria are less likely to be removed by washing or other decontamination procedures and may be more resistant to processing conditions. This view agrees for the incidence of HPVs in frozen fish products collected from the processing plants. Sudies of Sanjeev *et al.* (2000) have also shown the incidence of HPVs in frozen fish products collected from seafood processing plants.

Bacterial attachment to the muscle surfaces involves two stages (Firstenberg, 1981). The first is a loose, reversible sorption, which may be related to Vander Waals forces or other physicochemical factors (Marshall *et al.*, 1971). One of the

factors that influence attachment at this point is the population of the bacteria in the water film (Chung *et al.*, 1989, Firstenberg, 1981). The second stage consists of an irreversible attachment to surfaces involving the production of an extracellular polysaccharide layer known as a glycocalyx (Costerson *et al.*, 1981).

V. furnissii and *V. parahaemolyticus* showed three log reduction after third wash. This shows the sensitivity of these species to osmotic pressure difference. Findings of Sanjeev and Iyer (1986) indicate that the washing of the fish and equipment used to handle the fish in drinking water may reduce the number of viable cells of *V. parahaemolyticus*.

4.3. Effect of chilling on HPVs

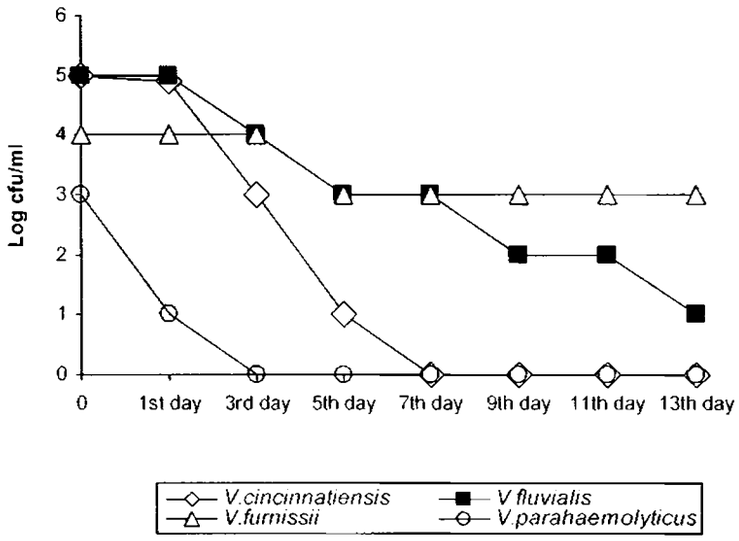
Studies on the effect of chilling on HPVs were conducted on cooked shrimps. Four species i.e., *V. cincinnatiensis*, *V. fluvialis*, *V. furnissii* and *V. parahaemolyticus* were selected for the study. Load of each species of vibrio was analyzed for a period of 13 days.

V. parahaemolyticus was found to be very sensitive to chilling followed by *V. cincinnatiensis*, *V. fluvialis* and *V. furnissii*. (Fig 2)

V. parahaemolyticus lost its viability within 3 days. *V. cincinnatiensis* survived chilling upto the seventh day although it showed four log reduction on the fifth day.

V. fluvialis showed four log reduction on the 13^{th} day few viable cells were detected even after the 15^{th} day. *V. furnissii* showed maximum resistance to chilling during the study. On the 13^{th} day the species showed only one log reduction.

Fig. 2 Effect of chilling on HPVs



Reduction in load of all the species of HPVs was observed during chill storage. Vibrios in general are sensitive to cold, although there are reports on the isolation of different HPVs from chilled and frozen foods.

In this study cooked, peeled and deveined shrimp was inoculated with different species of HPVs viz., *V. cincinnatiensis, V. fluvialis, V. furnissii and V. parahaemolyticus*. *V. parahaemolyticus* showed the maximum sensitivity to chilling. Muntada-Garriga *et al.* (1995) obtained similar results and indicates that high numbers of *V. parahaemolyticus* can be inactivated at chill temperatures. Gooch *et al.* (2002) have reported a 0.8 $\log_{10}$ decrease in number of *V. parahaemolyticus*, when the oysters were chilled at 3°C after 14 days. Andrews *et al.* (2000) have observed that low temperature pasteurization of raw oysters in ice was very effective in reducing *V. vulnificus* and *V. parahaemolyticus* from >100000 to non detectable levels in less than 10 min of processing.

Some workers reported that inactivation of *V.parahaemolyticus* occurred more rapidly when the organism was chilled to 1- 7°C than when it was frozen at –2 to –30°C. (Beuchat, 1977, Johnson and Liston, 1973). Quite the reverse was observed by Matches *et al.* (1971) who found that temperatures below 8°C will usually stop growth but it has been observed that the organism can still survive.

Similar results were reported by Vasudevan *et al.* (2002) when the fish fillets were chilled, although the decline in numbers was less pronounced than when the fillets were frozen.

Bradshaw *et al.* (1974) studied the survival of *V.parahaemolyticus* surface inoculated on cooked shrimp and kept at various temperatures for 48 h. On cooked shrimp, the vibrios grew well at 18.3°C, but their numbers declined gradually at 10°C and below. At 12.8°C, vibrios remained static for the most part. Thus it appeared that 12.8°C was the borderline temperature for growth of the organism on cooked seafood.

Kaysner *et al.* (1989) in a controlled study on *V. vulnificus* in oyster shellstock found that the organisim survived upto 2 weeks at 2°C, whereas *V. parahaemolyticus* has been observed to survive storage in shell stock oysters for at least 3 weeks at 4°C (Oliver and Kaper, 1997). Cook and Ruple (1992) found that holding the oyster meats in containers with crushed ice for 3 days significantly lowered the *V. vulnificus* counts. However, Quevedo *et al.* (2005) observed that although rapid chilling by immersion of unwashed whole oysters in ice for 3 h generally declined the *V. vulnificus* numbers, the method cannot be relied upon because of the relatively small decline in *V. vulnificus* number and the possibility of concomitant increases in fecal coliform and total bacterial contamination.

V. cincinnatiensis was also found to be sensitive to chilling in this study. No viable cells were detected on the 7th day of chilling. *V. fluvialis* and *V. furnissii* survived chilling on the 13th day. *V. fluvialis* showed 4 log reduction on the 13th day. *V.furnissii* showed the maximum resistance to chilling, and showed only one log reduction on the 13th day. Muntada-Garriga *et al.* (1995) have reported that, the time of total inactivation depends on the initial number of microorganisms and incubation temperature.

Chilling seafood was found not very effective against *V. furnissii* and *V. fluvialis* in this study. No information is available about the effect of chilling on *V. cincinnatiensis, V. fluvialis* and *V.furnissii* for comparison. Results obtained in the study indicate that, although there was decline in the number of vibrios during

chilling the method cannot be relied upon as an effective measure to control vibrios.

4.4. Effect of low temperature storage (4°C and 10°C) on HPVs

Effect of storage at low temperature ie. 4°C and 10°C on HPVs was studied on shrimp homogenate (SH) prepared with 3 different diluents - 3 % NaCl solution, normal saline (NS) and distilled water (DW).

Survival of *V. cincinnatiensis* in sterile SH prepared with 3 % NaCl solution and NS stored at 4°C is presented in Fig. 3. In SH prepared with 3 % NaCl solution log value of *V. cincinnatiensis* came down from 10^5 to 10^3 within 30 days and no viable cells were detected on the 90^{th} day. In SH prepared with NS log value came down from 10^5 to 10^2 within 30 days and from the 60^{th} day onwards no viable cells were detected. Drastic reduction in count was noticed in SH with DW. No viable cells were detected on the 30^{th} day. Complete destruction took place within 7 days (Fig.3 a).

Fig.3 Survival of *V.cincinnatiensis* in SH with 3% Nacl solution and NS stored at 4°C

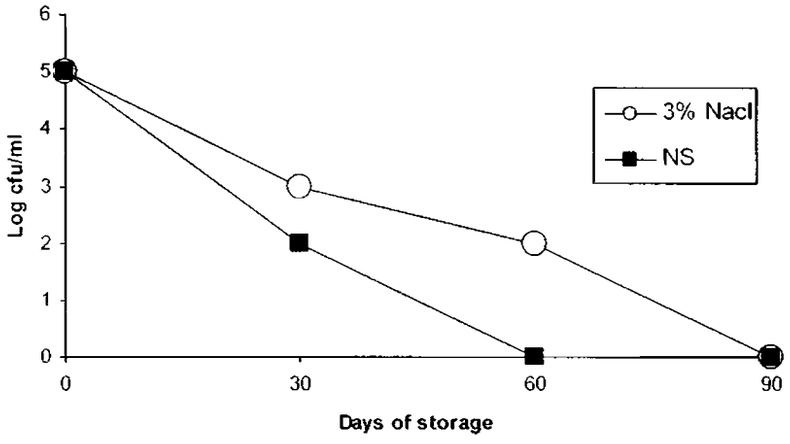


Fig.3 a Survival of *V.cincinnatiensis* in SH with DW stored at 4°C

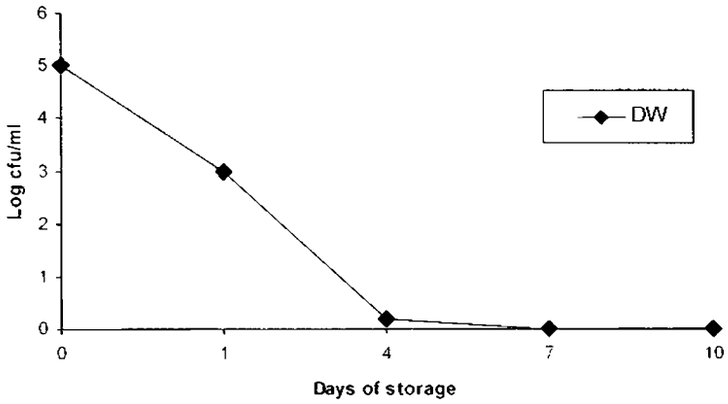


Fig. 4 shows the survival of *V. cincinnatiensis* in SH prepared with 3 % NaCl solution and NS stored at 10°C. Upto 30 days no reduction was observed in SH with 3 % NaCl solution. *V. cincinnatiensis* showed only 3 log reduction in SH with 3 % NaCl solution within 90 days. With NS log reduction from 10^5 to 10^3 was noticed upto 30 days, 3 log reduction on the 60th day and 4 log reduction was observed on the 90th day. In SH with 3 % NaCl solution and NS *V. cincinnatiensis* survived even after 3 months at 10°C. Viable cells of *V. cincinnatiensis* were detected upto 7 months in SH with 3 % NaCl solution, whereas with NS viable cells were detected upto 6th month. No viable cells of *V. cincinnatiensis* were detected in SH prepared with DW and stored at 10°C after 30 days; complete destruction took place within 7 days (Fig. 4 a)

Fig. 4 Survival of *V. cincinnatiensis* in SH prepared with 3% NaCl solution and NS stored at 10°C

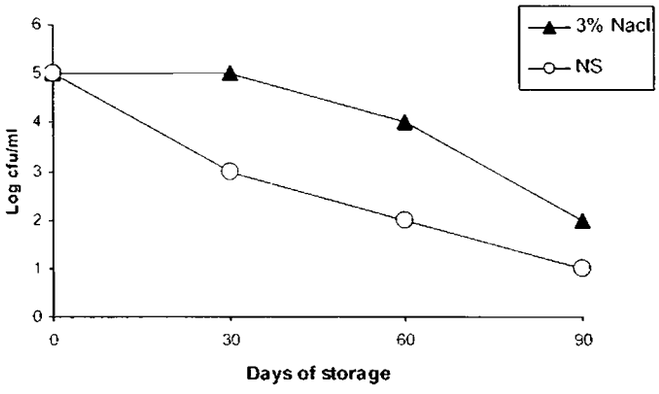


Fig. 4 a Survival of *V. cincinnatiensis* in SH prepared with DW stored at 10°C

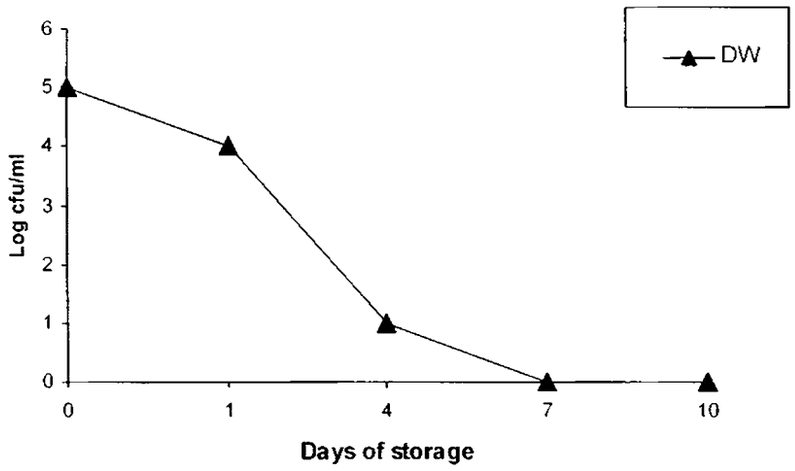


Fig. 5 illustrates the viability of *V. fluvialis* in SH with 3 % NaCl solution and NS stored at 4°C. In shrimp homogenate prepared with 3 % NaCl solution 1 log reduction was observed after 30 days followed by 2 log reduction on the 60th day and the load remained same Viable cells were detected upto the 7th month. With NS log value came down from 10^6 to 10^3 on the 90th day and viable cells were detected till the 6th month. In SH with DW no viable cells were detected on the 30th day, complete destruction took place within 7 days of incubation (Fig.5a).

Fig. 5 Survival of *V.fluvialis* in SH prepared with 3% NaCl Solution and NS stored at 4°C

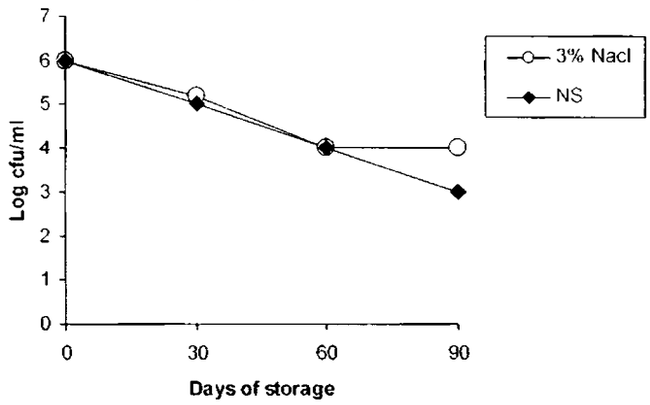
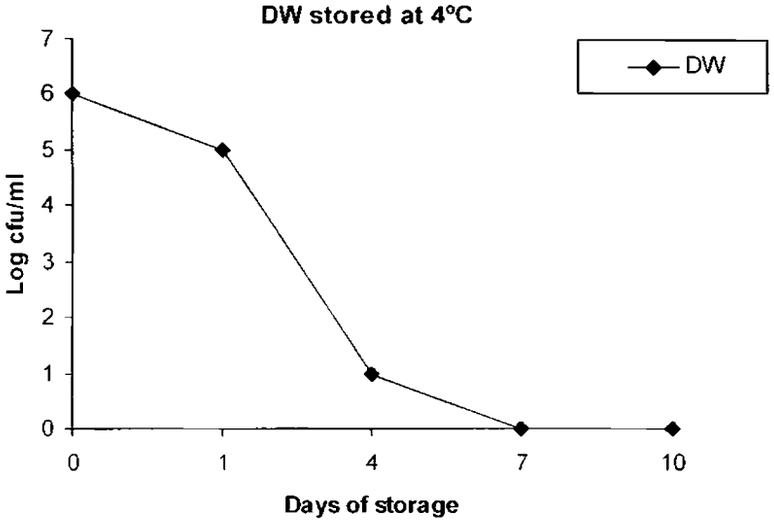


Fig. 5a Survival of *V.fluvialis* in SH prepared with DW stored at 4°C



Survival of *V. fluvialis* in SH prepared with 3 % NaCl solution, NS and DW stored at 10°C is plotted in Fig. 6. In SH prepared with 3 % NaCl solution *V. fluvialis* showed 1 log increase in count on the 30th day followed by 1 log reduction on the 60th day and the organism could not be detected on the 90thday. In SH prepared with NS, 5 log reduction was observed on the 60th day and on the 90th day onwards no viable cells were detected. In SH with DW stored at 10°C *V.fluvialis* cells were found viable upto 5th week (Fig. 6a). In contradictory to other species *V. fluvialis* in SH prepared with 3 % NaCl solution and stored at 10°C showed an increase of 1 log in count after storage. However after the 30th day count began to decrease in SH prepared with 3 % NaCl.

Fig. 6 Survival of *V.fluvialis* in SH prepared with 3% NaCl Solution and NS stored at 10°C

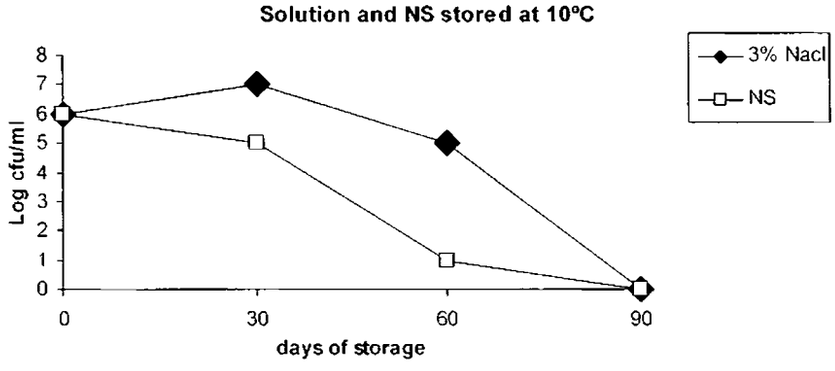


Fig. 6 a Survival of *V.fluvialis* in SH prepared with DW stored at 10°C

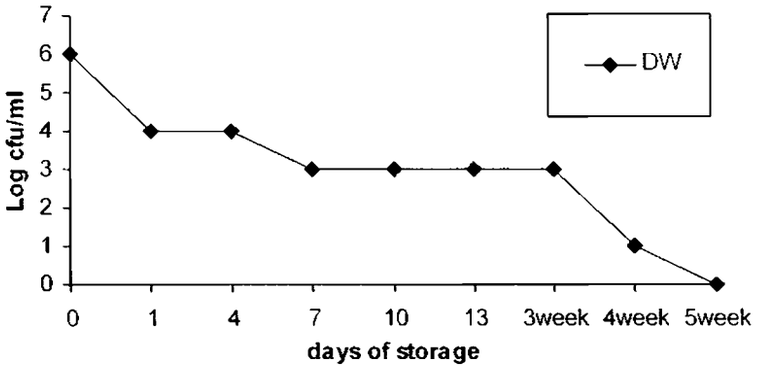


Fig. 7 shows the survival of *V. furnissii* in SH prepared with 3 % NaCl solution and NS stored at 4°C. In SH prepared with 3 % NaCl solution and NS 3 log reduction was observed on the 30th day, and no viable cells were detected from the 60th day onwards. In SH prepared with DW, cells were not detected on the 3rd week (Fig.7a).

Fig.7 Survival of *V. furnissii* in SH with 3%NaCl solution and NS stored at 4°C

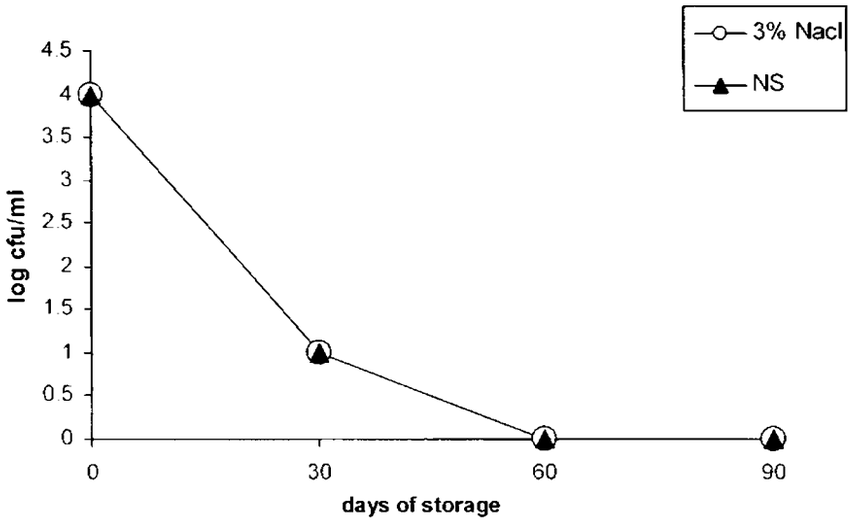
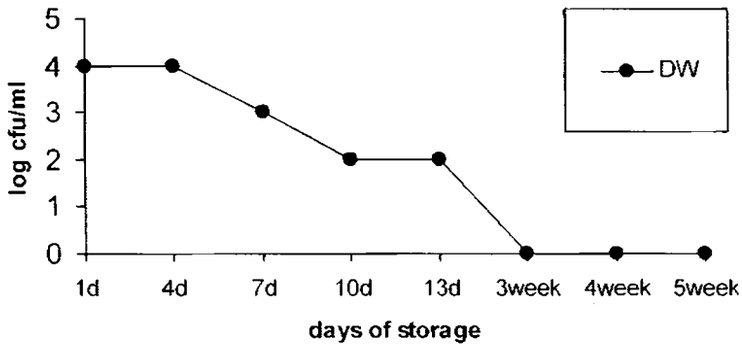


Fig. 7a Survival of *V. furnissii* in SH with DW stored at 4°C



Survival of *V. furnissii* in SH prepared with 3 % NaCl solution and NS stored at 10°C is depicted in Fig.8. In SH prepared with 3 % NaCl and NS log value came down from 10^4 to 10^1 on 30^{th} day. No surviving cells were detected from the 60^{th} day onwards. In SH prepared with DW no viable cells were detected from the 5^{th} week onwards (Fig.8a).

Fig. 8 Survival of *V. furnissii* in SH with 3%NaCl solution and NS stored at 10°C

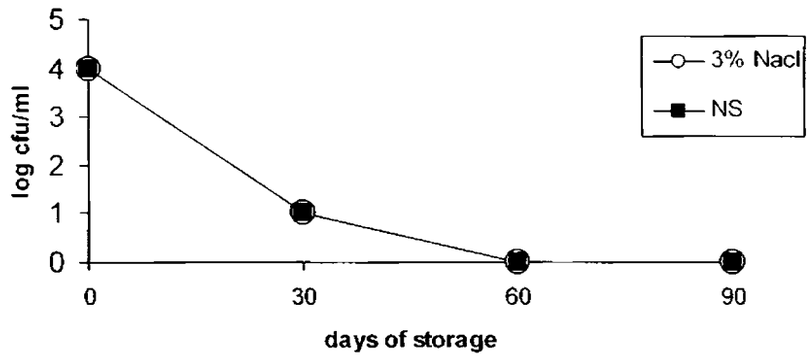


Fig.8a Survival of *V. furnissii* in SH with DW stored at 10°C

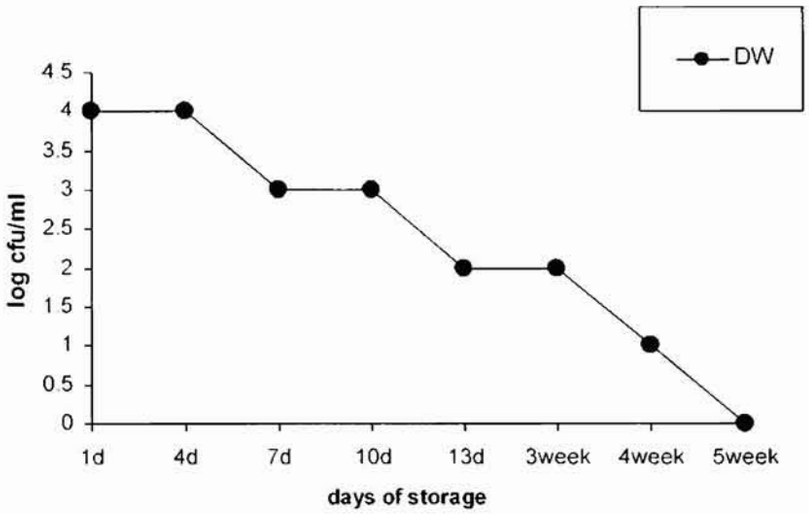
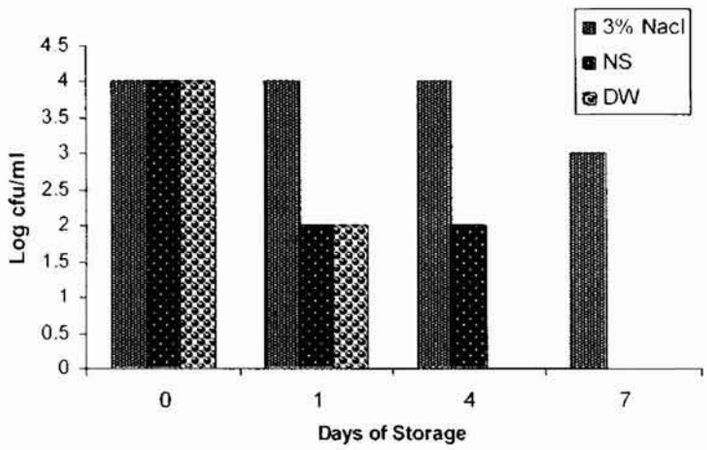


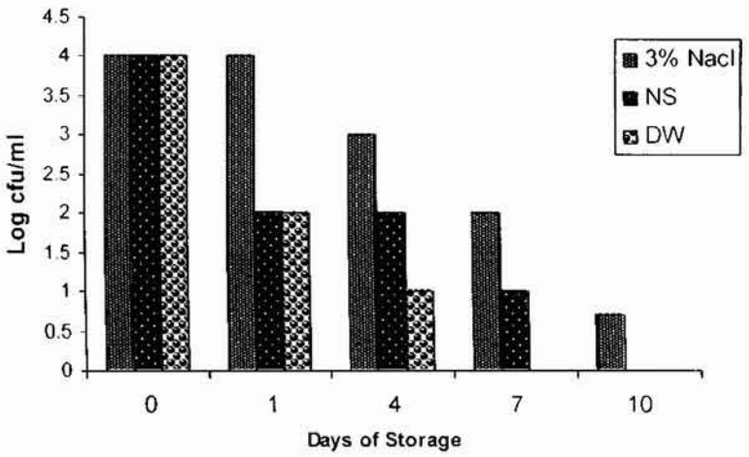
Fig. 9 shows the survival of *V. parahaemolyticus* at 4°C in SH prepared with 3 % NaCl solution, NS and DW. In SH prepared with 3 % NaCl, on 7th day log value came down from 10^4 to 10^3 and no surviving cells of *V. parahaemolyticus* were detected from 10th day onwards. In SH prepared with NS, 2 log reduction was observed on 4th day. No viable cells of *V. parahaemolyticus* were detected on 7th day onwards. In SH prepared with DW within 24 h 2 log reduction was observed and on 4th day onwards no viable cells were detected.

Fig. 9 Survival of *V.parahaemolyticus* in SH with 3% NaCl solution, NS and DW stored at 4°C



Survival of *V. parahaemolyticus* in SH prepared with 3 % NaCl solution, NS and DW, stored at 10°C is depicted in Fig. 10. In SH prepared with 3 % NaCl solution log value came down from 10^4 to 0.7 within 10 days. On 13th day onwards no surviving cells were detected. In SH prepared with NS log value came down from 10^4 to 10^1 on 7th day, complete destruction was observed on 10th day. In SH prepared with DW 3 log reduction was observed on 4th day and no surviving cells *V. parahaemolyticus* were detected on 7th day onwards.

Fig. 10 Survival of *V.parahaemolyticus* in SH with 3% NaCl solution, NS and DW stored at 10°C



Throughout out the studies HPVs showed higher survival rate in SH prepared with 3 % NaCl. Survival of HPVs at low temperatures is influenced by the concentration of salt in the suspending medium. SH was prepared in three different diluents, in order to expose vibrios to three different environment condition in seafood viz., a condition without salt, lesser salt concentration and optimum salt concentraton.The addition of NaCl to the suspending medium (upto 12 % NaCl) conferred a stabilizing effect on the organism (Covert and Woodburn, 1972; Temmyo, 1966) however the stabilizing effect was insufficient to protect cells for long-term storage at the low temperatures studied. In SH with 3 % NaCl solution and NS, viable cells of HPVs were detected, even after 6 months storage. Maximum survival upto 30 days was noticed in SH with DW at 4°C and 10°C. Covert and Woodburn (1972), Johnson *et al.* (1973) observed that seafoods were usually protective for vibrios at refrigeration temperature.

At 4°C no viable cells of *V. cincinnatiensis* was detected after 90 days storage (Fig. 3). But at 10°C viable cells were detected even after 90 days (Fig. 4). Irrespective of the temperature, survival was found to be maximum in SH prepared with 3 % NaCl, followed by NS. *V. cincinnatiensis* lost viability in SH with DW on the 7^{th} day at 4°C and 10°C. The studies have shown that low temperature storage at 4°C to be more effective against *V. cincinnatiensis*. Apparently no information is available regarding effect of low temperature storage on *V. cincinnatiensis* for comparison.

Compared to *V.cincinnatiensis*, *V. fluvialis* was found to be more resistant to refrigeration temperatures. In SH prepared with 3 % NaCl and NS viable cells were detected upto the 7^{th} month and 6^{th} month respectively at 4°C (Fig. 5). At 10°C in SH with 3 % NaCl and NS *V. fluvialis* showed 1 to 2 log increase in count during the first five weeks . This was followed by a gradual log reduction. On 90^{th} day no survivors were found (Fig. 6). This type of growth pattern was not shown by any other species studied. At 4°C, cells remained viable till the 7^{th} month. This peculiar behavior of *V. fluvialis* may be due to some inherent factors influencing the viability of the organism. Wong *et al.* (1994) have reported that psychrotrophic strain of *V. fluvialis* survived well at 4°C and 10°C.

It has been reported that the growth temperature range of an organism depends on how well the organism can regulate its lipid fluidity within a given range (Finne and Matches, 1976). Psychrotrophs contained increased amounts of unsaturated fatty acid residues in their lipids when grown at low temperatures. This maintains the lipid in a fluid and mobile state, thereby allowing membrane proteins to continue function (Jay, 1992). In addition, the transport permeases of psychrotrophs are apparently more operative at low temperatures than those of mesophiles (Baxter and Gibbons, 1962), and a cold resistant transport system characterizes psychrotrophic bacteria (Wilkins, 1973). In this study *V. fluvialis* had shown the maximum resistance to low temperatures compared to other species studied. This beyond doubt shows the psychrophilic nature of the organism.

In SH prepared with DW *V. fluvialis* were viable upto 30 days at 10°C and at 4°C viability lost within 7 days.

V. furnissii showed similar growth pattern in all the suspending media at 4°C. From the 60th day onwards no viable cells were detected (Fig.7). Similar results were obtained at 10°C (Fig.8) except for cells grown in SH with DW. At 10°C *V. furnissii* survived upto 5th week in SH with DW and at 4°C viable cells were not detected after 3rd week onwards. *V. furnissii* when compared to *V. cincinnatiensis* and *V. fluvialis* had shown a slightly higher rate of survival in DW. In general *V. furnissii* was found sensitive to refrigeration temperatures in this study. Apparently no information is available in this regard for comparison.

V. parahaemolyticus showed higher survival rate at 10°C compared to 4°C. More viable cells were detected from SH prepared with 3 % NaCl at both storage temperatures (Fig. 9 and 10). At 4°C viable cells were detected till 7th day and at 10°C the same were detected upto the 10th day. In SH with DW at 4°C cells lost viability on 4th day while at 10°C viable cells were detected till 4th day. Within two weeks viability was lost in all the suspending media irrespective of the storage temperature. In this study *V. parahaemolyticus* showed the maximum sensitivity to low temperature storage (4°C and 10°C) compared to other species studied. There is general agreement that *V. parahaemolyticus* exhibits poor cold resistance, but there are conflicting opinions over the years regarding the optimum temperature to be selected for storage of flesh foods like seafood. Some workers (Beuchat, 1977; Johnson and Liston, 1973) reported that inactivation occurred more rapidly when *V. parahaemolyticus* was held at chill temperature of 1 - 7°C than when it was frozen at –2 to –30°C. Others described a reverse effect. Matches *et al.* (1971) observed that inactivation of this organism was more at –34°C than at –18°C, which in turn was more than at 0.6°C. Such conflicting views have created an ambiguity regarding the selection of optimum storage temperature during seafood handling. Minimal temperature reported for *V. parahaemolyticus* multiplication was 5°C (Beuchat, 1973) and 8°C (Baross and Liston, 1970) in artificial media and 10°C in oyster homogenate (Thomson and Thacker, 1972).

Relatively low inoculum level of *V. parahaemolyticus* (10^4) was used because this is the range of this vibrio recovered from shellfish samples (Baross and Liston, 1970). Covert and Woodburn (1972) used similar inoculum level in their study on

the survival of *V.parahaemolyticus* in fish homogenate and in trypticase soya broth (TSB) with various NaCl concentrations during low temperature storage. They have noted a very sharp drop in survivors if NaCl was not added to the medium throughout the experiments. Similar results were obtained in this study also. They have also noted that temperatures of 5 ± 1, –5 ± 1, and –18 ± 1°C reduced the number of viable organisms regardless of the NaCl concentration. In the presence of NaCl viable cells ranging upto 580 per ml were detected at the end of 30 days of storage in fish homogenate. The first view is in agreement with the result obtained in this study. However viable cells could not be detected after 10 days storage. Fish homogenate might have provided better protection to *V. parahaemolyticus* than the shrimp homogenate used in this study. Sudha *et al.* (2003) studied the survival of *V. parahaemolyticus* at –18 ± 2°C and 6 ± 2°C in fish muscle homogenate, tryptic soya broth and 3 % NaCl solution and they found the maximum survival of *V. parahaemolyticus* in fish muscle substrate at both test temperatures and the cells were found to be viable till the end of study period of 90 days. Boutin *et al.* (1985) have reported the inactivation of *V. parahaemolyticus* at 4°C and –20°C in shrimp homogenate after 32 and 39 days respectively. They reported a higher inactivation rate for *V. parahaemolyticus* at 4°C compared to –20°C. The short period of survival of *V. parahaemolyticus* in SH in the present study might be due to the variation among the strains used, difference in menstura, difference in inoculam size, factors like salt content of the menstura etc.

Growth rate of *V. parahaemolyticus* at lower temperatures are naturally lower, but counts increased from10^2 to 10^8 cfu g^{-1} after 24 h storage at 25°C in homogenized shrimp, and from 10^3 to 10^8 cfu g^{-1} after 7 days storage at 12°C in homogenized oysters (Twedt, 1989). Similarly, cells of *V. parahaemolyticus* were reduced in cooked fish mince and surmi at 5°C for 48 h, but growth occurred when the products was held at 25°C (Oliver and Kaper, 1997). These results demonstrate that *V. parahaemolyticus* in seafood may grow to enormous numbers when held for short periods under improper refrigeration. The usual refrigeration temperatures permit growth of psychrophilic and psychrotrophic micro-organisms (Roberts *et al.*, 1981). If their initial population is high refrigerated foods can spoil in a short time.

In recent years it has been demonstrated that vibrios are able to respond to adverse environmental conditions by entering a viable, but non-cultuarable phase (Colwell and Huq, 1994). In this state they cannot be detected by standard bacteriological methods. However, when given optimal conditions they can return to normal "culturable" state. An obvious implication of this phenomenon is that routine examinations of environmental samples for these pathogens can be negative; while virulent bacteria are infact present. Wolf and Oliver (1992) had observed the low temperature sensitivity of *V. vulnificus*. They opined that the reduction in the viable cell count cannot be ascribed entirely to death, but it can also be due to the formation of viable but non cultivable (VBNC) cells. In this state, bacteria can survive for days at low storage temperatures. Jiang and Chai (1996) have reported VBNC state of *V. parahaemolyticus* cells starved at low temperature. Their studies had shown that VBNC cells were capable of growth and multiplication with limited nutrients at an extraordinary rate when the temperature was up shifted.

Results obtained in this study show that low temperature storage of seafood could not be relied upon as an effective method to eliminate vibrios. Although at these temperatures there was reduction in the count of various HPVs studied, viability was not completely lost in many cases and moreover these pathogenic vibrios are capable of multiplication with a slight increase in temperature. Time/ temperature control is of utmost importance as far as vibrios are concerned. In seafood processing plants, it is the usual practice to store fish and fish products under chilled conditions before processing and during processing, these conditions seem to be critical and any rise in temperature during storage should be avoided to minimize the growth of HPVs.

4.5. Effect of freezing and frozen storage at – 40°C and –20°C on HPVs

Effect of freezing and frozen storage on HPVs viz., *V.cincinnatiensis*, *V. fluvialis*, *V. furnissii* and *V. parahaemolyticus* were also studied in SH prepared with 3 different diluents viz., 3 % NaCl solution, NS and DW. Strains used in low temperature storage studies were employed in this study also. Inoculated samples were kept in a freezer at –40°C for 2 h. Sampling was done

at an interval of 90 min and 120 min. For frozen storage studies sampling was done until the viability was lost.

Fig. 11 Survival of *V. cincinnatiensis* in SH with 3% NaCl solution, NS and DW during storage at -40°C

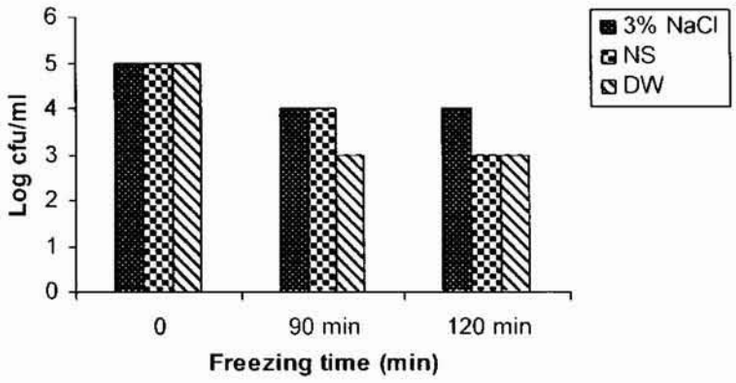
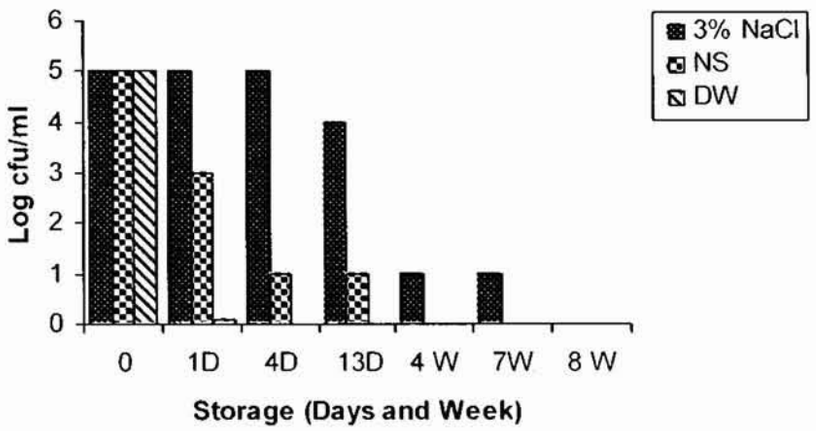


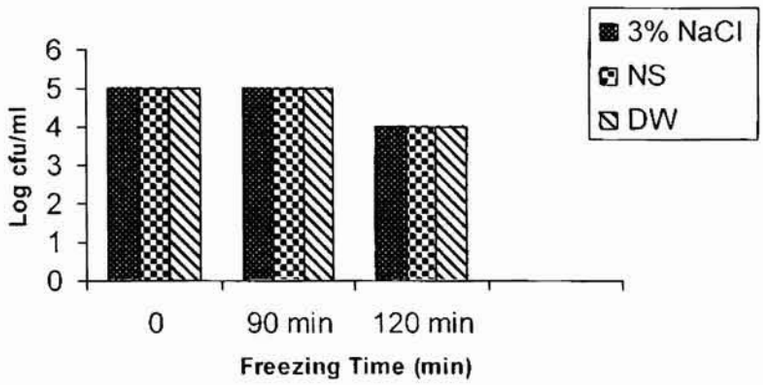
Fig. 11 shows the survival of *V. cincinnatiensis* during freezing at –40°C in SH prepared with three different diluents as mentioned above. *V. cincinnatiensis* showed 1 log reduction in SH with 3 % NaCl solution and NS and 2 log reduction in SH with DW within 90 min freezing. After 120 min freezing *V. cincinnatiensis* showed no log reduction and the log value remained at 10^4 in SH prepared with 3 % NaCl solution. However in SH with NS and DW 2log reduction was observed.

Fig. 12 Survival of *V. cincinnatiensis* in SH with 3% NaCl solution, NS and DW during storage at -20°C



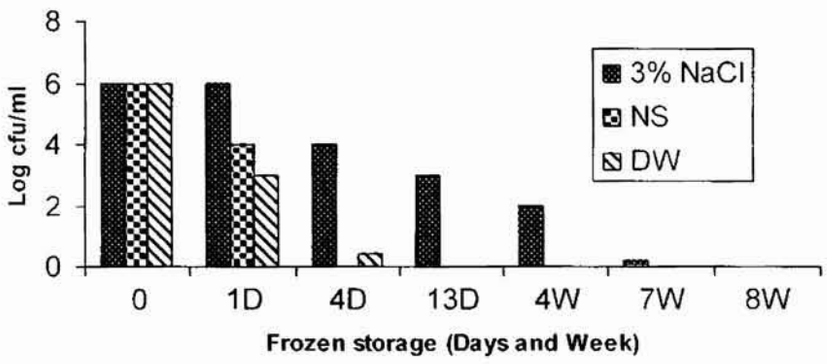
Survival of *V. cincinnatiensis* during storage at –20°C in SH prepared with 3 % NaCl solution, NS and DW is depicted in Fig. 12. In shrimp homogenate prepared with 3 % NaCl solution 4 log reduction was observed on the 7th week. In SH with NS *V. cincinnatiensis* survived upto 13th day. In SH prepared with DW complete reduction was observed within 4 days.

Fig. 13 Survival of *V. fluvialis* in SH with 3% NaCl solution, NS and DW during freezing at -40°C



During freezing at –40°C for 90 min *V. fluvialis* showed no log reduction irrespective of the diluents used for the preparation of shrimp homogenate. After 120 min freezing *V. fluvialis* showed 1 log reduction in SH prepared with 3 % NaCl solution, NS and DW (Fig .13).

Fig. 14 Survival of *V. fluvialis* in SH with 3% NaCl solution, NS and DW during storage at - 20°C



At –20°C *V. fluvialis* showed maximum survival in SH prepared with 3 % NaCl solution (Fig. 14). Log value came down from 10^6 to 0.2 within 7 weeks, and no surviving cells of *V. fluvialis* were detected after 8^{th} week. In SH prepared with NS and DW *V. fluvialis* could surviv only upto 4^{th} day.

Fig. 15 Survival of *V. furnissii* in SH with 3% NaCl solution, NS and DW during freezing at - 40°C

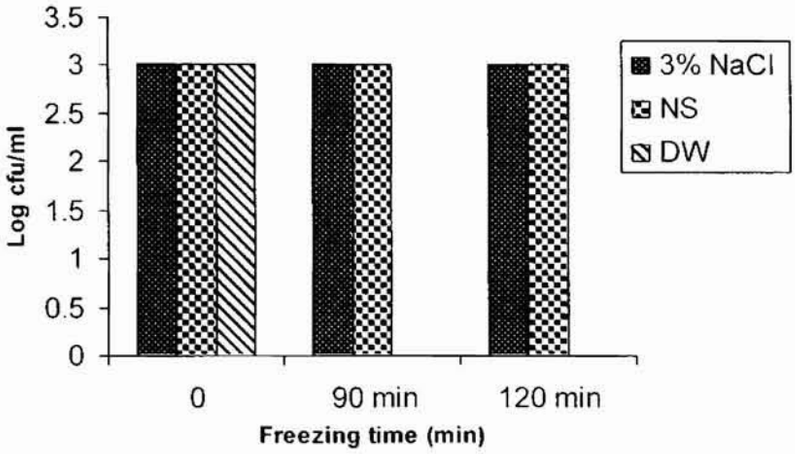
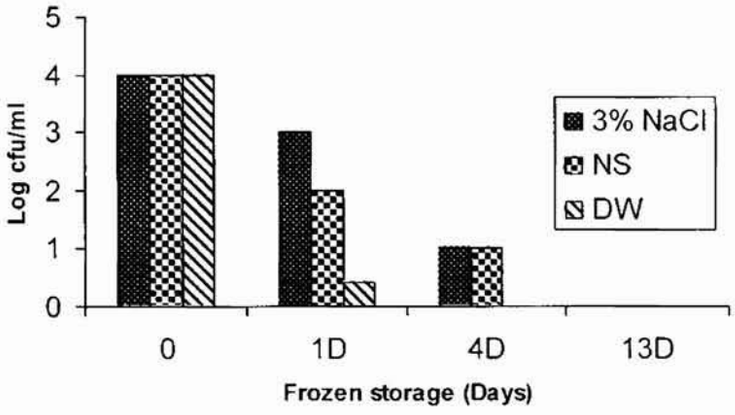


Fig. 15 shows the survival of *V. furnissi in* SH prepared with 3 % NaCl solution, NS and DW during freezing at –40°C. No log reduction was observed in SH with 3 % NaCl solution and NS in 90 min and 120 min. But in SH with DW complete reduction was observed within 90 min.

Fig. 16 Survival of *V. furnissii* in SH with 3% NaCl solution, NS and DW during storage at - 20°C



At –20°C *V. furnissii* showed 3 log reduction in SH with 3 % NaCl solution and NS on 4th day (Fig. 16), and no surviving cells of *V. furnissii* were detected on the 13th day. In SH with DW log value came down from 10^4 to 0.4 within 24 h, and on the 4th day no surviving cells were detected.

Fig. 17 Survival of *V. parahaemolyticus* in SH with 3% NaCl solution, NS and DW during freezing at - 40°C

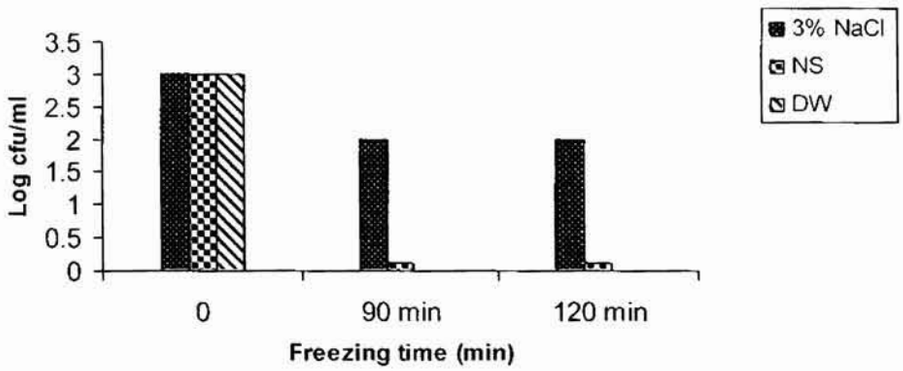
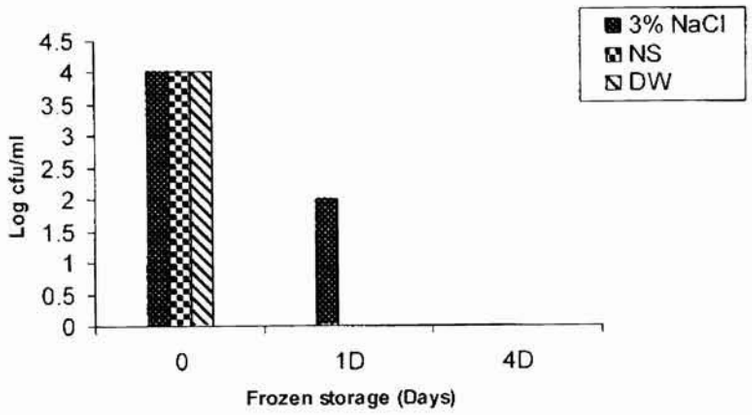


Fig. 17 shows the survival of *V. parahaemolyticus* in SH prepared with 3 % NaCl solution, NS and DW during freezing at –40°C. After 90 min freezing *V. parahaemolyticus* showed 1 log reduction in SH prepared with 3 % NaCl solution and and the count remained same. In SH with NS log value came down from 10^3 to 0.1 within 90 min. In SH with DW no surviving cells of *V. parahaemolyticus* were detected after 90 min freezing.

Fig. 18 Survival of *V. parahaemolyticus* in SH with 3% NaCl solution, NS and DW during storage at - 20°C



V. parahaemolyticus was found to be very sensitive to frozen storage temperatures. Viability of the organism was completely lost on the 4th day of frozen storage in SH with 3 % NaCl solution. While, with NS and DW, cells lost viability within 24 h (Fig. 18).

In general, freezing and frozen storage was found to be very effective against *V. cincinnatiensis*. *V. cincinnatiensis* was the dominant species isolated from iced and frozen fish products in this study. *V. cincinnatiensis* had shown 1-2 log reduction in different diluents during freezing at –40°C for 2 h. During frozen storage at –20°C no surviving cells of *V. cincinnatiensis* were detected on the 8th week. Muntada-gariga *et al.* (1995) have reported that the reduction of bacterial numbers at refrigeration and frozen temperatures were logarithmic function of log time. According to them inactivation time depends on initial bacterial level and incubation temperature. They have also reported that reduction in bacterial numbers were similar at refrigeration and frozen temperatures, though in all cases frozen temperatures were more effective than refrigeration temperatures.

Wong *et al.* (1994) studied the survival of psychotropic *V. fluvialis and V. parahaemolyticus* in culture broth at low temperature and found that both the organisims survived well at 10°C, 4°C and –30°C and could probably enhance the risk of vibrios in seafood. In the present study *V. fluvialis* showed only 1 log reduction during 120 min freezing, regardless of the medium suspended. However during frozen storage at –20°C complete destruction was observed within 8 weeks. Apparently no other information is available for comparision.

Freezing at –40°C for 2 h was found not effective against *V. furnissii* in SH prepared with 3 % NaCl and NS, infact no log reduction was observed (Fig. 15). But in SH prepared with DW viability was lost within 90 min. Probably the concentration of NaCl in the suspending medium conferred a stabilizing effect on the organism at freezing temperature (Covert and Woodburn, 1972). But frozen storage at –20°C was found very effective against *V. furnissii* in this study. 3 log reduction was observed within 4 days of frozen storage and lost viability on the 13th day (Fig. 16).

V. parahaemolyticus was found very sensitive to freezing and frozen storage at –20°C and –40°C. 1 log reduction was observed within 2 h in SH prepared with 3 % NaCl at –40°C (Fig. 17). In NS drastic reduction was observed, < 10 viable cells were isolated after 90 min, while in DW cells lost viability within 90 min. At –20°C *V. parahaemolyticus* survived only for 24 h (Fig. 18). Iyer (1985) reported that 99.9 % of *V. parahaemolyticus* were destroyed during freezing at –40°C and there was gradual reduction in numbers during storage at –20°C and all the cells lost viability within in 7 to 9 days. In general *V. parahaemolyticus* was found to be very sensitive to freezing compared to other species studied. Low survival of *V. parahaemolyticus* in this study during frozen storage could be due to small inoculums added to the suspending medium. Result obtained by Lampercht (1980) agrees with the above view, he could isolate the organism from lobster tails frozen at −18°C upto at least 3 months when the initial load was high (10^4-10^6 ml^{-1}). However the organism survived only upto one month if the inoculum was between 10^2 and 10^3 organisms per ml

Muntada-Garriga *et al.* (1995) studied the survival of *V. parahaemolyticus* in oyster meat homogenate at various frozen temperatures i.e., –18°C and –24°C and various loads i.e., 10^2, 10^4, 10^5 and 10^7 ml^{-1}. In all cases, the numbers of *V. parahaemo-lyticus* were a logarithmic function of log time, and the study indicated that high number of *V. parahaemolyticus* can be inactivated at low temperature. Thomson and Thacker (1972) reported that oysters held at –20°C for more than 2 weeks seldom contained viable *V. parahaemolyticus* cells. Matches *et al.* (1971) inoculated *V. parahaemolyticus* in fish homogenate and has shown that the log reduction values of 2.2 to 6.2 at –18°C were attained in 12 to 19 days, and the same reduction values at –34°C were reached before 12^{th} day. Sanjeev (1990) has reported the survival of *V. parahaemolyticus* at –20°C in crabmeat homogenate with 0 % NaCl and 3 % NaCl to be 16 and 30 days respectively. In similar studies the rate of destruction of Kanagawa +ve and Kanagawa –ve strains of *V. parahaemolyticus* was found to be more or less similar in shrimp homogenate with 3 % NaCl at –20°C (Sanjeev, 1990). Both the strains remained viable upto 21 days of storage.

Thompson and Trenholm (1971) reported that shellfish held in deep freezer at –20°C for more than 2 weeks seldom contained *V. parahaemolyticus*. Johnson and Liston (1973) could isolate this organism from frozen crabmeat and fish fillets stored at –15°C and –30°C, after 30 days and 60 days respectively. These investigators could also isolate the organism from inoculated oysters stored at –15°C and –30°C after 130 days.

Sudha *et al.* (2003) have reported the complete elimination of *V. vulnificus* from fish muscle homogenate within 3 months of storage (–18°C ± 1) whereas *V. parahaemolyticus* survived the period indicating better survival capacity for this pathogen. *V. parahaemolyticus* survived freezing at –20°C for 7 weeks in fish fillets (Vasudevan *et al.*, 2002). Despite general agreement that *V. parahaemolyticus* exhibits poor resistance to cold, there has been much disagreement over optimal long-term storage temperature. Wide variation in the results could be due to variation in the strains, inoculam level, substrates used, method used for the study etc.

Freezing and frozen storage was found very effective against HPVs in this study, although complete reduction could not be achieved it helps in reducing the count. HPVs in general showed one to two log reductions on freezing. Thampuran and Gopakumar (1990) had shown that the survival of vibrios is maximum in fish muscle homogenate during freezing at – 39 ± 2°C compared to other species viz., Moraxella, Acinetobacter, Micrococcus and Bacillus species.

During freezing and frozen storage, HPVs in general showed maximum survival in SH prepared with 3 % NaCl when compared to NS and DW. As with low temperature studies the presence of NaCl in the suspending medium conferred a stabilizing effect on the organism (Covert and Woodburn, 1972; Temmyo, 1966). Incidence of vibrios in frozen seafood was found to be less when compared to iced or refrigerated foods, however, the risk of pathogenic vibrios in frozen foods is enhanced by the presence of these psychrotrphic strains.

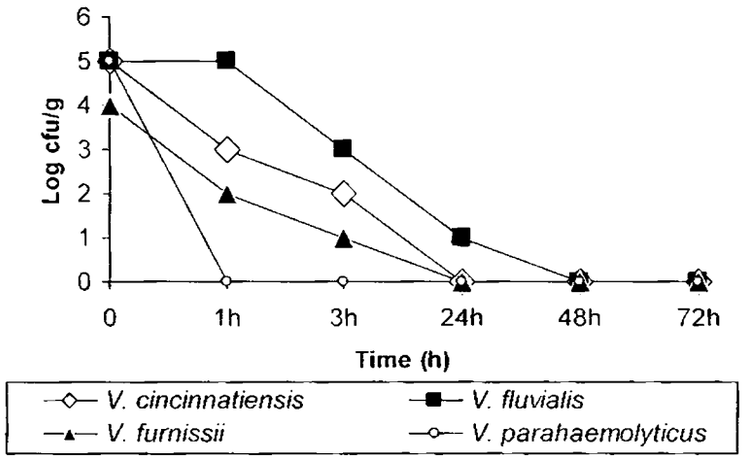
4.6. Effect of drying on HPVs

4.6.1. Effect of sun drying on HPVs

Effect of sun drying on HPVs viz., *V. cincinnatiensis*, *V. fluvialis*, *V. furnissii* and *V. parahaemolyticus* was studied on fresh samples of silver belly purchased from the market. Inoculated fish samples were dried in the sun for 3 consecutive days. Load of each species was analyzed at intervals of 1 h, 3 h, 24 h, 48 h and 72 h of drying. Moisture content and water activity of the inoculated fish sample was also measured at each interval.

Effect of sun drying on each species of HPVs is depicted in Fig 19. Immediately after 1 h drying, *V. cincinnatiensis* showed 2 log reduction. After 3 h drying 3 log reduction was observed and within 24 h complete inactivation was observed. Table 11 shows the moisture content and water activity (a_w) of the sample at various time intervals. Before drying, moisture content and water activity (a_w) of the sample were found to be 66.6 % and 0.987 and after 24 h drying values were 41.8 % and 0.629 respectively. No surviving cells of *V. cincinnatiensis* were noticed after 48 h drying.

Fig. 19 Effect of sun drying on HPVs



No log reduction in count was observed for *V. fluvialis* within 1 h drying (Fig. 19). 3 log reduction was observed after 3 h and 24 h drying. After 48 h complete inactivation took place. Table 11 shows the moisture content and a_w of the

sample, before and after drying. After 48 h drying the moisture content and a_w came down to 6.4 % and 0.521 respectively. No surviving cells of *V. fluvialis* were noticed after 48 h drying.

V. furnissii showed 2 log reduction within 1 h drying (Fig. 19). Within 3 h drying the load came down to <3 MPN g^{-1}. Complete destruction took place within 24 h. Moisture content and a_w of the sample after 24 h drying were 31.2 % and 0.862 respectively (Table 11). No surviving cells of *V. furnissii* were noticed after 48 h and 72 h drying.

Complete inactivation occurred within 1 h sun drying with respect to *V. parahaemolyticus*. Moisture content came down to 66 % from 68.6 %, a_w came down from 0.991 to 0.989 (Table 11) after 1 h. No surviving cells of *V. parahaemolyticus* were noticed after 24 h drying.

Table 11. Effect of sun drying on moisture content and water activity (a_w) of fish inoculated with different species of HPVs during different time intervals

Vibrio spp		After Inoculation	Period of sun drying				
			1 h	3 h	24 h	48 h	72 h
V. cincinnatiensis	Moisture content (%)	66.6	57.8	53.3	41.8	7.5	2
	Water activity (a_w)	0.987	0.979	0.942	0.629	0.561	0.4
V. fluvialis	Moisture content(%)	66.8	64.8	60.2	25	6.4	3.6
	Water activity (a_w)	0.988	0.981	0.979	0.73	0.521	0.485
V. furnissii	Moisture content(%)	66.3	56.1	52.2	31.2	5.3	2.4
	Water activity (a_w)	0.986	0.983	0.932	0.862	0.453	0.406
V. parahaemolyticus	Moisture content(%)	68.6	66	58.6	48.7	8.3	2.5
	Water activity (a_w)	0.991	0.989	0.982	0.878	0.624	0.407

Studies have shown that HPVs were very sensitive to sun drying. Complete inactivation was observed within 3days sun drying. *V. cincinnatiensis* showed 3 log reduction after 3 h drying and complete destruction took place within 24 h (Fig.19). a_w of the sample after 3 h drying was found to be 0.942. The moisture content observed for the sample was 53.3 % (Table 11). Jayaprakasha *et al.* (1997) have reported that as a_w is reduced, some bacteria stop growing at high values, while others are able to grow at much lower values. A decrease in a_w causes physiological problems within the microorganisms due to higher concentration of solutes in the external environment (Jayaprakasha *et al.*, 1997).

V. fluvialis showed slight resistance to sun drying compared to other species studied (Fig. 19). Jayaprakasha *et al.* (1997) have indicated that halophilic bacteria accumulate potassium ion (a compatible solute) within the cell to counteract osmotically hostile environment. Although the species showed 4 log reduction after 24 h drying complete inactivation was observed, only after 48 h drying. After 3 h drying cells at a level of 10^2 g^{-1} were found surviving in fish samples. The a_w and moisture content observed for the sample after 3 h drying was 0.979 and 60.2 % respectively (Table 11).

V. furnissii also survived 3 h sun drying, although drastic reduction in count was observed. Complete inactivation took place within 24 h (Fig. 19). After 1 h drying 2 log reduction was observed. The a_w and moisture content observed in the sample after 1 h drying were found to be 0.983 and 56.1 % respectively (Table 11). Apparently no other information is available on this aspect for comparison.

V. parahaemolyticus was found very sensitive to drying compared to other species. Within 1 h complete inactivation was observed. Venugopal *et al.* (1984) have got similar results. They found that even when the dry fish are contaminated at levels ranging from 10^{10} g^{-1}, *V. parahaemolyticus* does not survive for even 2 h after contamination. In another study (Venugopal *et al.*,1984) when the white baits inoculated with *V. parahaemolyticus* to the level of 10^3 g^{-1} were subjected to sun drying, it was observed that salted and unsalted fish were free from this organism on the 4^{th} day.

4.6.2. Effect of salt curing and sun drying on HPVs

Effect of salt curing and sun drying on HPVs were studied on Barracuda (*Sphyraena jello*). Load was analyzed immediately after inoculation, after curing for 48 h, after different periods of sun drying i.e., 1 h , 3 h , 24 h , 48 h and 72 h. Simultaneously moisture content and water activity were determined at each time interval.

Fig. 20 shows the effect of salt curing on different species of HPVs. After curing for 48 h *V. cincinnatiensis* showed 1 log increase in count. Fig. 21 shows the effect of salt drying on HPVs. No log reduction was observed after 1 h and 3 h drying. After 24 h 1 log decrease in count was noticed. 3 log reduction was observed within 48 h drying and complete inactivation took place within 72 h sun drying. Table 12 shows the moisture content and a_w of the sample during different time intervals. Moisture content and a_w of the sample came down to 67 % and 0.887 respectively after curing for 48 h. After sun drying for 72 h moisture content and a_w of the sample came down to18.6 %and 0.672 respectively.

Fig. 20 Effect of salt curing on HPVs

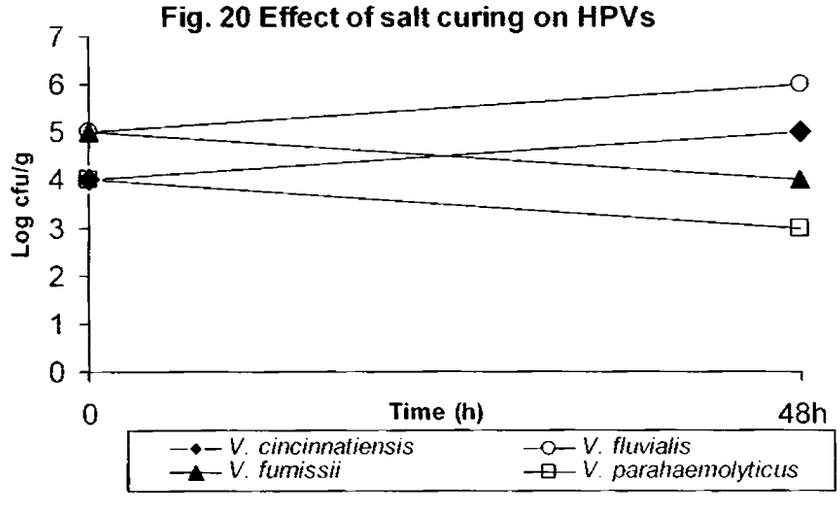
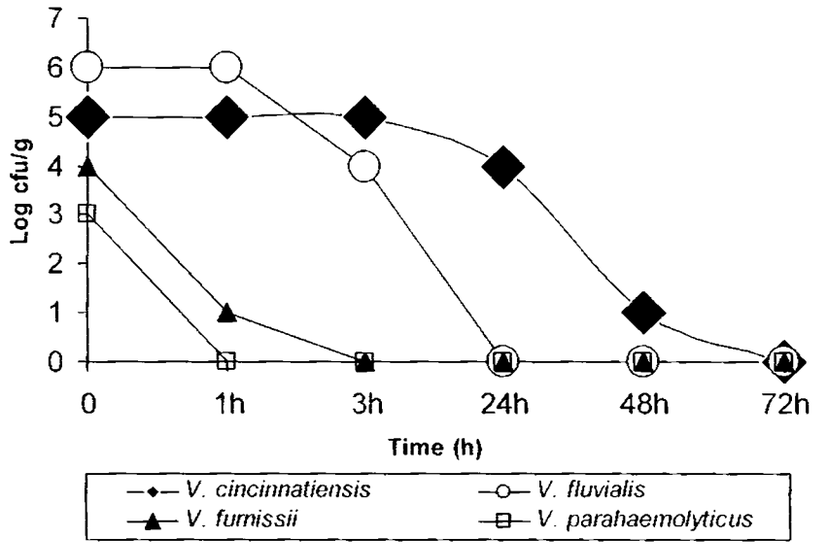


Fig. 21 Effect of salt drying on HPVs



Like *V. cincinnatiensis, V. fluvialis* showed 1 log increase in count after curing (Fig. 20). After 1 h drying no change in count was observed (Fig 21). After 1 h drying, moisture content and a_w came down to 55.4 % and 0.750 respectively (Table 12). 2 log reduction in count of *V. fluvialis* was observed after 3 h sun drying, moisture content and a_w came down to 53.6 % and 0.740 respectively. No viable cells were detected after 24 h and moisture content and a_w came down to 49.2 % and 0.735 respectively.

V. furnissii showed 1 log reduction after curing (Fig. 20), moisture content and a_w of the sample was found to be 67.4 % and 0.801 respectively (Table. 12). 3 log reduction in count was observed within 1 h sun drying, and within 3 h complete inactivation took place (Fig. 21). Moisture content and a_w came down to 63.6 % and 0.758 respectively after 1 h sun drying. After 3 h drying moisture content reduced to 55 % and a_w reduced to 0.751. No surviving cells of *V. furnissii* were detected after 24 h drying.

V. parahaemolyticus also showed 1 log reduction on curing (Fig. 20). Complete destruction took place within 1 h sun drying (Fig. 21). Moisture content and a_w of the sample before and after curing were 79.8 % and 67 %, 0.896 and 0.887

respectively (Table 12). After 1 h drying moisture content came down to 58 %. Similarly a_w reduced to 0.753 from 0.887 after 1 h drying. Surviving cells of *V. parahaemolyticus* could not be detected after 3 h drying.

Table 12. Effect of salt curing and sun drying on moisture content and water activity of fish inoculated with different species of HPVs during different time intervals

Vibrio spp		After Inocu-lation	After curing	Period of sun drying				
				1 h	3 h	24 h	48 h	72 h
V. cin.	Moisture content (%)	79.6	67	59.4	49	48	29	18.6
	Water activity (a_w)	0.895	0.887	0.837	0.759	0.74	0.734	0.672
V. flu.	Moisture content(%)	79.6	60	55.4	53.6	49.2	30	16
	Water activity (a_w)	0.895	0.772	0.75	0.74	0.735	0.729	0.532
V. fur.	Moisture content(%)	80	67.4	63.6	55	44.2	40.2	19
	Water activity (a_w)	0.892	0.801	0.758	0.751	0.745	0.701	0.621
V. par.	Moisture content(%)	79.8	67	58	55	48	28	18.2
	Water activity (a_w)	0.896	0.887	0.753	0.75	0.745	0.735	0.692

V. cincinnatiensis, in contrast to unsalted dried fish showed maximum survival in salted dried fish in this study. *Barracuda* spp. salted in the ratio 1:4 was used in this study. *V. cincinnatiensis* showed 1 log increase in count after curing. This is probably due to the exposure of the organism to elevated temperature during curing (Fig.20). a_w reduced to 0.887 from 0.895 (Table 12). No log reduction was observed (Fig. 21) despite reduction in a_w and moisture content during 1 h and 3 h drying. Complete inactivation was observed at 72 h. Salt curing was found not effective against *V. cincinnatiensis* in this study. Information regarding the survival of HPVs in unsalted and salted dried fish is scanty.

V. furnissii and *V. fluvialis* showed complete inactivation within 3 h and 24 h of salted drying respectively (Fig. 20 and 21).

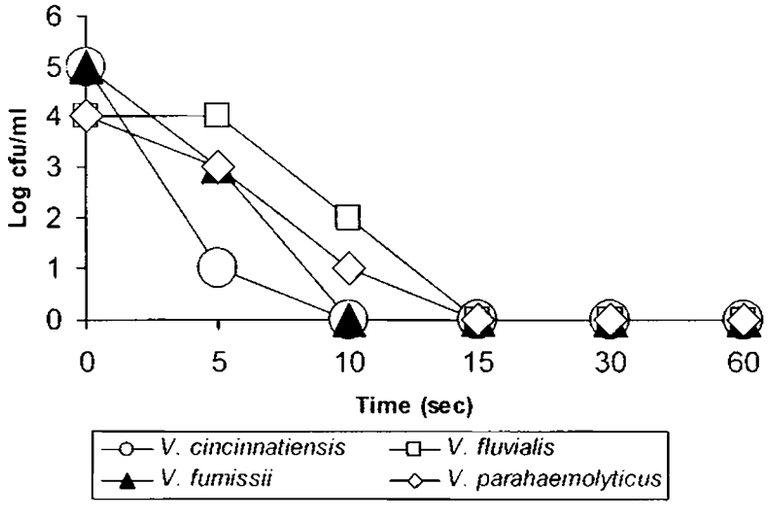
V. parahaemolyticus was found very sensitive to salt curing and sun drying. Within 1 h sun drying cells lost viability. Venugopal *et al.* (1984) observed that salted and unsalted fish inoculated with *V. parahaemolyticus* to the level of $10^3 g^{-1}$ and subjected to sun drying were free from this organism on the 4th day. Temmyo (1966) reported rapid death of *V. parahaemolyticus* when inoculated membrane filters were placed in a container with silica gel and an inoculated chopping board was allowed to dry.

The results of the present study indicate that the survival period of HPVs in salted and unsalted fish were very short. Complete inactivation was observed within 3 days sun drying. Venugopal *et al.* (1984) suggested that *V. parahaemolyticus* encountered in market samples of dry fish could be due to post process contamination. Studies have shown that even when the fish samples were contaminated with HPVs at a level of 10^5 g^{-1}, rate of destruction took place within 3 days, which is the usual time taken for drying in commercial practices. However there are ample opportunities for the contamination of dry fishes in the market environment. Drying fish for the required period of time and preventing cross contamination can avoid risk due to HPVs in dried fishes.

4.7. Effect of blanching on HPVs

Fig. 22 shows the effect of blanching at 100°C on various species of HPVs. *V. cincinnatiensis* after 5 sec of blanching showed 4 log reduction. Complete inactivation occurred within 10 sec of blanching. No log reduction was observed after 5 sec of blanching with respect to *V. fluvialis*. After 10 sec 2 log reduction was observed and in 15 sec complete inactivation occurred. *V. furnissii* showed 2 log reduction after 5 sec blanching and within 10 sec complete destruction was observed. *V. parahaemolyticus* showed 1 log reduction after 5 sec and a 3 log reduction after 10 sec. Complete inactivation of HPVs studied was observed within 15 sec.

Fig.22 Effect of blanching on HPVs



V. cincinnatiensis and *V. furnissii* showed the maximum sensitivity to blanching. Complete inactivation occurred within 10 sec. *V. fluvialis* and *V. parahaemolyticus* on the other hand showed slight thermal resistance but complete inactivation took place within 15 sec (Fig. 22). Varnam and Evans (1996) have indicated that resistance to heating depends on several factors, including heating menstrum and physiological condition.

Blanching at 100°C was found very effective against HPVs studied. Sakazaki (1983) has reported that raw seafoods are mostly implicated in out breaks of food poisoning, but if they are heated at 100°C shortly before consumption, infection with *V.parahaemolyticus* never occurs. The commercial practice of heat shocking oysters in boiling water to facilitate opening reduced counts of *V.parahaemolyticus* and often non *V. cholera* vibrios to undetectable levels (Hackney *et al.*, 1980). Vanderzant and Nickelson (1972) observed 6 $\log_{10}$ decline at 100°C in 1 min in shrimp homogenate supplemented with 3 % NaCl. Chang *et al.* (2004) in a study revealed that when *V.parahaemolyticus* were heat shocked at 42°C for 15, 30 or 45 min, it caused an increased demand for NaCl during recovery from heat injury. They also reported that heat shock generally increased the survival of the test organism during subsequent exposure to 47°C.

Studies have shown that vibrios are not heat resistant and are readily destroyed within 1 min by dipping in water at boiling temperatures. Risk associated with HPVs is more in countries were people have the habit of consuming raw or insufficiently cooked seafoods.

4.8. Effect of NaCl on HPVs

Sodium chloride concentration and the corresponding optical density of various species of HPVs are presented in Table.13. *V. cincinnatiensis* showed good growth between 2 % and 8 % NaCl and optimum level was observed at 7 % NaCl. *V. fluvialis* showed maximum growth at 3 % NaCl, good growth was observed between 2 and 4 % NaCl. *V. furnissii* showed maximum growth at 2 % NaCl .*V. parahaemolyticus* showed good growth between 2 and 6 % NaCl and maximum growth was observed at 3 % NaCl.

Table.14 shows the optimum level of NaCl for each species studied. Studies have shown that the optimum concentration of NaCl for different species of HPVs varied between 2 and 7 % (Table. 14). V. cincinnatiensis showed the maximum growth in tryptic soya broth with 7 % NaCl. Brayton *et al.* (1986) have reported that the species can tolerate 6 % NaCl, but not 8 % NaCl supplement to the growth medium. West and Colwell (1984) have also observed the growth of *V. cincinnatiensis* at 3 and 6 %.

Optimum level of NaCl noticed for *V. fluvialis* and *V. furnissii* in this study was 3 % and 2 % respectively. However, growth was observed between 1 - 10 % for *V. fluvialis* and between 1 - 6 % for *V. furnissii*. Similar results were reported by Brenner *et al.* (1983), West and Colwell (1984) and Alsina and Blanch (1994).

Table 13. Effect of NaCl on growth of HPVs in tryptic soya broth.

NaCl Conc. (%)	Optical Density (OD) and *Vibrio* spp.			
	V. cincinnatiensis	*V. fluvialis*	*V. furnissii*	*V. parahaemolyticus*
0	0.000	0.000	0.000	0.000
1.0	0.227	0.250	0.303	0.045
2.0	0.281	0.307	0.305	0.112
3.0	0.350	0.381	0.174	0.314
4.0	0.363	0.375	0.106	0.175
5.0	0.370	0.296	0.076	0.156
6.0	0.375	0.282	0.036	0.110
7.0	0.384	0.250	0.007	0.098
8.0	0.241	0.240	0.005	0.027
9.0	0.105	0.235	0.003	0.005
10.0	0.055	0.137	0.001	0.003

Table 14. Optimum level of NaCl for each species of HPVs

Vibrio spp.	Optimal level of NaCl (%)
V. cincinnatiensis	7.0
V. fluvialis	3.0
V. furnissii	2.0
V. parahaemolyticus	3.0

Maximum growth of *V. parahaemolyticus* was noticed at 3 % level and good growth was in the range of 2-6 %. Sakazaki and Shinoda (1986) reported good to fair growth at all salt concentrations between 0.5 and 8 %, with maximum growth at 3 % level According to Beuchat (1974) the fastest generation time observed for *V. parahaemolyticus* was 16.4 min in tryptic soya broth containing 2.9 % NaCl. Twedt (1989) reported tolerance of *V.parahaemolyticus* to low and high NaCl concentrations is a function of osmotic as well as ionic sensitivity. The optimal concentration of 3 % corresponds to a_w, of 0.992. They also found the minimum a_w found for growth of *V. parahaemolyticus* in trypticase soya broth with added NaCl was 0.948. The organism was readily inactivated in distilled water, with 90 % of the cells inactivated between 0.9 and 4.4 min (Lee. 1972). Covert and Woodburn (1972) found NaCl appeared to be protective to the cells of *V. parahaemolyticus* in tryptic soya broth and fish homogenate at 48 $\pm$1°C.

4.9. Effect of pH on HPVs in shrimp homogenate prepared with different diluents and incubated at 37 °C for 24 h

Effect of pH (1-14) on various species of HPVs was studied in shrimp homogenate (SH). SH prepared with three different diluents i.e. DW, NS and 3 % NaCl solution were used in this study. Counts were taken after inoculating into shrimp homogenate with different pH and incubated at 37°C for 24 h.

V. cincinnatiensis could not grow in any of the pH in SH prepared with DW after incubation at 37°C for 24 h. In SH prepared with NS and 3 % NaCl, cells survived in the pH range 5-12 (Table. 15). *V. cincinnatiensis* could not survive below pH 5 and above pH 12. In SH with NS maximum growth was observed at pH 8, a gradual reduction in count was observed at pH 6 and 5. At pH 9 and 10 count remained almost the same.

The lowest survival in SH prepared with NS was noticed at pH 12. In SH prepared with 3 % NaCl maximum growth was obtained at pH 8, and a gradual reduction was observed towards the acidic pH. At pH 9 and 10, log value remained the same and at pH 9, 10, 11 and 12 reduction in count took place at a slower rate.

Table. 16 shows the survival of *V. fluvialis* at various pH. In SH prepared with DW cells survived at pH 7 and 8. In SH prepared with NS and 3 % NaCl *V. fluvialis* survived at a pH range between 5 and 12. Maximum growth was observed at pH 7, 8 and 9 in SH with NS. Towards the lower pH *V. fluvialis* showed a rapid reduction in log count in NS and 3 % NaCl.On the alkaline side reduction was gradual. Minimum survival in SH with NS was observed at pH 5. In SH prepared with 3 % NaCl maximum growth was observed at pH 7,8,9 and 10, a gradual reduction in log count was observed towards the lower and higher pH. Lowest survival in SH with 3 % NaCl was observed at pH 5.

V. furnissii survived in SH prepared with DW at a pH range 6-12 (Table. 17) Maximum survival in DW occurred at pH 9. A gradual reduction was observed towards both the acidic and alkaline side. In SH with NS and 3 % NaCl growth was observed at a pH range 5-10. In NS maximum growth was observed at pH 9 and growth remained almost steady at pH 5, 6, 7, 8 and 10. Reduction in log count was observed as the pH increased in SH with NS. In SH with 3 % NaCl maximum growth was observed at pH 6 and 7 and the count remained almost the same at pH 5, 8, 9 and 10. Lowest survival in SH with 3 % NaCl was observed at pH 11 and 12.

Table. 18 shows the growth and survival of *V. parahaemolyticus* at various pH in SH with different diluents. In SH prepared with DW *V. parahaemolyticus* survived at a pH range 6-7. 3 log reduction was observed at pH 6 and 7 in DW. In SH prepared with NS growth was observed between pH 7 to 9. Maximum growth was observed at pH 7. At pH 8 and 9 count remained almost the same and the lowest survival in SH prepared with NS was obtained at pH 11. In SH prepared with 3 % NaCl *V. parahaemolyticus* showed growth at a pH range 6-11. Maximum growth was observed at pH 7. The count remained almost the same at pH 9, 10 and 11, and the lowest count in SH prepared with 3 % NaCl was observed at pH 12. At pH 6 growth of *V.parahaemolyticus* was very poor.

Table 15. Growth and survival of *V. cincinnatiensis* in shrimp homogenate prepared with different diluents and pH

pH	Diluents		
	DW	NS	3 %NaCl
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	1.5×10^4	7.2×10^4
6	0	8.9×10^6	1.1×10^8
7	0	3.5×10^7	7.6×10^7
8	0	6.0×10^7	1.8×10^9
9	0	7.3×10^6	2×10^8
10	0	6.2×10^6	2.1×10^8
11	0	5.2×10^5	2.8×10^7
12	0	1.3×10^3	3×10^5
13	0	0	0
14	0	0	0

(Initial load 7×10^5 cfu ml^{-1})

Table 16. Growth and survival of *V. fluvialis* in shrimp homogenate prepared with different diluents and pH

pH	Diluents		
	DW	NS	3 %NaCl
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	2×10^1	1.1×10^3
6	0	4×10^3	2×10^5
7	3.5×10^2	2.4×10^7	1.9×10^7
8	2.8×10^2	3.1×10^7	9.6×10^7
9	0	2.8×10^7	3.5×10^7
10	0	2×10^6	2.1×10^7
11	0	3.5×10^5	4×10^6
12	0	2.6×10^5	2.3×10^5
13	0	0	0
14	0	0	0

(Initial load 3.9×10^5 cfu ml^{-1})

Table 17. Growth and survival of *V. furnissii* in shrimp homogenate prepared with different diluents and pH

pH	Diluents		
	DW	NS	3 %NaCl
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	1.4×10^7	2×10^7
6	2×10^2	5×10^7	1×10^8
7	6×10^5	9.5×10^7	1.1×10^8
8	6.9×10^5	8.4×10^7	8.7×10^7
9	3.2×10^6	1×10^8	6.1×10^7
10	1.6×10^5	7.5×10^7	6.7×10^7
11	2×10^3	4.8×10^5	5×10^5
12	1×10^3	3.8×10^4	3.2×10^5
13	0	0	0
14	0	0	0

(Initial load 4.4×10^6 cfu ml^{-1})

Table 18. Growth and survival of *V. parahaemolyticus* in shrimp homogenate prepared with different diluents and pH

pH	Diluents		
	DW	NS	3 %NaCl
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6	1.8×10^1	1.1×10^4	1×10^5
7	3.5×10^1	9.6×10^5	1.1×10^6
8	4	4.3×10^4	1.3×10^5
9	2	3.9×10^4	7.9×10^4
10	0	6.1×10^3	5×10^4
11	0	5×10^2	3.2×10^4
12	0	0	4.2×10^2
13	0	0	0
14	0	0	0

(Initial load 3×10^4 cfu ml^{-1})

Studies have shown that growth of HPVs occurred at a PH range 6-11 and prefers an alkaline pH. Varnam and Evans (1996) have reported similar views and observed that enteropathogenic vibrios grew well at alkaline pH, the upper limiting values being pH 10-11.

V. cincinnatiensis couldn't survive in any of the pH in SH prepared with DW (Table. 15). This species was found to be very sensitive to low saline condition. More or less similar growth pattern was observed at various pH in SH with NS and 3 %NaCl solution. Maximum growth was observed at pH 8 in both the media .Lowest growth was obtained at pH 5. *V. cincinnatiensis* in SH with 3 % NaCl solution and NS showed better survival towards the alkaline side. Apparently no other information is available on this aspect for comparison.

V. fluvialis survived in SH prepared with DW at pH 7 and 8 although the log value came down from 5 to 2 at both pH (Table 16). In the present study it was observed that two species i.e., *V. fluvialis* and *V. furnissii* showed the maximum survival in SH prepared with DW at various pH. Another interesting point noted is the maximum survival of these species at low temperature (4°C and 10°C) in SH prepared with DW, these results show the potential of above mentioned pathogens in low saline condition in seafood. Although the studies on the incidence of HPVs in fish and fish products have shown a low percentage of incidence of these species, it is clear from the above studies that these species could survive very well at low temperatures at an optimum pH of 7 in seafood. In SH prepared with NS and 3 % NaCl solution *V. fluvialis* have shown more or less similar growth pattern at various pH i.e. 5-12. Maximum growth was observed at pH 7, 8, 9 and 10 (Table 16). Growth was not observed below pH 6.

Compared to other species *V. furnissii* showed the maximum survival at various pH in SH with DW. Table 17 shows that the species survived at a pH range 6-12. Maximum growth was observed at pH 9 in SH with NS and at pH 7 in SH with 3 % NaCl solution. In SH with NS and 3 % NaCl solution growth was observed at a pH range 5-10. One of the remarkable features of *V. furnissii* as compared to other species is the acid tolerance. One log increase in count was observed at pH 5 in SH with NS and 3 % NaCl solution. Audia *et al.* (2001) in a study on the

ability of acid tolerance in enteropathogens have opined that acid tolerance occurs by modulating the activities or levels of diverse regulatory proteins in response to pH stress. It resulted in induction of overlapping arrays of acid shock proteins that protect the cell against acid and other environmental stress.

V. parahaemolyticus survived in SH prepared with DW at a pH range 6-7.In SH prepared with NS and 3 % NaCl solution maximum growth was observed at pH 7 (Table 18). Vanderzant and Nickelson (1972) studied the survival of *V. parahaemolyticus* in shrimp homogenates at various pH values. In homogenates adjusted to pH 1, 2,3 and 4 no survivors could be detected. At pH 5.0, a sharp drop in viable count took place immediately, with no survivors detectable after 15 min. This is in agreement with the result obtained in this study. Sakazaki *et al.* (1963) have reported the optimal pH range of *V. parahaemolyticus* to vary from 7.5 to 8.5. Although *V. parahaemolyticus* has been reported as growing at pH 4.8 (ICMSF, 1980), they are generally sensitive to pH values below 7.0. The minimum growth temperature reported for *V. parahaemolyticus* is 5°C (Beuchat, 1973; Katoh, 1965), the lower limit is affected by pH and salt concentration (Beuchat, 1973). The minimum pH reported to allow growth at 5°C in trypticase soy broth with 3 % NaCl was 7.3; when salt concentration was increased to 7 %, the minimum pH rose to 7.6 (Beuchat, 1973).

Wong and Wang (2004) analyzed the susceptibility of the Viable but noncultiviable (VBNC) cells of *V. parahaemolyticus* to environmental stresses. According to them low salinity of the medium crucially and markedly shortened the induction of VBNC state of *V. parahaemolyticus*. They also found that VBNC cells were highly resistant to low salinity (0 % NaCl), or acid (pH 4.0) inactivation.

In general studies have shown that HPVs were sensitive to pH below 5 and above 12. They preferred an alkaline environment to grow. *V. furnissii* had shown remarkable capacity to grow at low pH in low saline condition in SH as compared to *V. cincinnatiensis* and *V. fluvialis*. There was also a strong interaction with NaCl concentration in the media and pH, the lower limiting pH values tending to rise as the NaCl concentration increases. The enhanced stress resistance of these pathogens is a threat to seafood consumers.

4.10. Effect of chlorine and chlorine dioxide on HPVs

4.10.1. Effect of chlorine

Table 19 shows the survival of *V. cincinnatiensis* in sterile distilled water (DW), normal saline (NS) and 3 % NaCl solution with various levels of chlorine. In DW *V. cincinnatiensis* could not survive at any levels of available chlorine studied even for 1 min. In NS with 1 ppm available chlorine *V. cincinnatiensis* survived upto 2 min and no viable cells were detected after 5 min. However at 2 ppm level in NS cells lost viability within 2 min. Viability lost completely at 5 ppm and 10 ppm level available chlorine within 1 min. In 3 % NaCl solution viable cells were detected upto 15 min at 1 ppm level and viability was lost within 30 min. At 2 ppm level in 3 % NaCl solution, within 10 min cells lost viability. At 5 ppm and 10 ppm levels in 3 % NaCl *V. cincinnatiensis* lost viability within 1 min. In control 3 % NaCl solution *V. cincinnatiensis* was isolated through out the period of study.

Effect of various levels of chlorine on *V. fluvialis* is given in Table 20. *V. fluvialis* could not survive at any levels of available chlorine in DW and NS even for 1 min. In 3 % NaCl solution at 1 ppm level *V. fluvialis* survived upto 15 min. At 2 ppm level in 3 % NaCl solution *V. fluvialis* lost viability within 5 min and lost viability completely within 1 min at 5 ppm and 10 ppm available chlorine level. In control cells survived through out the experiment period.

V. furnissii couldn't survive at any levels of available chlorine in DW and NS even for 1 min (Table 21). In 3 % NaCl solution *V. furnissii* survived only for 1 min at 1 ppm and 2 ppm level available chlorine. At 5 ppm and 10 ppm available chlorine *V. furnissii* could not survive even for 1 min in 3 % NaCl solution.

Table. 22 shows the effect of various levels of chlorine on *V.parahaemolyticus* in distilled water, normal saline and 3 % NaCl solution. *V.parahaemolyticus* was found very sensitive to chlorine. No viable cells could be isolated at any levels of available chlorine in DW, NS and 3 % NaCl solution. In the control 3 % NaCl solution *V.parahaemolyticus* could be isolated through out the period of study.

Table 19. Effect of various levels of chlorine on *V. cincinnatiensis* in sterile distilled water, normal saline and 3 % NaCl solution during different time intervals

Medium	Cl_2 levels (ppm)	Time (min)						
		1	2	5	10	15	30	60
Distilled water	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Normal saline	1	+	+	-	-	-	-	-
	2	+	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
3 % NaCl solution	1	+	+	+	+	+	-	-
	2	+	+	+	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Control: 3 % NaCl solution	Without Cl_2	+	+	+	+	+	+	+

(Initial load 2.4 x 10^{5} cfu ml^{-1})

Table 20. Effect of various levels of chlorine on *V. fluvialis* in sterile distilled water, normal saline and 3 % NaCl solution during different time intervals

Medium	Cl_2 levels (ppm)	Time (min)						
		1	2	5	10	15	30	60
Distilled water	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Normal saline	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
3 % NaCl solution	1	+	+	+	+	+	-	-
	2	+	+		-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Control: 3 % NaCl solution	Without Cl_2	+	+	+	+	+	+	+

(Initial load 1.6×10^5 cfu ml^{-1})

Table 21. Effect of various levels of chlorine on *V. furnissii* in sterile distilled water, normal saline and 3 % NaCl solution during different time intervals

Medium	Cl_2 levels (ppm)	Time (min)						
		1	2	5	10	15	30	60
Distilled water	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Normal saline	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
3 % NaCl solution	1	+	-	-	-	-	-	-
	2	+	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Control: 3 % NaCl solution	Without Cl_2	+	+	+	+	+	+	+

(Initial load 4 x 10^3 cfu ml^{-1})

Table 22. Effect of various levels of chlorine on *V. parahaemolyticus* in sterile distilled water, normal saline and 3 % NaCl solution during different time intervals

Medium	Cl_2 levels (ppm)	Time (min)						
		1	2	5	10	15	30	60
Distilled water	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Normal saline	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
3 % NaCl solution	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Control: 3 % NaCl solution	Without Cl_2	+	+	+	+	+	+	+

(Initial load 1.2 x 10^3 cfu ml^{-1})

The general conclusion is that HPVs could not survive at any levels of available chlorine even for 1 min in DW. Complete inactivation in DW could be due to the osmotic pressure difference of the media rather than the effect of chlorine. Available chlorine at a minimum level of 1 ppm and a contact time of 5 min was found very effective against *V. cincinnatiensis* in NS. At 2 ppm level in NS, complete inactivation took place within 2 min. In 3 % NaCl solution inactivation took place at a slower rate, sodium chloride in the medium might have supported for the increased rate of survival. A contact time of 30 min and an available chlorine level of 1 ppm were required for the complete inactivation of *V. cincinnatiensis* in 3 % NaCl solution. However, when the concentration of available Cl_2 was increased in the medium protective function of sodium chloride seemed ineffective. At 2 ppm available chlorine level in 3 % NaCl solution complete inactivation took place within a contact time of 10 min. At 5 ppm and 10 ppm available chlorine level in NS and 3 % NaCl solution *V. cincinnatiensis* lost viability within a contact time of 1 min.

V. fluvialis was found very sensitive towards chlorine. In NS this species was found not able to survive any levels of chlorine, even for 1 min. In 3 % NaCl solution a minimum level of 1 ppm available chlorine and a contact time of 30 min was found sufficient to inactivate the bacteria. A contact time of 5 min was required at 2 ppm level in 3 % NaCl solution for complete inactivation.

Like *V. fluvialis*, *V. furnissii* was also found to be very much sensitive towards chlorine. *V. furnissii* could not tolerate any level of available chlorine in NS, and complete inactivation took place within 1 min. In 3 % NaCl solution complete inactivation took place in 1 ppm and 2 ppm available chlorine within a contact time of 2 min. At 5 pm and 10 ppm level complete inactivation took place within 1min in NS and 3 % NaCl.

Chlorine was found very effective against *V. parahaemolyticus* in this study. This species was found not able to survive any levels of available chlorine in NS and 3 % NaCl solution even for 1 min. Venugopal *et al.* (2000) have reported that a minimum level of 0.5 ppm available chlorine was able to reduce the count of both Kanagawa Positive (K^+) and Kanagawa negative (K^-) *V.parahaemolyticus* in

phosphate buffered saline (PBS) by 90 % within 5 min and complete killing of both was achieved in 20 and 30 min, respectively. He also showed that *V. para haemolyticus* was not able to survive beyond 5 min in PBS containing 1 ppm chlorine level. In fish artificially contaminated with K^+ *V.parahaemolyticus* and exposed to 10 and 20 ppm available chlorine complete destruction of the cells was observed within 10 min, but at 30 ppm the time required was only 5 min (Venugopal *et al.*, 2000).

Information regarding the use of chlorine in process water against HPVs is limited to *V. parahaemolyticus*, nothing is known about the effectiveness of chlorine against other species of vibrios studied. Studies have shown that in DW HPVs couldn't survive at any levels of available chlorine. *V. cincinnatiensis* was found to be the most resistant to chlorine compared to other species. At 2 ppm level it could survive upto 5 min (Table 19).

The study also indicates that a minimum available chlorine level of 1 ppm and contact time of 30 min was found very effective against all the HPVs studied. Above results were well within the limits of EU requirement of < 2 ppm available chlorine for the process water in seafood factories. Results obtained in this study also suggests that water treatment in fish processing industries should give sufficient time for inactivation of pathogens. Available chlorine at 2 ppm and a contact time of 10 min was also found very effective against HPVs in this study.

To minimize chlorine waste and optimize its efficient use, chlorine concentration in sanitizing solutions should be monitored (Suslow, 2000). The concentration of the fast acting, antimicrobial hypochlorous acid, the chemical species providing free available chlorine to disinfect solutions, is a function of pH, between pH 6.5 and 7.0, HOCl exists as 80-95 % of the free chlorine concentration (Suslow, 2000). Free chlorine disinfects by chemically disrupting bacterial cell walls and membrane through oxidation of a chemical group known as the thiol group (WHO, 1998). The exposure of microbial cells to chlorine is known to cause disruption of cellular enzyme system (Wyss, 1961), protein synthesis (Benarde *et al.*, 1967), oxygen uptake and oxidative phosphorylation (Venkobackar *et al.*, 1977) resulting in death or inactivation of cells. Chlorination of water to required

level and giving sufficient time for inactivation was found very effective against HPVs studied.

4.10.2. Effect chlorine dioxide (ClO_2) on HPVs

Table 23. shows the survival of *V. cincinnatiensis* in DW, NS and 3 % NaCl solution with different levels of ClO_2 during different time intervals. In DW *V. cincinnatiensis* could not survive at any level of chlorine dioxide, complete inactivation took place within 1 min. In NS at 1 ppm level inactivation occurred within 2 min and at 2 ppm, 5 ppm and 10 ppm level *V. cincinnatiensis* lost viability within 1 min. In 3 % NaCl solution at 1 ppm level cells could not survive upto 5 min and at 2 ppm, 5 ppm and 10 ppm complete inactivation took place within 1 min. In control i.e. 3 % NaCl solution without ClO_2 *V. cincinnatiensis* survived through out the period of study.

Effect of various levels of chlorine dioxide on *V. fluvialis* in sterile DW, NS and 3 % NaCl solution is given in the Table 24. *V. fluvialis* could not survive at any levels of ClO_2 in DW and NS . Viability lost within 1 min. In 3 % NaCl solution at 1 ppm level *V. fluvialis* survived only upto 1 min. At 2 ppm, 5 ppm and 10 ppm level in 3 % NaCl solution *V. fluvialis* lost viability within 1 min. In the control sample i.e. 3 % NaCl solution without ClO_2 cells survived through out the period of study.

V. furnissii couldn't survive at any levels of chlorine dioxide in DW, NS and 3 % NaCl. Lost viability within 1 min in all the media studied (Table 25).In the control sample cells survived through out the study.

Table 26. shows the effect of various levels of chlorine dioxide on *V.parahaemolyticus* in distilled water, normal saline and 3 % NaCl solution. No viable cells could be isolated at any levels of chlorine dioxide in DW, NS and 3 % NaCl solution, complete inactivation occurred within 1 min in all the media studied. In control i.e. 3 % NaCl solution without ClO_2 *V. parahaemolyticus* was isolated through out the period of study.

Table 23. Effect of various levels of chlorine dioxide on *V. cincinnatiensis* in sterile distilled water, normal saline and 3 % NaCl solution during different time intervals

Medium	ClO_2 levels (ppm)	Time (min)						
		1	2	5	10	15	30	60
Distilled water	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Normal saline	1	+	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
3 % NaCl solution	1	+	+	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Control: 3 % NaCl solution	Without ClO_2	+	+	+	+	+	+	+

(Initial load 7.8 x 10^3 cfu ml^{-1})

Table 24. Effect of various levels of chlorine dioxide on *V. fluvialis* in sterile distilled water, normal saline and 3 % NaCl solution during different time intervals

Medium	ClO_2 levels (ppm)	Time (min)						
		1	2	5	10	15	30	60
Distilled water	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Normal saline	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
3 % NaCl solution	1	+	-	-	-	-	-	-
	2	-	-		-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Control: 3 % NaCl solution	Without ClO_2	+	+	+	+	+	+	+

(Initial load 3.12×10^5 cfu ml-1)

Table 25. Effect of various levels of chlorine dioxide on *V. furnissii* in sterile distilled water, normal saline and 3 % NaCl solution during different time intervals

Medium	ClO_2 levels (ppm)	Time (min)						
		1	2	5	10	15	30	60
Distilled water	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Normal saline	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
3 % NaCl solution	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Control: 3 % NaCl solution	Without ClO_2	+	+	+	+	+	+	+

(Initial load 2.8 x 10^2 cfu ml-1)

Table 26. Effect of various levels of chlorine dioxide on *V. parahaemolyticus* in sterile distilled water, normal saline and 3 % NaCl solution during different time intervals

Medium	ClO_2 levels (ppm)	Time (min)						
		1	2	5	10	15	30	60
Distilled water	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Normal saline	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
3 % NaCl solution	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Control: 3 % NaCl solution	Without ClO_2	+	+	+	+	+	+	+

(Initial load 3.1 x 10^4 cfu ml-1)

HPVs were found very sensitive towards chlorine dioxide in this study. None of the species could survive in DW at any levels of chlorine dioxide. ClO_2 at a minimum level of 1 ppm with 2min contact time was found very effective against all the HPVs in this study. In media with chlorine dioxide at 1 ppm level *V. cincinnatiensis* survived only for 1 min in NS and 2 min in 3 % NaCl solution. Complete inactivation within 1 min contact time was observed in 2 ppm, 5 ppm and 10 ppm level chlorine dioxide in 3 % NaCl solution. *V. fluvialis* showed complete inactivation within 1 min contact time in DW and NS at all levels of chlorine dioxide tested. In 3 % NaCl solution *V. fluvialis* survived for a short period of 1 min at 1 ppm level. This organism lost viability at all other ClO_2 level tested in 3 % NaCl solution. *V. furnissii* and *V. parahaemolyticus* showed the maximum sensitivity towards ClO_2. HPVs lost viability completely in all the media studied within 1 min contact time. No reported information is available on the effect of ClO_2 on HPVs.

Chlorine dioxide at low concentration and quick inactivation was found very effective against all the HPVs in this study. Information is limited regarding the usefulness of ClO_2 in seafood processing. No standards are available on its usage in seafood process water. Food and Drug Administration on March 3, 1995 amended the food additive regulations to provide a 3 ppm residual chlorine dioxide for controlling microbial populations in poultry processing water (FDA, 1995). Chlorine dioxide offers some unique advantages over chorine due to its selectivity, effectiveness over a wide pH range, and speed of kill, but safety and cost issues have restricted its use as a viable replacement. In light of the stringent quality control measures adopted by EU and USFDA, it is high time for our seafood industry to shift to more effective means of bacterial disinfection.

4.11. Antibiotic sensitivity of HPVs

Antibiotic sensitivity of 22 strains of *V. cincinnatiensis* isolated during the study is given in Fig. 23. Maximum sensitivity was shown towards chloramphenicol (87.5%) followed by trimethoprin (83.3 %), nalidixic acid, penicillin and streptomycin (41.6 %). Sensitivity towards other antibiotics like ceftriaxone,

amikacin, erythromycin and kanamycin were 33.3 %, 16.6 %, 8.33 % and 4.16 % respectively. All the strains were found resistant towards ampicillin.

Fig. 23 Antibiotic sensitivity of *V. cincinnatiensis*

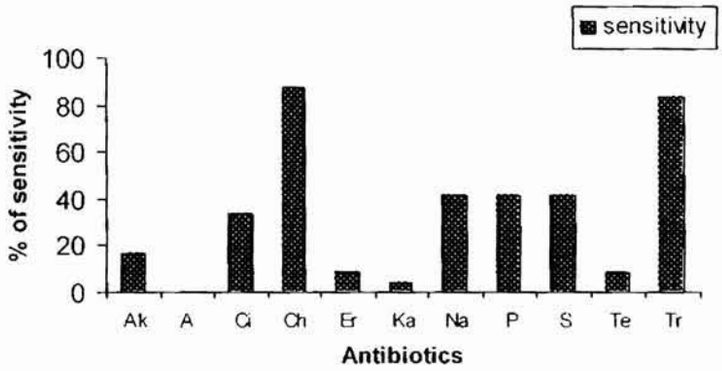
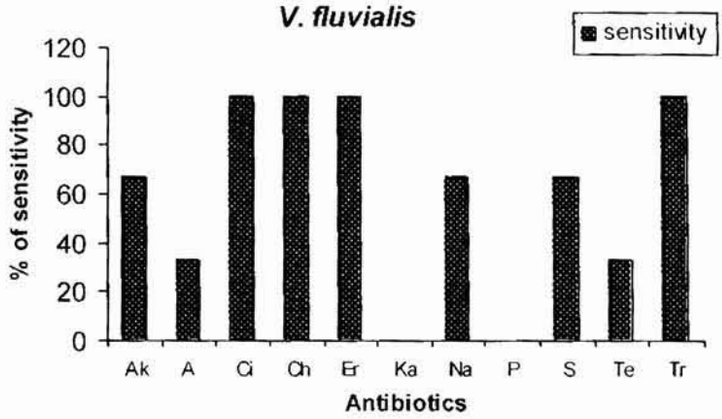


Fig. 24. shows the sensitivity of *V. fluvialis* towards various antibiotics. *V. fuvialis* showed maximum sensitivity (100 %) towards ceftriaxone, chloramphenicol, erythromycin and trimethorpin followed by amikacin, nalidixic acid and streptomycin (66.6 %), ampicillin and tetracycline (33.3 %). All the isolates were found resistant towards kanamycin and penicillin.

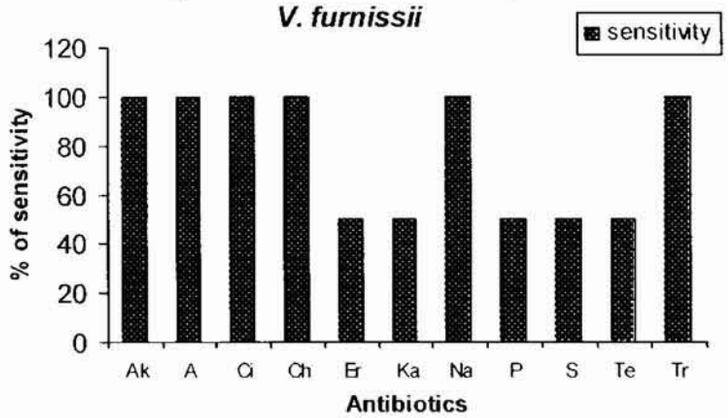
Fig. 24 Antibiotic sensitivity of *V. fluvialis*



Antibiotic sensitivity pattern of *V. furnissii* is given in Fig. 25. Maximum sensitivity (100 %) was observed towards amikacin, ampicillin, ceftriaxone,

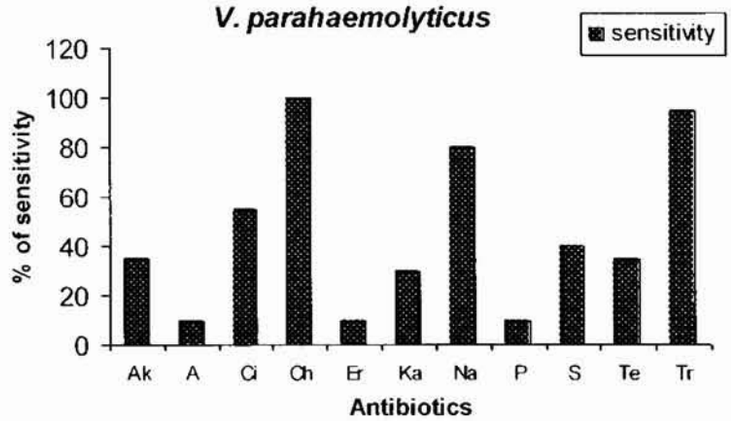
chloramphenicol, nalidixic acid and trimethoprin. 50 % sensitivity was shown towards other antibiotics like erythromycin, kanamycin, penicillin, streptomycin and tetracycline.

Fig. 25 Antibiotic sensitivity of *V. furnissii*



Antibiotic sensitivity pattern of *V. parahaemolyticus* is shown in Fig. 26. Maximum sensitivity was observed towards chloramphenicol (100 %) followed by trimethoprin (95 %) and nalidixic acid (80 %). Sensitivity towards other antibiotics like ceftriaxone, streptomycin, amikacin and tetracycline, kanamycin were 55 %, 40 %, 35 %, 30 %, 30 %, respectively. Minimum sensitivity was observed towards ampicillin (10 %), erythromycin (10 %) and penicillin (10 %).

Fig. 26 Antibiotic sensitivity of *V. parahaemolyticus*



HPVs in general showed maximum sensitivity towards chloramphenicol, trimethoprin and nalidixic acid in this study. Most of the species showed resistance towards ampicillin, kanamycin and penicillin. Li *et al.* (1999) viewed that different vibrio strains had similar antibiotic resistance profiles. They tested the antibiotic sensitivity of seven vibrio species viz *V. alginolyticus, V. vulnificus, V. parahaemolyticus, V. logei, V.pelagius II, V.fluavialis* and *V.mdeditterranei* by the agar dilution method. All isolates were sensitive to streptomycin, nalidixic acid, rifampicin and ceftriaxone and almost all were sensitive to chloramphenicol (98 %), sulphamethoxazole (98 %) and ceftazidime (96 %). A large number of strains were found to be resistant to ampicillin, amikacin, kanamycin, trimethoprin and cefuroxime. Ottaviani *et al.* (2001) studied the antimicrobial susceptibility of potentially pathogenic halophilic vibrios isolated from seafood, and confirmed that all isolates were uniformly sensitive to chloramphenicol. This is in agreement with the result obtained in this study

V. cincinnatiensis showed maximum sensitivity towards chloramphenicol and trimethoprin (Fig. 23). Bode *et al.* (1986) have reported the use of chloramphenicol and tetracycline in the successful treatment of *V.* meningitis caused by *V. cincinnatiensis* in an adult. Mao *et al.* (2001) have also reported the maximum sensitivity of *V. cincinnatiensis* isolated from diseased mud crab towards chloramphenicol.

In case of *V. fluvialis* and *V. furnissii* the number of strains used for the study was less. It is rather difficult to compare the results. However *V. fluvialis* had shown maximum sensitivity towards ceftriaxzone, chloramphenicol, erythromycin and trimethoprin (Fig. 24). Lee *et al.* (1981) while studying the taxonomy of *V. fluvalis* have reported that *V.fluvalis* biovar-I strains were sensitive to kanamycin, streptomycin, sulphonamide, tetracycline and trimethoprin. Similar antibiotic sensitivity patterns were reported for *V.furnissii* and *V fluvialis* (Brenner *et al.*, 1983). They were sensitive towards chloramphenicol, nalidixic acid, tetracycline and kanamycin and very much resistant towards penicillin and ampicillin. Except for ampicillin, more or less similar results were obtained in this study (Fig. 25).

V. parahaemolyticus had shown maximum sensitivity towards chloramphenicol (100 %), trimethoprin (95 %) and nalidixic acid (80 %) in this study (Fig. 26).

Sanjeev (1999) has reported similar results in a study of the antibiotic sensitivity of *V. parahaemolyticus* isolated from a brackishwater culture pond. They found all the 250 strains sensitive towards chloramphenicol, 68.4 % were sensitive to gentamycin, and 18 % were sensitive to tetracycline and 16.8 % to streptomycin. Pradeep and Lakshmanaperumalasamy (1985) in a study of antibiotic sensitivity of 120 strains of *V. parahaemolyticus* isolated from water, sediment, plankton, fish and prawns of Cochin backwaters, noted higher resistance to ampicillin by isolates from fish and prawns. Plate 8 shows the antibiotic sensitivity of HPVs.

4.12. Haemolytic activity of HPVs

Haemolytic activities of HPVs were studied on Wagatsuma agar. Kanagawa phenomenon of 22 strains of *V.cincinnatiensis*, 3 strains of *V. fluvialis*, 2 strains of *V. furnissii* and 22 strains of *V. parahaemolyticus* was studied. 5 strains of *V. cincinnatiensis* isolated from seafood samples showed haemolytic activity. One strain of *V. fluvialis* isolated from seafood sample and *V. fluvialis* (ATCC 33809) showed kanagawa positive reaction on the wagatsuma agar. Strains of *V. furnissii* showed negative reaction on Wagatsuma agar. Of the 22 strains of *V. parahaemolyticus* studied only two strains showed kanagawa positive phenomenon. All the other strains were found kanagawa negative. Table 27 shows name and number of *Vibrio* spp and its kanagawa phenomenon.

Table 27. *Vibrio* spp and its kanagawa phenomenon

***Vibrio* spp.**	**No. of strains tested**	**No. of strains found +ve**	**Percentage**
V. cincinnatiensis	22	5	22.72
V. fluvialis	3	2	66.66
V. furnissii	2	0	0.00
V. parahaemolyticus	22	2	9.09

Five strains of *V. cincinnatiensis* in this study showed haemolytic activity. *V. cincinnatiensis* was the most frequently isolated species from iced and frozen seafood samples.

One strain of *V. fluvialis* isolated from seafood and numbered strain (ATCC 33809) showed haemolytic activity in this study. Wong *et al.* (1992) reported that 97 % of *V. fluvialis* and 65.55 % of *V. parahaemolyticus* strains isolated from seafood and aquacultured foods in Taiwan showed haemolytic activity. In another study Wong *et al.* (1993) observed thermostable haemolytic activity of *V. fluvialis* strain after being heated at 100°C but not at 60°C.

Only two strains of *V. furnissii* were available for the study and they did not show the ability to produce haemolysin on wagatsuma agar.

In this study only two strains of *V. parahaemolyticus* showed ß haemolysis on wagatsuma agar. All the other strains were found Kanagawa negative. Bandekar *et al.* (1982) have isolated 2 strains of kanagawa positive *V. parahaemolyticus* out of the 17 strains isolated from fresh and frozen samples. Karunasagar and Mohankumar (1980) observed 25 % incidence of K^+ strains in the environment around Mangalore. Malathi *et al.* (1988) have reported the isolation of *V. parahaemolyticus* and *V. vulnificus* strains capable of producing haemolysins from seafoods. Sanjeev (1999) in a study of brackishwater culture pond isolated 12.4 % of K^+ *V. parahaemolyticus* strains. Hara-kudo *et al.* (2003) have reported the prevalence of pandemic tdh- positive *V. parahaemolyticus* O3:K6 from 10 % of shellfish samples in Japan.

Association between haemolysin production and virulence of *V. parahaemolyticus* has been noted by a number of workers. Sanyal and Sen (1974) observed symptoms of gastroenteritis in human volunteers who had injested 2 x 10^5 to 3 x 10^7 cells of KP^+ *V. parahaemolyticus* whereas volunteers receiving Kanagawa negative strains did not show symptoms even at 1.6 x 10^{10} levels.

Substantial evidence has been provided that TDH is a major virulence determinant of K^{+ve} *V. parahaemolyticus* and that the K^{+ve} phenotype makes a good marker for virulent strains (Nichibuchi and Kaper, 1995). However, recently a K^{-ve} *V. parahaemolyticus* strains that produce a toxin TDH related haemolysin (TRH) was found associated with gastroenteritis (Suthienkul *et al.*, 1995), and it appears that both TDH and TRH haemolysin are important virulence factors in the pathogenesis of *V. parahaemolyicus* (Suthienkul *et al.*, 1995).

According to Zhang and Austin (2005) there are four representive haemolysin families in Vibrio spp., including the TDH (thermostable direct haemolysin) family, the HlYA (El Tor haemolysin) family, the TLH (thermolabile haemolysin) family and the δ- VPH (thermostable haemolysin) family.

Haemolysins act on erythrocytes membranes thus lysing the cells which lead to the freeing up of iron-binding proteins namely haemoglobin, transferring and lactoferrin (Zhang and Austin, 2005). Lang *et al.* (2004) have reported that haemolysin induces cation permeability and activates endogenous Gardos K^+ channels, consequences include break down of phosphatidyl serine asymmetry, which depends at least partially on cellular loss of K^+. The pore - forming activity of haemolysin is not restricted to erythrocytes, but extends to a wide range of other cell types including mast cells, neutrophils, and polymorpho nuclear cells and enhances virulence by causing tissue damage (Zhang and Austin 2005).

Zhang and Austin (2005) reported that pathogenic Vibrio species were capable of producing various virulence factors consisting of enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and/ or haemagglutinins. Results obtained by Baffone *et al.* (2001) corroborates the above view, they observed vibrio strains consisting of *V. alginolyticus*, *V. parahaemolyticus*, *V. chlorea non-O1*, *V. vulnificus*, *V. fluvialis*, *V. furnissii* and *V. metschnikovii* were in general positive for lipase and gelatinase activity (100 %), haemolytic activity (7.2 %), urease activity (19.2 %), adhesiveness (63 %), cytotoxicity (57.6 %), 23 % of the strains gave positive results in the illeal loop test in rats and 23 % showed the ability to infect the laboratory animals and suggested that pathogenicity of vibrios could be the result of a combination of factors. Plate 9 shows the Kanagawa reaction of HPVs.

This study shows that HPVs demonstrating haemolytic activities could be isolated from seafood samples and can be a threat to seafood consumers and exporters.

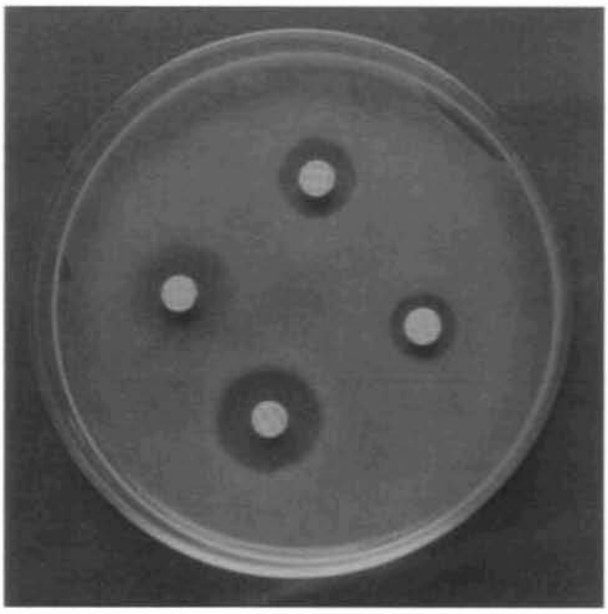


Plate 8. Antibiotic sensitivity test for Vibrios

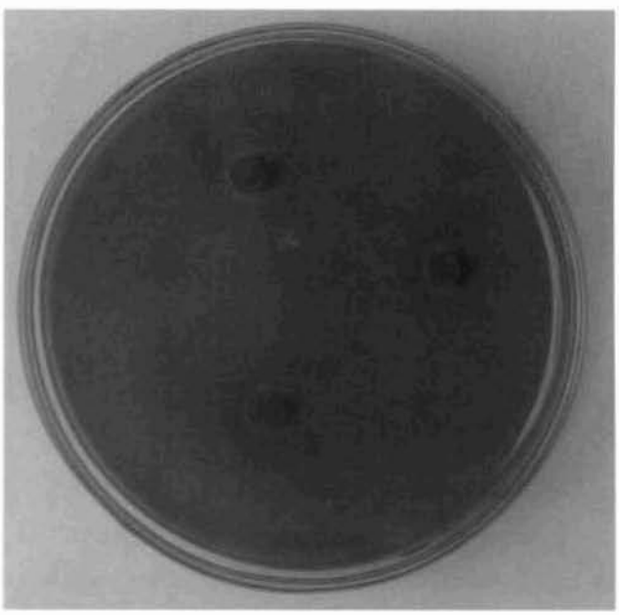


Plate 9. Kanagawa reaction of HPVs

5. Summary

Seafood is easily susceptible to contamination by pathogenic and toxigenic microorganisms.The quality of the processed seafood and their safety aspects are of great importance to the consumer. Halophilic vibrios are one of the major groups of bacteria found in marine and estuarine environment. It is therefore expected that freshly harvested finfish and shellfish from these environments might be harbouring some of the pathogenic halophilic *Vibrio spp.* Vibrios may cause a variety of diseases including gastroenteritis, wound infections, ear infection and septicemia. A number of halophilic *Vibrio spp.* viz., *V. alginolyticus, V. cincinnatiensis, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii, V. parahaemolyticus and V. vulnifius* are recognized as human pathogens. Data generated in this study will be useful for risk assessment and risk management of HPVs in seafood sector.

In this study, HPVs were isolated from 50.8 % of the fish and fish products collected from markets, landing centers and processing plants situated in and around Cochin. 66.7 % of the iced samples, 44.2 % of the block frozen samples and 22.9 % of IQF samples showed the incidence of HPVs. *V. cincinnatiensis* was the dominant species isolated (30.8 %) from the samples analyzed followed by *V. alginolyticus* (26.2 %), *V. vulnificus* (17.7 %), *V. parahaemolyticus* (7.7 %), *V. metschnikovii* (5.4 %), *V. fluvialis* and *V. damsela* (0.8 %). Presence of more sucrose positive vibrios than sucrose negative vibrios may be due to the relative abundance of this species in the coastal waters of Cochin. HPVs showed their maximum load in iced samples (<1-930 MPN g^{-1}) followed by block frozen samples (<1-11MPN g^{-1}) and IQF samples (<1-9.1MPN g^{-1}). *V.cincinnatiensis* was the dominant species isolated during the study and the load varied from <1-930 MPN g^{-1} followed by *V. vulnificus* (<1-530 MPN g^{-1}), *V. alginolyticus* (<1-360 MPN g^{-1}), *V. parahaemolyticus* (<1-36 MPN g^{-1}), V. furnissii (<1-30 MPN g^{-1}), *V. metschnikovii* (<1-10MPN g^{-1}), *V. damsela* (<10 MPN g^{-1}) and *V. fluvialis* (<1 MPN g^{-1}). Results obtained in this study indicate that HPVs could be isolated from iced and frozen seafood samples and highlights the risk associated with these pathogens. However the load of *V.parahaemolyticus* in the samples analyzed in this study is well within the limits of ICMSF specification of 10^2 *V.*

parahaemolyticus per gram of the sample (ICMSF, 1974). No such standards are available to compare the load of other species of HPVs. It's high time to evolve such standards for other species as well.

In general washing with tap water was found to be very effective against HPVs although complete destruction could not be achieved. One to three log reductions in counts were observed with different species. After third wash *V.furnissii* and *V. parahaemolyticus* showed the maximum reduction (3 log) followed by *V. fluvialis* and *V. cincinnatiensis* (1 log).

Reduction in load of all the species of HPVs was observed during chill storage although complete elimination could not be achieved.

Data obtained in this study shows that low temperature storage of seafood cannot be relied upon as an effective method to eliminate vibrios. Although at these temperatures there was reduction in the count of various HPVs studied, viability was not completely lost in many cases. In seafood processing plants, it is the usual practice to store fish and fish products under chilled condition before final processing, this stage seems to be critical and any rise in temperature during storage should be avoided to minimize the growth of HPVs.

Incidence of vibrios in frozen seafood was found to be less when compared to iced or refrigerated foods. *V. cincinnatiensis* had shown 1-2 log reduction after freezing at -40°C for 2 h. At –20°C no survivors were found on the 8th week. *V. fluvialis* showed 1 log reduction at –40°C regardless of the medium suspended. At –20°C in shrimp homogenate (SH) prepared with 3 % NaCl viable cells were detected till the 7th day. *V. furnissii* showed not much reduction at –40°C, except in SH prepared with distilled water (DW). But frozen storage at –20°C was found very effective against *V. furnissii* in this study. 3 log reduction was observed on the 4th day of storage and lost viability on the 13th day. *V. parahaemolyticus* showed 1 log reduction within 2 h at –40°C in SH prepared with 3 % NaCl. While in DW *V. parahaemolyticus* cells lost viability within 90 mts. At -20°C in SH prepared with 3 % NaCl cells were viable upto 4 days. In NS and DW within 24 h cells lost viability at -20°C.

Blanching at 100°C for 1 min was found very effective against HPVs studied. Studies have shown that vibrios are not heat resistant and are destroyed within 15 secs by dipping in water at boiling temperatures.

Studies have shown that HPVs were very sensitive to drying. The results of the present study indicate that the survival period of HPVs in salted and unsalted fish were very short. *V.parahaemolyticus* showed the maximum sensitivity to sun drying, within 1 h complete inactivation took place.

HPVs were sensitive to pH below 5 and above 12. They preferred an alkaline environment to grow. *V. furnissii* and *V. parahaemolyticus* had shown remarkable capacity to grow at low pH in low saline condition in SH as compared to *V. cincinnatiensis* and *V. fluvialis*.

Studies have shown that the optimum concentration of NaCl for different species of HPVs varied between 2 % and 7 %.

Chlorine at a minimum available chlorine level of 1 ppm and contact time 30 min was found very effective against all the HPVs studied. This chlorine level is well below the limits of EU requirement of < 2 ppm available chlorine for the process water in seafood factories. Similarly ClO_2 at a minimum level of 1 ppm and a contact time of 2 min was found effective against all the HPVs studied.

HPVs in general showed maximum sensitivity towards chloramphenicol, trimethoprin and nalidixic acid in this study.

Studies have shown that haemolytic HPVs could be isolated from seafood samples and these haemolytic HPVs even in small numbers poses risk to seafood consumers.

It is expected that these data will be usefull for HACCP based risk management programme in the seafood industry, for improving the processing strategies and thus broadening the vision for high quality and safe seafood in both domestic as well as international trade.

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