STUDIES ON RIBOTYPING, INTEGRON GENES AND PATHOGENICITY OF MARINE VIBRIOS

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By

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CERTIFICATE

This is to certify that the research work presented in the thesis entitled "**Studies on Ribotyping, Integron Genes and Pathogenicity of Marine *Vibrios***" is based on the original research work carried out by Mr. Bernard Rajeev S. W. under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any degree.

Sarita G. Bhat

SARITA G. BHAT

DECLARATION

I hereby declare that the work presented in this thesis entitled "**Studies on Ribotyping, Integron Genes and Pathogenicity of Marine *Vibrios***" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin, under the guidance of Dr. Sarita G Bhat, Sr. Lecturer, and the thesis or no part thereof has been presented for the award of any degree, diploma, associateship or other similar titles of recognition.

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Bernard Rajeev S. W.

Dedicated to My Parents

CONTENTS

Chapter 1

INTRODUCTION

The *Vibrios* are a group of Gram negative, curved or straight motile rods that are autochthonous to estuarine habitats. It is understood that they play important roles in the health of humans and many different marine hosts, in addition to being an abundant and virtually ubiquitous component of the aquatic microbiota. *Vibrios* contain nearly all known bacterial genetic elements, including (super) integrons, episomes, plasmids, transposons, and bacteriophages, making them an attractive model for the study of genome plasticity (Fabiano *et al.*, 2006).

The *Vibrios* are ubiquitous in the aquatic environment, free or in association with aquatic organisms. They occur in a wide range of aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide (Barbieri *et al.*, 1999; Urakawa *et al.*, 2000; Thompson *et al.*, 2001; Heidelberg *et al.*, 2002a; Vandenberghe *et al.*, 2003; Venter *et al.*, 2004). Several cultivation-dependent and independent studies have showed that *Vibrios* appear particularly in high densities in and/or on marine organisms such as corals (Rosenberg and Ben Haim, 2002), fish (Huys *et al.*, 2001), gorgonians (Martin *et al.*, 2002), shellfish (Sawabe *et al.*, 2003), seagrass (Weidner *et al.*, 2000), sponges (Hentschel *et al.*, 2001), shrimps (Gomez-Gil *et al.*, 1998), squids (Ruby, 1996; Nishiguchi, 2000), and zooplankton (Heidelberg *et al.*, 2002b). Halophilic *Vibrios* can represent as much as 40 % of the total microbiota of subtropical coastal water (Chan *et al.*, 1986). In the light organs of squids, for instance, there may be 10^{11} cells/organ (Fidopiastis *et al.*, 1998; Nishiguchi, 2000).

Hosts for many of these *Vibrios* are yet to be identified. *V. cholerae's* association with *Chironomid* egg mass was a recently discovered fact (Broza and Halpern, 2001), and this finding unfolds an amazing association between microbes and higher organisms. *Vibrio* derives food out of the hard gelatinous case of the *Chironomid* egg mass– a beautifully and intelligently chosen symbiotic association!

The environment acts as a reservoir for most of the *Vibrios*. Most of them require moderate salinity for their survival. *Vibrios* are found in their natural habitat as free living bacterium or in association with phyto or zoo plankton (Lipp *et al.*, 2003). Most of them remain as Viable but Non Cultivable organisms (VBNCs) in nature, but capable of retaining virulence properties (Gary *et al*, 2005). Unknown environmental factors favour their resuscitation.

Species concept

The concept of species is continuously being mooted in *Vibrionaceae* family, especially with respect to *V. harveyi, V. tubiashii*, and *V. splendidus* (Thompson *et al.*, 2005). Multilocus sequence typing is being projected as a reliable and near to reality method of classifying *Vibrios* (Thompson *et al.*, 2005). Today, at least 12 species of *Vibrios* are known to be associated with human disease. *V. cholerae, V. parahaemolyticus, V. fluvialis, V. furnissii, V. hollisae* and *V. mimicus* are primarily associated with diarrhoeal diseases. Most notorious *Vibrio* infection is that caused by *Vibrio cholerae*. This was originally described by Pacini in 1854 (Baumann *et al.*, 1984). *V. alginolyticus* and *V. damsela* cause wound infections (Cuevas *et al*, 1987; Goodell *et al.*, 2004). *V. vulnificus* is an important cause of septicaemia in alcoholics and immunosuppressed hosts. (Kim *et al.*, 2003).

Recent developments of group- or species-specific identification methods have been providing new insights into the ecology of *Vibrionaceae* in which the number of members is continuing to expand. According to the most recent outline of Bergey's Manual of Systematic Bacteriology (Holt and Krieg 1994), the family *Vibrionaceae* comprises six genera *i.e. Allomonas, Catenococcus, EnteroVibrio, Listonella, Photobacterium, SaliniVibrio* and *Vibrio*. The genera *Allomonas* and *Enhydrobacter* were tentatively allocated to the family *Vibrionaceae* based on phenotypic features. *Allomonas enterica* and *V. fluvialis* are very similar in the 16S rRNA sequence, DNA-DNA similarity, and phenotypically, and are therefore considered be a junior synonym of the latter species (Farmer, 1986). *Enhydrobacter aerosaccus* is placed in the family *Moraxellaceae* due to the high phenotypic and 16S rRNA sequence similarity with *M. osleoensis* (Thompson, 2003).

More recently, it was proposed that the current family *Vibrionaceae* should be split in to four families, *EnteroVibrionaceae, Photobacteriaceae, SaliniVibrionaceae*, and *Vibrionaceae* (Thompson, 2003; Thompson *et al.*, 2004a & b). The new family *Vibrionaceae* would then comprise only species of the genus *Vibrio*. The splitting of *Vibrios* into four families was based on 16S rRNA, *rpoA* (RNA polymerase A), *recA* (Recombination aiding protein A), *pyrH* (Pyrimidine Homohexamer- a gene coding for UTP-Mono Phosphate-kinase) gene sequences and phenotypic features.

Virulence/virulence related genes

Most of these *Vibrios* have specific virulence factors such as capsules, toxins, colonization factors and also many yet to be identified virulence molecules. Some of the most studied virulence factors are Cholera Toxin, TCP (Toxin Co-regulated Pili), ToxR (the transmembrane transcription regulator of many downstream virulence genes), Ace (Accessory cholera enterotoxin), Zot (Zonula occludens toxin), SXT (an

integrative conjugative element with many antibiotic resistance genes), integron systems, Nag-st (Non-agglutinating stable toxin), *tdh* (Thermostable direct haemolysin), *trh* (tdh related haemolysin), haemolysins, proteases, metalloproteins *etc*. *Vibrios* draw the attention of the general public as well as health care workers more than any other species, because they are pathogenic in nature and cause undesirable host interaction. All together they are very important members of aquatic microbiota, and consequently the study on such an important group of micro organisms in all respects is indispensable.

One of the most elaborately studied *Vibrio* infections is that by *Vibrio cholerae*. There has been a sharp increase in the number of cholera cases reported to WHO during 2005. A total of 131 943 cases, including 2272 deaths, have been notified from 52 countries. Overall, this represents a 30% increase compared with the number of cases reported in 2004 (WHO, 2006). The menaces of cholera still remain as a conundrum. *Vibrio cholerae* get toxigenic genes only when it gets transformed by a temperate phage called ctx phi (Waldor and Mekalanos 1996). This was a great finding which revolutionized the scientific communities' thinking process. Scientists began to look into the environmental phase of *Vibrio cholerae*, with more attention, since *Vibrionaceae* members are labile to frequent Horizontal Gene transfer events (Faruque and Mekalanos, 2003). Hence it is inevitable that a close watch on the frequent genetic shuffling events taking place in the nature, with respect to *Vibrios is* warranted in order to have a long term disease management programme.

Characterization of environmental isolates of *Vibrios* from many different locales, in terms of virulence genes, antibiotic resistance potential, and unknown toxigenic factors are the demand of the hour, both for deciding treatment options and taking in view the alarming emergence of antibiotic resistance among the microbes,

and towards the control of any new virulent strains that may evolve due to the continuous stress due to pollution of the aquatic environment; this kind of study will therefore be an important information source for any probable disaster management programmes. Consequently, there is an urgent need to draw an insight on this area and gather information on these highly diverse and important groups of microbes, so that they can be effectively controlled or managed.

OBJECTIVES OF THE STUDY

Horizontal Gene Transfer (HGT) among *Vibrionaceae* members is a well established fact (Purdy *et al.*, 2005). The environment acts as a reservoir of genes and as a triggering agent for HGT occasionally. The environmental phase of *Vibrios* is under explored, with respect to its ecology. Many genes get exchanged or lost during this period. Genome shuffling really occurs in nature, at paces that are unbelievable. Recent developments in the *Vibrio cholerae* genomics paved way for many new studies and had answered a lot of unanswered questions. The cholera toxin gene, a key virulent gene of *Vibrio cholerae*, was observed to be laterally acquired through a filamentous phage (ctxΦ) genome (Waldor and Mekalanos, 1996). Likewise a lot of new genes were recorded to be horizontally acquired. Integron system existing in *Vibrio cholerae*, called in other words as 'Super Integrons' are hot spot of such lateral gene transfer events. Super integrons in *Vibrio cholerae* extends to 126kb size and gathers 179 cassettes (Heidelberg *et al.*, 2000). Full genome sequence data of *Vibrio cholerae* is available for public access from year 2000 onwards (Heidelberg *et al.*, 2000).

Since HGT is an established phenomenon in *Vibrionaceae*, many individual genes were analysed, and found as horizontally acquired, especially in *Vibrio cholerae*

(Bik *et al.*, 1995). There is a dearth of information on the presence of certain established virulence genes among the *Vibrionaceae* members particularly from India, especially with respect to Kerala, except for the report from Italy, which presented data for a similar kind of approach (Sechi *et al.*, 2000). Hence, this present study was planned and executed towards understanding the role of the hitherto understudied environmental phase of *Vibrios*, especially with respect to their diversity, presence or absence of virulence genes, antibiotic resistance genes, Horizontal gene transfer (HGT) among the *Vibrionacea* and in order to study the *in vivo* pathogenicity of these microbes.

Specific objectives included the following:

1. Isolation and identification of *Vibrios* from the coastal environment of Kerala based on ribotyping using partial 16S rRNA to assign correct systematic position.
2. To evaluate the antibiotic sensitivity or resistance of *Vibrio* isolates.
3. To study the presence of selected virulence genes by Polymerase Chain Reaction (PCR).
4. To study the presence of integrons in environmental *Vibrios* by PCR.
5. To study the pathogenicity of selected *Vibrio* strains using the suckling mouse assay.

Chapter 2

REVIEW OF LITERATURE

2.1. Historical perspective

Vibrios are a group of gram negative, curved or straight motile rods that normally inhabit the aquatic environments. *Vibrios* can be found in their natural habitat as free living bacterium or in association with phyto or zoo plankton (Lipp *et al.*, 2003). Most of them remain as Viable but Non Cultivable organisms (VBNCs) in nature. Unknown environmental factors favour their resuscitation.

The family *Vibrionaceae* is classified into six genera, namely *Vibrio, Allomonas, Enhydrobacter, Listonella, Photobacterium* and *Salinivibrio*. Currently the genus consists of 51 species, of which at least 12 are known to be associated with human diseases.

The most notorious human *Vibrio* infection and the most extensively studied one is that caused by *Vibrio cholerae*. Although this was originally described by Pacini in 1854, it was Robert Koch who identified *Vibrio cholerae* as the etiological agent of cholera (1884). Koch also hypothesised the existence of a "special poison" now known as Cholera Toxin (CT) elaborated by pathogenic strains of *Vibrio cholerae*. It acts upon the mucosal cells present on the luminal surface of the small intestine causing excessive accumulation of the fluid in the lumen. This results in diarrhoea and fluid loss (Finkelstein, 1973). There have been several recorded pandemics of cholera of which the seventh one, the longest, began in 1961, and persisted over 40 years.

For many of the *Vibrios* in nature the hosts are yet to be identified. *V. cholerae's* association with *Chironomid* egg mass, a recently discovered fact (Broza and Halpern, 2001), unfolds an amazing association between microbes and higher organisms. *Vibrio* derives food out of gelatine by the action of hydrolytic enzymes, like gelatinase secreted by them on the hard gelatinous case of the *chironomid* egg mass, which would have been a difficult task for the developing embryo; - a beautifully chosen symbiotic association!

2.2. Role in nature

Vibrios are halophilic autochthonous flora of estuaries, lakes and ponds. Most of the earlier reports dealt with their survivability and incidence in natural habitats. The study conducted in Chesapeake Bay, estuaries and sewers in Louisiana that *Vibrio cholerae* survive and multiply in nature in the absence of human disease. It was reported that they can be in association with *Crassostrea*, the marine oyster, without causing disease to the host (Colwell *et al.*, 1981; Hood *et al.*, 1981; Huq *et al.*, 1983; Kaper *et al.*, 1979; Tamplin *et al.*, 1982; Tilton and Ryan, 1987).

Vibrios have a seasonal growth and decline in conjunction with complex ecological aspects. Their growth and decline is in association with higher copepods and other plankton. They also play a very vital role in the maintenance of the ecosystem. Water temperature and salinity are the most important modulators of *Vibrio* concentrations worldwide (Kelly, 1982; Singleton *et al.*, 1982). *Vibrios* were recorded at high concentrations in water with temperatures in the range 17-37^{0}C, and salinity 5-25%. However individual *Vibrio* species have different optimal requirements for growth. In any aquatic sources *Vibrio* concentrations may vary immediately, depending on the amount of rainfall and fresh water runoff. Physical and chemical attributes of the environment may also modulate toxin production by

Vibrio cholerae (Tamplin and Colwell, 1986). *Vibrio cholerae* due to its inherent way of living has to alternate between free living and host associated states. In the course of transition, this organism is exposed to varying environmental situations and some of them are known to be stressful to the bacteria. Increase in temperature, presence of bile salts, low levels of pH of the stomach, and so on. Several lines of evidence indicate that environmental signals co-ordinately regulate the expression of virulence determinants in *Vibrio cholerae*. Acidic pH (Miller and Mekalanos, 1988) and anaerobiosis (Shimamura *et al.*, 1985) have been reported to increase the expression of virulence genes and enterotoxin production. On the other hand, temperature elevations reduce the level of virulence determinants (Parsot and Mekalanos, 1990).

2.3. Clinically important *Vibrios*

Pathogenic *Vibrios* cause three major syndromes of clinical illness: gastroenteritis, wound infections, and septicaemia (Nicholas *et al.*, 2000). There are at least twelve pathogenic *Vibrio* species recognized to cause human illness (Janda *et al.*, 1988; Pierce *et al.*, 1969). Six of the *Vibrio* species -*V. cholerae, V. parahaemolyticus, V. fluvialis, V. furnissii, V. hollisae* and *V. mimicus*, are primarily associated with diarrhoeal disease; two generally cause wound infections - *V. alginolyticus* and *V. damsela*. *V. vulnificus* is an important cause of septicaemia in alcoholics and immunosuppressed hosts, while the significance of isolation of *V. cincinnatiensis, V. carchariae* and *V. metschnikovii* from humans, remain to be determined. Most of these pathogenic *Vibrios* have specific virulence factors such as capsules, toxins, colonization factors and some yet to be identified virulence molecules also.

Vibrio cholerae causes the characteristic cholera in humans. Cholera is thought to have its ancestral home in the Ganges Delta of the Indian subcontinent. In the nineteenth century, pandemic waves of cholera spread to many parts of the world.

In 1961, a massive epidemic began in Southeast Asia; this is now recognised as the beginning of the seventh cholera pandemic. This pandemic was caused by El Tor biotype of toxigenic *Vibrio cholerae* 01 (CDC 1999).

Vibrio vulnificus cause primary septicaemia, through the GI tract. The fatality of the primary septicaemic infections *by V. vulnificus* is 50% (Johnston *et al.*, 1985; Klontz *et al.*, 1988). Most of the outbreaks are associated with seafood consumption. *V. vulnificus* can also cause skin infections, when open wounds are exposed to warm seawater. These skin infections may lead to cellulitis, ulceration, necrotizing fasciitis and sepsis.

Vibrio parahaemolyticus is also a seafood-borne infective agent. The most common clinical manifestation of *Vibrio parahaemolyticus* infection is gastroenteritis (Levine and Griffin, 1993), characterized by acute watery diarrhoea, abdominal cramps, and nausea. Consumption of raw seafood gives the pathogenic *Vibrio parahaemolyticus* an edge over the humans. The shortest incubation period was reported to be about 1h, but in most cases it was between 2 and 6 h. The symptoms common to all patients were intense abdominal pain, which was expressed by many patients as a "burning sensation in the stomach", vomiting and diarrhoea that varied in frequency from a few to many discharges. In most cases stools were watery, but in some cases bloody discharges were reported and coldness of extremities, cyanosis and constriction of the chest, general lassitude, headache, sleeplessness and thirst were also observed in some cases. Clinically, increased pulse rate and constriction of peripheral blood vessels were observed (Miwatani and Takeda, 1976). The first report of *Vibrio parahaemolyticus* infection associated with consumption of raw oysters and clams from New York waters were reported by Centres for Disease control and prevention in 1998 (CDC, 1999).

2.4. Pathogens to other animals in nature, aquaculture

Vibrios are recognised as pathogens to finfish and shellfish. *Vibrio alginolyticus* have been isolated from moribund cultured sea bream and were reported to be associated with mass mortalities in aquacultured carpet shell clam (*Ruditapes decussatus*) larvae (Gómez-León *et al.*, 2005), and cultured *Sepia officinalis, Sepia apama,* and *Sepia pharaonis* (Sangster and Smolowitz, 2003).

Vibrio mimicus is a non-halophilic *Vibrio* named due to its similarity to *Vibrio cholerae.* It can cause sporadic episodes of acute gastroenteritis and ear infections in humans. They have been isolated from eggs of olive ridley sea turtles (*Lepidochelys olivacea*) (María *et al.*, 1999), implying an association between them.

Vibrio fluvialis is a halophilic *Vibrio* first identified in 1975 in a patient with diarrhoea in Bahrain (Furniss *et al.*, 1977). Their ability to grow well on media containing 6% to 7% sodium chloride makes them different from *Aeromonas hydrophila.*

Vibrio hollisae was described first in 1982, is a halophilic member of *Vibrionaceae*, causes gastroenteritis in humans; (Hlady and Klontz, 1996; Rank *et al.*,1988) and an attempt was made to reclassify *Vibrio hollisae* into *Grimontia hollisae* gen. nov., comb. nov. (Thompson *et al.*, 2003).

Vibrio furnissii was retrospectively implicated with an outbreak of gastroenteritis occurred on an aircraft in 1969 (Centers for Disease Control, 1969). In 1994, during a cholera surveillance program in Peru, *Vibrio furnissii* was isolated

from fourteen patients, six with diarrhoea and 8 without symptoms (Dalsgaard *et al.*, 1997).

Vibrio metschnikovi, not a common pathogen in human beings and mostly an environmental *Vibrio*, was isolated from five infants with diarrhoea during a cholera surveillance program in Peru (Dalsgaard *et al.*, 1996).

Vibrio cincinnatiensis is the most recently described pathogenic *Vibrio* Clinical evidences are yet to be reported for its pathogenesis mode and virulence genes (Brayton *et al.*, 1986).

Vibrio anguillarum is an established fin-fish pathogen and attempts were made to find out the virulence determinants in them (Ronan *et al.*, 1996). In such an attempt, even complete sequencing of a virulence plasmid from *V. anguillarum* was done (Wu *et al.*, 2004).

Vibrio harveyi is a luminous bacterial pathogen to tiger prawn and a cystein protease which is supposed to be a key virulence factor in them had been isolated and studied in detail (Liu and Lee, 1999).

Vibrio shiloi is the causative agent of bleaching of the coral *Oculina patagonica* in the Mediterranean Sea (Sussman *et al.*, 2003).

2.5. Isolation and maintenance

For most of the *Vibrios*,TCBS (Thiosulphate Citrate Bile salt Sucrose) agar is a reliable selective medium, although there are slight variations in the process of isolation of them. A combination of alkaline pH and bile salts gives the medium

selectivity, while sucrose fermentation is the reaction for differential segregation. A medium designed for isolation of *Vibrio cholerae* is taurocholate-tellurite gelatine agar (TTG). Tellurite is reduced to non toxic tellurium by *Vibrio cholerae* on this medium. Most of the *Vibrios* are salt loving and can tolerate between 2-20% NaCl in the medium (Janda *et al.*, 1988).

Laboratory maintenance of *Vibrios* can be done on nutrient agar stabs (0.8% agar) supplemented with sodium chloride. Alternatively they can be stored with glycerol (final conc. 15%) at -80^{0}C.

2.6. Identification

2.6.1. Phenotypic identification: Pitfalls of classical biochemical identification and dichotomous keys

Classical phenotypic identification techniques, including tests for arginine dihydrolase and lysine and ornithine decarboxylases, were among the most extensively used techniques to screen the diversity of *Vibrio* strains associated with marine animals and their habitat, and these tests have been proposed as reliable species identification schemes (Alsina and Blanch, 1994a; Alsina and Blanch, 1994b; Austin *et al.*, 1996; Austin *et al.*, 1997; Blanch *et al.*, 1997; Hidaka and Sakai, 1968; Lanyi, 1987; Macia´n *et al.*, 1996; Ortigosa *et al.*, 1989; Ortigosa *et al.*, 1994). Variable results, *e.g.*, for arginine dihydrolase of some species, have been reported, making their identification on this basis difficult (Pujalte *et al.*, 1992).

Biolog® has been one of the most widely used phenotypic technique for the identification of *Vibrionaceae* strains in the last decade (Austin *et al.*, 1995; Austin *et al.*, 1997; Klingler *et al.*, 1992; Miller and Rhoden, 1991; Vandenberghe, 2003. The

identification of strains is based on the pattern of utilization of the ninety-five carbon sources.

A very important diagnostic phenotypic feature for the identification of *Vibrio* species has always been the presence of flagella and thus motility (Allen and Baumann, 1971). Non-motile *Vibrio* species, *e.g.*, the *V. halioticoli* group, have described (Sawabe *et al.*, 2002; Sawabe *et al.*, 2004a; Sawabe *et al.*, 2004b), suggesting that the presence of flagella is not an essential diagnostic feature. Likewise, oxidase-negative *V. metschnikovii* and *V. gazogenes* strains have been documented, as *Vibrio* strains that fail to grow on TCBS (Alsina and Blanch, 1994a; Alsina and Blanch, 1994). The phenotypic identification of genera and species of the *Vibrionaceae* is problematic. Dichotomous keys (Alsina and Blanch, 1994a; Alsina and Blanch, 1994b; Holt and Krieg, 1994) are misleading for the identification of *Vibrionaceae* isolates.

2.6.2. Numerical and polyphasic taxonomy

The foundation of modern *Vibrio* taxonomy was laid by a number of numerical (phenetic) and/or polyphasic taxonomic studies (Bang *et al.*, 1978; Baumann and Baumann, 1973; Baumann *et al.*, 1971; Baumann *et al.*, 1973; Baumann and Baumann, 1977; Baumann *et al.*, 1980; Baumann *et al.*, 1983; Baumann and Schubert, 1984; Baumann *et al.*, 1984; Citarella and Colwell, 1970; Colwell, 1970; Fujino *et al.*, 1974; Hada *et al.*, 1984; Lee *et al.*, 1981; Reichelt *et al.*, 1976; Tubiash *et al.*, 1970; West *et al.*, 1983; Woolkalis and Baumann, 1981). Large collections of strains were clustered on the basis of their ability to utilize different (about fifty to one hundred and fifty) compounds as sources of carbon and/or energy, enzyme activity (gelatinase chitinase and DNase), salt tolerance, luminescence, growth at different temperatures, antibiograms, DNA base composition, morphological features and other

biochemical tests (*e.g.*, oxidase, catalase, Voges-Proskauer, indole, nitrate reduction, arginine dihydrolase and lysine and ornithine decarboxylases). The clusters defined by phenotypic features were further refined and validated by DNA-DNA hybridization experiments and phenotypic clusters with about eighty percent similarity were found to correspond to DNA-DNA homology clusters with more than eighty percent similarity (Baumann and Baumann, 1977; Baumann *et al.*, 1984). The method being extensive and time consuming, faced a natural death process.

2.6.3. Genotypic identification

Over the last three decades many new *Vibrio* identification techniques was devised by various workers. Ribotyping and PCR-based techniques for example, Amplified Fragment Length Polymorphism (AFLP), Fluorescence in situ Hybridization (FISH), Random Amplified Polymorphic DNA (RAPD), Repetitive Extragenic Palindrome PCR (REPP), Multilocus Enzyme Electrophoresis (MLEE), Multilocus Sequence Typing (MLST) and Colony Hybridization by species specific probes. These techniques have yielded the most valuable information about *Vibrionaceae* and certain new insights into their population structure.

2.6.4. Ribotyping

Ribotyping consists of four main steps: (i) restriction of the bacterial chromosome with an endonuclease, (ii) gel electrophoresis of the resulting fragments, (iii) transfer of the fragments to a membrane, and (iv) hybridization of the gel with a labelled probe complementary to the 16S and 23S rRNAs (Grimont and Grimont, 1986). Ribotyping was one of the first finger printing techniques to be successfully used in the taxonomy of *Vibrios* and it has been particularly useful in the study of

Vibrio cholerae (Grimont and Grimont, 1986; 2001; Lan and Reeves, 1998; Pourshafie *et al.*, 2000; Pourshafie *et al.*, 2002). More recently ribotyping has been used to assess the genomic diversity of environmental *Vibrio* strains associated with fish and oysters (Austin *et* al., 1997; Macia'n, 2000; Pujalte *et al.*, 1999). Ribotyping pattern is like a signature of each species. It can be used for identification as well as classification.

2.6.5. Amplified Fragment Length Polymorphism (AFLP)

This technique consists of three main steps (i) digestion of total genomic DNA with two restriction enzymes and subsequent ligation of the restriction-half site specific adaptors to all restriction fragments; (ii) selective amplification of these fragments with two PCR primers that have corresponding adaptor and restriction site sequences as their target sites; and (iii) electrophoretic separation of the PCR products on polyacrylamide gels with selective detection of fragments which contain the fluorescently labelled primer and computer assisted numerical analysis of the band patterns (Huys and Swings, 1999; Jobson, 1996; Vos *et al.*, 1995). AFLP was used to study various *Vibrios* (Thompson *et al.*, 2002) including *Vibrio cholerae* (Jiang *et al.*, 2000a; Jiang *et al.*, 2000b; Lan and Reeves, 2002) and *Vibrio harveyi* (Gomez-Gil *et al.*, 2004; Pedersen *et al.*, 1998).

2.6.6. Fluorescence in situ Hybridization (FISH)

Another efficient means of detecting, quantifying and identifying bacteria is called FISH. This is an easy to perform technique, and can be done with filter fixed cells. Here what we do is hybridization of fluorescently labelled specific 16S rRNA probes with filter fixed cells and their counting by epifluorescence microscopy. This

approach have shed the light on distribution and ecology of *Vibrios* in the marine environment and have overcome the problem of great plate count anomaly (Eilers *et.al.*, 2000; Eilers *et.al.*, 2000; Giovannoni and Rappe´, 2000; Rappe´ and Giovannoni, 2003). As several *Vibrio* species (*e.g.*, *V. harveyi, V. campbelii V. rotiferianus* and other closely phylogenetic neighbours) have very similar 16S rRNA sequences, it may be difficult to perform reliable species identification.

2.6.7. Microarrays

This method may be an alternative to DNA-DNA hybridization currently performed in bacterial taxonomy. (Stackebrandt *et al.*, 2002; Wilson *et al.*, 2002). Cho and Tiedje (Cho and Tiedje, 2001) successfully designed a microarray, containing up to ninety-six genomic fragments (about 1 kb long), for the identification of *Pseudomonas* species. It was suggested that a chip containing 100,000 genomic fragments would allow the identification of most gram-negative bacteria.

2.6.8. Multilocus Enzyme Electrophoresis and Multilocus Sequence Typing

Both techniques index the variation in housekeeping genes. MLST assigns alleles directly from the nucleotide sequences, while MLEE compares the electrophoretic mobility of the enzymes encoded by the genes (Feil and Spratt, 2001). MLST has several advantages over MLEE, *e.g.*, higher discriminatory power because it detects synonymous and non synonymous changes, accuracy and portability of the data, ease of performance and reproducibility (Maiden *et al.*, 1998).

2.6.9. Random Amplified Polymorphic DNA and Repetitive Extragenic Palindrome PCR

RAPD makes use of arbitrary primers and involves PCR amplification of random fragments of genomic DNA, while rep-PCR amplifies intervening sequences located between highly repetitive DNA motifs (Dijkshoorn *et al.*, 2001). This technique was used mainly to type within species. Rep-PCR was used to identify presumptive *V. harveyi* isolates responsible for luminous vibriosis in shrimp (Gomez-Gili *et al.*, 2004). In another study rep-PCR was used to analyze genomic diversity of *Vibrios* isolated from the abalone gut (*Haliotis* spp) (Sawabe *et al.*, 2002).

2.7. Current Concepts on *Vibrionaceae* classification

Although classical ribotyping has been used to assess the genomic diversity of environmental *Vibrios* until recently, 16S rRNA sequencing has revolutionized microbial taxonomy.

The 16S rRNA gene (about 1500 bp in length) consists of highly conserved regions which may reveal deep- branching (*e.g.*, classes, phyla) relationships but may also demonstrate variable regions which discriminate species within the same genus. This was the feature which prompted researchers to use 16S rRNA gene both as a phylogenetic marker and as an identification tool (Wiik *et al.*, 1995).

16SrRNA sequencing is considered the most reliable tool for the allocation of genera, species and strain into the family *Vibrionaceae*. The proposal to split the family *Vibrionaceae* into three new families was put forward by Thompson (Thompson, 2003). *Vibrios* differ among themselves on the basis of 16S rRNA gene

and other phenotypic features like growth in varying percentages of sodium chloride, D-mannitol fermentation, poly (3-hydroxy) butyrate accumulation, Voges-Proskauer reaction, indole production, arginine dihydrolase production, ornithine decarboxylase production, presence of certain fatty acids etc. The newly proposed family *Vibrionaceae* comprises only the genus *Vibrio* with sixty-three species.

Species would be entities with at least ninety-seven percent 16S rRNA gene similarity (Thompson, *et al.*, 2004b) and according to Thompson, bacterial taxonomy is moving toward a genomically based concept in which phenotypic data may not have a clear standing.

The *Vibrionaceae* is heterogeneous and may require further splitting. Several *Vibrionaceae* species have nearly identical 16S rRNA gene sequences. In these cases, the only alternatives for identification are genomic fingerprinting, *e.g.*, FAFLP and rep-PCR, DNA-DNA hybridizations or MLST.

As almost any phylogenetic marker has weakness and strengths because no single gene is completely resistant to lateral gene transfer, hidden paralogy and changes in the evolutionary clock among related species (Jobson, 1996; Gevers *et al.*, 2004); alternative phylogenetic markers should be identified that would pave the way for accuracy in identification of microbes. Alternative phylogenetic markers have to fulfil several criteria as put forward by Zeigler (Zeigler, 2003):

(i) The genes must be widely distributed among genomes.

(ii) The genes must be single copy within a given genome.

(iii) The individual gene sequences must be long enough to contain sufficient information but short enough to allow sequencing in a convenient way (900-2,250 nucleotides).

(iv) The sequences must predict whole-genome relationships with acceptable precision and accuracy that correlates well with the 16S rRNA and with whole-genome similarity measured by DNA-DNA hybridizations.

2.8. Plasticity of *Vibrio* genomes

Genomes of *Vibrios* are to a certain extent plastic in nature. Compared with the genome of pathogenic *Escherichia coli*, which shows numerous traces of horizontal gene transfer (Ohnishi *et al.*, 2001), *Vibrios* seem to have fewer mobile genetic elements *e.g.*, transposons and phages and DNA regions with a G + C content that differ from the whole genome average, which are indicative of recent horizontal transfer (Heidelberg *et al.*, 2000; Makino *et al.*, 2003).

2.9. Two chromosome concept

Several *Vibrio* species contain two chromosomes (Trucksis *et al.*, 1998; Yamaichi *et al.*, 1999). The size of the large chromosome is predominantly within a narrow range (3.0 to 3.3 Mb), whereas the size of the small chromosome varies considerably among the *Vibrios* (0.8 to 2.4Mb). This fact suggests that the structure of the small chromosome is more flexible than that of the large chromosome; during the evolution of *Vibrios* (Kazuhisa *et al.*, 2005). There are many theories put forth to explain the development of the two-chromosome structure of *Vibrios*. Heidelberg and co-workers (Heidelberg *et al.*, 2000) hypothesised that second chromosome of *V. cholerae* was a mega-plasmid, acquired by an ancestral *Vibrio*. Other researchers argue that the small chromosome may have arisen by excision from a single, large ancestral genome (Waldor and Chaudhuri, 2000).

But what could possibly be the advantage for *Vibrios* to have two separate chromosomes rather than to have the small chromosome to be integrated into the large

chromosome? Several plausible reasons have been given for above-Yamaichi and fellow workers (Yamaichi *et al.*, 1999) suggested that the split of the genome into two replicons would be advantageous for the rapid DNA replication normally observed in *Vibrio parahaemolyticus*, a species with a doubling time of only eight to nine min. Although the first chromosome contains most of the genes that are required for growth (Heidelberg *et al.*, 2000; Makino *et al.*, 2003), the second contains more genes for bacterial adaptation to environmental changes, indicating that the two chromosomes play different roles (Makino *et al.*, 2003).

2.10. Known virulence factors in *Vibrios*

2.10.1. *Vibrio cholerae*

(1) Cholera Toxin – The genes for CT (Cholera Toxin), the most important virulence factor of *Vibrio cholerae* have long been thought to be encoded in the chromosome of the bacterium. In 1996, Waldor and Mekalanos (Waldor and Mekalanos, 1996) reported that these genes are actually encoded in the genome of a newly identified bacteriophage, CTXΦ and that the phage genome is integrated into the bacterial chromosome as a prophage. DNA for cholera toxin has got two genes, *ctxA* and *ctxB*, coding for A and B subunits of the cholera toxin respectively. Since the pathogenic and pandemic potential of *Vibrio cholerae* is determined by the presence of *ctxA* and *ctxB* genes, in many surveillance studies these genes were sought for (Rivera *et al.*, 2003; Dalsgaard *et al.*, 2001; Erin *et al.*, 2003).

(2) TCP (Toxin Co-regulated Pili) – The *tcp* was first described by Taylor and fellow scientists in nineteen eighty seven (Taylor *et al.*, 1987). This pilus is under the same regulation as the cholera toxin operon (Renate *et al.*, 1989). The genes for *tcp* are responsible for the production of a type four pili which is the receptor for the

filamentous phage ctxΦ to enter into the bacterial cell. *Tcp* is coded by the host bacterial chromosome and it plays another vital role in the pathogenesis process; it is essential for the bacteria to get adhered to the gut wall, before the toxin production. Recently it was shown that TCP mediates bacterial interactions required for biofilm differentiation on chitinaceous surfaces and that undifferentiated TCP- biofilms have reduced ecological fitness. (Gemma and Roberto, 2005).

(3) **ToxR** system- Expression of more than seventeen virulence genes is under the co-ordinate control of the ToxR protein. ToxR is a transmembrane protein and it binds to and activates the promoter of the operon encoding cholera toxin. ToxR controls transcription of *toxT* whose product in turn is directly responsible for activation of several virulence genes under ToxR control (Victor *et al.*, 1991). Many environmental signals act via ToxR system.

(4) **Ace** – The gene encoding *ace* (Accessory cholera enterotoxin) is located immediately upstream of the gene encoding *zot* (Zonula occludens toxin) and cholera toxin. Ace increases short circuit current in the Ussing chambers and causes fluid secretion in ligated rabbit ileal loops. The predicted protein sequence of *ace* shows striking similarity to eukaryotic ion transporting ATPases, including the product of the cystic fibrosis gene and hence is being tried to use as a therapeutic agent for cystic fibrosis (Michele *et al.*, 1993).

(5) **Zot** – Zonula occludens toxin is a 44.8 kDa single polypeptide chain encoded by the filamentous bacteriophage CTXΦ of *Vibrio cholerae*. Zot binds a receptor on the intestinal epithelial cells and increases mucosal permeability by affecting the structure of epithelial tight junctions. This allows the passage of macromolecules through the para cellular route. The effect of Zot on tight junctions is reversible and does not

cause tissue damage (Fasano, 2000), because of these properties Zot is a promising tool for mucosal drug and antigen delivery.

(6) SXT – SXT is a 100kb integrative conjugative element (ICE) also called as a CONSTIN. It has its own integrase and a trio of antibiotic resistance genes apart from many other genes whose functions are unidentified. Generally SXT from *Vibrio cholerae* contain sulfamethoxazole, trimethoprim, streptomycin, chloramphenicol resistance genes clustered together near the 5' end of the element. There are two SXT regions that primarily contain genes with no detectable function. It has got a functional integrase gene and more than one transposase genes etc. SXT can be called as virulence associated genes, since in conjunction with virulence genes they can be dangerous.

(7) Nag-st- Non agglutinating stable toxin is a heat stable toxin found in some non-serum agglutinable *Vibrio cholerae* strains (Amit *et al.*, 1992).

(8) Integron system- Integrons are natural tools for bacterial evolution and innovation. They can be considered as DNA elements which are virulence associated in nature. Their involvement in capture and dissemination of antibiotic-resistance genes among gram-negative bacteria is well documented. Massive ancestral versions, the Super Integrons were discovered in the genome of *Vibrio cholerae*. Super integron gene cassettes with an identifiable activity encode proteins related to simple adaptive functions including resistance, virulence and metabolic activities and their recruitment was interpreted as providing the host with an adaptive advantage (Dean *et al.*, 2003).

Integrons are mobile DNA elements with the ability to capture genes, particularly those encoding antibiotic resistance, by site-specific recombination. They

1968). The haemolysis by *Vibrio parahaemolyticus* observed on Wagatsuma agar medium is called the Kanagawa phenomenon.

(2) **TDH**- Thermostable direct haemolysin is named so on the basis of the following characteristics: TDH was not inactivated by heating at 100^0C for 10 min, and the haemolytic activity was not enhanced by the addition of lecithin, indicating a direct action on erythrocytes (Sakurai *et al.*, 1973). TDH has been shown to be a homodimer protein with a molecular mass of 46 kDa, each peptide being composed of 165 amino acids with the sole disulfide bond between Cys 151 and Cys 161 (Tsunasawa *et al.*, 1987; Honda and Lida, 1993). TDH is encoded by *tdh* genes that usually, but not exclusively, located on the chromosome (Nishibuchi and Kaper, 1995).

(3) **TRH**-TDH-related haemolysin was first reported from Maldive Islands in 1987. In an outbreak of gastroenteritis in the Maldive Islands, Kanagawa phenomenon negative isolates of *Vibrio parahaemolyticus* were found to produce a TDH-related haemolysin (TRH) but not TDH (Hondo *et al.*, 1987; Honda *et al.*, 1988). Biological, immunological and physiological characteristics of TRH have been reported to be similar but not identical to those of TDH (Honda *et al.*, 1988). The *trh* gene encoding TRH contains a 567-bp open reading frame (Nishibuchi *et al.*, 1989).

2.10.3. *Vibrio vulnificus*

(1) **Capsular polysaccharide**- The most important virulence factor for *Vibrio vulnificus* is the capsular polysaccharide. *Vibrio vulnificus* is an extracellular pathogen that relies on its CPS to avoid phagocytosis by host defence cells and complement (Linkous and Oliver, 1999; Strom and Paranjpye, 2000). Presence of

capsule is related to the colony morphology, with encapsulated strains being opaque and unencapsulated strains being translucent (Yoshida *et al.*, 1985; Wright *et al.*, 1999).

(2) **Flagella and Motility**- Flagella in *Vibrio vulnificus* could act as a type III secretion system for toxins (Kim and Rhee, 2003). The gene which codes for flagella is *flgC*. The gene responsible for motility is *flgE*, coding for flagellar hook protein and a mutation in that particular locus made the strain non-motile lacking flagella (Lee *et al.*, 2004).

(3) **Haemolysin/Cytolysin**-Kreger and Lockwood (Kreger and Lockwood, 1981) and Johnson and Calia (Johnson and Calia, 1981) were the first to describe hemolytic and cytolytic activity of *V. vulnificus* in 1981. Activity of the toxin is inhibited by cholesterol, suggesting that this is part of the receptor (Gray and Kreger, 1985; Shinoda *et al.*, 1985). The gene encoding the haemolysin was cloned by Wright (Wright *et al.*, 1985). Injection of the haemolysin induced skin damage in mice that resembled damage caused by infection (Gray and Kreger, 1987). This result suggested that the cytotoxin could be responsible for most of the damage caused during infection. Several biochemical activities have been ascribed to the haemolysin/cytolysin.

(4) Metalloprotease- The *V. vulnificus* metalloprotease was originally discovered because of its collagenase activity (Smith and Merkel, 1982). The protease was subsequently purified and additional activities of elastase, caseinase, metalloprotease (Zn2+) were identified (Kothary and Kreger, 1985; Miyoshi *et al.*, 1987). As was the case for the haemolysin/ cytolysin, injection of the purified protease into mice caused dermal necrosis (Kothary and Kreger, 1987), increased vascular permeability and

edema (Molla *et al.*, 1989). The ability of the protease to cause oedema was explained by its ability to cause histamine release from mast cells, increased vascular permeability from activation of Hageman factor, pre kallikrein and generation of kinins (Miyoshi and Shinoda, 1988; Molla *et al.*, 1989; Miyoshi and Shinoda, 1992).

2.10.4. *Vibrio hollisae*

(1) **Enterotoxin**- An enterotoxin which elongated Chinese hamster ovary (CHO) cells was detected in extracts of experimentally infected-mouse intestines (Kothary and Richardson, 1987). It could produce fluid accumulation in suckling mouse assay. Not much work is done since then to isolate a single virulence factor responsible for cytotoxicity, but some suggest that the toxicity due to this bacterium may be multifactorial (Marianne *et al.*, 1995).

2.10.5. *Vibrio alginolyticus*

(1) **Tetrodotoxin**- It was found that the puffer fish *Fugu vermicularis vermicularis* harbour *Vibrio alginolyticus* strains which could produce tetrodotoxin (Noguchi *et al.*, 1987). Later studies by Chun-fai and fellow workers confirmed the finding (Chun-fai *et al.*, 2004).

(2) **Alkaline serine protease**- Bacterial proteases have been known as important virulence factors in many diseases. *Vibrio alginlolyticus* also produce an alkaline serine protease which acts as an exotoxin to Kuruma Prawn, *Penaeus japonicus*. The toxin has a maximal activity at pH 10, having a molecular weight of about 33 kDa and is inhibited by phenyl-methylsulfonylfluoride (PMSF), (Lee *et al.*, 1997).

(3) **Calcium-dependent, detergent-resistant alkaline serine exoprotease A-** a Calcium dependent exoprotease, also have been reported from *Vibrio alginolyticus* (Shelly *et al.*, 1989).

2.10.6. *Vibrio mimicus*

(1) **Enterotoxins** identical to cholera toxin- Spira and Fedorka-cray in 1984 reported the purification of enterotoxins from *Vibrio mimicus* that appear to be identical to cholera toxin (Spira and Fedorka-cray, 1984). This cholera toxin like enterotoxin was consisting almost entirely of a subunit which was proteolytically unnicked.

(2) **Haemolysin**- *vmhA* gene encodes a haemolysin in *Vibrio mimicus* which is a clear virulent factor present in them. The *vmhA* gene contains an open reading frame consisting of 2232 nucleotides which can code for a protein of 744 amino acids with a predicted molecular mass of 83.059 kDa. The similarity of amino acid sequence shows 81.6% identity with *Vibrio cholerae* El Tor haemolysin (Kim *et al.*, 1997).

(3) **Metalloprotease**- The *vmc* gene contained 1884 nucleotide sequence. The *vmc* gene of *Vibrio mimicus* encodes a metalloprotease of 628 amino acids and a predicted molecular mass of 71,275 Da. The deduced amino acid sequence had the similarity of 68.5% with *V. parahaemolyticus* metalloprotease, and is a virulence factor in itself (Lee *et al.*, 1998).

(4) **Phospholipase A**- Phospholipase (PhlA) protein shares a highly conserved amino acid sequence with the lecithinase (Lec) of *V. cholerae*. This is an established virulence factor in *Vibrio mimicus*. The rPhlA, the recombinantly expressed phospholipase A, could lyse the erythrocytes obtained from the sh such as rainbow

trout and tilapia. A signi cant cytotoxic activity on a sh cell line, CHSE-214, was observed after 24 h exposure to 401 g rPhlA protein (Lee *et al.*, 2002).

(5) **LT and ST** (Labile toxin and Stable toxin) - *Escherichia coli* heat-labile toxin (LT) and extracellular heat-stable enterotoxins (ST) were produced by clinical isolates of *V. mimicus* (Nishibuchi and Seidler, 1983; Kodama *et al.*, 1984; Sanyal *et al.*, 1983).

2.10.7. *Vibrio fluvialis*

(1) **Extra cellular cytotoxin**- *Vibrio fluvialis* produce an extra cellular cytotoxin or CHO cell-killing factor. The cell killing factor had an apparent molecular weight of 12,200 Da (estimated by gel filtration) (Wall *et al.*, 1984).

(2) **Haemolysin**- *Vibrio fluvialis* possess a haemolysin gene *vfh*. The purified VHF protein exhibited haemolytic activity on many mammalian erythrocytes with rabbit erythrocytes. Nucleotide sequence analysis of the *vfh* gene revealed an open reading frame (ORF) consisting of 2200 bp which encodes a protein of 740 amino acids with a molecular weight of 82 kDa. Molecular weight of the purified VFH was estimated to be 79 kDa by SDS-PAGE (Han *et al.*, 2002). In another study involving clinical isolates, partial sequence analysis of the *V. fluvialis* haemolysin gene revealed that it has 81 % homology with that of the El Tor haemolysin of *Vibrio cholerae*. This was the factor responsible for evoking a striking cytotoxic and vacuolation effects on HeLa cells (Rupa *et al.*, 2005).

(3) **Exocellular thermolysin-like metalloprotease**- An exocellular metalloprotease produced by *Vibrio fuvialis*, is very similar in characteristics to *V. vulnificus* protease.

It is having a molecular mass of 45 kDa. The deduced amino acid sequence confirmed that VFP (*Vibrio fluvialis* protease) was a member of the thermolysin family (Miyoshi *et al.*, 2002).

2.10.8. *Vibrio furnissii*

(1) **Haemolysin**- There are only scant reports about the presence of haemolysin in *Vibrio furnissii*. At least one reference for its presence could be gathered from literature (Esteve *et al.*, 1995). Many uncharacterized toxins are proposed to be present in *Vibrio furnissii* and much work need to be done to find out them.

2.11. Undiscovered virulence factors

A large number of genes are involved in virulence gene expression cascades in bacterial pathogens. Hence any one gene, although not responsible for direct virulence to host, but participating in the pathogen virulence gene/genes' expression cascade may be considered as a virulence/virulence associated gene and can therefore be targeted for drug development. As most of the virulence genes express themselves only when in contact with their host, in order to study their expression and regulation, modern techniques like In Vivo Expression Technology (IVET), Recombination based In Vivo Expression Technology (RIVET), Differential Fluorescence Induction (DFI), Signature Tagged Mutagenesis (STM) and for a wider view Micro array are essential. A lot of studies in this direction have already been carried out (Lee *et al.*, 2001; Wendy and Scott, 2005; Osorio *et al.*, 2005; Angelichio and Camilli, 2002).

2.12. Environmental parameters associated with virulence

Many serious diseases have recently emerged worldwide, mostly related to environmental factors. Many factors have contributed to the persistence and increase in the occurrence of infectious diseases.

Temperature is a key factor. Sunlight can affect the persistence and spread of a pathogen if it is associated with phytoplankton and/or algae. For example, when algae and phytoplankton increase in biomass, zooplankton blooms rapidly follow. Bacteria associated with zooplankton also increase. Also, humidity resulting from evaporation due to elevation of temperature may play an important role in the occurrence of many diseases. With appropriate humidity and moisture, most bacteria survive longer than they would in less humid or dry areas. There are many diseases common to tropical climates that are linked to water transmission. If transmission between hosts does not involve vectors, then water, or at least humid conditions, can be involved in transmission (Brinkman, 1994). For example, in warm and humid regions, where water is available as a transmitting medium, *Vibrio cholerae* may proliferate rapidly to the level of an infective dose. In general, it is impossible to separate environmental factors from biological factors, as can be seen from interrelationships in nature that play a significant role in the emergence of infectious diseases. Recent work of Lobitz and co-workers (Lobitz *et al.*, 2000) correlate very nicely the mean sea-surface temperature data from remote sensing satellite and the occurrence of Cholera in Bangladesh.

2.13. Viable, but non cultivable (VBNCs)

Viable, but non cultivable (VBNC) as the name implies are those bacteria which are Viable, but cannot be cultured on usual laboratory agar media. In 1985,

Colwell and coworkers (Colwell *et al.*, 1985) introduced the term "Viable, but non culturable (VBNC) bacterial cells" to distinguish particular cells that could not form colonies on solid media but maintained metabolic activity and the ability to elongate after the administration of nutrients. According to Oliver (Oliver, 1993) a VBNC bacterium would be defined as a metabolically active bacterial cell that crossed a threshold for known or unknown reasons, and became unable to multiply in or on a medium normally supporting its growth.

VBNC state is important, because, a lion's share of total bacterial diversity is in this phase and more than that, the pathogenic faction of the bacteria in VBNC state, is capable of producing disease in their hosts. They hold our attention mainly because of these two reasons, *Vibrios* in the VBNC state remain metabolically active, retain their virulence properties, and under the appropriate conditions (such as ingestion) recover to become fully vegetative, culturable (Colwell *et al.*, 1996) and pathogenic (Oliver and Bockian, 1995).

How to tap the rich bacterial biodiversity existing in VBNC state? Also how to escape from the VBNC bacteria related illness? For the first part of the question, the answer is Metagenomics (Voget *et al.*, 2003) and the answer for latter part of the question is technological advances like Microarray (Vora *et al.*, 2005).

2.14. Horizontal Gene Transfer (HGT) among *Vibrios*

HGT is any process in which an organism transfers genetic material (*i.e.* DNA) to another cell that is not its offspring. By contrast, vertical transfer occurs when an organism receives genetic material from its ancestor, *e.g.*, its parent or a species from which it evolved. Most thinking in genetics has focused on the more prevalent vertical transfer, but there is a recent awareness that horizontal gene transfer

is a significant phenomenon. Increasingly, studies of genes and genomes are indicating that considerable horizontal transfer has occurred between prokaryotes (Jain *et al.*, 1999). The means of HGT can be

- Plasmids
- Transposons
- Integrons and
- Phages

And the methods by which HGT occur can be conjugation, transformation and transduction.

2.14.1. Cross walk among family members

Horizontal Gene Transfer among *Vibrionaceae* is an established fact. Many virulence genes and Pathogenicity islands are acquired horizontally (Faruque and Mekalanos, 2003). Antibiotic resistance genes are also acquired in a similar manner (Hochhut *et al.*, 2000). Rampant HGT events taking place in the genosphere is responsible for the evolution of new combination of virulence genes and so new pathogens! Surveillance programs for HGT have relevance always. The results of such programs can be both informative as well as cautionary to the concerned people. The evolution of 0139, the new pandemic strain of *Vibrio cholerae* is an example of how HGT can directly influence human health. Later it was found that genetic exchange between *Vibrio cholerae* 01 and a non 01 strain resulted in the emergence of this new pandemic strain (Bik *et al.*, 1995).

How can we find out Lateral Gene Transfer? What are the recognised tests? to find out LGT? The suspicion of HGT usually emerges when a gene sequence from a particular organism shows the strongest similarity to a homolog from a distant taxon or in other words when unexpected ranking of sequence similarity among homologs

occur (Koonin *et al.*, 2001). Analysis of phylogenetic tree topologies is traditionally the principal means to decipher evolutionary scenarios, including horizontal gene transfer events (Syvanen, 1994). In a well-supported tree, if a bacterial protein groups with its eukaryotic homologs to the exclusion of homologs from other bacteria, and best of all, shows a reliable affinity with a particular eukaryotic lineage, the conclusion that HGT is at play can be drawn. The evolution of bacterial and archaeal genomes involved extensive gene shuffling, and there is little conservation of gene order between distantly related genomes (Dandekar *et* al., 1998; Itoh *et al.*, 1999; Mushegian *et al.*, 1996). It has been determined that the presence of three or more genes in the same order in distant genomes is extremely unlikely unless these genes form an operon (Wolf *et al.*, 2001) and if such an observation is made, it can be a probable HGT event. Genes whose nucleotide or codon composition are significantly different from the mean for a given genome are considered as probable horizontal acquisitions although the likely source of these alien genes generally can not be identified (Garcia-vallve *et al.*, 2000; Lawrence *et al.*, 1997; Medigue *et al.*, 1991).

Chapter 3

MATERIALS AND METHODS

3.1. Isolation of *Vibrios*

3.1.1. Sources of *Vibrios*

Vibrios were isolated from sea water, green mussel, prawns, and plankton. List of samples and sampling location is presented in **Table A**.

3.1.2. Collection of samples

Water samples were collected from different locations along the coastal areas of Kerala, and Aquaculture farms. Surface water samples were collected in sterile polythene bags, tied well and brought to the laboratory in icebox within 2- 6 hr of sampling.

Fresh samples of Green Mussel and prawn (*Penaeus monodon*) were obtained from market and used in the study. They were transported in sterile conditions to the laboratory within 6 hours.

Plankton were collected from Cochin estuary using a plankton collector (Bongo net) and brought to the laboratory in an icebox and processed for plating.

Table A. List of sampling stations and samples types

Sl. No.	Sampling Station	Sample Types
1.	Ernalulam, Alappuzha, Aquafarm, Aquafarm with fish kill, Conolly canal, Ponnani, Chettuva, Chavakkadu, Kasarakod, Omanapuzha	Water
2.	Vadakara	Green mussels
3.	Neendakara	Prawns (*P.monodon*)
4.	Cochin estuary	Plankton

3.1.3. Preparation of serial dilutions of the sample

(a) Water samples were serially diluted using physiological saline and 50µl of the prepared dilution was used as inoculum.

(b) The tissue samples from green mussel (*Perna viridis*) and prawns (*P. monodon)* were each homogenized with sterile physiological saline. 10 gm tissue (mussel and prawns) was weighed and transferred aseptically to a sterile mortar, ground well with a pestle and mixed with 90 ml physiological saline. After thorough mixing, the homogenate was centrifuged and the supernatant was used for serial dilution.

(c) Plankton was filtered from the water and plankton sample thus prepared was used for *Vibrio* isolation. Initially, the plankton suspension in physiological saline was vortexed in order to dislodge the adhered bacteria present as biofilm on it. The vortexed suspension was used for serial dilution.

3.1.4. Medium used for isolation and purification

After serial dilution, the corresponding samples were spread plated onto Thiosulphate Citrate Bile salt Sucrose (TCBS) agar plates and incubated at 37^0C for 24 hours. Isolated single colonies were picked, purified on NA plates, then sub-cultured and stocked for further characterisation

3.2. Identification of *Vibrios*

All the isolates obtained from Nutrient agar were repeatedly streaked on NA plates and confirmed their purity by performing Gram staining and microscopic observation. *Vibrios* were identified based on their,

(i) Morphological and biochemical characteristics and,

(ii) Molecular ribotyping using partial 16S rRNA.

3.2.1. Morphological and biochemical characteristics

All the isolates were assigned to various genera based on their morphological and biochemical characters outlined in Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974).

3.2.1.1. Gram staining

The bacterial isolates were Gram stained. Gram negative rods/cocci were segregated as *Vibrios* since most *Vibrios* are gram negative.

3.2.1.2. Oxidase test

Oxidase test, tests the ability of the organism to produce Cytochrome oxidase enzyme. Oxidase enzyme plays a vital role in the operation of the electron transport system during aerobic respiration. Cytochrome oxidase catalyzes the oxidation of a reduced cytochrome by molecular oxygen (O_2) resulting in the formation of H_2O or H_2O_2. The ability of bacteria to produce Cytochrome oxidase can be determined by the addition of the test Kovacs reagent, Tetramethyl-p-phenylenediamine dihydrochloride. The reagent serves as an artificial substrate, donating electrons and there by becoming oxidized to a blue compound indicating the formation of indophenol. No colour change indicates the absence of Cytochrome oxidase. This test is used for screening species of *Pseudomonas*, *Neisseria*, *Vibrio*, *Pasturella*, which gives a positive reaction and for excluding *Enterobacteriaceae*, all of which gives a negative reaction.

According to the methods recommended, the organisms were freshly grown on nutrient agar. A positive result was recorded when the smear turned violet within 10 seconds, the oxidation product of tetramethyl para phenylene diamine dihydrochloride.

3.2.1.3. Oxidation/ Fermentation reactions with glucose (MOF Test)

This characteristic is usually determined by inoculating the organisms into deep agar medium in test tubes. The incorporation of a pH indicator into the medium enables detection of changes in the pH resulting from the decomposition process of glucose to be observed in addition to visible signs of growth in different parts of the medium. Cultures are stab inoculated with a straight inoculation needle.

Acidic changes at or near the surface indicates that the substrate is being oxidized by aerobic bacteria, whereas the development of uniform acidity throughout the tube shows that facultative anaerobic organisms are both oxidizing and fermenting the substrate.

MOF medium (HIMedia) was used for the present work. The medium was sterilized by autoclaving at 15 lbs for 15 minutes. Added 1% glucose to the sterile basal medium and transferred 4 ml aliquots aseptically into sterile tubes and autoclaved at 10 lbs for 8 minutes. Converted to slants with a long butt. The tubes were then stab inoculated and streaked and incubated at 37°C for 24 hours. The results were recorded as follows:

O- Oxidation (yellow coloration in the butt)

F – Fermentation (yellow coloration throughout the tube)

(F)- Fermentation with gas production

Alkaline reaction- Pink coloration in the slant and no reaction in the butt.

The isolates, segregated as *Vibrios* after a presumptive screening by Gram staining, oxidase, and MOF tests, were confirmed as *Vibrios* by plating them onto TCBS agar plates. These isolates showed as yellow or green colonies on the TCBS agar.

Isolates which were purified were maintained as stock cultures employing three methods, viz. paraffin overlay, semisolid agar method and glycerol stock.

(a) **Paraffin overlay method-** Nutrient agar supplemented with 1%sodium chloride was made in glass stocking vial. A single celled colony was inoculated onto the same and grown for 18hr. Sterile liquid paraffin wax was added on top and the vials were covered with sterile rubber stopper. The vials were kept in dark until further use

(b) **Semisolid agar method of stocking-** Semisolid nutrient agar (0.8% agar) was made in 5ml BOROSIL® tubes and overnight bacterial culture was used to stab inoculate this semisolid agar. After 18hr growth, the tubes were stoppered with sterile rubber corks and stored at room temperature.

(c) **Glycerol stocking** - Cultures were grown in nutrient broth, supplemented with 1% sodium chloride. After 18hr growth, the broths were stored in 15% (final) glycerol conc. at -80°C.

3.2.2. Ribotyping using partial 16S rRNA gene

Identification of the *Vibrio* strains was done using a primer pair for 16SrDNA. A portion of the 16S rRNA gene (1.5kb) was amplified from the genomic DNA (Shivaji *et al.*, 2000; Reddy *et al.*, 2000, 2002a, 2002b). Products after PCR amplification was purified by gene clean kit (Bangalore Genei) and subsequent sequencing of the amplicons generated, followed by homology analysis.

Sequence	Amplicon	Reference
16SF 5' AGTTTGATCCTGGCTCA 3'	**1500** bp	Shivaji *et al.*, 2000
16SR 5' ACGGCTACCTTGTTACGACTT 3'		Reddy *et al.*, 2000, 2002a,b

3.2.2.1. DNA sequencing

Products after PCR amplification was purified by gene clean kit (Bangalore Genei) and nucleotide sequences determined by the ABI Prism 310 genetic analyzer by using the big dye Terminator kit (Applied Biosystems). The identity of the sequences determined were established by comparing the sequences obtained with the gene sequences in the database using BLAST software (Altschul *et al.*, 1980).

3.2.2.2. Multiple sequence alignment

Assembled nucleotide sequences were aligned using the Clustal X software program (Thompson *et al.*, 1997), which is a new Windows interface for the earlier Clustal W (Thompson *et al.*, 1994) sequence alignment program. Aligned sequences were imported into an alignment editor GeneDoc (Nicholas and Nicholas, 1997) and the ends of the alignment were trimmed to obtain equal lengths for all sequences. The trimmed, aligned nucleotide sequences were imported into the DAMBE software program for further Phylogenetic analysis as described below.

3.2.2.3. Phylogenetic tree construction

Phylogenetic trees were constructed using the neighbour joining methods implemented in the DAMBE (Data Analysis in Molecular Biology and Evolution) software program (Xia 2000). Trees were constructed using nucleotide based TN84 evolutionary model for estimating genetic distances based on synonymous and non-synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps and those nodes with a bootstrap value of 75 or more were

taken to represent nodes with significance. Trees were visualised using the TreeView program (version 1.6.1).

3.3. Antibiotic susceptibility test

The axenic strains of *Vibrios* were examined for resistance to nine commonly administered antibiotic drugs for treatment of gastroenteritis. **Table-1** shows the concentration of the various antibiotics incorporated in the commercial discs available from HIMedia, Bombay, India. Antibiotic susceptibility test was done according to Kirby-Bauer disc diffusion method (Bauer, *et al.*, 1966) as described below.

(i) A single celled colony of the test strain was transferred into 3ml of sterile normal saline. Turbidity of the cell suspension was adjusted to 0.5 Mc Farlands' standard, either by adding new colonies or adding more sterile normal saline.

(ii) With the help of a sterile cotton swab, a uniform bacterial smear was made on to Mueller-Hinton agar (HIMedia) plate.

(iii) Antibiotic discs were placed on the plate, each plate holding not more than five discs. The discs were spaced to provide room for the development of the zone of inhibition.

(iv) The plates were incubated at 37°C for 24 hours before examination.

(v) The result was interpreted as resistant, intermediate or sensitive based on the size of the inhibition zones as provided by the manufacturer (**Table B**).

Table B. Antibiotics used in this study and their zone size interpretation chart

Antimicrobial agent	Disc content (µg/disc)	Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
Ampicillin	10	13	14-16	17
Chloramphenicol	30	12	13-17	18
Ciprofloxacin	5	15	16-20	21
Co-Trimoxazole	25	10	11-15	16
Nalidixic acid	30	13	14-18	19
Norfloxacin	10	12	13-16	17
Tetracycline	30	14	15-18	19
Furazolidone	100	14	15-16	17
Trimethoprim	5	10	11-15	16

This chart is adapted from the zone-size interpretative chart published by HIMedia Pvt. Laboratories Ltd., India (2005).

3.3.1. MAR (Multiple Antibiotic Resistance) indexing

MAR index was calculated with the formula a/b, where 'a' is the number of antibiotics to which the isolate was resistant, and 'b' is the number of antibiotics to which the isolate was exposed (Krumperman, 1983).

3.4. Polymerase Chain Reaction (PCR)

3.4.1. Template preparation for PCR (Murray and Thompson, 1980)

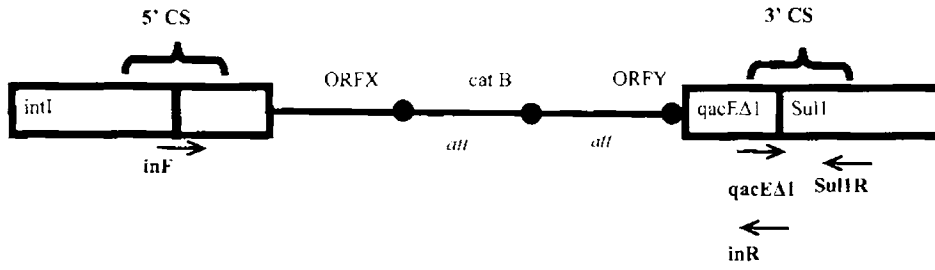
DNA isolation by various methods (Sambrook *et al.*, 2000 and Murray and Thompson, 1980) was tested and the method by Murray and Thompson was selected since it yielded good quality DNA for PCR. The procedure is described below.

1. A single celled colony was inoculated into 10ml Luria broth (HIMedia) and incubated at 37°C overnight with constant shaking (180 rpm).
2. 1.5ml of the resultant culture was taken in a sterile Eppendorf tube and centrifuged at 6000rpm for 5 min.
3. The pellet was suspended in 567µl of sterile Tris-EDTA buffer (pH 8.0) (**Appendix I**)
4. To the above, 30µl of 10% Sodium Dodecyl Sulphate (SDS) and 3µl (20mg/ml) of proteinase K was added to attain a final concentration of 100µg/ml of Proteinase K in 0.5% SDS.
5. The contents of the Eppendorf tube were mixed well and incubated at 37°C for 1hr.
6. Incubation was followed by the addition of 100µl of 5M sodium chloride, and 80µl Hexadecyltrimethylamonium bromide (CTAB) (10mg/ml) (**Appendix I**)
7. After thorough mixing, the tubes were incubated at 65°C for 10 min. in a water bath.
8. The tubes were allowed to cool down to room temperature before adding 720µl of chloroform and 30µl of isoamyl alcohol in the ratio 24:1.
9. The contents were mixed gently and centrifuged at 10,000rpm for 10 min.
10. The aqueous layer formed on top was pipetted out using a blunt end sterile tip and transferred into a fresh sterile microfuge tube.

11. To the above collected aqueous layer, a mixture of 375µl phenol: 360µl chloroform: 15µl isoamyl alcohol was added to obtain a ratio of 25:24:1.
12. The contents were mixed thoroughly by inverting several times and centrifuged at 10,000 rpm for 10 min.
13. The resulting aqueous layer was collected in the same way as above and transferred to a labelled fresh sterile microfuge tubes.
14. To the aqueous layer was added 0.6 volumes (~600µl) of isopropanol, mixed gently, and kept at room temperature for 30 min. followed by centrifugation at 12,000 rpm for 15 min. at 4°C.
15. The supernatant was removed and the pellet was washed in cold 70% ethanol.
16. The nucleic acid pellet was air-dried to remove all traces of ethanol and resuspended in sterile Tris-EDTA buffer (pH 8.0).
17. The concentration of genomic DNA thus prepared was estimated spectrophotometrically and appropriate dilutions (~80-100ng) were used as template for PCR reactions.

3.5. Screening for Integrons

Schematic representation of a typical Integron system denoting the relative positions of primers.



3.5.1. PCR with *qacEΔ1*F and *sul1*R (Dalsgaard *et al.*, 2000)

Integrons were screened using ***qacEΔ1*** and ***sul1*** (Quarternary ammonium compound resistance and Sulphonamide resistance) primers designed from integrons. Depending on the class and number of integrons present, these primers may yield one or more amplicons. These must amplify a conserved region from 3'end of integrons.

Primer sequences

Forward primer: 5' - ATCGCAATAGTTGGCGAAGT -3'

Reverse primer: 5' – GCAAGGCGGAAACCCGCGCC -3'

Amplicon size: ~800bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 60°C – 1 min.

Extension - 68°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.5.2. PCR with *in*F and *in*R (Dalsgaard *et al.*, 2000)

PCR was performed with ***in*F** and ***in*R** integron primers and depending on the number and length of gene cassettes, they may yield amplicons of varying length. '*In*' stand for primers designed from a portion of the integrase of the integron gene. This can be called a virulence-associated gene.

Primer sequence

Forward primer: 5' – GGCATCCAAGCAGCAAGC – 3'

Reverse primer: 5' – AAGCAGACTTGACCTGAT – 3'

Amplicon size: variable (Thungapathra *et al* 2002)

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 65°C - 1 min.

Extension – 68°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.6. Screening for Virulence/virulence related genes

Virulence related genes were screened employing PCR in which three marker genes and six virulence genes were tested. *ompw, O1rfb, O139 rfb* were the marker genes, and *tcpA, toxR, ace, zot, ctxA* and *sxt* were the virulence genes.

3.6.1. PCR with *ompw*F and *ompw*R (Bisweswar *et al.*, 2000)

ompw is a gene which can be of marker value (Outer membrane protein w) Ninety-seven percent of all ***ompw*** positive isolates can be spelled as *Vibrio cholerae* (Bisweswar *et al.*, 2000).

Primer sequence

Forward primer : 5' – CACCAAGAAGGTGACTTTATTGTG – 3'

Reverse primer: 5' – GAACTTATAACCACCCGCG – 3'

Amplicon size: 588bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 65°C – 1 min.

Extension – 68°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.6.2. PCR with *01rfb*F and *01rfb*R (Hoshino *et al.*, 1998)

01rfb is a gene of marker value. The gene codes for a component protein of bundle forming pili. A positive amplification indicates that the isolate belongs to 01 serotype of the pandemic causing *V. cholerae*.

Primer sequence

Forward primer: 5' – GTTTCACTGAACAGATGGG – 3'

Reverse primer: 5' – GGTCATCTGTAAGTACAAC – 3'

Amplicon size: 192 bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl

Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 62°C – 1 min.

Extension – 65°C – 1min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.6.3. PCR with *0139rfb*F and *0139rfb*R (Hoshino *et al.*, 1998)

0139rfb is a gene of marker value. The gene codes for a component protein of bundle forming pili of 0139 serotype of pandemic causing *Vibrio cholerae*. A positive amplification indicates that the isolate belongs to 0139 serotype of *V. cholerae*.

Primer sequence

Forward primer: 5' – AGCCTCTTTATTACGGGTGG – 3'

Reverse primer: 5' – GTCAAACCCGATCGTAAAGG – 3'

Amplicon size: 449 bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 62°C – 1 min.

Extension – 65°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.6.4. PCR with *tcpA* F and *tcpA* R (Keasler and Hall, 1993)

tcpA denote Toxin co-regulated pili, and is the key virulence factor in *Vibrio cholerae*. The presence of *tcpA* is indicated by a positive amplification.

Primer sequence

Forward primer: 5' – CACGATAAGAAAACCGGTCAAGAG – 3'

Reverse primer: 5' – ACCAAATGCAACGCCGAATGGAGC – 3'

Amplicon size: 618 bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 60°C – 1 min.

Extension – 68°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.6.5. PCR with *toxR* F and *toxR* R (Singh *et al.*, 2001)

ToxR is a direct virulence factor. A positive amplification indicates its presence.

Primer sequence

Forward primer: 5' – CCTTCGATCCCCTAAGCAATAC – 3'

Reverse primer: 5' – AGGGTTAGCAACGATGCGTAAG – 3'

Amplicon size: 779 bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 60°C – 1 min.

Extension – 68°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.6.6. PCR with *ace* F and *ace* R (Singh *et al.*, 2001)

Accessory cholera enterotoxin is called as ***ace***. A positive amplification will indicate its presence. This is also a direct virulence factor.

Primer sequence

Forward primer: 5'– TAAGGATGTGCTTATGATGGACACCC – 3'

Reverse primer: 5'– CGTGATGAATAAAGATACTCATAGG – 3'

Amplicon size: 289 bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 62°C – 1 min.

Extension – 68°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.6.7. PCR with *zot* F and *zot* R (Singh *et al.*, 2001)

Zonula occludens toxin is known as ***zot***. Positive amplification will denote its presence. It can be called a direct virulence factor.

Primer sequence

Forward primer: 5' – TCGCTTAACGATGGCGCGTTTT – 3'

Reverse primer: 5' – AACCCCGTTTCACTTCTACCCA – 3'

Amplicon size: 947 bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl

Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 60°C 1 min.

Extension – 68°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.6.8. PCR with *ctxA* F and *ctxA* R (Singh *et al.*, 2001)

ctxA is the most important virulence factor of *Vibrio cholerae*. It codes for cholera toxin A subunit.

Primer sequence

Forward primer: 5' – CTCAGACGGGATTTGTTAGGCACG – 3'

Reverse primer: 5' – TCTATCTCTGTAGCCCCTATTACG – 3'

Amplicon size: 302 bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 60°C – 1 min.

Extension – 68°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.6.9. PCR with *sxt* F and *sxt* R (Thungapathra *et al.*, 2002)

sxt is an integrative conjugative element, which is around 100kb in size. A part of *sxt*, the partial integrase gene will be amplified if the element is present. This is a virulence associated gene.

Primer sequence

Forward primer: 5' – TTATCGTTTCGATGGC – 3'

Reverse primer: 5' – GCTCTTCTTGTCCGTTC – 3'

Amplicon size: 803 bp

PCR Mix composition

10X PCR buffer	2.5µl
2.5mM each dNTPs	2.0µl
Forward primer (10 picomoles) -	1.0µl
Reverse primer (10 picomoles) -	1.0µl
*Taq*DNA polymerase	1U
Template DNA (As prepared above)	3.0µl
Sterile Distilled water	to a final volume of 25µl

PCR conditions

Annealing – 54°C – 1.5min.

Extension – 68°C – 1.5min.

PCR was performed in a thermal cycler (Eppendorf master cycler personal).

3.7. Conjugation experiments

Conjugation experiment was carried out with slight modifications from Li's protocol (Li *et al.*, 1999) using *Vibrio alginolyticus* strain FK4 as the donor, and *E.coli* HB101 as the recipient. HB101 has a streptomycin resistance marker. Donor and recipient cells were inoculated in LB broth separately and incubated overnight at 30°C. After overnight incubation, donor and recipient cells were mixed, and were

filtered through 0.2 µm membrane filter. The membrane filter containing the bacteria was then placed onto the MacConkey agar. The plates were incubated at 30°C for 24 hours. After incubation, the membrane filters containing bacteria were washed with normal saline. The conjugated bacterial suspension was plated onto MacConkey agar containing ampicillin and streptomycin. The inoculated plates were incubated for 24 hrs at 37°C. The transconjugants were selectively grown on MacConkey agar plates containing ampicillin and streptomycin. The transconjugants were subjected to antibiogram study. The transfer of SXT element to transconjugants was confirmed by PCR and the PCR amplicons were visualised on agarose gel.

3.8. Agarose gel electrophoresis (Sambrook *et al.*, 2000)

(i) Agarose gel with a concentration of 1.5% or 2% (Depending on the expected amplicon size) was prepared for electrophoresis of the PCR products.

(ii) 10 µl of the PCR products was loaded on to the gel and electrophoresed at 80 volts for 1 h or until the migrating dye (Bromophenol blue) had traversed two-thirds distance of the gel. 100 bp DNA ladder (Bangalore Genei) was used as a marker.

(iii) The gel was stained in freshly prepared 0.5mg/ml ethidium bromide solution for 20 min.

(iv) The gel was viewed on a UV transilluminator, and image captured with the help of Gel Doc system (Bio Rad).

3.9. Suckling mouse assay

Three day old Swiss Albino Suckling mice were separated from their mother immediately before use, and randomly divided into groups of three. About 100 l of cell free culture supernatant containing a drop of 0.02% Evan's Blue was administered orally using a fine polythene tube, connected to a 1 ml Tuberculin syringe. The inoculated mice were individually placed on a layer of filter paper in plastic Petri dishes. After three hours at room temperature, the mice were sacrificed by keeping them for 15 to 20 minutes in a closed container which was saturated with chloroform. A test is considered positive if the ratio of intestinal weight to remaining carcass weight (F/A) was >0.083. For each strain a minimum of three mice were used. (Takeda, *et al.*, 1978).

The following are the cultures used for suckling mouse assay. 1. CHV2(2), 2. CHV2(4), 3. CHV3(1), 4. CHV4(3), 5. CHAVA3, 6. CHAVA4(3), 7. CHAVA4(4), 8. P5, 9. P6, 10. P9, 11. C3, 12. C9, 13. MUS7, 14. MUS13, 15. PV8(1), 16. FK2, 17. FK4, 18. AF4, 19. AF7, 20. ALP(VC)11, 21. EKM14.

Chapter 4

RESULTS

4.1. Isolation and identification of *Vibrios*

4.1.1. *Isolation of Vibrios from various samples*

A total of 108 isolates of *Vibrio* like organisms were obtained from various source of samples collected from different locations and aqua farms. In many samples other than that presented in **Table 1**. *Vibrios* were absent.

Table 1. *Vibrio* like isolates obtained from different sampling stations

Sl.No.	Sampling station	Sample type	No. of isolates
1.	Ernakulam, Alappuzha, Aquafarm, Aquafarm with fish kill, Connolly canal, Ponnani, Chettuva, Chavakkadu, Kasarakod, Omanapuzha	Water	89
2.	Vadakara	Green mussels	14
3.	Neendakara	Prawns (*P.monodon*)	2
4.	Cochin estuary	Plankton	3

4.2. Identification of *Vibrios*

The isolates which are Gram negative, oxidase positive, fermentative on MOF media and which showed yellow/green coloured colonies on TCBS (Thiosulfate Citrate Bile salt Sucrose) agar were segregated as *Vibrios*.

All the isolates recognised as *Vibrios* were further subjected species level identification based on ribotyping using partial 16SrRNA gene.

Partial 16S rRNA gene could be amplified and sequenced from all 108 strains of *Vibrios*. Their identity could be confirmed by comparing the sequences with Genbank entries, by BLAST programme (Altschul *et al.*, 1980).

Table-2. Percentage of the different species segregated in this study

Sl. No.	*Vibrio* Species	No. of isolates obtained	Percentage of isolates N=108
1	*Vibrio cholerae*	24	22.222
2	*Vibrio alginolyticus*	22	20.370
3	*Vibrio parahaemolyticus*	19	17.592
4	Uncultured bacterium	12	11.111
5	*Vibrio harveyi*	8	7.407
6	*Vibrio species*	8	7.407
7	*Vibrio vulnificus*	6	5.555
8	*Vibrio proteolyticus*	2	1.851
9	*Vibrio natriegens*	2	1.851
10	*Vibrio furnissii*	1	0.925
11	Marine bacterium	1	0.925
12	*Photobacterium sp*	1	0.925
13	*Mucus bacterium*	1	0.925
14	Bacterium JB8	1	0.925

A portion of 16SrRNA gene was used for amplification and this amplicon was then sequenced from all isolates. Some of these sequences were deposited in the Genbank database. The accession numbers of these submissions are as given: DQ241795, DQ898173, DQ904445, DQ904444, DQ904443, DQ904442 and DQ904441. Sequences were compared with the previous database entries by BLAST. 16S rRNA gene based identification of all 108 isolates of *Vibrios* was carried out and they were compared amongst themselves on the basis of this gene sequence, by phylogenetic methods.

4.3. Phylogenetic Tree construction

Phylogenetic trees can tell a great deal about the interrelationship of strains in a sample. They are useful and reliable tools of molecular taxonomy. The relative position of an unknown strain in a well constructed phylogenetic tree can give a lot of information about the probable affinities of the strains and also its evolutionary progenitors.

The identity of the strains and their interrelationship based on the phylograms constructed are depicted in **Figures 1-10** and **Figure 11** depicts the interrelationship of all *Vibrio* strains isolated from various locales.

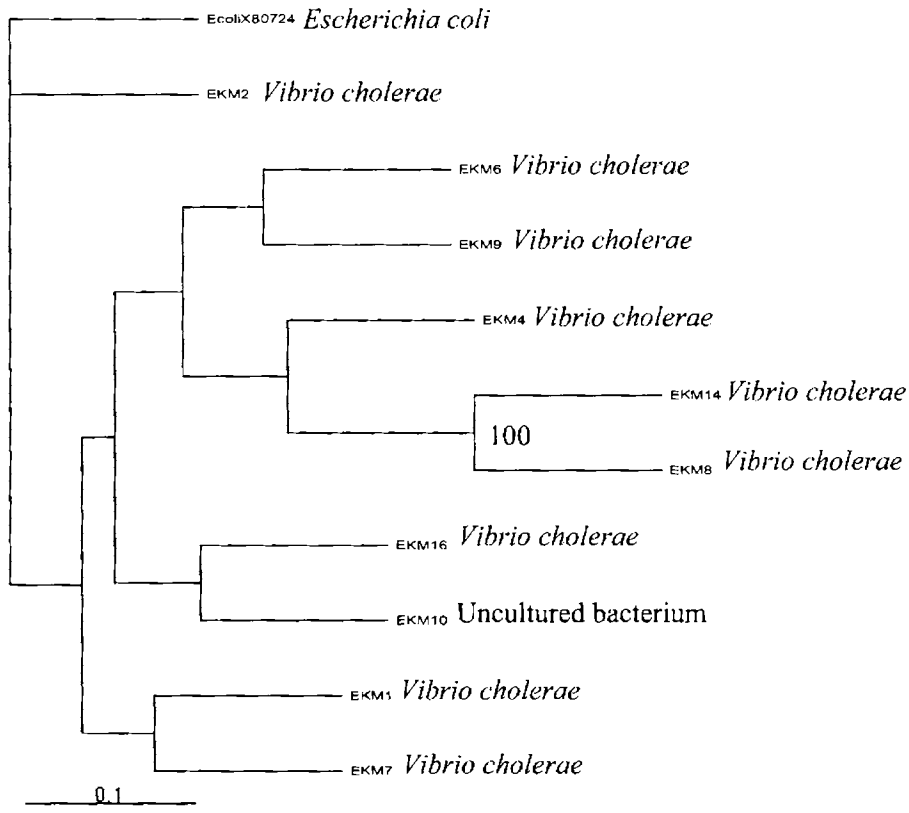


Fig. 1. Phylogram of *Vibrios* isolated from Ernakulam

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence. Node values more than 75 only is shown

All isolates from this station were *Vibrio cholerae*. The relationship between strains EKM14 and EKM8 are maximally supported. Even though EKM10 is designated as an 'uncultured bacterium', later it was confirmed as a *Vibrio cholerae* strain by the *ompw* PCR assay method.

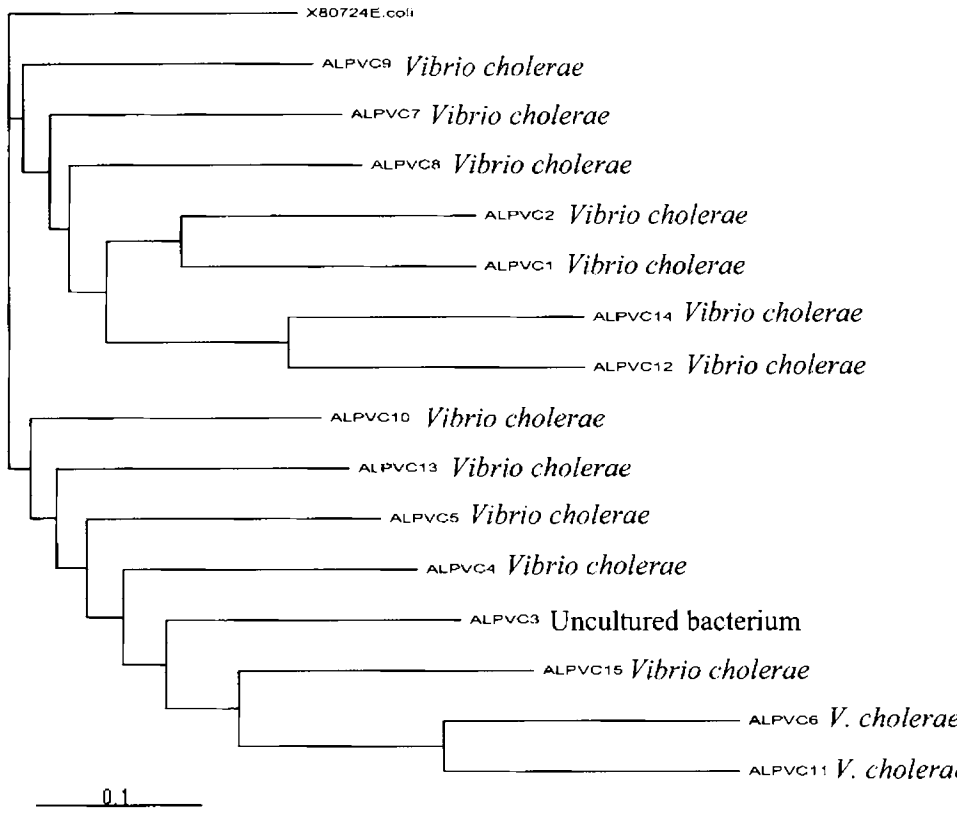


Fig. 2 Phylogram of *Vibrios* isolated from Alappuzha

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence. Node values more than 75 only is shown.

Most of the isolates from station Alappuzha belonged to the *Vibrio cholerae* group, and they clearly claded among themselves into two.

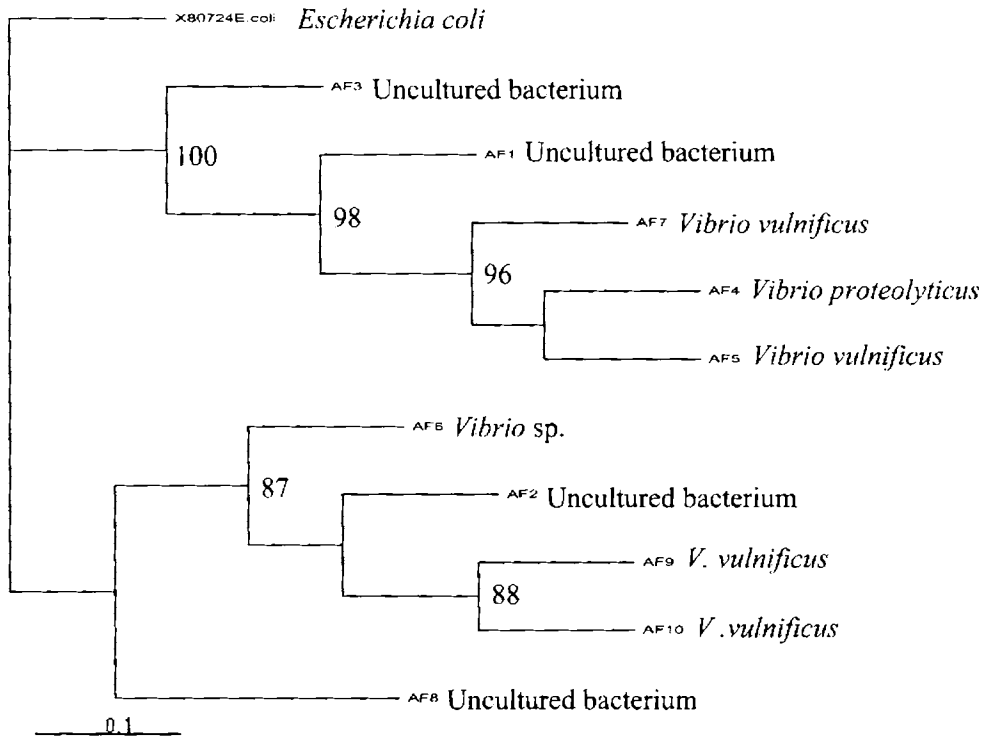


Fig. 3 Phylogram of *Vibrios* isolated from Aqua Farm

The numbers at the nodes indicate the levels of bootstrap support based on 1,000replicates.Bar = 10 % sequence divergence. Node values more than 75 only is shown.

Maximum number of isolates identified as 'uncultured bacterium' were from station Aqua Farm. Maximum number of *V.vulnificus* (n=4) were also isolated from the same station.

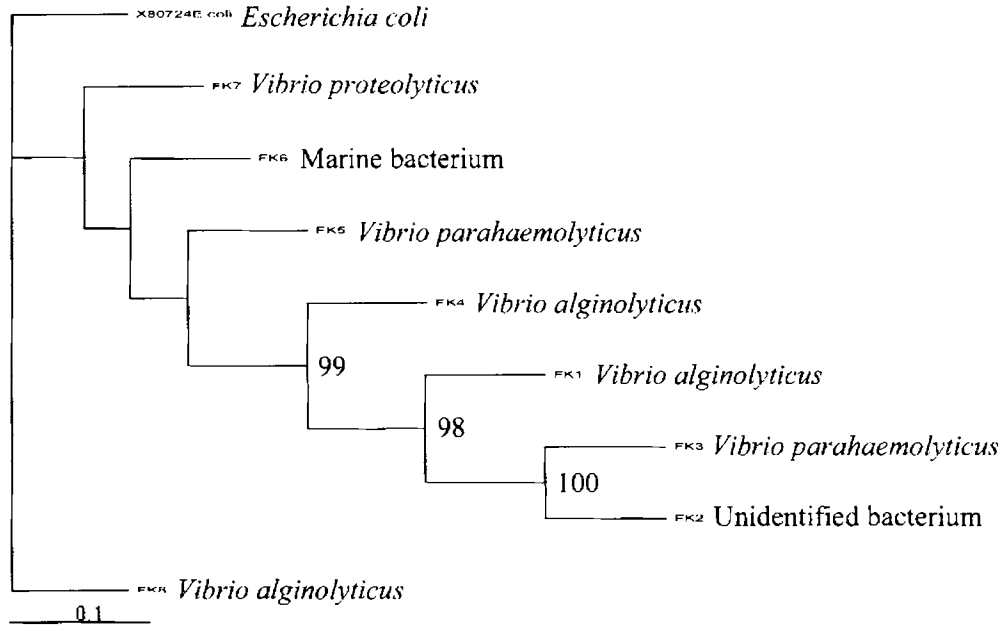


Fig. 4. Phylogram of *Vibrios* isolated from Aqua Farm where Fish kill occurred

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence. Node values more than 75 only is shown.

In this station, the *Vibrio* isolates were identified as *V.proteolyticus, V. parahaemolyticus & V. alginolyticus* - a potential fish pathogen; one strain each was identified as a marine bacterium and unidentified bacterium. .The strains showed an all together different pattern of inter relatedness. Strain FK8 was a stand alone among the rest of the isolates.

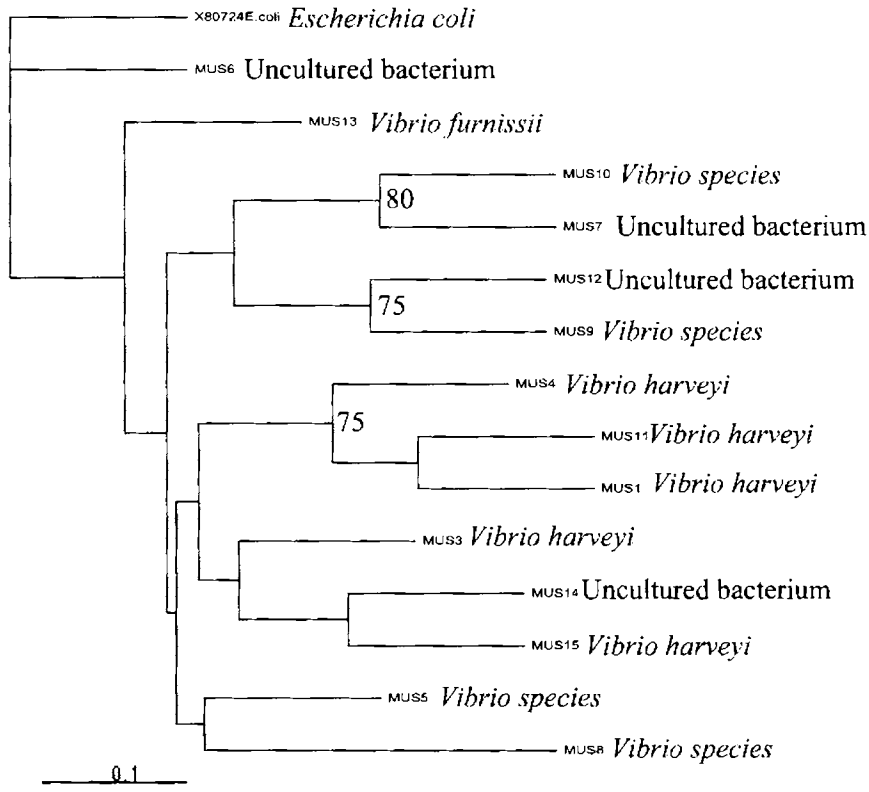


Fig. 5 Phylogram of *Vibrios* isolated from Green mussel

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence. Node values more than 75 only is shown.

The only isolate identified as *Vibrio furnissii* in this whole study is from this station. *Vibrio harveyi* was represented by five strains.

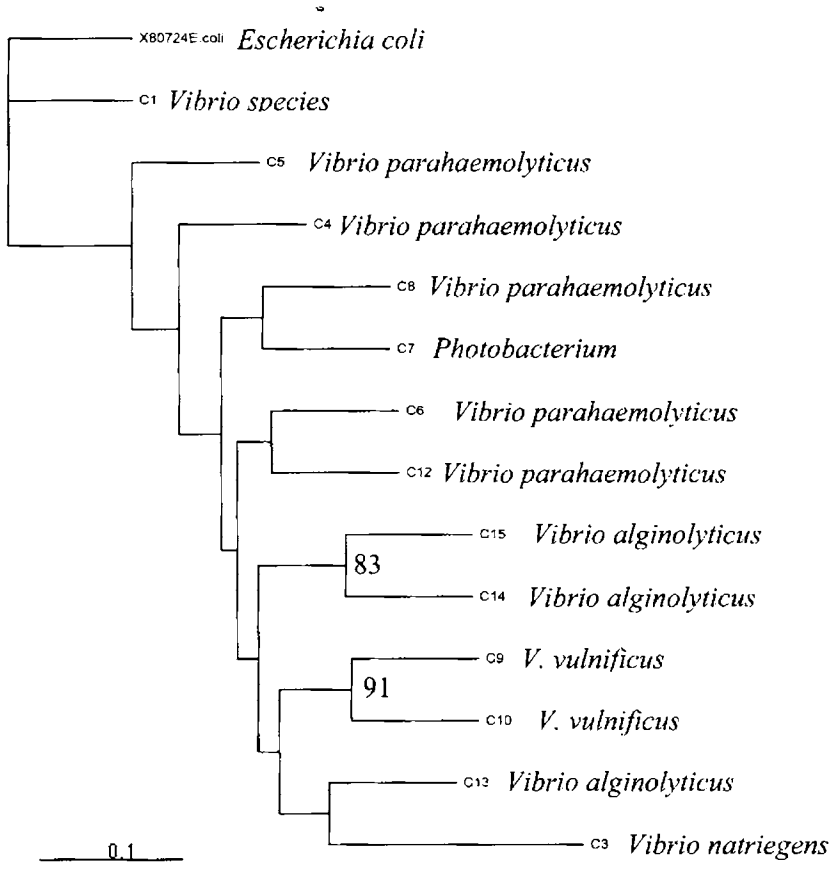


Fig. 6 Phylogram of *Vibrios* isolated from Connolly canal

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence. Node values more than 75 only is shown.

Strain C1 identified only as unspecified *Vibrio*, cladded separately from the rest of the isolates. Isolate C7 identified as *Photobacterium* cladded with *V. parahaemolyticus* and strain C3 identified as *V. natriegens* cladded with *V. alginolyticus* in this analysis.

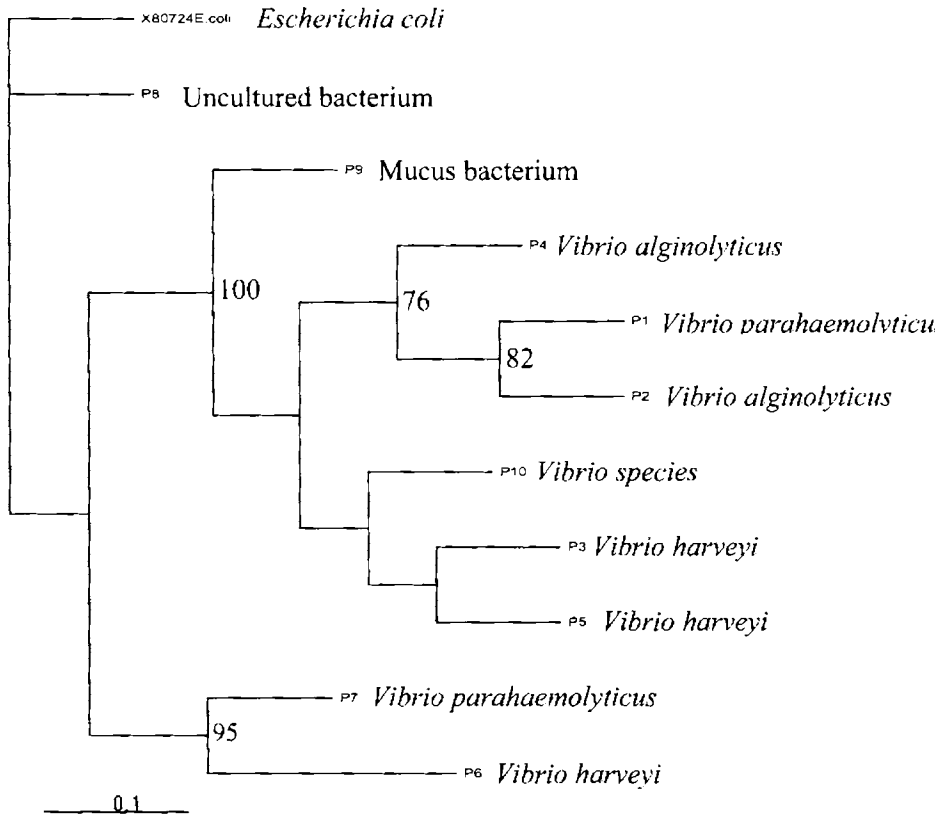


Fig. 7 Phylogram of *Vibrios* isolated from Ponnani

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence. Node values more than 75 only is shown.

This is the only station from where Mucus bacterium was isolated. The other isolates were identified as *V. parahaemolyticus*, *V. harveyi* and *V. alginolyticus*.

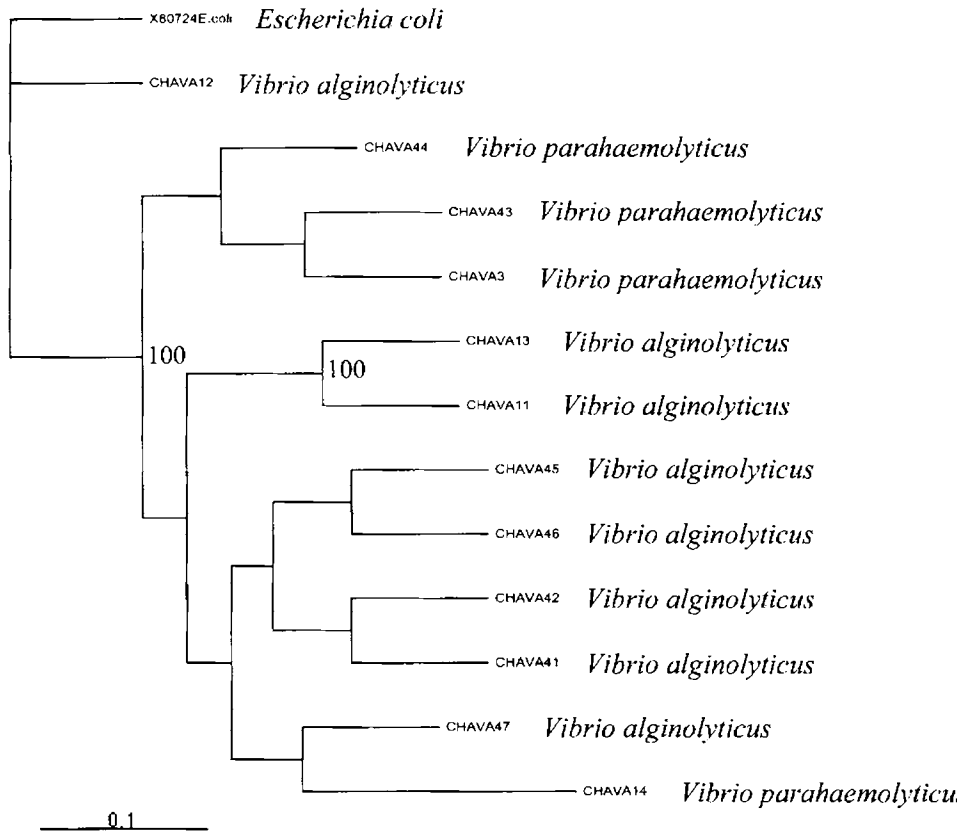


Fig. 8 Phylogram of *Vibrio* isolates from Chavakkadu

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence. Node values more than 75 only is shown.

The number of strains identified as *Vibrio alginolyticus* were maximum from this station, followed by *Vibrio parahaemolyticus*.

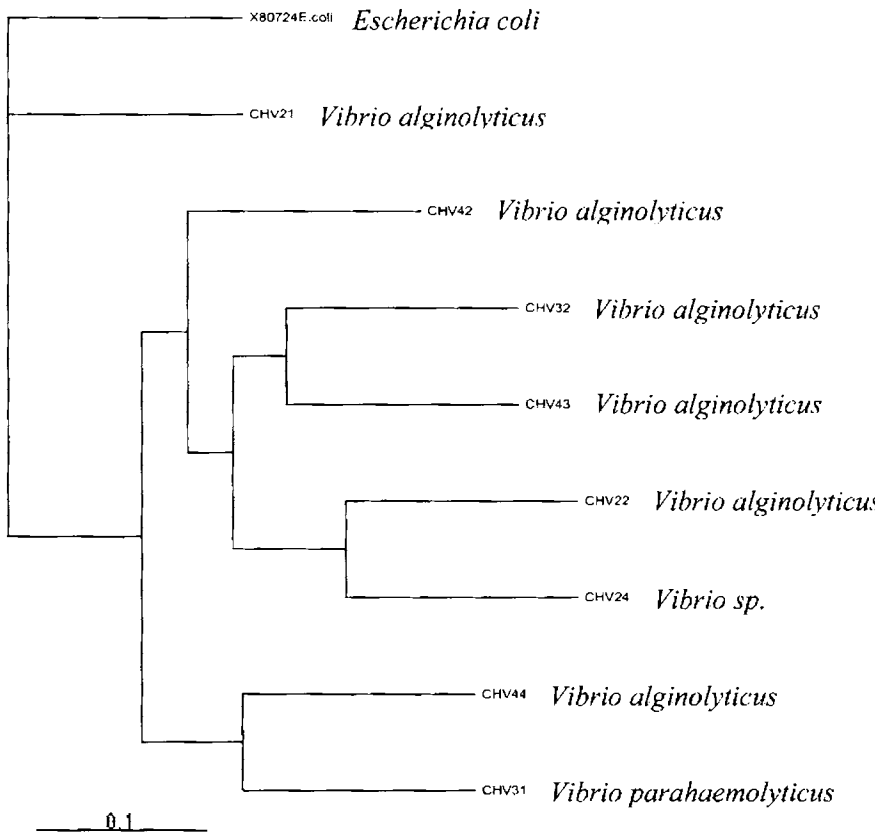


Fig. 9 Phylogram of *Vibrios* isolated from Chettuva

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence. Node values more than 75 only is shown.

Out of a total of eight strains isolated from this station, six were identified as *Vibrio alginolyticus*. Strain CHV24, identified only as *Vibrio* sp. cladded with the stain CHV22 (*V.alginolyticus*).

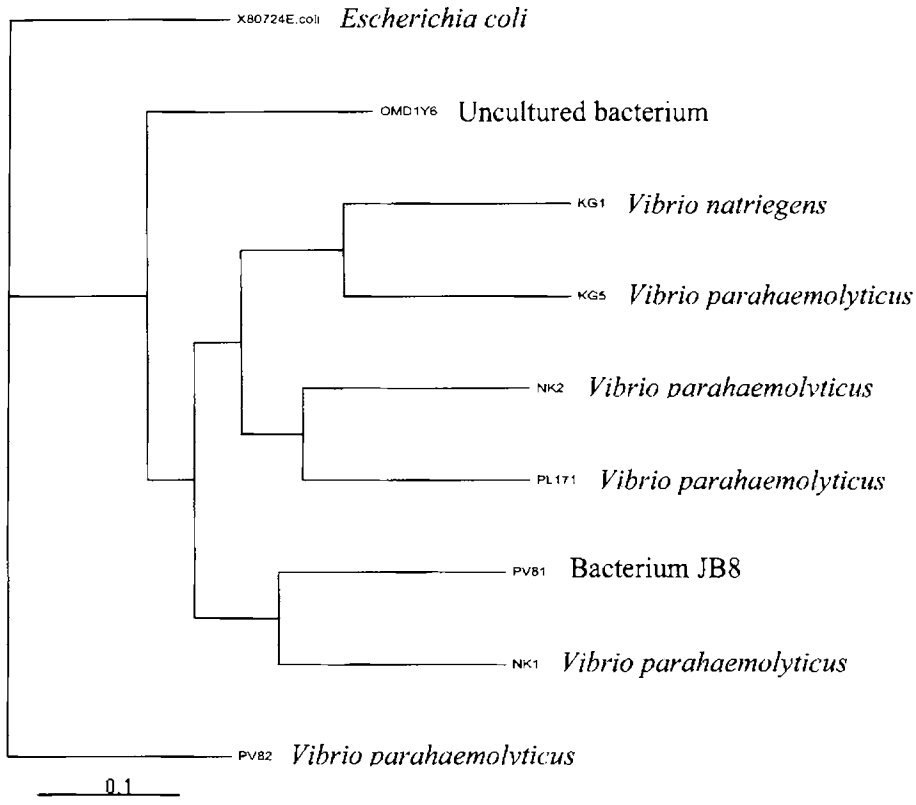
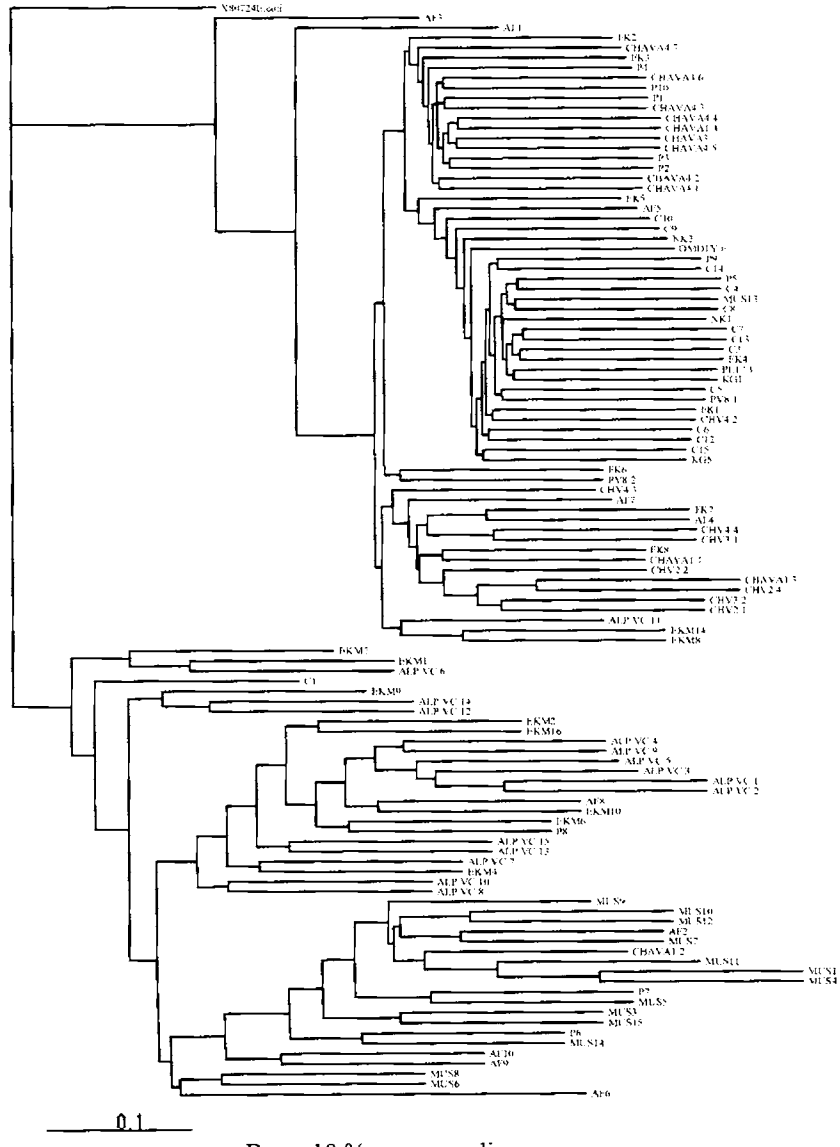


Fig. 10. Phylogram of *Vibrios* isolated from Kasaragod, Neendakara, Omanappuzha and Plankton

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence. Node values more than 75 only is shown.

Of the 8 strains isolated from these four stations, strain PV81 showed identity to a Bacterium JB8 in the database, while OMD1Y6 was shown as an 'uncultured bacterium'. The others were *V. parahaemolyticus,* except KG1 which was *V.natriegens.*



Bar = 10 % sequence divergence.

Fig. 11 Phylogram of *Vibrios* isolated from all stations

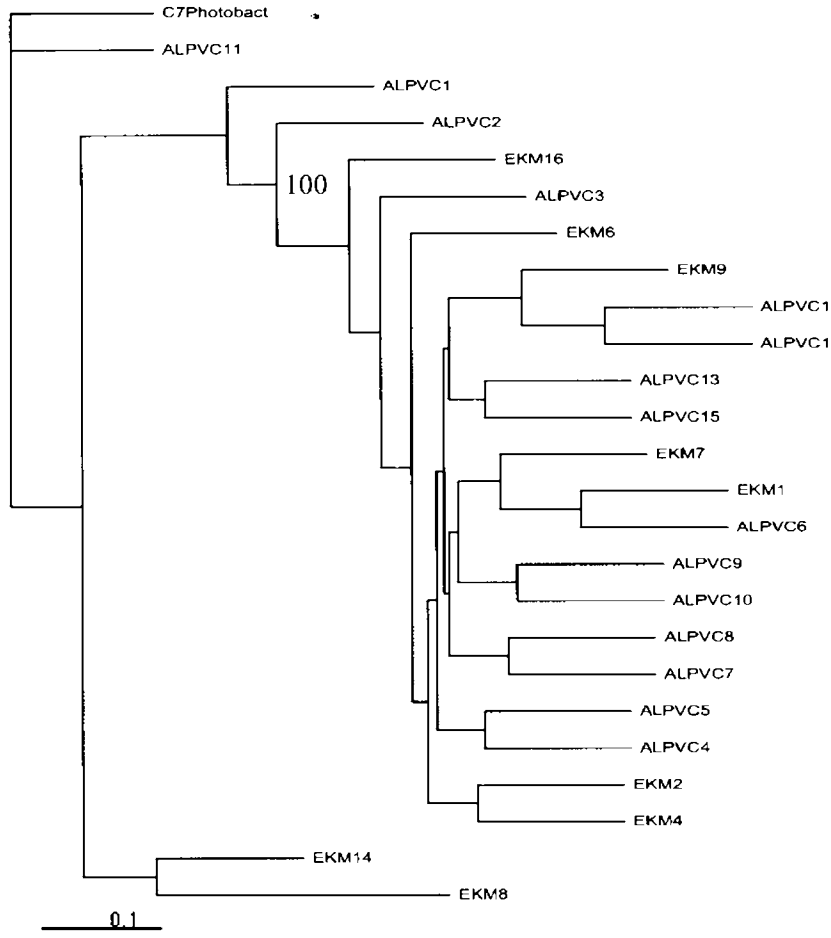


Fig. 12. Intra species variation among *Vibrio cholerae* isolates

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence.

Intra species variation for the 24 *Vibrio cholerae* strains is depicted. Strain ALP(V.C)11 claded separately, while strains EKM14 and EKM8 group together. Rest of the 22 strains grouped together to form a major clade.

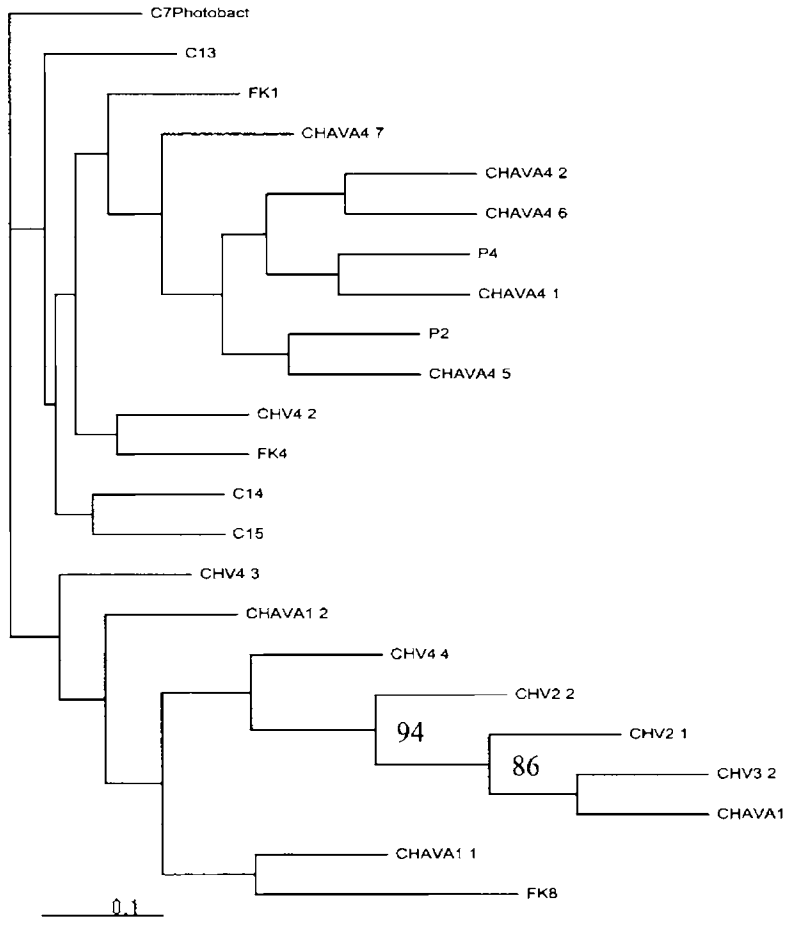


Fig. 13. Intra species variation among *Vibrio alginolyticus* isolates

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence.

Within the *Vibrio alginolyticus* obtained in this study, the strains are quite diverse and mainly group into two, viz, the first group comprising 13 strains and the second one with 9 strains. Only two nodes have supporting value of more than 75.

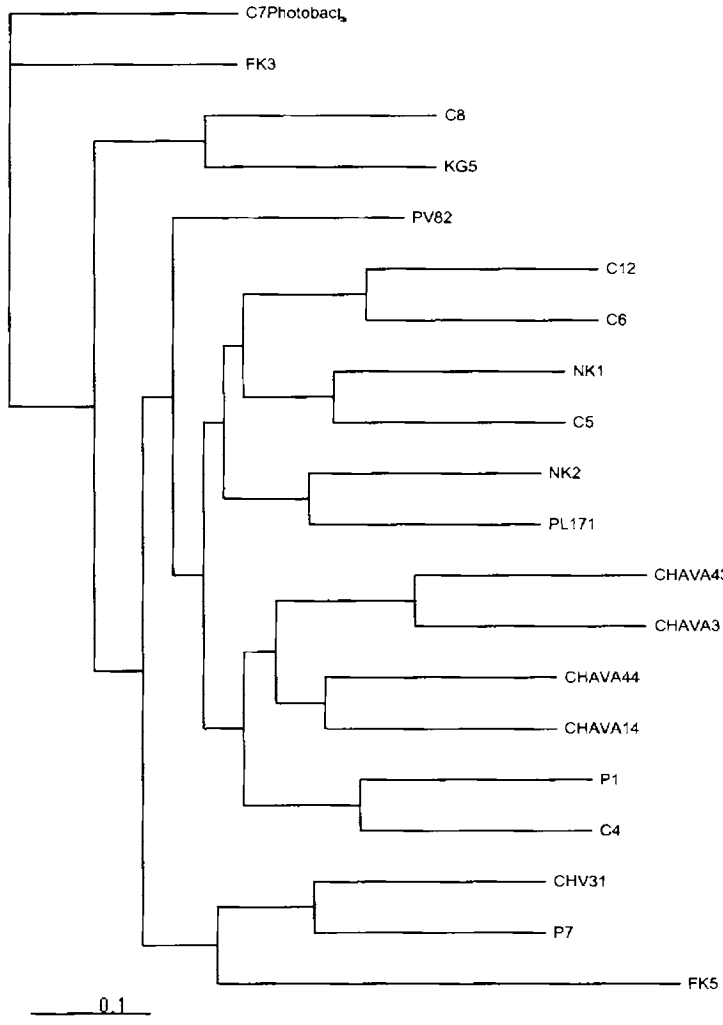


Fig. 14. Intra species variation among *Vibrio parahaemolyticus* isolates

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence.

Strain FK3 is the only stand alone member in this group, while all the other 18 strains of *V. parahaemolyticus* group together. This phylogram shows the diversity of the strains among themselves.

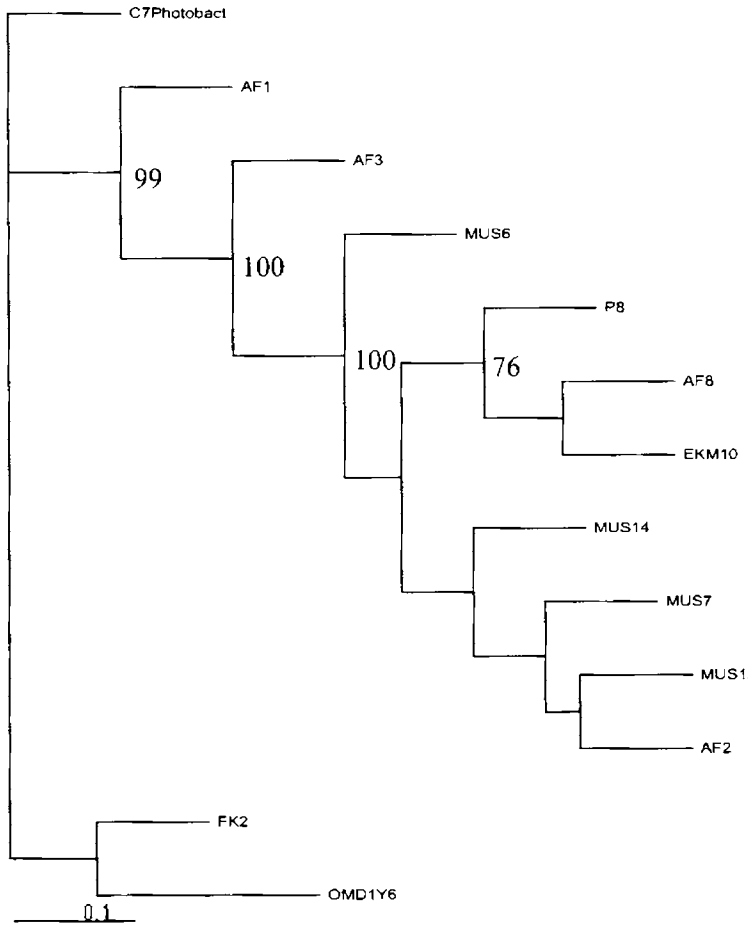


Fig. 15. Intra species variation among Uncultured bacterium isolates
The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence.

An attempt was made to understand the relatedness of the 12 strains of 'uncultured bacterium' obtained during this study. The designation 'uncultured' is solely based on the database identification. The phylogenetic analysis showed they were quite diverse in themselves. These 12 strains claded into two groups, one comprising FK4 and OMD1Y1, and the remaining 10 strains forming a separate group.

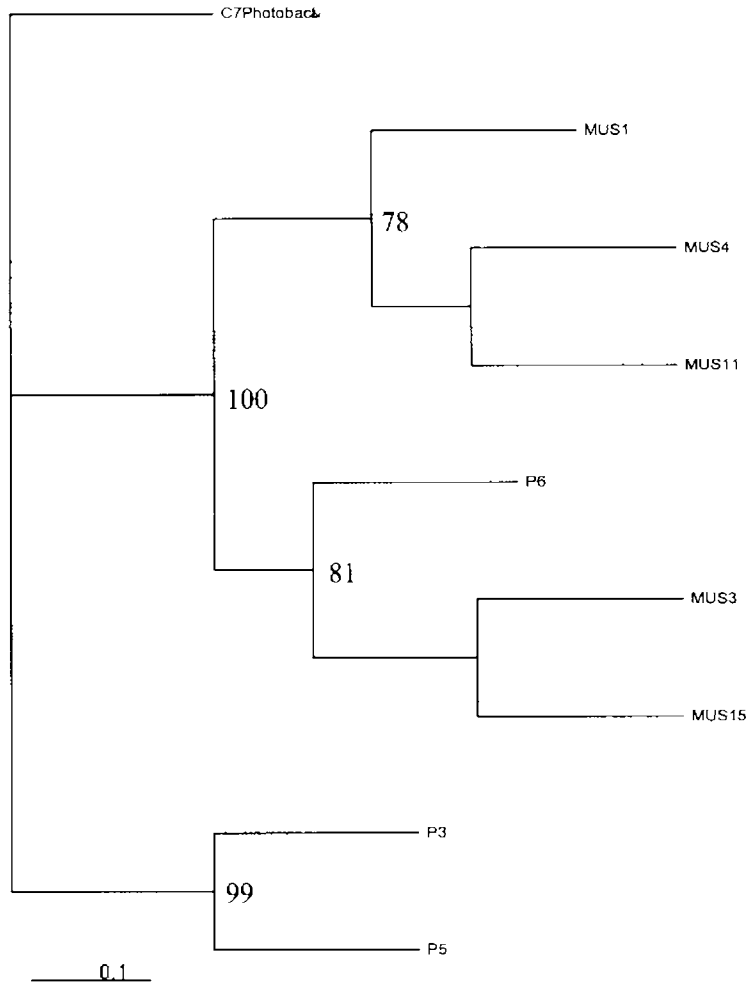


Fig.16. Intra species variation among *Vibrio harvevi* isolates

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence.

Vibrio harveyi isolates were mostly isolated from mussel (5 strains) and from Ponnani (3 strains). It was interesting to note that the strains from each station claded together to form two groups. One exception to this was P6, which claded with the isolates from mussel.

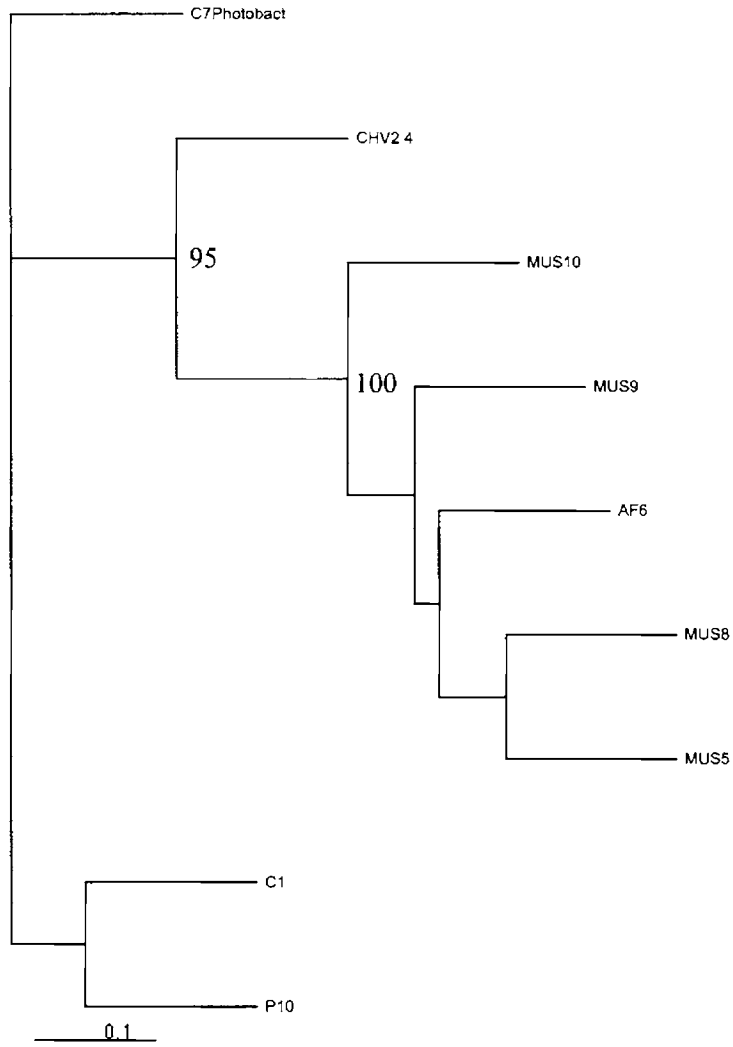


Fig. 17. Intra species variation among unspecified *Vibrio.* isolates

The numbers at the nodes indicate the levels of bootstrap support based on 1,000 replicates. Bar = 10 % sequence divergence.

Even though such an analysis doesn't carry weightage, it was interesting to see how these 8 strains identified only as *Vibrio* sp, were related to each other. Except for strains C1 and P10 which grouped together, all the other 6 strains claded together.

4.4. Antibiotic susceptibility test

Antibiotic resistance pattern can be used as a strain marker. It can also be of use in assessing the extent of drug resistance existing in nature. Multiple antibiotic resistances among microbes are to be viewed with extreme fear and caution, especially since knowledge about the different means and methods of horizontal gene transfer are emerging. Data generated in this field will prove to be of great value and use both to doctors and environmentalists.

The results of antibiotic susceptibility tests for the 108 isolates from different stations are presented in **Tables 3-12.**

Table 3: Antibiogram of *Vibrios* isolated from water samples of Ernakulam

Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
EKM 1	S	S	S	S	S	S	S	S	S
EKM 2	S	S	S	S	S	S	S	S	S
EKM 4	S	S	S	S	S	S	S	S	S
EKM 6	S	S	S	S	S	S	S	S	S
EKM 7	S	S	S	S	S	S	S	S	S
EKM 8	S	S	S	S	S	S	S	S	S
EKM 9	S	S	S	S	S	S	S	S	S
EKM10	**R**	S	**R**	S	S	S	S	S	S
EKM14	S	S	S	S	S	S	S	S	S
EKM16	S	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr-furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

As shown in **Table-3**, all the strains isolated from station Ernakulam were sensitive to all the antibiotics tested, except for the strain EKM10 which was resistant to ampicillin and trimethoprim.

Table 4: Antibiogram of *Vibrios* isolated from water samples of Alappuzha

Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
ALP(V.C) 1	S	S	S	S	S	S	S	S	S
ALP(V.C) 2	S	S	S	S	S	S	S	S	S
ALP(V.C) 3	S	S	S	S	S	S	S	S	S
ALP(V.C) 4	S	S	S	S	S	S	S	S	S
ALP(V.C) 5	S	S	**R**	**R**	S	S	S	S	S
ALP(V.C) 6	S	S	S	S	S	S	S	S	S
ALP(V.C) 7	S	S	S	S	S	S	S	S	S
ALP(V.C) 8	S	S	S	S	S	S	S	S	S
ALP(V.C) 9	S	S	S	S	S	S	S	S	S
ALP(V.C) 10	S	S	S	S	S	S	**R**	S	S
ALP(V.C) 11	S	S	S	S	S	S	S	S	S
ALP(V.C) 12	S	S	**R**	**R**	S	S	S	S	S
ALP(V.C) 13	S	S	S	S	S	S	S	S	S
ALP(V.C) 14	S	S	S	S	S	S	S	S	S
ALP(V.C) 15	S	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr-furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

The antibiogram of the 15 *Vibrio* strains isolated from station Alappuzha are as shown in **Table-4.** The two strains of *Vibrios*, ALP(V.C)5 and ALP(V.C)12 showed resistance to the antibiotics, trimethoprim and co-trimoxazole, while strain ALP(V.C)10 was resistant to furazolidone. All the other strains were susceptible to all the nine antibiotics used.

Table 5: Antibiogram of *Vibrios* isolated from water samples of aqua farm

Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
AF1	S	S	S	S	S	S	S	S	S
AF2	S	S	S	S	S	S	S	S	S
AF3	S	S	S	S	S	S	S	S	S
AF4	S	S	S	S	S	S	S	S	S
AF5	S	S	S	S	S	S	S	S	S
AF6	S	S	S	S	S	S	S	S	S
AF7	S	S	S	S	S	S	S	S	S
AF8	S	S	S	S	S	S	S	S	S
AF9	S	S	S	S	S	S	S	S	S
AF10	S	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr- furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

The results of the antibiotic sensitivity testing for the 10 strains of *Vibrios* isolated from water samples of station aqua farm are shown in **Table-5**. All the 10 *Vibrio* strains were found to be sensitive to all the nine antibiotics used in this study.

Table 6: Antibiogram of *Vibrios* isolated from an aqua farm where fish kill occurred

Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
FK1	**R**	S	S	S	S	S	S	S	S
FK2	**R**	S	S	S	S	S	S	S	S
FK3	**R**	S	S	S	S	S	S	S	S
FK4	**R**	S	**R**	S	S	S	**R**	S	S
FK5	**R**	S	**R**	S	S	S	**R**	S	**R**
FK6	**R**	S	S	S	S	S	S	S	S
FK7	**R**	S	S	S	S	S	S	S	S
FK8	**R**	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr- furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

The antibiotic sensitivity pattern of the *Vibrios* isolated from an aqua farm where fish kill occurred is shown in **Table-6**. All the 8 strains from this station showed resistance to ampicillin, the most commonly used beta lactam. Strain FK4 also showed resistance to two other antibiotics- trimethoprim and furazolidone. Strain FK5 was resistant to ampicillin, trimethoprim, furazolidone and ciprofloxacin.

Table 7: Antibiogram of *Vibrios* isolated from Green Mussel

Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
MUS1	**R**	S	S	S	S	S	S	S	S
MUS3	**R**	S	S	S	S	S	S	S	S
MUS4	**R**	S	S	S	S	S	S	S	S
MUS5	**R**	S	S	S	S	S	S	S	S
MUS6	**R**	S	S	S	S	S	S	S	S
MUS7	**R**	S	S	S	S	S	S	S	S
MUS8	**R**	S	S	S	S	S	S	S	S
MUS9	**R**	S	**R**	S	S	S	**R**	S	S
MUS10	**R**	S	S	S	S	S	S	S	S
MUS11	**R**	S	S	S	S	S	S	S	S
MUS12	**R**	S	S	S	S	S	S	S	S
MUS13	**R**	S	S	S	S	S	**R**	S	S
MUS14	**R**	S	S	S	S	S	S	S	S
MUS15	**R**	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr-furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

The antibiotic sensitivity pattern of the *Vibrios* isolated from green mussel samples obtained from Vadakara is as shown in **Table-7**. All the isolates tested showed resistance to ampicillin. Out of the 15 strains screened, only two strains (MUS9 and MUS13) were resistant to more than one antibiotic. MUS9 was resistant to ampicillin, trimethoprim and furazolidone and MUS13 to ampicillin and furazolidone.

Table 8: Antibiogram of *Vibrios* isolated from Connolly Canal

Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
C1	S	**R**	**R**	S	S	S	**R**	S	S
C3	**R**	S	S	S	S	S	S	S	S
C4	S	S	S	S	S	S	S	S	S
C5	**R**	S	S	S	S	S	S	S	S
C6	**R**	S	S	S	S	S	S	S	S
C7	S	S	S	S	S	S	S	S	S
C8	**R**	S	S	S	S	S	S	S	S
C9	S	S	S	S	S	S	S	S	S
C10	S	S	S	S	S	S	S	S	S
C12	**R**	S	S	S	S	S	S	S	S
C13	**R**	S	S	S	S	S	S	S	S
C14	**R**	S	S	S	S	S	S	S	S
C15	S	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr-furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

The antibiogram of *Vibrios* isolated from Connolly Canal is shown in **Table-8.** Most of the strains were resistant to ampicillin. Strain C1 displayed resistance to tetracycline, trimethoprim and furazolidone. The 7 strains that displayed beta lactam resistance were C3, C5, C6, C8, C12, C13, and C14.

Table 9- Antibiogram of *Vibrios* isolated from Ponnani

Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
P1	**R**	S	S	S	S	S	S	S	S
P2	S	S	S	S	S	S	S	S	S
P3	**R**	S	S	S	S	S	S	S	S
P4	**R**	**R**	S	S	S	S	S	S	S
P5	**R**	S	S	S	S	S	S	S	S
P6	**R**	**R**	**R**	S	S	S	**R**	S	S
P7	**R**	S	S	S	S	S	S	S	S
P8	**R**	S	S	S	**R**	S	S	S	**R**
P9	**R**	S	S	S	S	S	S	S	S
P10	**R**	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr- furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

The antibiotic sensitivity pattern of the 10 *Vibrio* strains isolated from station Ponnani, is as shown in **Table-9**. Strains P1, P3, P5, P7, P9 and P10 were resistant only to ampicillin. Strain P4 was resistant to ampicillin and tetracycline, while strain P6 is the one isolate from this station showing maximum antibiotic resistance. The strain P6 was resistant to 4 antibiotics -ampicillin, tetracycline, trimethoprim and furazolidone. Strain P8 was resistant to ampicillin, nalidixic acid and ciprofloxacin.

Table 10: Antibiogram of *Vibrios* isolated from Chavakkadu

Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
CHAVA1(1)	**R**	S	S	S	S	S	S	S	S
CHAVA1(2)	**R**	S	S	S	S	S	S	S	S
CHAVA1(3)	**R**	S	S	S	S	S	S	S	S
CHAVA1(4)	**R**	S	S	S	S	S	S	S	S
CHAVA3	**R**	S	S	S	S	S	S	S	S
CHAVA4 (1)	**R**	S	S	S	S	S	**R**	S	**R**
CHAVA4 (2)	**R**	S	S	S	S	S	S	S	S
CHAVA4 (3)	**R**	S	S	S	S	S	S	S	S
CHAVA4 (4)	**R**	S	S	S	S	S	S	S	S
CHAVA4 (5)	**R**	S	S	S	S	S	**R**	S	S
CHAVA4 (6)	**R**	S	S	S	S	S	S	S	S
CHAVA4 (7)	**R**	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr- furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

The antibiogram of *Vibrios* isolated from Chavakkadu is shown in **Table-10**. All isolates tested were resistant to ampicillin; but strain CHAVA4(5) was also resistant to furazolidone and strain CHAVA4(1) was resistant to furazolidone and ciprofloxacin.

Table 11: Antibiogram of *Vibrios* isolated from Chettuva

Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
CHV2 (1)	**R**	S	S	S	S	S	S	S	S
CHV2 (2)	**R**	S	S	S	S	S	S	S	S
CHV2 (4)	**R**	S	S	S	S	S	S	S	S
CHV3 (1)	**R**	S	S	S	S	S	**R**	S	S
CHV3 (2)	**R**	S	S	S	S	S	S	S	S
CHV4 (2)	**R**	S	S	S	S	S	S	S	S
CHV4 (3)	**R**	S	S	S	S	S	S	S	S
CHV4 (4)	**R**	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr- furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

The antibiogram of the 8 strains isolated from station Chettuva are as shown in **Table-11**. All strains isolated from Chettuva were resistant to ampicillin, and but the strain CHV3(1) was also resistant to furazolidone.

Table 12: Antibiogram of *Vibrios* isolated from Kasaragod, Neendakara, Omanappuzha and Plankton

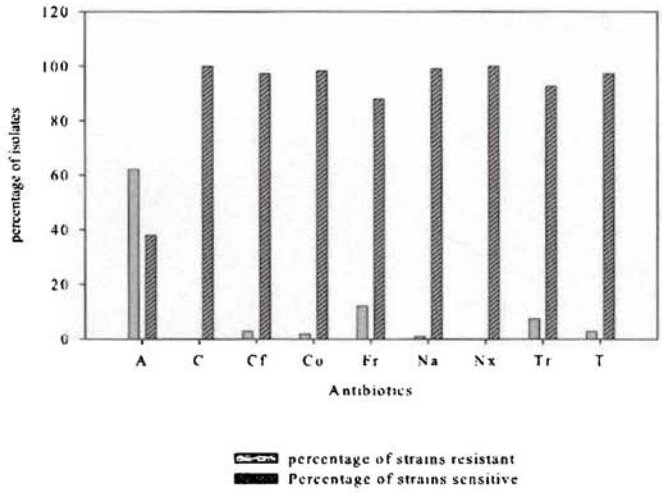
Culture identity	Antibiotics used								
	A	**T**	**Tr**	**Co**	**Na**	**Nx**	**Fr**	**C**	**Cf**
KG1	**R**	S	S	S	S	S	**R**	S	S
KG5	**R**	S	S	S	S	S	**R**	S	S
NK1	**R**	S	S	S	S	S	S	S	S
NK2	**R**	S	S	S	S	S	**R**	S	S
OMD1Y6	**R**	S	S	S	S	S	**R**	S	S
PV8(1)	**R**	S	S	S	S	S	S	S	S
PV8(2)	**R**	S	S	S	S	S	S	S	S
PL17(1)	**R**	S	S	S	S	S	S	S	S

A-ampicillin, T-tetracycline, Tr-trimethoprim, Co-cotrimoxazole, Na-nalidixic acid, Nx-norfloxacin, Fr- furazolidone, C-chloramphenicol, Cf-ciprofloxacin.

The results of the antibiotic sensitivity testing of the isolates from Kasaragod, Neendakara, Omanappuzha and Plankton are as shown in **Table-12.** All the strains in this group of isolates show a common pattern. They were all resistant to ampicillin; the commonly used beta lactam antibiotic. Two strains from Kasaragode (KG1 and KG5) and one strain each from Neendakara (NK2) and Omanappuzha (OMD1Y6) were resistant to furazolidone.

The percentage resistance/sensitivity of all the isolated *Vibrio* strains against the nine commonly administered antibiotics are as depicted in **Figure 18** (N=108).

Figure 18. Percentage resistance/sensitivity of *Vibrio* strains against the nine antibiotics



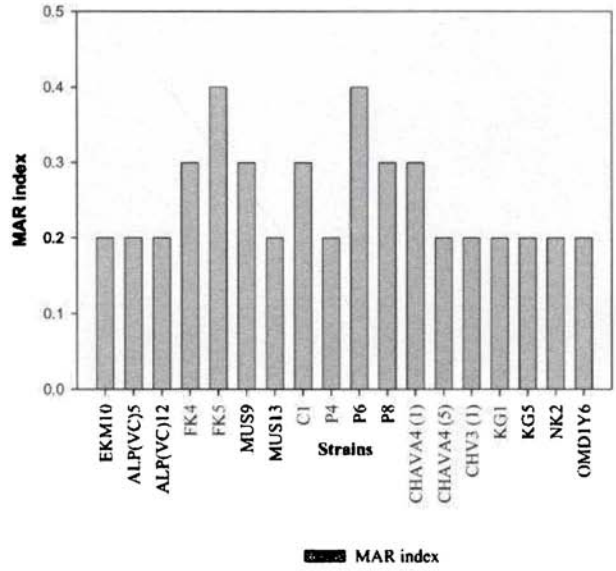
It is obvious from the graph that >60% of the strains tested were resistant to ampicillin, the most commonly used beta lactam antibiotic, while all the stains tested were sensitive to chloramphenicol and norfloxacin. A small percentage of strains showed resistance to nalidixic acid.

4.5. Multiple antibiotic resistance (MAR) indexing

Of the 108 isolates tested, 18 showed multiple antibiotic resistances. MAR index of the eighteen isolates that showed multiple antibiotic resistance are presented in **Figure 19**. A MAR index of more than 0.2 can be considered as contamination

from high risk sources and due to anthropogenic interference. MAR index of representative isolates indicate the multiple antibiotic resistance potential of the micro organisms in that locality.

Figure 19. MAR index of the *Vibrio* isolates



A highest MAR index of 0.4 was recorded for strains FK5 and P6. A MAR index of 0.3 was recorded for 5 strains i.e. strains FK4, MUS9, C1, P5, and CHAVA4(1). All the remaining eleven strains were resistant to at least two antibiotics, therefore showing a MAR index of 0.2.

4.6. Screening for virulence genes by Polymerase Chain Reaction (PCR)

PCR screening for eight genes directly or indirectly involved in the virulence processes and three genes which can be of marker value were carried out for all *Vibrio* strains isolated. This was the main test employed to detect obvious HGT events

among the *Vibrios* isolated from different stations. The result of this experiment, whether positive or negative, is extremely valuable information in terms of the virulence genes in the *Vibrionaceae* members isolated from different station.

4.6.1. Template preparation for PCR

Results of three different methods of template preparation for PCR are depicted in the **Figure 20**.

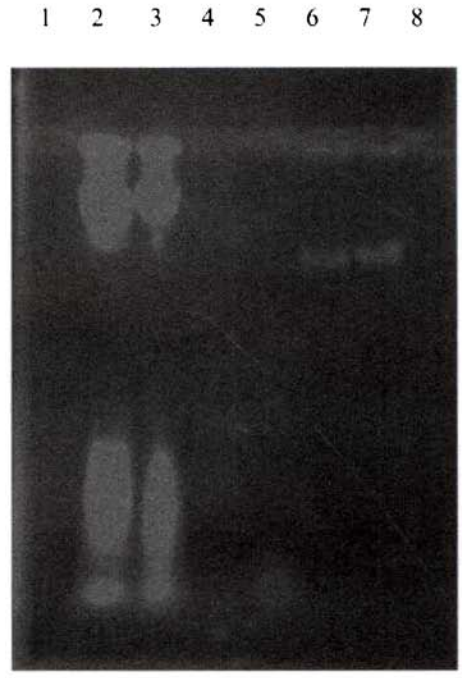


Fig. 20. Comparison of DNA isolation using various protocols. Lanes 2 and 3 - Genomic DNA isolated according to Sambrook *et al.* 2000; Lanes 4 and 5 - Genomic DNA isolated by Boiling Method; Lanes 6 and 7- Genomic DNA isolated according to Murray and Thompson, 1980.

It can be seen from Fig-20, that DNA isolated by using the method of Sambrook *et al,* 2000, and by the method of Murray and Thompson, 1980 yielded

good quality DNA that could be used for further study. DNA isolated by the boiling method got sheared and lost in the course of storage.

4.6.2. Screening for Integrons

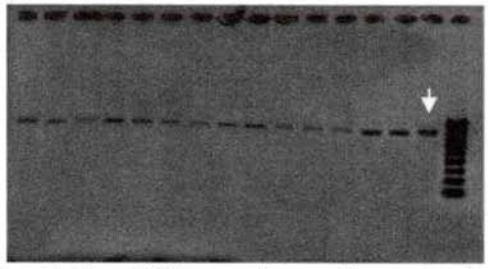
Two sets of primers were used for screening integrons in the *Vibrio* isolates. *qacEΔ1*, *Sul1* and *inF* and *inR*. The main aim was just to check for their presence in the *Vibrios* isolates by PCR.

4.6.2.1. PCR with *qacEΔ1*F and *sul1*R (Dalsgaard *et al.*, 2000)

Results of PCR screening for integron genes, *qacEΔ1* and *Sul*1 are presented in **Figures 21 & 22** and **Tables 13 & 14.**

Fig. 21 & Table 13. Screening for integron genes in *Vibrios* by using primers *qacEΔ1* and *Sul*1 by PCR

Figure 21



Lane1-14, PCR products from strains ALP(VC)1, 2, 3, 4 5,11; EKM14; ALP(VC)6,7,8,9,10,12.13. Lane 15 has positive control (*Vibrio cholerae* CO366 Eltor strain) and lane 16 has 100bp DNA ladder
↓- indicates the position of positive control amplicon at ~800bp.

*Strain (MUS13) is not shown in gel picture.

Table 13

Isolate code	Identity based on 16S rRNA Gene	*qacEΔ1 and sul1*
ALP(VC)1	*Vibrio cholere*	+
ALP(VC)2	*Vibrio cholere*	+
ALP(VC)3	Uncultured bacterium	+
ALP(VC)4	*Vibrio cholere*	+
ALP(VC)5	*Vibrio cholere*	+
ALP(VC)11	*Vibrio cholere*	+
EKM14	*Vibrio cholere*	+
ALP(VC)6	*Vibrio cholere*	+
ALP(VC)7	*Vibrio cholere*	+
ALP(VC)8	*Vibrio cholere*	+
ALP(VC)9	*Vibrio cholere*	+
ALP(VC)10	*Vibrio cholere*	+
ALP(VC)12	*Vibrio cholere*	+
ALP(VC)13	*Vibrio cholere*	+
MUS13*	*Vibrio furnissii*	+

Fig. 22 & Table 14. Screening for integron genes in *Vibrios* using primers *qacEΔ1* and *Sul*1 by PCR

Figure 22

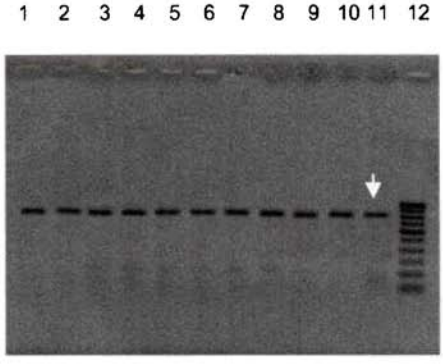


Table 14

Isolate code	Identity based on 16S rRNA Gene	*qacEΔ1 and sul1*
ALP(VC)14	*Vibrio cholere*	+
ALP(VC)15	*Vibrio cholere*	+
EKM1	*Vibrio cholere*	+
EKM2	*Vibrio cholere*	+
EKM4	*Vibrio cholere*	+
EKM6	*Vibrio cholere*	+
EKM7	*Vibrio cholere*	+
EKM8	*Vibrio cholere*	+
EKM9	*Vibrio cholere*	+
EKM16	*Vibrio cholere*	+

Lane1-10, PCR products from strains ALP(VC)14, 15; EKM1,2,4,6,7,8,9,16; Lane 11 has positive control (*Vibrio cholerae* CO366 Eltor strain) & lane 12 has 100bpDNA ladder
↓- indicate the positive control amplicon at ~800bp.

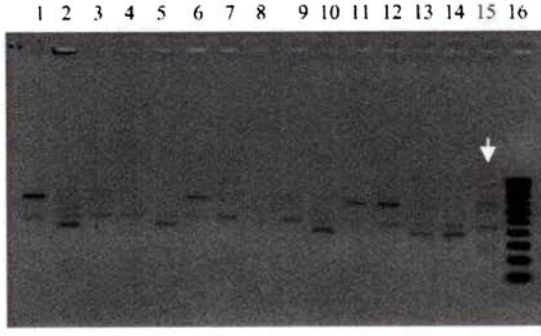
From **Figures 21 & 22** and **Tables 13 & 14,** it was observed that only 25 strains of *Vibrios* out of the 108 isolated from the different stations gave amplification and an amplicon size of ~800 bp was obtained. Out of the 25 strains that showed the presence of the integron genes, *qacEΔ1* and *Sul*1, the 23 strains identified as *V.cholerae,* and the strain ALP(VC)3 identified as 'Uncultured bacterium', were all isolated from two stations –Alappuzha and Ernakulam, whereas MUS13 which was identified as *V. furnissii* was from green mussel.

4.6.2.2. PCR with *in*F and *in*R (Dalsgaard *et al.*, 2000)

Another set of integron genes '*in*' was checked among all the 108 isolates and the results are as presented in **Figures 23** & **24** and **Tables-15** & **16.**

Fig.23 & Table 15. Screening for integron genes in *Vibrios* using *in*F and *in*R by PCR

Figure 23



Lane1-14,PCR products from strains ALP(VC)1, 2, 3, 4,5,11: EKM14: ALP(VC)6, 7, 8, 9,10, 12, 13. Lane 15 has positive control (*Vibrio cholerae* CO366 Eltor strain) and lane 16 has 500bp DNA ladder

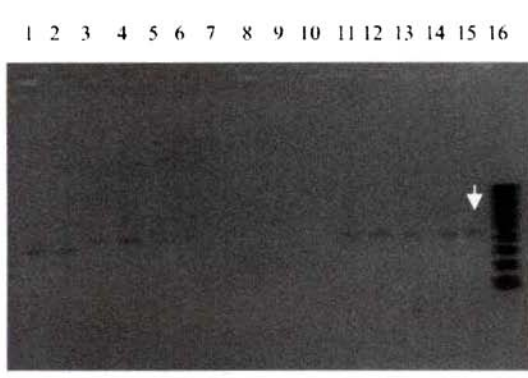
↓- indicates variable length positive control amplicons

Table 15

Isolate code	Identity based on 16S rRNA Gene	*inCS*
ALP(VC)1	*Vibrio cholere*	+
ALP(VC)2	*Vibrio cholere*	+
ALP(VC)3	Uncultured bacterium	+
ALP(VC)4	*Vibrio cholere*	+
ALP(VC)5	*Vibrio cholere*	+
ALP(VC)11	*Vibrio cholere*	+
EKM14	*Vibrio cholere*	+
ALP(VC)6	*Vibrio cholere*	+
ALP(VC)7	*Vibrio cholere*	+
ALP(VC)8	*Vibrio cholere*	+
ALP(VC)9	*Vibrio cholere*	+
ALP(VC)10	*Vibrio cholere*	+
ALP(VC)12	*Vibrio cholere*	+
ALP(VC)13	*Vibrio cholere*	+

Fig. 24 & Table 16. Screening for integron genes in *Vibrios* using *in*F and *in*R by PCR

Figure 24



Lane1-6-PCR products from strains ALP (VC)14, 15; EKM1,2,4,6. Lane11-14 EKM7, 8, 9, 16. Lanes 7, 8, 9 and 10 are empty. Lane 15 has positive control (*Vibrio cholerae* CO366 Eltor strain) & lane 16 has 500bpDNA ladder
↓ - indicate positive control amplicon.

Table 16

Isolate code	Identity based on 16S rRNA Gene	*inCS*
ALP(VC)14	*Vibrio cholere*	+
ALP(VC)15	*Vibrio cholere*	+
EKM1	*Vibrio cholere*	+
EKM2	*Vibrio cholere*	+
EKM4	*Vibrio cholere*	+
EKM6	*Vibrio cholere*	+
EKM7	*Vibrio cholere*	+
EKM8	*Vibrio cholere*	+
EKM9	*Vibrio cholere*	+
EKM16	*Vibrio cholere*	+

From the results of the PCR screening for the integron gene '*in*' shown in figures 23 & 24 and table-15 & 16, it is seen that only 24 isolates tested positive for the '*in*' gene. Variable length amplicons were observed. It was seen that except for strain ALP (VC) 3, which was identified to an 'uncultured bacterium' by using 16S rRNA sequencing, all the others were *V. cholerae*. It is also interesting to note that all these 24 strains were from station Alappuzha and Ernakulam.

4.6.3. Screening for Virulence/virulence related genes

Results of the screening for three marker genes and six virulence genes are presented here. *Ompw, 01rfb, 0133 rfb* are the marker genes screened for, and *tcpA, toxR, ace, zot, ctxA* and *sxt* are the virulence genes looked for in all the 108 isolates.

4.6.3.1. PCR with *ompw*F and *ompw*R (Bisweswar *et al.*, 2000)

Outer membrane protein w gene- *ompw* can be used as a reliable marker for the presence of *Vibrio cholerae*. This knowledge was made use of and its presence was screened for in the *Vibrio* isolates under study. The results of this screening are as depicted in **Figures 25 & 26** and **Tables 17 & 18.**

Fig. 25 & Table 17. Screening for the *ompw* gene in *Vibrios* by PCR

Figure 25

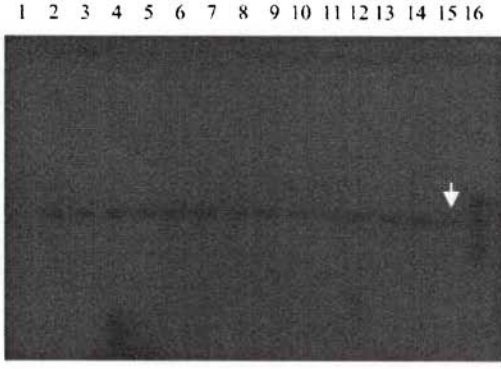


Table 17

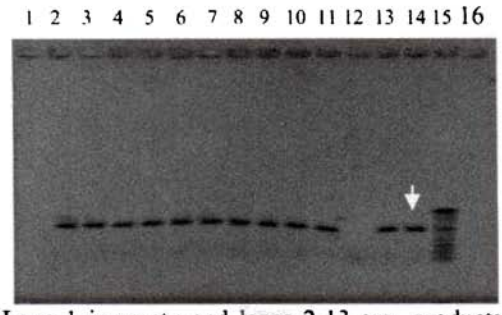
Isolate code	Identity based on 16S rRNA Gene	*ompw*
ALP(VC)1	*Vibrio cholere*	+
ALP(VC)2	*Vibrio cholere*	+
ALP(VC)3	Uncultured bacterium	+
ALP(VC)4	*Vibrio cholere*	+
ALP(VC)5	*Vibrio cholere*	+
ALP(VC)11	*Vibrio cholere*	+
EKM14	*Vibrio cholere*	+
ALP(VC)6	*Vibrio cholere*	+
ALP(VC)7	*Vibrio cholere*	+
ALP(VC)8	*Vibrio cholere*	+
ALP(VC)9	*Vibrio cholere*	+
ALP(VC)10	*Vibrio cholere*	+
ALP(VC)12	*Vibrio cholere*	+

Lane 1 is empty and lane 15 has positive control (*Vibrio cholerae* CO366 Eltor strain). Lanes 2-14 are products from strains ALP (VC) 1, 2, 3, 4, 5, 11; EKM14; ALP (VC) 6, 7, 8, 9, 10, and 12.

↓- indicate positive control amplicon at ~588bp. Lane 16 has 100bp DNA ladder.

Fig. 26 &Table 18 .Screening for the *ompw* gene in *Vibrios* by PCR

Figure 26



Lane 1 is empty and lanes 2-13 are products from strains ALP(VC)13, 14, 15; EKM1, 2, 4, 6, 7, 8, 9, 10, 16. Lane 14 has positive control (*Vibrio cholerae* CO366 Eltor strain,)
↓ indicate positive control amplicon at ~588bp. Lane 16 has 10bp ladder.

Table 18

Isolate code	**Identity based on 16S rRNA Gene**	***omp w***
ALP(VC)13	*Vibrio cholere*	+
ALP(VC)14	*Vibrio cholere*	+
ALP(VC)15	*Vibrio cholere*	+
EKM1	*Vibrio cholere*	+
EKM2	*Vibrio cholere*	+
EKM4	*Vibrio cholere*	+
EKM6	*Vibrio cholere*	+
EKM7	*Vibrio cholere*	+
EKM8	*Vibrio cholere*	+
EKM9	*Vibrio cholere*	+
EKM10	Uncultured bacterium	-
EKM16	*Vibrio cholere*	+

From the results of the screening for the *ompw* gene shown in Figures 25 & 26and Table-17 & 18, it was observed that only 25 strains showed the characteristic amplicon size of ~588bp. This included all the strains identified previously by 16S rRNA analysis as *V.cholerae* and two strains ALP (VC) 3 & EKM10, identified as 'Uncultured bacterium'.

4.6.3.2. PCR with *01rfb*F and *01rfb*R (Hoshino *et al.*, 1998); and ***0139rfb*F and *0139rfb*R** (Hoshino *et al.*, 1998)

01rfb and *0139rfb* are genes which can be used as marker genes for serotyping. Earlier serotyping was done with the help of antiserum raised against one particular antigen and now it is possible to check for the presence of the gene responsible for making that particular antigen. 01 and 0139 are the two serotypes of

Vibrio cholerae causing pandemics, and checking for their presence can tell us about the reliability of the genes as marker candidates, as well as give information about the serotype of the strains.

In the experiments conducted with the *Vibrios* isolates under study, none of the 108 strains, nor the strains identified previously as *V. cholerae* tested positive either for *01rfb* or *0139 rfb*. This indicates that all the *V. cholerae* strains identified as part of this study were non-01 and non-0139 serotypes.

4.6.3.3. PCR with *tcpA* F and *tcpA* R (Keasler and Hall, 1993)

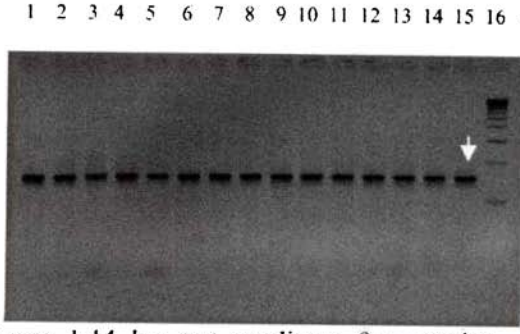
tcpA codes for the A subunit of a cluster of proteins making toxin-co-regulated pili. This is a gene which is directly responsible for virulence in *Vibrio cholerae*. PCR screening for checking its presence among the *Vibrio* isolates were all negative, indicating its absence.

4.6.3.4. PCR with *toxR* F and *toxR* R (Singh *et al.*, 2001)

toxR codes for transmembrane regulatory protein. ToxR is required for expression of virulence factors in the human diarrhoeal pathogen *Vibrio cholerae*, including the cholera toxin (CT) and the toxin co-regulated pilus (TCP). Results of PCR assay for detecting *toxR* are depicted in **Figures 27 & 28 and Tables 19 & 20.**

Fig. 27 & Table 19. Screening for *toxR* gene in *Vibrios* by PCR

Figure 27



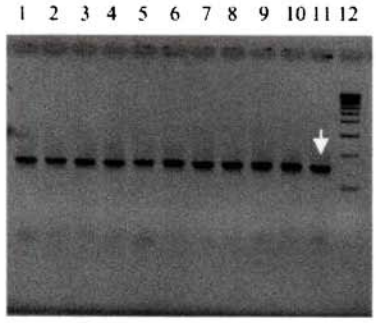
Lanes 1-14 has test amplicons from strains ALP(VC)1, 2, 3, 4, 5, 11; EKM14; ALP(VC) 6, 7, 8, 9, 10, 12, 13 and lane 15 contain positive control (Vibrio cholerae CO366 Eltor strain) ↓ indicate positive control amplification at~779bp. Lane 16 has 500bp DNA ladder.

Table 19

Isolate code	Identity based on 16S rRNA Gene	*toxR*
ALP(VC)1	*Vibrio cholere*	+
ALP(VC)2	*Vibrio cholere*	+
ALP(VC)3	Uncultured bacterium	+
ALP(VC)4	*Vibrio cholere*	+
ALP(VC)5	*Vibrio cholere*	+
ALP(VC)11	*Vibrio cholere*	+
EKM14	*Vibrio cholere*	+
ALP(VC)6	*Vibrio cholere*	+
ALP(VC)7	*Vibrio cholere*	+
ALP(VC)8	*Vibrio cholere*	+
ALP(VC)9	*Vibrio cholere*	+
ALP(VC)10	*Vibrio cholere*	+
ALP(VC)12	*Vibrio cholere*	+
ALP(VC)13	*Vibrio cholere*	+

Fig. 28 & Table 20. Screening for *toxR* gene in *Vibrios* by PCR

Figure 28



Lanes 1-10 has test amplicons from strains ALP (VC)14, 15; EKM1, 2 ,4, 6,7,8,9,10,16 and lane 11 has positive control (*Vibrio cholerae* CO366 Eltor strain). ↓ - indicates the positive amplicon at ~779bp. Lane 12 has 500bp DNA ladder.

Table 20

Isolate code	Identity based on 16S rRNA Gene	*toxR*
ALP(VC)14	*Vibrio cholere*	+
ALP(VC)15	*Vibrio cholere*	+
EKM1	*Vibrio cholere*	+
EKM2	*Vibrio cholere*	+
EKM4	*Vibrio cholere*	+
EKM6	*Vibrio cholere*	+
EKM7	*Vibrio cholere*	+
EKM8	*Vibrio cholere*	+
EKM9	*Vibrio cholere*	+
EKM16	*Vibrio cholere*	+

From the results of PCR assay for detecting *toxR* depicted in **Figures 27 & 28 and Tables 19& 20,** the positive amplicon of ~779bp was observed only in 24 strains. All of them, except strain ALP (VC)3 were identified as *V. cholerae*, all isolated from stations Alappuzha and Ernakulam.

4.6.3.5. PCR with *ace* F and *ace* R (Singh *et al.*, 2001)

ACE stands for accessory cholera enterotoxin. This is a newly found toxin present in *Vibrio cholerae* itself. Presence of *ace* was checked with PCR and it was found that *ace* was absent in all the isolates in the present study.

4.6.3.6. PCR with *zot* F and *zot* R (Singh *et al.*, 2001)

Zonula occludence toxin (ZOT) is a toxin affecting tight junctions of cells. Even though originally reported from *Vibrio cholerae*, as part of this study the

presence of *zot* was checked in the *Vibrio* strains. The outcome of the PCR screening was all negative for *zot* indicating its absence in all the 108 isolates under study.

4.6.3.7. PCR with *ctxA* F and *ctxA* R (Singh *et al.*, 2001)

The key virulence factor in *Vibrio cholerae*, the Cholera toxin gene has two subunits, linked together. The presence of either one subunit may indicate the presence of the other. Here the presence of 'A' subunit gene of cholera toxin was checked by PCR, and it was observed that none of the isolates tested in this study showed the presence of ***ctxA***.

4.6.3.8. PCR with *sxt* F and *sxt* R (Thungapathra *et al.*, 2002)

SXT is around 100kb, integrative conjugative element, which is indicated by the presence of sulfamethoxazole, trimethoprim, streptomycin and chloramphenicol resistance genes clustered together near the 5' end of the element. The results of the screening for the presence of this element in all the *Vibrio* isolates are presented in **Figure 29 and Table 21**.

Figure 29 & Table 21 .Screening for the *sxt* gene in *Vibrios* by PCR

Figure 29

1 2 3 4 5 6

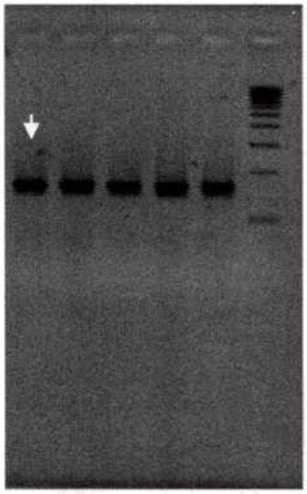


Table 21

Isolate code	Identity based on 16S rRNA Gene	*sxt*
ALP(VC)3	Uncultured bacterium	+
ALP(VC)4	*Vibrio cholere*	+
ALP(VC)6	*Vibrio cholere*	+
ALP(VC)7*	*Vibrio cholere*	+
FK4	*Vibrio alginolyticus*	+

Lanes 2-5 have test amplicons generated (ALP (VC)3, 4, 6, and FK4; and lane -1 has positive control (*Vibrio cholerae* CO366 Eltor strain). ↓– indicates positive amplicon at ~803bp. Lane 6 has 500bp DNA ladder.

*Strain ALP (VC)7 is not shown in gel picture

Only 5 strains, ALP(VC)3, ALP(VC)4, ALP(VC)6 & ALP(VC)7 and FK4 out of the 108 studied, showed the presence of ***sxt***. Three of these, ALP(VC)4, ALP(VC)6 & ALP(VC)7 were identified as *V. cholerae* , strain ALP(VC)3 was 'uncultured bacterium' and strain FK4 was *V. alginolyticus*.

These 4 strains, ALP(VC)3, ALP(VC)4, ALP(VC)6 & ALP(VC)7 had also shown the presence of the integron genes *qacEΔ1 and sul1 & in* and also *ompw and toxR*.

4.7. Conjugation experiments

Mating experiments with the *V.alginolyticus* carrying SXT element and *Escherichia coli* HB101 recipient could prove that the SXT element found in *Vibrio alginolyticus,* was indeed able to move to *Escherichia coli*, and was capable of expressing its antibiotic resistant genes in *E.coli.* The result of the conjugation studies are shown in **Figure 30**.

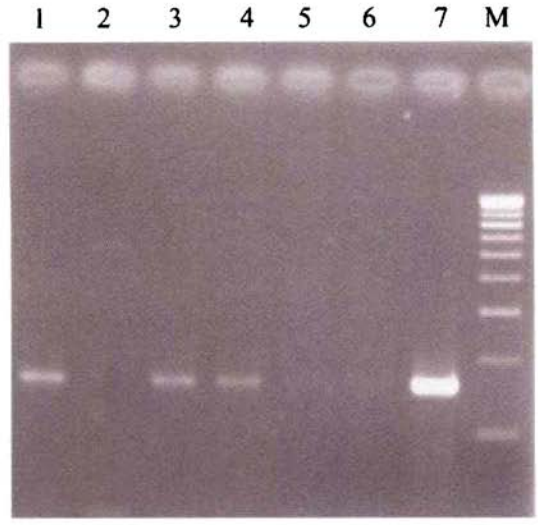


Figure 30. Agarose gel electrophoresis for the PCR products of sxt integrase gene in *Vibrio alginolyticus* strain FK4

Lane 1 has *V. alginolyticus* strain FK4; Lane2 has *E.coli* HB101; Lanes 3&4 has *E.coli* HB101 transconjugants; lanes 5 & 6) has negative control for PCR; Lane 7 positive control *Vibrio cholerae* CO366 Eltor strain. Lane M is 500bp size DNA marker (Bangalore genei).

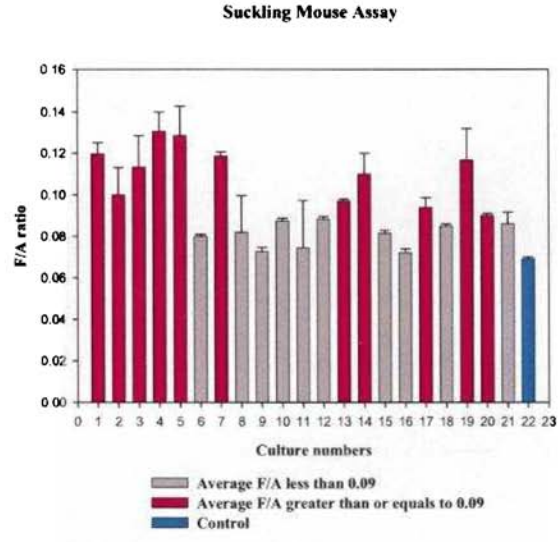
The antibiotic sensitivity test of the *E..coli* HB101 transconjugants, showed resistance to the antibiotics sulfamethoxazole, trimethoprim, streptomycin and chloramphenicol proving that the resistance markers not part of *E..coli* HB10. Plasmid isolation from FK4 had yielded no plasmids. FK4 was also negative for the

integron genes *qacEΔ1 and sul1 & in,* confirming that these 4 resistance markers were indeed part of the integrative conjugative element.

4.8.Suckling mouse assay

The suckling mouse assay could give more information about those *Vibrio* strains which were definitely negative for the virulence genes tested; but may have retained some intrinsic, uncharacterized virulence factors. Twenty one such strains were randomly chosen and subjected to suckling mouse assay and the results of this study are presented in **Figure 31** and **Table 22**.

Figure 31. The study of virulence in selected *Vibrio* isolates using the suckling mouse experiment



(The following are the cultures used for suckling mouse assay:- **1**. CHV2(2), **2**. CHV2(4), **3**. CHV3(1), **4**. CHV4(3), **5**. CHAVA3, **6**. CHAVA4(3), **7**. CHAVA4(4), **8**. P5, **9**. P6, **10**. P9, **11**. C3, **12**. C9, **13**. MUS7, **14**. MUS13, **15**. PV8(1), **16**. FK2, **17**. FK4, **18**. AF4, **19**. AF7, **20**. ALP(VC)11, **21**. EKM14)

Table 22. Characteristics of *Vibrio* strains that showed an F/A ratio ≥ 0.09 in the suckling mouse experiment

Culture numbers	Isolate code	Identity based on 16S rRNA Gene	*qac*	*in*	*sxt*
1	CHV2(2)	*Vibrio alginolyticus*	-	-	-
2	CHV2(4)	*Vibrio* sp.	-	-	-
3	CHV3(1)	*Vibrio parahaemolyticus*	-	-	-
4	CHV4(3)	*Vibrio alginolyticus*	-	-	-
5	CHAVA3	*Vibrio parahaemolyticus*	-	-	-
7	CHAVA4(4)	*Vibrio parahaemolyticus*	-	-	-
13	MUS7	Uncultured *Vibrio* sp.	-	-	-
14	MUS13	*Vibrio furnissii*	+	+	-
17	FK4	*Vibrio alginolyticus*	-	-	+
19	AF7	*Vibrio vulnificus*	-	-	-
20	ALP(V.C)11	*Vibrio cholerae*	+	+	-

Out of the 21 strains that were screened for their intrinsic virulence factors, only 11 showed an F/A ratio ≥ 0.09 in the suckling mouse experiment and the other characteristics of these strains are as shown in table-22. It was obvious that strains CHV4 (3) (*V. alginolyticus*) and CHAVA3 (*V. parahaemolyticus*) had the highest F/A ratio (>0.09).

Chapter 5

DISCUSSION

Vibrios are autochthonous flora of estuaries where the salinity varies from 28 to 32 ppt. Water, sediment, crustaceans, molluscs and plankton are the previously known sources of *Vibrios* (Juan and Alfonso, 2005; Rashid *et al.*, 1992; Arulampalam *et al.*, 1998). In the present study *Vibrios* could be isolated from water, mussel, prawns and plankton and thus the results of the present study also substantiated the above mentioned reports. Salinity is a prerequisite for the growth and surging of *Vibrios* in general. Not all *Vibrios* are halophilic. *Vibrio cholerae* can survive in less saline conditions (Falcao *et al.*, 1998). This is evident from the results of this study as well. The maximum numbers of *Vibrio cholerae* were isolated from water samples drawn from Ernakulam and Alappuzha. The source of water samples from Alappuzha was a land locked canal, where there is no saline intrusion or mixing with sea water. Naturally, the salinity expected was low in those areas. In the case of Ernakulam, the source of the water samples was connected to the sea via the Cochin estuary, and therefore the salinity could vary from 0.2-30ppt. (Menon *et al.*, 2000).

In the present study, when the incidences of *Vibrios* from all the four sample types were compared, it was obvious that the maximum number of isolation was from water. A total of 89 different strains could be isolated from all the water samples. Analysis of the species diversity of the isolates studied showed that water is an excellent source of different species of *Vibrios*. A total of 24 strains of *Vibrio cholerae*, 22 strains of *V. alginolyticus*, 15 strains of *V. parahaemolyticus*, 9 strains of uncultured bacterium, 8 strains of *V. harveyi*, 4 strains of unspecified *Vibrio* sp., 6

strains of *V. vulnificus*. 2 strains each of *V. proteolyticus* and *V. natriegens*, 1 strain each of Marine bacterium, *Photobacterium*, Mucus bacterium and Bacterium JB8 could be isolated from water samples.

Mostly *Vibrio cholerae* could be isolated from only two stations, viz. Ernakulam and Alappuzha, indicating that in those particular point sources, the inter species competition or probable other environmental factors were in favour of *Vibrio cholerae*, so that their chance of isolation was above that of other *Vibrio* species. This logic may be applicable even to other *Vibrios* isolated *i.e., Vibrio alginolyticus* was mostly isolated from Aqua farm where fish kill occurred, Connolly canal, Chavakkadu, Chettuva and Ponnani, denoting their better survival over a wide range of habitats.

An interesting observation made during the present study is the incidence of other non- *Vibrio* isolates on TCBS agar, with characteristic biochemical features of *Vibrios*. From this study, 9 uncultured bacterium, 1 strain each of Marine bacterium and Mucus bacterium, all of which showed typical *Vibrio* reactions on TCBS agar, were positive for cytochrome oxidase test, Grams negative rods/cocci and fermentative on MOF medium. Earlier workers doubted the reliability and reproducibility of TCBS, as a medium for isolation of environmental *Vibrios* (McLaughlin, 1995). The present study also leads one to doubt the reliability of TCBS for screening *Vibrios* since non *Vibrios* could also be isolated from TCBS.

Green mussel or *Perna viridis* was also a source of various *Vibrios* species (Kueh and Chan, 1985). In this study, the maximum representation of *Vibrios* from green mussel (*Perna viridis*) was by *Vibrio harveyi* (5 strains). There were 4 strains of unspecified *Vibrios* and 1 strain of *Vibrio furnissii*, also isolated from this

molluscan source. Here too, 4 strains were designated as the uncultured bacterial faction. The crustaceans, especially *Penaeus* sp. are a good source of *Vibrios* (Hosseini *et al.*, 2004). In this study, 2 strains of *Vibrio parahaemolyticus* could be isolated from the prawn sample. Plankton is another source of *Vibrios* (Maria *et al.*, 1999). In this study, 2 strains of *Vibrio parahaemolyticus* and a strain of Bacterium JB8 (unidentified) could be isolated from the plankton sample.

The designation 'uncultured' and 'unidentified' are with respect to the database entry. That is, these designations are simply based on 16S rRNA gene sequence homology search and the hits generated by BLAST programme. The 16S rRNA gene sequence of a culturable bacteria giving 100% homology to the 16S rRNA gene of an unculturable bacteria in DNA database, only indicates the existence of a previous entry to the DNA database, giving 100% homology to the sequence in query; it does not mean that the bacteria in the identity query was an unculturable one.

Since the identification of the strains were solely based on sequence similarity search from the DNA database, many of these strains initially identified as uncultured and unidentified may get specific names/identities, upon frequently repeating the BLAST analysis later; as the DNA databases get updated regularly with gene sequences submissions from all over the world.

5.1. 16S rRNA gene based taxonomy

The 16S rRNA gene sequence is ~ 1,550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient inter-specific polymorphisms of 16S rRNA gene, to provide distinguishing and statistically valid measurements. Universal primers are usually chosen as complementary to the

conserved regions at the beginning of the gene, and at either the 540-bp region or at the end of the whole sequence (~ the 1,550- bp region); and the sequence of the variable region in between is used for comparative taxonamy. (Chen *et al.*, 1989; Relman, 1999).

16S rRNA gene is a reliable method for identifying unknown bacterial isolates. The data generated using the universal 16S rRNA gene segment primers are of great accuracy and reproducibility. This method is less time consuming compared to the conventional phenotypic identification schemes. A 16S rRNA gene sequence similarity of ≥97% is a reasonable level for grouping bacteria into species. (Hagstrom *et al.*, 2000).

Even though there are other inherent problems and shortcomings of this method, it is being widely used even today. Phylogenetic methods can be employed for assigning systematic position of an unculturable organism. Phylograms were drawn with strains isolated from each locality, to study their relatedness or variability. All the trees were rooted with the out group. As part of this study, a total of 13 uncultured bacteria were identified. Their relative systematic positions could be fixed by employing phylogenetic methods. Neighbour joining method was successfully used for this purpose. In this study, the strains EKM10 and ALPVC (3) were first assigned the name 'uncultured bacterium'- based on BLAST homology search; whereas the phylogram analysis indicated that both these strains belong to the group of *V. cholerae.* Use of markers other than the phylogenetic markers, like the genetic markers (*ompw*), could be employed to conclusively state their systematic position. By using this PCR marker, it was found that strains ALPVC (3) is *V. cholerae*, as evidenced by a positive PCR reaction with *ompw* primer pairs (Bisweswar *et al.*, 2000), but the strain EKM10 was negative for *ompw.*

All isolates from Ernakulam were identified as *Vibrio cholerae.* In the tree drawn here, it can be seen how intra species variation plays a role in determining clades. Strain EKM2 was cladding as a separate entity and all the rest of the isolates went together.

From the 16S rRNA gene based phylogenetic tree constructed for *Vibrios* isolated from Alappuzha, it was obvious that the *Vibrio cholerae* isolates from this region were more related to each other than those from the previous station. Here they formed two major clades, one with 7 representing strains and the other with 8. It is to be noted here that the strain ALPVC (3) that was designated as unculturable bacterium claded with *Vibrio cholerae.*

The phylogenetic tree constructed to analyze the *Vibrio* strains isolated from the fish kill site, depicted an altogether different pattern. They divided among themselves as two separate groups comprising of strain FK8 as a distinct group, strains FK1, 2, 3, 4, 5, 6 and 7 as another group. Here, FK8, although identified as a *Vibrio alginolyticus* strain, did not clade with the rest of the *V. alginolyticus* in the group. The other point of interest here was the presence of strain FK2 designated 'unidentified bacterium'; by the nature of its 16S rRNA gene sequence similarity, branched with *V. .parahaemolyticus*, denoting that it might be a close relation of *V. parahaemolyticus.* Strain FK6 was also distinct in this matter with its identity as just a marine bacterium.

The phylogram of the strains from the Aqua farm site presented a different scenario. They were cladding into two main groups, with the maximum of uncultured bacterium', each group having two uncultured bacterium. Clade one having strains AF3 & AF1 and the second clade with strains AF2 &AF8. Another important factor

regarding this particular site is that maximum number of *V. vulnificus* strains could be isolated from this site (4 isolates). *V. vulnificus*, is a *Vibrioanaceae* member capable of causing septicaemia, wound infections and gastroenteritis in human beings (Blake *et al.*, 1980; Klontz *et al.*, 1988). The prevalence of this organism in coastal waters and shellfish in India has been reported earlier (Karunasagar *et al.*, 1987, 1990; Thampuran and Surendran, 1998; Parvathi *et al.*, 2004). An unspecified *Vibrio* sp. also could be isolated from this site. Yet another noted feature about the *Vibrio* representation from this site was that it accounted for 50% of the *V. proteolyticus* strains isolated from all stations.

The station Connolly canal was represented by 13 *Vibrio* isolates and showed significant species diversity. In fact, this station was represented by the maximum number of different *Vibrio* species (6 different species). The strain C1 claded as a separate entity, and the rest of the isolates grouped together. Out of the 13 isolates *V .parahaemolyticus* with 5 representative strains formed the predominant group.

Isolates from green mussel (*Perna viridis*) collected from Vadakara market (north Kerala) showed a distinct phylogram pattern. Among these isolates, one *V. furnissi* (MUS13) could be singled out, and 4 unspecified strains of *Vibrios*. *Vibrio harveyi* was represented by 5 strains in this sample. The strain MUS6 was far distant in relation to all other isolates from this site. Isolates from Ponnani were distinct in their interrelationship with reference to 16S rRNA gene. Here too, two major groups were formed, with isolate P8 as the only member of one group and the remaining 9 isolates as another group. One peculiarity of these 9 isolates is that they also included a non *Vibrio* member (i.e., Mucus bacterium.). But it was evident from the phylogram that the Mucus bacterium claded well outside the *Vibrio* group.

The phylogenetic tree drawn for station- Chavakkadu contained 12 *Vibrio* isolates, 8 of which were *V. alginolyticus* and the rest were *V parahaemolyticus* (4 isolates). Percentage representation of *V. alginolyticus* per station was 66% for station Chavakkad. CHAVA1 (2) was distantly related to the rest of the isolates.

Station Chettuva was represented by 8 isolates, of which 6 were identified as *Vibrio alginolyticus*. Here the percentage representation per station was 75, and 25% was claimed by an unspecified *Vibrio* i.e, isolate CHV2 (4) and a *Vibrio parahaemolyticus*, CHV3 (1). The isolate CHV2 (1), although identified as a *V. alginolyticus*, was very distant in its relation to the other strains of *V. alginolyticus* from the same station. This denoted that the *V.alginolyticus* group was diverse in nature.

The phylogram drawn for all the isolates from Kasargode, Neendakara, Omanappuzha and from plankton, showed cladding into two groups with isolates PV8 (2) as a distant relative of all the rest of the isolates. *V. parahaemolyticus* was the species represented maximally (i.e., 5 isolates). At least one *V. parahaemolyticus* was represented per sample, except in the case of station Omanappuzha, which is being represented only once with an uncultured bacterium.

The phylogram drawn with all the *Vibrio* isolates showed how inter related these strains were to one another. It also showed how a subset of *Vibrionaceae* members could be related to each other on the basis of 16S rRNA gene. They were cladding grossly into two groups. Wrong phylogram patterns may arise, because of wrongly identified database entries. Later studies depending on such identities will show unusual cladding pattern (Clarridge *et al.*, 2004).

Intra species variation was also analysed with all six major groups of *Vibrios*, which were obtained in isolation, namely *Vibrio cholerae* (24 isolates), *Vibrio alginolyticus* (22 isolates), *Vibrio parahaemolyticus* (19 isolates), Uncultured bacterium (13 isolates), *Vibrio harveyi* (8 isolates) and *Vibrio spp.* (8 isolates). For all these analysis, strain C7 (*Photobacterium*) was kept as an outgroup.

It was seen that *Vibrio cholerae*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus* were quite diverse among themselves with respect to 16srRNA gene. *Vibrio cholerae* grouped into two, with ALP (V.C) 11 as a separate entity and the rest of the isolates as a main group. Within this main group, EKM14 and EKM8 went together as a separate branch, while the rest of the 21 strains grouped together.

Vibrio alginolyticus strains also showed a distinct pattern of relatedness, among themselves, by cladding into two main groups, comprising of 13 strains in one group and 9 strains in the other.

Vibrio parahaemolyticus showed unique intraspecies variation. Strain FK3 claded alone and the remaining isolates formed a major group. Uncultured bacteria although distinct among themselves, showed a pattern of their relatedness, by cladding into two. *Vibrio harveyi* claded into two, with strains P3 and P5 in one group and the rest of the isolates in another group. Unspecified *Vibrios* also share a common pattern as in the case of *Vibrio harveyi*.

5.2. Antibiotic susceptibility tests

Antibiotic susceptibility/resistance data provides precious information for drug developers and for those medical practitioners who really worry about the ever

increasing drug resistance in bacteria. As of now, the first generation antibiotics like ampicillin have become useless against the armoury of the microbes. From this study it was obvious that 62.04% of the isolates studied were resistant to ampicillin. 12.04% were resistant to furazolidone and 7.41% were resistant to trimethoprim. All the isolates studied (i.e. 100%), were susceptible to chloramphenicol and norfloxacin and 99.07% of the isolates were sensitive to nalidixic acid. However small the number, the figures showing resistance to nalidixic acid, mark the beginning of an alarming situation in the development of multiple drug resistance, in environmental *Vibrios*. Even the Quinolones were being resisted! Nalidixic acid, being a broad spectrum quinolone antibiotic killed the bacteria by inhibiting DNA gyrase.

Multiple Antibiotic Resistance indexing is an easy way to group isolates which are resistant to more than one antibiotic. Out of 108 strains studies, only 16% showed multiple antibiotic resistance patterns. There are reports that multiple antibiotic resistances were increasing day by day (Threlfall *et al.*, 1997). A MAR index of more than 0.2 can be attributed to contamination from high risk sources, where antibiotics were frequently used and to high anthropogenic interference. A MAR index of 0.4 was shown by two *Vibrio* strains i.e., FK5- *Vibrio alginolyticus* and P6 - *Vibrio harveyi*. These strains were resistant mostly to beta lactams, tetracycline, trimethoprim, nitrofuran and Quinolone. A MAR index of 0.3 was shown by five strains i.e., FK4, MUS9, C1, P5 and CHAVA4 (1).

5.3. Screening for virulence genes by PCR

PCR screening is a rapid method to check for the presence of desired gene/genes. This method has been employed successfully in this study to check the presence of eight established virulence genes and three genes of marker value. Most

of the virulence genes tested were absent in the *Vibrio* isolates studied, except for the genes from integrons (*qacEΔ1*, *Sul1* and *inCS*), *ompw* (a marker gene for *V. cholerae*), *toxR* (the transcriptional regulator of most virulence cascades in *Vibrio*s) and *sxt*, an integrative conjugative element.

Integrons are mobile DNA elements capable of capturing genes, particularly those encoding antibiotic resistance, by site-specific recombination of their intergase gene (*int*). They have a recombination site (*attI*), and a promoter (*Pant*). Integrons may be classified based on the type of integrase gene they possess. Class 1 integrons are the most studied ones. They consist of a variable region bordered by 5' and 3' conserved regions. The 5' region is made up of the int gene, *attI*, and the promoter *Pant* which drives transcription of genes within the variable region. The 3' region consists of an ethidium bromide resistance locus (*qacEΔ1*), a sulfonamide resistance gene (*sulI*), and an open reading frame containing a gene of unknown function (Peter , 1999).

The antibiotic resistance genes that integrons capture are located on gene cassettes. The cassettes consist of a promoterless gene and a recombination site (*attC*). The cassettes can exist as free, circular DNA but cannot be replicated or transcribed in this form. A recombination event occurs between *attI* and *attC*, integrating the cassette into the integron. The gene on the cassette is then bound by the *attI* site on the 5' side and by *attC* on the 3' side. (Collis *et al.*, 1992).

Primers from integrons *qacEΔ1* and *Sul1* gave a positive amplification with only the *V. cholerae* isolates and with only one non cholera *Vibrio*, i.e., strain MUS13-*V.furnissii*. These genes are present at the 3'CS (conserved stretch) of class I integrons and their presence indicated the presence of the whole integron structure.

'inCS' are a primer set designed from integron structure itself, but directed to amplify the gene cassettes present in integrons. Depending on the number and length of gene cassettes, one or more bands may get amplified (Thungapathra *et al.*, 2002). All the isolates identified as *V. cholerae* responded to amplification with this primer pair denoting their presence. It could be inferred from the results that at least one gene cassette is present in all of the isolates identified as *V. cholerae* tested here. Apart from the *V. cholerae*, a *V. furnissii* (MUS13) also yielded positive amplification with this primer pair.

0mpw (Outer membrane protein) is a reliable marker gene for *V. cholerae* (Bisweswar *et al.*, 2000) and the outcome of this PCR amplification reconfirmed the reliability of this marker. Only those strains which are *V. cholerae* responded to amplification with this primer set , but strain EKM10 did not respond to this test denoting its identity as some other *Vibrio* species. The phylogenetic tree also testifies this view. This marker can be easily adapted for any quick check for the presence of *V. cholerae* in food or other samples (Bisweswar *et al.*, 2000).

01rfb, 0139rfb (bundle forming pili genes) are surface marker genes for pandemic strains of *V. cholerae*. 01 and 0139 are the two serotypes of *Vibrio cholerae* causing pandemics, and checking for their presence can endorse the reliability of the genes as marker candidates, as well as indicate the serotype of the strains. This PCR is an alternative for serotyping and hence called as serotyping PCR. Since none of the tested *Vibrio* isolates showed a positive amplification, it was inferred that all the isolates identified as *V. cholerae* belong to either the non01 or the non0139 group.

Toxin co-regulated pili are the sites of attachment for the temperate phage, ctxΦ in *V. cholerae*. Its expression is co-regulated by the expression of cholera toxin genes ctxA and ctxB. Whole pili structure are coded by many genes and *tcpA* codes for the A subunit of a cluster of proteins making toxin-co-regulated pili. This gene is directly responsible for virulence in *Vibrio cholerae*. Absence of positive amplification with the primers denoted their total absence in any of the *Vibrios* tested.

There was no positive amplification for *tcpA*, *ace*, *zot* and *ctxA* with any of the *Vibrios* tested. Most of these toxin genes tested here are key virulence factors of *Vibrio cholerae*. Their absences in the *Vibrio cholerae* isolates do not pronounce them as innocuous. This was evident from the *in vivo* suckling mouse assay done for *Vibrio cholerae* strain ALP (VC) 11. Although this strain does not carry any tested virulence genes like *tcpA, ace, zot*, and *ctxA*, it was capable of producing gastric fluid accumulation in animals, proving beyond doubt that there are other factors also, that contribute to their toxigenicity.

toxR code for a transmembrane transcription controlling protein. Expression of more than seventeen virulence genes is under the co-ordinate control of the ToxR protein. ToxR is a transmembrane protein and it binds to and activates the promoter of the operon encoding cholera toxin. ToxR controls transcription of toxT, whose product in turn is directly responsible for activation of several virulence genes under ToxR control (Victor *et al.*, 1991). Many environmental signals act via the ToxR system. Here in this analysis, the importance of *toxR* was highlighted by their presence in 24 *Vibrio cholerae* strains i.e., 22.22% of the total 108 strains harboured this upstream toxic cascade regulator.

Sxt, an integrative conjugative element of around 100kb size, was originally reported from *V. cholerae* (Waldor *et al.*, 1996). Strains FK4-*V.alginolyticus*, ALP (VC) 3-*V.cholerae*, ALP (VC) 4-*V.cholerae*, ALP (VC) 6-*V.cholerae*, ALP (VC) 7-*V.cholerae* were all positive for *sxt*. The SXT element has an integrase gene and many other genes of unknown function (John *et al.*, 2002). SXT integrase, *int* from *V. cholerae* is about 1200bp long. This was the gene that was targeted in order to check for the presence of SXT element in the isolates. Any positive results for this screen in isolates tested than in the *V. cholerae*, could only be due to lateral gene transfer. This study showed for the first time the presence of the SXT element in *V. alginolyticus*. . In order to check whether this SXT element in *V. alginolyticus* (strain FK4) was transferable, conjugation experiments were performed with *Escherichia coli*HB101 as the recipient. Testing the ex-conjugant *E. coli* DNA by PCR for the presence of *sxt*, confirmed, that indeed the element was transferable and that it was incorporated into the recipient genome. This finding may be the first report of its kind with reference to *V. alginolyticus*.

Out of the eight genes directly or indirectly involved in virulence, only *toxR* and *sxt* showed positive amplification, denoting their presence. There may be many other virulent genes, both reported and unreported in these isolates. Out of 108 strains studied, 24 strains of *V. cholerae* were positive for *qacEΔ1, Sul1 ,ompw* and *toxR*, i.e., 22.22% of the *V. cholerae* strains in this study carried potential virulent genes;. 16% of the *V. cholerae* isolates in this study were positive for sxt.

With respect to virulence/virulence related genes, isolates from stations Alappuzha and Ernakulam harboured the maximum compared to the rest. Within the MAR isolates, three belonged to station Alappuzha and Ernakulam (ALP (VC)5, ALP(VC)2 and EKM10). These strains claimed a MAR index of 0.2. In terms of

MAR index, station Ponnani could more anthropogenically contaminated and the station is represented by three MAR strains *i.e.*, P4, P6 and P8. Within which P6 has maximum MAR of 0.4, along with strain FK5.

5.4. Suckling mouse assay

In order to see the expression of any of the virulence factors *in vivo*, suckling mouse assay was performed with selected isolates and the results showed that an average F/A ratio greater than or equals to 0.09 was shown by eleven strains, out of twenty one strains tested; i.e. more than 50% of the strains tested showed the presence of gastric fluid accumulating type toxin/toxins. But out of these eleven strains, only three harboured any of the tested virulence factors/ virulence related factors. The strains MUS13 (*V. furnissii*) and ALP (V.C) 11 (*Vibrio cholerae),* harboured both the integron genes and the strain FK4 (*V. alginolyticus*) harboured the *sxt* integrase gene and the *toxR* gene. From the results of the suckling mouse assay, it was obvious that strains CHV4(3) (*V. alginolyticus*) and CHAVA3 (*V. parahaemolyticus*) have the highest F/A ratio (>0.09), denoting the probability of gastric virulence factors already reported but not studied as part of this study; or the presence of totally uncharacterized and unstudied gastric virulence factors in these isolates. The latter paves way for further study and characterization of the responsible factor/factors in various dimensions.

Out of all the characterized strains which were part of the sucking mouse assay, *V. cholerae* was the only *Vibrio* species which had tested positive for the maximum number of the virulence genes. All the other *Vibrio* species except *V. furnissii* and *V.alginolyticus* had coded for none of the tested virulence genes. *V. parahaemolyticus* (FK5-MAR-0.4) and *V.harveyi* (P6-MAR-0.4) showed the

maximum MAR index, followed by *V. alginolyticus* (FK4-MAR-0.3), Unspecified *Vibrio* (MUS9-MAR-0.3), Uncultured bacterium (P8-MAR-0.3) and *V. alginolyticus* (CHAVA4 (1)-MAR-0.3). *V.cholerae*, *V.furnissii*, *V.alginolyticus*, *V. parahaemolyticus*, *V. natriegens* were some representative species of *Vibrio* isolates with least MAR index of 0.2. An F/A ratio of ≥ 0.09 was shown by three strains each of *V. parahaemolyticus* (Strains CHV3 (1), CHAVA3 and CHAVA4(4) and *V. alginolyticus* (strains CHV2(2), CHV4(3) and FK4).

Briefly, this study was able to access the pathogenic potential of different *Vibrio* isolates from the coastal waters of Kerala and from other sources. This study showed the antibiotic resistance pattern existing/evolving among *Vibrio* members in the environment. It was also possible to spot a new Horizontal Gene Transfer event, i.e., movement of SXT from *V. cholerae to V. alginolyticus*. As part of this work many unknown *Vibrio* strains from the environment were proposed probable systematic positions based on partial 16S rRNA gene sequencing and homology studies, which also enabled a very basic study of the phylogenetic relationship among the *Vibrio* isolates. All the above work has developed substantial data on the *Vibrios* in the aquatic environment of Kerala and can therefore form the basis for further research in this area.

Chapter 6

SUMMARY AND CONCLUSION

This study involved the morphological, biochemical and molecular characterization of the *Vibrios* isolated from the marine environment. The study also included, the characterization of the virulence or virulence associated gene as most of these *Vibrios* have specific virulence factors such as capsules, toxins, colonization factors and many are known to harbour yet to be identified virulence molecules.

Many species of *Vibrio* could be isolated from water, crustaceans, molluscs and plankton, sampled from different locations along the coastal areas of Kerala, and Aquaculture farms. The sampling stations included Kasaragode, Vadakara, Chavakkadu, Chettuva, Ponnani, Connolly canal, Kochi, Alappuzha, Aqua farm, Aqua farm where fish kill occurred, Omanappuzha, Neendakara etc.

One hundred and eight strains of *Vibrios* could be isolated, identified and stocked before they were characterized.

Phylogenetic trees were constructed using the neighbour joining methods implemented in the DAMBE (Data Analysis in Molecular Biology and Evolution (Xia 2000) software program. Trees were constructed using nucleotide based evolutionary model TN84 method for estimating genetic distances. Statistical support for branching was estimated using 1000 bootstrap steps and nodes with a bootstrap value of 750 or more were taken to represent nodes with significance.

All the isolates were compared with respect to their stations of isolation and with respect to the species obtained.

Genetic relatedness of all isolates, based on 16SrRNA gene could be established. Phylogenetic tree method could be employed to find the correct identity of the strains. Maximum numbers of *Vibrio cholerae* were isolated from the two stations, Ernakulam and Alappuzha. The station Connoly canal (Kozhikode) was represented by 13 *Vibrio* isolates, and it was the only station represented by highest species diversity with 6 different species.

All the 108 isolates were tested for antibiotic susceptibility. It was found that ampicillin resistance was exhibited at the highest rate (62.04% were resistant against ampicillin). Chloramphenicol and norfloxacin were the most effective antibiotics in controlling the *Vibrios*. All the strains tested (n=108) were susceptible to both these antibiotics. Nalidixic acid resistance was shown by only 0.93% of the isolates tested. But even this small number indicating resistance to the quinolones is significant. In the current context, this information is both cautionary and alarming. Quinolones, a powerful treatment option which are now being used, may be rendered ineffective in the near future, if this seemingly insignificant value gradually increases to alarming proportions.

Multiple Antibiotic Resistance index was found out for those isolates which were resistant to more than one antibiotic. Only 16% of the tested isolates showed multiple antibiotic resistance pattern. A MAR index of 0.4 was shown by two strains of *Vibrios*- FK5 *(Vibrio alginolyticus)* and P6 (*Vibrio harveyi*). These strains were mostly resistant to ß-lactams, tetracycline, trimethoprim, nitrofuran and quinolone. A MAR index of 0.3 was show by five strains i.e, FK4, MUS9, CI, P5 and CHAVA4

(1). Within the MAR isolates, three belonged to the station Alappuzha and Ernakulam (ALP (VC)5, ALP(VC)2 and EKM10), from where the maximum numbers of *Vibrio cholerae* was isolated. These strains have a MAR index of 0.2. Station Ponnani was represented by three MAR strains i.e., P4, P6 and P8, of which P6 (*Vibrio harveyi*) had the highest MAR of 0.4, which was also observed in strain FK5 (*Vibrio parahaemolyticus*) isolated from the aquafarm where fish kill occurred.

Polymerase Chain Reaction was employed as a rapid screening method to check for the presence of selected virulence genes among the 108 strains of *Vibrios*. Most of the virulence genes tested were absent in the tested *Vibrio* isolates, except for the genes from integrons (*qacEΔ1,Sul1 and inCS*), *ompw* (a marker gene for *V. cholerae*), *toxR* (the transcriptional regulator of most virulence cascades in *Vibrios*) and *sxt*, an integrative conjugative element. Primers targeting genes *tcpA*, *ace*, *zot* and *ctxA* did not give any positive amplification in any of the *Vibrio* isolates.

A rare and unusual event of lateral gene transfer could be reported, between *V. cholerae*, and *V. alginolyticus*. SXT, an integrative conjugative element, which was previously reported only from *Vibrio cholerae*, was found in a non cholera *Vibrio* in this study. Strain FK4, identified as *Vibrio alginolyticus* was found to harbour this element. Conjugation experiments helped to prove that this element could be transferred to *Escherichia coli* HB101 along with its resistance markers, confirming its presence.

Suckling mouse assay was performed with 21 strains to observe the expression of other uncharacterized gastric toxin/toxins, which may express in a susceptible host. More than 50% of the strains tested showed the presence of gastric fluid accumulating type toxin/toxins. Out of eleven *Vibrio* species that showed gastric

fluid accumulation and a F/A ratio of ≥ 0.09, *V. parahaemolyticus* and *V. alginolyticus* had the maximum representation with three strains each. (Strains CHV3 (1), CHAVA3 and CHAVA4 (4) - *V. parahaemolyticus* and strains CHV2(2), CHV4(3) and FK4 –*V .alginolyticus*).

Conclusion

A rare horizontal gene transfer event could be traced. The movement of the SXT element among the *Vibrionaceae* could be followed. This element was first reported from *Vibrio cholerae* and in this study the same could be confirmed in *Vibrio alginolyticus*. Events such as these, which take place with respect to other virulence/virulence associated genes, may lead to the emergence of pathogenic strains from hitherto non-pathogens or may even give rise to new pathogens.

The results generated in the course of this study paves way for further characterization and detailed study, especially with respect to those strains which showed gastric fluid accumulation in the *in vivo* suckling mouse assay. Antibiotic resistance pattern shown by a sample population of *Vibrios* can be used for deciding treatment options. There is enough scope for further research on these topics towards generating basic knowledge, which can be of immense significance in human and aquaculture health.

Chapter 7

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APPENDIX I

Agarose gel

Agarose (SRL)	1.0 g
1X TBE (see TBE buffer)	100 ml

The agarose was melted in the microwave oven and allowed to cool to 50°C before pouring the gel.

Bromophenol blue loading dye

Bromophenol blue	0.05 g
Glycerol	5.0 ml
EDTA	0.186 g
1X TAE	volume to 10 ml

The loading dye was dispensed in 1 ml aliquots in fresh Eppendorf tubes and stored at 4°C.

Disodium ethylenediamine tetraactete (EDTA) - 0.5 M

EDTA	186.1 g
Distilled water	1000 ml
NaOH	~20 g

EDTA was dissolved in 800 ml of distilled water and stir vigorously on a magnetic stirrer. Adjust pH to 8.0 using NaOH pellets and make the final volume to 1000 ml. Autoclave before use.

Ethidium Bromide (10 mg/ml)

Ethidium Bromide	0.1 g
Distilled water	10 ml

The solution was stirred using a magnetic stirrer for several hours to ensure that the dye had completely dissolved. The container was wrapped in aluminium foil and stored at 4°C. For staining agarose gels, a working solution of 0.5 µg/ml was made.

Hexadecyltrimethylammonium bromide (CTAB) – 10 % in 0.7 M NaCl

CTAB	10 g
NaCl	4.1 g
Distilled water	80 ml

Dissolve 4.1 g NaCl in 80 ml distilled water and slowly add 10 g CTAB, while heating and stirring. If necessary, heat to 65°C to dissolve. Adjust final volume to 100ml.

Oxidase reagent

Tetramethyl p-phenyldiamine	1 g
Isoamyl alcohol	100 ml

Proteinase K (20 mg/ml)

Proteinase K	10 mg
Sterile distilled water	0.5 ml

Stored at -20°C in 50 µl aliquots.

Sodium dodecyl sulphate (SDS) – 10%

SDS	10 g
Distilled water	100 ml

Tris-Acetate EDTA (50 X)

Tris base	242 g
0.5 M EDTA (pH 8.0)	100 ml
Glacial acetic acid	57.1 ml
Distilled water	to 1000 ml

The stock solution was diluted to 1X for gel runs.

Tris-Borate EDTA (TBE) buffer - 5 X

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
Distilled water	to 1000 ml

The stock solution was diluted to 0.5X just before use as gel running buffer.

Tris EDTA (TE) buffer

Tris-HCl	10 mM	2 ml 1 M Tris-HCl (pH 8.0)
EDTA (pH 8.0)	1 mM	0.4 ml 0.5 M EDTA
Distilled water	to 200 ml	

APPENDIX II

GenBank submissions

DQ241795 – 16S rRNA Gene from strain FK4, *Vibrio alginolyticus.*

DQ898173 – 16S rRNA Gene from strain EKM14, *Vibrio cholerae.*

DQ904445 - 16S rRNA Gene from strain PV8(2), *Vibrio sp.*

DQ904444 – 16S rRNA Gene from strain CHV4(2), *Vibrio alginolyticus* strain RH2.

DQ904443 – 16SrRNA Gene from strain MUS13, *Vibrio furnissii* (ATCC 35016T).

DQ904442 – 16S rRNA Gene from stain C4, *Vibrio sp.*

DQ904441 – 16S rRNA Gene from strain P10, *Vibrio sp.*