Chapter 2

DIVERSITY OF *VIBRIO* SPECIES IN COCHIN ESTUARY

Contents

2.1 Introduction

Vibrios are Gram-negative halophiles occurring naturally in shallow coastal waters to the deepest parts of the ocean (Okada *et al.*, 2005). They are highly abundant in aquatic and marine environments, and aquaculture settings worldwide (Denner *et al.*, 2002). At present, there are more than 100 recognised species under the genus *Vibrio* (Okada *et al.*, 2010) and 12 of them are reported to be pathogenic to humans. Many *Vibrio* species have been implicated in water and seafood-related outbreaks of gastrointestinal infections in humans (Eiler *et al.*, 2006). *Vibrio* spp. have been also found to be pathogens of fish, coral, shellfish and shrimp and infections with these organisms have profound environmental and economic consequences (Rosenberg and Falkovitz, 2004; Le Roux *et al.*, 2009; Austin, 2010).

Cochin is a major fishing hub along the southwest coast of India, contributing over 90% of state-wide exports (Chakraborty *et al.,* 2013), and the Cochin estuary is a favourite tourist hotspot in Kerala. Cochin backwaters also act as nursery grounds of commercially important prawns and fishes. The fields around the backwater are suitable for aquaculture. The presence of specific pathogenic *Vibrio* species serve as an indicator of public health safety of water and food destined for human consumption. Vibriosis caused by *Vibrio* spp. has been identified as a serious disease problem in shrimp culture ponds (Jayasree *et al.,* 2006). Until the study commenced, only a limited number of other studies have been conducted on diversity of vibrios from Cochin estuary. Considering all these factors, the chapter aims to investigate the diversity of *Vibrio* species in Cochin estuary.

2.2 Review of literature

According to Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994) vibrios (*Vibrionaceae* strains) belong to the Gamma-proteobacteria. They are Gram-negative motile rods, mesophilic and chemoorganotrophic, and have facultative fermentative metabolism. The first *Vibrio* species, *V. cholerae* was discovered by Italian physician Filippo Pacini in 1854. This discovery was during his investigation on the outbreaks of cholera in Florence. Thirty years later, Robert Koch managed to obtain pure culture of this bacterium on gelatin plates. In the late 1880 the Dutch microbiologist Martinus Beijerinck discovered the first non pathogenic *Vibrio* species (*V. fischeri*, *V. splendidus* and *Vibrio phosphoreum*) from the aquatic environment. *Vibrio* diversity studies have been reported by many authors from various parts of the world

(Thompson *et al.,* 2004; Eiler *et al.,* 2006; Prashanthan *et al.,* 2011; Mansergh and Jonathan, 2014; Amin *et al.,* 2016). At present, more than 100 well identified *Vibrio* species have been discovered (Okada *et al.,* 2010).

2.2.1 Ecology and distribution of *Vibrio*

Vibrios are highly abundant in aquatic environments, including estuaries, marine coastal waters and sediments and aquaculture settings worldwide and also in association with eukaryotes (Denner *et al.,* 2002). Studies revealed that vibrios thrive more on/or in marine organisms such as corals, fish, molluscs, seagrass, sponges, shrimp and zooplankton (Suantika *et al.,* 2001; Hedelberg *et al.,* 2002; Rosenberg and Ben-Haim, 2002; Sawabe *et al.,* 2003). Hunt *et al.* (2008) reported that some species of *Vibrio* are found only in association with plankton and some are exclusively free-living. The wide ecological relationships and ability to cope with global climate changes may be a reflection of the high genome plasticity of vibrios (Lipp *et al.,* 2002). Moreover, vibrios have a broad metabolic range that helps them to use different types of carbon sources (Thompson and Polz, 2006).

The distribution of most *Vibrio* populations is influenced by environmental factors including salinity, temperature and pH (Thompson *et al.,* 2004). The distribution of pathogenic vibrios is mainly influenced by the physico-chemical parameters of the environment (Sedas, 2007). They grow abundantly in warm, low saline waters (DePaola *et al.,* 1990). Studies showed that temperature and salinity are the two major factors influencing the occurrence of *V. cholerae* in the aquatic environment (Barbieri *et al.,* 1999; Jiang, 2001). Vezzulli *et al.* (2012) reported that there was a significant increase in abundance of vibrios in the North Sea.

They also reported that the ocean warming observed in the last decades was the inducing factor behind this phenomenon. Another study by Fukui *et al.* (2010) showed an increase in growth of certain vibrios in northern Japan when the seawater temperature increased from 21 ^{o}C to 24.3 ^{o}C.

Vibrios are known to exist in viable-but-non-culturable (VBNC) under unfavorable environmental conditions (Huq *et al.,* 2000; Chaiyanan *et al.,* 2007; Sedas, 2007; Fernández-delgado *et al.,* 2015). In this state the cell size is reduced drastically and becomes coccoidal (Huq *et al.,* 2000). However, even in the VBNC state *Vibrio* maintains its metabolic activity, antibiotic resistance, specific gene expression and virulence potential for a prolonged time (Oliver and Bockian, 1995; Gonzalez-Escalona *et al.,* 2005; Zhong *et al.,* 2007; Oliver 2010).

2.2.2 Studies on *Vibrio*- Indian scenario

Vibrio studies have been reported from various parts of India. The diversity of pathogenic vibrios along the Palk Bay was previously monitored by Sneha *et al.,* 2016. Five *Vibrio* species namely *V. cholerae*, *V. hollisae*, *V. furnissii*, *V. alginolyticus* and *V. aestuarianus* were detected among which *V. cholerae* dominated. The isolation and identification of *Vibrio* spp. from cultured diseased shrimp from Andhra Pradesh was undertaken by Jayasree *et al.* (2006). In their study *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus* and *V. splendidus* were identified from diseased shrimps.

V. cholerae O1 belonging to the El Tor biotype is the most common serogroup found in India (Kanungo *et al.,* 2010). During the year 2015 alone, 889 outbreaks of *V. cholerae* were reported from India. Cholera

outbreak was also reported from Chandigarh during the years 2002 and 2003 (Kaistha *et al.,* 2005*).*

Incidence of *V. parahaemolyticus* in India has almost doubled in the last 5 years (Chowdhury *et al.,* 2000). The pandemic serovar O3:K6 that emerged in India in 1996 has since been found to account for many cases of *V. parahaemolyticus* outbreaks worldwide. *V. parahaemolyticus* is the causative agent of 10% of the *Vibrio* outbreaks from India (Deepanjali *et al.,* 2005). Recently, there was a report on the presence of multidrug resistant *V. parahaemolyticus* in seafood samples collected from Cochin (Sudha *et al.,* 2014). The diversity of the species associated with disease outbreak among *Litopenaeus vannamei* from the east coast of India was studied (Kumar *et al.,* 2014b). The study demonstrated *V. parahaemolyticus* as the organism responsible for the outbreak. The detection of *V. parahaemolyticus* from the Vellar estuary and adjoining shrimp ponds confirmed the presence of the species (Alagappan *et al.,* 2013). The species is also reported from clinical samples. Around 178 *V. parahaemolyticus* strains were isolated from diarrheal patients admitted in Infectious Diseases Hospital, Kolkata during 2001 to 2012 (Pazhani *et al.,* 2014).

V. vulnificus is pathogenic to humans, eels, shrimps and fish (Al-Mouqati *et al.,* 2012*).* The presence of *V. vulnificus* has been previously reported from coastal waters, shrimp and shellfish in India (Thampuran and Surendran, 1998; Parvathi *et al.,* 2004; Jayasree *et al*., 2006).

2.2.3 Studies on *Vibrio*- global scenario

Various studies have been conducted world-wide regarding the *Vibrio* diversity, its distribution and disease outbreaks. The abundance of

culturable vibrios was monitored along the west coast of Peninsular Malaysia and *V. alginolyticus* dominated in the study (Vijayan and Lee, 2014). A recent study was conducted to analyse the diversity of *Vibrio* species in seawater surrounding a coral reef in Ishigaki, Japan (Amin *et al.,* 2016). The results revealed *V. hyugaensis*, *V. owensii* and *V. harveyi* as the most prevalent species. A similar study was reported previously from Fiji (Singh *et al.*, 2012). A total of nine *Vibrio* spp. were detected in their study. Another diversity study was undertaken in the coastal marshes of Yucatan Peninsula (Ortiz-Carrillo *et al.,* 2015). The diversity of *Vibrio* spp. in two estuaries along the Italian Adriatic coast was studied. *V. alginolyticus* predominated followed by *V. parahaemolyticus*, non-O1 *V. cholerae* and *V. vulnificus* (Barbieri *et al.,* 1999). The diversity and dynamics of *Vibrio* in Monterey Bay, California was studied by Mansergh and Jonathan (2014).

Yearly, about 8000 *Vibrio* infections are reported in the United States (Mead *et al.,* 1999). During the year 2015, around 1, 72, 454 cholera cases and 1304 deaths due to cholera were reported from 42 countries (WHO, 2016). In 2016 a cholera outbreak was reported in Nepal which was caused by multidrug resistant *V. cholerae* serogroup O1 (Gupta *et al*., 2016).

Canigral *et al.* (2010) detected *V. vulnificus* in seafood and environmental samples from a coastal area of Spain. A study on occurrence of pathogenic vibrios in sea water and estuarine environments of the Caspian Sea in Iran revealed *V. vulnificus* as the predominant species observed (Amirmozafari *et al.,* 2005).

V. parahaemolyticus is attracting increasing interest worldwide where raw or undercooked seafood is often consumed (Chao *et al*., 2009). It is regarded as the primary source of rise in vibriosis incidence (Newton *et al*., 2012), and highly pathogenic serotypes of the species are emerging on a global scale. *V. parahaemolyticus* strains belonging to pandemic O3:K6 have been reported from environmental and clinical samples in several countries, including Bangladesh (Islam *et al.,* 2004), Japan (Hara-Kudo *et al.,* 2003), Taiwan (Yu *et al.,* 2013), China (Li *et al.,* 2014), Malaysia (Tan *et al.,* 2017) and Italy (Caburlotto *et al.,* 2010).

2.3 Objectives of the study

Considering the importance of Cochin estuary and the fact that *Vibrio* species are an emerging pathogen in human and the aquatic animals, the present study had been taken up with the broad objective of understanding the diversity of *Vibrio* from Cochin estuary which is influenced by urban, industrial, human and hospital waste water. The specific objectives are as follows:

1) To isolate and identify *Vibrio* species from water and sediment of Cochin estuary.
2) To find out the diversity of *Vibrio* species in water and sediment from Cochin estuary.
3) To study the seasonal variation in distribution of *Vibrio* species in water and sediment from Cochin estuary.
4) To study the spatial distribution of *Vibrio* species in Cochin estuary.

2.4 Materials and Methods

2.4.1 Description of sampling site

The sampling areas were selected based on their closeness to satellite townships and waste inputs. Samples were collected from ten stations in the Cochin backwaters (9°40′ and 10°12′ N and 76°10′ and 76°30′ E) located along the South west coast of India.

Figure 2.1 Map showing the location of sampling stations along the Cochin estuary

Figure 2.1 shows the sampling locations of the Cochin estuary. The stations include Marine science jetty (S1), Bolghatty (S2), Vaduthala (S3), Varapuzha (S4), Eloor (S5), Thevara (S6), Kumbalam (S7), Aroor (S8), Panavalli (S9), Murinjapuzha (S10).

2.4.2 Analysis of hydrographical parameters

Temperature, salinity and pH of the estuarine water were measured on field using centigrade thermometer, salinity refractometer (Atago, Japan), and hand-held digital pH meter (Eutech, Singapore), respectively.

2.4.3 Sample collection

Sediment and water samples were collected seasonally for a period of one year from various stations in and around Cochin estuary**.** Sampling was done during the pre-monsoon, monsoon and post-monsoon seasons of the year 2012. Water samples were collected using Niskin water sampler and sediment samples using Van-Veen grab on board research vessel King Fisher.

2.4.4 Isolation of *Vibrio* species from water and sediment of Cochin estuary

Five hundred millilitre of water sample from each station was filtered using 0.45 µ bacteriological filter and the filter was transferred to 100 ml alkaline peptone water and incubated at 37 °C for 18-24 h for pre enrichment. Sediment samples were analysed after making 10 fold dilutions of them in isotonic saline. 1 ml of the diluted sediment was transferred to 99 ml alkaline peptone water and enriched by incubation at 37 °C for 18-24 h. 100 µl of each enrichment broth was aseptically streaked on to

sterile surface dried Thiosulphate Citrate Bile salt Sucrose (TCBS-Himedia, India) agar plates and incubated at 37 °C for 24 h. Typical colonies were picked from TCBS plates and stored in nutrient agar slants for further identification.

2.4.5 Presumptive identification

The presumptive identification of *Vibrio* species was performed using Gram staining, oxidase test and oxidative-fermentative test. Gram-negative, oxidase-positive and glucose-fermentative without gas producing rods were considered as presumptive vibrios (Noguerola and Blanch, 2008).

2.4.5.1 Gram staining

The Gram staining technique was devised by Hans Christian Gram in the year 1882. It differentiates bacteria based on cell wall composition into Gram-positive and negative organisms. Gram-positive bacteria has a thick peptidoglycan layer in their cell wall whereas, cell wall of Gram-negative bacteria is made of lesser peptidoglycan layer and higher amount of lipo-polysaccharides. A thin bacterial smear was prepared on a clean glass slide by using an overnight bacterial culture. The smear was flooded with the primary stain crystal violet solution and allowed to stand for 1 min. It was rinsed with tap water and flooded with Gram's iodine solution and kept for 1 min. The slide was rinsed with tap water and decolouriser was added. It was washed with tap water after few seconds. Then, the secondary stain safranin was added and allowed to stain for 30 sec. The slide was washed in running tap water and air dried. The slides were

examined microscopically under 100 X objective. Gram-positive bacteria appear violet and Gram-negative bacteria appear pink in colour.

Both bacteria form a crystal violet-iodine complex during the staining procedure. The cell wall permeability of Gram-negative bacteria increases during decolourisation and it loses the crystal violet-iodine complex. It then takes the colour of secondary dye safranin and appears pink. Gram-positive bacteria resist decolourisation and retain crystal violet dye and appear violet in colour.

2.4.5.2 Oxidase test

The presence of the enzyme cytochrome oxidase was determined by this test, which is an important enzyme in the electron transport chain of organism. They catalyse the oxidation of reduced cytochrome by molecular oxygen, resulting in the formation of water or hydrogen peroxide. In oxidase test, sterile strips of filter paper soaked in N,N,N'N'-Tetramethylene paraphenylene diamine dihydrochloride (1% w/v in distilled water) were dried at 37 ^{o}C and stored in a dark, air tight bottle at 4 ^{o}C. Young bacterial cultures were picked with sterile toothpicks and spotted on the filter paper. The cultures which produced deep violet colour within 10 seconds were taken as positive for the presence of oxidase. The cultures which do not produce or those which take more than a minute to produce violet colour were taken as negative.

2.4.5.3 Oxidative-Fermentative test

Carbohydrates are degraded either aerobically or anaerobically by fermentation to obtain energy. Some use both the pathways

and some others do not oxidise glucose at all. The medium used for the Oxidative-fermentative test is the Oxidative-fermentative medium (OF basal medium) with peptone, beef extract, sodium chloride, bromocresol green as the pH indicator and agar agar as the gelling agent. 1% w/v glucose was also added to the basal medium as the substrate to study the organism's fermentative capacity. The medium was prepared as agar deep tubes and the organism was inoculated by stabbing the butt. The tubes were incubated at 37 ^{o}C for 24 h. The isolates were differentiated based on their ability to metabolize glucose either oxidatively or fermentatively.

2.4.6 Species level identification

The dichotomous key described by Noguerola and Blanch (2008) was used for the species level identification of *Vibrio* spp. The presumptive *Vibrio* isolates (Gram-negative, oxidase- positive, glucose-fermentative without gas production) were grouped into eight different A/ L/ O clusters based on the amino acid (L-Arginine, L-Lysine and L-Ornithine) utilisation pattern. Further identification was based on various biochemical tests as described in the key (Please refer Appendices 1 a-h).

2.4.6.1 Amino acids utilisation test (Decarboxylase/dihydrolase test)

The amino acid degradation by microorganisms were analysed by the test. The principle behind the test is the removal of carboxyl groups of the amino acids to produce alkaline end product. This process is termed as decarboxylation and the enzyme involved is called as decarboxylase. A pH indicator bromocresol purple added to the broth containing peptone, glucose and beef extract and the specific amino acid. The culture tubes were sealed with sterile mineral oil to provide an

anaerobic condition suitable for carboxylation. The tubes were incubated at 37 °C for 96 h.

The inoculated microbes grew well by utilising glucose, indicated by the colour change from purple to yellow. The medium was thus acidified and the enzymes were activated. The decarboxylase enzyme act on the amino acid in the medium resulting in the production of amines and carbon dioxide which turn the medium alkaline. This can be noted by change in colour of media from yellow to purple once again. Development of turbid purple colour indicated the presence of decarboxylase.

2.4.6.2 Carbohydrate fermentation test

Microorganisms utilise carbohydrates either aerobically or anaerobically. Facultative anaerobes like *Vibrio* fermentatively utilise glucose to produce acidic end products. The test medium for carbohydrate fermentation test contains nutrient broth, a pH indicator bromocresol purple and a specific carbohydrate substrate. Organic acids are produced if fermentation occurs, changing the colour of the medium from purple to yellow. The results should be noted in 24 h at 37 ^{o}C since prolonged incubation may affect fermentation by the production of alkaline products from the degradation of other substrates present in the medium. The carbohydrates (1% w/v) tested were D-sucrose, D-melibiose, D-mannose, D-mannitol, D-amygdalin and D-arabinose.

2.4.6.3 Carbon source utilisation test

Vibrio was tested for its ability to utilise sugars like L-arabinose and glucosamine as their carbon source in the absence of other sources. The test

medium should be a nutrient deficient mineral medium supplemented with an appropriate carbohydrate (1%). The cultures were streaked on them and incubated at 37 °C for 4 days. A visible growth on the medium was considered as a positive result.

2.4.6.4 Indole production

The enzyme tryptophanase present in some bacteria hydrolyse amino acid tryptophan to indole. To detect indole production, the culture was inoculated into tryptone (1%) supplemented with NaCl and incubated at 37 °C for 24 h. After incubation, a few drops of Kovac's reagent (p-dimethyl-aminobenzaldehyde), butanol and hydrochloric acid) was added to the culture. If tryptophanase is present in the bacterium, it hydrolyses tryptophan to indole. The indole is extracted from the medium into the upper layers by acidified butyl alcohol. It then combines with p-dimethyl-aminobenzaldehyde to form a cherry red ring at the upper layer showing the presence of indole. The formation of the cherry red colour was considered as a positive result confirming the presence of the enzyme.

2.4.6.5 Voges Proskauer (acetoin production) test

Certain microorganisms produce acetylmethylcarbinol from the organic acids by glucose metabolism, when grown in the Methyl Red-Voges Proskauer (MR-VP) medium at 37 °C for 48 h. After incubation, α-naphthol (Barritt's A) and 40% potassium hydroxide (Barritt's B) were added to detect the production of acetylmethylcarbinol. Acetylmethylcarbinol is oxidized to a diacetyl compound in the presence of the catalyst α-naphthol and potassium hydroxide. Diacetyl compound further combines with

guanidine present in peptone in the MR-VP medium and forms a pink complex. The formation of pink colour was taken as a positive result and its absence was considered as a negative result.

2.4.6.6 Salt tolerance test

For testing the tolerance of *Vibrio* species in various concentrations of NaCl (sodium chloride), the isolates were inoculated into Tryptone (1%) broth with varying NaCl concentrations (0, 1, 6, 12% NaCl). The cultures were incubated for 24 h at 37 °C. Growth was noted after incubation and heavy turbidity was taken as the positive result.

2.4.6.7 Growth at different temperatures

The ability of *Vibrio* spp. to grow at different incubation temperatures was tested. Tryptone (1%) broth supplemented with NaCl (1%) was used as the test medium. After 24 h incubation at different temperatures, a positive result was indicated by heavy turbidity in the medium.

2.4.6.8 Gelatinase production

Certain *Vibrio* produces gelatinase enzyme that hydrolyses the protein gelatin. For testing the enzyme production, the isolates were spot inoculated onto gelatin agar plates (nutrient agar supplemented with 2% w/v gelatin). The plates were incubated at 37 ^{o}C for 24 to 48 h. Zone of clearance around the colonies after the plates were flooded with saturated solution of mercuric chloride indicated that gelatin has been hydrolyzed.

2.4.6.9 Urease production

Urease production was determined by inoculating the bacteria in Christensen's urea agar (HiMedia, India) containing phenol red as the pH indicator. The urease enzyme hydrolyses urea to form alkaline ammonia which in turn changes the colour of the medium to pink due to the alkaline end product. Absence of pink colour was taken as a negative result. The incubation was done at 37 °C for 24-48 h.

2.4.6.10 Production of β- galactosidase (ortho-Nitrophenyl β- D-galactopyranoside (ONPG)) test

The lactose fermentation by microbes involves two enzymes namely β-galactosidase and permease. Some microorganisms lack permease. Such microbes do not ferment lactose in normal carbohydrate fermentation test media. In such microbes, the presence of β-galactosidase can be checked by inoculating the culture in 100 µL of sterile physiological saline containing a chromogenic substrate ONPG (incorporated in discs, HiMedia, Mumbai). The presence of β-galactosidase is indicated by yellow colour formed by utilisation of ONPG. The culture tubes were checked for the development of yellow colour for the first two hours of incubation at 37 °C and also after 24 h.

2.4.6.11 Nitrate reduction test

Nitrate reduction test was performed in a nutrient broth medium supplemented with potassium nitrate (0.1%) and agar (0.1%) to create an anaerobic condition required for the reduction of nitrate. The nitrate reductase enzyme present in certain microbes reduces inorganic substrate

(nitrate) to nitrite. Some organisms are able to further reduce nitrite to molecular nitrogen. After incubation of the cultures at 37 °C for 24-48 h, nitrate reduction was detected by the addition of sulfanilic acid (Solution A) and α-naphthylamine (Solution B) which results in the instant production of cherry red colour. Appearance of the cherry red colour was taken as a positive result. Absence of cherry red colour indicates that nitrate is not reduced to nitrite/nitrate is reduced to molecular nitrogen. To confirm this, zinc powder is added to the cultures (which already contains solution A and B). Zinc reduces nitrate and produces a red colour indicating that the nitrates in the medium were not reduced by the organisms. This was regarded as a negative result. Whereas, no colour change following the addition of zinc showed that nitrates have been reduced to nitrogen, which indicated a positive result.

2.4.6.12 Citrate utilisation test

Certain microorganisms have the capability to utilise citrate as sole carbon source in the absence of other carbon sources. To test this ability, the cultures were inoculated in a nutrient deficient medium with citrate as the sole carbon source and bromothymol blue as pH indicator. Incubation was done at 37 °C for 96 h. The enzyme citrate permease acts on citrate to produce oxaloacetic acid and acetate which are then converted enzymatically to pyruvic aid and carbon dioxide. Sodium and water in the medium combines with carbon dioxide to form the alkaline product sodium carbonate which turns the green colour of the medium to deep prussian blue. This was considered as a positive result. Negative result was noted by the absence of growth and the colour of the medium remaining unchanged.

2.4.6.13 Resistance to ampicillin

Resistance to the antibiotic ampicillin (10 mcg) was tested following the disc diffusion method (Bauer *et al.*, 1966). Briefly, a 24 h broth culture of *Vibrio* was swabbed on Mueller-Hinton agar plate supplemented with 1% NaCl. Ampicillin disc (AMP^{10}; HiMedia, India) was placed on the agar plate and incubated at 37 °C for 24 h. The results were noted by measuring the diameter of the zone of clearance formed around the disc, if any. The results were then compared with an interpretative chart provided by the manufacturer.

2.4.7 Molecular confirmation of *Vibrio* by 16S rRNA gene sequencing

2.4.7.1 DNA isolation

Genomic DNA of the isolates was extracted by boiling method (Devi *et al.*, 2009). The colonies were inoculated in the Luria Bertani (LB) broth supplemented with NaCl (2% w/v), and incubated at 37 ^{o}C under shaking (120 rpm) for 16-18 h. The broth cultures were centrifuged (10,000 rpm, 4 ^{o}C, 1 min) to obtain the pellet, which was then washed with normal saline (0.85% NaCl w/v) and re-suspended with DNA-free sterile distilled water (0.5 ml). The resulting suspension was heated at 98 ± 2 ^{o}C for 15-20 min in a water bath to lyse the cells, and release the DNA. The lysate was centrifuged to remove the cell debris (10,000 rpm, 4 ^{o}C, 5 min), and the supernatant was stored (-20 ^{o}C) until further use.

2.4.7.2 PCR amplification of 16S rRNA gene

Amplification was performed using the universal primer set (27F:5′-AGAGTT TGATCCTGGCTCAG-3′, 149R:5′-GGTTACCTTGTTA CGACTT-3). PCR amplification was optimized in a total reaction volume

of 25 µl consisting of sterile Milli Q water (15.5 µl), 10X PCR buffer (2 µl), primer (1 µl each), dntp mix (1 µl, 200 mM), template (4 µl), and Taq DNA polymerase (0.5 µl). The reaction conditions included an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 2 min, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min. A final extension at 72 °C for 10 min was also included.

The PCR amplified products were separated by electrophoresis on agarose (1.5% w/v) gel in 1X TBE Buffer (HiMedia, India) containing 0.5 µg/ml of ethidium bromide. The amplicon size was compared with a 100 bp DNA ladder. The gels were then visualized under UV transilluminator and recorded as tiff file by using Gel Documentation System (GelDoc EZ imager, Bio-Rad, USA).

The PCR products were sent for sequencing at Scigenom, Kochi and the obtained sequences were checked for homology with already identified 16S rRNA sequences in the GenBank database using basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/ BLAST) at NCBI (National Center for Biotechnology Information). The sequences were deposited in the Genbank and were allotted with accession numbers.

2.4.8 Isolation and identification of *V. parahaemolyticus*

V. parahaemolyticus is autochthonous to estuarine, marine, and coastal environments throughout the world. It is a leading cause of food-borne gastroenteritis in Asia. Hence, their prevalence and distribution in estuarine environments is of great public health importance. Considering

their role in disease outbreaks, we decided to carry out a detailed identification of the species from Cochin estuary using the HiCrome media and species-specific *tlh* and *toxR* genes.

2.4.8.1 Isolation of *V. parahaemolyticus* on HiCrome Vibrio agar

The sucrose non-fermenting colonies having green or bluish green colour on TCBS agar plates were picked. They were streaked onto HiCrome Vibrio agar (HiMedia, India) for further identification.The colonies showing typical *V. parahaemolyticus* specific bluish green colour on HiCrome Vibrio agar were aseptically picked and stored in Nutrient agar slants for further confirmation.

2.4.8.2 Detection of *V. parahaemolyticus* species-specific genes

The PCR detection of the *V. parahaemolyticus* species-specific *tlh* and *toxR* genes were carried out for the confirmation of the species.

2.4.8.2.1 Extraction of genomic DNA

Refer section 2.4.7.1

2.4.8.2.2 Detection of *tlh* gene

The detection of the *tlh* gene was performed using the primers TLHF (5'-AAAGCGGATTATGCAGAAGCACTG3') and TLHR (5'GCTACTTTCTAGCATTTTCTCTGC-3') (Bej *et al.,* 1999). PCR amplification was optimized in a total reaction volume of 25 µl consisting of sterile Milli Q water (15.5 µl), 10X PCR buffer (2 µl), primer (1 µl each), dntpmix (1 µl, 200 mM), template (4 µl), and Taq DNA polymerase (0.5 µl). The PCR conditions included an initial denaturation of 94 ^{o}C for

3 min, followed by 30 cycles of denaturation (94 ^{o}C for 1 min), primer annealing (58 ^{o}C for 1 min), primer extension (72 ^{o}C for 1 min) followed by a final extension (72 ^{o}C for 5 min).

2.4.8.2.3 Detection of *toxR* gene

The strains were further confirmed by checking for the presence of *V. parahaemolyticus* specific regulatory gene *toxR* (Kim *et al.,* 1999). PCR amplification was optimized in a total reaction volume of 25 µl consisting of sterile Milli Q water (15.5 µl), 10X PCR buffer (2 µl), primer (1 µl each), dntp mix (1 µl, 200 mM), template (4 µl), and Taq DNA polymerase (0.5 µl). The PCR conditions included an initial denaturation of 94 ^{o}C for 3 min, followed by 30 cycles of denaturation (94 °C for 1 min), primer annealing (63 °C for 1 min), primer extension (72 ^{o}C for 1 min) followed by a final extension (72 °C for 5 min).

2.4.8.2.4 Gel documentation and image analysis

The PCR amplified products were separated by electrophoresis on agarose (1.5% w/v) gel in 1X TBE Buffer (HiMedia, India) containing 0.5 µg/ml of ethidium bromide. The amplicon size was compared with a 100 bp DNA ladder. The gels were then visualized under UV transilluminator and recorded as tiff file by using Gel Documentation System (GelDoc EZ imager, Bio-Rad, USA).

2.4.9 Statistical analysis

The diversity indices Shannon-Wiener index (H') and Margalef species richness index (d) of various stations were calculated using the PRIMER 6 statistical software**.**

2.5 Results

2.5.1 Environmental parameters

The mean values of environmental parameters of water from various stations recorded during the pre-monsoon, post-monsoon and monsoon seasons of the study period are given in Table 2.1. Surface water temperature, salinity and pH fluctuated widely between various stations of the Cochin estuary with the mean temperature ranging between 28.7-32.3 °C, pH between 7.3-8.1 and salinity between 0-20.6 ppt. We observed that the salinity of station S4 (Varapuzha) and station S5 (Eloor) remained at 0 ppt throughout the study period.

Table 2.1: Environmental parameters observed in various stations of Cochin estuary (mean value ±standard deviation)

Parameter	Temperature (°C)	Salinity (ppt)	pH
S1	32 ± 2.6 (29-34)	20.6 ±14 (5-32)	8.1 ± 0.5 (7.5-8.5)
S2	29.7 ± 3.2 (26-31)	14.3 ±12 (2-26)	7.7 ± 0.6 (7.4-8.4)
S3	32.3 ± 1.5 (31-33)	5.2 ± 5.3 (0-10.7)	7.8 ± 0.6 (7.2-8.3)
S4	30.6 ± 4 (26-33)	0	8 ± 0.2 (7.8-8.2)
S5	28.7 ± 3.2 (25-31)	0	7.9 ± 1.1 (7.1-9.2)
S6	30 ± 1 (29-31)	16.7 ± 12.5 (5-30)	7.3 ± 0.4 (6.9-7.8)
S7	31.7 ± 2.1 (31-34)	14 ± 10.5 (3-24)	7.6 ± 0.9 (7.1-8.7)
S8	31 ± 1 (30-32)	12 ± 10.8 (0-21)	7.5 ± 0.8 (7.1-8.5)
S9	31 ± 1.7 (30-33)	10 ± 10 (0-20)	7.8 ± 0.6 (7.5-8.5)
S10	30.7 ± 2.1 (29-33)	2 ± 3.4 (0-6)	7.6 ± 1.0 (6.8-8.8)

*Value in parentheses indicates range

2.5.2 Species level identification and distribution of *Vibrio* species in Cochin estuary

Two hundred and thirty five colonies were isolated during the study period. Out of the total, 180 isolates were Gram-negative, oxidase-positive and capable of fermenting glucose without producing gas. This presumptive *Vibrio* isolates were further identified up to the species level using the Dichotomous key.

The isolates which produced bluish-green colour (specific for *V. parahaemolyticus*) on HiCrome Vibrio agar were picked (Plate 2.1).

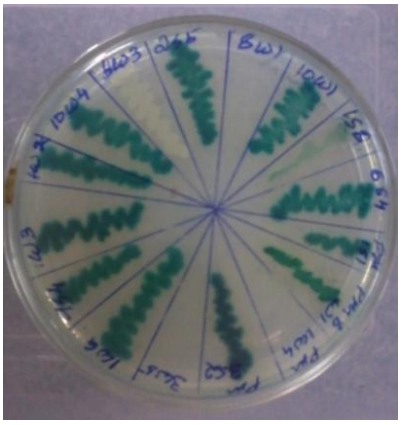

Plate 2.1 HiCrome Vibrio agar plate showing *V. parahaemolyticus* specific (bluish green colour) and non- specific colonies

They were confirmed by PCR detection of species specific genes. The isolates which showed positive result for presence of *tlh* (450 bp) and *toxR* (368 bp) genes were confirmed as *V. parahaemolyticus* species (Figure 2.2 a, b). A total of 90 isolates were thus identified as *V. parahaemolyticus*.

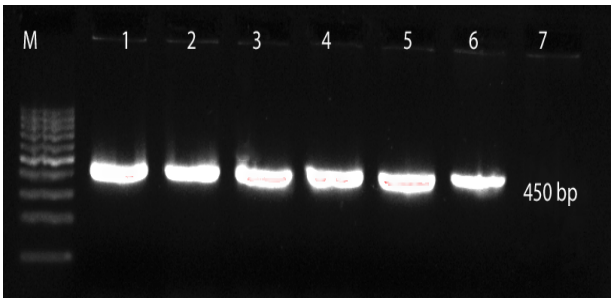

Figure 2.2 a. PCR amplified *V. parahaemolyticus* specific *tlh* gene (450 bp). Lane 1: 100 bp molecular weight ladder; Lanes 2-6: *V. parahaemolyticus* isolates from Cochin estuary: lane 7: negative control

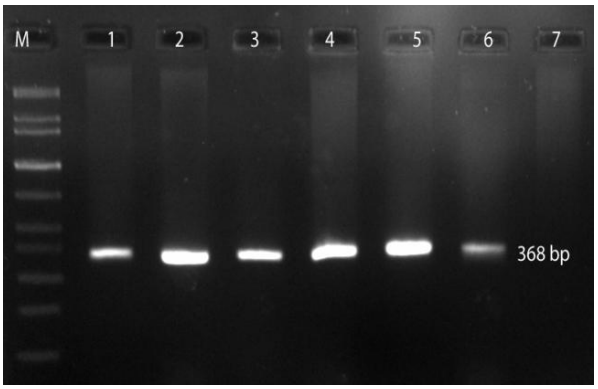

Figure 2.2 b. PCR amplified *V. parahaemolyticus* specific *toxR* (368 bp) gene products. Lane 1: 100 bp DNA ladder; lanes 2-6: *V. parahaemolyticus* isolates from Cochin estuary; lane 7: negative control

A total of 16 species were isolated from Cochin estuary. This included *Vibrio parahaemolyticus*, *V. coralliilyticus*, *V. proteolyticus*, *V. litoralis*, *V. rumoiensis*, *V. calviensis*, *V. superstes*, *V. natriegens*, *V. agarivorans*, *V. fischeri*, *V. pelagius*, *V. aestuarinus*, *V. mytilii*, *V. mimicus*, *V. pacinii*, *V. furnisii and Photobacterium damselae (earlier V. damselae).* The percentage distribution of *Vibrio* spp. in Cochin estuary is represented in Figure 2.3. *V. parahaemolyticus* was found to be the predominant species

(50%). It was followed by *V. coralliilyticus (*17.2%*)* and *V. proteolyticus* (10%).

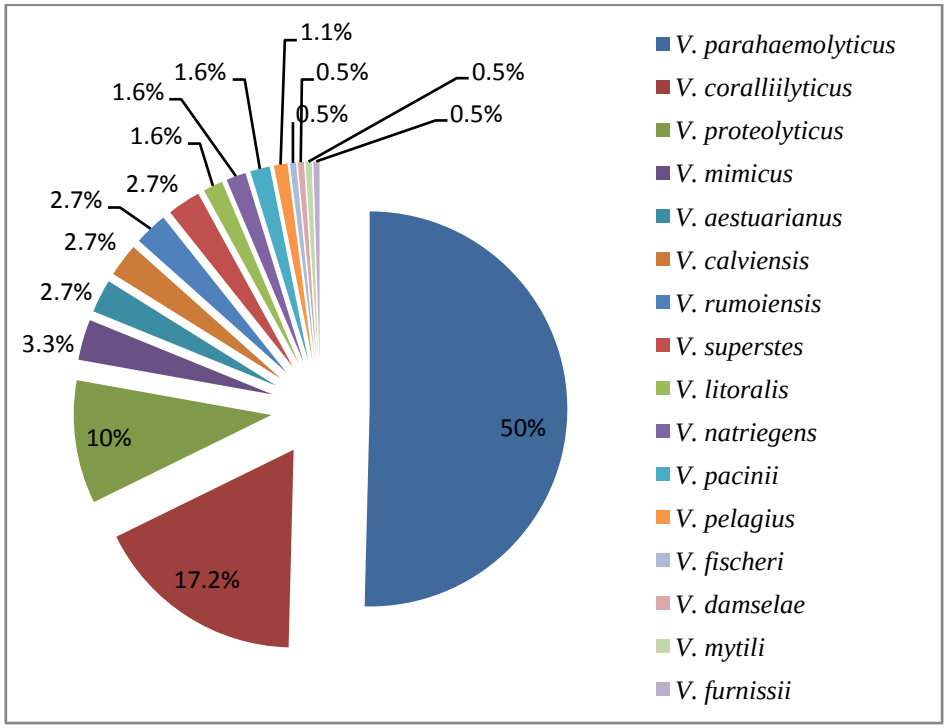

Figure 2.3 Percentage distribution of *Vibrio* species in Cochin estuary

Figure 2.4 shows the diversity and distribution of *Vibrio* in the ten stations of Cochin estuary. Among the stations, maximum abundance of *Vibrio* was observed in station 1(Marine science jetty) and the least in stations 4 (Varapuzha) and 5 (Eloor). Among the stations, maximum diversity (H') was observed in station 10 Murinjapuzha, followed by station 1 (Marine science jetty) with H' index values 1.556163 and 1.098852 respectively. Station 7 was the the least diverse (H'= 0.721464). H' index value of stations 2, 3, 4, 5, 6, 8 and 9 were 1.039720771,

1.096463643, 1.039720771, 1.060856947, 1.046630487, 1.365368986 and 1.239659392 respectively. From the lesser diversity index values of the stations (H' <4) it was clear that diversity was very less in Cochin estuary. A single species i.e. *V. parahaemolyticus* was dominant in the estuary. Species richness was also highest in station 10 (d=1.781845) followed by station 1 (d=1.406332). It was least in station 7 (d=0.804859). The d value of stations 2, 3, 4, 5, 6, 8 and 9 were 0.804859209, 0.985376216, 0.961796694, 0.910239227, 0.985376216, 1.358493088 and 1.136769545 respectively. *V. pelagius* and *V. fischeri* species were unique to station 1, *V. damselae* to station 7, *V. mytili* to station 8, *V. natriegens* to station 9 and *V. furnissii* to station 10 respectively. *Vibrio* was not isolated from the sediment of station 5 (Eloor).

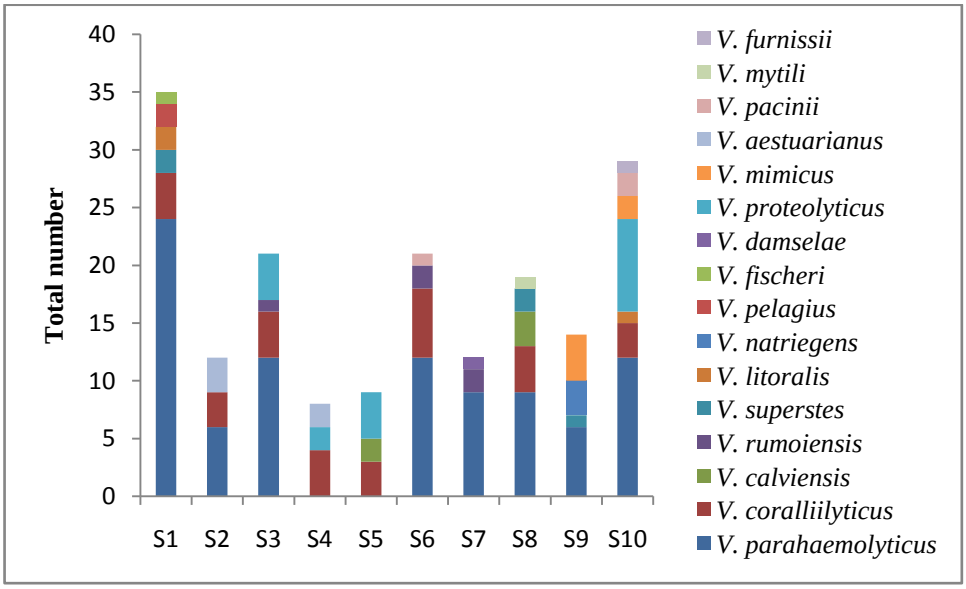

Figure 2.4 Station-wise distribution of *Vibrio* species in Cochin estuary

2.5.3 Relative diversity and distribution of *Vibrio* in the water and sediment of Cochin estuary

The distribution of *Vibrio* species in the water of Cochin estuary is given in Figure 2.5. Among the total 180 *Vibrio*, 55% (100/180) were isolated from water of Cochin estuary. Out of the total 16 species isolated, 11 species were present in the water. This included *V. parahaemolyticus*, *V. calviensis*, *V. rumoiensis*, *V. corralliilyticus*, *V. natriegens*, *V. superstes*, *V. pacinii*, *V. proteolyticus*, *V. mimicus*, *V. mytilii* and *V. damselae*. Among them, the species *V. calviensis*, *V. rumoiensis*, *V. natriegens*, *V. damselae* and *V. mytili* were unique to estuarine water. They were absent in the sediment.

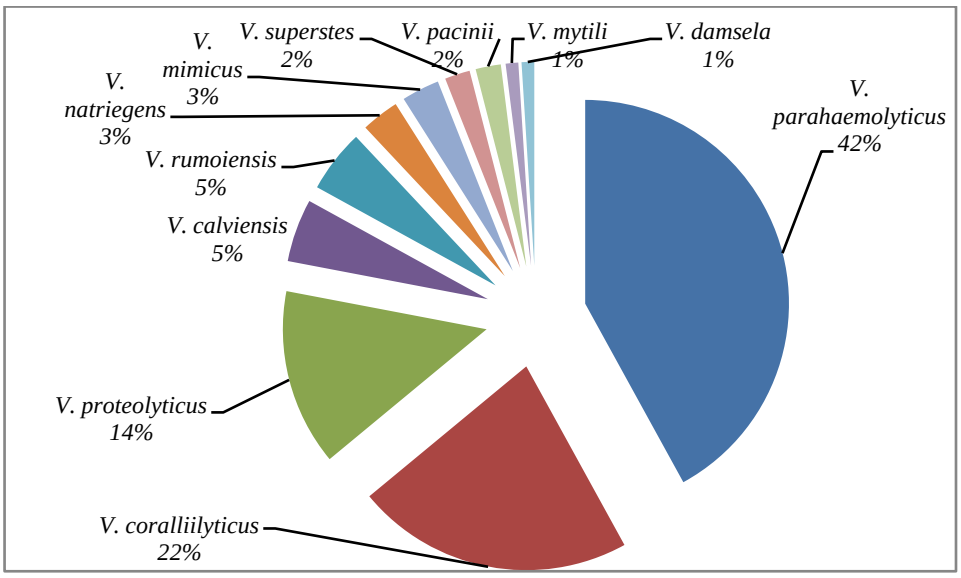

Figure 2.5: Distribution of *Vibrio* in water of Cochin estuary

The distribution of *Vibrio* species in sediment of Cochin estuary is given in Figure 2.6. Of the total *Vibrio*, 44% were isolated from the sediment. Eleven species were present in the estuarine sediment. This included *V. parahaemolyticus*, *V. litoralis*, *V. pelagius*, *V. corralliilyticus*, *V. furnissii*, *V. superstes*, *V. pacinii*, *V. proteolyticus*, *V. mimicus*, *V. aestuarianus* and *V. fischeri.* Among them, *V. litoralis*, *V. pelagius*, *V. furnissii*, *V. aestuarianus* and *V. fischeri* were unique to sediment. They were absent in the water.

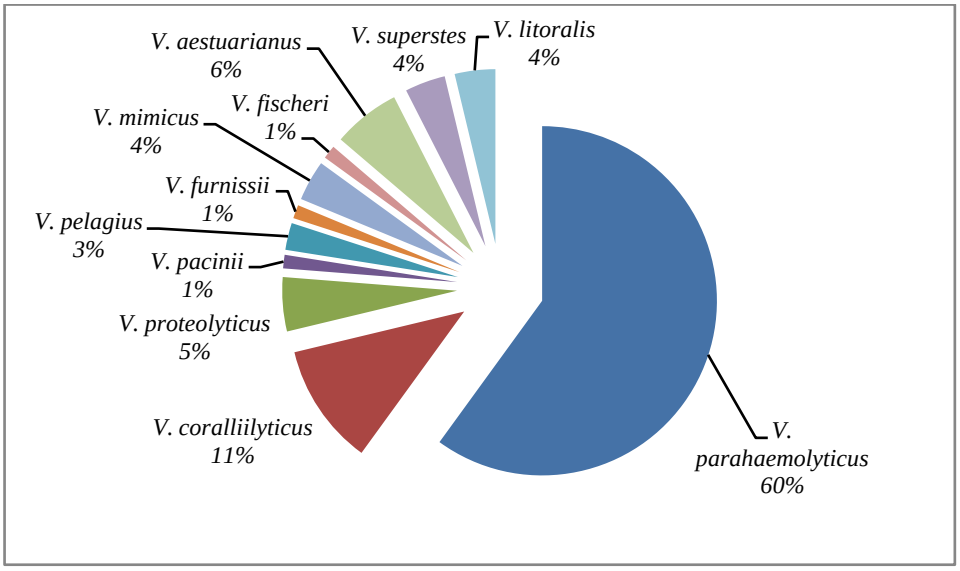

Figure 2.6 Distribution of *Vibrio* in sediment of Cochin estuary

2.5.4 Seasonal variation in the diversity and distribution of *Vibrio* in Cochin estuary

Sampling was done during the pre-monsoon, monsoon and post-monsoon seasons of the year 2012. When the seasonal distribution of

Vibrio was analysed, it was found that maximum abundance of *Vibrio* was observed during the pre- monsoon season.

Of the total 180 *Vibrio* strains, 100 of them were isolated during the pre-monsoon season. Of the total 16 species identified, 10 were observed during this period. The species included *V. parahaemolyticus, V. calviensis, V. rumoiensis, V. litoralis, V. coralliilyticus, V. natriegens, V. pelagius, V. superstes, V. fischeri* and *V. damselae.* Among them, *V. calviensis, V. rumoiensis, V. litoralis, V. natriegens, V. pelagius, V. superstes, V. fischeri* and *V. damselae* were observed only during the pre-monsoon period. Figure 2.7 shows the percentage distribution of each species in Cochin estuary during pre-monsoon season. *V. parahaemolyticus* was the dominant species during the period contributing to 52% of the total *Vibrio* isolated, followed by 23% of *V. coralliilyticus*.

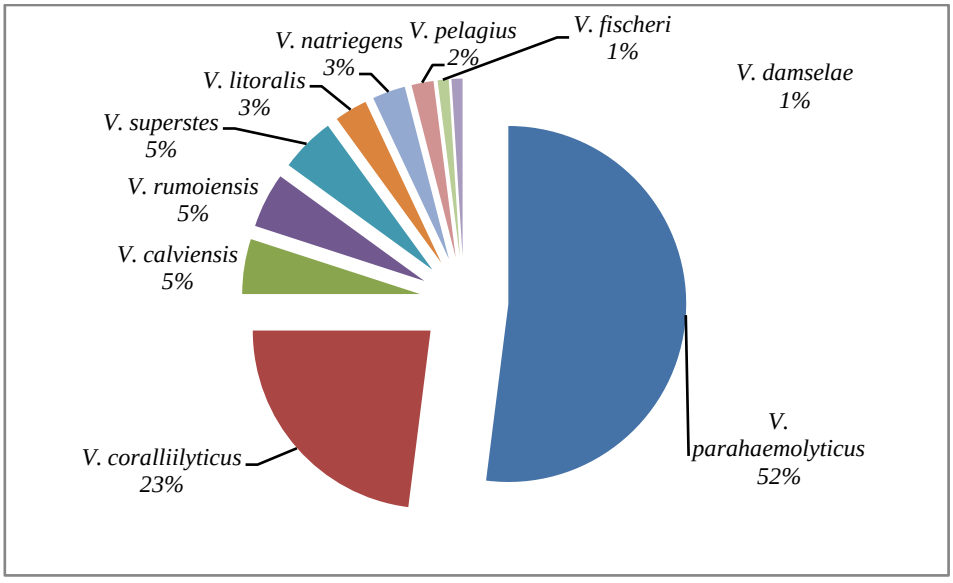

Figure 2.7 Distribution of *Vibrio* species in Cochin estuary during the pre-monsoon season

Out of the total 180 isolates, 60 were isolated during the monsoon season in Cochin estuary. Eight species of *Vibrio* were observed. This included *V. parahaemolyticus, V. coralliilyticus, V. furnissii, V. pacinii, V. proteolyticus, V. mimicus, V. aestuarianus* and *V. mytili*. The species *V. furnissii, V. pacinii, V. proteolyticus, V. mimicus, V. aestuarianus* and *V. mytili* were observed solely during the monsoon. Figure 2.8 shows the percentage distribution of *Vibrio* species in Cochin estuary during the monsoon season. *V. parahaemolyticus* was the dominant species followed by *V. proteolyticus* contributing to 36% and 30% respectively of total *Vibrio* isolated during monsoon from the estuary.

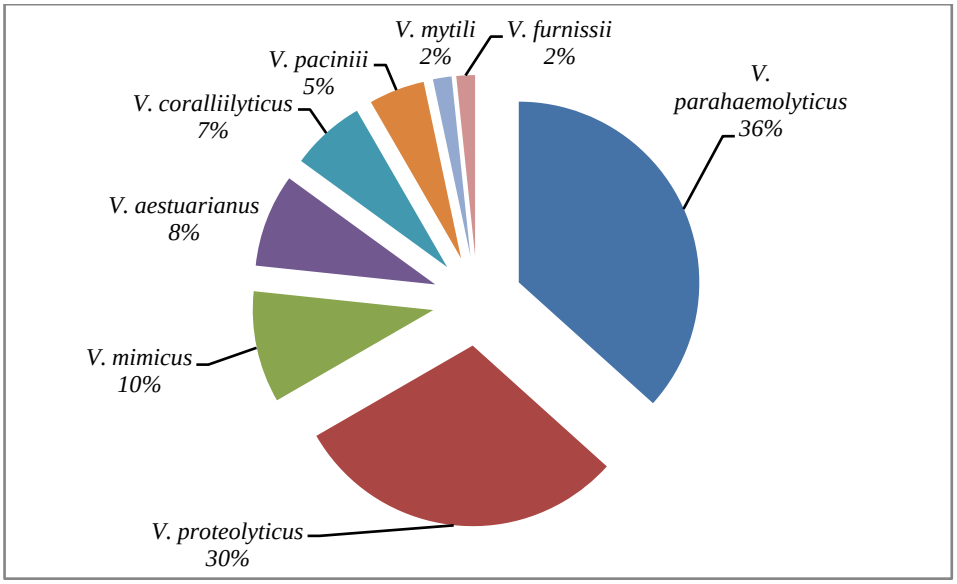

Figure 2.8 Distribution of *Vibrio* species in Cochin estuary during the monsoon season

The diversity of *Vibrio* was very less during the post-monsoon season. Of the total 180 isolates, only 20 *Vibrio* were isolated during this period. Two *Vibrio* species alone were observed during this season namely, *V. parahaemolyticus* and *V. coralliilyticus.* Among them the majority belonged to *V. parahaemolyticus*. Figure 2.9 shows the percentage distribution of both the species during post-monsoon season in Cochin estuary.

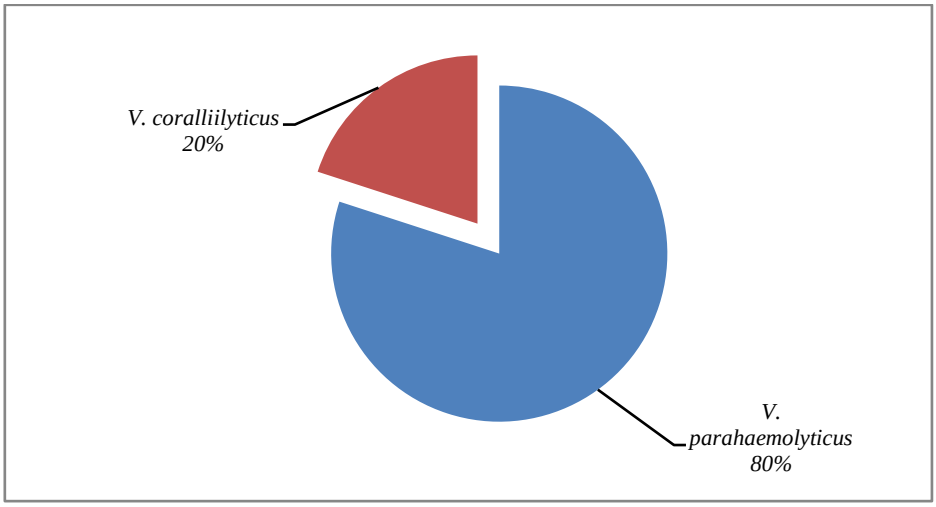

Figure 2.9 Distribution of *Vibrio* species in Cochin estuary during the post-monsoon season

2.5.5 Genbank accession numbers

The obtained 16S rRNA sequences were deposited in the NCBI Genbank. The Genbank accession numbers allotted are *V. parahaemolyticus* PM1S2- KM406325, *V. mimicus* M9W1- KT187246, *V. proteolyticus* M10W1- KT748656, *V. damselae (Photobacterium damselae*) M7W1- KY485151, *V. coralliilyticus* 2W9- KY485150. The sequence details are given in Appendix 4.

2.6 Discussion

In the present study, a total of 16 *Vibrio* species were isolated from Cochin estuary. This included *V. parahaemolyticus*, *V. coralliilyticus*, *V. proteolyticus*, *V. litoralis*, *V. rumoiensis*, *V. calviensis*, *V. superstes*, *V. natriegens*, *V. agarivorans*, *V. fischeri*, *V. pelagius*, *V. aestuarinus*, *V. mytili*, *V. mimicus*, *V. pacinii*, *V. furnissii and Photobacterium damselae (earlier V. damselae)*. The *Vibrio* spp. are widely distributed in marine environment and studied extensively by various authors (Thompson *et al.*, 2004; Eiler *et al.*, 2006; Mansergh and Jonathan 2014; Amin *et al.*, 2016). Prashanthan *et al.* (2011) reported that the inshore coastal waters of Kerala replenish with indigenous *Vibrio* spp. because of ecological imbalance effected by intrusion of untreated sewage and land river runoff.

There are 12 *Vibrio* species reported to be pathogenic to humans which includes *Vibrio alginolyticus, V. cholerae, V. cincinnatiensis, Photobacterium damselae (earlier V. damselae), V. harveyi, Grimontia hollisae (earlier V. hollisae), V. fluvialis, V. furnissii, V. metschnikovii, V. mimicus, V. parahaemolyticus* and *V. vulnificus* (Oliver *et al.*, 2013). Hence, in the present study, the isolation of *V. parahaemolyticus, V. mimicus, Photobacterium damselae (earlier V. damselae) and V. furnissii* from Cochin estuary is a matter of serious concern. The presence of human pathogenic *Vibrio* spp. serve as an indicator of public health safety of water and food destined for human consumption. As these human pathogenic vibrios are mesophiles with growth temperature optima around 37 °C, maximum human health risks ought to be expected

in permanently warm tropical environments. Yet, currently published health hazards arising from non-cholera mesophilic vibrios have raised particular awareness of global warming in non-tropical climates (Vezzulli *et al.*, 2012, 2015). Whereas in tropics the situation is neglected, either since health hazards by vibrios are not properly reported here or since it is regarded as less alarming.

Growth and virulence of mesophilic vibrios can rise with increasing temperature even beyond 30 °C (Farmer and Hickman-Brenner, 2006; Mahony *et al.,* 2010). The temperature of all the stations in our study sites was observed to be equal to or greater than 30 °C. This provokes a dismaying situation since a temperature of 30 °C has already been considered as an upper threshold for detecting maximum abundance of classic clinical pathogens (Tantillo *et al.,* 2004). *V. harveyi*, *V. parahaemolyticus*, *V. anguillarum*, *V. campbelli*, *V. fischeri*, *V. damselae*, *V. pelagius*, *V. orientalis*, *V. ordalii*, *V. mediterrani*, *V. logei* etc. are some of the *Vibrio* spp. reported as shrimp pathogens (Lavilla-Pitogo, 1995; Jayasree *et al.*, 2006; Austin, 2010; Raissy *et al.,* 2012 b). Among them, we isolated *V. damsela, V. fischeri, V. pelagius* and *V. parahaemolyticus* from our study area. They are reported as common inhabitants of shrimp hatcheries, pond water and sediment and they turn pathogenic under poor environmental conditions (Jayasree *et al.,* 2006).

In our study, *V. parahaemolyticus* was found to be the predominant species isolated from water and sediment of Cochin estuary. It was present throughout the year during the study. Highest abundance of the species was noted during the pre-monsoon period and least during post-

monsoon period. Our findings corroborated with Deepanjali *et al.* (2005) who reported an increased prevalence of *V. parahaemolyticus* during the pre-monsoon and showed a decreased trend during the post-monsoon season. Even though *V. parahaemolyticus* was the dominant species in the estuary, it was not detected from Eloor and Varapuzha stations indicating the obligate requirement of NaCl for *Vibrio* species with the exception of *V. cholerae*. The salinity of both the stations remained at 0 ppt throughout the study. Since, *V. parahaemolyticus* is a halophile, fresh water condition at the above stations could be a possible reason for its absence in these stations. *V. parahaemolyticus* is a part of the natural estuarine microflora and coastal marine waters and are usually present in seafood, especially shellfish and bivalve molluscs (DePaola *et al.,* 2003; Zorrilla *et al.,* 2003; Krishna *et al.,* 2014; Sudha *et al.*, 2014). It has been recognized as one of the most important food borne pathogens and the leading causal agent of human acute gastroenteritis, following the consumption of raw, undercooked or mishandled seafood and marine products (Su and Liu 2007; Pal and Das 2010; Velazquez-Roman *et al.*, 2012). The organism has also been reported as the causative agent of early mortality syndrome that caused large-scale economic losses in farmed shrimp production in India and other countries as well (FAO 2013; Tran *et al.*, 2013; Krishna *et al.*, 2014). By 16S rRNA sequencing, we observed that our isolate had 100% similarity to the pandemic clone *V. parahaemolyticus* O3:K6. Infection associated with this clone was first reported in Kolkata in 1996 (Velazquez-Roman *et al.,* 2012). Strains belonging to pandemic O3:K6 have been previously isolated from environmental samples in several countries, including Bangladesh (Islam *et al.*, 2004), Japan (Hara-Kudo *et*

al., 2003), India (Deepanjali *et al.,* 2005), and Italy (Caburlotto *et al.,* 2010), Africa (Ansaruzzaman *et al.,* 2005), North, Central and South America (Daniels *et al.,* 2000; Gonzalez-Escalona *et al.,* 2005; Velazquez-Roman *et al.,* 2012).

V. coralliilyticus was the second dominant species in the estuary. *V. coralliilyticus* is a global marine pathogen that has been associated with coral disease from geographically distinct global regions. First isolated from diseased and bleaching corals of the Zanzibar coast (Ben-Haim *et al.,* 2003 a, b) this species has also been implicated in white syndrome disease outbreaks in the Indo-Pacific (Sussman *et al.,* 2008). It causes fatal infections in a wide range of organisms, including unicellular algae, corals, oysters, shrimp, rainbow trout and flies during experimental infection assays (Austin *et al.,* 2005; De Santos *et al.,* 2011). Previous reports show that *V. coralliilyticus* displays a tightly temperature related virulence. It is found to be avirulent at temperatures less than 24 ^{o}C and is considered virulent at temperatures above 24.5 ^{o}C (Ben- Haim *et al.,* 2003a).

V. proteolyticus was another dominant species observed in the estuary during the study. *V. proteolyticus* was originally isolated from the intestine of the wood borer *Limnoria tripunctata* (Merkel *et al.,* 1964). Pathogenicity study on *Artemia* animal model had revealed the virulence potential of the organism (Verschuere *et al.,* 2000). A recent study by Ray *et al.* (2016) demonstrated that the organism causes cytotoxicity in both *HeLa* cells and macrophages. On proteomic analysis of *V. proteolyticus* they found that a secreted hemolysin was the toxin responsible for its virulence character.

V. mimicus was isolated from both water and sediment of Cochin estuary during this study. It is an opportunistic pathogen and is closely related to *V. cholerae.* Reports revealed that *V. mimicus* can cause gastroenteritis in humans (Campos *et al.,* 1996; Takahashi *et al.,* 2007; Mizuno *et al.,* 2009). It is also known to cause secondary infections in red claw crayfish (Eaves and Ketterer, 1994). One strain of *V. damselae* was isolated in the present study. The species was found only in water of station 7 (Kumbalam). The species was initially isolated from ulcers of damselfish *(*Lov*e et al.,* 1981). *P. damsela* is also known to cause wound infections (Austin, 2010). *V. furnissii* was unique to sediment of station 10 (Murinjapuzha). The species has been found to be associated with mass mortality in tiger shrimps and diseases in eels (Sung *et al.,* 2001; Austin and Austin, 2007). Other shrimp pathogens like *V. fischeri* and *V. pelagius* were also isolated from our study area. Both the species were detected only in sediment from station 1 (Marine science jetty).

From the diversity index (H') values, we could draw a conclusion that among the various stations in Cochin estuary, Murinjapuzha and Marine science jetty had the highest species diversity. Almasi (2005) reported that highest population of vibrios were at polluted areas and their habitat decreased with increase in distance from the source of pollutants. Moreover, plankton composition plays an important and independent role as a driver of the total culturable *Vibrio* community in natural estuarine systems (Turner *et al.,* 2009). Microbial contamination occurs secondary to point source sewage dumping, as well as from indirect contaminated run off. Humans are exposed to microbial contamination by consuming

contaminated seafood and through recreational and occupational exposure to contaminated marine waters (Fleming *et al.*, 2006).

The present study reveals that pathogenic *Vibrio* species are present in the water and sediments of Cochin estuary; it is likely that they could also be present in the fishes and shrimps itself, with the implicit consumer health risk, particularly in regions where raw seafood is consumed. They could also become a major vector for cross contamination when not properly handled. In spite of their public health significance, pathogenic vibrios have not been extensively monitored, in contrast to other pathogens. The coastal waters receives huge quantum of fresh water from rivers and large quantities of waste water from cities besides variety of industrial effluents, harbour and partially treated/untreated municipal sewage water which carries enormous level of pathogens. Degrading the estuarine water quality by such actions leads to many environmental consequences. Under such poor environmental conditions bacteria like *Vibrio*, autochthonous to the estuary, turns pathogenic and causes disease outbreaks. Hence, a regular monitoring of the prevalence of pathogens such as vibrios is needed to ensure seafood safety and also to prevent the potential spread of any outbreaks. Simultaneously, the pollution control board and environmentalists should together initiate necessary remedial measures to instantly spot and regulate the alleged discharge of untreated wastes into the natural water bodies.

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