GENOTYPING, VIRULENCE CHARACTERIZATION AND SURVIVAL KINETICS OF *VIBRIO* SPP. FROM FOOD AND ENVIRONMENTAL SOURCES ALONG THE SOUTH WEST COAST OF INDIA

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By

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Genotyping, virulence characterization and survival kinetics of *Vibrio* spp. from food and environmental sources along the South west coast of India

Ph.D. Thesis under the Faculty of Marine Sciences

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Certificate

This is to certify that the thesis entitled **"Genotyping, virulence characterization and survival kinetics of *Vibrio* spp. from food and environmental sources along the South west coast of India"** is an authentic record of research work carried out by **Ms. Reshma Silvester** under my supervision and guidance in the Department of the Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Microbiology of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any other University or Institution.

All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral committee have been incorporated in the thesis.

Kochi - 682 016
December 2017

Prof. (Dr.) A. A. Mohamed Hatha
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Declaration

I hereby declare that the thesis entitled **"Genotyping, virulence characterization and survival kinetics of *Vibrio* spp. from food and environmental sources along the South west coast of India"** is a genuine record of research work done by me under the supervision and guidance of Dr. A. A. Mohamed Hatha, Professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology and no part thereof has been presented for the award of any other degree, diploma or associateship in any other University or Institution earlier.

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Contents

List of Abbreviations

ANOVA	-	Analysis of variance
ARGs	-	Antibiotic resistance genes
ARP	-	Antibiotic resistance pattern
AW	-	Autoclaved water
bp	-	Base pair
CDC	-	Centre for Disease Control and Prevention
CDDEP	-	Center for Disease Dynamics, Economics and Policy
CFU	-	Colony forming unit
CLSI	-	Clinical and Laboratory Standards Institute
DNA	-	Deoxyribonucleic acid
DNase	-	Deoxyribonuclease
EDTA	-	Ethylene diamine tetraacetic acid
ERIC	-	Enterobacterial Repetitive Intergenic Consensus
g	-	Gram
HCl	-	Hydrochloric acid
h	-	Hours
klx	-	Kilolux
L	-	Litre
LB	-	Luria Bertani
LPS	-	Lipopolysaccharide
M	-	Molar
MAR	-	Multiple antibiotic resistance
mcg	-	Microgram
MgCl2	-	Magnesium chloride
mM	-	Milli molar
min	-	Minutes
ml	-	milli litre
mol/L	-	moles per litre
N	-	Normal
NaCl	-	Sodium chloride
NAOH	-	Sodium hydroxide
NEB	-	New England Biolabs
PCR	-	Polymerase chain reaction
PFGE	-	Pulse Field Gel Electrophoresis

PFU	-	Plaque forming units
ppt	-	parts per thousand
PRIMER	-	Plymouth Routines in Multivariate Ecological Research
RAPD	-	Random Amplified Polymorphic DNA
REP	-	Repetitive Extragenic Palindromic
RFLP	-	Restriction Fragment Length Polymorphism
rRNA	-	Ribosomal ribonucleic acid
rpm	-	revolutions per minute
sec	-	Seconds
SDS	-	Sodium Dodecyl Sulphate
spp.	-	Species
SPSS	-	Statistical Package for the Social Sciences
TCBS	-	Thiosulfate citrate bile salts sucrose
TE	-	Tris EDTA
T3SSs	-	Type three secretion system genes
UV	-	Ultraviolet
WHO	-	World Health Organization
w/v	-	weight by volume
µg	-	Microgram
µl	-	Microlitre
^{o}C	-	Degree Celsius
%	-	Percentage

Chapter 1

GENERAL INTRODUCTION

Contents

1.1 Introduction

Vibrios are Gram-negative halophilic bacteria found naturally in shallow coastal waters to the deepest parts of the ocean and are highly abundant in aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide (Thompson *et al.*, 2004). Many *Vibrio* species are recognized as human pathogens and have been implicated in water and seafood-related outbreaks of gastrointestinal infections in humans (Eiler *et al.*, 2006, Austin, 2010). 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. It 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). Among them *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are serious human pathogens (Thompson *et al.,* 2004). Many *Vibrio* spp. have been found to be pathogens of fish (*V. anguillarum*), coral (*V. shiloi*, *V. coralliilyticus*), shellfish (*V. splendidus*), and shrimp (*V. harveyi*, *V. penaeicida* and *V. nigripulchritudo*), and infections with these organisms have profound environmental and economic consequences (Rosenberg and Falkovitz, 2004; Le Roux *et al.,* 2009; Austin, 2010).

1.2 Major pathogenic vibrios

1.2.1 *Vibrio cholerae*

V. cholerae is the causative agent of deadly cholera disease. The original reservoir of *V. cholerae* was in the Ganges delta in India. By the 19th century it spread to various continents across the world. There are various serogroups of *V. cholerae* among which the serogroups O1 and O139 are responsible for the deadly diarrhoeal cholera disease. This toxigenic strain carries a filamentous bacteriophage (CTXΦ) which encodes the cholera toxin (Waldor and Mekalano, 1996). Secretion of this toxin into the host intestine leads to watery diarrhoea commonly known as "rice-water stool disease". The disease is usually acquired by ingestion of food or water contaminated with the bacterium *V. cholerae*. In the environment, the bacterium normally exists in a VBNC (Viable but non-culturable) form (Alam *et al.,* 2007).

Annually 3-5 millions of people are affected by cholera cases resulting in 100,000–120,000 deaths worldwide (Gupta *et al.,* 2016). Figure 1.1 shows the cholera case reported to World health organisation

(WHO) from four continents during 1989-2015. During the year 2015, around 1,72,454 cases and 1304 deaths were reported from 42 countries (WHO, 2016) and from India alone 889 cases were reported.

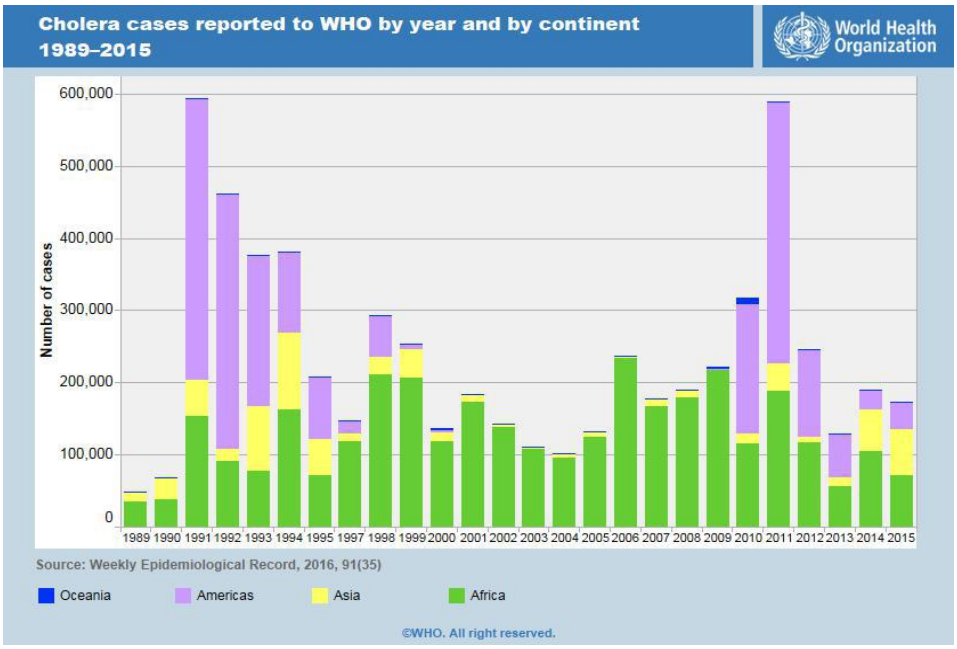

Figure 1.1 Cholera cases reported to WHO during 1989-2015 (retrieved from Cholera annual report of WHO, 2016)

1.2.2 *Vibrio parahaemolyticus*

V. parahaemolyticus, autochthonous to estuarine, marine and coastal environments throughout the world, is a leading cause of food borne gastroenteritis in Asia as well as in other countries (Chiou *et al*., 2000; Wong *et al*., 2000; Mc Laughlin *et al*., 2005; Matsuda *et al*., 2012; Odeyemi, 2016). *V. parahaemolyticus* is attracting increasing interest worldwide where raw or undercooked seafood is often consumed (Chao

et al., 2009). *V. parahaemolyticus* is regarded as the primary source of increase in vibriosis incidence (Newton *et al*., 2012), and highly pathogenic serotypes of the species are emerging on a global scale. 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. It causes wound infections in those exposed to contaminated water (Miyoshi *et al*., 2008). It also causes septicemia, particularly in immuno-compromised people (Daniels *et al*., 2000). It is also the causal agent of mass mortality among marine fishes and invertebrates, causing huge economic losses to the aquaculture industry.

1.2.3 *Vibrio vulnificus*

V. vulnificus is pathogenic to humans, eels, shrimps and fish (Al-Mouqati *et al.,* 2012*).* It is an etiologic agent of wound infections and septicemia in humans (Finkelstein *et al.,* 2002). It is commonly known as 'the flesh eating bacteria'. It is the rare causative agent of necrotising fasciitis (Madiyal *et al.,* 2016). A capsular polysaccharide (CPS) is the main virulence factor responsible for the pathogenesis of the organism (Thompson *et al.,* 2004). Besides CPS, cytotoxins, hydrolytic enzymes, lipopolysaccharide, pili and flagellum also plays a role in the virulence of the bacterium (Horseman and Surani, 2011; Hor and Chen, 2013).

1.3 Role of *Vibrio* in nutrient cycling

Vibrios play a major role in nutrient cycling and biogeochemical cycles and degradation of organic matter in aquatic environments (Vijayan and Lee, 2014). They act as a major link to transfer dissolved organic carbon to higher trophic levels in the marine ecosystem (Mourino-Perez

et al., 2003). Due to its broad metabolic range vibrios can utilize a variety of carbon sources for its food (Thompson and Polz, 2006). Many of them are able to degrade toxic polycyclic aromatic hydrocarbons and chitin present in the polluted marine sediments and oceans (Thompson *et al.,* 2004).

In the marine environment, members of genus *Vibrio* play a major role in remineralization of organic matter in the sea (Fukami *et al.,* 1985). Organic substances in marine ecosystems consist mainly of starch, proteins, lipids, cellulose, chitin, pectin, nucleic acids etc. These are subjected to degradation by exoenzymes produced by marine microorganisms. The ability to produce extracellular enzymes such as gelatinase, DNase, pectinase, cellulase, lipase etc. has already been reported in vibrios (Raghul and Sarita, 2011). The sources of organic material that *Vibrio* utilises as energy source may influence the *Vibrio* dynamics in natural environments.

1.4 *Vibrio* phenotyping *versus* genotyping

Phenotypic methods have a number of practical limitations, making it less suitable for detailed studies of bacterial population, for infection control and surveillance. A given phenotype may not always exactly reflect the genotype of a microorganism. Thus, it cannot be employed as a reliable and stable epidemiological marker. The biochemical methods currently used to identify *Vibrio* species are time-consuming and laborious because of high phenotypic similarity between closely related species. Hence, new genotyping methods have largely replaced the traditional phenotyping.

Genotyping is the process of analysing the genetic constitution of an individual by studying its DNA sequence. Even the smallest variation at the sequence level can be detected and thus can be applied efficiently for studying the intra-specific genetic variation among various organisms. It can also be used for studying variation between closely related bacterial species. It can effectively predict occurrence of any past mutation events. It is widely used for characterizing the epidemiological spreads of pathogenic bacteria. These are specifically useful for source tracking of the strains dispersed in the environment and to provide information on the genetic relatedness of strains and detection of particularly virulent strains, as well as the study of the geographical and host distribution of possible variants of a specific pathogen (Olive and Bean, 1999).

Typing pathogens at the strain level is very important for diagnosis, treatment and epidemiological surveillance of bacterial infections. It is also employed to examine the level of genetic diversity among different strains within the same species. Several polymerase chain reaction (PCR)-based techniques such as RFLP, RAPD, REP, ERIC-PCR etc. exist for both molecular typing and differentiation of bacterial species. In recent years, these molecular-based techniques have been shown to be useful methods for discriminating among isolates of various pathogens including *Vibrio* (Tsen and Lin, 2001).

1.5 Antibiotic resistance among vibrios

Antibiotic resistance is a serious global threat. Yearly, at least 700,000 people around the world die from infections caused by multi-drug resistant bacteria. It is predicted that the death toll due to superbugs

will rise to 10 million by the year 2050. During the past few decades, there is rapid emergence in antimicrobial resistance in many bacterial genera due to the overuse/misuse of antibiotics in humans, agriculture and aquaculture systems. Humans acquire these drug resistant bacteria through food, environment or through direct human-animal contact. Unprescribed, over-the-counter use of antibiotics has also accelerated the emergence of antibiotic resistance and this is a serious threat to the public health. Currently, the situation is such that the antibiotics have started losing their effectiveness. The world is running out of effective antibiotics to treat deadly diseases and this brings humans to a medical dilemma.

The overuse of antibiotics has resulted in the antibiotics entering our water system, making wastewater a perfect breeding ground for superbugs. Release of sewage results in entry of large number of drug resistant bacteria from various sources into the environment. Resistant genes are further transferred from non-pathogens to pathogens through horizontal gene transfer *via* conjugation, transduction and transformation. This could lead to transfer of drug resistance features to extremely autochthonous microflora such as *Vibrio*. Thus, the search for genetic elements like plasmids, transposons and integrons associated to antibiotic resistance in microorganisms also becomes important.

In spite of their public health significance, pathogenic vibrios have not been extensively monitored for their antimicrobial resistance in contrast to other pathogens. The multiple drug resistance among *Vibrio* in estuarine-marine environments may have future implications for those who consume seafood contaminated with these pathogenic vibrios and

also for the recreational and commercial users of these environments. Hence, as a part of risk assessment, it is crucial to study the antibiotic resistance of pathogens like *Vibrio*.

1.6 Significance of Cochin estuary

Estuarine ecosystems are one of the most important coastal life support systems and an ideal breeding ground of various economically important marine and freshwater organisms, particularly fishes. The brackish water area in India is approximately about 1.2 million hectares (Heran *et al*., 1992) of which 65,000 hectares area is now used for shrimp farming. In the south west coast of India, there is an extensive estuarine system of backwaters, covering an area of 2,42,600 ha (Abdul Aziz and Nair, 1978) of which Vembanad Lake is the largest. Vembanad lake is the largest brackish, tropical wetland ecosystem in India and is of extraordinary importance for its hydrological function, biodiversity and rich fishery resources. The Vembanad lake was designated as a Ramsar Site in November 2002 and is the largest of the 3 Ramsar sites located in Kerala. It is linked to the Arabian Sea through the Cochin estuary.

Cochin estuary is situated at the tip of the northern Vembanad lake. It is a tropical estuary and lies between 9° 40′-10° 12′N and 76°10' -76° 30′ E. It has its northern boundary at Azheekode and southern boundary is situated at Thannirmukham bund. Water from Periyar and Muvattupuzha rivers drains into this estuary. Cochin estuary forms the life line of people around the estuary as it provides livelihood for large number of people. It is rich in fishery resources and also acts as breeding grounds of commercially important shrimps and fishes. The fields around the backwater are suitable

for aquaculture. These areas support traditional, seasonal and perennial prawn fishery (Menon *et al*., 2000). It is one of the famous tourist hotspots and shrimps grown in these areas are exported worldwide. It is interesting to note that the estuary is frequently subjected to seasonal and diurnal variations in salinity. Thus, the estuary could provide an ideal habitat for a range of vibrios, which may vary in their preference to NaCl.

Population explosion and urbanisation has resulted in the alleged discharge of untreated domestic and industrial waste into the natural water bodies. Marine ecosystem is being continuously threatened by the accidental/ careless waste discharge from various sources. Cochin backwaters receive partially treated/untreated sewage from many points throughout its tidally mixed zone (Menon *et al*., 2000). The seafood industry is the major industry which contributes to the microbial pollution of Cochin estuary and results in entry of nutrient rich effluents from various fish processing units situated in and around the estuary (Hatha *et al*., 2004). The considerable organic pollution results in prevalence of diverse pathogens such as *Salmonella*, *Shigella*, *Vibrio*, *Escherichia coli* O157: H7 etc. in the estuary, posing a negative impact to the marine resources and the public health.

1.7 Significance of the study

Vibrios are ubiquitous in marine and estuarine ecosystems as well as aquaculture farms. Vibriosis caused by this bacterium is one of the most serious diseases in fishes and aquatic animals, thus posing a major challenge to food security and economy of the country. The safety of the exported products is also under challenge. All the seafood and shrimps exported from our country must be pathogen free. Failure to meet the

food safety standards due to contamination with pathogens like *Vibrio*, leads to export rejection.

The aquaculture ponds in and around Cochin estuary is dependent on the estuary for its water and seed supply. Government of Kerala has recently introduced *Penaeus vannamei* farming, which would help shrimp farmers reduce cost and increase production. With *Vibrio* being a major pathogen of *P. vannamei*, it is relevant to study the prevalence of pathogenic vibrios in estuarine environment. The poor quality of the harvest water is a main factor to trigger *Vibrio* infections in the aquaculture ponds. People of Kerala, especially in my study area Cochin, love seafood and consumes them on a daily basis. Fishing is the main economic activity of the local people in this area. Being popular food items in the study area, shellfishes and shrimps are the potential vehicles for food borne illnesses mediated by *Vibrio*. Moreover, prevalence of human pathogenic *Vibrio* species in estuary also poses a major challenge to the public health.

Cochin estuary is considered as a eutrophic system. It is well documented that nutrient rich environments are ideal for horizontal gene transfer among bacteria. *Vibrio* being autochthonous to marine and estuarine waters co-exist with allochthonous pathogenic bacteria such as diarrhegenic *E. coli*, *Salmonella*, *Shigella* etc. in the environment, where exchange of drug resistance/virulence genes are quite possible.

Diseases caused by multidrug resistant bacteria are very difficult to treat and is a serious issue plaguing the health care practitioners and public health officials worldwide. Though *Vibrio* being an autochthonous

organism, co-existence with human-derived pathogens would considerably influence the drug resistance and virulence features.

Similarly, the survival capabilities of pathogenic vibrios in the estuary is of importance to assess the threat proved by them to fish and shellfish as well as the recreational users of the system. Considering all these aspects the following broad objectives were set for the present research.

1.8 Broad objectives

1) To study the diversity and distribution of *Vibrio* species in Cochin estuary.

2) To determine the prevalence of antibiotic resistance among the *Vibrio* from food and environmental sources and to understand the molecular mechanisms underlying resistance.

3) To screen the *Vibrio* strains from food and environmental sources for their pathogenicity potential.

4) To analyse the genetic diversity within the different strains of *Vibrio* using genotyping.

5) To study the survival of pathogenic *Vibrio* species as a function of biological, physical and chemical factors in water and sediment of Cochin estuary.

6) To develop a tool to differentiate pathogenic *Vibrio* species.

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).

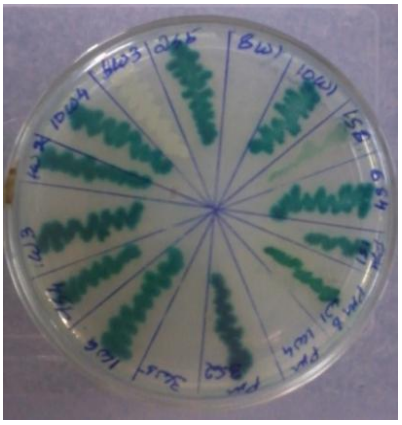

Plate 2.1 HiCrome Vibrio agar plate showing *V. parahaemolyticus* specific (bluish green colour) and non- specific colonies

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*.

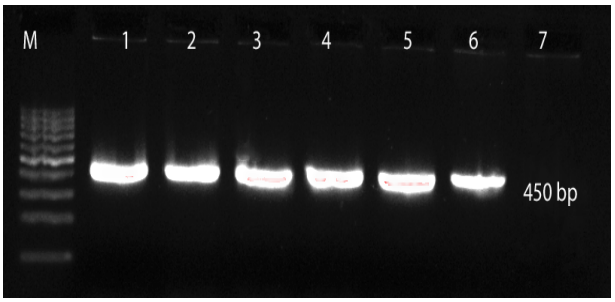

Figure 2.2 a. PCR amplified *V. parahaemolyticus* specific *tlh* gene (450 bp). Lane 1: 100 bp molecular weight ladder; Lanes 2-6: *V. parahaemolyticus* isolates from Cochin estuary: lane 7: negative control

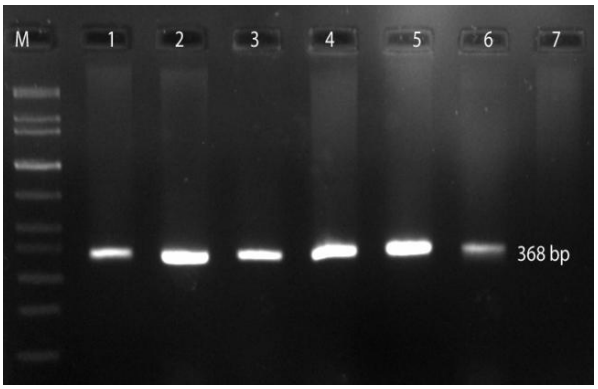

Figure 2.2 b. PCR amplified *V. parahaemolyticus* specific *toxR* (368 bp) gene products. Lane 1: 100 bp DNA ladder; lanes 2-6: *V. parahaemolyticus* isolates from Cochin estuary; lane 7: negative control

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%).

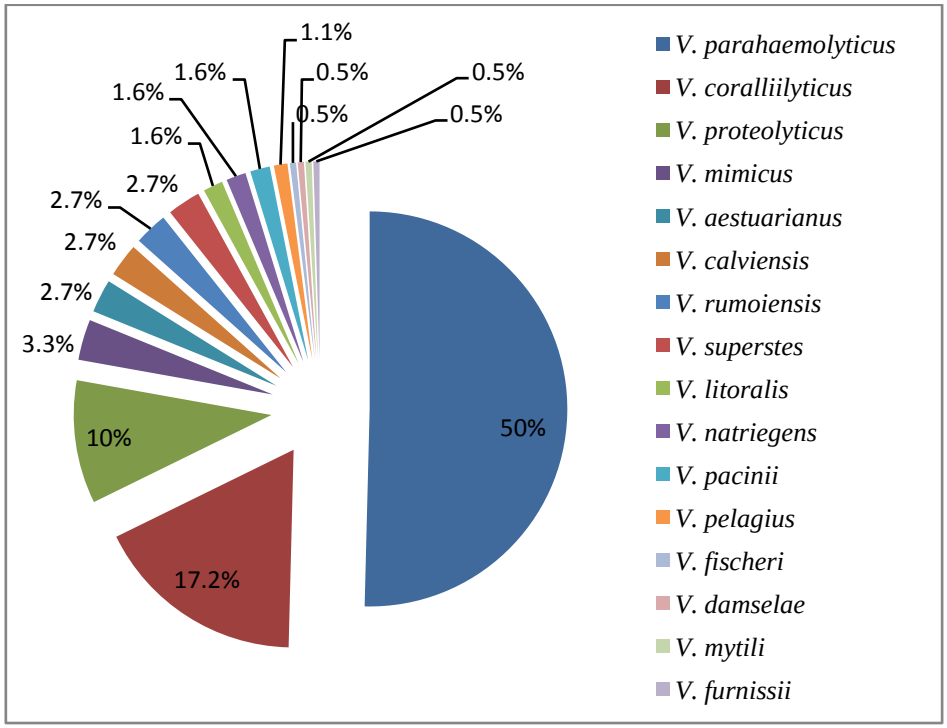

Figure 2.3 Percentage distribution of *Vibrio* species in Cochin estuary

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).

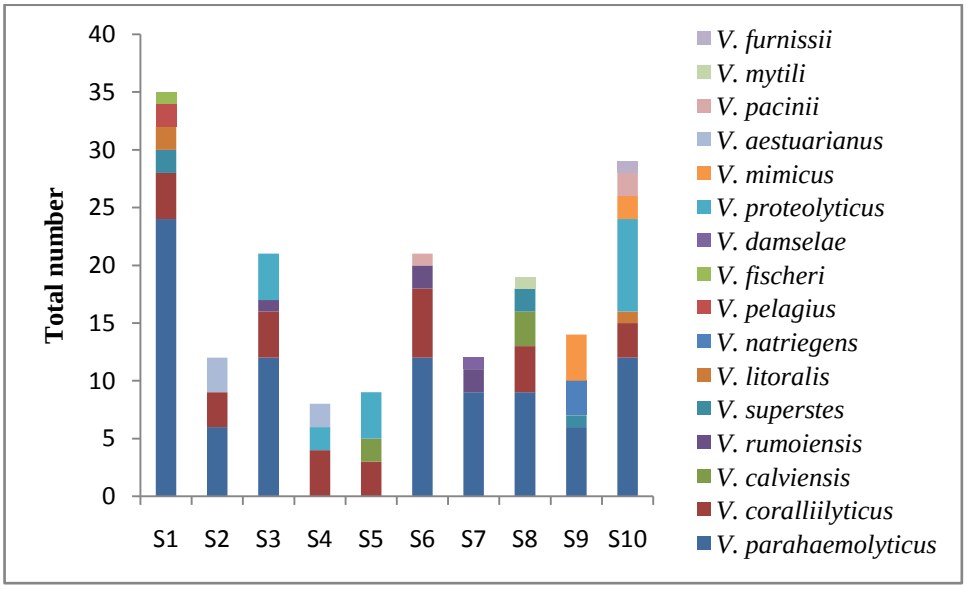

Figure 2.4 Station-wise distribution of *Vibrio* species in Cochin estuary

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.

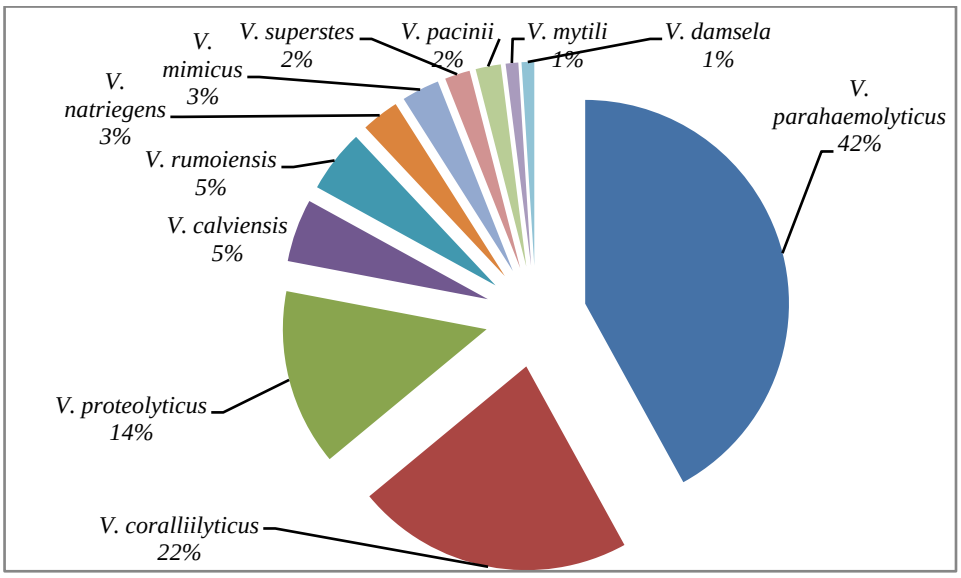

Figure 2.5: Distribution of *Vibrio* in water of Cochin estuary

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.

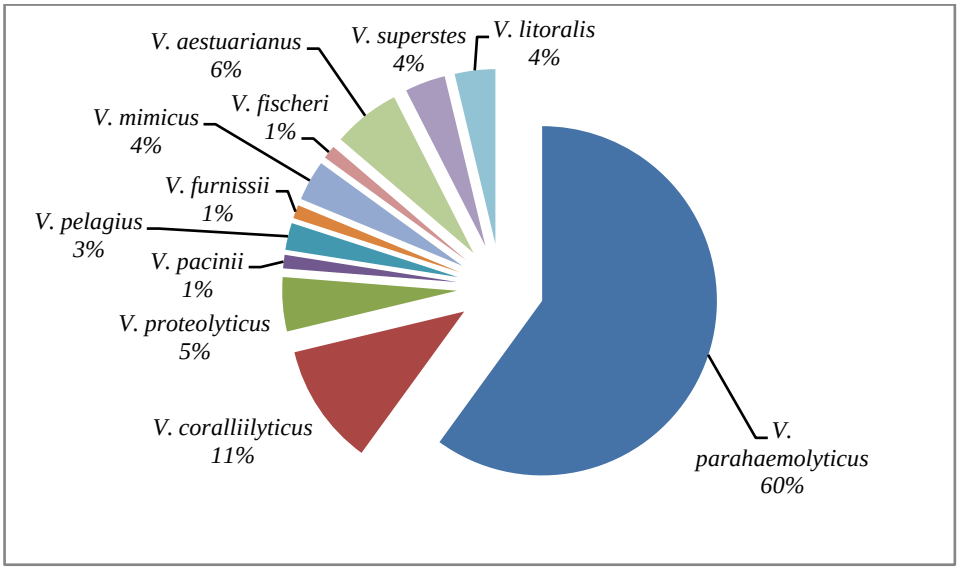

Figure 2.6 Distribution of *Vibrio* in sediment of Cochin estuary

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*.

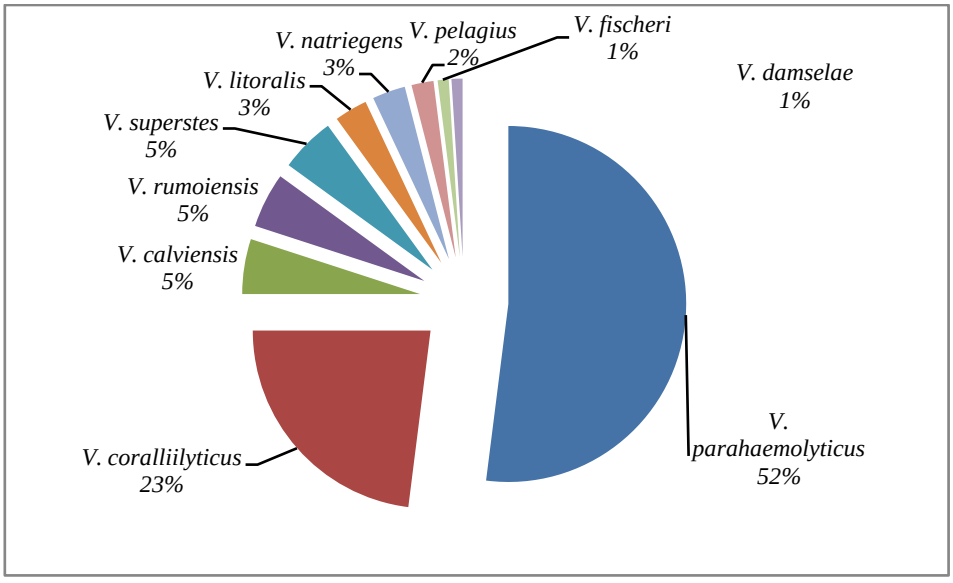

Figure 2.7 Distribution of *Vibrio* species in Cochin estuary during the pre-monsoon season

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.

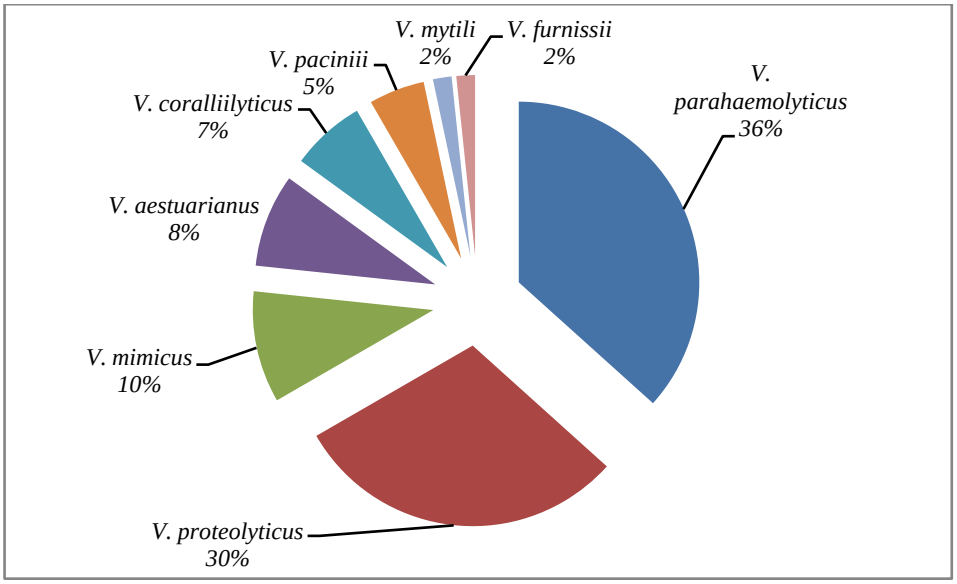

Figure 2.8 Distribution of *Vibrio* species in Cochin estuary during the monsoon season

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.

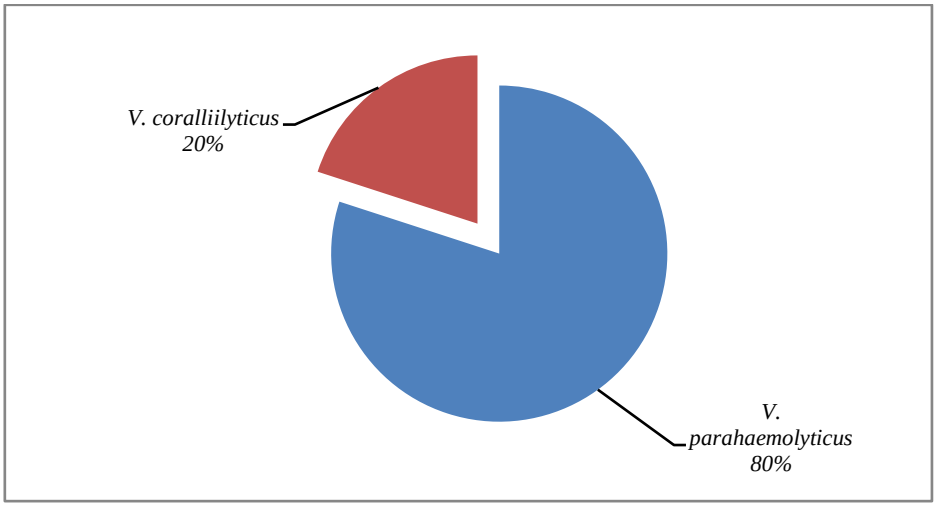

Figure 2.9 Distribution of *Vibrio* species in Cochin estuary during the post-monsoon season

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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Chapter 3

PREVALENCE OF ANTIBIOTIC RESISTANCE AND PLASMID PROFILES OF *VIBRIO* FROM FOOD AND ENVIRONMENTAL SOURCES ALONG THE SOUTH WEST COAST OF INDIA

Contents

3.1 Introduction

During the past, research has been mainly focused on antibiotic resistant bacteria in clinical environments. But, recently the rapid increase in community-acquired infections due to resistant bacteria has driven the interest to antibiotic resistance in natural environments (Martínez, 2008; Forsberg *et al.*, 2012). Natural environments are reservoirs of antibiotic resistance genes. So far, the occurence and emergence of antibiotic resistance in marine environment has been given little attention. There are three different ways through which antibiotic resistant bacteria occurs in marine environment such as surface runoff of antibiotic resistant bacteria from terrestrial sources, selection of antibiotic resistant strains due to anthropogenic antibiotic discharge and selection for antibiotic resistance as a result of antibiotic production in marine environments (Hatosy and Martiny, 2015).

The current trend of increasing antibiotic resistance is a crisis in global scale and it is a major health and economic issue. During the past few decades, there is rapid emergence in antimicrobial resistance in many bacterial genera due to the excessive use of antibiotics in humans, agriculture and aquaculture systems (Cabello, 2006). Release of sewage also results in entry of large number of drug resistant bacteria from various sources into the environment. Resistant genes are further transferred from non-pathogens to pathogens through horizontal gene transfer *via* conjugation, transduction and transformation. This could lead to transfer of drug resistance features to autochthonous microorganisms such as *Vibrio*. Thus, the search for genetic elements such as plasmids, transposons and integrons associated with antibiotic resistance in microorganisms has also become very important. Multiple drug resistance among *Vibrio* spp. in estuarine/marine environments may have further implications for those who consume seafood contaminated with these pathogenic vibrios and also for the recreational and commercial users of these environments (Shaw *et al.*, 2014).

3.2 Review of literature

3.2.1 Antibiotics and their mode of action

The first antibiotic, penicillin, was discovered by Alexander Fleming in 1928. Later, streptomycin was discovered by Selman Waksman in 1943 from *Streptomyces griseus* and in 1944 it was introduced for treatment of tuberculosis. Further, many new antibiotics were discovered. The discovery of antibiotics has revolutionized healthcare in many aspects, and since then numerous lives have been saved (Davies and Davies, 2010).

The modes of action of the antibiotics are not similar. Different antibiotics have difference mechanisms by which it inhibits the growth or kills the bacteria. Based on the mode of action, antibiotics are mainly grouped into four, which includes those that:

1) Inhibit cell wall biosynthesis
2) Inhibit protein synthesis
3) Alter nucleic acid metabolism
4) Inhibit folate metabolism

Table 3.1 Mode of action of commonly used antibiotics (adapted from Davies and Davies, 2010)

Antibiotic class	**Mode of action**	**Examples**
Beta lactams	Inhibits peptidoglycan biosynthesis	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)
Aminoglycosides	Inhibits translation	Gentamicin, amikacin, streptomycin, spectinomycin
Glycopeptides	Inhibits peptidoglycan biosynthesis	Vancomycin, teicoplanin
Macrolides	Inhibits translation	Erythromycin, azithromycin
Tetracyclines	Inhibits translation	Minocycline, tigecycline, oxytetracycline
Phenicols	Inhibits translation	Chloramphenicol
Quinolones	Affects nucleic acid metabolism	Ciprofloxacin, nalidixic acid
Pyrimidines	Inhibition of C1 metabolic pathways	Trimethoprim
Sulfonamides	Inhibition of C1 metabolic pathways	Sulfamethoxazole
Cationic peptides	Disrupts cell membrane	Colistin

All beta-lactam antibiotics are bactericidal and disrupt the bacterial cell wall synthesis by inhibiting the peptidoglycan synthesis. Sulphonamides competitively bind to dihydropteroate synthase (DHPS), an enzyme in the folic acid biosynthesis pathway, and inhibit the formation of dihydrofolicacid (Sköld, 2000). Trimethoprim competitively inhibits the dihydrofolate reductase (DHFR) enzyme and interrupts the folic acid pathway thereby disrupting the nucleic acid synthesis (Davies and Davies, 2010). The drug binds to the dihydrofolic acid reductase enzyme which is involved in the synthesis of folic acid. Aminoglycosides and tetracyclines bind to the aminoacyl site of 16S ribosomal RNA (rRNA) within the 30S ribosomal subunit and inhibits protein synthesis pathway. Macrolides on the other hand binds to the 50S ribosomal subunit to inhibit protein synthesis (Davies and Davies, 2010). Quinolones act by inhibiting the activities of DNA gyrase and topoisomerase IV. The initial target site of action for colistin antibiotic is the bacterial cell membrane. Colistin binds to lipopolysaccharides and phospholipids in the outer cell membrane of Gram-negative bacteria (Falagas and Kasiakou, 2005). It displaces the divalent cations (Ca^{2+} and Mg^{2+}) from the phosphate groups of membrane lipids and ultimately disrupts the bacterial outer cell membrane and leads to bacterial death (Landman *et al.*, 2008).

3.2.2 Antibiotic resistance mechanism in bacteria

Antimicrobial resistance is the ability of a microbe to survive and multiply in the presence of an antimicrobial agent that normally inhibits or kill the particular microorganism. Any use of antimicrobials, contributes to the emergence of antibiotic resistance. Its widespread unnecessary and

indiscriminate use makes the situation worse. Bacteria and other microbes evolve rapidly to resist to newly developed drugs. The pace at which novel antibiotics are being discovered has slowed drastically, whereas the antibiotic use is rising rapidly.

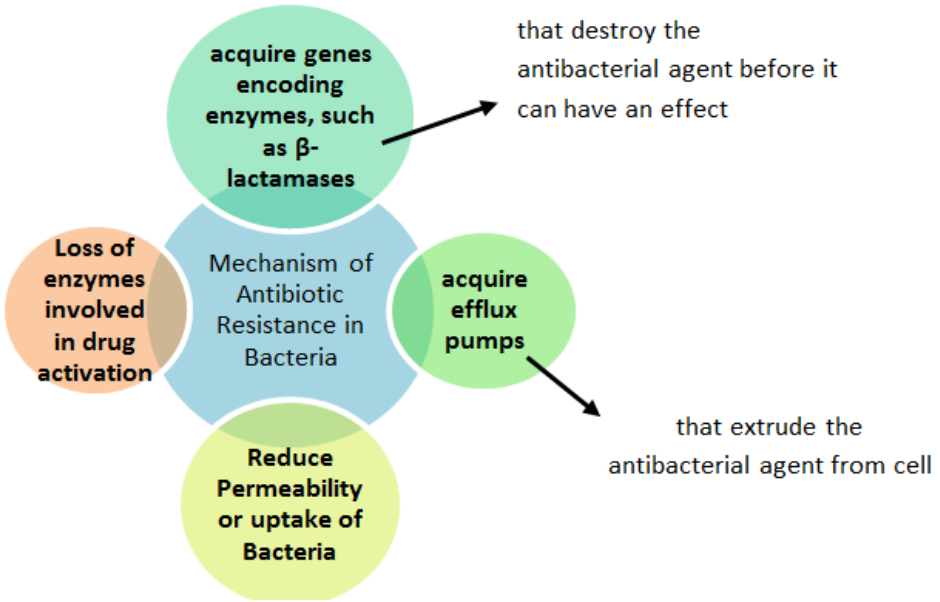


Figure 3.1 Mechanism of antibiotic resistance (adapted from Verma and Rawat, 2014)

There are four main mechanism of bacterial resistance to antibiotics: (1) enzymatic degradation of drugs, (2) alteration of bacterial proteins that are targets for the antimicrobial agent, (3) changes in cell membrane permeability to antibiotics and (4) by expulsion of the antimicrobial agents from the cell through bacterial efflux pumps (Dever and Dermody, 1991). A bacterium attains antibiotic resistance through intrinsic or acquired mechanisms. Intrinsic resistance is the innate ability of bacteria to resist antibiotics and is attained by naturally occurring genes

found on the bacterial genome. Acquired resistance is through mutations in genes targeted by the antibiotic and by transfer of resistance determinants through transformation, transduction or conjugation (Levy and Marshall, 2004; Alekshun and Levy, 2007).

Bacteria resist the action of β-lactam antibiotics by producing β-lactamases (penicillinases), which hydrolyze the β-lactam ring and inactivates the antibiotic (Davies and Davies, 2010). Bacterial resistance to aminoglycosides is accomplished by acetyltransferases, phosphotransferases and nucleotidyltransferases which modifies the aminoglycosides (Alekshun and Levy, 2007; Zhang *et al.*, 2009). Quinolones are resisted by bacteria by expelling the antibiotic through the efflux proteins, by mutation in the quinolone target molecule (DNA gyrase) and also by blocking the entry of quinolones (Fonseca *et al.*, 2008). Mutations in the trimethoprim target dihydrofolate reductase enzyme are the main mechanism through which bacteria acquire very high-level trimethoprim resistance (Skold, 2001). Other mode of resistance to pyrimidine antibiotics is through efflux proteins and development of permeability barriers. Resistance to sulphonamides is primarily attributed to possession of mutated chromosomal dihydropteroate synthase (DHPS) gene (Enne *et al.*, 2001; Davies and Davies, 2010). Tetracycline resistance occurs by monooxygenation, efflux pumps and modifying target molecule (Chopra and Roberts, 2001). The expulsion of chloramphenicol by chloramphenicol specific efflux proteins (Davies and Davies, 2010) and production of chloramphenicol acetyltransferases (Alekshun and Levy, 2007; Dang *et al.*, 2008) are the major resistance mechanisms adopted by chloramphenicol resistant bacteria. Glycopeptide resistance is achieved through reprogramming the

peptidoglycan synthesis and production of a modified peptidoglycan (Courvalin, 2006). Macrolide resistant bacteria have enzymes that modifies the RNA and protects the ribosome by preventing the entry of the antibiotic (Alekshun and Levy, 2007; Aminov and Mackie, 2007).

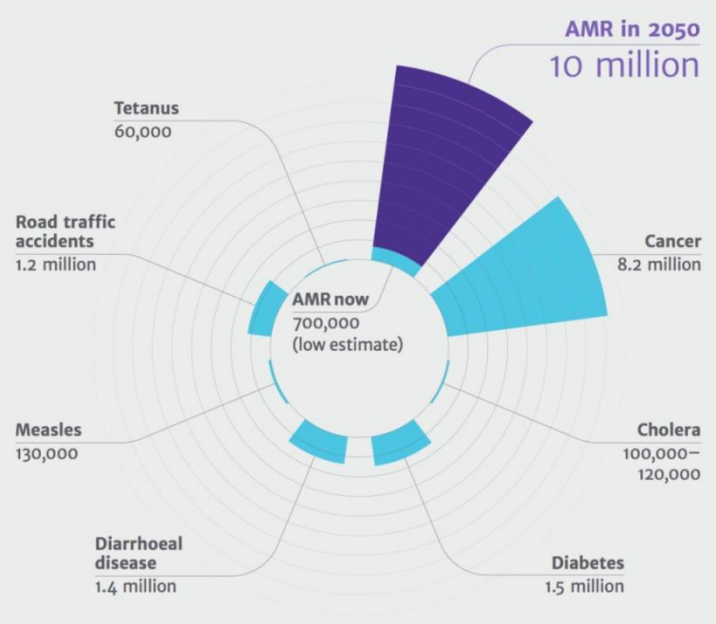


Figure 3.2 Deaths caused due to antimicrobial resistance infections every year compared to other major causes of death **(Adapted from 'The Review on Antimicrobial Resistance' by UK government, December 2014)**

Above figure shows a recent report published by the UK government. It reveals an alarming prediction. They report that by 2050, antimicrobial resistant infections could kill 10 million people across the world which is more than the current death toll from cancer.

3.2.3 Spread of antibiotic resistance genes among bacteria

The spread of antibiotic resistance genes from environment to pathogenic bacteria is a global issue. Natural environment is known to be a major reservoir of potential antibiotic resistant genes (ARGs) and plays a pivotal role in the dissemination of these genes (Hatosy and Martiny, 2015). The presence of antibiotics in the environment due to misuse and overuse of antibiotics creates a selective pressure for the emergence and transmission of these antibiotic resistance genes in mutants (Canton, 2009). The pathogenic bacteria acquire the ARGs from environmental gene pool through horizontal gene transfer processes such as transduction, transformation and conjugation. Natural water bodies are the hotspots for horizontal gene transfer (HGT) of ARGs between environmental bacteria and the human and animal pathogens. This can lead to serious public health issue complicating the disease treatment (Igbinosa and Odjadjar, 2015).

Conjugative transfer of ARGs was initially discovered in 1950s (Davies and Davies, 2010). It was then the first plasmids were isolated and plasmid-mediated antibiotic resistance was discovered. Plasmids and mobile DNA elements belonging to the class integrative conjugative elements are the transferable genetic elements. For example *V. cholerae* acquires ARGs from intrinsically resistant environmental bacteria through these mobile genetic elements (Martinez, 2008). It further transfers these ARGs with other commensals or other pathogens in the human gut, thereby making the treatment of many infections complicated (Sedas, 2007). ARG transfer is possible even between phylogenetically distant

organisms like species of Gram-positive and Gram-negative bacteria (Kruse and Sørum, 1994). Among various bacteria, *Escherichia coli* have been recognized as a major carrier of antibiotic resistance genes (Zhao and Dang, 2012). Larger plasmids usually harbour a number of mobile genetic elements (MGEs) like IS elements, transposons, integrons, gene cassettes and conjugative transposons (Osborn *et al.*, 2000; Toussaint and Merlin, 2002). These accessory elements give plasmids a selective advantage in transferring ARGs.

Plasmid-mediated antibiotic resistance is causing great trouble in the treatment of infectious diseases. Curing or elimination of plasmids from bacterial strains is a way to determine the antibiotic resistance mediation. Curing occurs either naturally through cell division or through any physical or chemical agent (Elias *et al.*, 2013). Chemical agents such as acridine orange (AO) and ethidium bromide (intercalating agents) and sodium dodecyl sulfate (anionic detergent) are widely used for plasmid curing (Molina-Aja *et al*., 2002; Manjusha and Sarita, 2011; Costa *et al.*, 2014; Yano *et al.*, 2014; Letchumanan *et al.*, 2015a). Intercalating agents are known to eliminate plasmids by inhibition of plasmid replication. In a study by Reboucas *et al.* (2011), 0.2 mg/ml AO was used to cure plasmid from *Vibrio* spp. from shrimps. Oxytetracycline resistance was lost in many isolates after curing, indicating plasmid mediated resistance to oxytetracycline. All the isolates exhibited resistance to ampicillin even after curing, indicating it to be chromosomal mediated. In another study, penicillin G, ampicillin and aztreonam resistant *Vibrio* isolates were subjected to plasmid curing with 0.1 mg/ml of AO. After the curing treatment, 11 resistant isolates became susceptible to the antibiotics

(Costa *et al.,* 2014). In a study on antibiotic resistant *Vibrio* spp. from shrimps in Thailand, ethidium bromide (0.2 mg/ml) was used to eliminate plasmids. Results suggested that oxytetracycline resistance in the isolates was lost after the plasmid curing (Yano *et al.,* 2014). In a study from India, sodium dodecyl sulfate was used to cure plasmids from *V. parahaemolyticus* strains. The results concluded all strains were resistant to the antibiotics even after curing, thus revealing the resistance of *V. parahaemolyticus* isolates to be chromosomal borne (Devi *et al.,* 2009). Among the physical agents, elevated growth temperature is most commonly used in *Vibrio* plasmid curing (Letchumanan *et al.,* 2015b). Incubation of bacteria at higher temperature leads to complete or partial deletion of plasmid DNA (Letchumanan *et al.,* 2015 b).

Uptake of naked extracellular DNA from environment is achieved through transformation. In a study from China it was found that extracellular DNA carrying ARGs was more abundant than intracellular DNA. This revealed that extracellular DNA is also an important environmental reservoir for ARGs that can be transferred through transformation (Mao *et al.,* 2014). Transduction or the DNA transfer between bacteria *via* bacteriophages also plays an important role in the spread of antibiotic resistance genes in the environment (Muniesa *et al.,* 2013). Bacteriophages have higher survival in water bodies than inside the host body, which makes them suitable for dissemination of ARGs among bacteria (Duran *et al.,* 2002). Through metagenomic studies, bacteriophages carrying beta-lactamase genes and methicillin-resistance gene (*mecA*) have been detected in activated sludge, urban sewage and wastewater treatment plants (Colomer-Lluch *et al.,* 2011; Rolain *et al.,*

2012). Recently, high levels of beta-lactam antibiotic and fluoroquinolone resistance genes were detected in phage DNA from hospital and urban treated effluents using qPCR assays (Marti *et al.*, 2014).

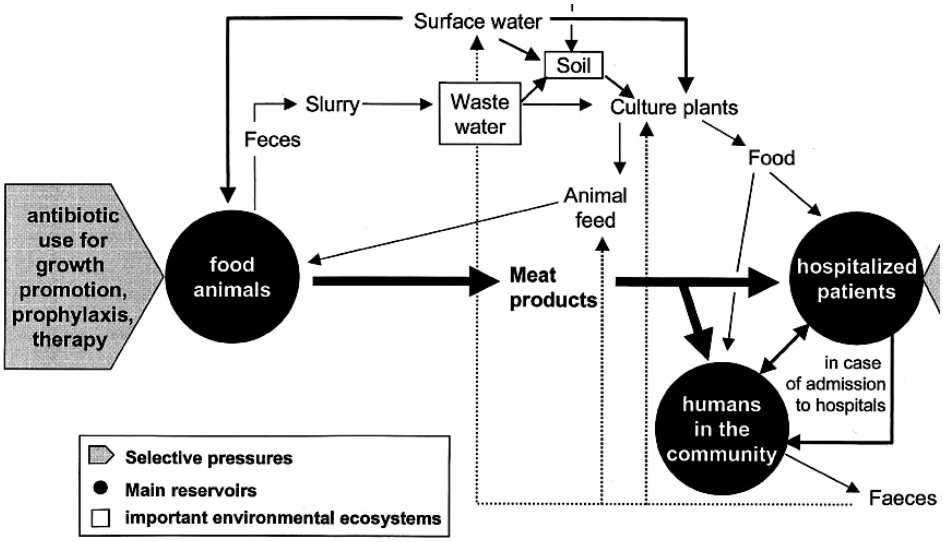


Figure 3.3 Routes of transmission of antibiotic resistance genes (adapted from Witte, 2000)

There are about 38 different tetracycline resistance (*tet*) genes and three oxytetracycline resistance (*otr*) genes which are characterised (Roberts, 2005; Thompson *et al.*, 2007). Many of the tetracycline resistance genes are found either on non-mobile plasmids or incomplete transposons in the chromosome (Roberts, 2005). The *aac*, *aph* and *ant* genes encoding acetyltransferases, phosphotransferases and nucleotidyltransferases respectively are the major genes involved in aminoglycoside resistance (Chandrakanth *et al.*, 2008). Sulfonamide and trimethoprim resistance are usually encoded by *sul* and *dfr* genes (Skold 2000, 2001). The environmental reservoir of these genes include various wastewater treatment plants (da

Silva *et al.,* 2007; Moura *et al.,* 2007), river water (Mohapatra *et al.,* 2008) and the aquaculture ponds (Jacobs and Chenia, 2007). The *bla* gene encoding the beta- lactamase confers beta-lactam resistance in bacteria. Currently, nearly 400 different β-lactamases encoded by different *bla* genes have been characterised (Li *et al.,* 2007). The *bla* gene is often reported in environment from animal derived pathogens such as *Vibrio, Aeromonas, Enterobacter, Salmonella* etc. (Volkmann *et al.,* 2004; Taviani *et al.,* 2008; Zhang *et al.,* 2009). Reports show that different types of genetic elements are involved in the transfer of $bla_{CTX\text{-}M}$ genes (Bou *et al.,* 2002; Chanawong *et al.,* 2002; Saladin *et al.,* 2002). Among them ISEcp1-like insertion sequences are most common (Lartigue *et al.,* 2004). The *erm* gene confers resistance to macrolide, lincosamide and streptogramin antibiotics (Roberts, 2008). The *erm* gene can be easily spread from one host to another as they are usually located on plasmids (Liu *et al.,* 2007) and transposons (Okitsu *et al.,* 2005).

3.2.4 Antibiotic use in India

Van Boeckel *et al.* (2014) conducted a survey on the global antibiotic consumption from 2000-2010. Their study revealed an alarming report that, India is the largest consumer of antibiotics in the world in 2010 followed by China. Figure 3.4 gives a picture about the consumption of antibiotics in India from 2000 to 2010. Overall the consumption of cephalosporins, quinolones and broad spectrum penicillins is very high and has been increasing considerably over the years. The carbapenem resistance in *E. coli* isolates increased from 10% to 13% and in the *Klebsiella pneumoniae* isolates fluoroquinolone resistance increased from

57% to 73% (CDDEP, 2015). High population density and lack of proper public health measures are the main reasons for rapid spread of antibiotic resistant pathogens in India (Laxminarayan and Chaudhury, 2016).

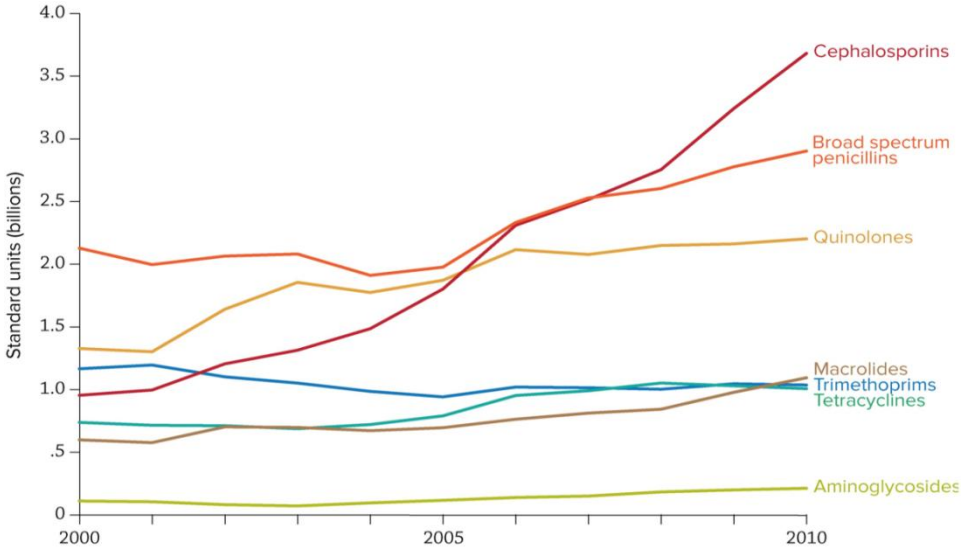


Figure 3.4 Trends in antibiotic consumption in India, 2000–2010 (Retrieved from Laxminarayan and Chaudhury, 2016)

In order to prevent or regulate the over-the-counter sale of antibiotics in India, the Central Drugs Standard Control Organization (CDSCO) included 24 important antibiotics under Schedule H1 in 2014 (Laxminarayan and Chaudhury, 2016). These antibiotics could be sold only with a valid prescription from a registered medical practitioner.

In India, antibiotics are not only overused/misused in clinical sectors. It is also exploited indiscriminately in various animal production sectors also. Recent study revealed presence of high load of antibiotic residues in poultry meat and milk samples (Kakkar and Rogawski, 2013).

3.2.5 Antibiotic resistance in *Vibrio*

Vibrios are generally known to be highly susceptible to most of the clinically used antibiotics (Shaw *et al.,* 2014). Commonly used antibiotics for treatment for *Vibrio* infections include cephalothin, cefuroxime, cefotaxime, ceftazidime, tetracycline, doxycycline, fluoroquinolone, amikacin, gentamicin and trimethoprim-sulfamethoxazole (Al-Othrubi *et al.,* 2014; Letchumanan *et al.,* 2015a).

The first report on antimicrobial resistant *V. cholerae* was from Tanzania (Mhalu *et al.,* 1979) and later from Bangladesh (Glass *et al.,* 1980). French *et al.* (1989) studied antibiotic susceptibility of 244 halophilic vibrios isolated from Hong Kong and reported most of the strains to be resistant to sulphamethoxazole, trimethoprim, penicillins and older cephalosporins. In Africa, the *V. cholerae O1* strain that caused 1996-97 epidemic, carried a conjugative multiple-resistance plasmid with class 1 integrons that encoded resistance to trimethoprim and aminoglycosides (Dalsgaard *et al.,* 2000).

Integrating conjugative elements (ICE) carry many ARGs and the first *V. cholerae* ICE was described in 1992 in a *V. cholerae* O139 isolate from Madras, India (Waldor *et al.,* 1996). Presence of class 1 integrons carrying antibiotic resistance genes was also reported in *V. cholerae* non-O1 and O139 strains from India (Thungapathra *et al.,* 2002).

There are numerous studies on antibiotic susceptibility of *Vibrio* from India. Devi *et al.* (2009) tested the antibiotic susceptibility of *V. parahaemolyticus* isolated from shrimp farms along the southwest

coast of India and showed that more than 50% of the strains were sensitive to chlortetracycline, chloramphenicol and nitrofurantoin. In another similar study, potentially pathogenic *Vibrio* strains isolated from retail markets in Cochin, India were tested for their susceptibility to various antibiotics (Sudha *et al.,* 2014). A high incidence of resistance was exhibited by the strains towards ampicillin, colistin, cephalothin, amoxycillin, carbenicillin and ceftazidime. In another study by Vaseeharan *et al.* (2005), *Vibrio* strains isolated from shrimp culture hatcheries and ponds in India were highly resistant to ampicillin, furazolidone, neomycin B and penicillin G. Multiple antibiotic resistant *Vibrio* spp. were isolated from Palk Bay, India (Sneha *et al.,* 2015). Chowdhury *et al.* (2016) screened *Vibrio fluvialis* from hospitalized patients with acute diarrhoea in Kolkata, India and found 27 *Vibrio fluvialis*-harbouring $bla_{\text{NDM-1}}$ gene coding for New Delhi metallo-β-lactamase.

Lesley *et al.* (2011) studied the antibiotic resistance of *V. parahaemolyticus* isolated from cockles in Malaysia and found that all the tested strains showed resistance to streptomycin, tobramycin, carbenicillin, teicoplanin, cephalothin, clindamycin, rifampicin, sulfamethoxazole and oflaxacin. A study on the antibiotic resistance in *Vibrio* sp. isolated from seafoods collected from Persian gulf, revealed all the strains to be multiple drug resistant (Raissy *et al.,* 2012a). Ottaviani *et al.* (2013) reported high antibiotic resistance in *V. parahaemolyticus* isolated from wild shrimps in Italy. The strains exhibited resistance to amoxicillin, ampicillin, cefalexin, colistin, erythromycin, cefalothin and streptomycin. Costa *et al.* (2014) reported that *V. parahaemolyticus* isolates from shrimps were resistant to penicillin, tetracycline, ampicillin and cephalothin.

V. parahaemolyticus strains isolated from shrimps in Louisiana Gulf in Mexico were resistant to ampicillin, oxytetracycline and tetracycline (Han *et al.,* 2007). A recent study on antibiotic susceptibility profile of *Vibrio cholerae* isolated from catfish in Malaysia showed that all isolates were multiple antibiotic resistant (Norshafawati *et al.,* 2017).

3.3 Objectives of the study

1) To determine the prevalence of antibiotic resistance in vibrios isolated from the Cochin estuary, pokkali cum shrimp farm and seafood collected from retail markets.
2) To evaluate the extent of multiple antibiotic resistance (MAR) and to delineate the resistance profiles.
3) To screen the vibrios for the presence of antibiotic resistance genes.
4) To screen the antibiotic resistant vibrios for presence of plasmids.
5) To carry out plasmid curing experiments to ascertain any possible plasmid-mediated antibiotic resistance.

3.4 Materials and Methods

A total of 280 *Vibrio* strains were subjected to antibiotic sensitivity screening. This included 180 isolates from Cochin estuary, 70 from pokkali cum shrimp farm and 30 from seafood collected from retail markets.

3.4.1 Antibiotic sensitivity test

The antibiotic sensitivity of the strains were analysed and compared using the disc diffusion method (Bauer *et al.,* 1966).Twenty-five different

antibiotic discs (Himedia, India) of 8-mm diameter were used for the test. The antibiotics belonged to eleven different classes according to their chemical structure. The concentration of each antibiotic used, their class and abbreviations are given in Table 3.2.

Table 3.2 Details of the antibiotics used in the study

Antibiotic class	**Antibiotics used**	**Abbreviations**	**Concentration (mcg)**
Aminoglycosides	Amikacin	Ak	30
	Streptomycin	S	10
	Gentamicin	Gen	10
	Netillin	Net	10
Beta-lactams	Amoxycillin	Amx	10
	Ampicillin	Amp	10
	Carbenicillin	Cb	100
	Cephalothin	Cep	30
	Ceftriaxone	Ctr	30
	Ceftazidime	Caz	30
Fluoroquinolones	Ciprofloxacin	Cip	5
	Enrofloxacin	Ex	5
	Norfloxacin	Nx	10
Folate pathway inhibitors	Trimethoprim	Tr	5
Macrolides	Erythromycin	E	15
Nitrofurans	Furazolidone	Fr	50
	Nitrofurantoin	Nit	100
Polymyxins	Colistin	Cl	10
Quinolones	Nalidixic acid	Na	30
Sulfonamides	Cotrimoxasole	Cot	25
	Sulphamethoxazole	Sm	100
Tetracyclines	Doxycycline hydrochloride	Do	30
	Oxytetracycline	O	30
	Tetracycline	Te	30
Miscellaneous class	Chloramphenicol	C	30

Discs containing the following antibacterial agents were placed on the plates swabbed with enriched bacterial culture and incubated over-night at 37 °C. After incubation, the diameter of the zone of inhibition was measured and the results were interpreted based on recommendations of Clinical Laboratory Standards Institute (CLSI, 2012).

3.4.2 MAR indexing

Isolates that are resistant to 3 or more antibiotics were grouped as multiple antibiotic resistant. Multiple antibiotic resistance (MAR) indexing of the isolates was determined by calculating the ratio between the number of antibiotics to which an isolate is resistant and the total number of antibiotics to which the isolate was exposed (Krumperman, 1983).

3.4.3 Detection of beta-lactam antibiotic resistance genes

3.4.3.1 DNA isolation

Refer section 2.4.7.1

3.4.3.2 Detection of *bla*$_{TEM}$ gene

Primers used were forward 5' GAGTATTCAACATTTTCGT 3' and reverse 5' ACCAATGCTTAATCAGTGA 3' (Marynard *et al.*, 2003). 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). PCR condition included 1 cycle of initial denaturation for 5 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 1.5 min; 1 cycle of final extension for 5 min at 72 °C.

3.4.3.3 Detection of *bla*$_{\text{CTX-M}}$ gene

The primers used were CTX-M F (5′ CGATGTGCAGTACCAGTAA 3′) and CTX-M R (5′ TTAGTGACCAGAATCAGCGG 3′) (Batchelor *et al.*, 2005). 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). PCR conditions included an initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec, extension at 72 °C for 1.5 min; final extension at 72 °C for 5 min.

3.4.3.4 Detection of *bla*$_{\text{NDM-1}}$ gene

The primers used in this study were NDM-Fm (5′-GGTTTGGCG ATCTGGTTTTC-3′) and NDM-Rm (5′-CGGAATGGCTCATCACGATC-3′, positions), which amplified an internal fragment of 621 bp of the *bla*$_{\text{NDM-1}}$ gene (Nordmann *et al.*, 2011). 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). PCR conditions included an initial denaturation for 10 min at 94 °C; 36 cycles of amplification consisting of 30 sec at 94 °C, 40 sec at 52 °C, and 50 sec at 72 °C; and 5 min at 72 °C for the final extension.

3.4.3.5 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).

3.4.4 Plasmid profiling of the drug resistant strains

The drug resistant strains were screened for the presence of plasmids. Bacterial strains were grown in 10 ml Luria Bertani broth (Himedia, India) containing 1% sodium chloride and ampicillin and incubated overnight at 37 °C in a shaker incubator (200 rpm) (Scigenics Biotech, India) for 16–18 h. About 1.5 ml of this culture was used for plasmid extraction following the alkaline lysis method (BirnBoim and Doly, 1979). Briefly, 1.5 ml of the culture was transferred into a microfuge tube and centrifuged at 6000 rpm for 5 min. Supernatant was removed and the pellet was re-suspended in 100 µl distilled water followed by 100 µl lysis buffer (10% SDS, 0.5 M EDTA, 10 N NAOH). The tubes were kept in boiling water bath for 10 min, then 50 µl of 1 mM $MgCl_2$ was added into it in the hot condition itself. Tubes were kept in ice for 2 min, and then centrifuged at 12000 rpm for 2 min. Then 3 mM potassium acetate was added and kept for 2 min in ice and then centrifuged at 12000 rpm for 2 min. The supernatant was transferred to a new tube and 600 µl of isopropanol was added into it and kept in ice for 10 min. Tubes were then centrifuged at 10000 rpm for 10 min. Supernatant was discarded and the pellet was rinsed in ice-cold 70% ethanol at 5000 rpm for 5 min and air-dried for about 10 min to allow the ethanol to evaporate. The pellet was re-suspended in 30 µl distilled water and kept at 4 °C overnight for dissolving.

The extracted plasmids were subjected to electrophoresis in 0.8% agarose gel (Agarose, Himedia, Mumbai, India) 1% (w/v) in 1X TBE

Buffer (HiMedia, India) containing 0.5 μg/ml of ethidium bromide. Electrophoretic separation was carried out at 75 V for 1 hour and a molecular weight marker (Supercoiled DNA ladder, HiMedia, India) was included. The gels were then visualized under UV transilluminator and recorded as tiff file by using Gel Documentation System, (GelDoc EZ imager, Bio-Rad). The obtained plasmid profiles were noted.

3.4.5 Plasmid curing experiment

Plasmid curing treatments were carried out using acridine orange (Molina-Aja *et al.,* 2002). An overnight culture of plasmid containing resistant *Vibrio* strain (200 μl) was added into five different 5 ml cultures of LB broth supplemented with 0.1 mg mL^{-1} of acridine orange and incubated at 35 °C for 24 h under constant agitation. Subsequently, the plasmid cured strains were tested for the antibiogram pattern, for the antibiotics to which they were originally resistant. The resistance was considered chromosomal DNA mediated when observed after the curing procedure; otherwise, it was characterized as mediated by plasmid.

3.5 Results

3.5.1 Antibiotic resistance among *Vibrio*

3.5.1.1 Antibiotic resistance among *Vibrio* from Cochin estuary

All the *Vibrio* strains from Cochin estuary were found to be multiple drug resistant. All the strains (100%) were resistant to amoxicillin, ampicillin, cephalothin (beta-lactam) and colistin (polymyxin). A high percentage of resistance was also observed towards furazolidone and nitrofurantoin-91.6% (nitrofurans), carbenicillin-70% (beta-lactam),

sulphamethoxazole-82.8% (sulphonamide) and enrofloxacin-84.2% (fluoroquinolone). A medium-to-low level resistance was exhibited towards ceftazidime (beta-lactam), erythromycin (macrolide), cotrimoxasole (sulphonamides), trimethoprim (folate pathway inhibitors), streptomycin, gentamicin and amikacin (aminoglycoside), doxycyline hydrochloride, oxytetracycline (tetracycline), nalidixic acid (quinolone) and norfloxacin (fluoroquinolone). All the strains were sensitive to netillin (aminoglycoside), ciprofloxacin (quinolone) and ceftriaxone (beta-lactam) (Figure 3.5).

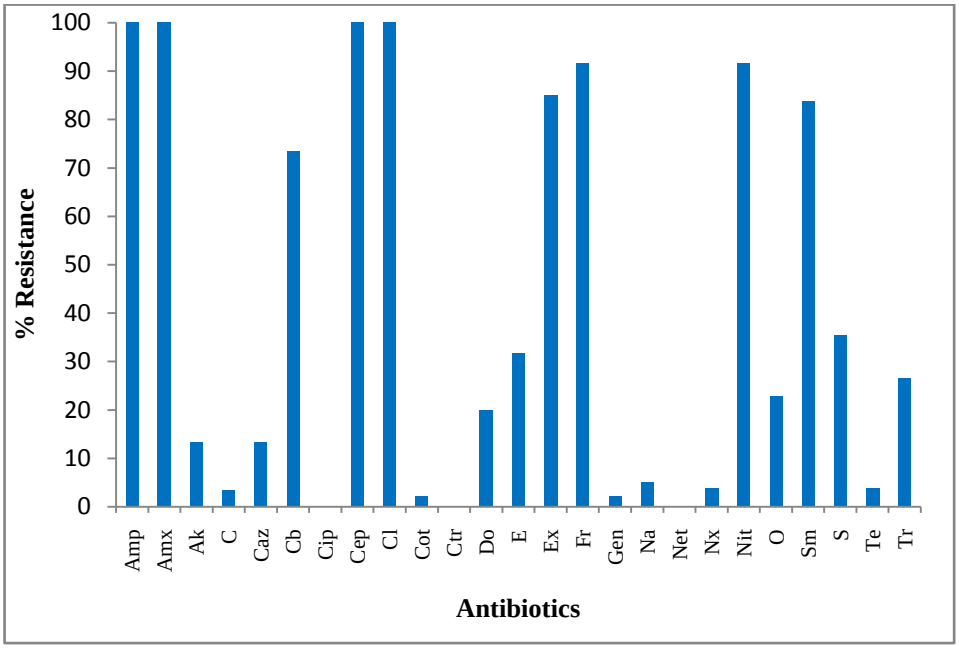


Figure 3.5 Percentage of antibiotic resistance among *Vibrio* from Cochin estuary

3.5.1.2 Antibiotic resistance among *Vibrio* from shrimp farm

All the *Vibrio* strains from shrimp farm exhibited multiple drug resistance. All the strains were resistant to cephalothin (beta-lactam).

High level of resistance was observed towards amoxicillin (88%), ampicillin (78%) and colistin (72%) (Figure 3.6). All were sensitive to ciprofloxacin, cotrimoxasole, netillin and tetracycline. Medium to low level resistance was observed towards all other tested antibiotics.

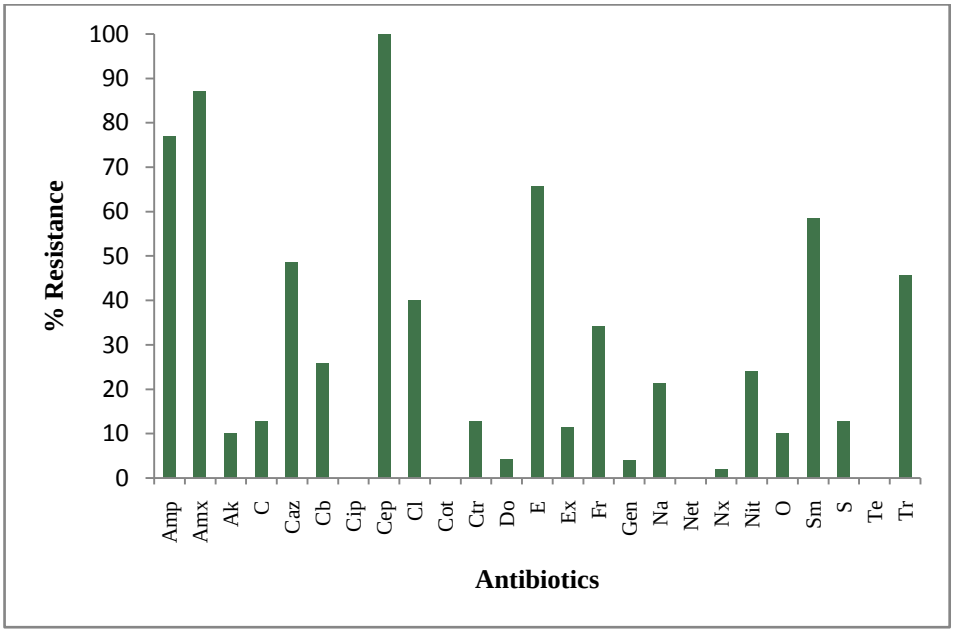


Figure 3.6 Percentage of antibiotic resistance among *Vibrio* from shrimp farm

3.5.1.3 Antibiotic resistance among *Vibrio* from seafood

All the *Vibrio* strains isolated from seafood were multiple drug resistant. All the strains were resistant to enrofloxacin, furazolidone and trimethoprim. A majority also exhibited resistance to amoxicillin (86%), ampicillin (80%), cephalothin (90%) and colistin (93%). All were sensitive to cotrimoxasole, ceftriaxone, doxycycline hydrochloride, gentamycin, nalidixic acid, netillin, norfloxacin and sulphamethoxazole (Figure 3.7).

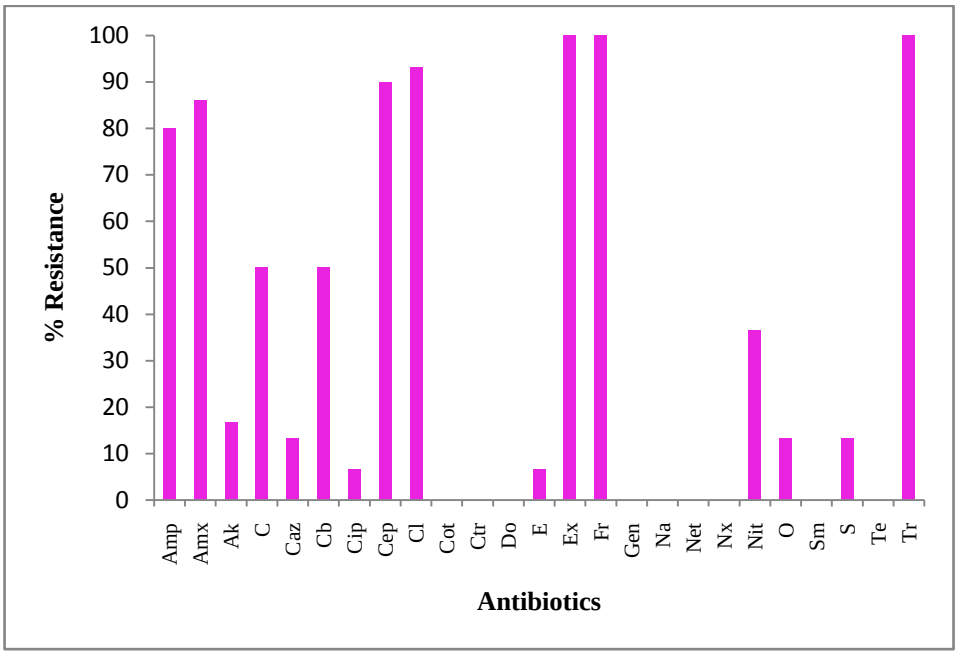


Figure 3.7 Percentage of antibiotic resistance among *Vibrio* from seafood

3.5.1.4 Relative antibiotic resistance among *Vibrio* isolated from Cochin estuary, shrimp farm and seafood

All the *Vibrio* strains from Cochin estuary, shrimp farm and seafood were multiple drug resistant. Figure 3.8 shows the relative antibiotic resistance among *Vibrio* from the three sources. Prevalence of antibiotic resistance was relatively high among the *Vibrio* isolates from Cochin estuary. Among the 25 different antibiotics tested, strains from Cochin estuary showed resistance towards 22 antibiotics. All the strains were sensitive towards netillin, ciprofloxacin and ceftriaxone. *Vibrio* strains from shrimp farm exhibited resistance towards 21 out of the 25 antibiotics tested and all exhibited sensitivity to ciprofloxacin, cotrimoxasole, netillin and tetracycline. Among the *Vibrio* from seafood, resistance was observed towards 17 out of the 25 antibiotics tested. All were sensitive to

cotrimoxasole, ceftriaxone, doxycycline hydrochloride, gentamycin, nalidixic acid, netillin, norfloxacin and sulphamethoxazole. Ciprofloxacin resistance was observed only among the *Vibrio* from seafood.

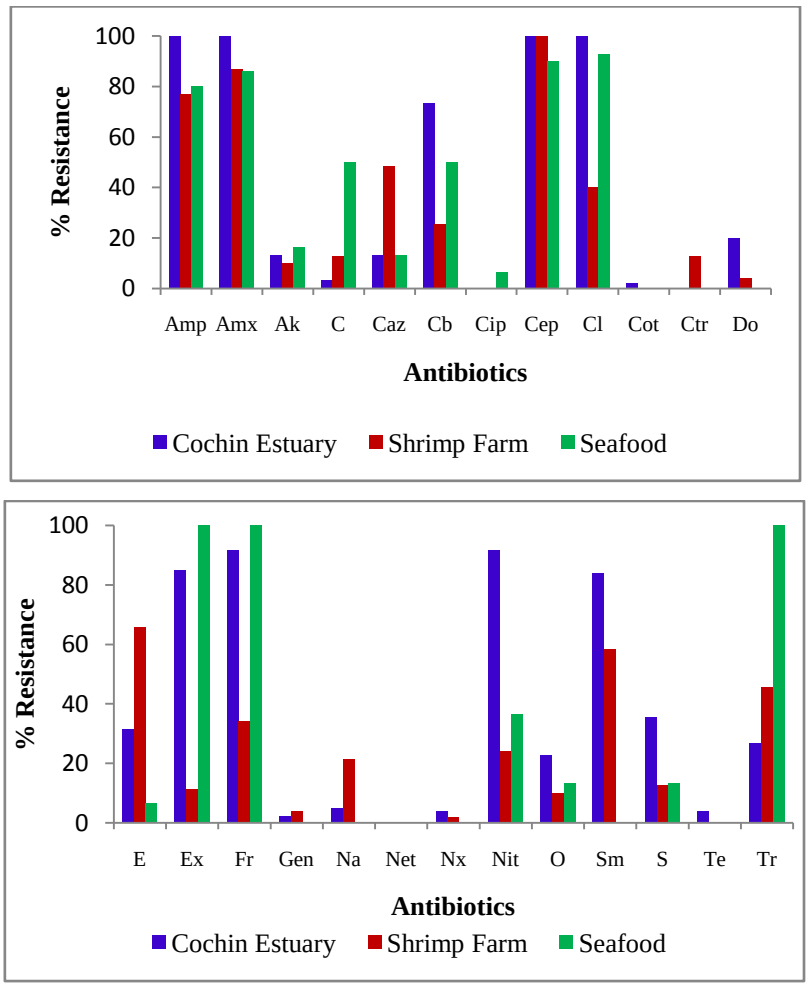


Figure 3.8 Relative antibiotic resistance among *Vibrio* isolated from Cochin estuary, shrimp farm and seafood

Resistance towards cotrimoxasole and tetracycline was exhibited by *Vibrio* from Cochin estuary alone. Similarly, ceftriaxone resistance was

observed only among *Vibrio* from seafood. Overall, *Vibrio* strains from the 3 sources showed significant difference in their resistance towards Amp ($p<0.001$), Amx ($p<0.001$), C ($p<0.001$), Caz ($p<0.001$), Cb ($p<0.01$), Cep ($p<0.01$), Cip ($p<0.01$), Cl ($p<0.001$), Ctr ($p<0.001$), Do ($p<0.001$), E ($p<0.001$), Ex ($p<0.001$), Fr ($p<0.001$), Na ($p<0.001$), Nit ($p<0.001$), O ($p<0.01$), Sm ($p<0.001$), S ($p<0.001$), Te ($p<0.05$) and Tr ($p<0.001$) (Appendix 3.1).

3.5.2 MAR indexing and antibiotic resistance pattern among *Vibrio*

The MAR index of *Vibrio* isolated from the three sources is shown in Tables 3.3 a, b, c. MAR index among *Vibrio* from Cochin estuary ranged from 0.24 to 0.6 and among the shrimp farm isolates it ranged from 0.16 to 0.48. The MAR index among *Vibrio* from seafood ranged from 0.16 to 0.44. The highest MAR index of 0.6 was observed among the *Vibrio* strains from Cochin estuary.

There was variation in the antibiotic resistance pattern among the *Vibrio* from estuary, shrimp farm and seafoods. The different antibiotic resistant patterns observed among the *Vibrio* are shown in Tables 3.3 a, b, c. A total of 34 different antibiotic resistance patterns were observed among the *Vibrio* strains from estuary. The most frequently observed pattern was Amp, Amx, Cb, Cep, Cl, Ex, Fr, Nit, Sm. A total of 35 different antibiotic resistance patterns were observed among the strains from shrimp farm. The most repeated pattern was Amp, Amx, Caz, Cb, Cep, E, Sm, Tr. Among the *Vibrio* from shrimp farm a total of 19 different antibiotic resistance patterns were observed and the pattern Amp, Amx, C, Cb, Cep, Cl, Ex, Fr, Nit, Tr was the most frequently observed.

Table 3.3(a) MAR indexing and antibiotic resistant patterns of *Vibrio* strains from Cochin estuary

MAR index	Antibiotic resistance pattern	No. of strains showing the pattern
0.24	Amp, Amx, Cep, Cl, Fr, Sm	2
0.24	Amp, Amx, Cep, Cl, Nit, Sm	2
0.28	Amp, Amx, Caz, Cep, Cl, S, Sm	2
0.32	Amp, Amx, Cep, Cl, E, Ex, Fr, Nit	7
0.32	Amp, Amx, Cb, Cep, Cl, E, S, Sm	7
0.32	Amp, Amx, Cep, Cl, Ex, Fr, Nit, Sm	11
0.32	Amp, Amx, C, Cb, Cep, Cl, Fr, Sm	2
0.32	Amp, Amx, Cep, Cl, E, Fr, Nit, Sm	2
0.32	Amp, Amx, Cb, Cep, Cl, Ex, Nit, Nx	2
0.36	Amp, Amx, Cep, Cl, Ex, Fr, Nit, O, Sm	7
0.36	Amp, Amx, Cep, Cl, Ex, Fr, Nit, O, Sm	2
0.4	Amp, Amx, Cb, Cep, Cl, Do, Ex, Fr, Nit, Sm	27
0.4	Amp, Amx, Ak, Cep, Cl, Cb, Ex, Fr, Nit, Sm	11
0.4	Amp, Amx, Cb, Cep, Cl, Ex, Fr, Nit, S, Sm	7
0.4	Amp, Amx, Ak, Cb, Cep, Cl, Ex, Fr, Nit, Tr	7
0.4	Amp, Amx, Cb, Cep, Cl, E, Ex, Fr, Gen, O, Nit, Tr, Sm	2
0.4	Amp, Amx, , Cb, Cep, Cl, Cot, E, Ex, Fr, Nit	2
0.4	Amp, Amx, Cb, Cep, Cl, E, Ex, Fr, Nit, Tr	2
0.4	Amp, Amx, Cep, Cl, Fr, Ex, Nit, Te, Tr, Sm	2
0.4	Amp, Amx, Cep, Cl, E, Ex, Fr, Nit, Tr, Sm	2
0.44	Amp, Amx, Caz, Cep, Cb, Cl, Ex, Fr, Nit, Tr, Sm	19
0.44	Amp, Amx, Cb, Cep, Cl, E, Ex, Fr, Nit, S, Sm	15
0.44	Amp, Amx, Cb, Cep, Cl, Ex, Fr, S, Nit, O, Sm	11
0.44	Amp, Amx, Cb, Cep, Cl, Do, Ex, Fr, Nit, S, Sm	7
0.44	Amp, Amx, Cb, Cep, Cl, Fr, Ex, Nit, Nx, O, Tr	2
0.44	Amp, Amx, Ak, Cb, Cep, Cl, Ex, Fr, Nit, S, Sm	2
0.44	Amp, Amx, Ak, Cep, Cl, E, Ex, Fr, Nit, Nx, O	2
0.48	Amp, Amx, Caz, Cb, Cep, Cl, Ex, Fr, Na, Nit, O, Tr	2
0.48	Amp, Amx, Cb, Cep, Cl, E, Ex, Fr, Nit, O, Tr, Sm	2
0.48	Amp, Amx, Cb, Cep, Cl, Cot, E, Ex, Fr, Nit, S, Sm	2
0.48	Amp, Amx, Ak, C, Cb, Cep, Cl, Ex, Fr, Nit, S, Sm	2
0.52	Amp, Amx, C, Cep, Cl, E, Fr, Na, Nit, O, Te, Tr, Sm	2
0.6	Amp, Amx, Cb, Cep, Cl, E, Ex, Fr, Na, Nit, O, Te, Tr, S, Sm	2
0.6	Amp, Amx, Cb, Cep, Cl, Do, E, Ex, Fr, Na, Nit, O, S, Sm, Tr	2

Table 3.3 (b) MAR indexing and antibiotic resistant patterns of *Vibrio* strains from shrimp farm

MAR index	Antibiotic resistance pattern	No. of strains showing the pattern
0.16	Cep, Cl, Fr, Sm	1
0.16	Amp, Amx, Cep, Na	8
0.2	Amp, Amx, E, Cep, Do	1
0.2	Amp, Amx, E, Cep, Tr	1
0.2	Amp, Amx, Cep, E, Tr	1
0.2	Cep, Cl, Fr, Nit, Sm	5
0.24	Amp, Amx, Caz, Cep, Cl, Ctr	3
0.24	Ak, Cep, Do, Ex, S, Tr	1
0.24	Cep, Do, E, Gen, S, Tr	2
0.24	Amp, Amx, Cep, E, Na, Sm	1
0.24	Amp, Amx, Cep, E, Na, Nx	1
0.24	Amp, Amx, Ak, Cep, E, Tr	1
0.24	Cep, Ex, Fr, Gen, Sm, Tr	1
0.28	Amp, Amx, Caz, Cb, Cep, Cl, Ctr	1
0.28	Amx, Caz, Cb, Cep, Cl, Do, Tr	1
0.28	Ak, Amx, Cep, Cl, Fr, Nit, Tr	1
0.28	Amp, Amx, Caz, Cb, Cep, E, Fr	1
0.28	Amp, Amx, Caz, Cb, Cep, E, Sm	1
0.28	Amp, Amx, Cep, E, Ex, Fr, Nit	1
0.28	Amp, Amx, Cep, Ex, Fr, O, Nit	1
0.28	Amp, Amx, Cep, E, Fr, Nit, Sm	1
0.32	Amx, C, Caz, E, Nit, S, Sm, Tr	2
0.32	Amp, Amx, Ak, Cep, E, Fr, Nit, Sm	1
0.32	Amp, Ak, Amx, Cb, Cep, E, Fr, Sm	1
0.32	Amp, Amx, Caz, Cep, Cl, E, Fr, Sm	4
0.32	Amp, Amx, Cb, Cep, E, Ex, Na, Tr	1
0.32	Amp, Ak, Amx, Cep, E, Ex, Fr, Sm	1
0.32	Amp, Amx, Caz, Cb, Cep, E, Sm, Tr	10
0.36	Amp, Amx, C, Caz, Cep, Cl, Ctr, E, Sm	4
0.36	Amp, Amx, Caz, Cep, Cl, E, Na, O, Tr	4
0.36	Amx, Nx, Cb, Cep, E, Fr, O, Sm, Tr	1
0.4	Amx, Ak, Cep, Sm, Nit, E, Ex, O, Fr, Tr	1
0.4	Amp, Amx, Cb, Cep, Cl, Ctr, E, Fr, Sm, Tr	1
0.44	Amp, Amx, Cb, Cep, Ex, Fr, Nit, O , S, Sm, Tr,	1
0.48	Amp, Amx, C, Caz, Cep, Cl, E, Fr, Nit, S, Sm, Tr	3

Table 3.3(c) MAR indexing and antibiotic resistant patterns of *Vibrio* strains from seafood

MAR index	Antibiotic resistance pattern	No. of strains showing the pattern
0.16	Cl, Ex, Fr, Nit	2
0.16	Caz, Ex, Fr, Tr	1
0.2	Amp, Amx, Caz, Cep, Cl	1
0.2	Amp, Amx, Cb, Cep, Cl	1
0.24	Amp, Amx, Ex, Cl, Cep, Nit	1
0.28	Amp, Amx, Cb, Cep, Cl, Ex, Nit	2
0.32	Amp, Amx, Cep, Cl, Ex, Fr, Nit, Tr	2
0.32	Amp, Amx, C, Cep, Cl, Ex, Fr, O	1
0.32	Amp, Amx, C, Cep, Cip, Cl, Ex, Fr	2
0.32	Amp, Amx, Ak, Cep, Cl, Ex, Fr, Tr	2
0.32	Amp, Amx, Cb, Cep, Cl, Ex, Fr, O	1
0.36	Amp, Amx, Ak, Cb, Cep, Cl, Ex, Fr, Tr	1
0.36	Amp, Amx, Caz, C, Cb, Cep, Ex, Fr, S	1
0.36	Ak, C, Cep, Cl, Ex, Fr, Nit, O, Tr	1
0.36	Amp, C, Cep, Cl, E, Ex, Fr, S, Tr	2
0.36	Amp, Amx, C, Cb, Cep, Cl, Ex, Fr, Tr	2
0.4	Amp, Amx, C, Cb, Cep, Cl, Ex, Fr, Nit, Tr	5
0.4	Amp, Amx, C, Cb, Cep, Cl, Ex, Fr, O, Tr	1
0.44	Amp, Amx, C, Caz, Cb, Cep, Cl, Ex, Fr, Tr, S	1

3.5.3 Distribution of antibiotic resistance genes in *Vibrio* from Cochin estuary, shrimp farm and seafood

All the *Vibrio* strains from Cochin estuary, shrimp pond and seafoods were further screened for the presence of antibiotic resistance genes. Since majority of the strains exhibited resistance towards beta-lactam antibiotics they were further screened for the presence of beta-lactam antibiotic resistance genes such as bla_{TEM}, bla_{CTXm} and $bla_{\text{NDM-1}}$ genes.

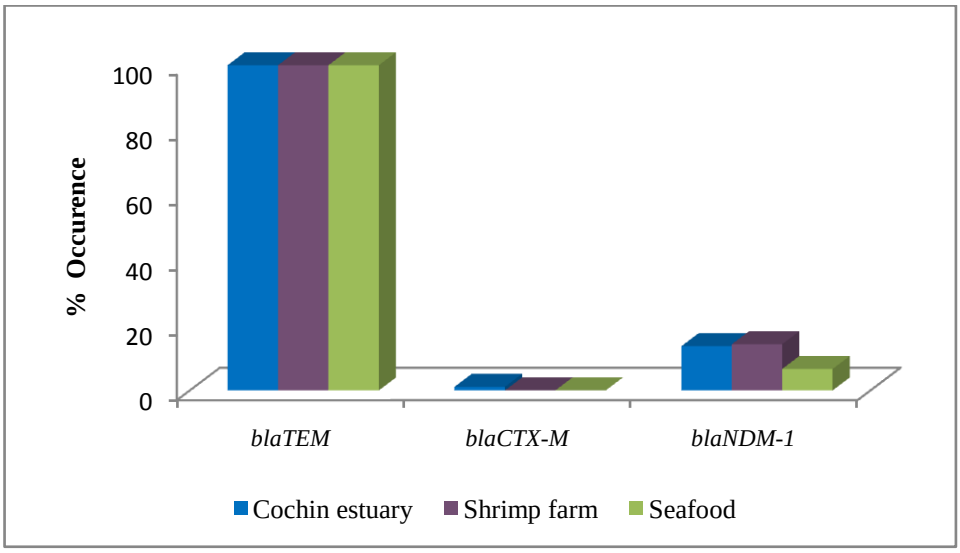
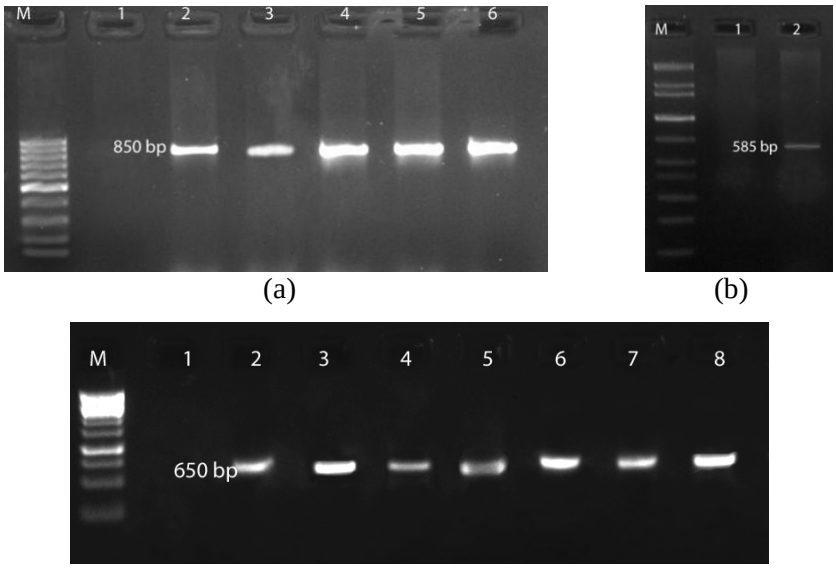


Figure 3.9 Percentage distribution of antibiotic resistance genes in *Vibrio* from Cochin estuary, shrimp farm and seafood

Figure 3.9 shows the percentage distribution of antibiotic resistance genes in *Vibrio* from three sources. All the *Vibrio* isolated from the three sources harboured the bla_{TEM} gene. The bla_{CTX-M} gene was present in 1.1% of strains from Cochin estuary. None of the strains from seafood and shrimp farm harboured bla_{CTX-M} gene. New Delhi metallo-beta lactamase (bla_{NDM-1}) gene was present in 13.3% of strains from Cochin estuary, 6.6% from seafood and 14.2% strains from shrimp farm. Figure 3.10 displays the agarose gel images of PCR amplified $bla_{TEM,}$ bla_{CTX-M} and bla_{NDM-1} genes.



(a) (b)

(c)

Figure 3.10 Agarose gel image showing PCR amplified (a) bla_{TEM} Lane M: 100 bp DNA ladder; lane 1: negative control; lanes 2-6 *Vibrio* strains (b) bla_{CTX-M} lane M: 100 bp DNA ladder; lane 1 negative control; lane 2 *Vibrio* strain (c) bla_{NDM-1} gene Lane M: 200 bp to 10 kb ladder; lane 1: negative control; lanes 2-8: *Vibrio* strains.

3.5.4 Plasmid profiles among *Vibrio* from Cochin estuary, shrimp farms and seafood

Plasmid profiling revealed the presence of plasmids in 58 *Vibrio* strains. The size of plasmids ranged from 0.5 to 33 kb. Plasmids were present in 30 strains (16.6%) from Cochin estuary, 23 strains (32.8%) from shrimp farm and 5 strains (16.6%) from seafoods. Plasmid of size 33 kb was the most frequently encountered.

Among the 30 *Vibrio* strains from Cochin estuary that carried plasmids, 23 of them harboured single plasmid of 33 kb in size, 6 strains had 2 plasmids, 4 strains had 3 plasmids and 3 strains revealed the presence of 4 plasmids. Among the 23 strains from shrimp farm, 21

harboured a single plasmid of 33 kb and 2 had 2 plasmids each. Among the 5 strains from seafood all harboured a single plasmid of 33 kb. The plasmid profiles of the strains are given in Table 3.4.

Table 3.4 Table showing the plasmid profiles observed among the *Vibrio* strains from Cochin estuary, shrimp farms and seafood

Isolates from Cochin estuary	**Plasmid size in kb**	**Isolates from Shrimp farm**	**Plasmid size in kb**
M8W2	33	FSV5	33
PM1S2	33	MWE10	33
1W5	33, 2, 1.5	MWV11	33
PM6W6	33, 8.9	MAYSV7	33
6S4	33, 2, 1.5	SSV24	33
2S5	33	MSV8	33
M9W1	33	AWV20	33
PM 1S1	33	MSA1	33
M10W4	33	MWV1	33
3S4	33	AWA3	33
10W2	33, 8.9	4G	33
M6W4	33	WA197	33
3W6	33	JULYWA8	33
9S4	33	DWA218	33
M7S3	33, 4	WV151	33
PM1W3	33	MWV3	33
1S3	33	MSA3	33
1W1	33	MWV1	33
M7S2	33, 4	MSA11	33
8W3	33	MSV14	33
10W1	33	AST5	33, 8.9
PM8W1	33, 5, 2, 1.5	SV178	33, 1
6S3	33, 1.5, 1	ASA17	33, 0.75
1W3	33	**Isolates from seafood**	**Plasmid size in** kb
M9S2	7, 3, 1.6, 1.8	M37	33
M10W1	33	M91	33
PM 1S5	5, 1, 2, 3	M10	33
1W3	7, 8.9	M4	33
PM3W5	33, 8.9, 7	M175	33
PM3S2	33, 15		

3.5.5 Plasmid curing of *Vibrio* from Cochin estuary, shrimp farms and seafood

All the plasmid harbouring strains were further subjected to plasmid curing experiments for detection of plasmid mediated antibiotic resistance. Among the 30 *Vibrio* strains from Cochin estuary 24 revealed the presence of plasmid mediated antibiotic resistance (Table 3.5). Among the *Vibrio* from shrimp farm 13 and all the 5 isolates from seafood exhibited plasmid mediated antibiotic resistance (Table 3.6, 3.7). Plasmid mediated resistance was shown towards 13 antibiotics (Ak, Amp, Amx, Caz, Cb, Cl, E, Ex, Fr, Nit, S, Sm, Tr). Resistance to carbenicillin was the most frequently lost phenotype after plasmid curing. Eight *Vibrio* isolates from Cochin estuary, 6 from shrimp farm and 1 from seafood lost their carbenicillin resistance after curing treatment. Resistance towards ceftazidime was plasmid mediated in 4 isolates from Cochin estuary, 3 from shrimp pond and 1 from seafood. Plasmid mediated nitrofurantoin resistance was observed in 3 isolates from Cochin estuary, 5 from shrimp farm and 1 from seafood. Sulphamethoxasole resistance was plasmid mediated in 9 isolates from Cochin estuary and 3 from shrimp farm. Amoxycillin resistance was plasmid borne in 1 isolate from Cochin estuary, 6 from shrimp farm and 3 from seafood. Plasmid mediated ampicillin resistance was observed among 2 isolates from Cochin estuary and one from shrimp pond. Two isolates from Cochin estuary exhibited plasmid mediated erythromycin resistance. One isolate each from Cochin estuary showed plasmid borne resistance to enrofloxacin, streptomycin, amikacin, furazolidone and trimethoprim. Similarly one isolate from shrimp pond had plasmid mediated colistin resistance.

Table 3.5 Changes in antibiotic resistance pattern (ARP) after plasmid curing in *Vibrio* from Cochin estuary

Strain	ARP before curing	ARP after curing	Plasmid size	Plasmid mediated resistance
M10S2 (CONTROL)	Amp, Amx, Cb, Cep, Cl, E, Ex, Na, O, S, Te, Tr, Nit, Sm	Amp, Amx, Cb, Cep, Cl, E, Ex, Na, O, S, Te, Tr, Nit, Sm	No plasmid	None
M8W2	Amp, Amx, Cb, Cep, Cl, E, Ex, Sm, Nit	Amp, Amx, Cep, Cl, E, Ex, Sm, Nit	33	Cb
PM1S2	Amp, Amx, Caz, Cb, Cep, Cl, Ex, Nit, Sm	Amp, Amx, Cep, Cl, Ex, Nit, Sm	33	Caz, Cb
1W5	Amp, Amx, Cb, Cep, Cl, Ex, Nit, O, Tr	Amp, Amx, Cep, Cl, Ex, Nit, O, Tr	33, 2, 1.5	Cb
PM6W6	Amp, Amx, Cb, Cep, Cl, E, Nit, O, Sm	Amp, Amx, Cep, Cl, E, Nit, O, Sm	33, 8.9	Cb
6S4	Amx, Cb, Cep, Cl, Ex, Nit, Sm,	Amx, Cep, Cl, Ex, Nit, Sm,	33, 2, 1.5	Cb
2S5	Amx, Amp, C, Cb, Cep, Cl, Fr, Sm	Amx, Amp, C, Cep, Cl, Fr, Sm	33	Cb
M9W1	Amp, Amx, Cb, Cep, Cl, Ex, Fr, Nit, Sm	Amx, Cb, Cep, Cl, Ex, Fr, Nit, Sm	33	Amp
PM 1S1	Amp, Amx, Ak, Cb, Cep, Cl, E, Nit, O, S, Sm	Amp, Ak, Cb, Cep, Cl, E, Nit, O, S, Sm	33	Amx
M10W4	Amp, Amx, Cep, Cl, Sm, Nit	Cep, Cl, Nit , Sm	33	Amp, Amx
3S4	Amp, Amx, Ak, Caz, Cb, Cep, Cl, Nit, S, Sm	Amp, Ak, Cb, Cep, Cl, Nit, S	33	Amx, Sm, Caz
10W2	Amp, Amx, Cb, Cep, Cl, Ex, Nit, Sm, Tr	Amp, Amx, Cb, Cep, Cl, Ex, Nit, Tr	33, 8.9	Sm
M6W4	Amp, Amx, Cep, Cl, Nit, Sm	Amp, Amx, Cep, Cl, Nit	33	Sm
3W6	Amp, Amx, Cb, Cep, Cl, E, Sm	Amp, Amx, Cb, Cep, Cl, E	33	Sm

Table 3.5 Continued ….

9S4	Amp, Amx, Cep, Cl, E, Ex, O, Te, Tr, Nit, Sm	Amp, Amx, Cep, Cl, Ex, O, Te, Tr, Nit, Sm	33	E
M7S3	Amp, Amx, Cb, Cep, Cl, E, Nit, S, Sm	Amp, Amx, Cb, Cep, Cl, S	33,4	E, Nit, Sm
PM1W3	Amx, Amp, Ak, Cb, Cep, Cl, Ex, Fr, Nit, S, Sm, Tr	Amx, Amp, Ak, Cb, Cep, Cl, Ex, Fr, Nit, S, Tr	33	Sm
1S3	Amx, Amp, Ak, Cb, Cep, Cl, Ex, Fr, Nit, Sm	Amx, Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm	33	Ak
1W1	Amx, Amp, Cb, Cep, Cl, E, Ex, Fr, Nit, S, Sm	Amx, Amp, Cb, Cep, Cl, E, Ex, Fr, Nit, Sm	33	S
M7S2	Amx, Cep, Cl, Ex, Fr, Nit, Sm, Tr	Cep, Cl, Ex, Fr, Nit, Tr	33,4	Amx, Sm
8W3	Amx, Cb, Cep, Cl, E, Ex, Fr, Gen, Nit, O, S, Sm, Tr	Amx, Cep, Cl, E, Ex, Fr, Gen, Nit, O, S, Tr	33	Cb, Sm
10W1	Amx, Cep, Cl, Ex, Fr, Nit, Sm	Cep, Cl, Sm	33	Amx, Ex, Fr, Nit
PM8W1	Amx, Cb, Cep, Cl, Ex, Fr, O, Nit, Tr	Cep, Cl, Ex, Fr, O, Nit	33	Amx, Cb, Tr
6S3	Amx, Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm	Amx, Amp, Cb, Cep, Cl, Ex, Fr,	33	Nit, Sm
1W3	Amx, Amp, Caz, Cb, Cep, Cl, Ex, Fr, Nit, Sm	Amp, Amx, Cb, Cep, E, Ex, Fr, Nit, Sm	33	Caz
M9S2	Amp, Amx, Cb, Cep, S, Sm	Amp, Amx, Cb, Cep, S, Sm	7, 3, 1.6, 1.8	None
M10W1	Amp, Amx, Cep, S, Sm	Amp, Amx, Cep, S, Sm	33	None
PM1S5	Amp, Amx, Cb, Cep, E, Ex, Nit, Sm	Amp, Amx, Cb, Cep, E, Ex, Nit, Sm	5, 1, 2, 3	None
1W3	Amp, Amx, Cb, Cep, Ex, Nit, Tr	Amp, Amx, Cb, Cep, Ex, Nit, Tr	7, 8.9	None
PM3W5	Amp, Amx, Cep, E, Nit, Sm	Amp, Amx, Cep, E, Nit, Sm	33, 8.9, 7	None

Table 3.6 Changes in antibiotic resistance pattern (ARP) after plasmid curing in *Vibrio* from shrimp farm

Strain	ARP before curing	ARP after curing	Plasmid size	Plasmid mediated resistance
FSV5	Amp, Cep, Cl, Cb, Ex, Fr, Nit, Sm, Tr	Amp, Cep, Cb, Ex, Fr, Sm, Tr	33	Cl, Nit
MWE10	Amp, Cep, Cl, Cb, Ex, Fr, Nit, Sm, Tr	Cep, Cb, Ex, Fr, Tr	33	Amp, Cl, Nit, Sm
MWV11	Amp, Cl, Cb, Ex, Fr, Nit, Tr	Amp, Cl, Ex, Fr, Nit, Tr	33	Cb
MAYSV7	Amp, Caz, Cb, Cep , Cl, Nit, Sm, Tr	Amp, Caz, Cep, Cl, Tr	33	Cb, Nit, Sm
SSV24	Amp, Amx, Cb, Cep, E, Ex, Fr, Nit	Amp, Amx, Cb, Cep, E, Ex, Fr	33	Nit
MSV8	Amp, Amx, Cb, Cl, E, Ex, Nit	Amp, Amx, Cb, Cl, E, Ex	33	Nit
AWV20	Amp, Cb, Cep, Fr, Nit, Sm, Tr	Amp, Cb, Cep, Fr, Nit, Tr	33	Sm
MSA1	Amp, Amx, Caz, Cb, Cep, Sm, Tr	Amp, Amx, Cep, Sm, Tr	33	Cb, Caz
MWV1	Amp, Amx, Caz, Cb, Cep, Cl, Fr, Tr	Amp, Amx, Cb, Cep, Cl, Fr, Tr	33	Caz
AWA3	Amp, Caz, Cb, Cep, Sm	Amp, Caz, Cep, Sm	33	Cb
4G	Amp, Caz, Cb, Cep, Sm, Tr	Amp, Cb, Cep, Sm, Tr	33	Caz
WA197	Amp, Cb, Cep, Ex, Fr, Nit, Sm	Amp, Cep, Ex, Fr, Nit, Sm	33	Cb
JULYWA8	Amp, Caz, Cb, Cep, Sm	Amp, Caz, Cep, Sm	33	Cb
DWA218	Amx, Cb, Cep, E, Fr, Nx, O, Sm, Tr	Amx, Cb, Cep, E, Fr, Nx, O, Sm, Tr	33	None
WV151	Amp, Amx, Caz, Cb, Cep, Ex, Na, Tr	Amp, Amx, Caz, Cb, Cep, Ex, Na, Tr	33	None
MWV3	Amp, Cb, Cep, Sm, Tr	Amp, Cb, Cep, Sm, Tr	33	None
MSA3	Amp, Cb, Cep, Ex, Fr, Nit, Sm, Tr	Amp, Cb, Cep, Ex, Fr, Nit, Sm, Tr	33	None
MWV1	Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm, Tr	Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm, Tr	33	None
MSA11	Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm, Tr	Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm, Tr	33	None
MSV14	Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm, Tr	Amp, Cb, Cep, Cl, Ex, Fr, Nit, Sm, Tr	33	None

Table 3.7 Changes in antibiotic resistance pattern (ARP) after plasmid curing in *Vibrio* from seafood

Strain	ARP before curing	ARP after curing	Plasmid size	Plasmid mediated resistance
M37	Amx, Amx, C, Cep, Cip, Cl, Ex, Fr	Amx, C, Cep, Cip, Cl, Ex, Fr	33	Amx
M91	Amx, Caz, Ex, Fr, Tr	Ex, Fr, Tr	33	Amx, Caz
M10	Amp, Amx, Cep, Cl, Ex, Nit	Amp, Amx, Cep, Cl, Ex,	33	Nit
M4	Amp, Amx, Cb, Cep, Cl	Amp, Amx, Cep, Cl	33	Cb
M175	Amp, Amx, C, Cb, Ex, Fr, S	Amp, C, Cb, Ex, Fr, S	33	Amx

3.6 Discussion

3.6.1 Antibiotic resistance of *Vibrio* from Cochin estuary, shrimp farm and seafood

Vibrios are generally considered to be highly susceptible to most clinically used antimicrobials (Oliver, 2006). However in the past few decades, antimicrobial resistance has emerged and evolved in many bacterial genera due to the excessive use of antimicrobials in human, agriculture and aquaculture systems (Mazel and Davies, 1999; Cabello, 2006). This emerging issue has gained great concern due to increased resistance of pathogenic *V. parahaemolyticus* towards clinically used antibiotics. Recently, higher frequency of drug-resistant *Vibrio* has been reported (Okoh and Igbinosa, 2010; Hua and Apun, 2013).

The presence of multiple antibiotic resistance among environmental microorganisms may be due to long-term exposure to antibiotic containing

effluents discharged from hospitals, agriculture and aquaculture farms or through horizontal transfer of antibiotic resistant genes from human pathogens (Martinez, 2012). It was alarming to note that in our study all the *Vibrio* strains from Cochin estuary, shrimp farm and seafood have acquired multiple antibiotic resistance. However, strains from the three sources varied widely in their resistance pattern. This could be due to the difference in the type and amount of antibiotic residues to which the isolates were exposed. As pointed out by Hsu *et al*. (1992) differences in percentage of bacterial resistance to various antibiotics may reflect the history of antibiotic application and hence there is a possibility of using bacterial drug resistance as an indicator of antibiotic application.

High percentage of β-lactam resistance was exhibited by our isolates. Resistance towards β- lactam antibiotics have been previously reported in *V. parahaemolyticus* and other vibrios from different sources (Molina-Aja *et al*., 2002; Manjusha *et al.,* 2005; Devi *et al.,* 2009). In a previous study sensitivity of *V. parahaemolyticus* from the south west coast towards nitrofurantoin and trimethoprim has been reported (Devi *et al.,* 2009). Similarly, in another study from Tunisia, more than 70% of isolates of *Vibrio* showed susceptibility to trimethoprim-sulfamethoxazole (Lajnef *et al.,* 2012). In the present study, isolates from Cochin estuary and shrimp farm demonstrated resistance towards sulphamethoxazole and low level resistance towards trimethoprim. While all the *Vibrio* strains isolated from seafoods were resistant towards trimethoprim and none of them were resistant to sulphamethoxasole. Resistance towards enrofloxacin was shown by all the strains from seafood and 85% strains from Cochin estuary, while only 14% of strains from shrimp farm had acquired

resistance towards it. All the strains from shrimp farm were sensitive towards gentamicin whereas strains from other sources exhibited low level resistance towards both the drugs.

A recent study on antibiotic resistance of *Vibrio* spp. isolated from Palk Bay revealed widespread distribution of multidrug resistant *Vibrio* across the Palk Bay (Sneha *et al.,* 2016). In their study, the *Vibrio* isolates exhibited resistance towards beta-lactams, vancomycin, nitrofurantoin, gentamicin, azithromycin, oxytetracycline, tetracycline and chloramphenicol. Antibiotics like chloramphenicol, ampicillin, tetracycline, chlortetracycline, nalidixic acid, gentamycin, sulfafurazole and trimethoprim are commonly used in aquaculture farms to ensure continuous production of seafood (Roque *et al.,* 2001; Manjusha and Sarita, 2011; Yano *et al*., 2014). In addition, treatment recommendations for *Vibrio* infections include cephalothin, cefuroxime, cefotaxime, ceftazidime, tetracycline, doxycycline, fluoroquinolone, amikacin, gentamicin and trimethoprim-sulfamethoxazole (Daniels and Shafaie, 2000; CDC, 2013; Letchumanan *et al.*, 2015a). In the present study, most of the *Vibrio* strains have attained resistance towards life saving drugs such as cephalothin, ceftazidime, doxycycline, enrofloxacin, nitrofurantoin, trimethoprim, sulphamethoxasole, streptomycin, amikacin and nalidixic acid. Treatment of infections caused by such *Vibrio* has major clinical implications as this may lead to therapeutic failure and finally death of the patient. Even though our findings are of local in nature, it may cause global consequences, if these antibiotic resistant strains are transported through ballast water, seafoods and ocean water currents to other parts of the world (Ruiz *et al*., 2000; Ge *et al.*, 2010, 2012).

All the isolates from Cochin estuary and majority from other two sources showed a MAR index higher than 0.2, and it ranged up to 0.6. MAR indices higher than 0.2 are often considered to have originated from higher-risk sources (Krumperman, 1983) of contamination, such as those from hospital sewage, commercial poultry farm waste etc., that somehow find their way to the open sea *via* illegal dumping of waste or transferred by infected humans. The study area is a famous tourist hot spot and shrimps grown in this area are exported to various countries, making the findings of our study all the more important. Therefore, continued monitoring of both the prevalence and the antimicrobial susceptibility profile of *Vibrio* is important to better ensure seafood and public health safety from our study area.

3.6.2 Distribution of antibiotic resistance genes in *Vibrio* from Cochin estuary, shrimp farm and seafood

The bla_{TEM} gene was present in all the *Vibrio* strains from Cochin estuary, shrimp farm and seafood. This gene encodes resistance to penicillins. The $bla_{\text{CTX-M}}$ gene was found only in a few *Vibrio* strains isolated from Cochin estuary. The gene encodes for extended spectrum β- lactamases conferring resistance to extended spectrum beta-lactam antibiotics (cephalosporins, penicillin and monobactams). Reports suggest antibiotic resistance genes are very diverse (Letchumanan *et al.,* 2015a). Currently, there are forty β-lactamases that encodes for plasmid-mediated CTX-M enzymes alone (Tzouvelekis *et al.,* 2000). Therefore, the absence of $bla_{\text{CTX-M}}$ in beta-lactam resistant *Vibrio* strains could be due to presence of other encoding genes in isolates in the present study. Their resistance may possibly be mediated by the efflux systems also.

In a previous study it was found that the resistance of *V. parahaemolyticus* towards ampicillin was not mediated by the *bla* gene instead it was conferred by an efflux system (Pazhani *et al.,* 2014). In a recent study on prevalence of β-lactamase genes in clinical *E. coli* and *K. pneumoniae* isolates from Northeast India, $bla_{\text{CTX-M}}$ was the most prevalent gene in *E. coli* and bla_{TEM} in *K. pneumonia* (Bora *et al.,* 2014). In a previous report, Enterobacterial strains were isolated during 2000-2003 from different countries like France, India, Poland and Turkey (Lartigue *et al.,* 2004). The strains produced emerging CTX-M-type extended-spectrum-β-lactamases and among the genes $bla_{\text{CTX-M-15}}$ was the most frequently observed gene. The New Delhi metallo-β-lactamase (NDM-1) gene, $bla_{\text{NDM- 1}}$ was detected in 13.3% of strains from Cochin estuary, 14.2% of strains from shrimp farm and 6.6% of strains from seafoods. This is indeed an alarming situation since strains harbouring $bla_{\text{NDM-1}}$ is resistant to carbapenems, extended spectrum cephalosporins, penicillin and monobactams. Carbapenems are the last drug of choice for infections caused by extended spectrum β-lactamase producing bacteria. In a previous study on antibiotic resistance in *Vibrio* isolated from Palk Bay, bla_{NDM1} gene was not detected in any of the isolates studied (Sneha *et al.,* 2015). *Vibrio fluvialis* harbouring $bla_{\text{NDM-1}}$ gene was isolated from acute diarrhoea patients in Kolkata, India (Chowdhury *et al.,* 2016).

The *Vibrio* strains from our study areas may act as potential reservoirs of drug resistance genes in the environment. Resistance genes can be further transferred from non-pathogens to pathogens through horizontal gene transfer *via* conjugation, transduction and transformation.

3.6.3 Plasmid profiling and plasmid curing of antibiotic resistant vibrios from Cochin estuary, shrimp farm and seafood

The plasmid is known to be one of the most important mediators facilitating the fast spread of antibiotic resistance among bacteria (Dale and Park, 2004). Plasmids were present in 30 strains (16.6%) from Cochin estuary, 23 strains (32.8%) from shrimp farm and 5 strains (16.6%) from seafoods. Plasmid of size 33 kb was the most frequently observed profile from all the environments; this is similar to the finding by Zhang *et al*. (2006), stating the presence of >30 kb plasmids in environmental *Vibrio* isolates. Bacterial antibiotic resistance patterns are known to sometimes be associated with the presence of large plasmids, as well as the abilities of plasmids in conjugation. Transferable R plasmids are usually as big as 30 kb, and the indispensable components of a conjugative plasmid make it big in size compared to other plasmids (Guiney and Landa, 1989).

In our study, when we compared the antibiotic resistance patterns and the plasmid profiles, we could not find any correlation. Even among the strains with same resistance pattern, the plasmid profiles were different and some strains even lacked plasmids, which was similar to findings by Lajnef *et al*. (2012). So, in some strains resistance may be plasmid coded, and in some it may be chromosomally borne. Plasmid profiles have been previously studied in *Vibrio* species such as *V. parahaemolyticus* (Devi *et al.*, 2009), *V. ordalii* (Tiainen *et al.*, 1995), *V. vulnificus* (Radu *et al.*, 1998) and *V. salmonicida* (Sorum *et al.*, 1990), and most extensively in *V. anguillarum* (Pedersen *et al.*, 1996, 1999), where a high diversity of profiles have been observed. In a study on

Vibrio spp. isolated from tropical waters of Malaysia 32 different plasmid profiles with size ranging from 2.2 to 24.8 kb were detected among the resistant isolates (You *et al.,* 2016). In the present study, the size of plasmids ranged from 0.5 to 33 kb. A high incidence of plasmids in *Vibrio* spp. of both polluted and pristine environments may be ecologically important to the survival of these bacteria in the environment (Zhang *et al.,* 2006).

Acquired antibiotic resistance in bacteria is generally plasmid mediated and is easily transferred to other bacteria in the environment through horizontal/vertical gene transfer mechanisms (Manjusha and Sarita, 2011). The extrachromosomal DNA may be responsible for the rapid emergence of multiple antibiotic resistance in bacteria (Schelz *et al.,* 2006). In order to ascertain the antibiotic resistance mediation, plasmid curing experiments are performed. In the present study, acridine orange was used as curing agent to eliminate plasmids from our *Vibrio* strains. Plasmid mediated resistance was observed towards 13 antibiotics (Ak, Amp, Amx, Caz, Cb, Cl, E, Ex, Fr, Nit, S, Sm, Tr). Plasmid mediated carbenicillin resistance was the most frequently observed phenotype in our study. In a previous study by Reboucas *et al.* (2011), plasmid mediated oxytetracycline resistance was the frequently observed profile in *Vibrio* species isolated from marine shrimp. Similarly, in a study on *Vibrio* from shrimp farm in Thailand, oxytetracycline resistance was eliminated through plasmid curing using ethidium bromide (Yano *et al.,* 2014). In another study on vibrios isolated from coastal waters of Kerala, chromosomal borne resistance was observed towards amoxicillin, ampicillin, furazolidone and tetracycline after plasmid curing with 0.05 to

0.5 mg/ml of ethidium bromide (Manjusha and Sarita, 2011). The presence of antibiotic resistance genes in the bacterial plasmid may lead to rapid dissemination of drug resistance among pathogenic strains in our environment.

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Chapter 4

VIRULENCE FEATURES OF *VIBRIO* FROM FOOD AND ENVIRONMENTAL SOURCES ALONG THE SOUTH WEST COAST OF INDIA

Contents

4.1 Introduction

The genus *Vibrio* is a normal inhabitant of the aquatic environments and plays an important role in maintaining the aquatic system. It contributes significantly to the nutrient recycling and decomposition of the organic matter in the aquatic environments. The nutrients and organic matter are used by *Vibrio* as their food source. The organism secretes various extracellular enzymes to degrade this organic matter. There are reports showing the presence of extracellular enzymes such as chitinase, caseinase, amylase, lipase, pectinase, cellulase, DNase, gelatinase, alginate lyase in vibrios (Raghul and Sarita, 2011). These enzymes also contribute to virulence of the organism. Apart from contributing to their virulence, they also help the organism to survive in various aquatic environments. Pathogenic bacteria have adopted a wide range of strategies to colonize and invade host cells. Secretion of extracellular

products, namely proteinases, lecithinases, gelatinase, lipases, DNase, hemolysins etc. are some of the strategies used by pathogenic bacteria to establish an infection. These enzymes are normally considered as virulence factors as they help the pathogen to damage the host tissues and make the host susceptible to infection (Edberg *et al.,* 1996; Pavlov *et al.,* 2004). Some authors report that the production of extracellular products help in the nutrition of the host (Balcazar *et al.,* 2006), whereas others suggest that the overproduction of these enzymes contribute to virulence of the pathogenic strains as such strains are reported to have high extracellular enzyme activity (John and Hatha, 2013). Thus, these virulence factors are used by vibrios as a means of survival, self-defence mechanism and establishment of pathogenicity.

Vibrio parahaemolyticus is a leading cause of seafood borne gastroenteritis in Asia and as well as in many other countries (Nair *et al.,* 2007; Matsuda *et al.,* 2012). The organism has also been reported as the causative agent of early mortality syndrome (EMS) in shrimps and caused large-scale economic losses in farmed shrimp production in India and other countries as well (Tran *et al.,* 2013; Krishna *et al.,* 2014). Many researchers have reported the abundance of *V. parahaemolyticus* throughout the year in tropical zones (Elhadi *et al.,* 2004). India being a tropical country has favourable conditions for the rapid dissemination of this pathogenic species. It thus increases the risk of disease outbreaks associated with the organism. The pathogenic potential of *V. parahaemolyticus* is not solely governed by a given virulence function (Klein *et al.,* 2014). A single factor alone does not contribute to the establishment of infection in the host cell. It is a complex process with different strains employing

different strategies. Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) and type III secretion systems (T3SSs) are considered as major factors associated with pathogenicity of the organism (Honda *et al.*, 1987 a, b; Noriea *et al.*, 2010).

4.2 Review of literature

4.2.1 Extracellular enzymes

Enzyme studies are usually carried out to study the nutrient cycling in the aquatic environments (Mallet and Debroas, 1999). Heterotrophic microbes secrete various extracellular enzymes to hydrolyse recalcitrant organic compounds present in the environment (Arnosti, 2011). The organic compounds present in aquatic environments include high molecular weight proteins, starch, lipids, pectin, cellulose, chitin, nucleic acids, or lignin (Unanue *et al.*, 1999). Environmental bacteria utilise this organic matter as a source of carbon and nitrogen (Patel *et al.*, 2000). They also acquire energy for its various metabolic activities through hydrolysis of these compounds (Patel *et al.*, 2000).

Pathogenic *Vibrio* species secrete several extracellular enzymes, which are characterised as direct virulence factors causing skin damage (Miyoshi, 2013). Cell wall degrading enzymes play a major role in pathogenesis as they facilitate the bacterial penetration and tissue colonization (Prasannath, 2013). Proteases digest cell membranes and degrade host surface molecules. These hydrolytic enzymes are able to attack cells and molecules of the host immune system thus weakening host immune response. The presence of extracellular products has been used as an indicator of health risk associated with bacteria isolated from

clinical, environment and food sources (Lafisca *et al.,* 2008). These enzymes can help the pathogens to trigger infections in humans and aquatic animals under favourable conditions. Most of the extracellular enzymes like gelatinase, chitinase, DNase, amylase, lipase etc. are known to be associated with the pathogenicity of *V. parahaemolyticus* (Costa *et al.,* 2013). The urease and hemolysin production is typical for *V. parahaemolyticus* isolates from clinical samples, hence it is also considered as a virulence marker (Okuda *et al.,* 1997).

Experimental studies by previous researchers have already recognised the role of extracellular enzymes in virulence among vibrios (Rodrigues *et al.,* 1993; Costa *et al.,* 2013; Miyoshi, 2013). Raghul and Sarita (2011) have reported presence of hydrolytic enzymes in *Vibrio* from marine sediments of South coast of India. Bunpa *et al.* (2016) studied the production of extracellular enzymes by *Vibrio alginolyticus* isolated from environments and diseased aquatic animals in Thailand. There are reports suggesting the role of extracellular enzymes in virulence of many other bacterial pathogens such as *Aeromonas* (Bagyalakshmi *et al.,* 2009; John and Hatha, 2013), *Erwinia* (Py *et al.,* 1998), *Pseudomonas aeruginosa* (Jaeger, 1994), *Staphylococcus aureus* (Dinges *et al.,* 2000) etc.

4.2.2 Virulence related genes in *V. parahaemolyticus*

Pathogenicity of *V. parahaemolyticus* is not dependant solely on a given virulence function; rather, virulence is a complex process and different strains employ different strategies (Klein *et al.,* 2014). The virulence properties of *V. parahaemolyticus* have been usually found to be

associated with thermostable direct haemolysin (TDH) and TDH- related haemolysin (TRH) (Honda *et al.*, 1987 a, b). Several studies reported the absence of *tdh* and *trh* in clinical strains of *V. parahaemolyticus* (Li *et al.*, 2014; Pazhani *et al.*, 2014); thus suggesting that many other factors also contribute to pathogenicity of these strains. Recently, it was found that the type III secretion systems (T3SSs) also play a pivotal role in virulence machinery in *V. parahaemolyticus* (Ham and Orth, 2012).

4.2.2.1 Thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*) genes

V. parahaemolyticus is the causative agent of seafood borne gastroenteritis. It colonizes the human gut *via* the gastrointestinal route through consumption of contaminated raw or undercooked seafood. The most common symptoms include gastroenteritis, diarrhea, headache, nausea and vomiting. *V. parahaemolyticus* infections can be life threatening to the infants or immuno-compromised persons. The pathogenicity of *V. parahaemolyticus* is rather complex and involves multiple factors (Broberg *et al.*, 2011). Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are the most frequently used indicators of pathogenicity of the organism (Honda *et al.*, 1987 a, b; Nishibuchi and Kaper, 1995; Raghunath, 2015). Both the TDH and TRH are tetrameric proteins (Yanagihara *et al.*, 2010; Broberg *et al.*, 2011) and they share nearly 70% homology (Kishishita *et al.*, 1992). They act as porins and helps in the efflux of divalent cations and other solutes from and influx of water molecules into intestinal cells (Takahashi *et al.*, 2000; Ohnishi *et al.*, 2011). TDH forms large pores on erythrocyte membrane, allowing both water and ions to flow through the membrane (Matsuda *et al.*, 2012). TRH also

alters the ion flux by activating ion channels (Takahashi *et al.,* 2000). The change in the ion flux of the intestine results in diarrhea during infection.

Detection of *tdh* in *V. parahaemolyticus* is conventionally studied by beta haemolysis assay on a blood agar called Wagatsuma agar (Nishibuchi *et al.,* 1985) and the presence of *trh* gene in *V. parahaemolyticus* by urease phenotype (Cai and Ni, 1996). Previous reports state that most of the environmental *V. parahaemolyticus* strains are not pathogenic to humans, and only 1 to 2% of the environmental strains have shown to be positive for these genes while 90% of the clinical strains carry them (Nishibuchi and Kaper 1995; Hervio-Heath *et al.,* 2002; Robert-Pillot *et al.,* 2004). However, some investigators have reported a higher prevalence of *tdh* and *trh* genes in *V. parahaemolyticus* from environmental samples (DePaola *et al.,* 2003; Deepanjali *et al.,* 2005; Raghunath *et al.,* 2008; West *et al.,* 2013). Reports also reveal that these genes are not present in all the clinical strains of *V. parahaemolyticus* (Meador *et al.,* 2007). Hence, they alone are not responsible for *V. parahaemolyticus* pathogenicity (Makino *et al.,* 2003; Hiyoshi *et al.,* 2010). A study by Jones *et al.* (2012a) revealed that even in the absence of these genes *V. parahaemolyticus* strains exhibited pathogenicity. Thus, it was clear that other virulence factors also exist which contributes to the organism's virulence.

4.2.2.2 Type III secretion systems in *V. parahaemolyticus*

The type III secretion systems (T3SSs) have been widely reported in many Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Yersinia* sp., *Salmonella* sp., *Shigella* sp. and *Escherichia coli* (Ono *et al.,* 2006) and the bacteria use this to induce pathogenicity in the host cells

(Kumar *et al.,* 2014 a, b). Two non-identical and non-redundant T3SS clusters, T3SS1 and T3SS2, were identified during the genome mapping of *V. parahaemolyticus* RIMD2210633 (Makino *et al.,* 2003). Recently, it was found that toxins secreted by T3SS have a profound role in pathogenicity of *V. parahaemolyticus* (Noriea *et al.,* 2010; Broberg *et al.,* 2011; Karunasagar *et al.,* 2012). The organism carries two type III secretion systems namely T3SS1 and T3SS2. T3SS1 causes cytotoxicity and T3SS2 is mainly associated with enterotoxicity (Ham and Orth, 2012). They are needle-like apparatus that enable injection of bacterial effector proteins directly into host cells resulting in modulation of numerous host processes (Noriea *et al.,* 2010). The apparatus is made of about 20 proteins and is highly conserved among the bacteria (Ono *et al.,* 2006). Once the effector enters the host cells, it disrupts the host's immune response (Coburn *et al.,* 2007). These effectors modify the host signalling pathways and make it beneficial for the pathogen (Ham and Orth, 2012). The effectors secreted from T3SS1 include VopQ, VopS, VopR and VPA045 while those from T3SS2 include VopA/VopP, VopL, VopT, VopV and VopC (Ham and Orth, 2012; Matsuda *et al.,* 2012). All these effector proteins have a distinct role and they work in an orchestrated manner to help the pathogen in colonising the host cell and establishing an infection (Ham and Orth, 2012).

T3SS1 is located on chromosome 1 (Caburlotto *et al.,* 2009). T3SS2 genes are located on the pathogenicity island in Chromosome II of *V. parahaemolyticus* (Ham and Orth, 2012). This location of the gene on a pathogenicity island (Vp-PAI) may facilitate the transfer of this specific DNA fragment from a pathogenic strain to non-pathogenic autochthonous microorganism in the marine environment (Caburlotto *et al.,* 2009).

Noriea *et al.* (2010) studied the distribution of type III secretion systems in 130 *V. parahaemolyticus* isolates from the Gulf of Mexico. Kumar *et al.* (2014 a, b) reported the presence of T3SS2β genes in *trh*+ *V. parahaemolyticus* from seafood collected from Mangalore coast, India. Klein *et al.* (2014) found genes similar to the *V. parahaemolyticus* virulence-related genes to occur in other *Vibrionaceae* species that were isolated from a pristine estuary.

4.3 Objectives of the study

The specific objectives set for the present study are:-

1) To screen the *Vibrio* strains isolated from Cochin estuary, shrimp farm and seafood for extracellular virulence factors.
2) To detect the *tdh* and *trh* virulence genes in *V. parahaemolyticus* isolated from Cochin estuary, shrimp farm and seafood.
3) To detect the type III secretion system virulence genes in *V. parahaemolyticus* isolated from Cochin estuary, shrimp farm and seafood.

4.4 Materials and Methods

4.4.1 Screening of *Vibrio* strains for extracellular enzymes

4.4.1.1 Bacterial strains used

A total of 276 *Vibrio* strains previously isolated from Cochin estuary (n=180), shrimp farm (n= 66) and seafood (n= 30) were used for the present study.

4.4.1.2 Production of Lipase

Tributyrin is commonly used for studying lipolytic activities. Pure cultures of the isolates were spot inoculated on tributyrin agar plates (nutrient agar incorporated with 1% tributyrin). The plates were incubated at 37 ^{o}C for 24-48 h. A positive result was indicated by zone of clearance around the colonies of lipolytic organisms, where the tributyrin has been hydrolyzed.

4.4.1.3 Production of amylase

The isolates were spot inoculated onto starch agar plates (nutrient agar supplemented with 1% starch). The plates were incubated at 37 ^{o}C for 24 to 48 h. A positive result was indicated by the presence of clear zones around the colonies on addition of Lugol's iodine solution.

4.4.1.4 Production of gelatinase

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.

4.4.1.5 Production of DNase

The isolates were spot inoculated onto DNA agar plates (nutrient agar containing 0.2% DNA). The plates were incubated at 37 ^{o}C for 24 to 48 h. After incubation the plates were flooded with 1M HCl. Zone of clearance around the colonies indicated that bacteria have produced DNase and hydrolysed the DNA.

4.4.1.6 Production of chitinase

The isolates were spot inoculated onto chitin agar (2% w/v colloidal chitin). The plates were incubated at 37 ^{o}C for 7 days. Degradation of chitin was confirmed by clearance zone around the test colonies after incubation which indicated the production of chitinase enzyme by the bacteria.

4.4.1.7 Production of phosphatase

To detect phosphatase production the isolates were spot inoculated onto phosphate agar (1% phenolphthalein diphosphate). The plates were incubated at 37 ^{o}C for 24 to 48 h. After incubation, the colonies were exposed to ammonia vapour. Upon exposure, phosphatase producing colonies turn pink whereas the non-phosphatase producing colonies remained unchanged.

4.4.1.8 Production of caseinase

The isolates were spot inoculated on skim milk agar plates. The plates were incubated at 37 ^{o}C for 24 to 48 h. Caseinase production was detected by presence of clear zone around the colonies after incubation.

4.4.1.9 Detection of hemolytic activity

Hemolytic activity was analysed on blood agar containing 5% human blood. The isolates were spot inoculated onto blood agar plates. The hemolytic activity was checked after incubation at 37 °C for 24 h.

4.4.1.10 Statistical analysis

Any significant difference in the prevalence of extracellular enzymes among *Vibrio* from different sources was analysed using Chi- Square test in SPSS statistical software. Significance level was set at $\alpha = 0.05$.

4.4.2 Screening for virulence genes in *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood

V. parahaemolyticus was the most predominant *Vibrio* species observed in our study areas. Hence, we planned to undertake a detailed study on the virulence potential of the *V. parahaemolyticus* strains.

4.4.2.1 Bacterial strains used

A total of 85 *V. parahaemolyticus* strains were used in the present study. This included isolates from various stations of the Cochin estuary (n=45), from a traditional pokkali cum shrimp farm (n=25) situated at Edavanakkad, adjoining the Cochin estuary and seafoods (n=15) collected from retail markets situated in and around Cochin.

4.4.2.2 DNA isolation

Genomic DNA was extracted from the *V. parahaemolyticus* strains using the method described in the previous section 2.4.7.1.

4.4.2.3 Detection of virulence genes *tdh* and *trh* by multiplex PCR

The detection of *tdh* and *trh* genes were performed using previously described primers (Bej *et al.*, 1999). The details of the primer pairs are given in Table 4.1. PCR amplification was performed 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 ^{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).

4.4.2.4 Detection of type III secretion system genes

For detection of T3SS1 gene, the primer VP1669 previously described by Noriea *et al.* (2010) was used. To detect T3SS2α and T3SS2β genes, the primers VPA1346 and VPA1376 (Caburlotto *et al.*, 2009) respectively were used. The details of the primer sequences used are given in the Table 4.1. 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 at 95 ºC for 5 min, followed by 33 cycles of amplification consisting of denaturation at 95 ºC for 45 sec, annealing at 60 ºC for 40 sec (for T3SS1 and T3SS2 α)/ 50 ºC for 45 sec (for T3SS2 β), extension at 72 ºC for 45 sec, followed by final extension at 72 ºC for 3 min.

Table 4.1 Details of the primer sequences used for the study

Gene	Forward primer	Reverse primer
tdh/trh	*tdh* -F 5' ccatctgtccctttcctgcc 3' *trh*- F 5' ttggcttcgatattttcagtatct 3'	*tdh*-R 5 'ccactaccactctcatatgc 3' *trhR* 5'cataacaaacatatgcccatttccg 3'
T3SS1	VP1669 5' taccgagttgccaacgtg 3'	VP1669 5' gattgttccgcgatttcttg 3'
T3SS2α	VPA1346 5' ggctctgatcttcgtgaa 3'	VPA1346 5′gatgtttcaggcaactctc 3'
T3SS2β	VPA1376 5' gctctccttggtaccaatcac 3'	VPA1376 5' ctgggatcttgatgtcaaggt 3'

4.4.2.5 Gel documentation and image analysis

Refer section 3.4.3.5

4.5 Results

4.5.1 Prevalence of extracellular virulence factors in *Vibrio* from Cochin estuary

Results revealed that gelatinolytic *Vibrios* dominated in the Cochin estuary. DNase and Phosphatase producers were the second and third dominant groups respectively in the estuary. Figure 4.1 shows the percentage distribution of extracellular enzymes among *Vibrio* from Cochin estuary. Out of the 180 strains screened, 83.8% (n=151) produced gelatinase, 83.3% (n=150) produced DNase, 80% (n=144) produced phosphatase, 76% (n=137) produced lipase and 67.7% (n=122) were amylase producers. Comparatively lesser number of chitinase (n=94), caseinase (n=79) and hemolysin (n=39) producers were detected in the estuary.

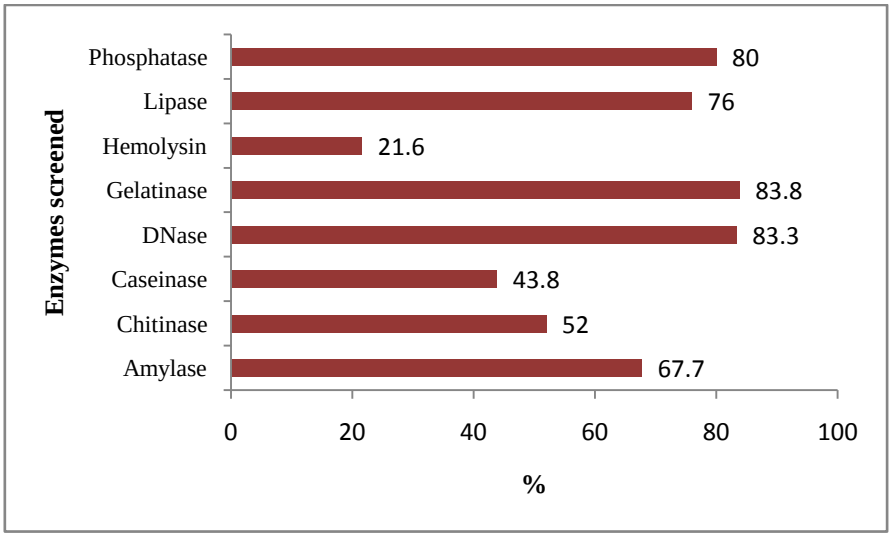

Figure 4.1 Percentage distribution of extracellular enzymes among *Vibrio* from Cochin estuary

4.5.2 Prevalence of extracellular virulence factors in *Vibrio* from shrimp farm

A total of 66 *Vibrio* strains from shrimp farm were screened for production of extracellular enzymes. Figure 4.2 shows the percentage distribution of extracellular enzymes among *Vibrio* from shrimp farm. The gelatinolytic vibrios dominated followed by lipase producers. Out of the 66 strains screened, gelatinase was produced by 89.3% (n=59) strains, lipase by 80.3% (n=53), DNase by 75.7% (n=50), phosphatase by 72.7% (n=48), cellulase by 68% (n= 45), caseinase by 51.5% (n=34) and amylase by 42.4% (n=28) strains. Chitinase producers constituted only 25.7% (n=17). Hemolysin was produced by only 10.6% (n=7) of the strains.

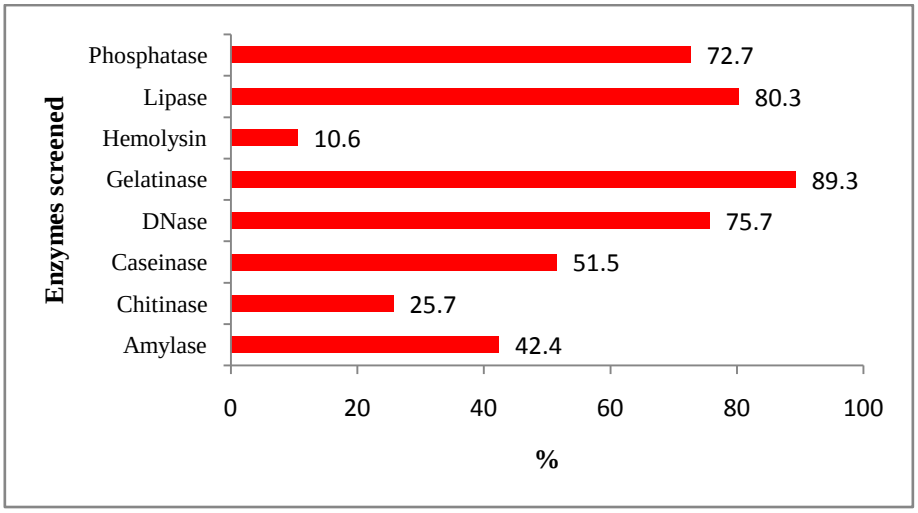

Figure 4.2 Percentage distribution of extracellular enzymes among *Vibrio* from shrimp farm

4.5.3 Prevalence of extracellular virulence factors in *Vibrio* from seafood

A total of 30 *Vibrio* strains from seafood were screened. Figure 4.3 gives the percentage distribution of extracellular enzymes among *Vibrio*

from seafood. Gelatinase and DNase producers were the dominant groups. All the strains produced gelatinase and DNase. About 27 strains (90%) were phosphatase and amylase producers while 80% (n=24) of the strains produced lipase and amylase. Only a few strains were positive for chitinase, caseinase and hemolysin.

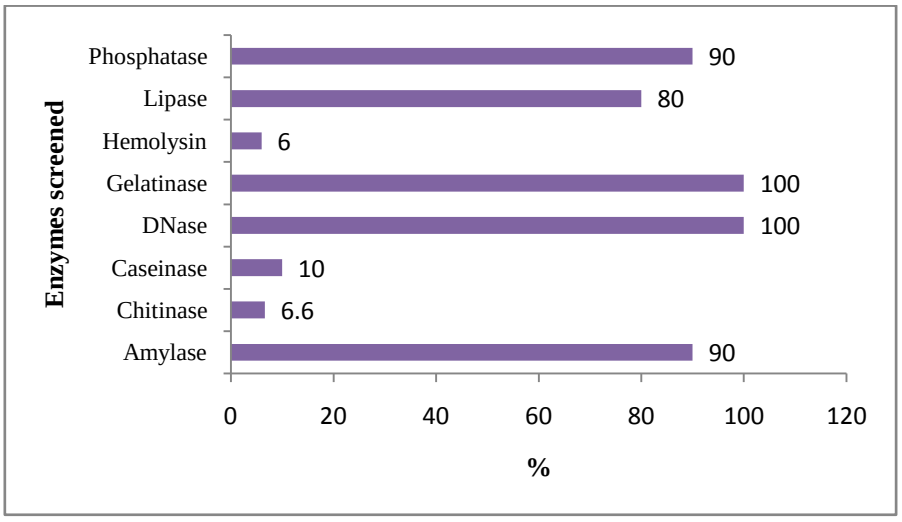

Figure 4.3 Percentage distribution of extracellular enzymes among *Vibrio* from seafood

4.5.4 Relative prevalence of extracellular virulence factors among *Vibrio* from Cochin estuary, shrimp farm and seafood

There was significant variation in the prevalence of DNase, gelatinase, phosphatase, amylase, chitinase, caseinase and hemolysin among *Vibrio* from the three sources ($p<0.01$). However, there was no significant difference in the distribution of lipase among *Vibrio* from the three sources ($p>0.05$). Almost 80% of the *Vibrio* from all the three sources produced lipase. The relative prevalence of extracellular virulence factors among *Vibrio* from Cochin estuary, shrimp farm and

seafood is represented in Figure 4.4. DNase and gelatinase producers were the dominant groups from Cochin estuary and seafood. Gelatinase producing *Vibrio* dominated in shrimp farm followed by lipolytic vibrios. The extracellular enzyme profile of the *Vibrio* from Cochin estuary showed the following dominance pattern: DNase= gelatinase> phosphatase>lipase>amylase>chitinase>caseinase>hemolysin producers. *Vibrio* from shrimp farm revealed the following extracellular enzyme profile: Gelatinase> lipase> DNase> phosphatase> caseinase> amylase> chitinase> hemolysin producers. Among the *Vibrio* from seafood the following pattern was revealed; DNase= gelatinase > phosphatase, amylase>lipase>caseinase>chitinase>hemolysin producers. Five enzymes namely gelatinase, DNase, amylase and phosphatase were frequently expressed in *Vibrio* from seafood. Hemolysin was the least expressed enzyme in all the three sources.

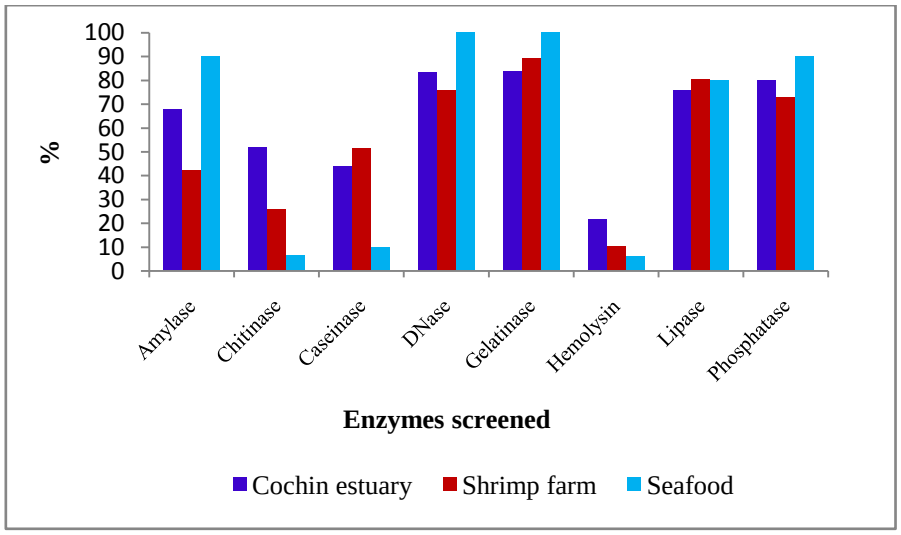

Figure 4.4 Relative prevalence of extracellular virulence factors among *Vibrio* from Cochin estuary, shrimp farm and seafood

4.5.5 Prevalence of *tdh* and *trh* genes among *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood

A total of 85 *V. parahaemolyticus* strains from three different environmental sources were screened for the presence of *tdh* and *trh* genes. Results revealed very low prevalence of the genes among the strains.

By multiplex PCR, the *tdh* and *trh* positive strains produced 270 bp and 500 bp amplicons respectively (Figure 4.5). Among the 45 strains screened from Cochin estuary, 2 strains revealed the presence of *tdh* gene and 7 strains revealed the presence of *trh* gene. None of the strains from shrimp farm and seafood were positive for *tdh* and *trh* genes.

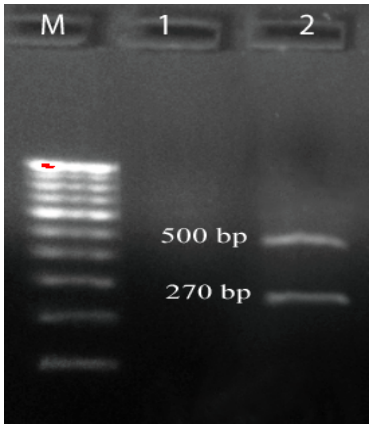

Figure 4.5 Agarose gel showing PCR amplified *tdh* and *trh* genes. Lane M: 100 bp DNA ladder; lane 1: negative control; lane 2: *V. parahaemolyticus* strain carrying *tdh* and *trh* genes

4.5.6 Prevalence of type III secretion system genes among *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood

A total of 85 *V. parahaemolyticus* strains from three different sources were screened for the presence of type III secretion system genes (T3SS) genes.

This included 45 strains from Cochin estuary, 25 strains from shrimp farm and 15 strains from seafood collected from retail market in Cochin. All the strains showed the presence of T3SS1 gene and yielded a 300 bp PCR product (Figure 4.6).

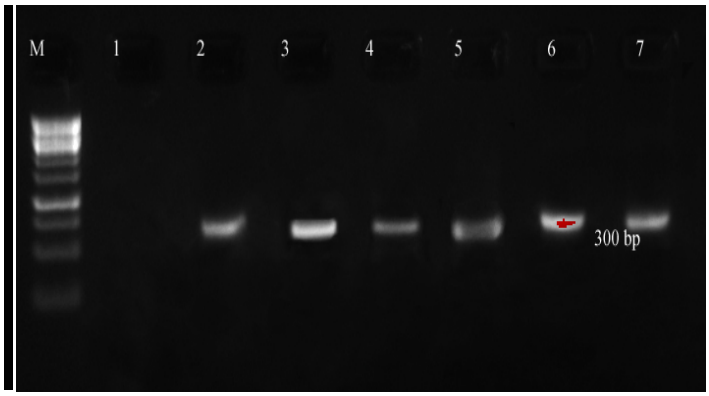

Figure 4.6 Agarose gel showing PCR amplified T3SS1 gene. Lane M: 100 bp DNA ladder; lane 1: negative control; lanes 2 to 7: *V. parahaemolyticus* strains

Among the T3SS2 genes, T3SS2α gene was not detected in any of the strains from the three sources. The T3SS2β positive strains produced a 1067 bp PCR product (Figure 4.7). T3SS2β gene was present in 30 out of the 45 strains from Cochin estuary. Among the 15 strains isolated from fishes, 9 were positive for the T3SS2β gene. Among the 25 strains from shrimp farm, 7 revealed the presence of T3SS2β gene.

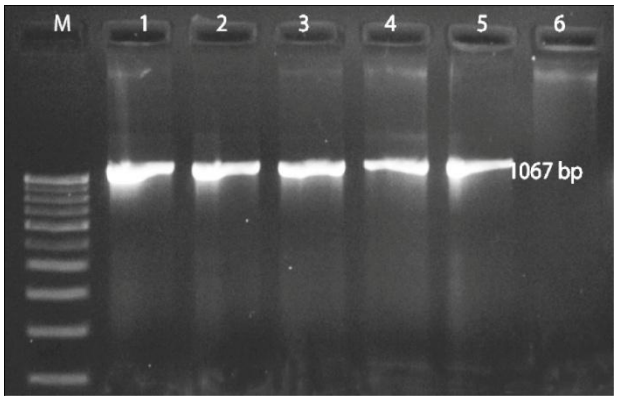

Figure 4.7 Agarose gel showing PCR amplified T3SS2β gene. Lane M: 100 bp DNA ladder; lane 1-5: *V. parahaemolyticus* strains; lane 6: negative control

4.5.7 Relative distribution of virulence genes among *V. parahaemolyticus* from Cochin estuary, shrimp farm and sea food

The distribution of virulence genes in *V. parahaemolyticus* isolated from the three sources was compared. *V. parahaemolyticus* strains from Cochin estuary were comparatively more virulent than those from shrimp farm and seafood.

Figure 4.8 shows the relative distribution of the virulence genes among *V. parahaemolyticus* from various sources. The *tdh* and *trh* genes were detected only in strains from Cochin estuary. T3SS1 gene was detected in all the *V. parahaemolyticus* strains isolated from the three sources. T3SS2α gene was not present in any of the strains screened. T3SS2β gene was most frequently observed in Cochin estuary isolates (66.6%), followed by seafood isolates (60%) and it was least frequent in the shrimp farm isolates (28%).

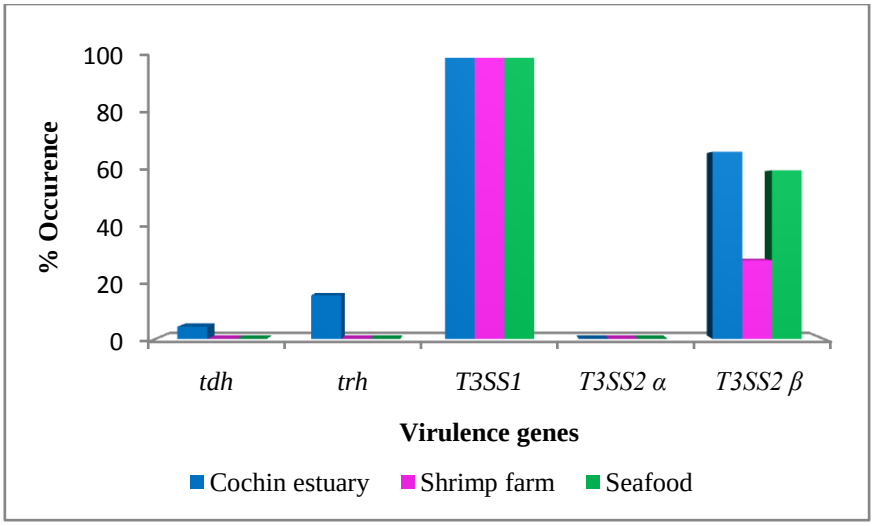

Figure 4.8 Relative distribution of virulence genes among *V. parahaemolyticus* from various sources

4.6 Discussion

4.6.1 Screening of *Vibrio* from Cochin estuary, shrimp farm and seafood for extracellular enzymes

Enzyme assays can be used for understanding the organic matter degradation and nutrient cycling in the aquatic systems. The mineralisation process in the marine environments is carried out mainly by the extracellular enzymes secreted by heterotrophic bacteria (Belanger *et al.*, 1997). Presence of hydrolytic enzymes in *Vibrio* from marine sediments of South west coast of India has been reported by Raghul and Sarita (2011). Gelatinolytic activity observed by these authors among marine vibrios (80.1%) was comparable to our results (84-89%). *Vibrio* from our study areas showed much higher cellulolytic and chitinolytic activity when compared to those from marine environment (Raghul and Sarita, 2011). In contrary to our results, phosphatase producers were not detected in the

previous study (Raghul and Sarita, 2011), while in our study they constituted one among the dominant groups. Though Cochin estuary and adjoining pokkali-cum-shrimp farms are physically linked to the south east Arabian Sea, the nutrient dynamics in these environments seems to be quite different, which in turn might have affected the microbial communities and their functionalities.

The screening of extracellular microbial enzymes is also important to understand their role in pathogenesis. Most of the *Vibrio* in our study belonged to pathogenic species such as *V. parahaemolyticus, V. cholerae, V. vulnificus, V. fischeri, V. mimicus, V. alginolyticus* etc. The enzymes produced by pathogenic species occasionally act as virulence factors (Hase and Finkelstein, 1993; Harrington, 1996). They help the organism to establish an infection in the host. Pathogenic *Vibrio* species produce and secrete several enzymes that are characterised as direct virulence factors causing skin damage (Miyoshi, 2013). The production of urease and hemolysin are considered as typical virulence markers for *V. parahaemolyticus* isolates from clinical samples (Okuda *et al.,* 1997; Miyoshi, 2013). Extracellular DNases acts as endonucleases and cause DNA hydrolysis. Gelatinase production has already been recognized as a virulence factor in bacteremia cases in humans (Vergis *et al.,* 2002). Bacterial lipase activity is mainly involved in nutrient acquisition from the host by degrading the membrane lipids. Phospholipases are also associated with virulence in bacteria and may act as haemolysins (Costa *et al.,* 2013).

Previous studies have already recognised the role of amylase, chitinase and caseinase in the virulence of *Vibrio* (Rodrigues *et al.,* 1993). Previous reports suggest that proteolytic enzymes of fish pathogens such as *Aeromonas* and *Vibrio* have a major role in causing massive tissue damage in the host (Rodrigues *et al.,* 1993; John and Hatha, 2013). Previous report on multi-enzymatic profile of *V. parahaemolyticus* from oysters supported our findings (Costa *et al.,* 2013). They also reported that gelatinase and DNase producers were the dominant groups and caseinase producers were the least (Costa *et al.,* 2013). Shrimp mortality due to viral/bacterial infections was frequent in the farms chosen for present study, for which the aforementioned virulence factors might have contributed significantly.

Our previous studies (Selvam *et al.,* 2012) revealed considerable environmental stress existing in these farms. Under such circumstances, environmental vibrios with potential virulence features could act as opportunistic pathogens, playing major role in disease outbreaks in the estuarine and shrimp farm systems.

Apart from that, we have also observed the presence of enzymes in *Vibrio* isolated from seafood samples, which further evokes the problem. Thus, the presence of these extracellular virulence factors in the *Vibrio* isolated from our study areas may play a substantial role in triggering infections in humans and aquatic animals under favourable conditions.

4.6.2 Screening for virulence genes in *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood

The *tdh* and *trh* genes are considered as major virulence associated genes in *V. parahaemolyticus* (Ceccarelli *et al.*, 2013). In our study, the virulence genes *tdh* could be detected in 4.4% and *trh* in 15.5% strains isolated from Cochin estuary. A previous study revealed much higher prevalence of *tdh* (8.4%) and *trh* (25.3%) genes in *V. parahaemolyticus* isolated from seafoods harvested from the south west coast of India (Raghunath *et al.,* 2008). In another study, the *tdh* and *trh* genes were detected in 4.3% and 0.3% of environmental *V. parahaemolyticus* strains from South Carolina and Georgia coasts (Baker- Austin *et al.,* 2008) while none of the strains from seafood and shrimp were positive for these genes. Previous reports state that most of the environmental strains are not pathogenic to humans, and only 1 to 2% of the environmental strains have shown to be positive for *tdh* and *trh* genes (Hervio-Heath *et al.,* 2002; Robert-Pillot *et al.,* 2004). However, in a recent study, *tdh* and *trh* genes were recovered from 48% and 8.3% of *V. parahaemolyticus* respectively (West *et al*., 2013).

In the present study, all the *V. parahaemolyticus* strains isolated from Cochin estuary, shrimp farm and seafood collected from retail markets were positive for the T3SS1 gene. This was in agreement with previous studies stating that T3SS1 are ubiquitous in *V. parahaemolyticus* (Park *et al.,* 2004 a; Noriea *et al.,* 2010). T3SS1 gene induces cytotoxicity in the host cells (Park *et al.,* 2004 a).

T3SS2 genes are involved in enterotoxicity (Park *et al.,* 2004 a) and helps in the environmental fitness of strains (Hiyoshi *et al.,* 2010; Matz *et al.,* 2011). Among the two T3SS2 genes screened, T3SS2α was not detected in any of the strains from the three sources. T3SS2β was detected in 66.6% strains from Cochin estuary, 28% strains from shrimp farm and 60% strains from seafood. A previous study reported the presence of T3SS2α and T3SS2β genes in *V. parahaemolyticus* isolated from Gulf of Mexico (Noriea *et al.,* 2010). T3SS2 genes are located on the pathogenicity island in Chromosome II of *V. parahaemolyticus* (Ham and Orth, 2012). This location of the gene on a pathogenicity island (Vp-PAI) may facilitate the transfer of this specific DNA fragment from a pathogenic strain to non-pathogenic autochthonous microorganism in the marine environment (Caburlotto *et al.,* 2009). The presence of T3SS genes in our strains suggests the ability of these pathogens to cause infection in humans and marine animals.

Previous studies reported the existence of a correlation of *tdh* with T3SS2α and *trh* with T3SS2β (Park *et al.,* 2004 a; Noriea *et al.,* 2010). However, in the present study we could not observe any such correlation between the occurrence of these genes. Our results were supported by a previous report stating the presence of T3SS2 in *tdh* and *trh* negative environmental strains of *V. parahaemolyticus (*Paranjpye *et al.,* 2012*)*. The current study thus revealed the pathogenic potential of *V. parahaemolyticus* strains from our study areas which enable them to act as opportunistic pathogens and play major role in disease outbreaks in this system. This increases the risk of disease outbreak in Cochin estuary and adjacent shrimp farms.

Hence, the present study emphasises the necessity to implement continuous surveillance of water bodies in Cochin estuary by regulatory agencies for human pathogens, in order to ensure the seafood and public health safety of the study area. The study also highlights the urgent need for adoption of proper water quality management in the aquaculture systems surrounding the estuary.

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Chapter 5

GENOTYPING OF *VIBRIO PARAHAEMOLYTICUS* FROM ESTUARINE, SHRIMP FARM AND SEAFOOD SOURCES BY RAPD AND ERIC-PCR

Contents

5.1 Introduction

Genotyping is the process of determining the genetic constitution or the genotype of an individual by examining its DNA sequence. Even the smallest variation at the sequence level can be detected by this method and thus can be applied efficiently for studying the intra-specific genetic variation among the microorganisms. It can be even used to predict occurrence of any past mutation events. It is widely used for characterizing the epidemiological spreads of pathogenic bacteria. These are specifically useful for source tracking of the strains dispersed in the environment and to provide information on the genetic relatedness of strains and detection of particularly virulent strains, as well as the study of the geographical and host distribution of possible variants of a specific pathogen (Olive and Bean, 1999). Typing pathogens at the strain level is very important for diagnosis, treatment and epidemiological surveillance of bacterial

infections. It is also employed to examine the level of genetic diversity among different strains within the same species.

Several polymerase chain reaction (PCR) based techniques exist for molecular typing of bacterial species. These include Random Amplified Polymorphic DNA (RAPD) and Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR, Restriction Fragment Length Polymorphism (RFLP), Pulse-Field Gel Eletrophoresis (PFGE), Repetitive Sequence-based (REP) PCR etc. In recent years, these molecular-based techniques have been proven to be useful for discriminating among various isolates of pathogens (Tsen and Lin, 2001). RAPD and ERIC-PCR are two widely used methods for typing of *Vibrio parahaemolyticus* (Maluping *et al.,* 2005; Sadok *et al.,* 2013).

Cochin estuary is a major fishing hub of south west coast of India and seafoods from the estuary are exported world-wide. Our previous study revealed high prevalence of *V. parahaemolyticus* in the Cochin estuary (Silvester *et al.*, 2015). Subspecies typing of *V. parahaemolyticus* isolates may be useful for studying the ecology of the bacterium as well as in source tracking the disease causing organism (Chakraborty *et al.,* 2013). Analysis of genetic variation is necessary to determine the epidemiology of these strains.

5.2 Review of literature

Vibrio 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).

It is a leading cause of bacterial gastroenteritis in Asia as well as in other countries (Chiou *et al.,* 2000; Daniels *et al.,* 2000; Wong *et al.,* 2000; McLaughlin *et al.,* 2005) and is attracting increasing interest worldwide where raw or undercooked seafood is often consumed (Chao *et al.,* 2009). Besides wound infections in those exposed to contaminated water (Miyoshi *et al.,* 2008), it also causes septicemia particularly in immunocompromised people (Daniels *et al.,* 2000). It is also the causative agent of acute hepatopancreatic necrosis disease (AHPND) in shrimps that has led to large scale losses in farmed shrimp production in China, Vietnam, Thailand and Malaysia (FAO, 2013; Tran *et al.,* 2013).

Subspecies typing of *V. parahaemolyticus* isolates may be useful for studying the ecology of the bacterium as well as in source tracking the disease-causing organism (Chakraborty *et al.,* 2013). The biochemical methods widely used to identify *Vibrio* species are time-consuming, confusing and laborious because of high phenotypic variation and diversity between closely related species (Alsina and Blanch, 1994). The molecular typing methods are more reliable than the biochemical and serological methods for determining the epidemiological spreads of *V. parahaemolyticus* (Xiao *et al.,* 2011). Hence, at present more focus has been given on the application of molecular-based techniques.

5.2.1 RAPD-PCR

Randomly amplified polymorphic DNA (RAPD) is a PCR-based genotyping technique, using arbitrary primers to detect changes in the DNA sequence at various sites in the genome (Maiti *et al.,* 2009). Analysis of the genetic variation by RAPD reveals proper genetic

diversity due to its ability to generate random markers from the entire genome (Lin *et al.,* 2014). It is one of the simplest methods used to demonstrate DNA variation by producing unique fingerprints of individual strains (Small *et al.,* 2014) with very little template DNA and an unlimited number of primers (Leal *et al.,* 2004). Thus, the main advantages of RAPD-PCR are (i) its applicability where limited quantities of DNA are available (ii) its suitability for working on unknown genomes and (iii) its low expense and high efficiency (Hadrys *et al.,* 1992).

Researchers across the world have applied RAPD-PCR technique in genetic diversity studies of many pathogenic *Vibrio* species including *V. parahaemolyticus* (Shangkuan *et al.,* 1997; Somarny *et al.,* 2002; Sudheesh *et al.,* 2002; Szczuka and Kaznowski, 2004; Maluping *et al.,* 2005; Mahmud *et al.,* 2006; Fouz *et al.,* 2007; Lu and Levin, 2008; Maiti *et al.,* 2009; Teh *et al.,* 2011; Chakraborty *et al.,* 2013; Sahilah *et al.,* 2014; Munirah *et al.,* 2015). Vincent *et al.* (2015) characterised *V. parahaemolyticus* strains from retail fishes in Malaysia using RAPD- PCR and found it as an efficient molecular epidemiological tool to study the temporal distribution of *V. parahaemolyticus*. Molecular typing of *V. parahaemolyticus* isolated from marine finfish/shellfish landed along south-west coast of India was performed by Chakraborthy *et al.* (2013).

RAPD-PCR has also been used in molecular typing of many other pathogenic bacteria like *E. coli* (Dhanashree *et al.,* 2012), *Salmonella* (Rezk *et al.,* 2012), *Flavobacteria* (Sahoo *et al.,* 2010), *Mycobacterium tuberculosis* (Singh *et al.,* 2006) etc.

5.2.2 ERIC-PCR

The ERIC sequence was first described in *Enterobacteriaceae* and later in many other bacterial species (Hulton *et al.,* 1991). These sequences are 126 bp long and are known to be restricted to transcribed regions of the genome (Hulton *et al.,* 1991). ERIC-PCR consists of amplification of genomic DNA enclosed between conserved repetitive regions scattered all over the bacterial genome, utilizing primers targeting highly conserved repetitive sequence elements in the whole bacterial genome (Loubinoux *et al.,* 1999; Wolska *et al.,* 2014). The number and the location of ERIC sequences vary between species and also between strains of the same species (Hulton *et al.,* 1991). Agarose gel electrophoresis of amplified fragments provides band patterns which helps in the differentiation of strains.

The ERIC sequences are present in many copies in the genomes of *Enterobacteriaceae* and *Vibrionaceae* (Hulton *et al.,* 1991; Thompson *et al.,* 2004). This technique is usually employed as molecular marker by many researchers to characterize isolates within *Vibrio* species (Marshall *et al.,* 1999; Ashraf *et al.,* 2001; Ben Kahla-Nakbi *et al.,* 2006). Researchers worldwide have reported the efficiency of ERIC-PCR for genotyping and characterisation of *V. parahaemolyticus* (Maluping *et al.,* 2005; Tanil *et al.,* 2005; Zulkifli *et al.,* 2009; Chakraborthy *et al.,* 2013; Sahilah *et al.,* 2014; Xie *et al.,* 2016) and other pathogenic *Vibrio* species like *V. cholerae* (Dalusi *et al.,* 2015; Ramazanzadeh *et al.,* 2015), *V. alginolyticus* (Rim *et al.,* 2012), *V. tapetis* (Rodriguez *et al.,* 2006) etc. *V. parahaemolyticus* isolates from ready-to-eat food samples in China was divided into 5 clusters when characterised by ERIC-PCR (Xie *et al.,*

2016). *V. cholerae* from clinical samples in Iran were subjected to molecular characterisation by ERIC-PCR and the strains were divided into different subtypes (Ramazanzadeh *et al.,* 2015). Dalusi *et al.* (2015) compared the genetic relation between clinical and environmental isolates of *V. cholerae* and found high degree of relationship between them. In another study by Waturangi *et al.* (2012) was used to analyse genetic heterogeneity of *V. cholerae* from edible ice in Indonesia.

ERIC-PCR has also been widely used in typing of many other pathogens like *Escherichia coli* (Wan *et al.,* 2011; Durmaz *et al.,* 2015), *Bacillus cereus* (Haitao *et al.,* 2011), *Corynebacterium pseudotuberculosis* (Dorneles *et al.,* 2014), *Pseudomonas aeruginosa* (Wolska *et al.,* 2014), *Salmonella typhi* (Nath *et al.,* 2010), *Shigella sonnei* (Ranjbar and Ghazi, 2013) etc. Strains of *Xanthomonas* spp. isolated from vegetable farms in China were discriminated using ERIC-PCR (Asgarani *et al.,* 2015).

5.3 Objectives of the study

Cochin is a major fishing hub along south-west coast of India, contributing over 90% of state-wide exports (Chakraborty *et al.,* 2013) and the Cochin estuary is a favourite tourist destination in Kerala. Since *V. parahaemolyticus* is a predominant seafood borne pathogen in many Asian countries, it is important to discriminate between strains of *V. parahaemolyticus* that are food-borne pathogens, to trace the routes of disease transmission and initiate suitable control measures. Hence, in the present investigation RAPD and ERIC-PCR based approach was undertaken to assess the genomic diversity within *V. parahaemolyticus* strains isolated from Cochin estuary, an adjoining traditional shrimp farm and from seafood

samples collected from retail markets in Cochin for surveillance of possible public health risk. Any possible discrimination in the genetic profiles of the strains based on the isolation source was also analysed.

The specific objectives set are:-

1) To investigate the genetic relatedness and diversity among *V. parahaemolyticus* strains from Cochin estuary, shrimp farm and seafood sources using RAPD-PCR.

2) To investigate the genetic relatedness and diversity among *V. parahaemolyticus* strains from Cochin estuary, shrimp farm and seafood sources using ERIC-PCR.

5.4 Materials and Methods

5.4.1 Bacterial cultures used

A total of 85 *V. parahaemolyticus* were used in the present study. This included isolates from various stations of the Cochin estuary (n=45), from a traditional pokkali cum shrimp farm (n=25) situated at Edavanakkad, adjoining the Cochin estuary and seafood (n=15) collected from retail markets situated in and around Cochin.

5.4.2 Extraction of genomic DNA

The genomic DNA was extracted using a standard DNA extraction method (Ausubel *et al.*, 1987). Briefly, *V. parahaemolyticus* were inoculated into Luria–Bertani (LB) broth medium supplemented with 3% NaCl and incubated overnight at 37 ^{o}C. Bacterial culture (1.5 ml) was centrifuged for 10 min at 12,000 g and the cell pellets were resuspended in 567 µl of Tris–EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), followed by

addition of 30 µl of 10% (w/v) sodium dodecylsulfate and 8 µl of proteinase K (Sigma) (20 mg/ml) and incubation for 1 h at 37 ^{o}C. The samples were treated with 100 µl of 5 M NaCl and 80 µl of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl, and incubated at 65 ^{o}C for 10 min. The mixture was extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, v/v) and DNA was precipitated with 0.6 volume of cold isopropanol and washed with 1 ml of 70% cold ethyl alcohol. The DNA pellet was dried at room temperature and resuspended in TE (10 mM Tris–HCl, 100 mM EDTA, pH 8) buffer and stored at -20 ^{o}C for further use.

5.4.3 RAPD-PCR typing of *V. parahaemolyticus* strains

Two random primers used for the present study were primer 1(5'-AGAAGCGATG-3') (Sahilah *et al.*, 2014) and primer 2 (5'-GAGATGACGA-3') (Bilung *et al.*, 2005). The primers were chosen based on their discriminatory ability, repeatability and stability. 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 at 94 ^{o}C for 2 min, followed by 35 cycles of denaturation (94 ^{o}C for 1 min), primer annealing (36 ^{o}C for 1 min) and primer extension (72 ^{o}C for 2 min). A final primer extension step (72 ^{o}C for 5 min) was also included. The reproducibility of the fingerprints produced by both the primers were analysed by repeating the assay thrice and comparing the banding pattern. All fragments generated by RAPD-PCR using the two primers were analysed separately for determining the RAPD types.

5.4.4 ERIC-PCR typing of *V. parahaemolyticus* strains

ERIC 1R (5'-ATGTAAGCTCCTGGGGATTCA C-3') and ERIC 2(5'-AAGTAAGTGACTGGGGTGAGCG-3') primers were used as previously described by Versalovic *et al.* (1991). 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 program consisted of initial denaturation at 95 ^{o}C for 5 min followed by 35 cycles of denaturation (92 ^{o}C for 45 sec), annealing (52 ºC for 1 min), and extension (70 ^{o}C for 10 min), with a final extension step at 70 ^{o}C for 10 min.

5.4.5 Gel documentation and image analysis

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

5.4.6 Cluster analysis and dendrogram construction

All fragments generated by RAPD-PCR using the two primers and ERIC-PCR were analysed separately for determining the genotypes. The presence (1) or absence (0) of each band was recorded in a spreadsheet and the data was exported into the PRIMER 6 statistical software for Cluster analysis. The relationships between the banding pattern profiles are displayed as dendrogram and expressed as percentage similarity.

5.5 Results

5.5.1 Genotyping of *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood by RAPD-PCR

A total of 85 *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafoods were analysed by RAPD-PCR method. Two random primers were used and PCR amplification by both primers generated multiple fragments of size raging from 300 bp to 3000 bp (Figure 5.1 a, b). Dendrogram constructed using PRIMER 6 software revealed the genetic relatedness among the strains.

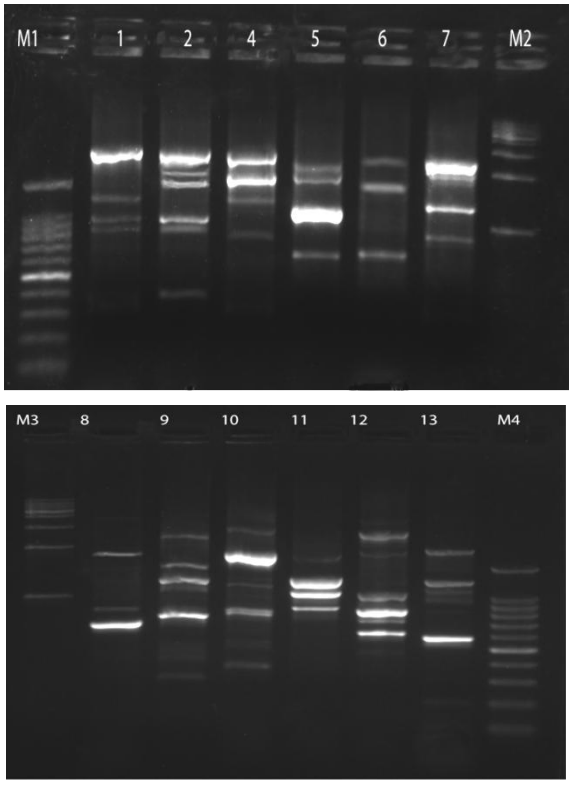

Figure 5.1 Agarose gel showing RAPD-PCR fingerprints of *V. parahaemolyticus* using (a) primer A (b) primer B. Lanes M1 and M4: 100 bp DNA ladder; Lanes M2 and M3: 1 kb DNA ladder; lanes 1-13: *V. parahaemolyticus* strains

5.5.1.1 RAPD-PCR typing of *V. parahaemolyticus* from Cochin estuary

A total of 45 *V. parahaemolyticus* strains from Cochin estuary were analysed by RAPD- PCR. Out of the 45 strains, 38 strains were typable using Primer A. Seven strains were untypable. Dendrogram revealed clustering of the strains into many RAPD profiles (Figure 5.2). At 14% genetic similarity, the strains were clustered into a minor and a major cluster. At 16% similarity, the major cluster was sub-divided, thus separating a single strain (PM8W1) from rest of the strains. Majority of the strains were genetically heterogeneous. RAPD using Primer A also revealed 100% genetic similarity among few strains from estuary. The strains 1W5, M6W2, PM1S1, 10S4, M8W2, 3W6 were genetically 100% similar. Similarly, strains 9S5 and M7S3 were also similar. There was no discrimination in the RAPD profiles of the strains based on geographical region or seasonality.

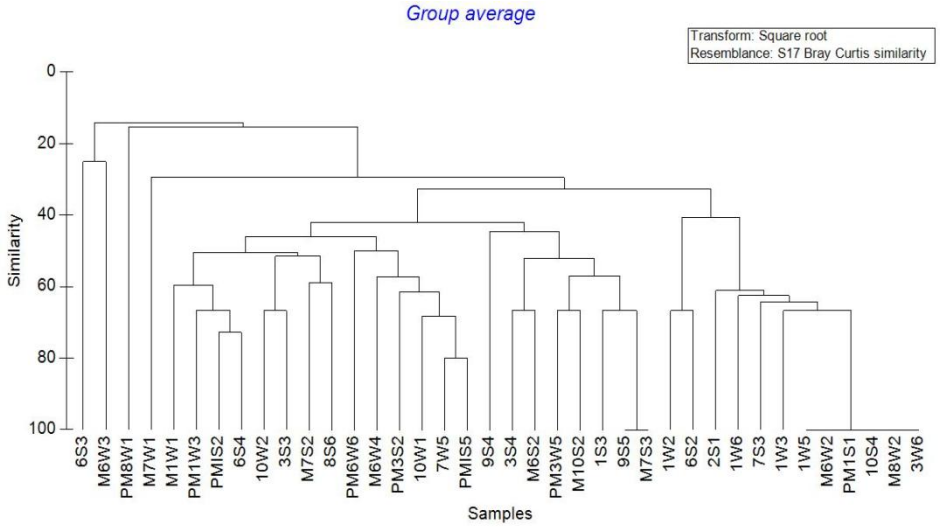

Figure 5.2 Dendrogram revealing clustering of *V. parahaemolyticus* from Cochin estuary based on RAPD-PCR using primer A

RAPD analysis using primer B also generated multiple fragments (Figure 5.3). Out of 45 strains, only 28 were typable using primer B. Rest of the 12 strains were untypable. At 18% similarity, the strains were grouped into one major cluster and one minor cluster. Only few strains were genetically similar. One isolate from station 1 (1W2) shared 100% genetic similarity with an isolate from station 7 (M7S2). Similarly, isolates 2S5 (station 1) and 8S6 (station 8) were similar. There was no season-wise or geographic-wise effect on the genotypic distribution of the strains. Both the primers clearly projected the genetic diversity among the strains.

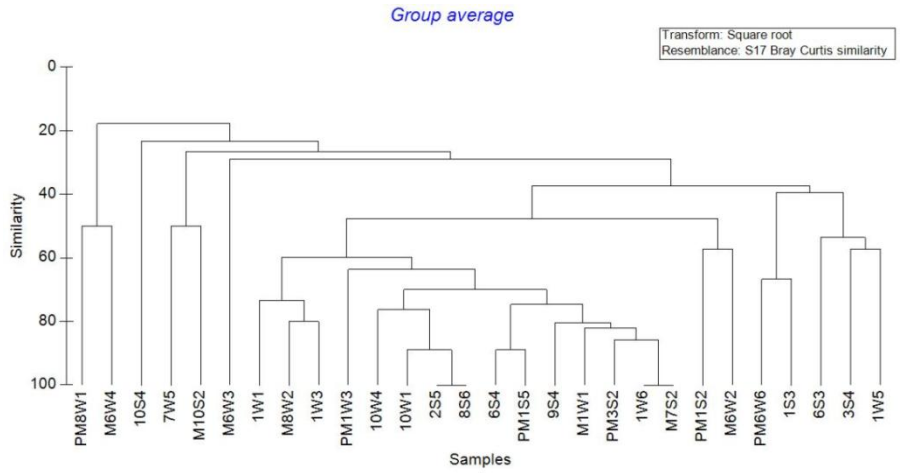

Figure 5.3 Dendrogram revealing clustering of *V. parahaemolyticus* from Cochin estuary based on RAPD-PCR using primer B

5.5.1.2 RAPD-PCR typing of *V. parahaemolyticus* from shrimp farm

A total of 25 strains were used for the study. All of them were typable using primer A. Dendrogram revealed the percentage genetic similarity among the strains (Figure 5.4). At 9% genetic similarity, the 25 strains were grouped into one major cluster with 22 strains and one minor cluster with 3 strains. Three strains MWA2, MAYWV3, MWE14 were

genetically 100% similar. Rest of the 22 strains belonged to different RAPD types and hence were genetically diverse.

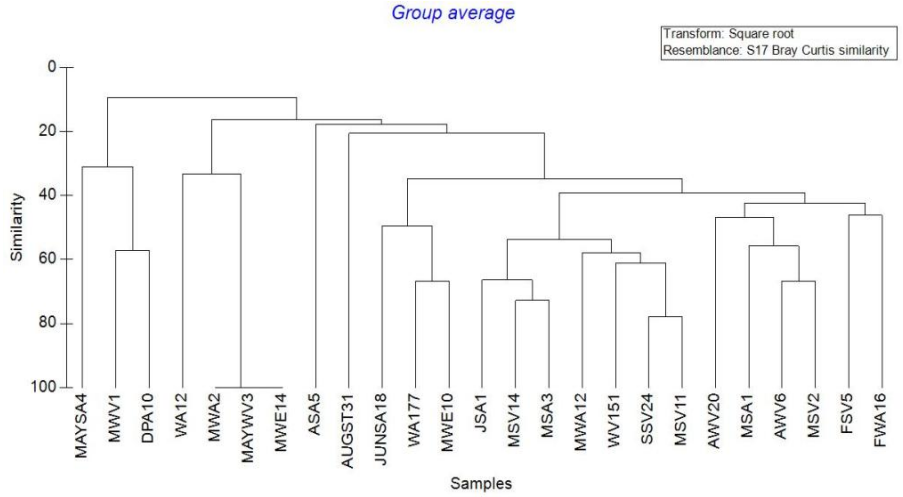

Figure 5.4 Dendrogram revealing clustering of *V. parahaemolyticus* from shrimp farm based on RAPD-PCR using primer A.

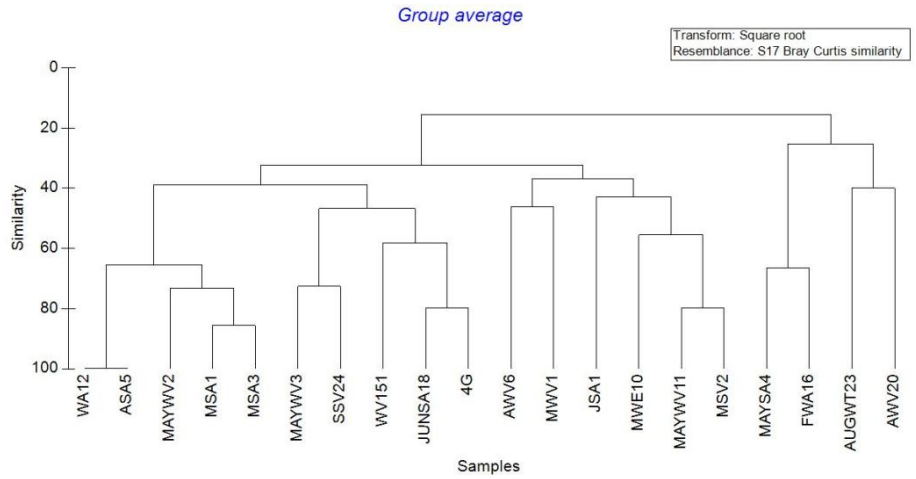

Figure 5.5 Dendrogram revealing clustering of *V. parahaemolyticus* from shrimp farm based on RAPD-PCR using primer B

Out of the 25 strains only 20 strains were typable using primer B. At 16% genetic similarity the 20 strains were divided into one major

cluster and one minor cluster (Figure 5.5). All the 20 strains were genetically heterogeneous and belonged to different RAPD types.

5.5.1.3 RAPD-PCR typing of *V. parahaemolyticus* from seafood

Randomly selected 15 strains isolated from seafood obtained from retail markets were subjected to RAPD-PCR using primer A and B.

All the 15 strains were typable using primer A. Dendrogram revealed the genetic similarity among the strains (Figure 5.6). At 25% genetic similarity, the 15 strains were grouped into one major cluster and one minor cluster. None of the strains were genetically 100% similar. All belonged to different RAPD types and thus were genetically heterogeneous.

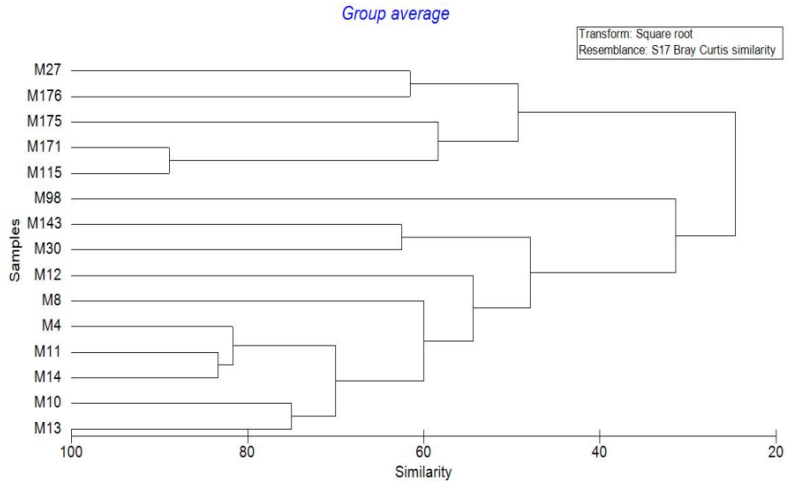

Figure 5.6 Dendrogram revealing clustering of *V. parahaemolyticus* from seafood based on RAPD- PCR using primer A

Only 13 out of 15 *V. parahaemolyticus* strains were typable using primer B. At 49% similarity, the strains were grouped into a major and a minor cluster (Figure 5.7). Strains M8 and M12, M40 and M176 were genetically 100% similar.

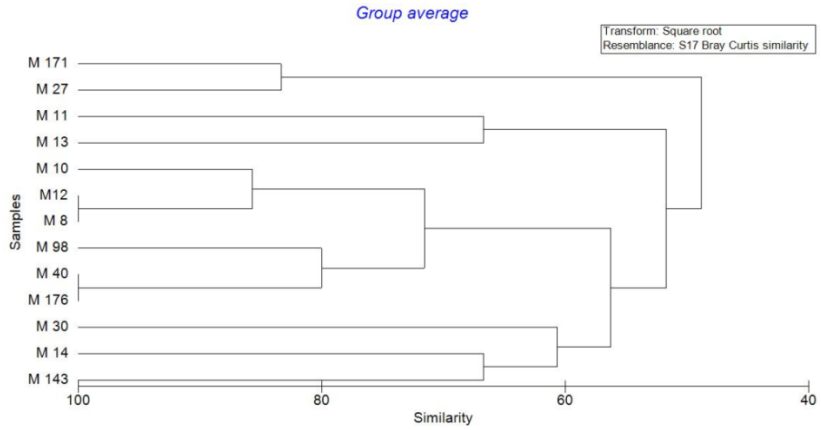

Figure 5.7 Dendrogram revealing clustering of *V. parahaemolyticus* from seafood based on RAPD-PCR using primer B

5.5.1.4 Comparison of RAPD-PCR typing of *V. parahaemolyticus* from the three sources

Dendrogram of RAPD-PCR using primer A revealed clustering of *V. parahaemolyticus* strains from the three sources into many RAPD profiles (Figure 5.8). *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood were grouped into one main cluster and one single isolate (ASA5) at 9% genetic similarity. At 11% similarity the major cluster was divided into sub- clusters A and B. Sub- cluster A included 3 strains from shrimp pond. B was sub-clustered to B1 and B2. It was found that 6 *V. parahaemolyticus* strains from Cochin estuary (PM1S1, 10S4, M8W2, 3W6, 1W5, M6W2, 1W3) and 3 strains from shrimp farm (MWA2, MAYWV3 and MWE14) shared similar RAPD fingerprints. They were 100% identical genetically.

Combined RAPD-PCR analysis using primer B generated fragments of size ranging from 300 bp to 3000 bp (Figure 5.9). At 15% similarity the strains were grouped into one major cluster A and one minor cluster B. Cluster A contained isolates from all the three sources. Few shrimp farm and estuarine isolates alone formed the cluster B.

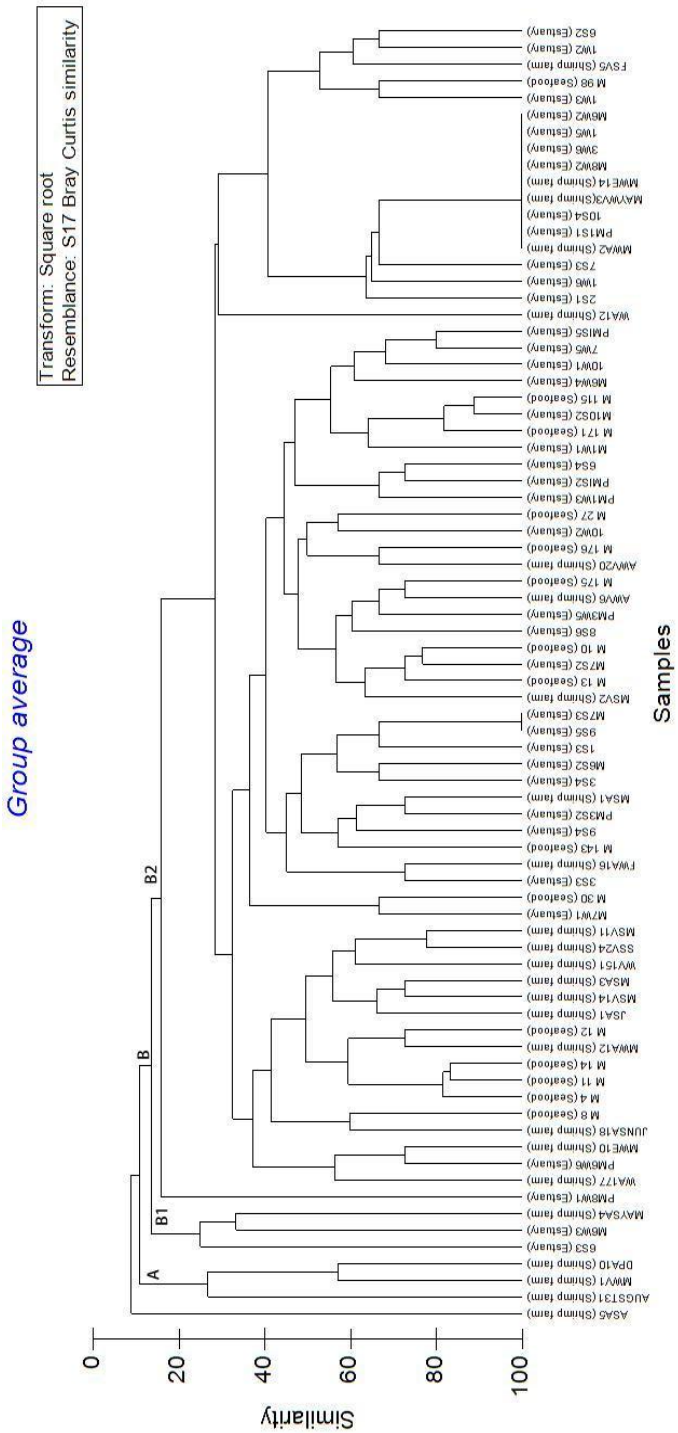

Figure 5.8 Combined dendrogram revealing clustering of *V. parahaemolyticus* from Cochin estuary and shrimp farm based on RAPD-PCR using primer A

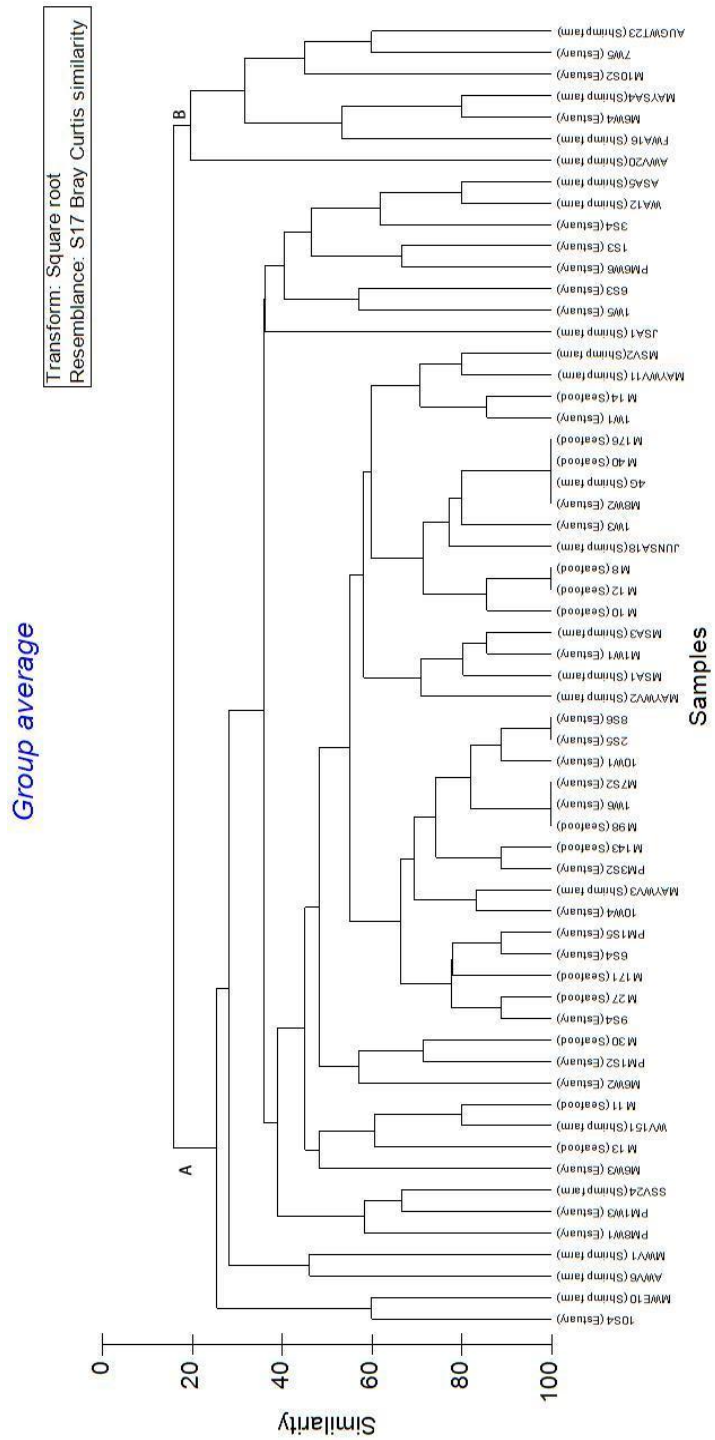

Figure 5.9 Combined dendrogram revealing clustering of *V. parahaemolyticus* from Cochin estuary and shrimp farm based on RAPD-PCR using primer B

5.5.2 Genotyping of *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood by ERIC-PCR

The method also revealed high level of genetic heterogeneity among the tested *V. parahaemolyticus* strains. ERIC-PCR generated fragments of size ranging from 200 to 3000 bp. It was interesting to note a common band of 700 bp in all the strains regardless of the different ERIC-PCR profiles observed.

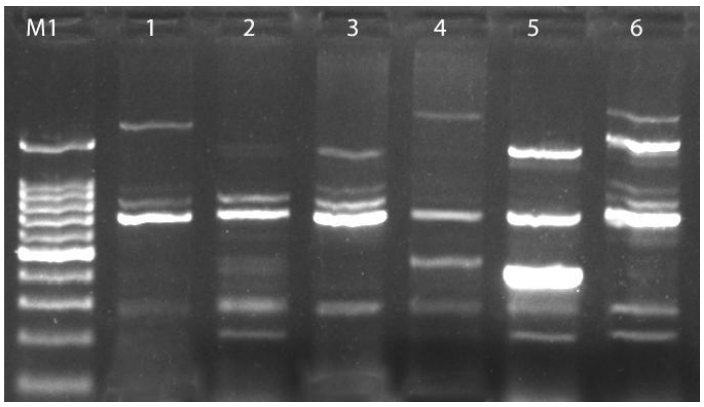

Figure 5.10: Agarose gel showing ERIC-PCR fingerprints of *V. parahaemolyticus*. Lane M1: 100 bp DNA ladder; lane 1-6 *V. parahaemolyticus* strains

5.5.2.1 ERIC-PCR typing of *V. parahaemolyticus* from Cochin estuary

A total of 45 strains isolated from various stations of Cochin estuary were subjected to ERIC-PCR typing. Only 37 were typable by ERIC-PCR. At 32% similarity, the strains were grouped into a major cluster and a single strain (8S6) (Figure 5.11). At 36% similarity, the major cluster was divided into two sub-clusters. Majority of the strains were genetically diverse. Few strains isolated from same stations during different seasons

belonged to same genotype (M7W1 and 7W5; 6S2 and M6W4). ERIC-PCR could also project 100% genetic similarity among few strains isolated from different stations during different seasons (1W2 and PM3W5; 1W3 and PM8W1; 3S4 and PM6W6; M1W4 and M10S2). The dendrogram thus revealed that *V. parahaemolyticus* strains in the present study were not grouped based on their source of isolation or seasonality of collection.

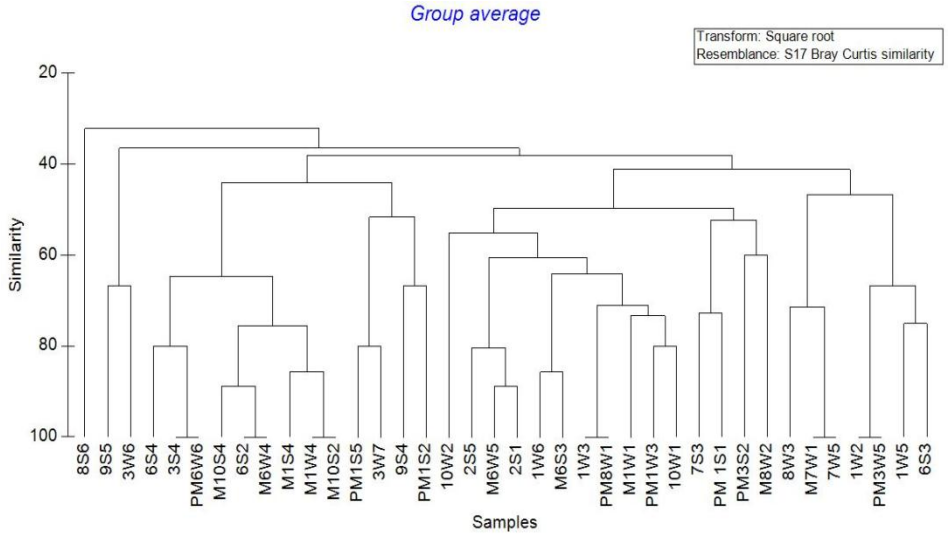

Figure 5.11 Dendrogram revealing clustering of *V. parahaemolyticus* from Cochin estuary based on ERIC-PCR

5.5.2.2. ERIC-PCR typing of *V. parahaemolyticus* from shrimp farm

All the 25 *V. parahaemolyticus* strains from shrimp farm were typable by ERIC-PCR. At 27% genetic similarity the strains were divided into a major cluster and a minor cluster with a single isolate (4G) (Figure 5.12). At 28% similarity, the major cluster was subdivided into 2 sub clusters, one cluster with 22 strains, a minor cluster with 2 strains. Strains

DWA218 and SV178; MSA3 and MWA12 shared 100% genetic similarity. Rest of the 21 strains had different ERIC types and thus were genetically heterogeneous.

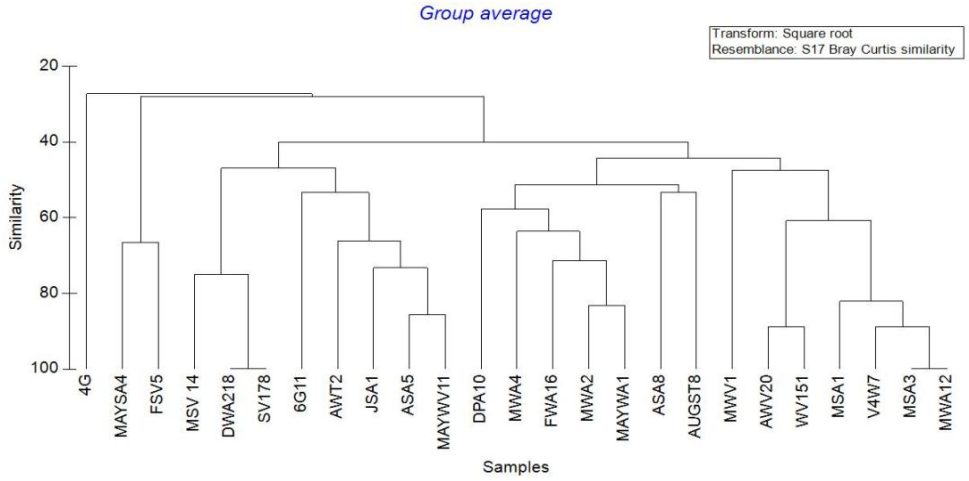

Figure 5.12 Dendrogram revealing clustering of *V. parahaemolyticus* from shrimp farm based on ERIC-PCR

5.5.2.3 ERIC-PCR typing of *V. parahaemolyticus* from seafood

All the 15 *V. parahaemolyticus* strains from seafood were typable by ERIC-PCR. At 33% similarity the strains were grouped into 2 clusters (Figure 5.13). Strains M12 and M176 shared 100% genetic similarity. Similarly, strains M11 and M13 were also similar. Rest of the 11 strains had different ERIC patterns and were of different genotypes.

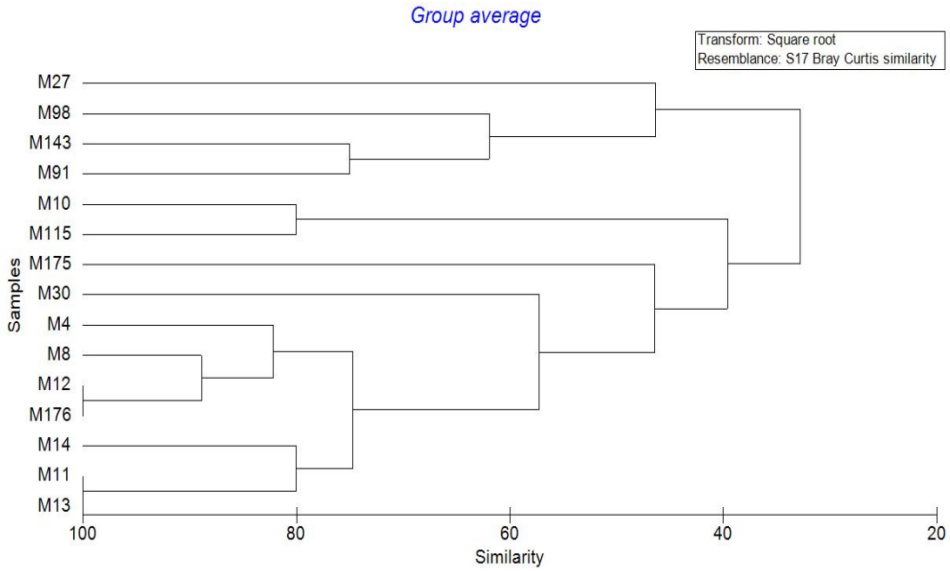

Figure 5.13 Dendrogram revealing clustering of *V. parahaemolyticus* from seafood based on ERIC- PCR

5.5.2.4 Comparison of ERIC-PCR typing of *V. parahaemolyticus* from the three sources

At 28% genetic similarity all strains were grouped into a large cluster except for a single isolate (4G) (Figure 5.14). At 29% similarity this cluster was divided to 2 subclusters A and B. Cluster A was again grouped into 2 sub-clusters A1 and A2. The A1 cluster had strains from all the three sources whereas all the strains in A2 cluster was from Cochin estuary except for a strain each from seafood and shrimp farm. Most of the shrimp farm isolates were grouped into cluster B along with a single isolate from estuary and 4 from seafood.

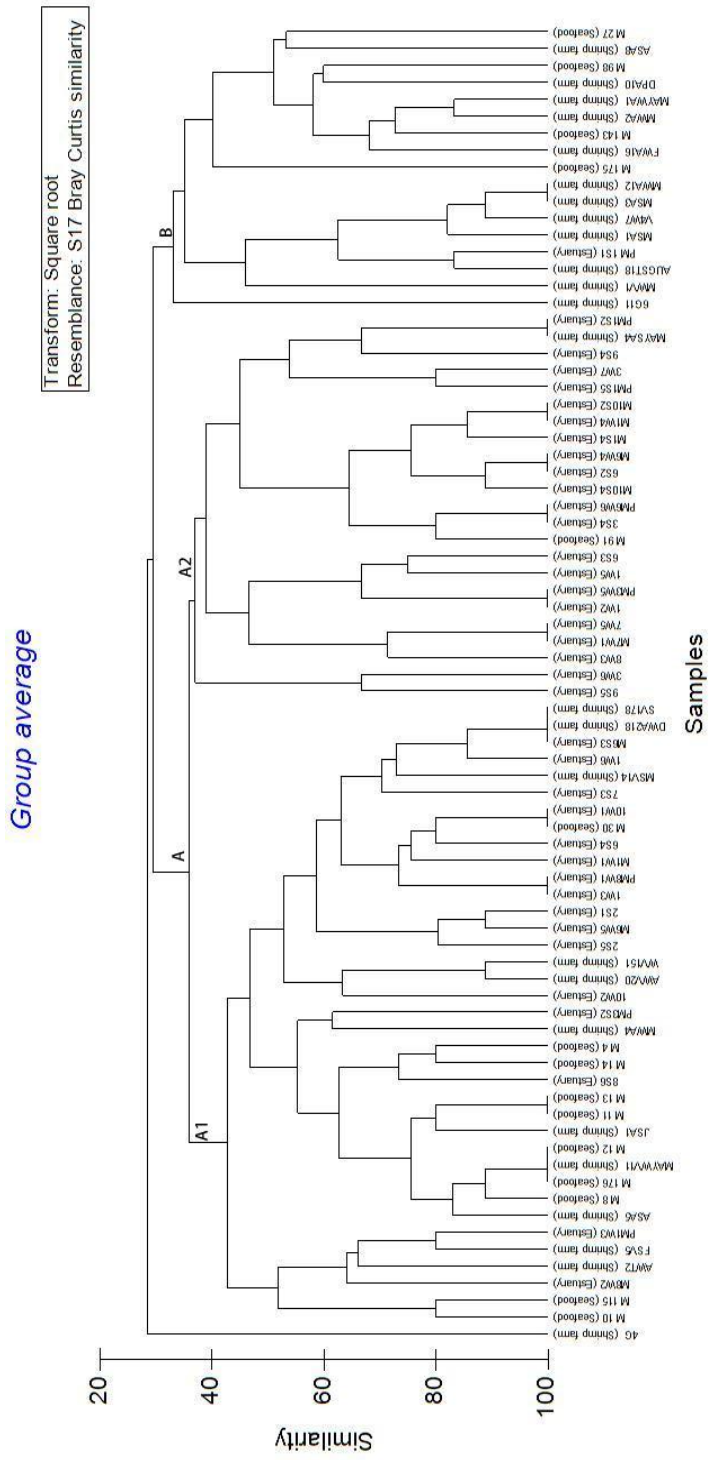

Figure 5.14 Combined dendrogram revealing clustering of *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood based on ERIC- PCR

5.6 Discussion

5.6.1 Genotyping of *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood by RAPD-PCR

In the present study, RAPD-PCR revealed high level of genetic diversity within the *V. parahaemolyticus* isolated from different sources. There was diversity even among isolates from same source. Various molecular typing methods have been developed and applied for analysing the genetic variation of this bacterium (Letchumanan *et al.*, 2014). The methods like RAPD-PCR have been used to demonstrate genetic diversity of this organism (Ansaruzzaman *et al.*, 2008). The molecular typing methods are more reliable than the biochemical and serological methods for determining the epidemiological spreads of *V. parahaemolyticus* (Xiao *et al.*, 2011). Moreover, The RAPD-PCR and ERIC-PCR techniques are widely used in diversity studies of several *Vibrio* spp. (Shangkuan *et al.*, 1997; Somarny *et al.*, 2002; Sudheesh *et al.*, 2002; Szczuka and Kaznowski 2004; Maiti *et al.*, 2009; Chakraborty *et al.*, 2013; Sadok *et al.*, 2013; Hatje *et al.*, 2014; Sahilah *et al.*, 2014). Chakraborty *et al.* (2013) evaluated the genetic relationships of *V. parahaemolyticus* strains from seafood samples collected from fish landing centres located in and around Cochin using RAPD- PCR. They reported grouping of strains based on the source of isolation. On the contrary, in the present study, grouping of strains from different locations into the same clusters indicates that there is no bio-geographical effect on the genotypic distribution of *V. parahaemolyticus* populations from various stations of the Cochin estuary, at the scale of meters and kilometres. Even though few sub-clusters were formed based on isolation

source there was no major clustering of strains based on isolation source. Similarly, no seasonal effect was observed in their genotypic distribution. Another study on *V. parahaemolyticus* from seafoods from South west coast of India reported clustering of the strains based on the host of isolation rather than seasonality (Chakraborty *et al.,* 2008).

We could observe presence of strains with similar genotype from different isolation sources suggesting the cycling of pathogens between different environments. Hence, there is a possibility these pathogens can enter humans through various food vehicles and, thus, cause clinical implications. The presence of similar clones from various environments also indicates there is no bio-geographical effect on the distribution of *V. parahaemolyticus* populations at the scale of metres and kilometres. In a similar study on *V. harveyi* strains from the Southwest coast of India the presence of heterogeneous genotypes within the isolates were highlighted (Maiti *et al.,* 2009). Another study from the Southwest coast of India on RAPD typing of *V. parahaemolyticus* from crustaceans, cephalopods and finfish revealed two major clusters, such as one comprising all the isolates from crustaceans and the other comprising fish and cephalopod isolates (Chakraborty *et al.,* 2008). The fish and cephalopods isolates were grouped together, thus indicating their genetic similarity. In another study from tiger shrimp culture environments along the Southwest coast of India, the toxigenic and non toxigenic *V. parahaemolyticus* isolates were grouped into different clusters (Chakraborty and Surendran, 2009).

Previous reports on genotyping of *V. parahaemolyticus* strains from local cockles in Malaysia also highlighted genetic heterogeneity among

the strains, thus supporting our findings (Bilung *et al.*, 2005; Sahilah *et al.*, 2014). Szczuka and Kaznowski, (2004) reported that strains with similarities below 90% were considered genetically unrelated. In the present study majority of the strains exhibited similarity below 90% and hence we conclude that the *V. parahaemolyticus* strains from our study areas are genetically diverse.

5.6.2 Genotyping of *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood by ERIC-PCR

Random Amplified Polymorphic DNA (RAPD) and Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR are two widely used methods for typing of *V. parahaemolyticus* (Maluping *et al.*, 2005; Sadok *et al.*, 2013). Subspecies typing of *V. parahaemolyticus* isolates may be useful for studying the ecology of the bacterium as well as in source tracking the disease-causing organism (Chakraborty *et al.*, 2013). Analysis of genetic variation is necessary to determine the epidemiology of these strains.

In the present study, ERIC-PCR also clearly demonstrated the level of genetic diversity among the *V. parahaemolyticus* strains isolated from estuary, shrimp farm and seafood collected from retail market. Genetic similarity between certain strains of *V. parahaemolyticus* isolated from various environmental sources was also observed. Presence of genetically similar clones from various sources demonstrates the cycling of pathogen between various environments. Strains that are genetically related are usually derived from a single cell, and its progeny expresses the same biochemical properties, virulence factors, and genomic traits. These strains belong to the same clone (Olive and Bean, 1999).

Chakraborty *et al.* (2013) carried out genotyping of *V. parahaemolyticus* strains isolated from marine finfish/shellfish along south-west coast of India by ERIC-PCR. The dendrogram generated two major clusters, one with the finfish and cephalopod isolates and the other with shellfish isolates suggesting that the strains isolated from finfish samples showed higher genetic similarity with the strains from cephalopods than that of shellfish isolates. Maluping *et al.* (2005) performed molecular typing of *V. parahaemolyticus* isolates from Philippines and reported genetic variation within the *V. parahaemolyticus* strains. Another study on ERIC-PCR typing on 126 *V. parahaemolyticus* isolates from clinical and aquatic products was reported from China (Xie *et al.*, 2016). In the particular study, the isolates were classified into eight clusters and the authors concluded that the ERIC-PCR method clearly revealed genetic variation and relatedness between the clinical and aquatic isolates. In a previous report on molecular characterisation of *V. cholerae* strains isolated from an outbreak in southern India, similar banding patterns were obtained among the strains. This revealed the clonal dissemination of a single predominant *V. cholerae O1* strain throughout the 2004 outbreak in Chennai (Goel *et al.*, 2010).

Our study disclosed ERIC-PCR as a better tool to type *V. parahaemolyticus* strains compared to RAPD-PCR. All the strains from shrimp farm and seafood were not typable using RAPD-PCR, whereas, ERIC-PCR was efficient enough to type all the strains from the 2 sources. Similarly, majority of the isolates from estuary was typed by ERIC-PCR. RAPD-PCR using primer A could type majority of the estuarine isolates whereas, very few were typable using primer B.

From the present study, it could be concluded that *V. parahaemolyticus* strains are genetically heterogeneous. Tremendous genomic variation was observed among the co-occurring *Vibrio* populations. The factors leading to the genomic variation observed in our study are unknown, but might involve both intracellular processes (such as rearrangements and point mutations) and horizontal gene transfer processes. Future studies are needed to determine the factors contributing to the genetic diversity. Further studies are also required to determine an association between a particular genotype to virulence and disease, which might also help in establishing the origin of a pathogenic strain. The study of genetic diversity of such pathogens could help in molecular epidemiology for tracing the route of infection, which, in turn, could result in better implementation of public health intervention strategies. Such studies will be also useful in treatment of bacterial diseases, whereby similar treatment can be applied for bacteria belonging to same clone.

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Chapter 6

IDENTIFICATION AND DIFFERENTIATION OF PATHOGENIC VIBRIOS BY *GROEL* PCR-RFLP METHOD

Contents

6.1 Introduction

Proper disease diagnosis and treatment relies largely on accurate diagnostics of the pathogen. Moreover, precise detection of the pathogen is very crucial in epidemic-preventing surveillances. 16S rRNA based identification has been found to be ineffective for identification of closely related pathogenic *Vibrio* spp. (Thompson *et al.,* 2005). 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 that 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). Some of the major aquaculture pathogens include *V. harveyi, V. anguillarum, V. fischeri* etc. (Thompson *et al.,* 2004).

Most of the closely related *Vibrio* spp. have similar phenotypic characters making it difficult to differentiate them biochemically (Croci *et al.,* 2007). Phenotypic approaches also have many drawbacks such as being time consuming, labour intensive and inaccurate detection specificity (Izumiya *et al.,* 2011). 16S rRNA-RFLP has proved to be inefficient to differentiate between closely related species with minimum difference in their nucleotide sequences. Alternative phylogenetic markers are needed for reliable and accurate species-specific identification of pathogenic vibrios.

6.2 Review of literature

6.2.1 Phylogenetic analysis of *Vibrio*

At present, there are more than 100 recognised species under the genus *Vibrio* (Okada *et al.*, 2010). They are highly abundant in aquatic, estuarine and marine environments and aquaculture settings worldwide (Thompson *et al.*, 2004; Letchumanan *et al.*, 2014). Most of the *Vibrio* spp. have similar phenotypic characters making it difficult to differentiate them biochemically (Croci *et al.,* 2007). 16S rRNA sequencing has been considered as the most reliable tool for the allocation of genera, species and strains into the family *Vibrionaceae*. In the past, many researchers have applied 16S rRNA sequences for phylogenetic analysis of *Vibrionaceae* (Dorsch *et al.,* 1992; Kita-Tsukamoto *et al.,* 1993; Ruimy *et al.,* 1994; Hoffmann *et al.,* 2010). However, the genus *Vibrio* contains a large number of closely related species sharing high level of sequence similarity with just 0.1 or 0.2% difference in the nucleotide sequence of 16S rRNA (Montieri *et al.,* 2010). Many pathogenic *Vibrio* species such

as *V. mimicus and V. cholerae, V. parahaemolyticus and V. alginolyticus, V. fluvialis and V. furnissii, V. anguillarum and V. ordalii* share nearly identical 16S rRNA gene sequences (Thompson *et al.,* 2004). The application of 16S rRNA gene sequencing is not precise for the discrimination of such closely related species (Thompson *et al.,* 2005). Alternative phylogenetic markers are needed for reliable species level identification. Different other loci such as 23S rRNA, *gapA, gyrB*, hsp60 and *recA* have been used for phylogenetic studies and the identification of *Vibrionaceae* species (Thompson *et al.,* 2004). Thompson *et al.* (2007) applied *atpA* gene sequences for the phylogenetic analysis of vibrios and related species**.** The efficiency of 16S rRNA and hemolyisn genes in phylogenetic analysis of *V. alginolyticus, V. harveyi, V. parahaemolyticus* and *V. mimicu*s was compared previously (Wang *et al.,* 2009). They concluded hemolysin to be superior to 16S rRNA in phylogenetic taxonomy of vibrios. Montieri *et al.* (2010) studied the usefulness of *toxR* gene for phylogenetic analysis of closely related *V. parahaemolyticus* and *V. alginolyticus* and proved it as an efficient phylogenetic marker than the 16S rRNA gene. Phylogenetic analysis of *V. cholerae* was studied on basis of three house-keeping genes *sd, hlyA* and *recA* and showed the similarity of strains based on *asd* and *hlyA* phylogenetic trees were higher than *recA* (Farhadi *et al.,* 2015). Li *et al*. (2016) performed $bla_{CARB\text{-}17}$-specific PCR-based detection of *V. parahaemolyticus* and concluded the gene to be more conserved than *tlh, toxR* and *atpA* genes in the species. *GroEL* gene is a potential candidate as general phylogenetic marker because of its ubiquity and conservation in nature (Yushan *et al.,* 2010).

6.2.2 Restriction fragment length polymorphism

Molecular markers also known as genetic markers are a powerful genetic tool. It has been widely used in genetic studies over years. It reveals polymorphism at the DNA level. RAPD and RFLP are widely used as molecular markers. Restriction fragment length polymorphism (RFLP) was invented in 1984 by the English scientist Alec Jeffreys during his studies on hereditary diseases. The technique involves fragmenting a DNA sample by a restriction enzyme that can recognize and cut DNA wherever a specific short sequence occurs in a process known as a restriction digest (Lin *et al.,* 2014). Restriction endonucleases or restriction enzymes are called as molecular scissors as they cut DNA. They make double stranded cuts or incisions, once through each (i.e. each strand) of the DNA double helix. The PCR-RFLP technique consists of PCR amplification of genes, e.g., 16S rRNA, *gyrB* and *rpoD* and subsequent restriction of the amplified products by restriction enzymes to obtain band patterns (Thompson *et al.,* 2004). The obtained patterns are visualised on an agarose gel. PCR-RFLP has been widely used by many researchers in molecular typing, differentiation and detection of pathogenic bacteria.

16S rRNA PCR-RFLP of *flic* gene was applied by Moreno *et al. (*2006) to type *E. coli* strains. Mehndiratta *et al.* (2009) successfully performed the molecular typing of *Staphylococcus aureus* by PCR-RFLP of *spa* gene. 16S rRNA PCR-RFLP was applied to identify *Enterococcus* strains (Scheidegger *et al*., 2009). Differentiation of *Xanthomonas* species was performed by PCR-RFLP of *rpfB* and *atpD* genes (Simões *et al*.,

2007). The authors found *rpfB* and *atpD* genes to be better than 16S and 23S rRNA genes in order to identify and classify *Xanthomonas* sp. Ghorbanalizadgan *et al.* (2016) found that PCR-RFLP of *flaA* gene can be applied as cheap, rapid and reliable method for epidemiological study of *C. jejuni* isolates. The efficiency of PCR-RFLP of MAM-7 gene to differentiate between virulent and environmental *V. parahaemolyticus* strains was proved previously (Lopez *et al.*, 2015). Yoon *et al.* (2003) genotyped six pathogenic *Vibrio* spp. by 16S rRNA PCR-RFLP. PCR-RFLP of *groEL* gene was applied by Chang *et al.* (2003) for detection and differentiation of *Bacillus cereus* strains.

6.2.3 *GroEL* gene

The *groEL* gene encodes a 60 kDa subunit known as HSP60, 60 kDa chaperonin or heat shock protein. This protein is indispensable for maintaining normal physiological function of the cell and is also a major antigen of many bacterial pathogens (Yushan *et al.*, 2010).

The *groEL* gene is reported to be one of the most conserved genes in nature (Giuseppe *et al.*, 2008). However, greater level of interspecies variation in its sequence makes it an ideal target for species classification. Previous reports suggest that it has already been used in the typing and identification of *Salmonella* sp., *Staphylococcus* sp., *Campylobacter jejuni*, *Bacillus cereus* and *Vibrio anguillarum* (Chang *et al.*, 2003; Kim *et al.*, 2010; Yushan *et al.*, 2010). Lee *et al.* (2003a) used *groEL* gene for differentiation of *Rickettsiae* from *Ehrlichia*. A study on *groEL* gene analysis of *Borrelia burgdorferi* sensu showed that the gene can be used for differentiation of the bacterium (Lee *et al.*, 2003b). A study by Park

et al. (2004b) successfully employed *groEL* gene for intraspecies differentiation of *Borrelia afzelii* and *Borrelia garinii*. Reports indicate that *groEL* gene is more heterogeneous than 16S and 23S rRNA genes (Yushan *et al.,* 2010) and hence, can be used for *Vibrio* identification (Nishibuchi, 2006).

6.3 Objectives of the study

Accurate and rapid identification of human pathogenic *Vibrio* spp. in samples from disease outbreak areas and environment is very important as far as public health is concerned. It is crucial for effective diagnosis and treatment of diseases. Similarly, control and prevention of disease outbreaks caused by *Vibrio* in aquaculture farms is also feasible only if the pathogen is detected rapidly and accurately. Successful diagnosis depends largely on the detection specificity and accuracy of the diagnostic method employed. In the current chapter an attempt was made to apply *groEL* PCR-RFLP method to identify and differentiate pathogenic *Vibrio* spp. The objectives set are:-

- To evaluate the effectiveness of *groEL* PCR-RFLP method for rapid differentiation of some of the major pathogenic *Vibrio* species.
- To validate the method *in silico* using NEBcutter tool.
- To compare the interspecies phylogenetic relationship of *Vibrio* based on *groEL* and 16S rRNA genes.

6.4 Materials and Methods

6.4.1 Bacterial strains used

Eight strains previously isolated and maintained in the laboratory were used. It included eight *Vibrio* spp. namely *V. parahaemolyticus* PM1S2, *V. mimicus* M9W1, *V. cholerae* AWT62, *V. diazotrophicus* JWV30, *V. vulnificus* JWV13, *V. anguillarum* JWA3, *V. alginolyticus* VKF44 and *V. fluvialis* 8M1 with Genbank accession numbers KM406325, KT187246, KT187245, KT005559, KT005560, KC549801, KT005561 and KT163389 respectively. A non-*Vibrio* species *Aeromonas hydrophila* SA7 (KC549803) was also included.

6.4.2 PCR amplication of the *Vibrio* specific *groEL* gene

Genomic DNA of the bacterial strains was extracted using the Bacterial genomic DNA prep kit (Origin diagnostics, India). A universal primer set Rflp-up 5′- TCCARAACATGGGCGCACAA- 3′ and Rflp-rp 5′-ACGTTTTGYTCTTCGTTGTCRC-3′ previously designed by Hossain *et al.* (2014) using the most conserved region of *groEL* gene of *Vibrio* spp. was used in the study. 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). PCR conditions included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation (94 °C for 30 sec), annealing (69 °C for 30 sec) and extension (72 °C for 30 sec), with a final extension at 72 °C for 7 min (Hossain *et al.*, 2014).

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 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 sequenced at Scigenom sequencing facility (Kochi, India) and the sequences were deposited in Genbank and allotted with accession numbers.

6.4.3 *GroEL* PCR-RFLP analysis

The amplicons were digested separately at 37 °C using five different restriction enzymes such as Alu1, Rsa1, Mbo1, Dde1 and Hha1 (Thermo Fisher Scientific, India), according to the manufacturer's instruction. About 6-8 µl of PCR products were subjected to restriction digestion in a total reaction volume of 30 µl with 2 µl of the respective enzyme. The resulting fragments were resolved by agarose gel (2%) electrophoresis. Detection of restriction fragments shorter than 100 bp from 2% agarose gels would be inaccurate (Yoon *et al.,* 2003). Hence, these fragments were excluded when comparing RFLP patterns.

6.4.4 *In silico* restriction pattern analysis and validation of the method

The *groEL* sequences were exported to computer-simulated RFLP analysis and virtual gel plotting program, NEB cutter online software version: 1.0.0.4028 (http://www.neb.com) (Vincze *et al.,* 2003). It is a program available *via* a web server (http://tools.neb.com/NEBcutter) that will accept an input sequence and performs theoretical digests of the sequence with the enzyme of our choice. The sequences were digested *in silico* using the Alu1, Rsa1, Mbo1, Dde1 and Hha1 enzymes. The pattern produced for each *Vibrio* species was compared with the pattern obtained experimentally.

Since nucleotide sequence variation in different genes is very common among vibrios, it is very important to validate the method by screening multiple isolates within the same species. Hence, available *groEL* gene sequences of the *Vibrio* spp. were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/Genbank/)and subjected to *in silico* RFLP analysis. Their RFLP patterns were compared with the already obtained patterns to check for the reproducibility and reliability of the method. The details of the sequences used for the validation study are given in Table 6.1.

Table 6.1 Details of *groEL* gene sequences retrieved from Genbank for validation of *groEL* PCR-RFLP method

Vibrio sp.	**Strain**	**Genbank accession number**
V. parahaemolyticus	ATCC 17802	CP014046
V. parahaemolyticus	BB220P	CP003972
V. parahaemolyticus O1:KUK	FDA R31	CP006004
V. parahaemolyticus	FORC 004	CP009847
V. parahaemolyticus	RIMD 2210633	BA000031
V. cholerae	MS6	AP014524
V. cholerae	IEC244	CP0033301
V. cholerae O1	KW3	CP0069471
V. mimicus	ATCC 33654	CP014043
V. mimicus	ATCC 33809	CP014035
V. alginolyticus	ATCC 33787	CP013484
V. alginolyticus	ATCC 17749	CP006718
V. vulnificus	YJ016	BA000037
V. vulnificus	93U204	CP009261
V. vulnificus	FORC_017	CP012739
V. fluvialis	ATCC 33809	CP014035
Vibrio diazotrophicus	NBRC 103148	NZBBJY00000000
V. anguillarum	90-11-286	CP0022841
V. anguillarum	NB10	LK021130
Listonella anguillarum	EU360592	EU360592

6.4.5 Construction of phylogenetic tree

Phylogenetic trees were constructed using the *groEL* and 16S rRNA gene sequences of the eight *Vibrio* species. The evolutionary history was inferred using the Neighbor-Joining method in MEGA6. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method. The genetic variation produced at 16S rRNA and *groEL* regions were analysed and compared to determine the evolutionary genetic relationship between the *Vibrio* species.

6.5 Results

6.5.1 PCR amplication of the *Vibrio* specific *groEL* gene

PCR amplification yielded a 1117-bp amplicon for each *Vibrio* species (Figure 6. 1). No amplicon was produced in *Aeromonas hydrophila* (non-*Vibrio* species), thus proving the specificity of the primer towards *Vibrio*. The Genbank accession numbers allotted for the *groEL* sequences were KX094895- KX094898, KX086220- KX086222 and KX528017 for *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. fluvialis, V. mimicus, V. diazotrophicus, V. cholerae* and *V. anguillarum respectively*. The details of the Genbank submissions are given in Appendix 4.

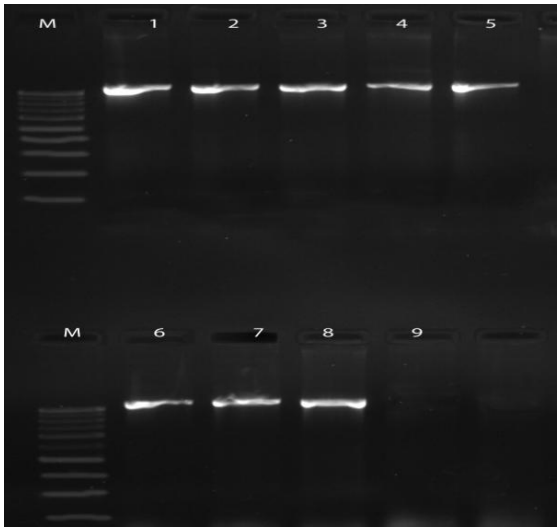

Figure 6.1 PCR amplification of the *Vibrio* species-specific *groEL* gene. Lane M: 100 bp DNA ladder; lanes 1-8: *Vibrio* spp. producing 1117 bp PCR product; lane 9: *Aeromonas* sp. (negative control)

6.5.2 *GroEL* PCR-RFLP

Each of the enzyme produced unique digestion pattern for each *Vibrio* sp. under study (Figure 6.2 a, b, c, d, e). Restriction site for Dde1 was absent in *V. anguillarum* and hence, *V. anguillarum groEL* gene remained uncut by Dde1. Table 6.2 shows the result of the computational restriction analysis after digesting with five restriction enzymes.

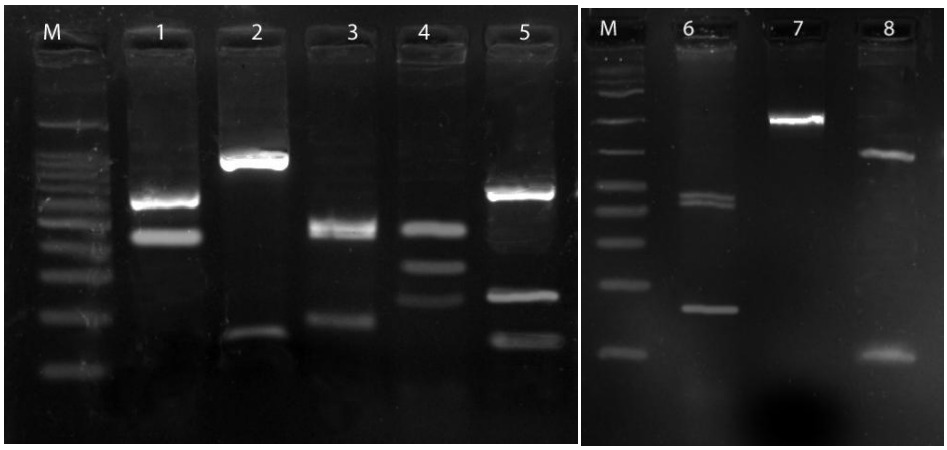

Figure 6.2 (a) Restriction patterns produced by digestion of *groEL* gene fragment of *Vibrio* with Rsa1. Lane M: 100 bp DNA ladder; lane 1:*V. vulnificus*; lane 2*: V. diazotrophicus*; lane 3: *V. parahaemolyticus*; lane 4: *V. anguillarum*; lane 5: *V. mimicus*; lane 6: *V. alginolyticus*; lane 7: *V. fluvialis;* lane 8: *V. cholerae*

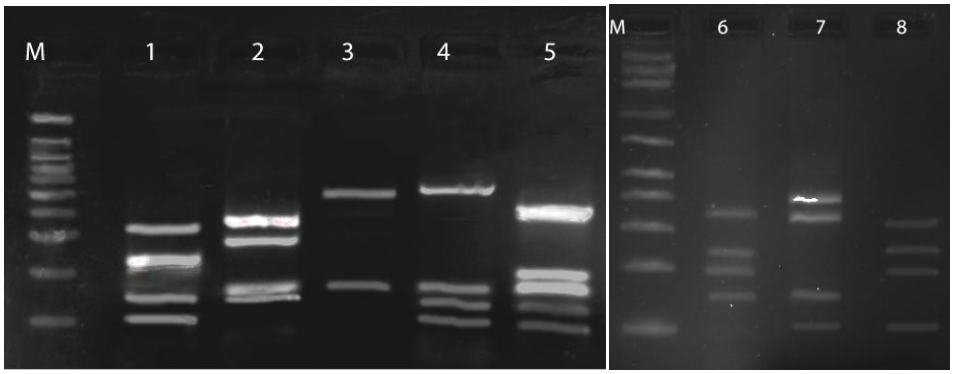

Figure 6.2(b) Restriction patterns produced by digestion of *groEL* gene fragment of *Vibrio* with Hha1. Lane M: 100 bp DNA ladder; 3:*V. parahaemolyticus*; lane 4: *V. anguillarum*; lane 5: *V. mimicus*; lane 6: *V. alginolyticus*; lane 7: *V. fluvialis;* lane 8: *V. cholerae*

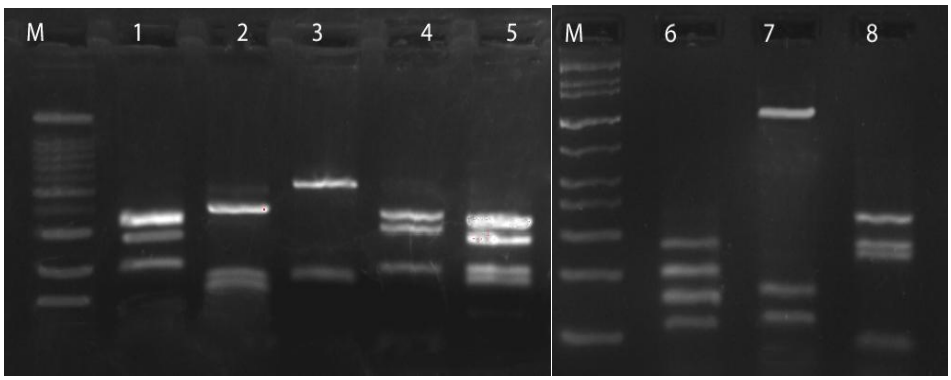

Figure 6.2(c) Restriction patterns produced by digestion of *groEL* gene fragment of *Vibrio* with Alu1. Lane M: 100 bp DNA ladder; lane 1: *V. vulnificus*; lane 2*: V. diazotrophicus*; lane 3: *V. parahaemolyticus*; lane 4: *V. anguillarum*; lane 5: *V. mimicus*; lane 6: *V. alginolyticus*; lane 7: *V. fluvialis;* lane 8: *V. cholerae*

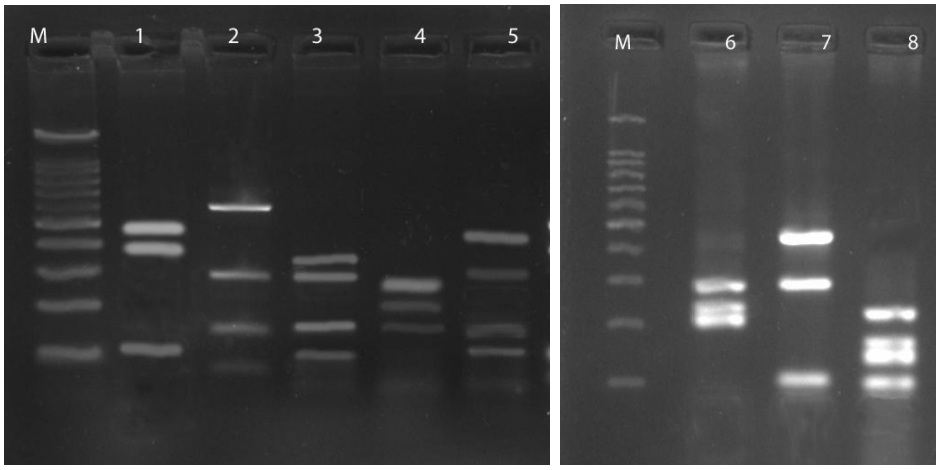

Figure 6.2(d) Restriction patterns produced by digestion of *groEL* gene fragment of *Vibrio* with Mbo1. Lane M: 100 bp DNA ladder; lane 1: *V. vulnificus*; lane 2*: V. diazotrophicus*; lane 3: *V. parahaemolyticus*; lane 4: *V. anguillarum*; lane 5: *V. mimicus*; lane 6: *V. alginolyticus*; lane 7: *V. fluvialis;* lane 8: *V. cholerae*

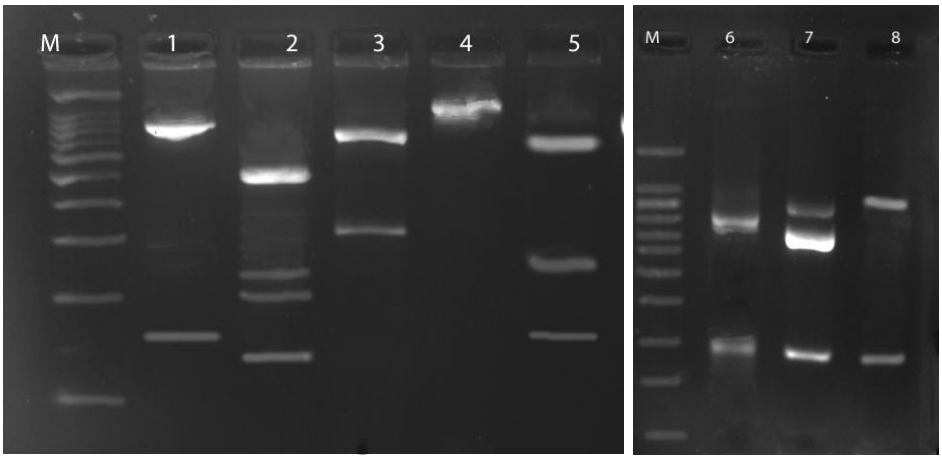

Figure 6.2(e) Restriction patterns produced by digestion of *groEL* gene fragment of *Vibrio* with Dde1. Lane M: 100 bp DNA ladder; lane 1: *V. vulnificus*; lane 2*: V. diazotrophicus*; lane 3: *V. parahaemolyticus*; lane 4: *V. anguillarum*; lane 5: *V. mimicus*; lane 6: *V. alginolyticus*; lane 7: *V. fluvialis;* lane 8: *V. cholerae*

Table 6.2 Computational restriction fragment length analysis of 1117 bp *groEL* gene product treated with restriction enzymes *viz*. Alu1, Rsa1, Hha1, Dde1 and Mbo1

***Vibrio* species**	**Digestion fragment length of *groEL* gene after restriction digestion by (in bp)**				
	Alu1	**Hha1**	**Rsa1**	**Mbo1**	**Dde1**
V. parahaemolyticus	539,184,159	481,137, 131	450,420, 163	335,288, 160, 100	805,312
V. alginolyticus	261,209,159 157,115	329,228, 193,128	450,406, 163	278,234, 211	800,270
V. fluvialis	759,168, 138	375,314, 132,105	423,263, 187,163	418,295, 104, 103	753,277
V. cholerae	334,302,274	298,228, 193,103	613,163, 126,100	222,165, 133,103,100	861,235
V. mimicus	366,296, 168, 167	419, 181, 147, 130, 103	607,263, 163	401, 271, 156, 119, 112	694, 236, 161
V. vulnificus	391, 301, 191	310, 228, 147, 111, 105	613, 421	452, 370, 103	857, 164
V. diazotrophicus	405, 153, 123	378, 288, 147, 130	876, 126	603, 298, 119	500, 236, 213,148
V. anguillarum	328, 216, 197, 195	530, 147,138, 123, 117	450, 361, 246	251, 243, 207, 188	Undigest ed (UD)

6.5.3 *In silico* restriction pattern analysis and validation of the method

The specificity and discriminatory ability of the method was further validated by *in silico* analysis in NEBcutter tool using multiple strains of each *Vibrio* species. Each of the five enzymes produced consistent banding patterns for multiple strains of the same species, confirming the reliability of our method. Thus, we could reach a conclusion that this method can be applied effectively to differentiate or identify the pathogenic *Vibrio* spp. under study.

6.5.4 Phylogenetic analysis

Phylogenetic analysis using *groEL* and 16S rRNA genes clearly demonstrated the *groEL* to be a better phylogeny marker for *Vibrio* spp. especially for differentiation of closely related species. The phylogenetic tree constructed using *groEL* genes of the *Vibrio* spp. (Figure 6.3) displayed greater divergence of nucleotide sequences than the 16S rRNA gene based tree (Figure 6.4).

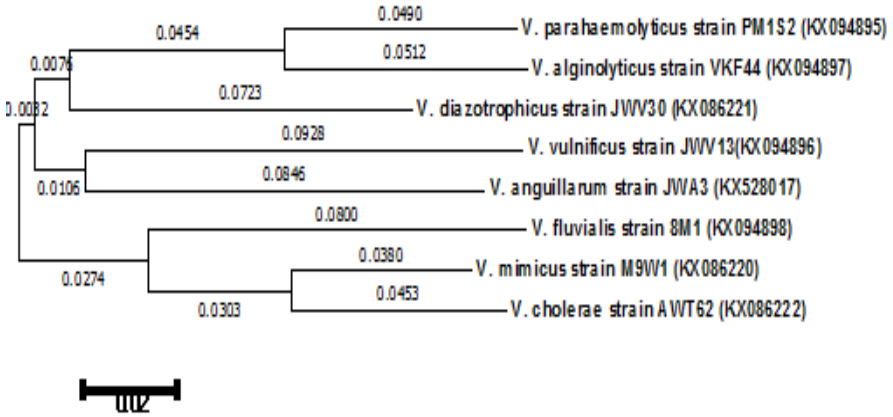

Figure 6.3 Phylogenetic tree displaying the relationship among the 8 *Vibrio* spp. based on *groEL* gene.

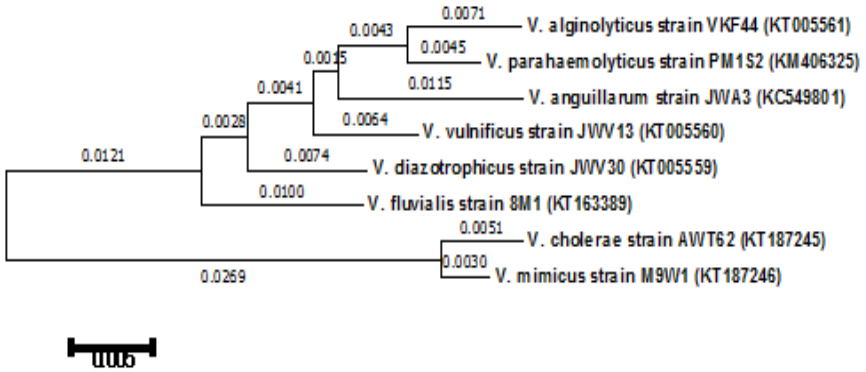

Figure 6.4 Phylogenetic tree displaying the relationship among the seven *Vibrio* spp. based on 16S rRNA gene.

The 16S rRNA based tree expressed high degree of genetic relatedness between nucleotide sequences of different *Vibrio* species. The genetic distance between *groEL* gene sequences of the closely related species was found to be much higher than that of 16S rRNA gene.

6.6 Discussion

Using *groEL* PCR-RFLP analysis, we could obtain differentiating patterns even among closely related species like *V. mimicus* and *V. cholerae, V. parahaemolyticus and V. alginolyticus*. Hossain *et al.* (2014) performed *groEL* PCR-RFLP of certain *Vibrio* species using Nru1 and Xba1 enzymes. However, the authors were unable to discriminate between *V. cholerae* and *V. mimicus* using those restriction enzymes. They obtained similar banding pattern for both the species. Whereas, in the present study each of the enzyme mentioned above produced clear differentiating pattern for the two species. Yoon *et al.* (2003) differentiated six pathogenic *Vibrio* spp. using 16S rRNA PCR-RFLP, but they did not attempt to compare between closely related species.

Genus *Vibrio* comprises of many human and aquaculture pathogens (Thompson *et al.*, 2004). Rapid detection of these pathogens and their differentiation from non-pathogens is very crucial for proper control of disease outbreaks. DNA-based diagnostic methods, especially PCR, have been studied and developed for accurate and rapid identification of *Vibrio* spp. (Jones *et al*., 2012 b). Recently, many multiplex PCR studies have been reported for the identification of the major pathogenic *Vibrio* species; however, these assays could not provide the diagnostic level required to be inclusive of the genus *Vibrio* (Nhunga *et al.,* 2007; Izumiya *et al.,* 2011). 16S rRNA gene has generally been accepted as the best target for classification and identification of bacterial species, but there have been reports where the 16S rRNA gene sequence is found to be insufficient, especially for differentiation of closely related species. Montieri *et al.* (2010) studied the usefulness of *toxR* gene for phylogenetic analysis of closely related *V. parahaemolyticus* and *V. alginolyticus* and proved it as an efficient phylogenetic marker than the 16S rRNA gene. Another study by Yushan *et al.* (2010) compared the effectiveness of 16S rRNA and the *groEL* genes for species identification and revealed that *groEL* sequences have higher divergence for species. Reports suggest *groEL* gene is a good candidate for classification and identification of bacterial species (Woo *et al.,* 2002; Montieri *et al.,* 2010; Hossain *et al.,* 2014). Studies reveal the *groEL* gene encoding the heat shock protein to be the most conserved component in evolution (Giuseppe *et al.,* 2008; Yushan *et al.,* 2010). Our study reconfirmed the above findings.

The current method is cost effective and time saving as well when compared to the cost and time spent on sequencing each one of the

strains. It is especially applicable when large numbers of isolates are to be identified. It is not feasible to identify each one of the isolates by sequencing. Instead, researchers can initially cluster the strains based on *groEL* PCR-RFLP pattern. By comparing the pattern with the pattern predicted in our study, they can accurately and rapidly identify the species. Later, if needed one representative isolate from each cluster can be subjected to further confirmation by sequencing. We recommend researchers and medical laboratories to apply the *groEL* gene based RFLP method along with 16S rRNA sequencing for more reliable and accurate identification and differentiation of *Vibrio* species.

Rapid and accurate diagnosis of the causative organism is very important for effective disease treatment and to implement proper disease preventive and control measures. Accuracy and detection specificity of the diagnostic method employed is crucial for proper disease diagnosis and treatment. Inaccurate or false identification of a pathogen may cause the treatment to be ineffective and consequently result in the death of the patient. This is a serious issue and can be tackled only through accurate identification of the pathogenic species. This can be achieved only through application of methods that are rapid and accurate and have high detection specificity. The *groEL* PCR-RFLP method employed in the present study is such a method that can be used in effective diagnostics of pathogenic *Vibrio* species and epidemic-preventing surveillance.

In conclusion, *groEL* PCR-RFLP method employed in this study can be applied as an efficient tool for rapid and accurate differentiation and identification of *Vibrio* spp. including the closely related species.

Chapter 7

EFFECT OF PHYSICO-CHEMICAL AND BIOLOGICAL FACTORS ON SURVIVAL OF PATHOGENIC *VIBRIO* SPECIES IN WATER AND SEDIMENT OF COCHIN ESTUARY

Contents

7.1 Introduction

Survival is defined as the ability to maintain the viability under adverse circumstances. *Vibrio* bacterium is autochthonous to estuarine and marine environments. Survival of *Vibrio* in estuarine environments depends on various factors. In these environments, it is exposed to frequent environmental variations in temperature, salinity, pH, nutrient levels, and presence of pollutants. It includes biotic factors (competition, phage mediated lysis, protozoan predation), physical factors (temperature, sunlight) and chemical factors (salinity, chemical composition of estuarine water etc.). Among the physical parameters, temperature is the major factor influencing its survival and virulence, salinity is the major chemical factor whereas protozoan and bacteriophage predators constitute the predominant biological factors.

Vembanad Lake connected to Arabian Sea through Cochin estuary, is the largest brackish, tropical wetland ecosystem along the south west coast of India. It is of extraordinary importance for its hydrological function, biodiversity and rich fishery resources. Evaluation of the survival capabilities of pathogenic bacteria like *Vibrio* in the Cochin estuary is significant as this estuary is one of the very popular tourist hot spots along the south west coast of India, and shrimps grown here are exported worldwide. Moreover, a number of seafood industries depend on the estuary and it is a means of livelihood for the local fisher folk. However, the studies on removal kinetics of this pathogen in this estuarine environment are scarce. Hence, it is essential to study the level of survival and understand the effect of environmental factors which enhance the elimination of these pathogenic organisms in estuarine environments. This study will also throw light into the self purifying capability of the estuary.

7.2 Review of literature

7.2.1 Survival of pathogenic bacteria in aquatic environments

Survival of pathogenic bacteria in aquatic environment has always assumed great public health significance. Various studies have been conducted worldwide to study the fate of pathogens in water and sediments of various aquatic environments (Davies-Colley *et al.,* 1999; Rozen and Belkin, 2001; Abhirosh *et al.,* 2011a, b; Pachepsky *et al.,* 2014; Abia *et al.,* 2016; Vezzulli *et al.,* 2016; Muhling *et al.,* 2017). The fate of bacteria in seawater is influenced by combination of various physico-chemical and biological factors (Waksman and Hotchkiss, 1937;

Carlucci and Pramer, 1960). The specific factors that influence the survival of bacteria in aquatic environments include (i) the adsorption of bacteria and their sedimentation (ii) the destructive effect of sunlight (iii) starvation (iv) the presence of toxic substances (v) predatory role played by protozoa and other predators like bacteriophages (vi) the competitive and antagonistic effects of other microorganisms (Waksman and Hotchkiss, 1937).

7.2.1.1 Role of biotic factors on survival of pathogens in the aquatic environments

The natural components of the microbial food web play a major role in controlling the bacterial density in the aquatic ecosystems. Among the biological factors, protozoan grazing takes an important role as bacterial predators that removes allochthonous bacterial community from the aquatic environments (Abhirosh and Hatha, 2005; Pomeroy *et al.,* 2007). Bacteriovory by protozoans, especially ciliates and heterotrophic nanoflagellates are major predators involved (Hahn and Hofle, 2001; Hisatugo *et al.,* 2014). Protozoans can easily track down hot spots of bacterial growth even in vast ocean/narrowest soil crevices because of their mobility, small size and high abundance. Previous studies suggest that protozoan grazing is largely dependent on the prey density and its physiological state (Barcina *et al.,* 1992; Menon *et al.,* 1996). The digestion capacity of the grazer also determines the extent of predation (Hisatugo *et al.,* 2014). Starved cells are easy to be ingested due to their smaller size (Christoffersen *et al.,* 1995). Similarly Gram-negative bacteria are more susceptible to predation compared to Gram-positive bacteria (Nilsson, 1987). This may be due to the thicker cell walls in

Gram-positive bacteria which is difficult to be digested. This can be the reason for *Escherichia coli* being eliminated more frequently in aquatic systems than *Enterococcus faecalis* and *Salmonella epidermidis* (Nilsson, 1987; Barcina *et al.*, 1992).

Bacteriophage induced mortality and competition with other autochthonous microflora are the other significant biotic factors. Bacteriophages are dynamic partners in microbial food webs (Bratbak *et al.*, 1994). They also have a major role in determining bacterial community composition, diversity and mortality in aquatic ecosystems (Bouvier and Giorgio, 2007). A previous study published an interesting report that about 10–20% of the marine bacterial community is lysed daily by viruses (Suttle, 1994). The significant effect of viral lysis and protozoan grazing on removal of bacteria in a eutrophic lake in Germany was previously reported (Weinbauer and Hofle, 1998). Jacquet *et al.* (2005) also investigated similar mortality of bacteria in a lake in France. Their results also showed a significant effect of virus and protozoa on mortality of bacterial communities in the lake. In another study from India, the authors studied the effect of biotic factors on survival of pathogens like *E. coli*, *S. paratyphi* and *V. parahaemolyticus* in a tropical estuary (Abhirosh *et al.*, 2009). They reported protozoan grazing as the major factor involved. The effect of predation and competition with autochthonous flora on survival of faecal indicator bacteria in aquatic habitats was studied previously (Wanjugi and Harwood, 2013). The researchers commented that the interplay of faecal indicator bacteria and other pathogens with indigenous microbiota is a complex process. A recent study reported that predation and competition had significant negative

effects on survival of *E. coli* in aquatic environments (Wanjugi *et al.,* 2016). The role of free-living protozoa on the survival of *V. cholerae* O1 in aquatic environments was studied (Nawar and Naser, 2015). Results showed that ciliates and flagellates have a major role in controlling the abundance of cholera bacteria in aquatic systems.

7.2.1.2 Role of abiotic factors on survival of pathogens in the aquatic environments

Aquatic and marine bacteria are exposed to frequent seasonal and geographic variations in temperature, salinity, nutrient input etc. Temperature, sunlight, salinity (osmotic stress), nutrients and other chemical factors are the major abiotic factors that influence the survival of bacteria in aquatic environments.

7.2.1.2.1 Effect of temperature and salinity

The effect of temperature and salinity on growth and survival of vibrios have been previously studied by many authors (Biosca *et al.,* 1996; Yoon *et al.,* 2008; Burnham *et al.,* 2009; Oliver, 2010; Kim *et al.,* 2012; Urquhart *et al.,* 2016). There are various reports highlighting higher incidence of *Vibrio* infections in coastal waters with warming temperatures (Khan *et al.,* 2002; Elhadi *et al.,* 2004; Martinez-Urtaza *et al.,* 2010; Le Roux *et al.,* 2015; Vezzulli *et al.,* 2016; Muhling *et al.,* 2017). The survival of *V. vulnificus* in seawater is greatly influenced by warm water temperatures and low-to-moderate salinities (Kaspar and Tamplin, 1993). *V. vulnificus* also entered a VBNC state in water temperatures below 15 °C (Oliver *et al.,* 1991). The cold stress induced VBNC state in *V. parahaemolyticus* (Kaneko and Colwell, 1975; Colwell

and Grimes, 2000). Similarly, low salinity also induced VBNC state in *V. parahaemolyticus* (Oliver, 2010). In this state the cells maintained their respiratory activity and membrane integrity, but remain non-culturable (Oliver, 2010). A study was conducted to investigate the effect of water temperature and salinity on survival of toxigenic *Vibrio cholerae* O1 in laboratory microcosms (Huq *et al.*, 1984). Among the 3 different salinities tested (5%, 10% and 15%), maximum growth was observed at 15%. Rapid growth was observed by increasing water temperature up to 30 °C. The effect of temperature and salinity on survival of *Vibrio splendidus* was performed by Susana *et al.* (2003). Salinity range chosen was (33–0.9%) and the results showed maximum survival at 3.3% salinity. The temperatures studied were 4 °C, 10 °C and 22 °C. These temperatures did not have any significant effect on the survival of the strains.

7.2.1.2.2 Effect of nutrients

Nutrient deprivation is the most common stress faced by environmental bacteria in aquatic environments**.** Stresses such as starvation or nutrient deprivation lead bacteria to decrease in their size and activity (Stevenson, 1978). One of the survival strategies of bacteria to withstand starvation effect is the reduction of its endogenous metabolic rate rapidly. *Vibrio* species have the capability of long term survival even during starvation. They withstand starvation through sequential changes in their cell physiology and morphology (Morita, 1993). They also have the ability to enter VBNC state under starvation (Amel *et al.*, 2008; Fernández-Delgado *et al.*, 2015). A study was conducted recently in order

to find the survival of *V. cholerae* in the Southern Caribbean Sea (Fernández-Delgado *et al.,* 2015). The recovery rate of *V. cholerae* cells declined gradually due to nutrient depletion and the cells entered VBNC state. Resuscitation experiments showed *V. cholerae* cells could revert to culturable state when favourable conditions were provided. *V. alginolyticus* strain exhibited long term survival during starvation in natural seawater (Jiabo *et al.,* 2008). The survival strategy of *V. fluvialis* in seawater with and without sediment under starvation was studied by Amel *et al.* (2008). Long term survival was observed in microcosms containing sediment. *Salmonella typhimurium* strains were resuscitated even after 5 years of incubation in seawater microcosms (Dhief *et al.,* 2001). A study demonstrated *Pseudomonas aeruginosa* cells detached from the biofilm due to lack of energy because of the nutrient starvation (Hunt *et al.,* 2004).

7.2.1.2.3 Effect of sunlight

Sunlight plays a major role in inactivation of pathogenic bacteria in aquatic environments (Walker *et al.,* 2004; Whitman *et al.,* 2004; Anuar and Chan, 2013). The spectrum of solar radiation reaching the Earth's surface includes ultraviolet radiation, the photosynthetically active and infrared radiation (Ruiz- Gonzalez *et al.,* 2012). The UV region is classified into UV-A and UV-B. The UV-B (280-315 nm) fraction of sunlight exerts strong bactericidal action causing direct DNA damage. It directly affects bio-molecules and cellular structures and blocks various enzymatic reactions. It also induces the formation of reactive oxygen species. Microorganisms adopt various mechanisms to withstand the

effect of UV-B radiation. This includes vertical migration, increasing the frequency of division, quenching of reactive oxygen species etc. UV-A (320–400 nm) and IR fraction (>700 nm) of sunlight also inactivate pathogens to some extent. UV-A absorbance leads to thymine dimer formation causing premature termination of DNA replication (Reed, 1997). The photosynthetically active radiation (400–700 nm) and UV-radiation have negative impact on the bacterial enzyme activities (Santos *et al.,* 2011; Ruiz- Gonzalez *et al.,* 2012). Reports state that bacteria and viruses are more sensitive to UV radiation than phytoplankton since they lack the UV-absorbing pigments (Hader *et al.,* 2011). Even though bacteria lack efficient physical protection from sunlight (García-Pichel, 1994), they can cope up with high radiation by immediately repairing the DNA damage caused upon exposure to solar radiation (Kaiser and Herndl, 1997; Ruiz-González *et al.,* 2012). The physiochemical properties of the water (salinity, aeration, presence of UV absorbing materials etc.) also influence the antimicrobial efficiency of sunlight (Reed, 1997; Davies *et al.,* 1999).

The effect of sunlight on inactivation of pathogens from various environments have been studied by many authors (Jeffrey *et al.,* 2000; Rijal and Fujioka, 2001; Hockberger, 2002; Abhirosh and Hatha, 2005; Mani *et al.,* 2006; Pakulski *et al.,* 2007; Djurdjevic-Milosevic *et al.,* 2011; Anuar and Chan, 2013). The negative impact of UV-A on survival of surface marine bacteria have been studied by Sieracki and Sieburth (1986). In another study, there was considerable reduction in the diversity of fresh water and estuarine bacteria when exposed to UV-B for 9 hours (Santos *et al.,* 2011). The combined effect of temperature, salinity and

solar radiation on survival of *E. coli* in seawater was studied recently (Jozić *et al.*, 2014). Results showed sunlight had a significant effect on the survival of the pathogen. The pathogen died-off rapidly upon exposure to sunlight.

7.3 Objectives of the study

The objectives set are:

1) Microcosm studies to find the effect of biological factors on survival of pathogenic vibrios in water and sediments of Cochin estuary.
2) Microcosm studies to find the effect of physical factors (temperature and sunlight) on the survival of pathogenic vibrios in estuarine water.
3) Microcosm studies to find the effect of chemical factors on survival of pathogenic vibrios in estuarine water.

7.4 Materials and Methods

7.4.1 Bacterial strains used

Five strains previously isolated from Cochin estuary and shrimp farm and maintained in the laboratory were used for the survival study. It included *V. parahaemolyticus* PM1S2, *V. vulnificus* JWV13, *V. alginolyticus* VKF44, *V. proteolyticus* M10W1 and *V. mimicus* M9W1 with Genbank accession numbers KM406325, KT005560, KT005561, KT748656 and KT187246 respectively.

7.4.2 Preparation of bacterial inoculum

The selected strains were inoculated into Tryptone Soy Broth and incubated for 24 h at 37 °C. After incubation the cells were concentrated by centrifugation at 13,000 rpm for 15 min. The pellet was re-suspended in 10 ml physiological saline after proper washing using physiological saline. One ml (approximately 10^8 CFU/ml) of the culture was inoculated into 250 ml Erlenmeyer flasks with 100 ml test solution to give initial inoculum density of 10^6 CFU/ml.

7.4.3 Microcosm design

Water and sediments samples were freshly collected from the Cochin estuary and brought to the laboratory for setting up the microcosms. Water samples were collected using Niskin sampler and sediment using Van Veen grab. Water microcosms were prepared by adding 100 ml water into sterile 250 ml Erlenmeyer flasks. Sediment microcosms were prepared with 50 gm sediment and 50 ml overlaying estuarine water in sterile 250 ml Erlenmeyer flasks (Hood and Ness, 1982). The bacterial load at the time of inoculation (time zero) was taken as the initial count. Survival assay was carried out upto 28 days with sampling intervals at 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 14^{th}, 21^{st} and 28^{th} day.

7.4.3.1 Microcosm to study the effect of biotic factors

Raw water (RW) and raw sediment (RS) freshly collected from estuary were used as test solutions to study the effect of the self-contained biological factors such as protozoa, bacteriophages and other competing microflora on the test organisms. Autoclaved water and sediment devoid of biotic factors were used as control microcosm.

The effect of protozoan predation on the test organisms was studied by addition of 500 mg/l of eukaryotic inhibitor cycloheximide into the raw water and sediment. The protozoans present were identified by microscopy.

Bacteriophages in the water sample were detected by double layer agar method (Kennedy *et al.*, 1986). Briefly, 45 ml of the sample and 5 ml of *V. parahaemolyticus*/ *V. mimicus*/ *V. proteolyticus*/ *V. vulnificus*/ *V. alginolyticus* were inoculated into 45 ml of Deca Strength Phage Broth (DSPB) and incubated at 37 °C for 24 h. The cells were then centrifuged at 2500 rpm for 10 min and the supernatant was filtered through bacteriological filter (0.45 μm). The filtrate (0.1 ml) was mixed with 1 ml of *V. parahaemolyticus*/ *V. mimicus*/ *V. proteolyticus*/ *V. vulnificus*/ *V. alginolyticus* culture and 5 ml of nutrient agar (0.6%) which is used as top agar and poured over basal agar (nutrient agar containing 1.2% agar). After 24 h of incubation at 37 °C the plaque formation was noted. The plaques were counted and expressed as plaque forming units (PFU)/mL.

The competing autochthonous bacteria present in water and sediment was enumerated by spread plating method. Briefly, 0.1 ml of serially diluted water/sediment sample was evenly spread over the surface of nutrient agar using sterile glass spreader. After overnight incubation at 37 °C the total heterotrophic count was expressed as CFU/ ml. The total heterotrophic bacteria were identified up to the genus level using the identification key described in Appendix 2.

7.4.3.2 Microcosm to study the effect of temperature

Autoclaved estuarine water (AW), devoid of all biological factors was used as test solution to study the effect of temperature on the survival of the test organisms. Three different temperatures 25 °C, 30 °C and 35 °C were chosen. The temperatures were chosen based on the temperature range that was observed in the Cochin estuary during the three seasons such as monsoon, post-monsoon and pre-monsoon respectively.

7.4.3.3 Microcosm to study the effect of chemical factor

Filter sterilised (0.22 µm) estuarine water microcosm was used to study the effect of chemical factors on the survival of the test organisms. Isotonic saline (0.85% NaCl solution) was used as the control.

Nutrient analysis of the estuarine water was done following the protocol by Grasshoff *et al.,* (1983). For heavy metal analysis, water samples were collected in acid-washed polythene cans and kept in iceboxes until filtration. Known volume was filtered through millipore filter paper (0.45 µ) and the filtrate was acidified to pH >2 using concentrated HCl. The dissolved metals were extracted using Ammonium Pyrrollidine Dithocarbamate (APDC) and Methyl Isobutyl Ketone (MIBK) at pH 4.5 and brought back to aqueous layer by back-extraction with concentrated nitric acid and made up to 20 ml with milli-Q water (Brooks *et al.,* 1967; Smith and Windom, 1972). The extracts were analysed in the ICP-AES (IRIS INTREPID ll XSP, Thenno Electron Corporation).

7.4.3.4 Microcosm to study the effect of salinity

Autoclaved estuarine water (AW), devoid of all biological factors was used as test solution to study the effect of varying salinity (0 ppt, 15 ppt and 30 ppt) on the survival of the test organisms.

7.4.3.5 Microcosm to study the effect of sunlight

Filter sterilised (0.22 µm) estuarine water was used as microcosm to study the effect of sunlight on the survival of the test organisms. The microcosms were set in glass bottles. Inoculated glass bottles were immersed in a tank filled with estuarine water and exposed to sunlight. All the control flasks were covered with black paper and incubated in dark to prevent exposure to light. The experiment started at 8 am and continued up to 6 pm. The microcosms were analysed and plated at 2 h interval. The intensity of sunlight was measured using Luxmeter.

7.4.4 Enumeration of microcosms and plotting of survival curves

All microcosms except for the sunlight experiment were analysed and sampled for 28 days. Time zero (inoculation time) and subsequent samples were taken for plate counts. Samples were taken and enumerated after 1^{st}, 3^{rd}, 5^{th}, 7^{th}, 14^{th}, 21^{st} and 28^{th} day by the drop plate method (Hoben and Somasegaran, 1982) using *Vibrio* selective media TCBS agar (Himedia, India). Dilution of the samples if needed was done in isotonic saline solution. The plates were incubated at 37 °C; the number of colonies was counted after 24 h. The microcosms to study effect of sunlight were analysed and plated at 2 h interval.

All the plating were done in triplicates. The mean value and standard deviation were used to plot the survival curves.

7.4.5 Statistical analysis

Any significant difference in survival of each species in the test and control microcosms was analysed using the T-test in SPSS statistical package. Significant difference in survival of the species at varying temperatures and also at varying salinities was analysed using one-way ANOVA in SPSS statistical package. The significance level was set at $\alpha = 0.05$.

7.5 Results

7.5.1 Relative survival of *Vibrio* spp. in water and sediment of Cochin estuary

There was highly significant difference in the survival of each of the species in water and sediment ($p<0.05$). Survival was better in sediment compared to water. In water microcosm, *V. parahaemolyticus* cells were non-culturable by end of 5th day itself (Figure 7.1 a). In sediment microcosm, the cells remained culturable till the end. *V. proteolyticus* remained culturable until end of 21st day and by end of 28th day it became non-culturable in both water and sediment (Figure 7.1 b). In water, *V. mimicus* showed a slight growth in the beginning and later a rapid decline in count was observed and reached non-detectable level by end of 5th day. In sediment it survived until the end (Figure 7.1 c). *V. alginolyticus* also exhibited prolonged survival in sediment compared to water. In water it survived only up to 7th day (Figure 7.1 d). *V. vulnificus* became non-culturable by end of 21st day in water, whereas in sediment it survived until the end (Figure 7.1 e). Overall, all the species exhibited better and extended survival in raw sediment compared to water.

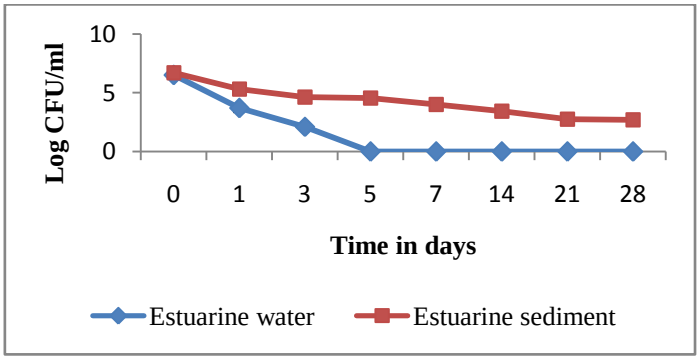

Figure 7.1 a Survival curves of *V. parahaemolyticus* in water and sediment of Cochin estuary

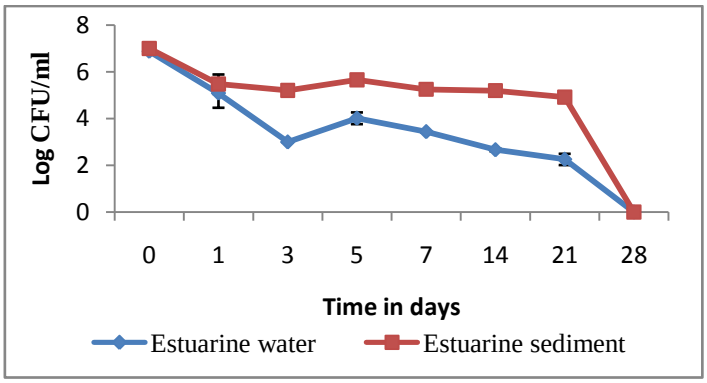

Figure 7.1 b Survival curves of *V. proteolyticus* in water and sediment of Cochin estuary

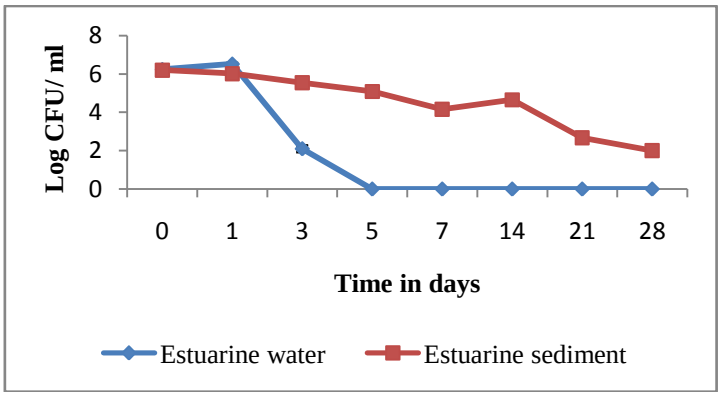

Figure 7.1 c Survival curves of *V. mimicus* in water and sediment of Cochin estuary

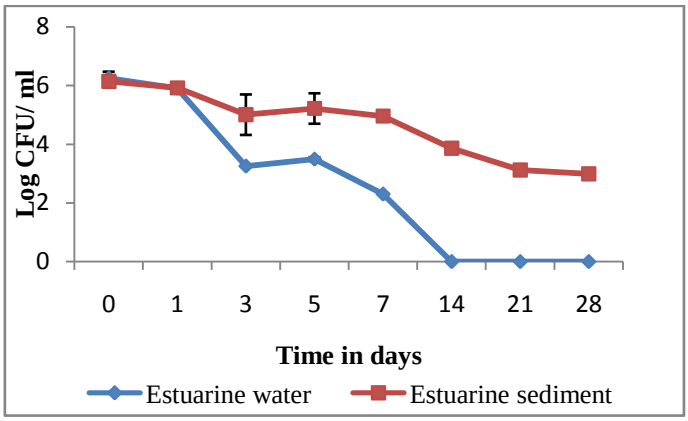

Figure 7.1 d Survival curves of *V. alginolyticus* in water and sediment of Cochin estuary

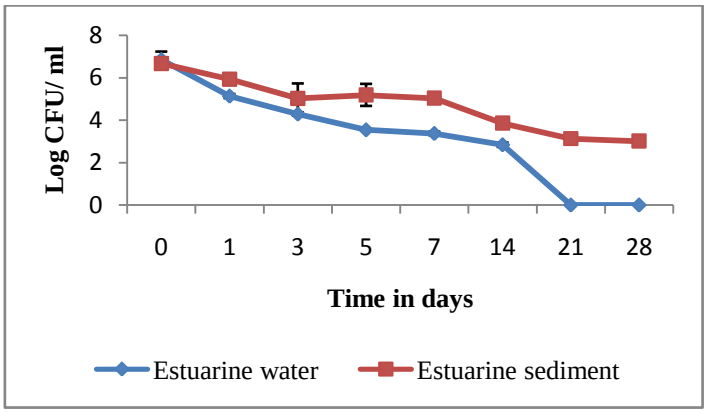

Figure 7.1 e Survival curves of *V. vulnificus* in water and sediment of Cochin estuary

Among the five *Vibrio* species, *V. proteolyticus* showed an extended survival in estuarine water, followed by *V. vulnificus*, *V. alginolyticus and V. mimicus*. *V. parahaemolyticus* showed the least survival capability in estuarine water (Figure 7.2). It survived only for 5 days. However, the difference in their survival rates were not statistically significant ($p>0.05$). In the sediment there was no significant difference in the survival of the five species ($p>0.05$). All the species showed almost

similar survival pattern (Figure 7.3). All survived until the end of the experiment period except for *V. proteolyticus* which became non-culturable by the end.

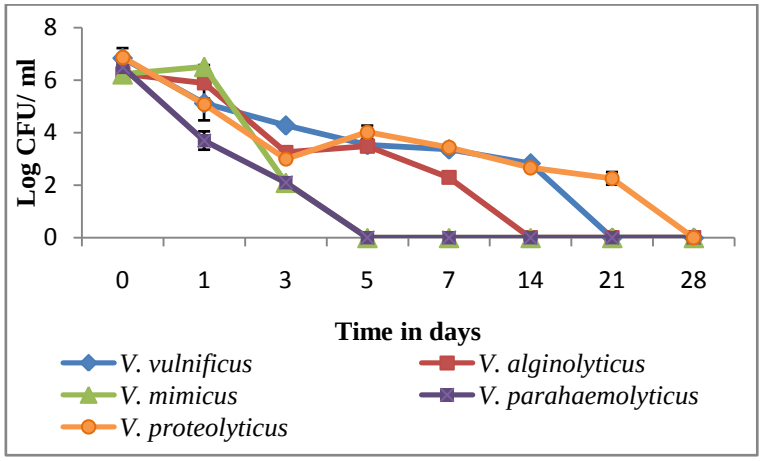

Figure 7.2 Survival curves showing relative survival of *Vibrio* species in water of Cochin estuary

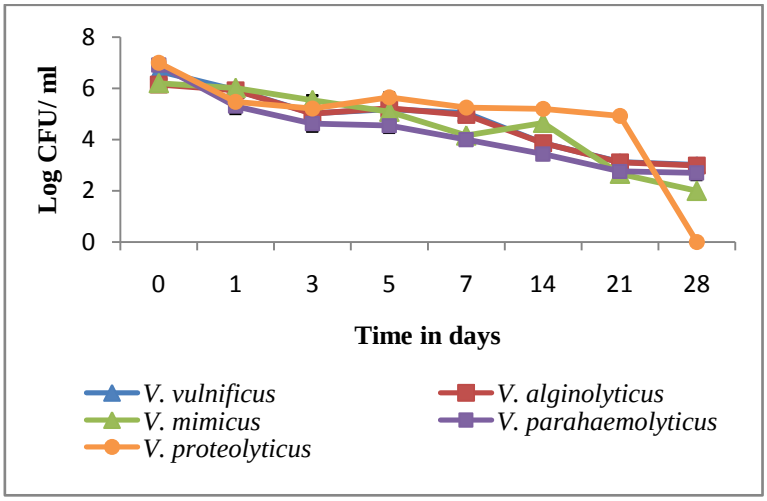

Figure 7.3 Survival curves showing relative survival of *Vibrio* species in sediment of Cochin estuary

7.5.2 Effect of biotic factors on survival of *Vibrio* spp. in water microcosms

All the species survived better in autoclaved microcosms (devoid of biotic factors) than raw microcosms. There was a highly significant difference in the survival rate of each of the *Vibrio* species in raw and autoclaved water ($p<0.05$) (Figure 7.4 a-e). The survival of all the species was extended in the autoclaved microcosms. This clearly shows the biotic factors have an effect on survival of *Vibrio* spp. in water of Cochin estuary.

Among the five species, *V. alginolyticus*, *V. vulnificus* and *V. proteolyticus* could withstand the effect of biotic factors to a great extent and remained culturable in raw water up to 14th, 21st and 28th days respectively. However, the cell counts of *V. parahaemolyticus* and *V. mimicus* reached zero by the end of 5th day itself.

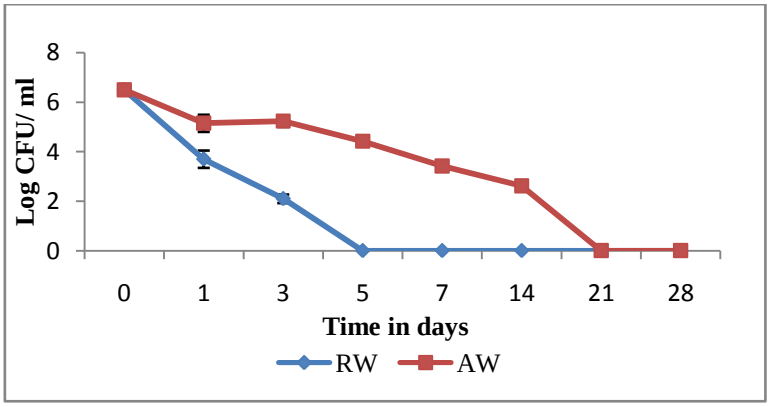

Figure 7.4 a Survival curves of *V. parahaemolyticus* as a function of biotic factors in water of Cochin estuary

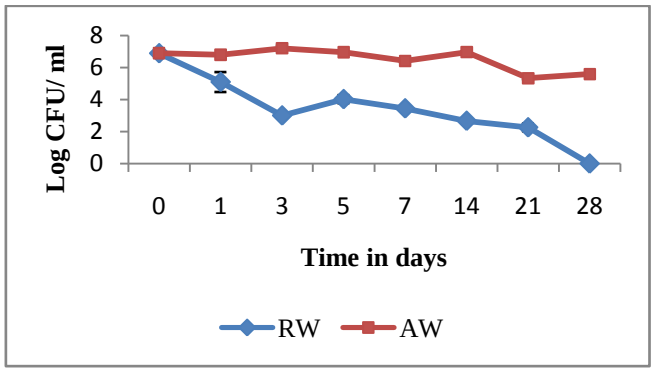

Figure 7.4 b Survival curves of *V. proteolyticus* as a function of biotic factors in water of Cochin estuary

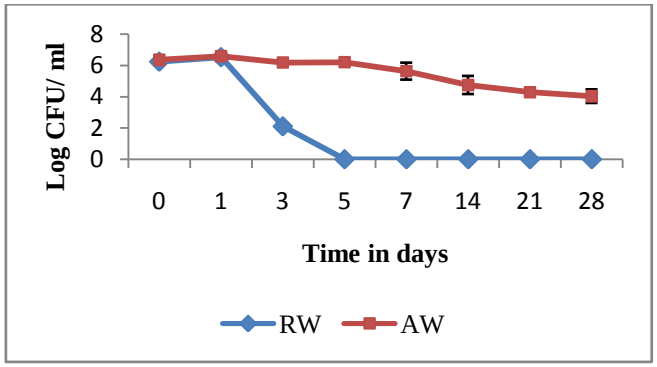

Figure 7.4 c Survival curves of *V. mimicus* as a function of biotic factors in water of Cochin estuary

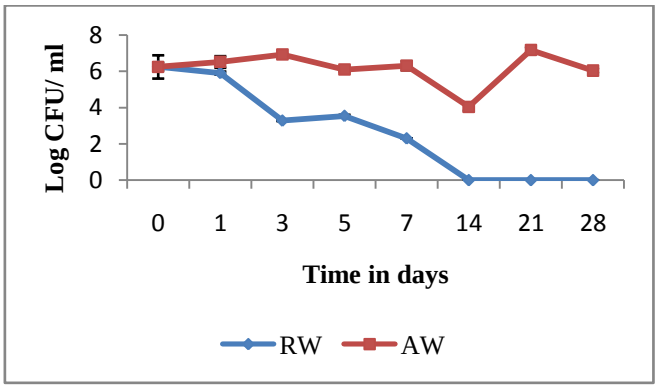

Figure 7.4 d Survival curves of *V. alginolyticus* as a function of biotic factors in water of Cochin estuary

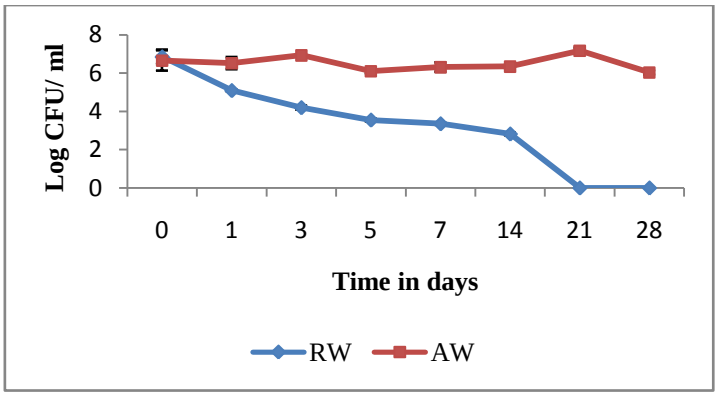

Figure 7.4 e Survival curves of *V. vulnificus* as a function of biotic factors in water of Cochin estuary

7.5.3 Effect of biotic factors on survival of *Vibrio* spp. in sediment microcosms

Each of the five species exhibited a significant difference in their survival pattern in autoclaved and raw sediments ($p<0.05$). A better growth and survival was observed in autoclaved sediment compared to raw sediment microcosms (Figure 7.5 a-e). A stable survival was shown by all the species until the end of the experiment in autoclaved microcosms. In raw microcosms, a gradual decline was observed in the cell count of all the species throughout the experiment.

All the species except *V. proteolyticus* remained culturable in raw sediment until the end. A rapid 5 log reduction was observed in the cell count of *V. proteolyticus* after the 21st day and count reached zero by 28th day.

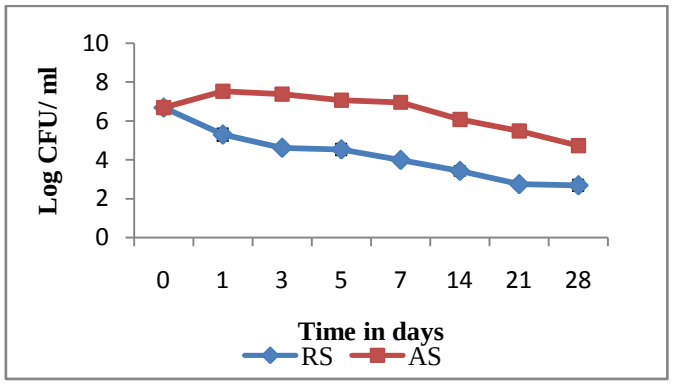

Figure 7.5 a Survival curves of *V. parahaemolyticus* as a function of biotic factors in sediment of Cochin estuary

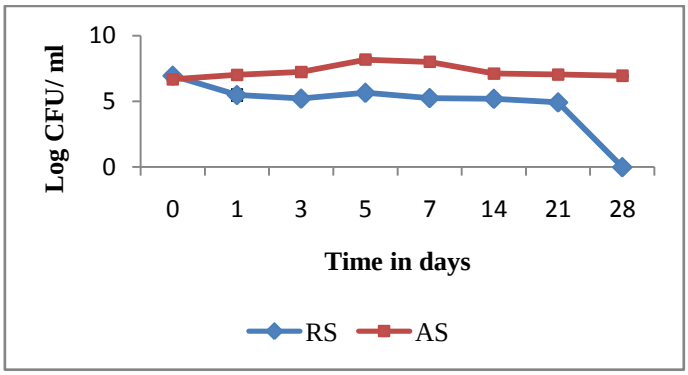

Figure 7.5 b Survival curves of *V. proteolyticus* as a function of biotic factors in sediment of Cochin estuary

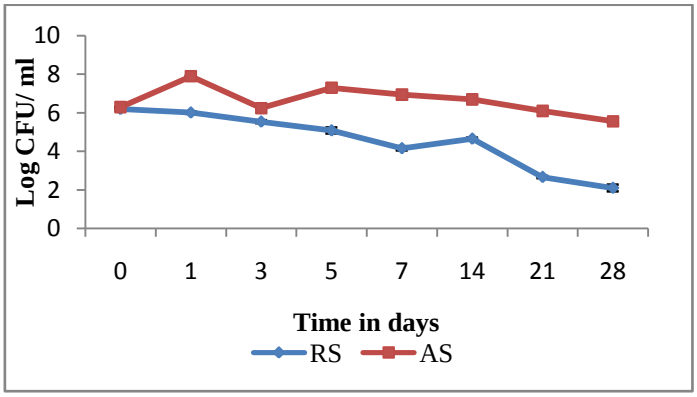

Figure 7.5 c Survival curves of *V. mimicus* as a function of biotic factors in sediment of Cochin estuary

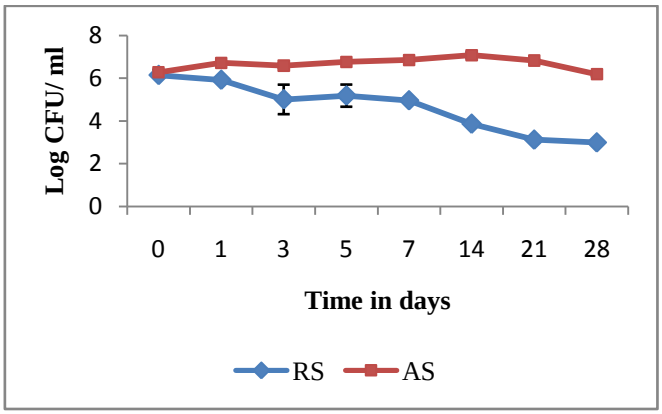

Figure 7.5 d Survival curves of *V. alginolyticus* as a function of biotic factors in sediment of Cochin estuary

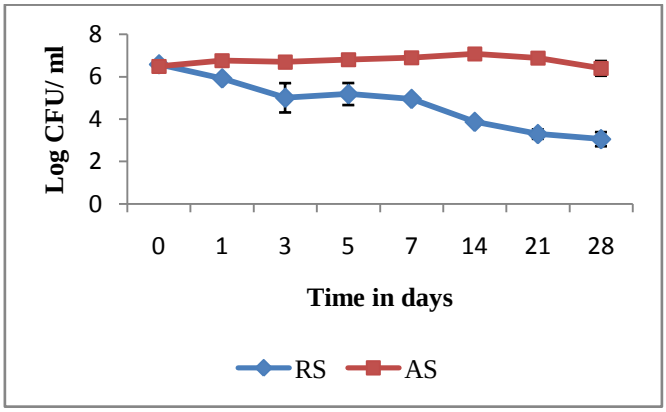

Figure 7.5 e Survival curves of *V. vulnificus* as a function of biotic factors in sediment of Cochin estuary

7.5.4 Determination of biotic factors present in Cochin estuary

7.5.4.1 Enumeration of competing autochthonous microflora

The competing autochthonous microflora present in Cochin estuary was enumerated by standard plate count method. The load of THB obtained from water and sediment of Cochin estuary were 6 x 10^{3} CFU/ ml and 2 x 10^{8} CFU/ ml respectively. Bacterial genera identified from

water include Gram-positive *Staphylococcus* and Gram-negative *Moraxella*, *Flavobacterium* and *Enterobacteriaceae*. Bacterial genera identified from sediment are Gram-positive *Bacillus* and *Staphylococcus*, and Gram-negative *Cytophaga*, *Moraxella*, *Flavobacterium* and *Alkaligenes*. Plate 1 shows photograph of nutrient agar plate with total heterotrophic bacterial colonies from Cochin estuary.

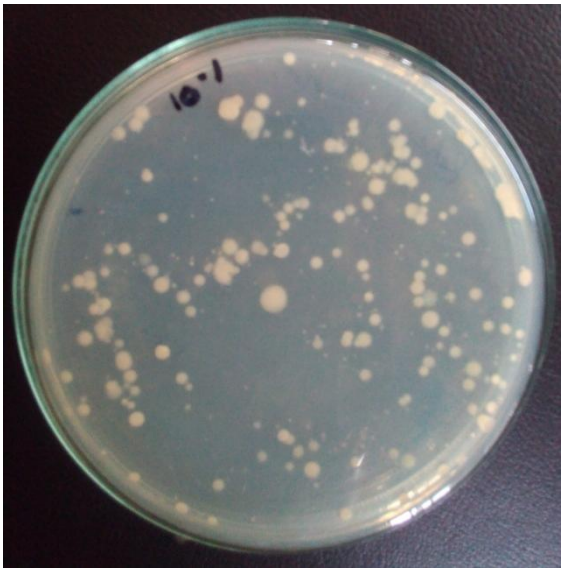

Plate 1 Total heterotrophic bacteria on Nutrient agar plate

7.5.4.2 Protozoans encountered in Cochin estuary

In the present study, protozoans from Cochin estuary were identified using microscopy. The protozoans encountered in Cochin estuary are *Vorticella, Tintinnids, Euglena, Phacus, Strombidium, Favella, Tintinnopsis, Centropyxis, Difflugia, Stylonichia*. Plate 2 shows microscopic images of few of the protozoans encountered.

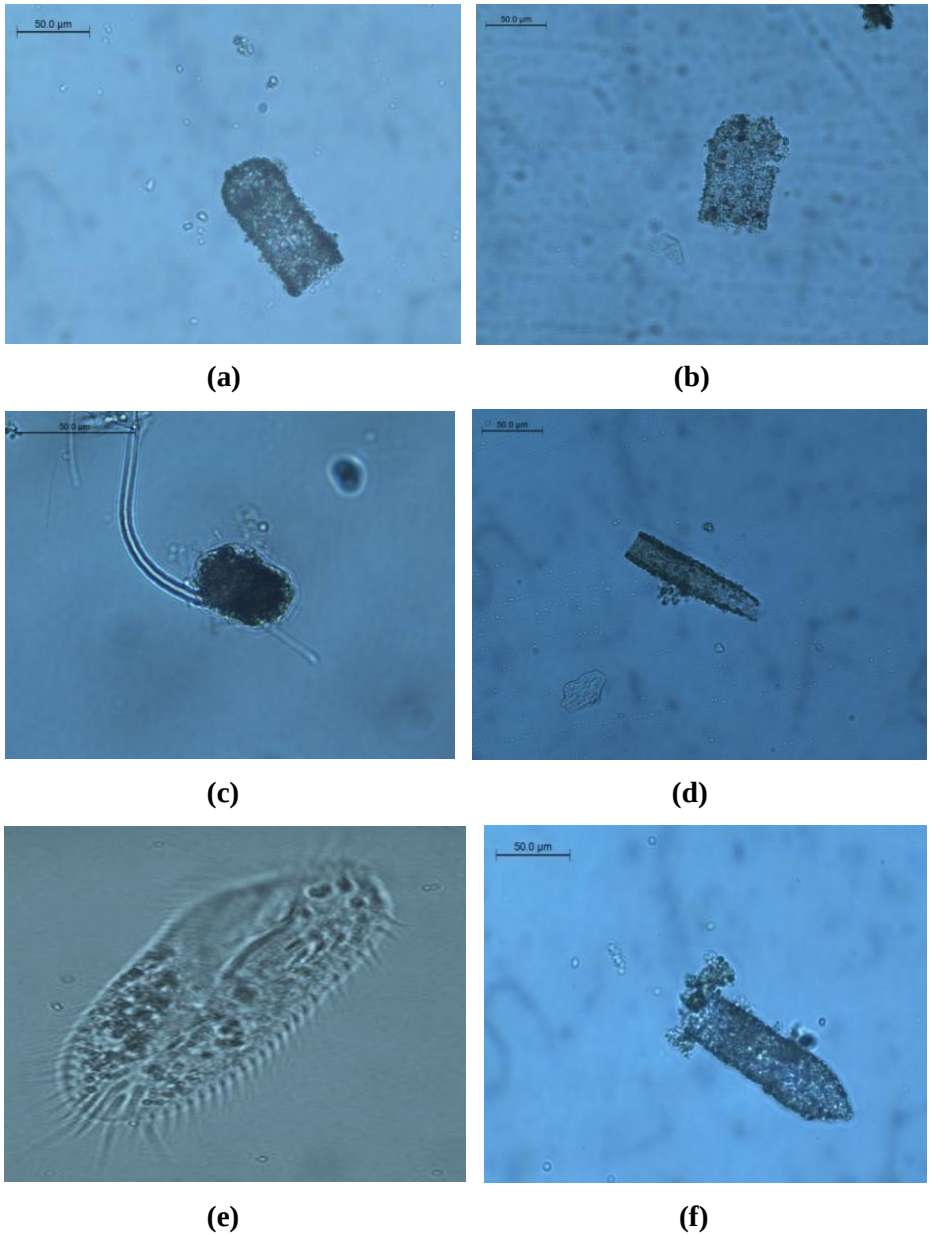

Plate 2 Microscopic images of few protozoans encountered in Cochin estuary (a) *Tintinnopsis* sp. (b) *Stenosemella* sp. (c) *Strombidium* sp. (d) *Tintinnidium* sp. (e) *Stylonichia* sp. (f) *Tintinnopsis* sp.

7.5.4.3 Enumeration of Vibriophages from Cochin estuary

Vibriophages in the water sample were detected by double layer agar method and formation of plaques was noted. Bacteriophages specific to *V. parahaemolyticus* alone were detected in our study (Plate 3). The mean vibriophage count observed was 2×10^3 PFU/ml. Bacteriophage specific to *V. vulnificus*, *V. mimicus*, *V. alginolyticus* and *V. proteolyticus* was not detected in our study.

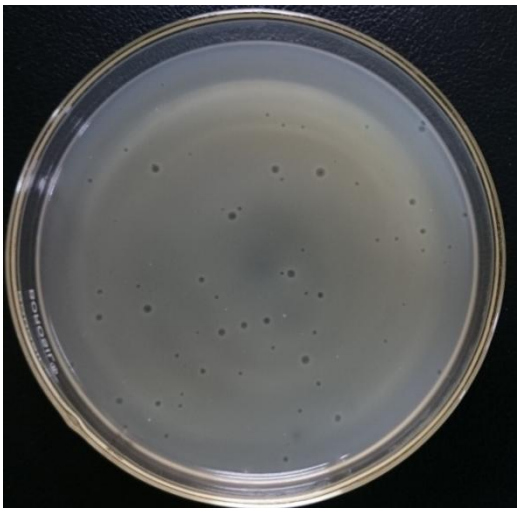

Plate 3 Plate showing plaque formation by phage specific to *V. parahaemolyticus*

7.5.5 Effect of protozoan predation on survival of *Vibrio* spp. in water and sediment microcosms

In the previous section it was revealed that protozoan predators are present in Cochin estuary (Section 7.5.4.2). Microcosms to study the effect of protozoan predation on the survival of *Vibrio* spp. in Cochin estuary was prepared by adding cycloheximide to raw water and

sediments. The protozoan effect was more visible in the estuarine water compared to the sediment.

Figures 7.6 a-e show the survival pattern of the five *Vibrio* species in cycloheximide treated and raw water microcosms. *V. parahaemolyticus* showed different survival pattern in the raw and treated microcosms However, the difference was not statistically significant ($p>0.05$). In cycloheximide treated water, the survival of *V. parahaemolyticus* was extended up to 7 days Similarly, *V. mimicus* also exhibited difference in the survival pattern in raw and treated water. However, the difference was not statistically significant ($p>0.05$). The survival of *V. mimicus* was extended up to 14 days in treated water. Both the species survived only for 5 days in raw untreated water. In other words mortality was more in the raw water microcosms. *V. proteolyticus* showed a statistically significant difference in the survival pattern in treated and untreated water (RW) ($p<0.05$). Protozoan predation did not have a significant effect on the survival of *V. vulnificus* and *V. alginolyticus* in estuarine water ($p >0.05$).

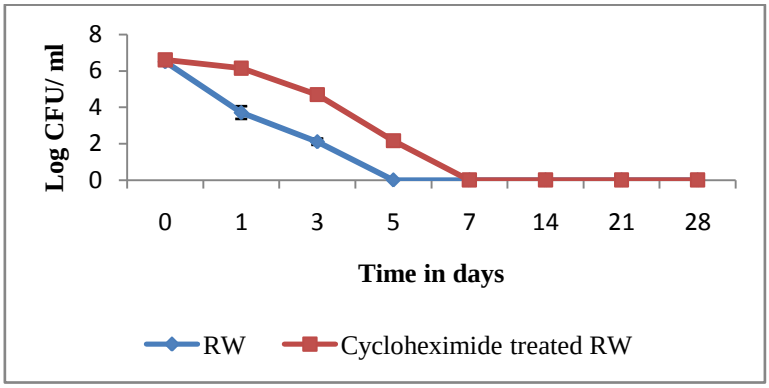

Figure 7.6 a Survival curves showing effect of protozoan predation on survival of *V. parahaemolyticus* in water of Cochin estuary

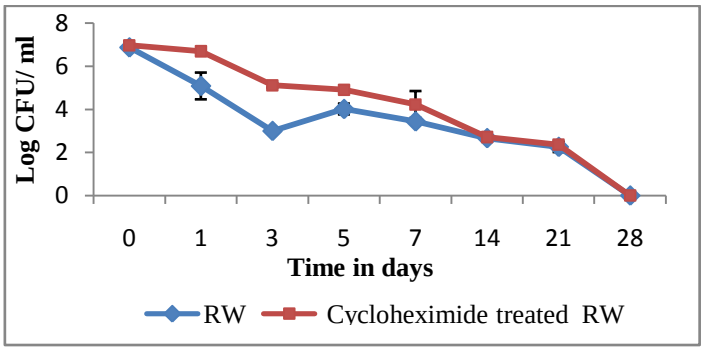

Figure 7.6 b Survival curves showing effect of protozoan predation on survival of *V. proteolyticus* in water of Cochin estuary

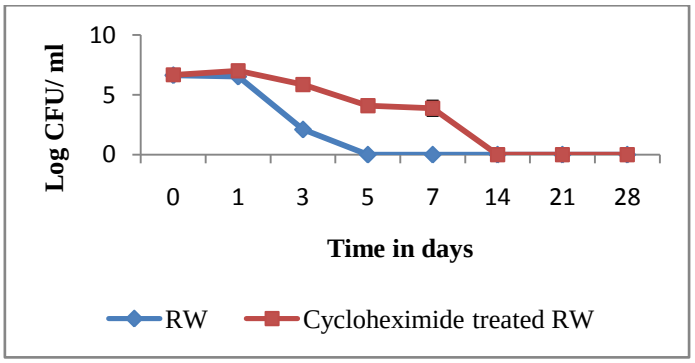

Figure 7.6 c Survival curves showing effect of protozoan predation on survival of *V. mimicus* in water of Cochin estuary

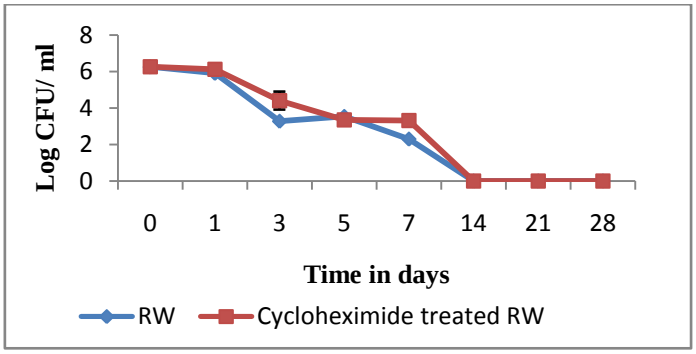

Figure 7.6 d Survival curves showing effect of protozoan predation on survival of *V. alginolyticus* in water of Cochin estuary

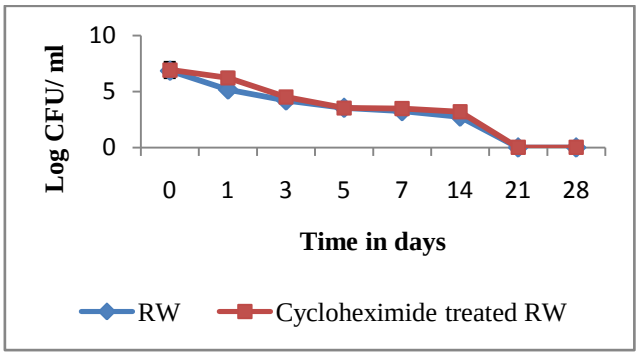

Figure 7.6 e Survival curves showing effect of protozoan predation on survival of *V. vulnificus* in water of Cochin estuary

Figures 7.7 a-e show the survival curves of the five *Vibrio* spp. in the cycloheximide treated and untreated (RS) sediments. There was no significant difference in the survival pattern of the *V. proteolyticus, V. vulnificus, V. alginolyticus* and *V. mimicus* in the treated and untreated (RS) sediments ($p > 0.05$). More or less similar pattern was exhibited by each species in the test and control microcosms. However, *V. parahaemolyticus* exhibited a statistically significant difference in its survival in treated and raw sediments ($p<0.05$).

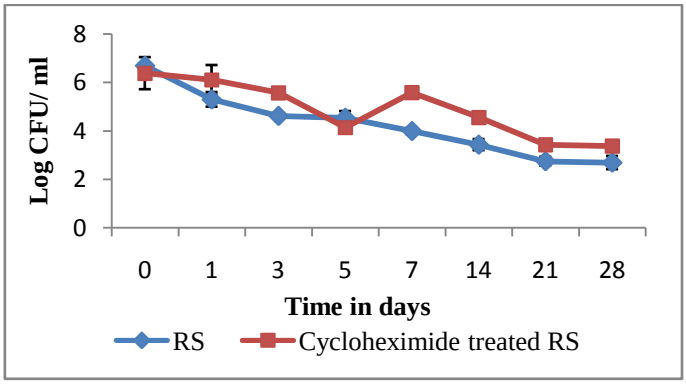

Figure 7.7 a Survival curves showing effect of protozoan predation on survival of *V. parahaemolyticus* in sediment of Cochin estuary

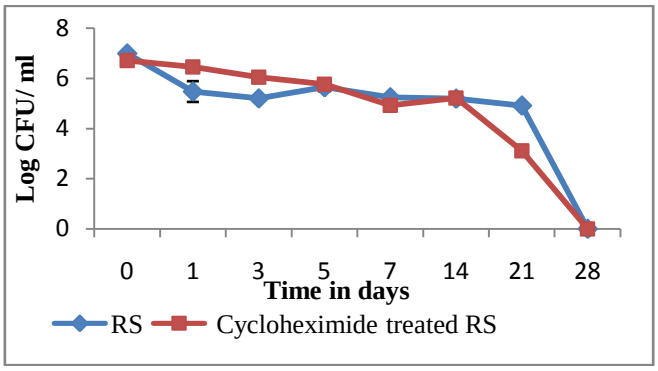

Figure 7.7 b Survival curves showing effect of protozoan predation on survival of *V. proteolyticus* in sediment of Cochin estuary

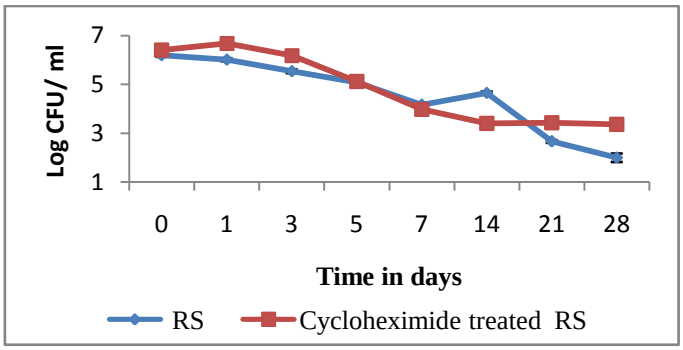

Figure 7.7 c Survival curves showing effect of protozoan predation on survival of *V. mimicus* in sediment of Cochin estuary

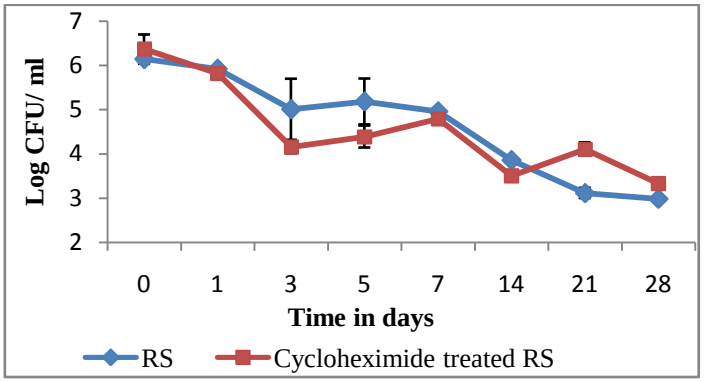

Figure 7.7 d Survival curves showing effect of protozoan predation on survival of *V. alginolyticus* in sediment of Cochin estuary

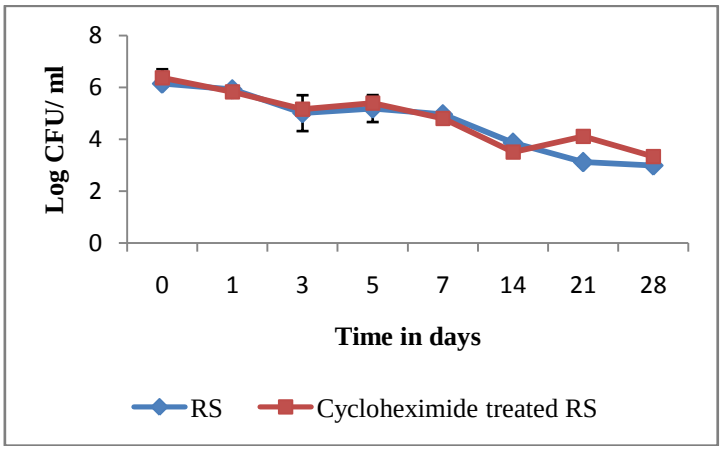

Figure 7.7 e Survival curves showing effect of protozoan predation on survival of *V. vulnificus* in sediment of Cochin estuary

7.5.6 Effect of sunlight on survival of *Vibrio* spp. in Cochin estuary

Among the five *Vibrio* spp. tested, *V. parahaemolyticus* was relatively more tolerant to the effect of sunlight. There was no significant difference in the survival of *V. parahaemolyticus* in the test and control microcosms (p=0.6). It showed similar survival pattern both in the presence and absence of sunlight (Figure 7.8 a). Thus, the species showed tolerance to the effect of sunlight. Sunlight showed a significant effect on the survival of *V. proteolyticus*, *V. mimicus*, *V. alginolyticus* and *V. vulnificus* (Figure 7.8 b-e). There was significant difference in the survival pattern of *V. vulnificus* (p=0.03), *V. mimicus* (p=0.01), *V. alginolyticus* (p=0.0007) and *V. proteolyticus* (p=0.008) in the control (kept in dark) and test (kept in sunlight) microcosms. They showed better growth and survival in control compared to the test microcosms.

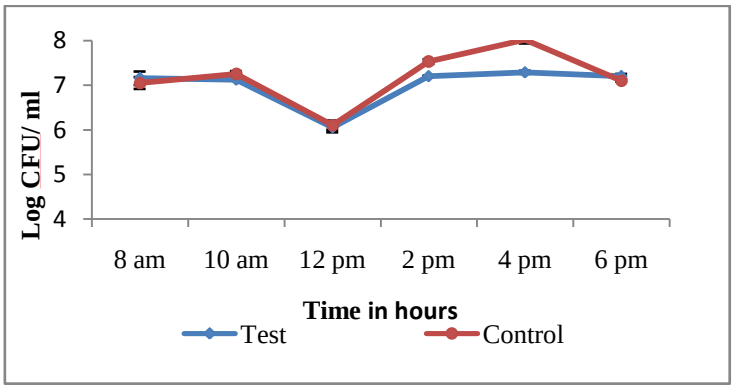

Figure 7.8 a Survival curves showing effect of sunlight on survival of *V. parahaemolyticus* in Cochin estuary

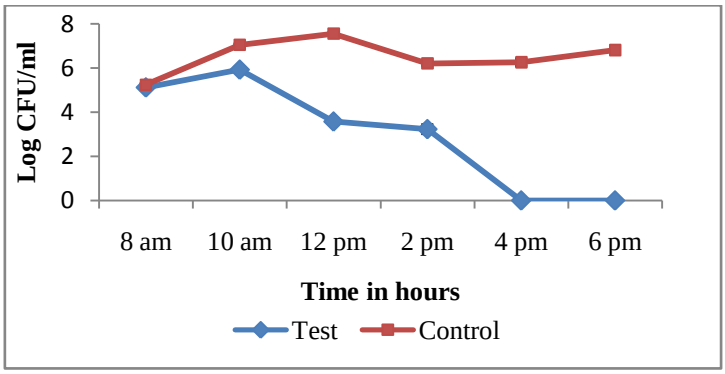

Figure 7.8 b Survival curves showing effect of sunlight on survival of *V. proteolyticus* in Cochin estuary

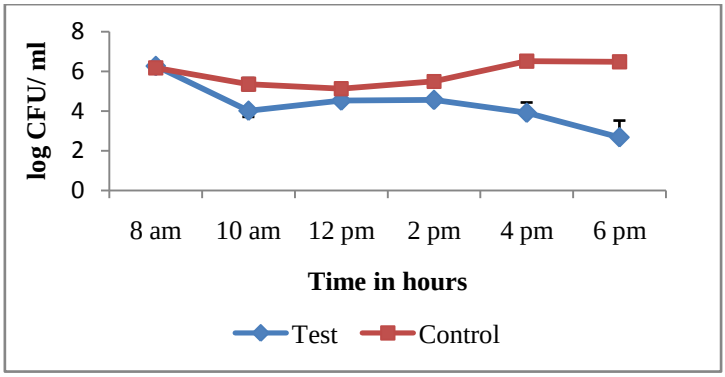

Figure 7.8 c Survival curves showing effect of sunlight on survival of *V. mimicus* in Cochin estuary

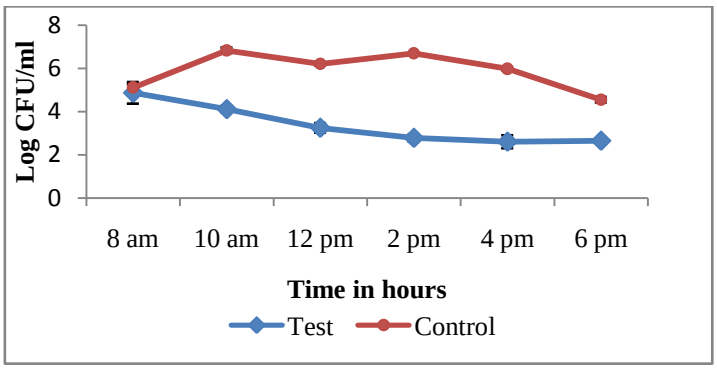

Figure 7.8 d Survival curves showing effect of sunlight on survival of *V. alginolyticus* in Cochin estuary

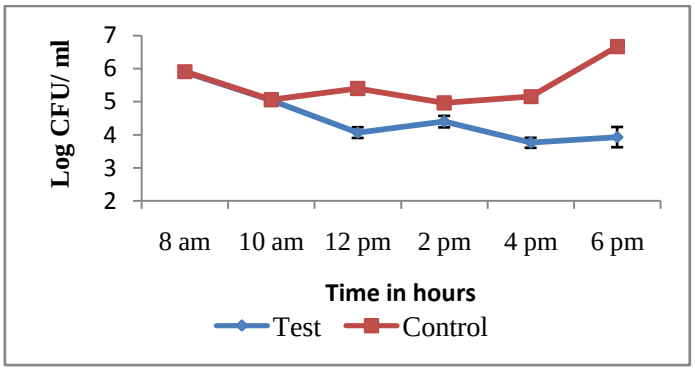

Figure 7.8 e Survival curves showing effect of sunlight on survival of *V. vulnificus* in Cochin estuary

The intensity of the sunlight during the experiment was measured using Luxmeter. Table 7.1 gives the intensity of sunlight measured during various hours starting from 8 am until 6 pm. The highest intensity (98 klx) was observed at 2 pm and the least at 6 pm (4 klx).

Table 7.1 Intensity of sunlight measured during various sampling intervals

Time	Illuminance
8 am	10 klx
10 am	83 klx
12 pm	90 klx
2 pm	98 klx
4 pm	53 klx
6 pm	4 klx

7.5.7 Effect of temperature on the survival of *Vibrio* spp. in Cochin estuary

Three different temperatures (25 ^{o}C, 30 °C and 35 °C) were selected for the present study. The survival pattern of each species at different temperatures was compared. Results revealed that all the species survived better at lower temperatures (Figure 7.9 a-e).

There was no significant difference in the survival of *V. proteolyticus* at 25 °C, 30 °C and 35 °C ($p>0.05$) (Figure 7.9 a). *V. parahaemolyticus* count started declining gradually at all temperatures after the 1st day. Initially, *V. parahaemolyticus* survived better at 35 °C for up to 7 days after which the count declined rapidly and the cells were non-culturable by end of 14th day. However, extended survival was observed at lower temperatures (25 °C and 30 °C). The cell count declined to zero by end of 21st day at 30 °C, while at 25 °C the cells remained culturable until the end.

No significant difference was observed in the survival kinetics of *V. proteolyticus* at 25 °C, 30 °C and 35 °C ($p>0.05$). Similar survival pattern was observed at all the temperatures until 7th day (Figure 7.9 b). After 7th day a slight growth was observed at 25 °C and 30 °C and a one log reduction was observed by end of 14th day. The count was stable afterwards till the end of the experiment. At 35 °C the survival was steady until 14th day after which the count dropped steeply by 3 logs by 28th day.

V. mimicus showed similar survival pattern at all the 3 temperatures until the 5th day (Figure 7.9 c). At 25 °C a steady count was observed until the 14th day. At 30 °C after 7th day there was a sudden drop in the count. At 35 °C a 5 logs reduction from the initial load was observed in the *V. mimicus* count by end of 21st day. After 21st day the count remained stable until the end of the experiment. Even though, the survival was better at lower temperatures compared to higher, there was no significant difference in the survival of *V. mimicus* at 25 °C, 30 °C and 35 °C ($p>0.05$).

V. alginolyticus showed similar survival pattern at all the three temperatures until the end of 7th day (Figure 7.9 d). After the 7th day, 2 logs reduction was found in the cell count at 30 °C and 35 °C. After 21st day, the *V. alginolyticus* count declined further at 35 °C whereas at 25 °C and 30 °C a slight increase was observed. Overall, there was no significant difference in the survival of *V. alginolyticus* at 25 °C, 30 °C and 35 °C ($p>0.05$).

Even though there was no significant difference in the survival kinetics of *V. vulnificus* at different temperatures ($p>0.05$), overall

survival was better at lower temperatures. The species exhibited occasional recovery and growth during the study period. At 25 °C and 35 °C almost similar pattern was observed while at 30 °C the pattern varied (Figure 7.9 e).

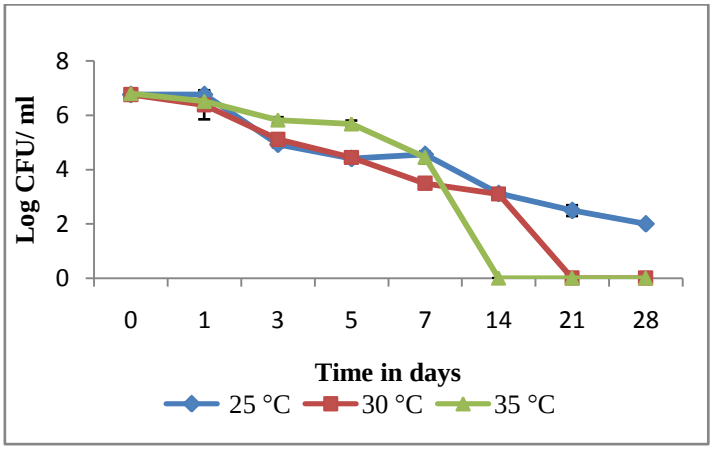

Figure 7.9 a Survival curves showing effect of different temperatures on survival of *V. parahaemolyticus* in Cochin estuary

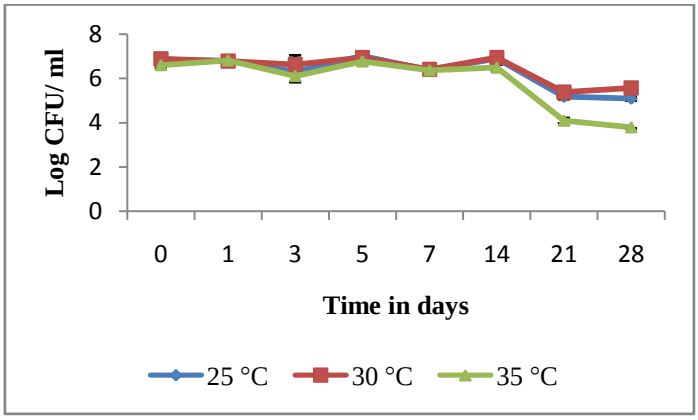

Figure 7.9 b Survival curves showing effect of different temperatures on survival of *V. proteolyticus* in Cochin estuary

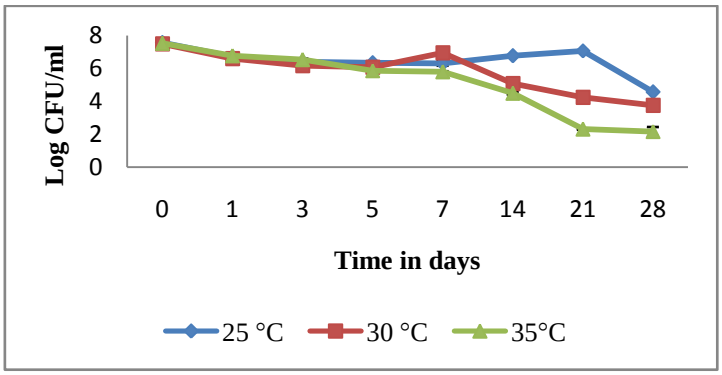

Figure 7.9 c Survival curves showing effect of different temperatures on survival of *V. mimicus* in Cochin estuary

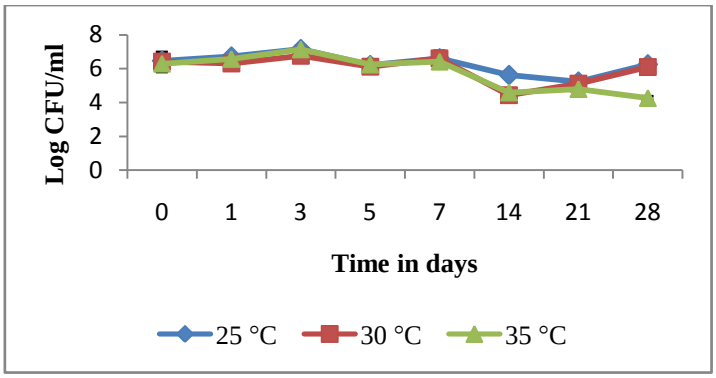

Figure 7.9 d Survival curves showing effect of different temperatures on survival of *V. alginolyticus* in Cochin estuary

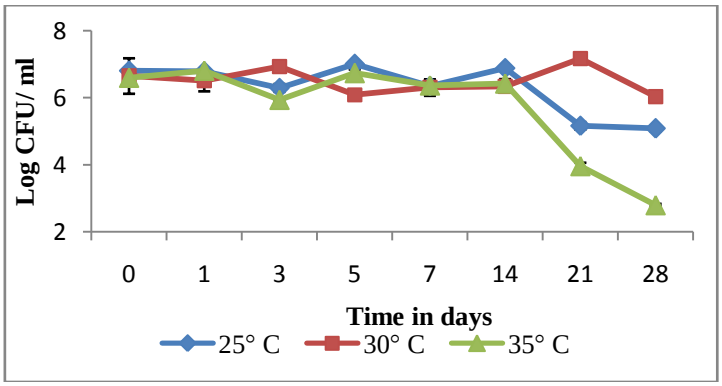

Figure 7.9 e Survival curves showing effect of different temperatures on survival of *V. vulnificus* in Cochin estuary

7.5.8 Effect of chemical factors on the survival of *Vibrio* spp. in Cochin estuary

The effect of chemical composition of water on the survival of *Vibrio* spp. in Cochin estuary was studied. In our study, a pronounced effect of chemical factor was found on the survival of all the 5 *Vibrio* species.

A significant difference was found on the survival of *V. parahaemolyticus* in the test and control microcosms (p=0.001) (Figure 7.10 a). In the test solution the cell were non-culturable by the end of 28th day. In the control (saline) survival persisted until the end. Better growth and survival was observed in the control microcosms.

V. proteolyticus exhibited enhanced growth and survival in the control microcosm (Figure 7.10 b). It exhibited pronounced difference in survival kinetics in the test and control and the difference was highly significant (p=0.0007). In the control, growth was observed initially up to the 3rd day. A slight decline was shown later and then a steady survival was observed until 14th day. Again a reduction in count was revealed followed by growth by end of 28th day. In the test solution after the 1st day itself a steep 3.5 logs drop was observed. The count was stable afterwards until 14th day and again a slight decline was observed.

V. mimicus also showed a significant difference in the survival pattern in the test and control microcosms (p=0.001). A steady and extended survival was observed in the control until the end of the experiment compared to test solution (Figure 7.10 c). In the test the cell count reached zero by end of 28th day.

There was a significant difference on the survival pattern of *V. alginolyticus* in the test and control microcosms (p=0.004) (Figure 7. 10 d). Initially, the cell count declined steeply in the test solution. After the 3rd day a steady count was observed till the end of the study. In the control the count dropped gradually after 3rd day until the end.

The test solution did not support any growth of *V. vulnificus*. From the initial day the count declined rapidly and culturability was lost by the end of 21st day itself. Whereas, growth was favoured in the control microcosm and survival was observed until the end of the experiment (Figure 7.10 e). The difference in the survival pattern of *V. vulnificus* in both the microcosms was statistically significant (p=0.002).

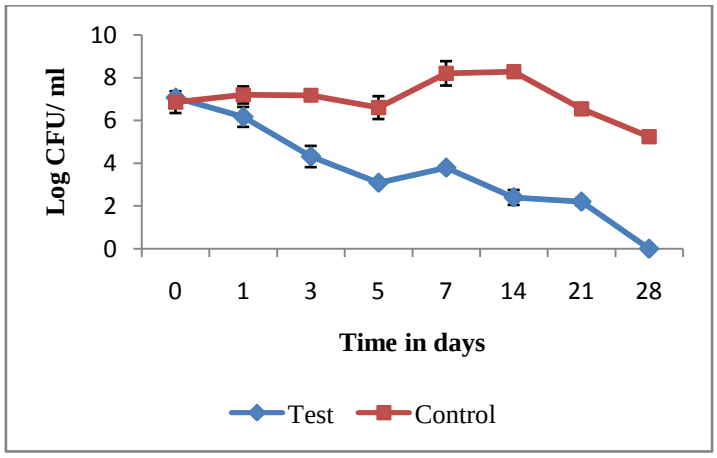

Figure 7.10 a Survival curves of *V. parahaemolyticus* as a function of chemical factors in Cochin estuary

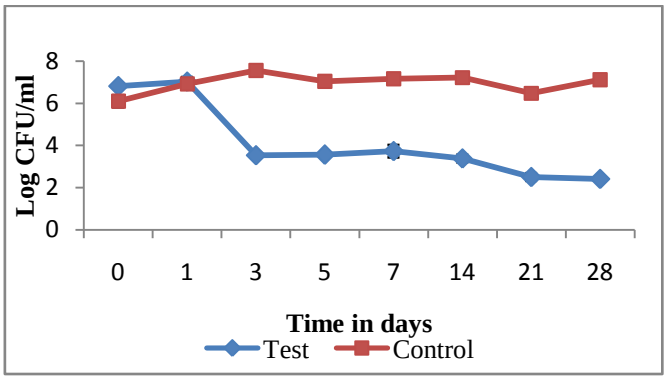

Figure 7.10 b Survival curves of *V. proteolyticus* as a function of chemical factors in Cochin estuary

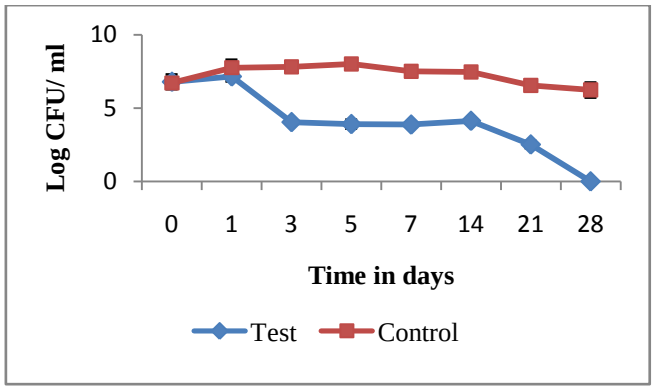

Figure 7.10 c Survival curves of *V. mimicus* as a function of chemical factors in Cochin estuary

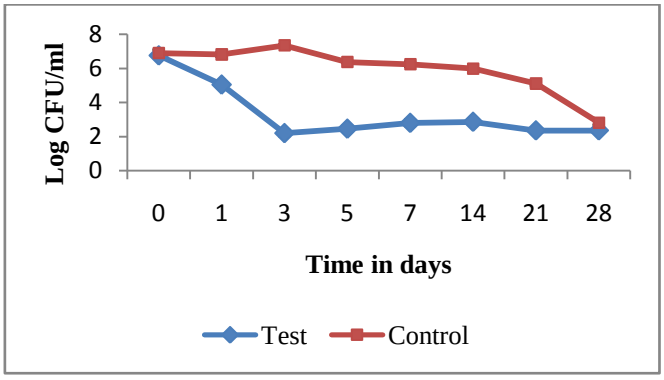

Figure 7.10 d Survival curve of *V. alginolyticus* as a function of chemical factors in Cochin estuary

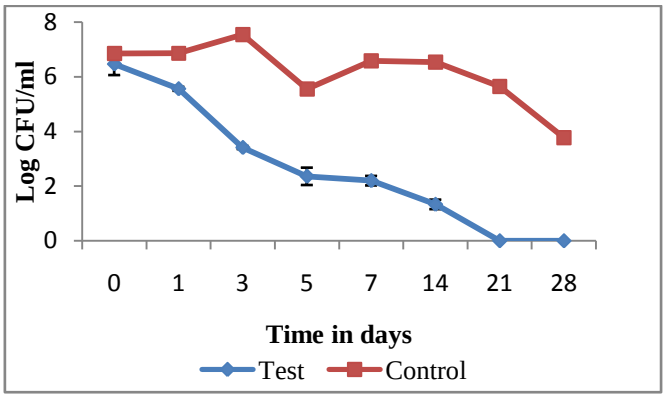

Figure 7.10 e Survival curves of *V. vulnificus* as a function of chemical factors in Cochin estuary

The nutrients levels and heavy metal concentrations of the estuarine water are given in Table 7.2.

Table 7.2 Nutrients and heavy metal concentration of water sample from Cochin estuary

Nutrients (mg/L)	
Ammonium	5.73
Nitrite	0.34
Nitrate	1.50
Phosphate	5.16
Silicate	10.37
Heavy metals (ppm)	
Lead	0.0045
Cadmium	0.00045
Iron	0.93724
Nickel	0.00635
Zinc	0.01135
Antimony	BDL*
Arsenic	BDL
Chromium	BDL
Mercury	BDL

*BDL Below detection limit

7.5.9 Effect of varying salinity on survival of *Vibrio* spp. in Cochin estuary

The survival kinetics of the five *Vibrio* spp. was analysed at 0 ppt, 15 ppt and 30 ppt. Results revealed that all the species exhibited enhanced survival at higher salinities. All the five species showed better and stable survival at 15 ppt and 30 ppt when compared to 0 ppt (Figure 7.11 a-e).

There was no significant difference in the survival pattern of *V. parahaemolyticus* at 15 ppt and 30 ppt salinities (Figure 7.11 a). At 0 ppt initially a slight growth was observed up to 3rd day, after which a gradual decline was observed in the cell count until the 28th day. The difference was however not statistically significant ($p>0.05$).

V. proteolyticus showed almost similar survival rate at 0 ppt and 15 ppt salinities until end of 3rd day (Figure 7.11 b). There was no significant difference in the survival pattern at varying salinities ($p>0.05$). At 35 ppt, an initial growth was observed and after 3rd day similar pattern was observed at 15 ppt and 35 ppt until the end of the experiment. At 0 ppt the cell count declined after 5th day and then it remained stable upto 21st day. It was followed by a rapid 1.8 logs reduction in the count.

There was no significant difference in the survival pattern of *V. mimicus* at varying salinities ($p>0.05$) (Figure 7.11 c). It showed a slight growth at 0 ppt initially up to the 3rd day. The count declined until the 7th day and then remained stable until 21st day. The count again declined after the 21st day. Almost similar survival pattern was observed at 15 ppt and 30 ppt microcosms.

V. alginolyticus exhibited similar survival trends at 15 ppt and 30 ppt salinities (Figure 7.11 d). Survival was better at higher salinities compared to 0 ppt. However, the difference in survival at three salinities was not statistically significant ($p>0.05$).

There was significant difference in the survival of *V. vulnificus* at varying salinities ($p<0.05$). Enhanced survival was observed at 30 ppt followed by 15 ppt and the least at 0 ppt (Figure 7.11 e). The cell count declined steeply after the 5th day at 0 ppt. At higher salinities an initial growth was shown. At 35 ppt the cell count reduced slowly from the 3rd day till the end. At 15 ppt the count reduced after 3rd day and a stable survival was exhibited afterwards.

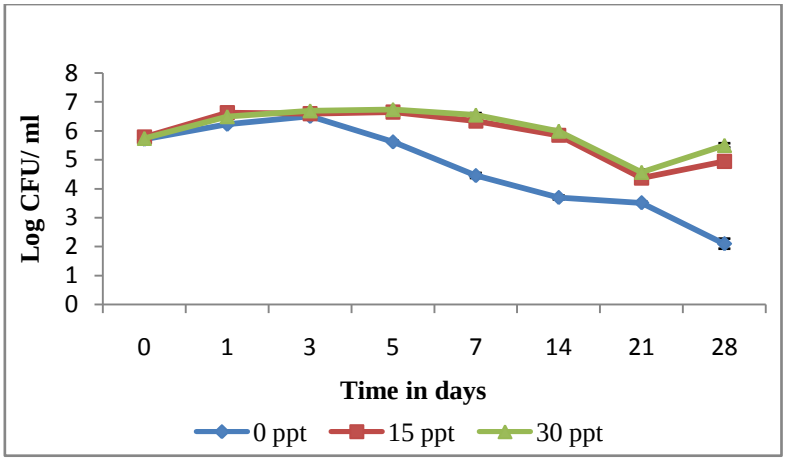

Figure 7.11 a Survival curves of *V. parahaemolyticus* at varying salinities

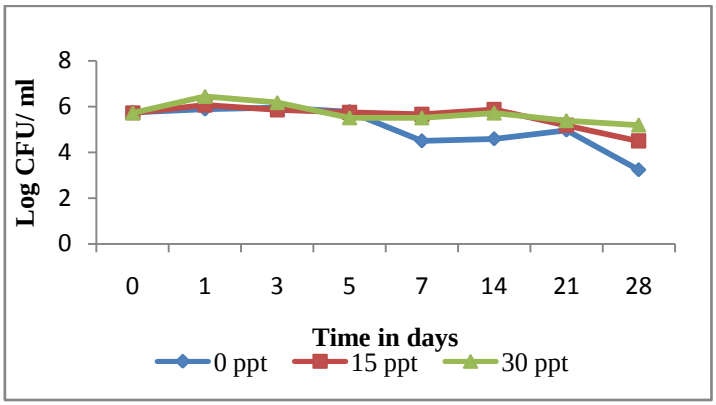

Figure 7.11 b Survival curves of *V. proteolyticus* at varying salinities

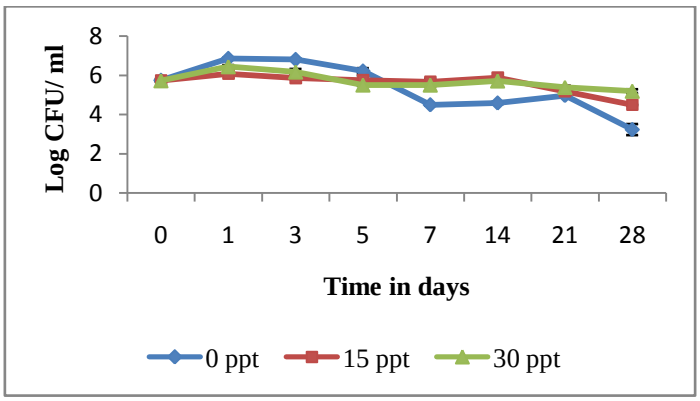

Figure 7.11 c Survival curves of *V. mimicus* at varying salinities

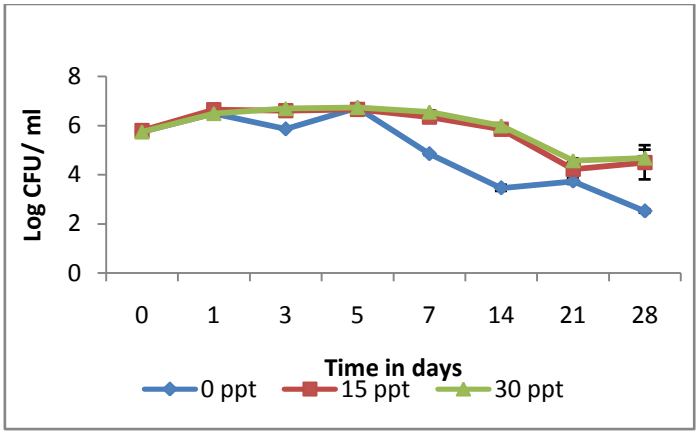

Figure 7.11 d Survival curves of *V. alginolyticus* at varying salinities

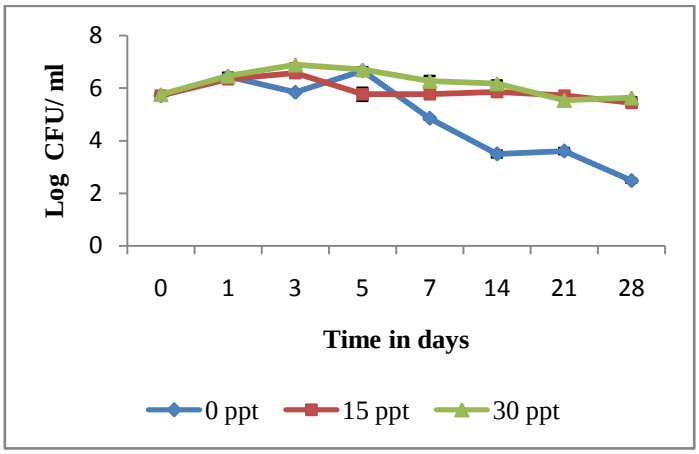

Figure 7.11 e Survival curves of *V. vulnificus* at varying salinities

7.6 Discussion

7.6.1 Survival of *Vibrio* spp. in water and sediment of Cochin estuary

All the five *Vibrio* species exhibited an extended and better survival in the sediment compared to water. This could be attributed to the better concentration and availability of nutrients in the sediment, which could support growth and survival. The sediments are also known to protect the bacteria from the deleterious effect of UV radiation from the sun, predation and even high salinity in marine environments (Abia *et al.*, 2016). This is in agreement with a previous report where *V. fluvialis* showed prolonged survival in sediment microcosms (Amel *et al.*, 2008). This highlights that sediments of Cochin estuary could act as permanent reservoirs or repository for these pathogenic bacteria as it showed extended survival in estuarine sediments, which in turn would pose serious public health concern. Similar results were also reported from Vembanad Lake where the lake sediments acted as repository for pathogens (Abhirosh *et al.*, 2011 b).

In addition, the better growth of *Vibrio spp.* in autoclaved sediments compared to raw may be possibly due to the absence of competing organisms and abundance of available nutrients. This is in agreement with previous report by Hood and Ness (1982). Autoclaving results in release of those nutrients from the sediments that are usually in bound form in the natural environments (Gerba and McLeod, 1976). Lack of nutrients is the most common stress faced by environmental bacteria in aquatic environments**.** Starvation leads bacteria to decrease in their size and activity (Stevenson, 1978). One of the survival strategies of bacteria to withstand starvation effect is the reduction of its endogenous metabolic rate rapidly. *Vibrio* spp. have the capability of long term survival even during starvation. They withstand starvation through sequential changes in their cell physiology and morphology (Morita, 1993). They also have the ability to enter VBNC state (non-culturable) under starvation (Amel *et al.,* 2008; Fernández-Delgado *et al.,* 2015).

Sediment borne bacteria may also be easily available to shellfishes during re-suspension while dredging. As the shipping channel in the Cochin estuary is dredged on a regular basis in order to facilitate movement of ships to Cochin port, the re-suspension of sediment is real. Extended survival of pathogenic *Vibrio* species in water and sediment of Cochin estuary is a major threat to the public health.

7.6.2 Role of biotic factors on survival of *Vibrio* species in Cochin estuary

Even though, there was variation in the survival kinetics between each *Vibrio* spp., all of them exhibited higher mortality in raw water and

sediment microcosms compared to autoclaved microcosms (which is devoid of any biological factors). This suggests the role played by various biological factors on the removal of the test organisms. According to Abhirosh and Hatha (2005) there are various physico-chemical and biological factors involved in the disappearance of pathogenic microorganisms in the aquatic environment. They highlighted the role of biotic factors on the removal of *E. coli* and *Salmonella* from Cochin estuary.

In cycloheximide treated microcosms that is devoid of protozoans, the species survived better compared to the non-treated microcosms. The protozoans identified in the present study include *Vorticella, Tintinnids, Euglena, Phacus, Strombidium, Favella, Tintinnopsis, Centropyxis, Difflugia, Stylonichia*. Previous studies have already documented that protozoan grazing was the significant factor responsible for the removal of bacterial population in aquatic environments (Hahn and Hofle, 2001; Abhirosh and Hatha, 2005). In the study by Abhirosh and Hatha (2005) 19 protozoan species were identified from the estuarine water. It included *Amoeba* spp., *Difflugia* spp., *Vorticella* spp., *Lionotus* spp., *Phacus* spp., *Oxytricha* spp., *Trachelomonas*, *Paramoecium* spp., *Stylonichia* spp., *Euplotes* spp., *Euglena* spp. etc.

Bacteriophage specific to *V. parahaemolyticus* species was found in the estuary. The mortality that was observed in cycloheximide treated microcosms may be considered to be induced by bacteriophage or by competing autochthonous bacteria. Thus, in the present study besides protozoans, bacteriophages and competition with other autochthonous bacteria were also identified to be other major biological factors responsible

for the mortality of the test organisms. There was significant level of autochthonous heterotrophic bacteria that could compete with the test organisms. The autochthonous bacteria identified in the study were *Cytophaga*, *Moraxella*, *Flavobacterium*, *Alkaligenes*, *Bacillus* and *Staphylococcus.* This clearly indicated the role played by biological factors in the removal of these pathogens from the aquatic environments. This is in agreement with the previous study on survival *of V. parahaemolyticus* from Vembanad Lake (Abhirosh *et al.,* 2009).

7.6.3 Role of physico-chemical factors on the survival of *Vibrio* species in Cochin estuary

The survival pattern of each species varied at different temperatures. However, all the species showed extended survival at 25 ^{o}C and 30 ^{o}C. Higher temperature (35 ^{o}C) was not favourable for its prolonged survival. Our findings are in agreement with the previous studies stating that lower temperatures favour extended survival of bacteria in fresh and marine waters (Rozen and Belkin, 2001; An *et al.,* 2002). Previous studies have shown that *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* are frequent in warm temperatures (>20 °C) compared to lower temperatures (5 °C) (Pfeffer *et al.,* 2003, Elhadi *et al.,* 2004; Fukushima and Seki, 2004).

Vibrio species are frequently observed during warm months in environmental waters (Pfeffer *et al.,* 2003; Parvathi *et al.,* 2004; Martinez-Urtaza *et al.,* 2010; Le Roux *et al.,* 2015; Vezzulli *et al.,* 2016; Muhling *et al.,* 2017). Higher temperature (35 ^{o}C) favoured the growth of the species for short durations. Deepanjali *et al.* (2005) and Julie *et al.* (2010) reported an increased prevalence of *Vibrio* during the pre-monsoon season (summer)

compared to winter months. In our study also, maximum abundance of *Vibrio* was observed during the summer season. They are known to enter VBNC state in water temperatures below 15 °C (Oliver *et al.,* 1991). The cells maintained their respiratory activity and membrane integrity in the VBNC state, but remained non-culturable (Oliver, 2010).

Sunlight plays a major role in inactivation of pathogenic bacteria in aquatic environments (Walker *et al.,* 2004; Whitman *et al.,* 2004; Anuar and Chan, 2013). In the present study, *V. parahaemolyticus* was tolerant to the effect of sunlight. The survival of *V. mimicus*, *V. proteolyticus*, *V. alginolyticus* and *V. vulnificus* was significantly affected by exposure to sunlight. The photosynthetically active region and UV regions (UV-A and B) of the sunlight have strong bactericidal activities (Santos *et al.,* 2011; Ruiz- Gonzalez *et al.,* 2012). Numerous research have been carried out to study the effect of sunlight on inactivation of pathogens from various environments (Jeffrey *et al.,* 2000; Rijal and Fujioka, 2001; Hockberger, 2002; Walker *et al.,* 2004; Whitman *et al.*, 2004; Abhirosh and Hatha, 2005; Mani *et al.,* 2006; Pakulski *et al.,* 2007; Djurdjevic-Milosevic *et al.*, 2011; Anuar and Chan, 2013).

The present study revealed that the chemical composition of estuarine water had a significant deleterious effect on the survival of all the five *Vibrio* spp. The chemical factors responsible for bactericidal activity in the natural waters include secondary metabolites like antibiotics produced by autochthonous bacteria, toxins, heavy metals etc. (Burgess *et al.*, 1991; Sharma, 2000). In the present study, heavy metals namely nickel, lead, zinc, cadmium and iron were detected in water from Cochin

estuary. A previous study also reported Cochin estuary to be highly polluted with heavy metals such as nickel, lead, zinc and cadmium (Bindu *et al.*, 2015).

All the species tested exhibited better survival at higher salinities compared to zero saline condition. Deepanjali *et al.* (2005) reported an increased prevalence of *Vibrio* during the pre-monsoon season (summer). During this season the salinity is higher compared to the monsoon. In monsoon, heavy rains and freshwater influx causes strong desalination of the estuarine and sea water (Colwell and Grimes, 2000; Oliver, 2010). All *Vibrio* spp. being halophiles (except *V. cholerae* and *V. mimicus*) are under stress at low salinities and its membrane integrity is lost (Oliver, 2010). The observed initial growth of *V. mimicus* at zero saline microcosms in our study can be justified by its capacity to tolerate fresh water conditions. Low salinities have also shown to induce VBNC state in *V. parahaemolyticus* (Oliver, 2010). The effect of water salinity on survival of toxigenic *Vibrio cholerae* O1 was investigated in laboratory microcosms (Huq *et al.*, 1984). Among the 3 different salinities tested (5%, 10% and 15%), maximum growth was observed at 15%. The effect of salinity on survival of *Vibrio splendidus* was studied (Susana *et al.*, 2003). Salinity range chosen was (33–0.9%) and the results showed maximum survival at 3.3% salinity.

Cochin backwaters are responsible for the rich fishery potential of Kerala and are a major source of livelihood for the local fishermen community. The estuary is also a famous tourist hot spot for recreational activities. Estuary being a receptacle for various kinds of domestic and

industrial effluents, is subjected to considerable pollution and poses health hazard to people who use this natural water body for recreation and livelihood. Hence, the persistence of pathogenic species of *Vibrio* in the estuary raises serious concern about the public health. Even though the study highlights the role of biological and physico-chemical factors in the self-purification of the estuarine environments, introduction of high load of pathogens into the system through untreated sewage may disrupt this balance. Hence, the concerned authorities should take necessary control measures to check the pollution to ensure seafood safety and to prevent the potential spread of any outbreaks in the present study areas.

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Chapter 8

SUMMARY AND CONCLUSION

In the present study a detailed investigation into the diversity and distribution of *Vibrio* species in Cochin estuary was undertaken. The vibrios were characterised both phenotypically and genotypically. Prevalence of multiple antibiotic resistance and virulence potential among *Vibrio* from Cochin estuary, shrimp farm and seafood were evaluated as part of risk assessment. Since pathogenic vibrios are a cause of major concern, their survival kinetics in Cochin estuary as a function of physico-chemical and biological factors were also studied. A tool to differentiate between closely related *Vibrio* species was developed based on *groEL* PCR-RFLP method.

The salient findings of the study are summarised below:

- A total of 16 *Vibrio* species were encountered in Cochin estuary among which *V. parahaemolyticus* was the dominant species (50%).
- Temporal and spatial variation was observed in the distribution of *Vibrio* species in Cochin estuary. Maximum diversity of *Vibrio* species was observed during the pre-monsoon and the least during post-monsoon season. A total of 12 species were

isolated during pre-monsoon, 8 during monsoon and only 2 species were isolated during the post-monsoon season.

- Among the 10 stations in Cochin estuary, Murinjapuzha exhibited maximum *Vibrio* diversity. Eloor and Varapuzha were the least diverse.
- Multiple antibiotic resistant strains were prevalent in most stations of estuary as well as in shrimp farm and seafood samples.
- Strains from different sources varied widely in their antibiotic resistance pattern.
- There was high prevalence of beta-lactam resistance among the strains from Cochin estuary, shrimp farm and seafood samples.
- Among the 25 antibiotics tested, netillin (aminoglycoside) was found to be effective against all *Vibrio* strains studied.
- MAR index of *Vibrio* isolates from Cochin estuary, shrimp farm and seafood ranged from 0.24-0.64, 0.2-0.48 and 0.16-0.44 respectively.
- All the *Vibrio* isolated from the three sources harboured the bla_{TEM} gene.
- The $bla_{\text{CTX-M}}$ gene was present in 1.1% of strains from Cochin estuary. None of the *Vibrio* strains from seafood and shrimp farm harboured the gene.

- New Delhi metallo-beta-lactamase (bla_{NDM-1}) gene was present in 13.3% of *Vibrio* strains from Cochin estuary, 14.2% of strains from shrimp farm and 6.6% of strains from seafood.
- Prevalence of plasmids among *Vibrio* from Cochin estuary, shrimp farm and seafood were 16.6%, 32.8% and 16.6% respectively. Plasmid of size 33 kb was the most frequently observed.
- Plasmid mediated resistance was shown towards 13 antibiotics (Ak, Amp, Amx, Caz, Cb, Cl, E, Ex, Fr, Nit, S, Sm, Tr). Resistance to carbenicillin was the most frequently lost phenotype after plasmid curing.
- Majority of the *Vibrio* from Cochin estuary and all from seafood produced gelatinase and DNase. Gelatinase and lipase producers dominated among shrimp farm isolates.
- The virulence related *tdh* and *trh* genes were detected only in *V. parahaemolyticus* from Cochin estuary.
- Detection of virulence related T3SS genes in *V. parahaemolyticus* strains revealed the presence of T3SS1 in all the strains from the three sources. T3SS2α was not detected in any of the strains. The prevalence of T3SS2β in *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood were 66.6%, 28% and 60% respectively.

- Genotyping by RAPD and ERIC-PCR revealed high level of genetic heterogeneity among *V. parahaemolyticus* strains from Cochin estuary, shrimp farm and seafood.
- No seasonal or bio-geographic effect was observed on the genotypic distribution of *V. parahaemolyticus* strains from Cochin estuary, shrimp farm and seafood.
- *GroEL* PCR-RFLP method employed in our study was highly efficient for detection and differentiation of closely related *Vibrio* species.
- Phylogenetic analysis revealed *groEL* gene to be a better phylogenetic marker for *Vibrio* compared to 16S rRNA gene.
- Species-wise variation was observed in the survival of pathogenic vibrios in water and sediment of Cochin estuary.
- All the five species (*V. parahaemolyticus, V. mimicus, V. alginolyticus, V. vulnificus* and *V. proteolyticus*) showed extended and better survival in the sediment compared to water. Thus, sediment of Cochin estuary could act as permanent reservoirs of such pathogenic vibrios.
- Biological factors revealed a major role on the removal of pathogenic vibrios from the estuarine environment.
- There was significant reduction in the survival of *V. mimicus, V. alginolyticus, V. vulnificus* and *V. proteolyticus* when exposed to sunlight. However, *V. parahaemolyticus* was tolerant to sunlight.

- Even though temperature did not have much significant effect on the survival of the five *Vibrio* species, lower temperatures favoured extended survival.
- Chemical factors had a pronounced effect on the survival of all the five *Vibrio* species.
- All the five species (*V. parahaemolyticus, V. mimicus, V. alginolyticus, V. vulnificus* and *V. proteolyticus*) had comparatively better survival at higher salinities.

The present study highlights that pathogenic *Vibrio* species are present in Cochin estuary. Hence, it is likely that they could be also present in the fishes and shrimps itself, with the implicit consumer health risk, particularly in regions where raw seafood is consumed. The study emphasizes the local health authorities to implement continuous surveillance of water systems in Cochin estuary for pathogenic vibrios, to ensure the seafood and public health safety of the study area. The study also urges the need for adoption of proper water quality management in the aquaculture systems surrounding the estuary.

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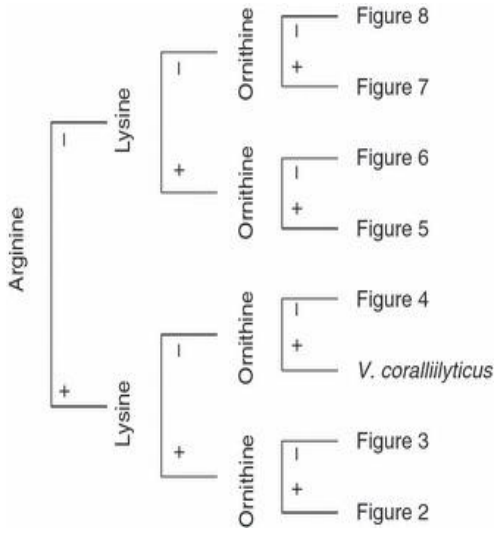
Appendices

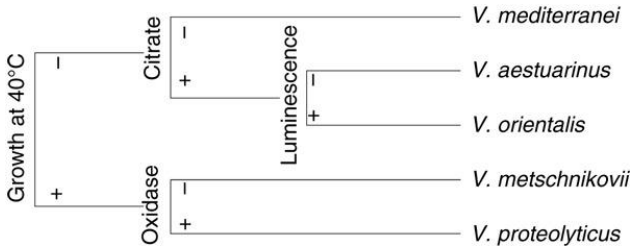
Appendix 1

DICHOTOMOUS KEYS FOR IDENTIFICATION OF *VIBRIO* SPECIES

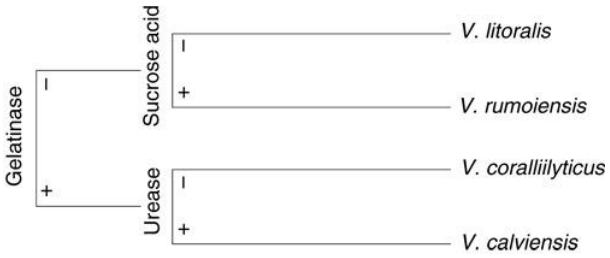


Appendix 1(a) The primary key defined by arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase (A / L / O). Every cluster leads to another figure (i.e. the next identification key)

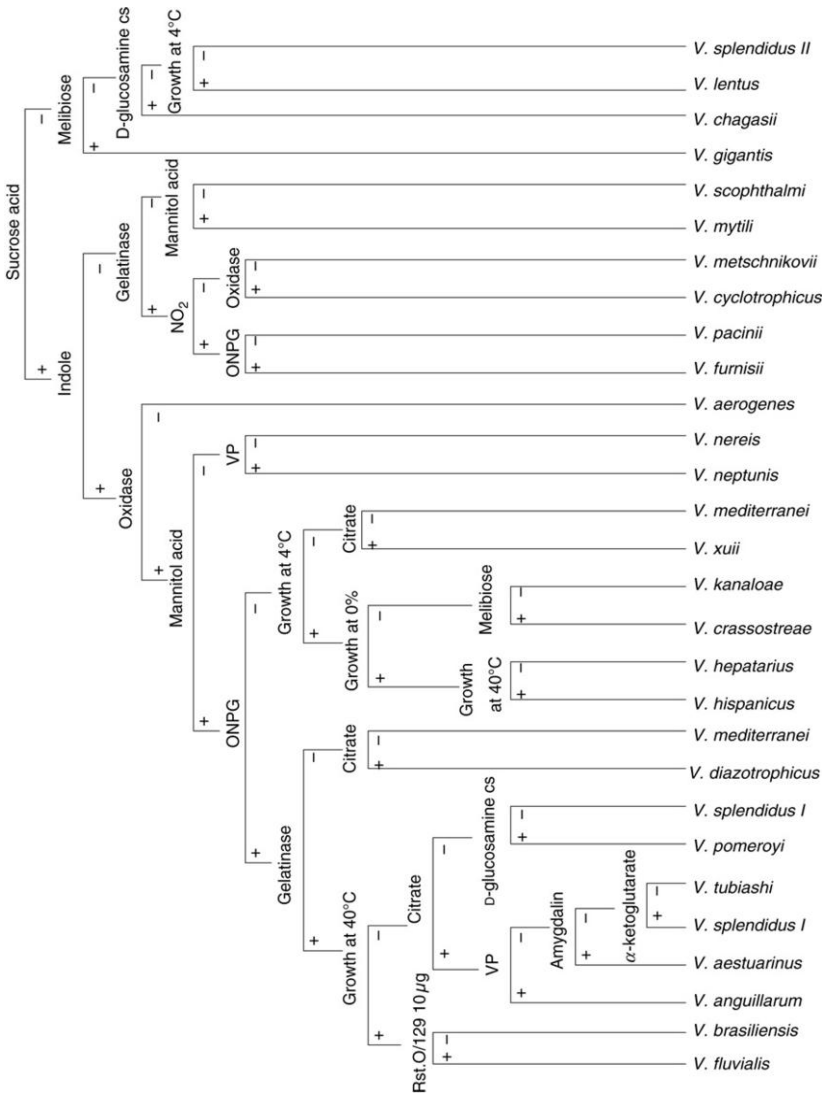
Appendix 1(b) Identification key for the cluster A+/L+/O+



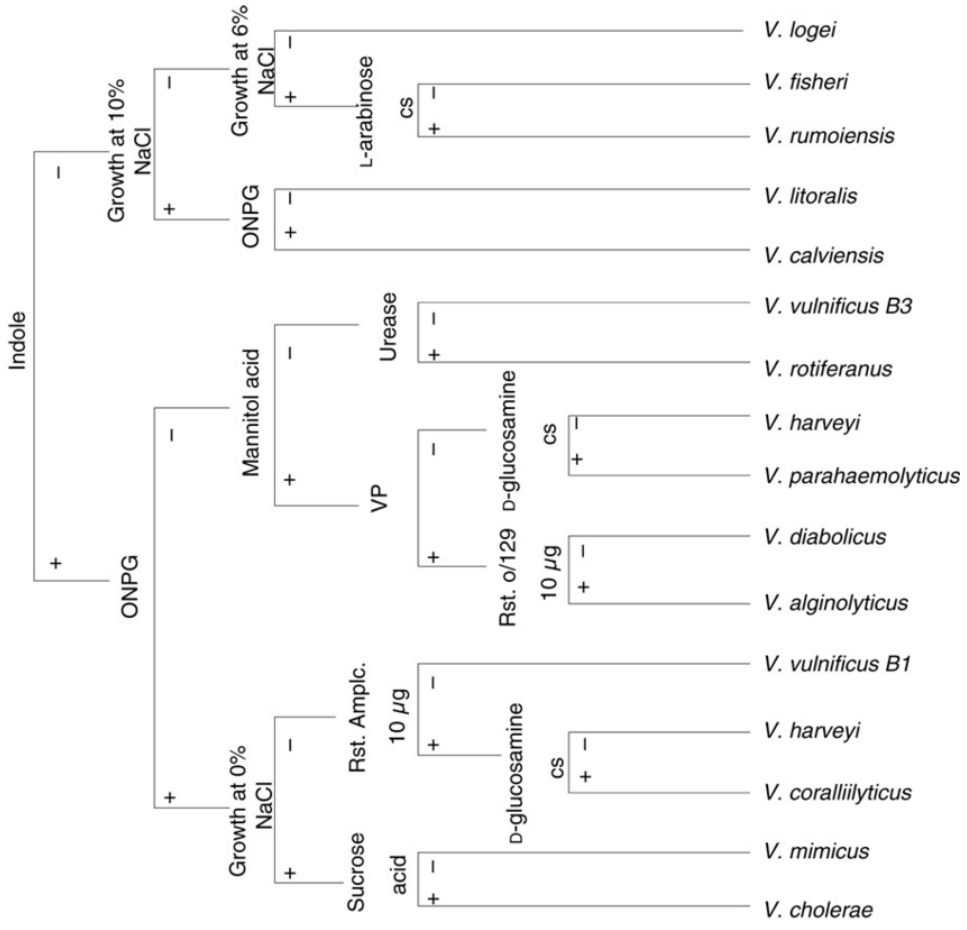
Appendix 1(c) Identification key for the cluster A+/L+/O-



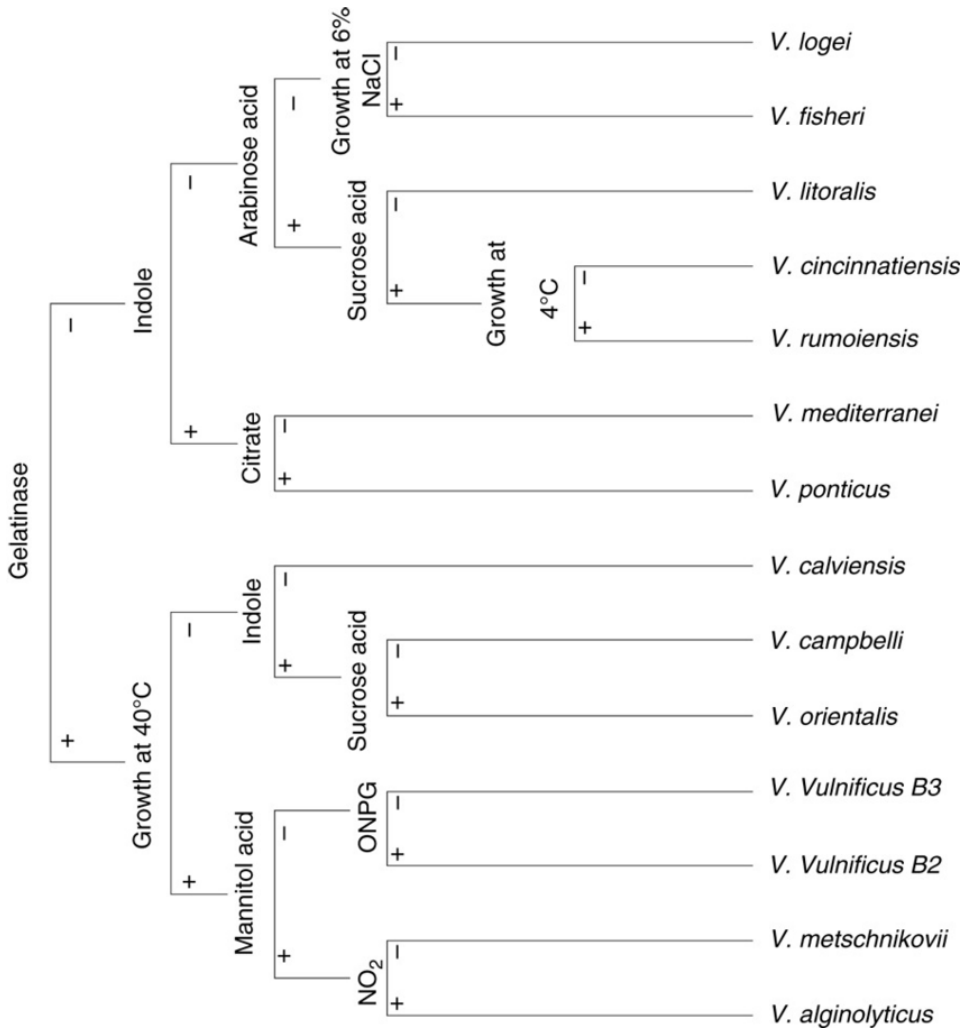
Appendix 1(d) Identification key for the cluster A-/L-/O+



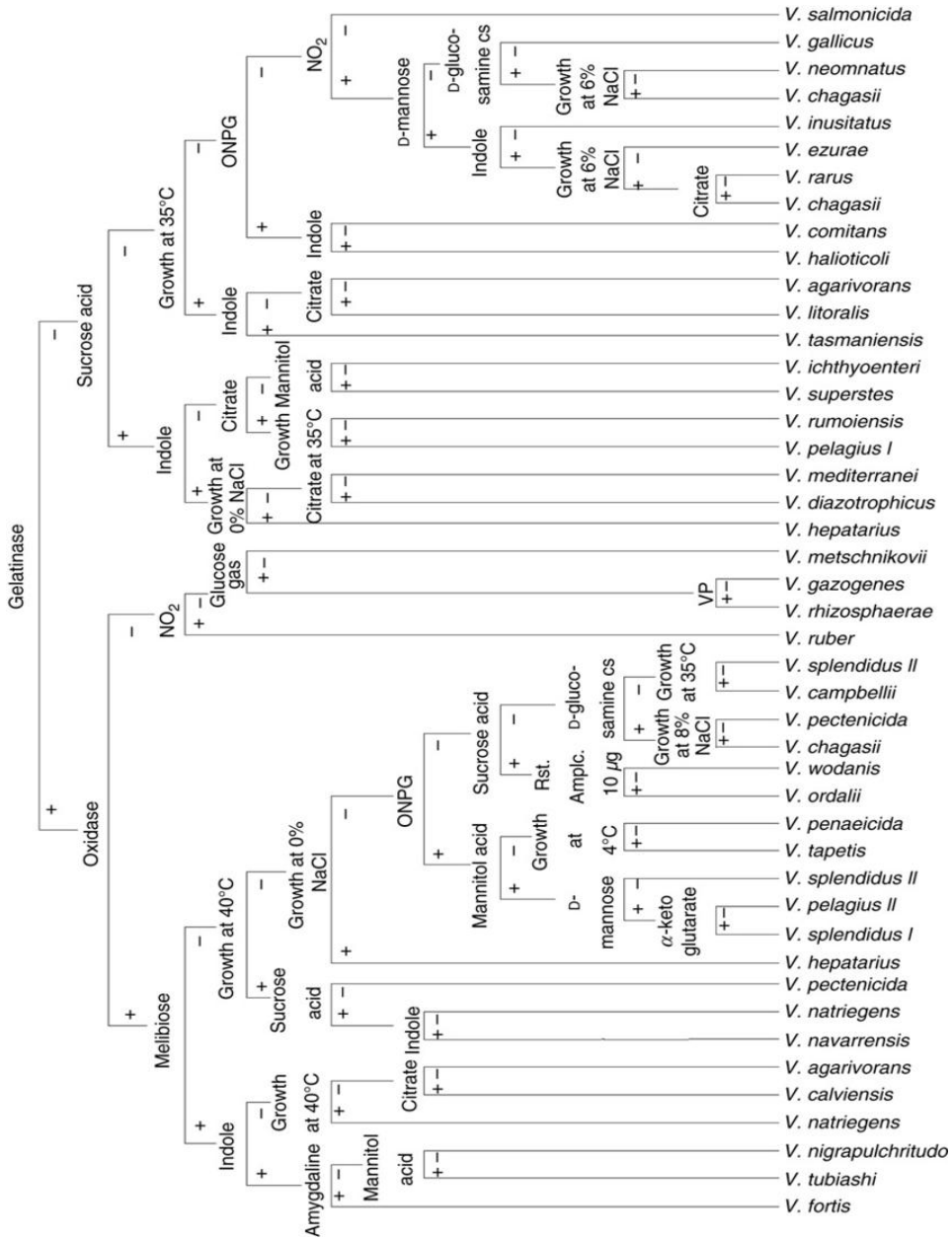
Appendix 1(e) Identification key for the cluster A+/L-/O-



Appendix 1 (f) Identification key for the cluster A-/L+/O+



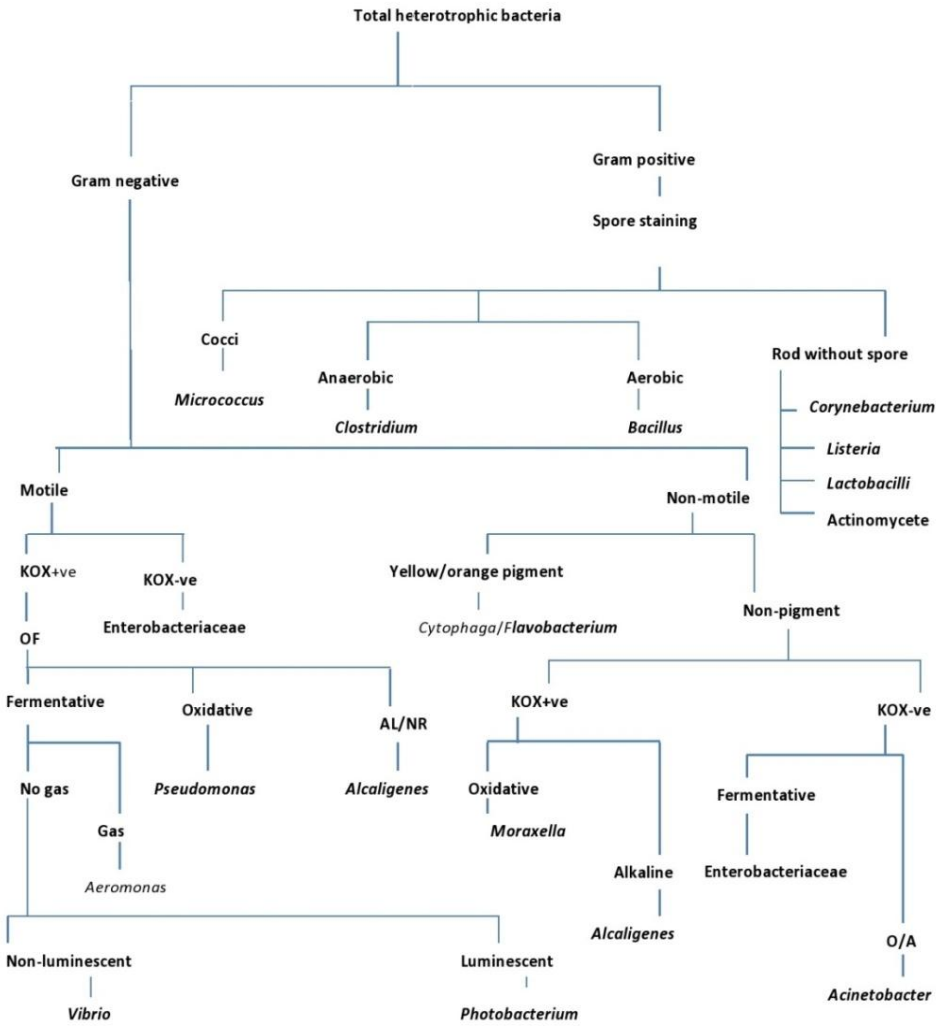
Appendix 1 (g) Identification key for the cluster A-/L+/O-



Appendix 1(h) Identification key for the cluster A-/L-/O-

Appendix 2

IDENTIFICATION CHART FOR TOTAL HETEROTROPHIC BACTERIA



Appendix 3

STATISTICAL ANALYSIS

Appendix 3.1 Chi-Square test to find any significant difference in the antibiotic resistance of *Vibrio* from Cochin Estuary, Shrimp Farm and Seafood

Antibiotics	Cochin Estuary	Shrimp Farm	Seafood	p -value
Amp	180 (100.0%)	54 (77.1%)	25 (83.3%)	0.000
Amx	180 (100.0%)	61 (87.1%)	26 (86.7%)	0.000
Ak	24 (13.3%)	8 (11.4%)	4 (13.3%)	0.917
C	6 (3.3%)	7 (10.0%)	15 (50.0%)	0.000
Caz	24 (13.3%)	43 (61.4%)	4 (13.3%)	0.000
Cb	32 (17.8%)	18 (25.7%)	15 (50.0%)	0.001
Cip	0 (0.0%)	0 (0.0%)	2 (6.7%)	0.011
Cep	180 (100.0%)	70 (100.0%)	27 (90.0%)	0.001
Cl	180 (100.0%)	50 (71.4%)	28 (93.3%)	0.000
Cot	4 (2.2%)	0 (0.0%)	0 (0.0%)	0.168
Ctr	0 (0.0%)	11 (15.7%)	0 (0.0%)	0.000
Do	36 (20.0%)	3 (4.3%)	0 (0.0%)	0.000
E	57 (31.7%)	40 (57.1%)	2 (6.7%)	0.000
Ex	153 (85.0%)	9 (12.9%)	30 (100.0%)	0.000
Fr	165 (91.7%)	23 (32.9%)	30 (100.0%)	0.000
Gen	4 (2.2%)	2 (2.9%)	0 (0.0%)	0.482
Na	9 (5.0%)	15 (21.4%)	0 (0.0%)	0.000
Net	0 (0.0%)	0 (0.0%)	0 (0.0%)	--
Nx	7 (3.9%)	1 (1.4%)	0 (0.0%)	0.224
Nit	165 (91.7%)	19 (27.1%)	11 (36.7%)	0.000
O	41 (22.8%)	5 (7.1%)	4 (13.3%)	0.007
Sm	151 (83.9%)	41 (58.6%)	0 (0.0%)	0.000
S	64 (35.6%)	5 (7.1%)	4 (13.3%)	0.000
Te	7 (3.9%)	0 (0.0%)	0 (0.0%)	0.043
Tr	48 (26.7%)	29 (41.4%)	30 (100.0%)	0.000

Appendix 3.2 Chi-Square test to find any significant difference in extracellular enzyme production among *Vibrio* from Cochin Estuary, Shrimp Farm and Seafood

	Cochin Estuary	Shrimp farm	Seafood	p-value
Amylase	68	43	90	0.000
Chitinase	52	26	7	0.000
Caseinase	44	52	10	0.000
DNase	84	76	100	0.000
Gelatinase	84	90	100	0.000
Hemolysin	22	11	6	0.003
Lipase	76	80	80	0.728
Phosphatase	80	73	90	0.009

Appendix 3.3 T-Test to find significant difference in survival of *V. vulnificus* in water and sediment

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Source	1.5000	16	.51640	.12910
	Count	3.9894	16	1.99232	.49808

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	source & count	16	-.383	.143

Paired Samples Test					
		Paired Differences			
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference
					Lower
Pair 1	source - count	-2.48942	2.24150	.56037	-3.68383

Paired Samples Test					
		Paired Differences	t	df	Sig. (2-tailed)
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	source – count	-1.29501	-4.442	15	.000

Appendix 3.4 T-Test to find significant difference in survival of *V. alginolyticus* in water and sediment

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	Source	1.5000	16	.51640	.12910
	Count	3.6504	16	2.19033	.54758

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	source & count	16	.471	.065

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	source – count	-2.15041	1.99948	.49987	-3.21586

Paired Samples Test					
		Paired Differences	**t**	**df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	source – count	-1.08496	-4.302	15	.001

Appendix 3.5 T-Test to find significant difference in survival of *V. mimicus* in water and sediment

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	source	1.5000	16	.51640	.12910
	count	3.1974	16	2.62375	.65594

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	source & count	16	.528	.035

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	source - count	-1.69738	2.39158	.59790	-2.97177

Paired Samples Test					
		Paired Differences	**t**	**Df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	source – count	-.42300	-2.839	15	.012

Appendix 3.6 T-Test to find significant difference in survival of *V. parahaemolyticus* in water and sediment

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	source	1.5000	16	.51640	.12910
	count	2.8957	16	2.36125	.59031

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	source & count	16	.594	.015

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	source - count	-1.39569	2.09603	.52401	-2.51258

Paired Samples Test					
		Paired Differences			
		95% Confidence Interval of the Difference			
		Upper	**T**	**Df**	**Sig. (2-tailed)**
Pair 1	source – count	-.27880	-2.663	15	.018

Appendix 3.7 T-Test to find significant difference in survival of *V. proteolyticus* in water and sediment

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	source	1.5000	16	.51640	.12910
	count	4.1269	16	2.10484	.52621

Paired Samples Correlations				
		N	**Correlation**	**Sig.**
Pair 1	source & count	16	.347	.188

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	source - count	-2.62692	1.98551	.49638	-3.68492

Paired Samples Test					
		Paired Differences	**t**	**Df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	source – count	-1.56892	-5.292	15	.000

Appendix 3.8 ANOVA for significant difference in survival of 5 *Vibrio* spp. in sediment

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	1.431	4	.358	.153	.960
Within Groups	81.685	35	2.334		
Total	83.116	39			

Appendix 3.9 ANOVA for significant difference in survival of 5 *Vibrio* spp. in water

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22.108	4	5.527	.907	.470
Within Groups	213.229	35	6.092		
Total	235.337	39			

Appendix 3 .10 T-Test for survival of *V. parahaemolyticus* in RW and AW

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	RW	1.5374	8	2.43269	.86009
	AW	3.4177	8	2.41426	.85357

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RW & AW	8	.736	.037

Paired Samples Test						
		Paired Differences				
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference	
					Lower	Upper
Pair 1	RW - AW	-1.88030	1.76195	.62294	-3.35333	-.40728

Paired Samples Test				
Pair 1	RW – AW	t	Df	Sig. (2-tailed)
		-3.018	7	.019

Appendix 3.11 T-Test for survival of *V. parahaemolyticus* in RS and AS

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	RS	4.2540	8	1.34627	.47598
	AS	6.4921	8	.98333	.34766

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RS & AS	8	.707	.050

Paired Samples Test						
		Paired Differences				
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference	
					Lower	Upper
Pair 1	RS - AS	-2.23807	.95263	.33680	-3.03449	-1.44166

Paired Samples Test				
Pair 1	RS – AS	t	Df	Sig. (2-tailed)
		-6.645	7	.000

Appendix 3.12 T-Test for survival of *V. proteolyticus* in RW and AW

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RW	3.4193	8	2.03171	.71832
	AW	6.5185	8	.69273	.24492

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RW & AW	8	.606	.111

Paired Samples Test						
		Paired Differences				
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**	
					Lower	**Upper**
Pair 1	RW - AW	-3.09913	1.70316	.60216	-4.52301	-1.67526

Paired Samples Test				
Pair 1	RW – AW	**t**	**df**	**Sig. (2-tailed)**
		-5.147	7	.001

Appendix 3.13 T-Test for survival of *V. proteolyticus* in RS and AS

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RS	4.8280	8	2.04690	.72369
	AS	7.2702	8	.53015	.18744

Paired Samples Correlations				
		N	**Correlation**	**Sig.**
Pair 1	RS & AS	8	.143	.736

Paired Samples Test						
		Paired Differences				
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**	
					Lower	**Upper**
Pair 1	RS - AS	-2.44211	2.03974	.72116	-4.14738	-.73685

Paired Samples Test				
		t	**Df**	**Sig. (2-tailed)**
Pair 1	RS – AS	-3.386	7	.012

Appendix 3.14 T-Test for survival of *V. mimicus* in RW and AW

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RW	1.8558	8	2.88169	1.01883
	AW	5.4981	8	1.00426	.35506

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RW & AW	8	.680	.063

Paired Samples Test						
		Paired Differences				
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**	
					Lower	**Upper**
Pair 1	RW - AW	-3.64235	2.31838	.81967	-5.58057	-1.70413

Paired Samples Test				
		t	Df	Sig. (2-tailed)
Pair 1	RW – AW	-4.444	7	.003

Appendix 3.15 T-Test for survival of *V. mimicus* in RS and AS

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RS	4.5515	8	1.50392	.53172
	AS	6.6214	8	.73880	.26121

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RS & AS	8	.612	.107

Paired Samples Test						
		Paired Differences				
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference	
					Lower	Upper
Pair 1	RS - AS	-2.06982	1.20323	.42541	-3.07575	-1.06389

Paired Samples Test				
		t	**Df**	**Sig. (2-tailed)**
Pair 1	RS – AS	-4.866	7	.002

Appendix 3.16 T-Test for survival of *V. alginolyticus* in RW and AW

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RW	2.6598	8	2.56226	.90590
	AW	6.1655	8	.94934	.33564

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RW & AW	8	.301	.469

Paired Samples Test						
		Paired Differences				
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**	
					Lower	**Upper**
Pair 1	RW - AW	-3.50566	2.45033	.86632	-5.55419	-1.45713

Paired Samples Test				
		t	**Df**	**Sig. (2-tailed)**
Pair 1	RW – AW	-4.047	7	.005

Appendix 3 .17 T-Test for survival of *V. alginolyticus* in RS and AS

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RS	4.6457	8	1.20077	.42454
	AS	6.6615	8	.29953	.10590

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RS & AS	8	-.081	.850

Paired Samples Test						
		Paired Differences				
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**	
					Lower	**Upper**
Pair 1	RS - AS	-2.01579	1.26075	.44574	-3.06980	-.96178

Paired Samples Test				
		t	**df**	**Sig. (2-tailed)**
Pair 1	RS – AS	-4.522	7	.003

Appendix 3 .18 T-Test for survival of *V. vulnificus* in RW and AW

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RW	3.2319	8	2.34935	.83062
	AW	6.5055	8	.39667	.14024

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RW & AW	8	.039	.927

Paired Samples Test						
		Paired Differences				
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**	
					Lower	**Upper**
Pair 1	RW - AW	-3.27366	2.36727	.83696	-5.25275	-1.29457

Paired Samples Test				
		t	**df**	**Sig. (2-tailed)**
Pair 1	RW – AW	-3.911	7	.006

Appendix 3 .19 T-Test for survival of *V. vulnificus* in RS and AS

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	RS	4.7325	8	1.24001	.43841
	AS	6.7408	8	.22122	.07821

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RS & AS	8	-.164	.698

Paired Samples Test						
		Paired Differences				
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference	
					Lower	Upper
Pair 1	RS - AS	-2.00832	1.29481	.45778	-3.09081	-.92584

Paired Samples Test				
		t	df	Sig. (2-tailed)
Pair 1	RS – AS	-4.387	7	.003

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Appendix 3.20 T-Test for effect of protozoan predation on survival of *V. parahaemolyticus* in water

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RW	1.5374	8	2.43269	.86009
	CYCLOHEXIMIDE	2.4510	8	2.92992	1.03588

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RW & CYCLOHEXIMIDE	8	.910	.002

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	RW - CYCLOHEXIMIDE	-.91366	1.23719	.43741	-1.94798

Paired Samples Test					
		Paired Differences	**t**	**Df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	RW - CYCLOHEXIMIDE	.12066	-2.089	7	.075

Appendix 3.21 T-Test for effect of protozoan predation on survival of *V. parahaemolyticus* in sediment

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RS	4.2540	8	1.34627	.47598
	CYCLOHEXIMIDE	4.8951	8	1.17971	.41709

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RS & CYCLOHEXIMIDE	8	.863	.006

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	RS - CYCLOHEXIMIDE	-.64114	.68051	.24060	-1.21006

Paired Samples Test					
		Paired Differences	**t**	**df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	RS - CYCLOHEXIMIDE	-.07223	-2.665	7	.032

Appendix 3 .22 T-Test for effect of protozoan predation on survival of *V. proteoyticus* in water

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RW	3.4193	8	2.03171	.71832
	CYCLOHEXIMIDE	4.1264	8	2.34516	.82914

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RW & CYCLOHEXIMIDE	8	.943	.000

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	RW - CYCLOHEXIMIDE	-.70710	.80371	.28416	-1.37902

Paired Samples Test					
		Paired Differences	**t**	**Df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	RW - CYCLOHEXIMIDE	-.03518	-2.488	7	.042

Appendix 3.23 T-Test for effect of protozoan predation on survival of *V. proteoyticus* in sediment

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RS	4.8345	8	2.05459	.72641
	CYCLOHEXIMIDE	4.7826	8	2.23538	.79033

Paired Samples Correlations				
		N	**Correlation**	**Sig.**
Pair 1	RS & CYCLOHEXIMIDE	8	.924	.001

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	RS - CYCLOHEXIMIDE	.05188	.85301	.30159	-.66126

Paired Samples Test					
		Paired Differences	**t**	**df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	RS - CYCLOHEXIMIDE	.76502	.172	7	.868

Appendix 3.24 T-Test for effect of protozoan predation on survival of *V. vulnificus* in water

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RW	3.2188	8	2.36010	.83442
	CYCLOHEXIMIDE	3.4771	8	2.52425	.89246

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RW & CYCLOHEXIMIDE	8	.992	.000

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	RW - CYCLOHEXIMIDE	-.25829	.35627	.12596	-.55614

Paired Samples Test					
		Paired Differences			
		95% Confidence Interval of the Difference			
		Upper	**t**	**Df**	**Sig. (2-tailed)**
Pair 1	RW - CYCLOHEXIMIDE	.03957	-2.051	7	.079

Appendix 3 .25 T-Test for effect of protozoan predation on survival of *V. vulnificus* in sediment

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RS	4.6457	8	1.20077	.42454
	CYCLOHEXIMIDE	4.8075	8	1.08931	.38513

Paired Samples Correlations				
		N	**Correlation**	**Sig.**
Pair 1	RS & CYCLOHEXIMIDE	8	.941	.000

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	RS - CYCLOHEXIMIDE	-.16182	.40907	.14463	-.50381

Paired Samples Test					
		Paired Differences	**t**	**df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	RS - CYCLOHEXIMIDE	.18016	-1.119	7	.300

Appendix 3 .26 T-Test for effect of protozoan predation on survival of *V. alginolyticus* in water

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RW	2.6598	8	2.56226	.90590
	CYCLOHEXIMIDE	2.9338	8	2.66251	.94134

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RW & CYCLOHEXIMIDE	8	.982	.000

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	RW - CYCLOHEXIMIDE	-.27399	.50432	.17831	-.69562

Paired Samples Test					
		Paired Differences	**t**	**df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	RW - CYCLOHEXIMIDE	.14764	-1.537	7	.168

Appendix 3.27 T-Test for effect of protozoan predation on survival of *V. alginolyticus* in sediment

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	RS	4.6457	8	1.20077	.42454
	CYCLOHEXIMIDE	4.5575	8	1.06592	.37686

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RS & CYCLOHEXIMIDE	8	.861	.006

Paired Samples Test					
		Paired Differences			
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference
					Lower
Pair 1	RS - CYCLOHEXIMIDE	.08818	.61078	.21594	-.42245

Paired Samples Test					
		Paired Differences	t	df	Sig. (2-tailed)
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	RS – CYCLOHEXIMIDE	.59880	.408	7	.695

Appendix 3 .28 T-Test for effect of protozoan predation on survival of *V. mimicus* in water

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	RW	1.9058	8	2.97053	1.05024
	CYCLOHEXIMIDE	3.4346	8	3.04575	1.07683

Paired Samples Correlations				
		N	**Correlation**	**Sig.**
Pair 1	RW & CYCLOHEXIMIDE	8	.784	.021

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	RW - CYCLOHEXIMIDE	-1.52881	1.97778	.69925	-3.18227

Paired Samples Test					
		Paired Differences	**t**	**df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	RW - CYCLOHEXIMIDE	.12465	-2.186	7	.065

Appendix 3.29 T-Test for effect of protozoan predation on survival of *V. mimicus* in sediment

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	RS	4.5390	8	1.52751	.54006
	CYCLOHEXIMIDE	4.8196	8	1.44955	.51249

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	RS & CYCLOHEXIMIDE	8	.862	.006

Paired Samples Test					
		Paired Differences			
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference
					Lower
Pair 1	RS - CYCLOHEXIMIDE	-.28060	.78421	.27726	-.93621

Paired Samples Test					
		Paired Differences			
		95% Confidence Interval of the Difference			
		Upper	t	df	Sig. (2-tailed)
Pair 1	RS - CYCLOHEXIMIDE	.37502	-1.012	7	.345

Appendix 3 .30 T-Test for effect of sunlight on survival of *V. alginolyticus*

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	sunlight	1.5000	12	.52223	.15076
	COUNT	4.6371	12	1.57977	.45604

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	sunlight & COUNT	12	-.835	.001

Paired Samples Test					
		Paired Differences			
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference
					Lower
Pair 1	sunlight - COUNT	-3.13715	2.03625	.58781	-4.43091

Paired Samples Test					
		Paired Differences	t	df	Sig. (2-tailed)
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	sunlight - COUNT	-1.84338	-5.337	11	.000

Appendix 3 .31 T-Test for effect of sunlight on survival of *V. parahaemolyticus*

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	sunlight	1.5000	12	.52223	.15076
	count	7.0928	12	.53956	.15576

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	sunlight & count	12	-.166	.606

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	sunlight - count	-5.59276	.81087	.23408	-6.10797

Paired Samples Test					
		Paired Differences			
		95% Confidence Interval of the Difference			
		Upper	**t**	**df**	**Sig. (2-tailed)**
Pair 1	sunlight - count	-5.07756	-23.893	11	.000

Appendix 3 .32 T-Test for effect of sunlight on survival of *V.vulnificus*

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	Sunlight	1.5000	12	.52223	.15076
	Count	5.0222	12	.87875	.25367

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	sunlight & count	12	-.601	.039

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	sunlight - count	-3.52224	1.26346	.36473	-4.32500

Paired Samples Test					
		Paired Differences			
		95% Confidence Interval of the Difference			
		Upper	**T**	**df**	**Sig. (2-tailed)**
Pair 1	sunlight - count	-2.71948	-9.657	11	.000

Appendix 3 .33 T-Test for effect of sunlight on survival of *V. mimicus*

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	Sunlight	4.3170	6	1.17188	.47842
	Count	5.8551	6	.60936	.24877

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	sunlight & count	6	-.210	.689

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	sunlight - count	-1.53811	1.43003	.58381	-3.03883

Paired Samples Test					
		Paired Differences	**t**	**df**	**Sig. (2-tailed)**
		95% Confidence Interval of the Difference			
		Upper			
Pair 1	sunlight - count	-.03738	-2.635	5	.046

Appendix 3 .34 T-Test for effect of sunlight on survival of *V. proteolyticus*

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	Sunlight	1.5000	12	.52223	.15076
	Count	4.7494	12	2.56546	.74059

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	sunlight & count	12	-.722	.008

Paired Samples Test					
		Paired Differences			
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**
					Lower
Pair 1	sunlight - count	-3.24942	2.96442	.85576	-5.13292

Paired Samples Test					
		Paired Differences			
		95% Confidence Interval of the Difference			
		Upper	**T**	**df**	**Sig. (2-tailed)**
Pair 1	sunlight - count	-1.36591	-3.797	11	.003

Appendix 3 .35 T-Test for effect of chemical factors on survival of *V. proteolyticus*

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	CF	1.5000	16	.51640	.12910
	COUNT	5.5406	16	1.93491	.48373

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	CF & COUNT	16	.755	.001

Paired Samples Test						
		Paired Differences				
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference	
					Lower	Upper
Pair 1	CF - COUNT	-4.04060	1.58142	.39536	-4.88328	-3.19792

Paired Samples Test				
		t	df	Sig. (2-tailed)
Pair 1	CF - COUNT	-10.220	15	.000

Appendix 3 .36 T-Test for effect of chemical factors on survival of *V. alginolyticus*

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	CF	1.5000	16	.51640	.12910
	COUNT	4.6469	16	2.00200	.50050

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	CF & COUNT	16	-.666	.005

Paired Samples Test						
		Paired Differences				
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference	
					Lower	Upper
Pair 1	CF - COUNT	-3.14694	2.37755	.59439	-4.41385	-1.88004

Paired Samples Test				
		t	df	Sig. (2-tailed)
Pair 1	CF - COUNT	-5.294	15	.000

Appendix 3 .37 T-Test for effect of chemical factors on survival of *V. parahaemolyticus*

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	CF	1.5000	16	.51640	.12910
	COUNT	5.3185	16	2.42390	.60597

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	CF & COUNT	16	-.720	.002

Paired Samples Test						
		Paired Differences				
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference	
					Lower	Upper
Pair 1	CF - COUNT	-3.81854	2.81874	.70468	-5.32054	-2.31654

Paired Samples Test				
		t	Df	Sig. (2-tailed)
Pair 1	CF - COUNT	-5.419	15	.000

Appendix 3 .38 T-Test for effect of chemical factors on survival of *V. mimicus*

Paired Samples Statistics					
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	CF	1.5000	16	.51640	.12910
	COUNT	5.6518	16	2.30789	.57697

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	CF & COUNT	16	-.715	.002

Paired Samples Test						
		Paired Differences				
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference	
					Lower	Upper
Pair 1	CF - COUNT	-4.15176	2.70139	.67535	-5.59123	-2.71229

Paired Samples Test				
		t	df	Sig. (2-tailed)
Pair 1	CF - COUNT	-6.148	15	.000

Appendix 3 .39 T-Test for effect of chemical factors on survival of *V. vulnificus*

Paired Samples Statistics					
		Mean	**N**	**Std. Deviation**	**Std. Error Mean**
Pair 1	CF	1.5000	16	.51640	.12910
	COUNT	4.4176	16	2.55987	.63997

Paired Samples Correlations				
		N	Correlation	Sig.
Pair 1	CF & COUNT	16	-.706	.002

Paired Samples Test						
		Paired Differences				
		Mean	**Std. Deviation**	**Std. Error Mean**	**95% Confidence Interval of the Difference**	
					Lower	**Upper**
Pair 1	CF - COUNT	-2.91755	2.94743	.73686	-4.48812	-1.34698

Paired Samples Test				
		t	**Df**	**Sig. (2-tailed)**
Pair 1	CF - COUNT	-3.959	15	.001

Appendix 3 .40 ANOVA for effect of varying salinities on survival of *V.mimicus*

Anova: Single Factor						
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.469899	2	0.23495	0.346466	0.711149	3.4668
Within Groups	14.24075	21	0.678131			
Total	14.71065	23				

Appendix 3 .41 ANOVA for effect of varying salinities on survival of *V. parahaemolyticus*

Anova: Single Factor						
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	8.237318	2	4.118659	3.372089	0.053703	3.4668
Within Groups	25.64934	21	1.221397			
Total	33.88665	23				

Appendix 3 .42 ANOVA for effect of varying salinities on survival of *V. vulnificus*

Anova: Single Factor						
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	7.402552	2	3.701276	4.052888	0.032473	3.4668
Within Groups	19.17812	21	0.913244			
Total	26.58068	23				

Appendix 3 .43 ANOVA for effect of varying salinities on survival of *V. alginolyticus*

Anova: Single Factor						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	4.952687	2	2.476343	1.827149	0.185537	3.4668
Within Groups	28.4614	21	1.355305			
Total	33.41408	23				

Appendix 3.44 ANOVA for effect of varying salinities on survival of *V. proteolyticus*

Anova: Single Factor						
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	1.730314	2	0.865157	1.943505	0.168108	3.4668
Within Groups	9.348213	21	0.445153			
Total	11.07853	23				

Appendix 3.45 ANOVA for effect of varying temperatures on survival of *V. parahaemolyticus*

Anova: Single Factor						
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.763501	2	1.38175	0.211814	0.810823	3.4668
Within Groups	136.9918	21	6.523417			
Total	139.7553	23				

Appendix 3 .46 ANOVA for effect of varying temperatures on survival of *V. mimicus*

Anova: Single Factor						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	6.617956	2	3.308978	1.177489	0.327581	3.4668
Within Groups	59.01418	21	2.810199			
Total	65.63214	23				

Appendix 3 .47 ANOVA for effect of varying temperatures on survival of *V .proteolyticus*

Anova: Single Factor						
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	1.678471	2	0.839236	1.004117	0.383291	3.4668
Within Groups	17.55169	21	0.835795			
Total	19.23017	23				

Appendix 3.48 ANOVA for effect of varying temperatures on survival of *V. alginolyticus*

Anova: Single Factor						
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.848289	2	0.424144	0.586833	0.564949	3.4668
Within Groups	15.17815	21	0.722769			
Total	16.02643	23				

Appendix 3.49 ANOVA for effect of varying temperatures on survival of *V. vulnificus*

Anova: Single Factor						
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	2.746619	2	1.37331	1.385771	0.272082	3.4668
Within Groups	20.81115	21	0.991007			
Total	23.55777	23				

Appendix 4

GENBANK SUBMISSIONS

Vibrio parahaemolyticus strain PM1S2 16S ribosomal RNA gene, partial sequence

GenBank: KM406325.1

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VERSION     KM406325.1
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  ORGANISM  Vibrio parahaemolyticus
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  AUTHORS   Silvester,R., Alexander,D. and Ammanamveetil,M.H.A.
  TITLE     Prevalence, antibiotic resistance, virulence and plasmid profiles
            of Vibrio parahaemolyticus from a tropical estuary and adjoining
            traditional prawn farm along the southwest coast of India
  JOURNAL   Ann. Microbiol. 65 (4), 2141-2149 (2015)
REFERENCE   2  (bases 1 to 1333)
  AUTHORS   Silvester,R., Alexander,D., Sruthy,K.S., Ajin,A.M., Rahiman,M.
and
            Hatha Abdulla,M.
  TITLE     Prevalence and multiple antibiotic resistance of Vibrio
            parahaemolyticus from shrimp pond and estuary along South West
            coast of India
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 1333)
  AUTHORS   Silvester,R., Alexander,D., Sruthy,K.S., Ajin,A.M., Rahiman,M.
and
            Hatha Abdulla,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-AUG-2014) Department of Marine Biology,
Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
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Vibrio mimicus strain M9W1 16S ribosomal RNA gene, partial sequence

GenBank: KT187246.1

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  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Genotyping of Vibrio sp. using RFLP PCR
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1381)
  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-JUN-2015) Department of Marine Biology,
Microbiology
            and Biochemistry., Cochin University of Science and Technology,
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Vibrio proteolyticus strain M10W1 16S ribosomal RNA gene, partial sequence

GenBank: KT748656.1

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  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Genotyping of Vibrio sp. using groEL RFLP
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 821)
  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-SEP-2015) Department of Marine Biology,
Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
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Photobacterium damselae strain M7W1 16S ribosomal RNA gene, partial sequence

GenBank: KY485151.1

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  AUTHORS   Silvester,R. and Hatha,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JAN-2017) MARINE BIOLOGY, MICROBIOLOGY AND
            BIOCHEMISTRY, COCHIN University OF SCIENCE AND TECHNOLOGY, Fine
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      361 gaagaaggcc ttcgggttgt aaagcacttt cagtagggag gaaggtagtg tagttaacac
      421 ctgcattatt tgacgttacc tacagaagaa gcaccggcta actccgtgcc agcagccgcg
      481 gtaatacgga gggtgcgagc gttaatcgga attactgggc gtaaagcgca tgcaggcggc
      541 ctgttaagtc agatgtgaaa gcccggggct taacctcgga attgcatttg aaactggcag
      601 gctagagtct tgtagagggg ggtagaattt caggtgtagc ggtgaaatgc gtagagatct
      661 gaaggaatac cagtggcgaa ggcggccccc tggacaaaga ctgacgctca gatgcgaaag
      721 cgtggggagc aaacaggatt agataccctg gtagtccacg ccgtaaacga tgtctacttg
      781 gaggttgtgg ccttgagccg tggctttcgg agctaacgcg ttaagtagac cgcctgggga
      841 gtacggtcgc aagattaaaa ctcaaatgaa ttgacggggg cccgcacaag cggtggagca
      901 tg
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Vibrio coralliilyticus strain 2W9 16S ribosomal RNA gene, partial sequence

GenBank: KY485150.1

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LOCUS       KY485150            1178 bp    DNA     linear   BCT 26-JAN-2017
DEFINITION  Vibrio coralliilyticus strain 2W9 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KY485150
VERSION     KY485150.1
KEYWORDS    .
SOURCE      Vibrio coralliilyticus
  ORGANISM  Vibrio coralliilyticus
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 1178)
  AUTHORS   Silvester,R., Madhavan,A. and Hatha,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-JAN-2017) MARINE BIOLOGY, MICROBIOLOGY AND
            BIOCHEMISTRY, COCHIN University OF SCIENCE AND TECHNOLOGY, Fine
            arts avenue, COCHIN, KERALA 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..1178
                     /organism="Vibrio coralliilyticus"
                     /mol_type="genomic DNA"
                     /strain="2W9"
                     /isolation_source="COCHIN ESTUARY WATER"
                     /db_xref="taxon:190893"
     rRNA            <1..>1178
                     /product="16S ribosomal RNA"
ORIGIN
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       61 ataacggcgt cgagcggcgg acgggtgagt aatgcctggg aaattgccct gatgtggggg
      121 ataaccattg gaaacgatgg ctaataccgc ataatagctt cggctcaaag agggggacct
      181 tcgggcctct cgcgtcagga tatgcccagg tgggattagc tagttggtga ggtaatggct
      241 caccaaggcg acgatcccta gctggtctga gaggatgatc agccacactg gaactgagac
      301 acggtccaga ctcctacggg aggcagcagt ggggaatatt gcacaatggg cgcaagcctg
      361 atgcagccat gccgcgtgta tgaagaaggc cttcgggttg taaagtactt tcagcagtga
      421 ggaaggtgga tgtgttaata gcgcattcat ttgacgttag ctgcagaaga agcaccggct
      481 aactccgtgc cagcagccgc ggtaatacgg agggtgcgag cgttaatcgg aattactggg
      541 cgtaaagcgc atgcaggtgg tttgttaagt cagatgtgaa agcccggggc tcaacctcgg
      601 aattgcattt gaaactggca gactagagta ctgtagaggg gggtagaatt tcaggtgtag
      661 cggtgaaatg cgtagagatc tgaaggaata ccggtggcga aggcggcccc ctggacagat
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      781 gccgtaaacg atgtctactt ggaggttgtg gccttgagcc gtggctttcg gagctaacgc
      841 gttaagtaga ccgcctgggg agtacggtcg caagattaaa actcaaatga attgacgggg
      901 gcccgcacaa gcggtggagc atgtggttta attcgatgca acgcgaagaa ccttacctac
      961 tcttgacatc ctcagaagag actggagaca gtcttgtgcg ttcgggaact gagagacagg
     1021 tgctgcatgg ctgtcgtcag ctcgtgttgt gaaatgttgc gttaagtccc gcaacgagcg
     1081 caacccttat ccttgtttgc cagggagtca tgtcgggaac tccagggaga ctgccggtga
     1141 taaaccggag aaggtgggga cgacgtcaag tcatcatg
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Vibrio cholerae strain AWT62 16S ribosomal RNA gene, partial sequence

GenBank: KT187245.1

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LOCUS       KT187245            900 bp    DNA     linear   BCT 07-OCT-2015
DEFINITION  Vibrio cholerae strain AWT62 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KT187245
VERSION     KT187245.1
KEYWORDS    .
SOURCE      Vibrio cholerae
  ORGANISM  Vibrio cholerae
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 900)
  AUTHORS   Silvester,R., Alexander,D., Madhavan,A.A. and Hatha,A.A.M.
  TITLE     Genotyping of vibrio sp. by rflp
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 900)
  AUTHORS   Silvester,R., Alexander,D., Madhavan,A.A. and Hatha,A.A.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-JUN-2015) Department of Marine Biology,
Microbiology
            and Biochemistry., Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /strain="AWT62"
                     /isolation_source="shrimp pond water"
                     /db_xref="taxon:666"
                     /country="India: Edavanakkad, Kochi"
                     /collection_date="03-Apr-2011"
     rRNA            <1..>900
                     /product="16S ribosomal RNA"
ORIGIN
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       61 tgcctgggaa attgcccggt agagggggat aaccattgga aacgatggct aataccgcat
      121 aacctcgcaa gagcaaagca ggggaccttc gggccttgcg ctaccggata tgcccaggtg
      181 ggattagcta gttggtgagg taagggctca ccaaggcgac gatccctagc tggtctgaga
      241 ggatgatcag ccacactgga actgagacac ggtccagact cctacgggag gcagcagtgg
      301 ggaatattgc acaatgggcg caagcctgat gcagccatgc cgcgtgtatg aagaaggcct
      361 tcgggttgta aagtactttc agtagggagg aagatggtta agttaatacc ttaatcattt
      421 gacgttacct acagaagaag caccggctaa ctccgtgcca gcagccgcgg taatacggag
      481 ggtgcaagcg ttaatcggaa ttactgggcg taaagcgcat gcaggtggtt tgttaagtca
      541 gatgtgaaag ccctgggctc aacctaggaa tcgcatttga aactgacaag ctagagtact
      601 gtagaggggg gtagaatttc aggtgtagcg gtgaaatgcg tagagatctg aaggaatacc
      661 ggtggcgaag gcggcccccc ggacagatac tgacactcag atgcgaaagc gtggggagca
      721 aacaggatta gataccctgg tagtccacgc cgtaaacgat gtctacttgg aggttgtgcc
      781 ctagaggtgt ggctttcgga gctaacgcgt taagtagacc gcctggggag tacggtcgca
      841 agattaaaac tcaaatgaat tgacgggggc ccgcacaagc ggtggagcat gtggtttaat
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Vibrio diazotrophicus strain JWV30 16S ribosomal RNA gene, partial sequence

GenBank: KT005559.1

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LOCUS       KT005559           1359 bp    DNA     linear   BCT 05-OCT-2015
DEFINITION  Vibrio diazotrophicus strain JWV30 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KT005559
VERSION     KT005559.1
KEYWORDS    .
SOURCE      Vibrio diazotrophicus
  ORGANISM  Vibrio diazotrophicus
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 1359)
  AUTHORS   Silvester,R., Alexander,D., Rahiman,M. and A A M,H.
  TITLE     Genotyping of Vibrio sp. using GRO EL RFLP
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1359)
  AUTHORS   Silvester,R., Alexander,D., Rahiman,M. and A A M,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-JUN-2015) Department of Marine Biology,
Microbiology
            and Biochemistry., Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /strain="JWV30"
                     /isolation_source="shrimp pond water"
                     /db_xref="taxon:685"
                     /country="India"
     rRNA            <1..>1359
                     /product="16S ribosomal RNA"
ORIGIN
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       61 gggggataac cattggaaac gatggctaat accgcatgat agcttcggct caaagagggg
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      181 tggctcacca aggcgacgat ccctagctgg tctgagagga tgatcagcca cactggaact
      241 gagacacggt ccagactcct acgggaggca gcagtgggga atattgcaca atgggcgcaa
      301 gcctgatgca gccatgccgc gtgtgtgaag aaggccttcg ggttgtaaag cactttcagt
      361 cgtgaggaag gtggtgttgt taatagcagc atcatttgac gttagcgaca gaagaagcac
      421 cggctaactc cgtgccagca gccgcggtaa tacggagggt gcgagcgtta atcggaatta
      481 ctgggcgtaa agcgcatgca ggtggtttgt taagtcagat gtgaaagccc ggggctcaac
      541 ctcggaatag catttgaaac tggcaagcta gagtactgta gaggggggta gaatttcagg
      601 tgtagcggtg aaatgcgtag agatctgaag gaataccggt ggcgaaggcg gccccctgga
      661 cagatactga cactcagatg cgaaagcgtg gggagcaaac aggattagat accctggtag
      721 tccacgccgt caacgatgtc tacttggagg ttgtggcctt gagccgtggc tttcggagct
      781 aacgcgttaa gtagaccgcc tggggagtac ggtcgcaaga ttaaaactca aatgaattga
      841 cgggggcccg cacaagcggt ggagcatgtg gtttaattcg atgcaacgcg aagaacctta
      901 cctactcttg acatctacag aatcctgcgg agacgcggga gtgccttcgg gaactgtaag
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     1201 ccaaaaagtg cgtcgtagtc cggattggag tctgcaactc gactccatga agtcggaatc
     1261 gctagtaatc gtgaatcaga atgtcacggt gaatacgttc ccgggccttg tacacaccgc
     1321 ccgtcacacc atgggagtgg gctgcaaaag aagcaggta
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Vibrio vulnificus strain JWV13 16S ribosomal RNA gene, partial sequence

GenBank: KT005560.1

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LOCUS       KT005560            921 bp    DNA     linear   BCT 05-OCT-2015
DEFINITION  Vibrio vulnificus strain JWV13 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KT005560
VERSION     KT005560.1
KEYWORDS    .
SOURCE      Vibrio vulnificus
  ORGANISM  Vibrio vulnificus
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 921)
  AUTHORS   Silvester,R., Alexander,D., Antony,A.C. and Mohamed,H.
  TITLE     Genotyping of Vibrio sp. using GRO EL RFLP
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 921)
  AUTHORS   Silvester,R., Alexander,D., Antony,A.C. and Mohamed,H.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-JUN-2015) Department of Marine Biology,
Microbiology
            and Biochemistry., Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /db_xref="taxon:672"
                     /country="India"
                     /collection_date="05-Jun-2012"
     rRNA            <1..>921
                     /product="16S ribosomal RNA"
ORIGIN
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      181 gggattagct agttggtgag gtaagggctc accaaggcga cgatccctag ctggtctgag
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      361 ttcgggttgt aaagcacttt cagttgtgag gaaggtggtg tcgttaatag cggcatcatt
      421 tgacgttagc aacagaagaa gcaccggcta actccgtgcc agcagccgcg gtaatacgga
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      541 agatgtgaaa gcccggggct caacctcgga actgcatttg aaactggcag actagagtac
      601 tgtagagggg ggtagaattt caggtgtagc ggtgaaatgc gtagagatct gaaggaatac
      661 cggtggcgaa ggcggccccc tggacagata ctgacactca gatgcgaaag cgtggggagc
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      901 ttcgatgcaa cgcgaagaac c
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Vibrio alginolyticus strain VKF44 16S ribosomal RNA gene, partial sequence

GenBank: KT005561.1

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LOCUS       KT005561               1379 bp    DNA     linear   BCT
05-OCT-2015
DEFINITION  Vibrio alginolyticus strain VKF44 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   KT005561
VERSION     KT005561.1
KEYWORDS    .
SOURCE      Vibrio alginolyticus
  ORGANISM  Vibrio alginolyticus
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 1379)
  AUTHORS   Antony,A.C., Silvester,R., Alexander,D. and Hatha,M.
  TITLE     Genotyping of Vibrio sp. using GRO EL RFLP
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1379)
  AUTHORS   Antony,A.C., Silvester,R., Alexander,D. and Hatha,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-JUN-2015) Department of Marine Biology,
Microbiology
            and Biochemistry., Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /collection_date="05-Jan-2014"
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     1021 gttgggttaa gtcccgcaac gagcgcaacc cttatccttg tttgccagcg agtaatgtcg
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Vibrio parahaemolyticus strain PM1S2 molecular chaperone GroEL (groEL) gene, partial cds

GenBank: KX094895.1

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LOCUS       KX094895            780 bp    DNA     linear   BCT 28-JUN-2016
DEFINITION  Vibrio parahaemolyticus strain PM1S2 molecular chaperone GroEL
            (groEL) gene, partial cds.
ACCESSION   KX094895
VERSION     KX094895.1
KEYWORDS    .
SOURCE      Vibrio parahaemolyticus
  ORGANISM  Vibrio parahaemolyticus
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 780)
  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Genotyping of Vibrio sp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 780)
  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-APR-2016) Department of Marine Biology,
Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /isolation_source="cochin estuary sediment"
                     /db_xref="taxon:670"
                     /country="India"
     gene            <1..>780
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     CDS             <1..>780
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                     /product="molecular chaperone GroEL"
                     /protein_id="ANN44211.1"

/translation="MNPMDLKRGIDKAVAAAVEQLKELSVECNDTKAIAQVGTISANS

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VELENPFILLVDKKISNIRELLPTLEAVAKASRPLLIIAEDVEGEALATLVVNNMRGI

VKVAAVKAPGFGDRRKAMLQDIAILTGGTVISEEIGLELEKVTLEDLGQAKRVSITKE
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ORIGIN
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      121 tctgcgaact ctgacgcaag cgtaggtaat atcattgctg aagcaatgga acgcgttggc
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      661 accatcatcg atggtgcggg tgaagaagcg atgatccaag gccgcgttgc tcaaattcgt
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Vibrio vulnificus strain JWV13 molecular chaperone GroEL (groEL) gene, partial cds

GenBank: KX094896.1

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LOCUS       KX094896           711 bp    DNA     linear   BCT 28-JUN-2016
DEFINITION  Vibrio vulnificus strain JWV13 molecular chaperone GroEL (groEL)
            gene, partial cds.
ACCESSION   KX094896
VERSION     KX094896.1
KEYWORDS    .
SOURCE      Vibrio vulnificus
  ORGANISM  Vibrio vulnificus
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 711)
  AUTHORS   Silvester,R., Alexander,D. and Abdulla,M.H.
  TITLE     Genotyping of Vibrio sp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 711)
  AUTHORS   Silvester,R., Alexander,D. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-APR-2016) Department of Marine Biology,
Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
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                     /isolation_source="shrimp farm water"
                     /db_xref="taxon:672"
                     /country="India"
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     CDS             <1..>711
                     /gene="groEL"
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                     /transl_table=11
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                     /protein_id="ANN44212.1"

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DSSVGKIIAEAMEKVGRDGVITVEEGQALHDELDVVEGMQFDRGYLSPYFINNQESGS

VELESPFILLVDKKISNIRELLPALEAVAKASRPLLIIAEDVEGEALATLVVNNMRGI

VKVAAVKAPGFGDRRKAMLQDIAILTGGTVISEEVGLELEKATLEDLGQAKRVSITKE
                     NTTIIDGVGEEAMIQGRVA"
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ORIGIN
        1 atgaacccaa tggaccttaa gcgtggtatc gacaaagctg tggcggctgc agttgaagaa
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      121 tctgcgaact ccgattcaag cgtgggtaaa atcattgcgg aagcaatgga aaaagtgggc
      181 cgtgacggtg ttatcacggt agaagaaggt caagcactgc acgatgagct agacgttgtt
      241 gaaggtatgc agtttgaccg tggttaccta tcaccatact tcatcaacaa tcaagagtct
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      481 gtgaaagtgg cggcggtgaa agcgcctggc tttggtgatc gtcgtaaagc tatgctacaa
      541 gacattgcta tcctaacagg tggtacggtg atttctgaag aagtgggtct tgagcttgaa
      601 aaagcaactc tagaagatct aggtcaggcg aagcgtgttt ctatcaccaa agaaaacacc
      661 accatcattg atggcgtggg tgaagaagcg atgattcaag gccgtgttgc t
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Vibrio alginolyticus strain VKF44 molecular chaperone GroEL (groEL) gene, partial cds

GenBank: KX094897.1

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LOCUS       KX094897             777 bp    DNA     linear   BCT 28-JUN-2016
DEFINITION  Vibrio alginolyticus strain VKF44 molecular chaperone GroEL
(groEL)
            gene, partial cds.
ACCESSION   KX094897
VERSION     KX094897.1
KEYWORDS    .
SOURCE      Vibrio alginolyticus
  ORGANISM  Vibrio alginolyticus
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 777)
  AUTHORS   Silvester,R., Antony,A.C. and Abdulla,M.H.
  TITLE     Genotyping of Vibrio sp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 777)
  AUTHORS   Silvester,R., Antony,A.C. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-APR-2016) Department of Marine Biology,
Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /product="molecular chaperone GroEL"
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/translation="MNPMDLKRGIDKAVAAAVEQLKELSVECNDTKAIAQVGTISANS

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VDLENPFILLVDKKISNIRELLPTLEAVAKASRPLLIIAEDVEGEALATLVVNNMRGI

VKVAAVKAPGFGDRRKAMLQDIAILTGGTVISEEIGLELEKVALEDLGQAKRVSITKE
                     NTTIIDGIGEEEMISGRVAQIRQQIEDATSDYDKEKLQERV"
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ORIGIN
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      121 tctgcaaact cagacgcaag cgtaggtaac atcattgctg aagcaatgga gcgtgtgggt
      181 cgtgatggcg ttatcacggt tgaagaaggt caggcgctac aagacgagtt agacgtagta
      241 gaaggtatgc agttcgaccg cggctaccta tctccttact tcatcaacaa ccaagagtca
      301 ggcagtgttg atctagaaaa cccattcatt cttctagtag acaagaagat ctcaaacatt
      361 cgtgaacttc ttccaacgct agaagctgta gcgaaagcgt ctcgtccact gcttatcatt
      421 gcagaagatg tagaaggcga agcgctagca acattggttg tgaacaacat gcgtggcatc
      481 gtaaaagttg cagctgtgaa agcacctggc ttcggtgacc gtcgtaaagc aatgctacaa
      541 gacatcgcga tcctaactgg cggtactgtg atttctgaag agatcggtct agagctagaa
      601 aaagttgcac tagaagatct aggtcaagcg aagcgtgttt ctatcacgaa agaaaacacg
      661 actatcatcg atggtatcgg tgaagaagag atgatctctg gccgtgttgc tcagattcgt
      721 caacaaatcg aagacgcaac ttcagactac gacaaagaga aactacaaga gcgcgta
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Vibrio fluvialis strain 8M1 molecular chaperone GroEL (groEL) gene, partial cds

GenBank: KX094898.1

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LOCUS       KX094898            612 bp    DNA     linear   BCT 28-JUN-2016
DEFINITION  Vibrio fluvialis strain 8M1 molecular chaperone GroEL (groEL)
gene,
            partial cds.
ACCESSION   KX094898
VERSION     KX094898.1
KEYWORDS    .
SOURCE      Vibrio fluvialis
  ORGANISM  Vibrio fluvialis
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 612)
  AUTHORS   Silvester,R., Antony,A.C. and Abdulla,M.H.
  TITLE     Genotyping of Vibrio sp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 612)
  AUTHORS   Silvester,R., Antony,A.C. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-APR-2016) Department of Marine Biology,
Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..612
                     /organism="Vibrio fluvialis"
                     /mol_type="genomic DNA"
                     /strain="8M1"
                     /isolation_source="shellfish harvesting water"
                     /db_xref="taxon:676"
                     /country="India"
     gene            <1..>612
                     /gene="groEL"
     CDS             <1..>612
                     /gene="groEL"
                     /codon_start=1
                     /transl_table=11
                     /product="molecular chaperone GroEL"
                     /protein_id="ANN44214.1"

/translation="MNPMDLKRGIDKAVIAAVEELKALSVPCEDTKAIAQVGTISANS

DLSVGNIIAEAMEKVGRDGVITVEEGQALQDELDVVEGMQFDRGYLSPYFINNQEAGS

VDLESPFILLVDKKVSNIRELLPVLEGVAKASRPLLIVAEDVEGEALATLVVNNMRGI
                     VKVAAVKAPGFGDRRKAMLQDIAILTGGTVISEEIGLELEKAVL"
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ORIGIN
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      121 tctgcaaact cggatttaag cgttggcaac atcatcgcag aagcgatgga aaaagtgggt
      181 cgcgacggcg ttatcactgt tgaagaaggt caggctctac aagacgagct ggacgtggta
      241 gaaggtatgc agtttgaccg cggttacctg tctccttact tcatcaacaa ccaggaagca
      301 ggcagcgtgg atctggaaag cccgttcatc ctgctggttg acaaaaaagt atcgaacatt
      361 cgcgaactgc tgccagtact ggaaggcgtc gcgaaggcct ctcgtccact gctgatcgtc
      421 gcagaagacg tggaaggtga agcgctggca acgttggttg tgaacaacat gcgtggcatc
      481 gtgaaagttg cggcagtgaa agcgcctggc tttggcgatc gtcgtaaagc gatgctgcaa
      541 gatatcgcga ttctgactgg cggtacggtg atttctgaag agatcggtct ggaactggaa
      601 aaagcagtgc tt
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Vibrio mimicus strain M9W1 GroEL molecular chaperone HSP60 (groEL) gene, partial cds

GenBank: KX086220.1

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LOCUS       KX086220            645 bp    DNA     linear   BCT 28-JUN-2016
DEFINITION  Vibrio mimicus strain M9W1 GroEL molecular chaperone HSP60 (groEL)
            gene, partial cds.
ACCESSION   KX086220
VERSION     KX086220.1
KEYWORDS    .
SOURCE      Vibrio mimicus
  ORGANISM  Vibrio mimicus
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 645)
  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Genotyping of Vibrio sp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 645)
  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-APR-2016) Department of Marine Biology,
Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /strain="M9W1"
                     /isolation_source="cochin estuary water"
                     /db_xref="taxon:674"
                     /country="India"
     gene            <1..>645
                     /gene="groEL"
     CDS             <1..>645
                     /gene="groEL"
                     /codon_start=1
                     /transl_table=11
                     /product="GroEL molecular chaperone HSP60"
                     /protein_id="ANN44206.1"

/translation="MNPMDLKRGIDKAVVAAVEELKALSVICADTKAIAQVGTISANS

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VELDNPFILLVDKKISNIRELLPVLEGVAKASRPLLIVAEDVEGEALATLVVNNMRGI

VKVAAVKAPGFGDRRKAMLQDIAILTGGVVISEEIGLELEKATLEDLGQAKRVSI"
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      121 tctgctaact cagactctag cgtgggcaac atcattgctg aagcaatgga aaaagtcggt
      181 cgcgacggcg ttatcactgt tgaagaaggc caagcgctgc aagacgagct ggatgttgta
      241 gaaggtatgc agtttgaccg tggctacctg tcaccatact tcatcaacaa ccaagaatca
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      421 gcagaagatg tggaaggcga agcgttggca actctggttg tgaacaacat gcgtggcatc
      481 gtgaaagtag cggcagtcaa agcacctggc tttggtgacc gtcgtaaagc gatgctgcaa
      541 gatatcgcga ttctgactgg tggcgtagtg atctctgaag agattggtct ggagctggaa
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Vibrio diazotrophicus strain JWV30 GroEL molecular chaperone HSP60-like (groEL) gene, partial sequence

GenBank: KX086221.1

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LOCUS       KX086221           747 bp    DNA     linear   BCT 28-JUN-2016
DEFINITION  UNVERIFIED: Vibrio diazotrophicus strain JWV30 GroEL molecular
            chaperone HSP60-like (groEL) gene, partial sequence.
ACCESSION   KX086221
VERSION     KX086221.1
KEYWORDS    UNVERIFIED.
SOURCE      Vibrio diazotrophicus
  ORGANISM  Vibrio diazotrophicus
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 747)
  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Genotyping of Vibrio sp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 747)
  AUTHORS   Silvester,R. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-APR-2016) Department of Marine Biology, Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     GenBank staff is unable to verify sequence and/or annotation
            provided by the submitter.

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:685"
                     /country="India"
     gene            <1..>747
                     /gene="groEL"
     misc feature    <1..>747
                     /gene="groEL"
                     /note="similar to GroEL molecular chaperone HSP60"
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      181 cgcgacggtg ttatcactgt tgaagaaggt caagcgcttc aagacgaact agacgttgtt
      241 gaaggtatgc aattcgaccg tggttaccta tcaccatact tcatcaacaa ccaagaagcg
      301 ggcagtgttg acctagacaa tccattcatc ctattggttg ataagaaagt ctctaacatc
      361 cgtgaacttc ttccagttct tgaaggtgtt gcaaaagctt cacgtccact gctaatcgta
      421 gcggaagacg tcgaaggtga agcactagca acgctagttg tgaacaacat gcgtggcatc
      481 gtgaaagtag ctgcggttaa agcgcctggt ttcggtgacc gtcgtaaagc tatgctacaa
      541 gacatcgcta tcctaactgg cggcactgtg atttctgaag aaatcggtct tgagctagag
      601 aaagcgacac tagaagatct aggtcaagct aagcgtgtga ctatcactaa agaaaacacc
      661 actattatcg acggtgctgg cgaagaagac gcgatcaagg tcgtgtatct caaattcgtc
      721 aacaaatcga agaagcaact tcagact
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Vibrio cholerae strain AWT62 GroEL molecular chaperone HSP60 (groEL) gene, partial cds

GenBank: KX086222.1

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LOCUS       KX086222         639 bp    DNA     linear   BCT 28-JUN-2016
DEFINITION  Vibrio cholerae strain AWT62 GroEL molecular chaperone HSP60
            (groEL) gene, partial cds.
ACCESSION   KX086222
VERSION     KX086222.1
KEYWORDS    .
SOURCE      Vibrio cholerae
  ORGANISM  Vibrio cholerae
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 639)
  AUTHORS   Silvester,R., Alexander,D. and Abdulla,M.H.
  TITLE     Genotyping of Vibrio sp.
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 639)
  AUTHORS   Silvester,R., Alexander,D. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-APR-2016) Department of Marine Biology,
Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
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                     /country="India"
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     CDS             <1..>639
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/translation="MNPMDLKRGIDKAVIAAVEELKALSVPCADTKAIAQVGTISANS

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VELDNPFILLVDKKISNIRELLPVLEGVAKASRPLLIVAEDVEGEALATLVVNNMRGI

VKVAAVKAPGFGDRRKAMLQDIAILTGGVVISEEIGLELEKATLEDLGQAKRV"
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      421 gctgaagatg tggaaggcga agcgctggcg actctggttg tcaacaacat gcgcggcatc
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Vibrio anguillarum strain JWA3 molecular chaperone GroEL (groEL) gene, partial cds

GenBank: KX528017.1

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LOCUS       KX528017                 810 bp    DNA     linear   BCT
20-JUL-2016
DEFINITION  Vibrio anguillarum strain JWA3 molecular chaperone GroEL (groEL)
            gene, partial cds.
ACCESSION   KX528017
VERSION     KX528017.1
KEYWORDS    .
SOURCE      Vibrio anguillarum (Listonella anguillarum)
  ORGANISM  Vibrio anguillarum
            Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales;
            Vibrionaceae; Vibrio.
REFERENCE   1  (bases 1 to 810)
  AUTHORS   Silvester,R., Alexander,D. and Abdulla,M.H.
  TITLE     Genotyping of pathogenic Vibrios
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 810)
  AUTHORS   Silvester,R., Alexander,D. and Abdulla,M.H.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-JUL-2016) Department of Marine Biology,
Microbiology
            and Biochemistry, Cochin University of Science and Technology,
            School of Marine Sciences, Lake Side Campus, Fine Arts Avenue,
            Ernakulam, Kerala 682016, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
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                     /country="India"
     gene            <1..>810
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     CDS             <1..>810
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                     /codon_start=1
                     /transl_table=11
                     /product="molecular chaperone GroEL"
                     /protein_id="ANS91075.1"

/translation="MNPMDLKRGIDKAVVAAVEALKELSVPCSDTKAIAQVGTISANS

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VDLDSPFILLVDKKISNIRELLPALEAVAKASRPLLIVAEDVEGEALATLVVNNMRGI

VKVAAVKAPGFGDRRKAMLQDIATLTGGTVISEEIGLELEKVTLEDLGQAKRISITKE
                    NTTIIDGAGEEQAIKGRVAQIRQQIEDATSDYDKEKLQERVAKLAGGVAVIK"
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      601 aaagtgacct tggaagatct aggccaagcg aagcgcatct ctatcactaa agaaaacacc
      661 accatcattg atggtgcagg tgaagagcaa gccattaaag gtcgcgttgc acaaattcgt
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      781 aaactggcag gtggcgttgc agtgatcaaa
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