MOLECULAR APPROACHES FOR CHARACTERIZATION AND DETERMINATION OF PATHOGENICITY OF VIBRIOS WITH SPECIAL REFERENCE TO VIBRIO HARVEYI FROM PENAEUS MONODON LARVAL PRODUCTION SYSTEMS

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BY

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November 2011

CERTIFICATE

This is to certify that the research work presented in this thesis entitled "**MOLECULAR APPROACHES FOR CHARACTERIZATION AND DETERMINATION OF PATHOGENICITY OF VIBRIOS WITH SPECIAL REFERENCE TO *VIBRIO HARVEYI* FROM *PENAEUS MONODON* LARVAL PRODUCTION SYSTEMS"** is based on the original work done by Mrs. B.Sreelakshmi (Reg. No. 3071) under my supervision and guidance at National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi 682016, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy** and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Declaration

I hereby do declare that the work presented in this thesis entitled **"MOLECULAR APPROACHES FOR CHARACTERIZATION AND DETERMINATION OF PATHOGENICITY OF VIBRIOS WITH SPECIAL REFERENCE TO *VIBRIO HARVEYI* FROM *PENAEUS MONODON* LARVAL PRODUCTION SYSTEMS"** is based on the original work done by me under the guidance of Prof. I. S. Bright Singh (Co-ordinator), National Centre for Aquatic Animal Health Cochin University of Science and Technology, Kochi – 682 016 and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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B.Sreelakshmi

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General Introduction and Review of Literature

General Introduction

Aquaculture deals with broad spectrum of activities, related to husbanding of aquatic organisms in controlled environments with appropriate propagation methods in the rearing medium, which assures a reliable supply of food. Owing to the highly advanced technology of livestock management, habitat conservation, the challenges of providing food for the ever-growing human population, shrinkage of land area for production and huge investments required for meeting even marginal increase in marine food products, man has turned his attention to aquatic animal farming. The role of aquaculture for augmenting protein food production, improving rural economy and providing large-scale employment opportunities has been well recognized. The increase in demand for cultured prawns, shrimps, fishes, mussels and other aquatic animals had led to research in this area all over the world which has resulted in the development of newer methods of culture and culture practices. Developing countries are the foremost contributors, where aquatic resources are utilized for the livelihood of the population, poverty alleviation, income generation, employment and trade. Aquaculture has emerged as one of the most promising industries in the world with substantial growth. About 63.1% of brackish water production in India is contributed by penaied shrimp (FAO, 2006). India has an exquisite potential of 1.12 million hectare of potential shrimp farming areas, mainly contributed by vast stretches of highly productive brackish water, and tropical climate favoring faster shrimp growth. Aquaculture production statistics of 2009 describes China with 34.78 million tonnes and followed by India 3.79 million tonnes as the major contributors of freshwater carp and brackish water shrimp production (FAO, 2009).

The increasing demand for cultured shrimp has led to intense farming practices. Disease loss both by attrition of chronic infection or sudden catastrophic epizootics, poor soil and water quality, high stocking density, accumulation of unutilized feed and fluctuating environmental conditions are the problems confronted by today's aquaculture sector. Though an extensive development of the culture systems has emerged in most of the Southeast Asian countries, successful cultivation is increasingly hampered by environmental pollution, mismanagement, nutritional imbalances, toxicants, stress, diseases and genetic agents. Hence, sustainable development is largely at stake, faced with numerous ecological and pathological problems augmented by environmental degradation and emergence of infectious and non-infectious diseases (Bache're, 2000).

Disease of aquatic organisms is a major concern. Ecosystems do not respond linearly to environmental changes, nor do the microorganisms that live there. Infectious diseases have distinctive biographies, and each one has a complex relationship with the environment. Complexity of these factors emerges at each level ranging from the cell, organism, community and ecosystem to induce a pathogenic response. Infectious diseases in penaied shrimp include viral, bacterial, fungal, rickettsial, protistan and metazoan etiologies (Lightner, 1996). The incidence of microbial diseases has increased dramatically in accordance with the growth of aquatic larvae production (Toranzo *et al*., 1993).

Shrimps are subjected to various diseases and are stressed and weakened under adverse environmental conditions. It was reported that high mortalities occur during first feeding stage of larvae due to emergence of pathogenic and opportunistic bacteria, through food chain (Campbell and Buswell, 1983; Muroga *et al*., 1987), especially while feeding with *Artemia* (Chair *et al*., 1994). High density, high nutrient conditions of aquaculture systems facilitate rapid spread of virulent strains. Artificial conditions in aquaculture environments serve as reservoirs for the growth and spread of

pathogenic vibrios. Of the infectious diseases, bacterial and viral infections, either as single or multiple pathogenic conditions, cause extensive production losses.

Viral pathogens reported in shrimp include Monodon Baculovirus (MBV), Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) also called as White Spot Disease Virus (WSDV), Hepatopancreatic Parvo-like Virus (HPV), Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) and Yellow Head Virus (YHV) (Flegel, 1997). Mortalities caused by virus in acute phase can be as high as 95% but surviving shrimp remain infected and become potential source of virus transmission (Kiatpathomchai *et al*., 2008, Walker and Winton, 2010).

Of the reported bacterial pathogens till date, vibrios are the most important among cultured shrimps responsible for a number of diseases and mortalities upto 100% (Lightner, 1983). Mass mortality caused by luminescent vibrios contributed largely to the collapse of shrimp grow-out activities. Vibrios were isolated from mussels, scallops, oysters, sea urchins, *Artemia*, rotifers, seaweeds, algae, aquaculture market products, from tank-water, seawater, sediments, diseased or dead larvae, and adult organisms (Johan *et al*., 2003). Internal signs of disease in fish and shellfishes caused by vibrios include intestinal necrosis, anaemia and liquid accumulation in the air bladder, hemorrhages in muscle wall, in or on the internal organs, mouth and or bloody exudates in the peritoneum, swollen intestine, pale mottled liver and gill damage. External symptoms include sluggish behaviour, spiral or erratic movement, damages in the gill and eyes, white and/or dark nodules on the gills and/or skin, fin rot and hemorrhaging at the base. Of the bacterial infection, pathogenicity caused to penaeid shrimps is mainly by vibrios, especially by *V.harveyi*, the diseases commonly referred to as luminous vibriosis. External signs of *V.harveyi* infection in diseased prawns include brittle shells, brown or black spots on the shells, darkened or red body surfaces, pink or brown gills, murky whitish muscle, lack of food

in the midgut and folded base of the tail (Lavilla-Pitogo *et al*., 1990, Adams, 1991). Affected larvae develop luminescence, reduced feeding, show sluggish swimming, reduced escape mechanisms, degeneration of hepatopancreatic tissues, formation of necrotic bundles and increased mortality (Robertson *et al*., 1998).

Several approaches have been proposed to increase aquaculture production, by improving nutritional quality of feed, repress the growth if pathogens in rearing environment (Nogami and Maeda, 1992), treatment with UV, use of nonspecific immunostimulants or vaccines (Anderson, 1992), phage therapy and probiotic bacteria to exclude or inhibit pathogens (Gatesoupe, 1999). The frequent use of high concentrations of these antibiotics poses significant disadvantages like the development of resistant strains and accumulation of antibiotics in crops, thereby causing problems regarding food safety. Antibiotics pose serious threats to human health, by transmitting the resistant microbes from animals to man via the food chain. The most promising prophylactic measure is the use of beneficial or probiotic bacteria (Dalmin *et al*., 2001). However, several screening methods and field trials are required to select the most appropriate probiotic. Other alternatives are the use of immunostimulants and vaccines which activates the immune system of animals imparting resistance to infections caused by viruses, bacteria, fungi and parasites. Bacterial capsule and extracellular products serve as essential protective antigens, and are effective candidates for vaccine preparations. Bacteriophages are thought to play a major role in the regulation of bacterial population in aquatic environments. Phages are the natural enemies of bacteria, and can be used for biocontrol without interfering with the natural microflora or the cultures in fermented products. The efficacy of phage preparation as therapeutics is ceased by the ever increasing use of antibiotics. The need of the hour is to develop new approaches to control the disease causing pathogens, which are

cost-effective, ecologically sustainable, industrially durable and safe to administer.

Major problem is the diagnosis of pathogenic vibrios from the non-pathogenic benevolent counterparts and eliminating them from the aquaculture systems. However, the hurdles in identification of these environmental isolates are the elevated levels of phenotypic heterogeneity amidst vibrios, inappropriate routine methods of analysis and biochemical variability amongst the species (Vandenberghe *et al*., 2003). The phenotypic heterogeneity is further mystified by the evidence that some vibrios harbor mobile genetic elements, plasmids and bacteriophages that influence phenotypic characteristics (Munro et al., 2003) leading to continuous revision of the taxonomy of *Vibrio*. Lateral gene transfer (LGT) can result in anomalous placement of a particular taxon, as a result of homologous recombination occurring at intraspecies (*recA*, *dnaE* in *V.cholerae*), interspecies (*asd* from *V.mimicus* to *V.cholerae* non-O_1) and intergenera (*gmd* from *E.coli* to *V.cholera*e O139) levels. New species are being added based on the information gained using various molecular tools which establishes highly informative measure of intra and interspecific genomic relatedness between strains; enabling reproducible and stable classification (Sawabe *et al*., 2007). Nevertheless, numerical taxonomy of a family, genus or species has its own importance in phenotypically grouping the isolates.

Various virulent factors expressed by vibrios such as adhesion, colonizing factors, extracellular proteases and protective antigens promote their pathogenecity (Austin and Zhang, 2006). A comprehensive search for virulence factors among vibrios revealed unequivocally the role of proteases, lipases, chitinase and plasmid coding for iron chelators apart from haemolysins in initiating an infectious death (Reid *et al*., 1980, Nottage and Birkbeck, 1987). Non pathogenic and benevolent forms have also been identified to co-exist as part of the natural flora amidst the large number of pathogenic forms. These non- pathogenic forms are essential for nutrient

cycling, degradation of complex molecules such as chitin. Use of antibiotics, chemotherapeutics etc, have broad spectrum of activity and they are not capable of targeting the pathogenic forms specially and killing them. The treatment with the above mentioned agents kills even the beneficial non-pathogenic forms. Therefore a foolproof diagnostic system to differentiate pathogens from non-pathogenic ones is essential.

Review of Literature

1.1. Distribution of vibrios

Vibrios are widely distributed in aquatic environments from brackish to deep sea waters, commonly found associated with marine organisms and as the important pathogens to farmed animals and human consuming contaminated seafood grown in polluted waters. Vibrios are thought to evolve from marine environments as they require sodium as an important growth factor. Vibrios are frequently detected in summer than winter, probably because they enter into viable but non culturable (VBNC) stage (Barer *et al*., 1993). Vibrios are frequently found in the digestive tract and on the skin of marine animals. The composition of bacterial population in digestive tract of marine animals differs from that of the surrounding environments, as magnitude of nutrients available in the animal gut is much higher than the surrounding seawater. Healthy *L.vannamei* harbored 10^4 to 10^5 vibrios/g tissue in the hepatopancreas (Gomez-Gil *et al*., 1998), showing that vibrios also exhibit symbiotic association with the host species. Vibrios attach preferentially to substrates, whereby they colonize and establish themselves. *V.alginolyticus* carries chitin-binding proteins enabling it to adhere to chitin surfaces of copepods and colonize.

Distribution of vibrios in freshwater environments is sparse, as salinity acts as a limiting factor. Vibrios require sodium ions for Na^+- proton antiports in the energy-transducing cytoplasmic membrane, to maintain cell membrane and cell wall integrity. Some vibrios such as *V.cholerae* can

survive in low salinity, making use of organic nutrients or divalent cations instead of Na^+. Isolates belonging to family Vibrionaceae obtained from seasonally cold coastal waters indicated variations in morphotypes compared to the other vibrios. This suggests that a large genetic difference in species composition exist among vibrios isolated from seasonally cold or permanently cold environments and their normal counterparts. Distribution and dynamics of *Vibrio* populations are influenced by the biotic and abiotic environment, ecosystems with optimal temperature, salinity, nutrient flow, abundance of host organisms and limited predation stress (Ben-Haim *et al*., 2003. Heidelberg *et al*., 2002b)

1.2. Taxonomy of vibrios

1.2.1 Phenotypic characterization of vibrios

Prokaryotic taxonomy deals with the classification (taxa description), identification (strain allocation) and nomenclature of the isolates (Vandamme *et al*., 1996). Taxonomy of microbes has a sound framework enabling stable, predictable and informative observations. Vibrios are important inhabitants of the riverine, estuarine and marine environments. Vibrios have received the attention of marine microbiologist when majority of the cultured bacterial populations in near-shore waters and those associated with fish and shellfishes were predominantly *Vibrio* spp. (Liston, 1954). The taxonomic group of Vibrionaceae is extremely diverse and can be traced back to the beginning of prokaryotic taxonomy, as vibrios were the first groups of microbes recognized in nature by Pacini, (1854). Shared characteristics of vibrios include NaCl concentration for growth, chitin digestion, morphological features and fermentative metabolism. Phenotypic heterogeneity amidst *Vibrio* spp. make their identification extremely difficult and time consuming especially when conventional bacteriological tests or kits which rely fully upon the phenotypic characters are employed (Vandenberghe *et al*., 2003; Alsina and Blanch, 1994a, b).

Currently the family Vibrionaceae has eight genera: *Vibrio*, *Allomonas*, *Catenococcus*, *Enterovibrio*, *Grimontia*, *Listonella*, *Photobacterium* and *Salinivibrio*.

Advent of various molecular tools has resulted in identification of new species based on the sequence information of the house keeping genes including *16S rRNA*, *recA*, *rpoA*, *gyrB*, *gapA*, *ftsZ*, *mreB*, *pyrH*, *toxR*, *23S rRNA* and 16S–23S intergenic spacer region (IGS) (Sawabe *et al*., 2007). Genetic markers that are unique to a species such as the virulence-associated genes, conserved gene primers and/or probes can be used to quantify the expressed gene and also to determine the taxonomic position. Numerous methods including ribotyping, RFLP, AFLP, RAPD, AP-PCR, ERIC-PCR, PFGE and MLSA are developed for typing and differentiating strains within the same species. Sequencing of the molecular chronometers such as the *5S* and *16S rRNA* has revolutionized prokaryotic taxonomy (Thompson *et al*., 2005). Additional phylogenetic markers within the 50-100 genes in the bacterial core genome are analyzed to complement the phylogenetic information obtained using the molecular chronometers (Harris *et al*., 2003). It has been shown that a polyphasic approach based on phenotypic, chemotaxonomic and genomic data, improves bacterial taxonomy and classification (Vandamme *et al*., 1996) of the genus *Vibrio*. This will most probably increase the number of species in future, as the genus has many new species that are still undescribed (Pedersen *et al*., 1998; Urakawa *et al*., 1999a, b; Thompson *et al*., 2001). Nevertheless, numerical taxonomy of a family, genus or species has its own importance in phenotypically grouping the isolates. Bacterial taxonomy could be performed by sequencing the whole genome, but it is not feasible yet, however, application of MLSA (Multi Locus Sequence Analysis) is a better step towards positioning of vibrios into the varied taxa (Sawabe *et al*., 2007).

1.2.2. Genomic Characterization of vibrios

The complete genome sequencing revealed that genus *Vibrio* possesses two circular chromosomes, a large chromosome and a small chromosome. The presence of two chromosomes is common among *Vibrionaceae*, but many do not extend to other families outside this group such as *Aeromonadaceae* and *Enterobacteriaceae*. Okada *et al*., (2005) suggested that all vibrios have two chromosomes and none of the isolates till date has one chromosome. The presence of essential genes on both the chromosomes, suggests that the small chromosome is an indispensable part of these bacteria (Heidelberg *et al*., 2000; Makino *et al*., 2003). The spilt of the genome into two replicons is advantageous for those bacteria where DNA replication takes place every 8-9min, as in the case of *V.parahaemolyticus* (Joseph *et al*., 1982). The large chromosome contains genes required for growth, while the small chromosome contains more genes involved in bacterial adaptations to environmental changes, transcriptional regulation and genes coding for transport of various substrates than the large chromosome (Heidelberg *et al*., 2000; Makino *et al*., 2003). The small chromosome is thought to have arisen from the large ancestral genome by a single excision (Waldor and Raychaudhuri, 2000). The distribution of functional genes between the large and small chromosomes of vibrios suggests how the two-chromosomal configuration mediates various functions in the organisms and confers evolutionary advantages. The large chromosome contained all the rRNA operons and atleast one copy of all tRNAs, while the small chromosome has intergrons and the third part is the plasmid (Mazel *et al*., 1998). Examination of the chromosome size in different *Vibrio* species demonstrated that, the size of the large chromosome remained almost stable when compared to the small chromosome, which was variable (Okada *et al*., 2005).

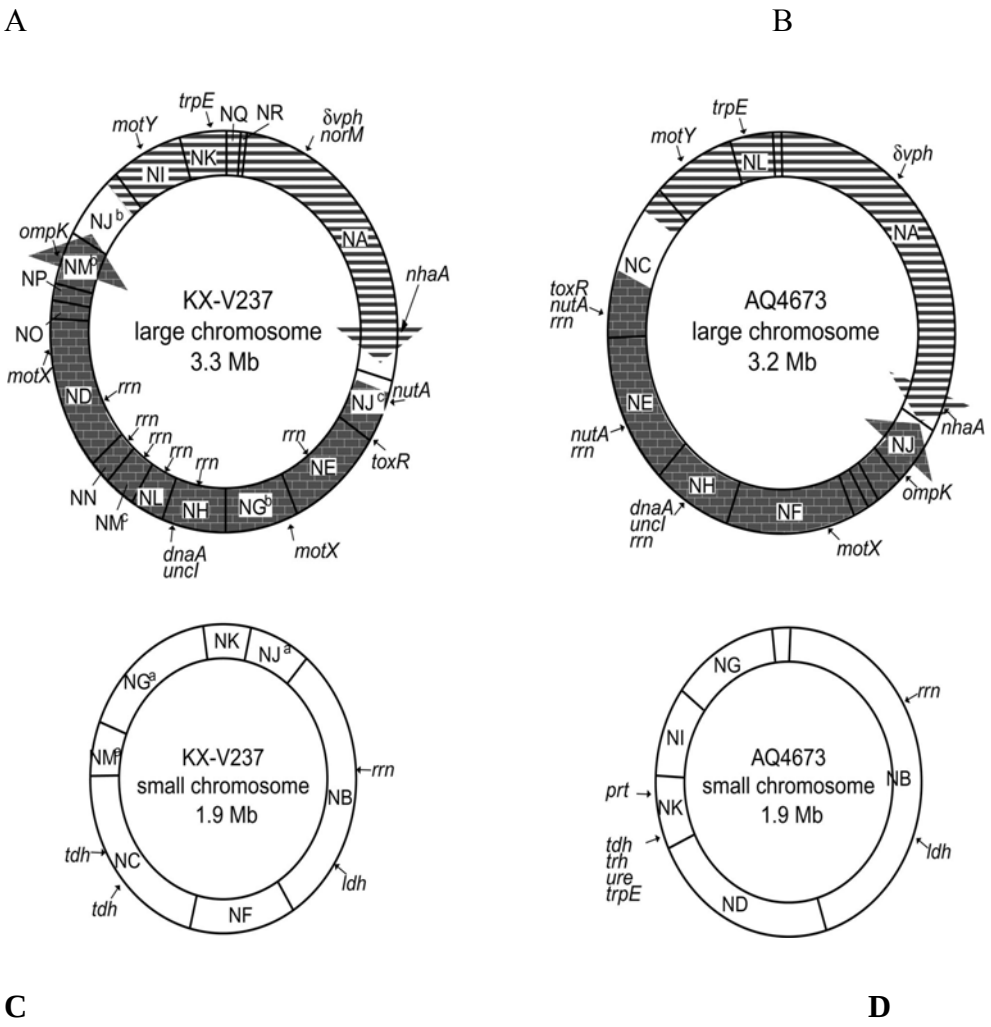


Fig 1.1: Comparison of the large and small chromosome of *V.cholerae* (A, B) and *V.parahaemolyticus* C, D (Okada *et al.*, 2005)

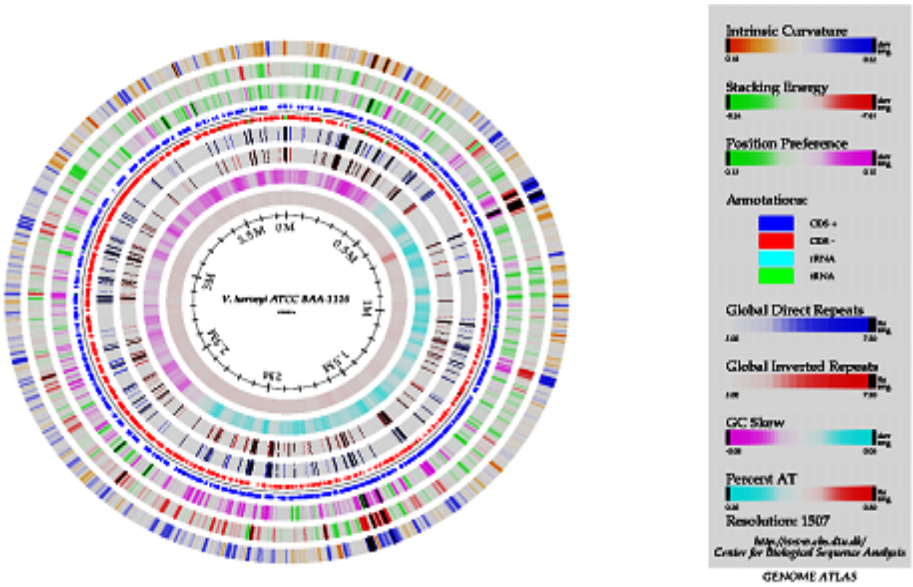
The size of the large chromosome except for a few strains, clustered at the range of 3 to 3.3Mb, where as that of the small chromosome varied considerably from 0.8 to 2.4Mb, suggesting that the small chromosome is more flexible. The large chromosomes of *V.parahaemolyticus* and *V.cholerae* are 3.4 and 3Mb, respectively, where as the small chromosomes are 1.9 and 1.1Mb, which suggest that the small chromosome has high proportions of genes unique to each *Vibrio* species (Makino *et al.*, 2003).

Both the chromosomes undergo extensive genome rearrangement, however, the location of the conserved regions of either chromosome remains unaltered; suggests interchromosomal rearrangements are less frequent than intrachromosomal rearrangements in *Vibrio* evolution. This view proposed that the ancestral *Vibrio* was diversified into various species retaining the most essential genes in the large chromosome.

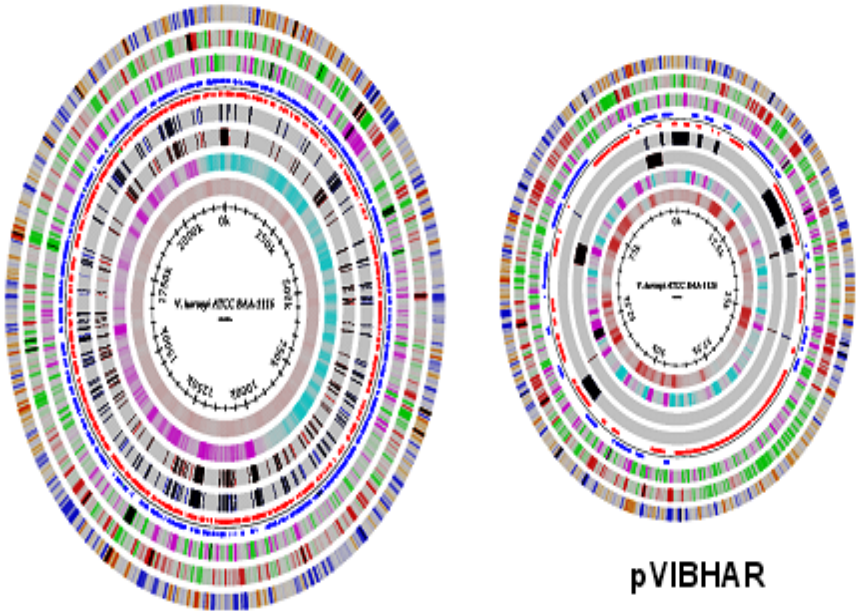
The genome of *Vibrio harveyi* has been sequenced to 8X coverage using a combination of plasmid and fosmid end sequences. The genome has undergone automated sequence improvement (pre-finishing) followed by manual finishing, and automated annotation. The National Science Foundation (NSF) provided funding for the complete sequencing of *Vibrio harveyi* genome. The genome consisted of two chromosomes (Chromosome I and II) and a plasmid (pVIBHAR).

Table 1.1: Details of *V.harveyi* Genome

Characters	**Chromosome I**	**Chromosome II**	**Plasmid (pVIBHAR)**
Accession No:	NC009783	NC009784	NC009777
Basepairs	37,65,351bps	22,04,018 bps	89,008 bps
Genes	3706	2411	120
Proteins Coding	3546	2373	120
Structural RNA	143	24	0
tRNA count	105	16	0
5S rRNA	10	1	0
16S rRNA	10	1	0
23 S rRNA	9	1	0
Pseudogenes	17	14	0
Others	23	11	0
GC content	45.5	45.3	43.8
% Coding	85%	86%	79%



V.harveyi chromosome I



V.harveyi chromosome II

Fig 1.2: Genome map of *V.harveyi*

(genome.wustl.edu/genomes/detail/*Vibrioharveyi*)

1.2.3. Serological characterization of vibrios

Immunological methods for detection of pathogen have been one of the powerful tools used in human and veterinary medicines and application of this technique has percolated into aquaculture also. Serological techniques are used for preparing standard antigens, to obtain antiserum for specific use, purification and labeling of antibodies, which are used in the diagnosis of several diseases and determining the serological properties of major pathogens. Scope and application of these techniques are very high but has to be developed carefully to meet the requirements of aquatic systems. The type and specificity of antibodies produced are direct reflections of the antigens used to produce them. The internal soluble antigen of isolates from the same species tend to be similar but the outer membrane proteins, lipopolysaccharides and capsular antigens tend to be variable (Caugant *et al*., 1988), that some induce the formation of neutralizing antibody while others induce only binding antibodies.

Serological methods using antibodies targeting the flagellar H (Chen *et al*., 1992) and LPS (Grisez and Ollevier, 1995) antigens have been developed for the rapid identification of certain pathogenic vibrios. The outer membrane protein-OmpK has been considered as a vaccine candidate for the prevention of infections due to *Vibrio harveyi, Vibrio alginolyticus* and *Vibrio parahaemolyticus* in fish. Polyclonal antibody raised against the recombinant OmpK from *V. harveyi* could recognize the OmpK homologues from other strains of *Vibrio* species by immunoblotting. Orange-spotted groupers vaccinated with recombinant OmpK were more tolerant to infection by virulent *Vibrio* strains and their relative percentage survival (RPS) was correlative with the degree of the identity of deduced amino acid sequences of their OmpK (Zhang *et al*., 2007). OmpK is a conserved protective antigen among tested *Vibrio* species and might be a potential vaccine candidate for the prevention of infections caused by *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus*.

Polyclonal antibody based immunodiagnostic kits for detection of different bacteria (*Aeromonas hydrophila, Pseudomonas fluorescens, Vibrio alginolyticus and Edwarsiella tarda*) in finfish and shellfishes have been developed. However, polyclonal antisera have limitations in terms of cross-reaction, lack of specificity and inability to discriminate antigen at epitope level, and hence monoclonal antibodies (MAbs) are preferred.

Monoclonal antibodies are sensitive to detect antigens at picogram level and scope for false positive is very less as the antibodies detect the existing copies of antigens. Monoclonal antibodies are used for development of simple, rapid and cheap field level tests such as immunoblot for use by the farmers with little training and with simple gadgets. The test is sensitive mostly at 500 picogram level, requiring a detection time of 3hrs for completion, however, it can vary based on the samples. Monoclonal antibodies (MAbs) that recognized distinct species-specific antigenic epitopes which included O-antigens from *Vibrio anguillarum* O2, O2a and certain O2b strains (MAb 7B4) and from *Vibrio ordalii* strains (MAbs A16 and 7D11) were generated. The generated MAbs that react with O-antigens from *V. anguillarum* serotype O1 (MAbs 7B8, 7B5 and 1C3) and serotype O3 (MAbs 13A1 and 14C5) strains (Mutharia and Amo, 2002). These MAbs provide rapid and accurate diagnostic reagents for serological differentiation of *V. ordalii* from serotype O2 strains of *V. anguillarum* (Mutharia and Amo 2002). Monoclonal antibodies (MAbs) developed against four different *Vibrio* spp. that infect humans, fish and shellfish (Phianphak *et al*., 2005), were tested for their potential application in immunohistochemistry (IHC). Six MAbs (VH1, VH2, VH3, VH4, VH5 and VH6) produced against *V. harveyi* ATCC 14126 were selected. MAb H5 raised against *V. harveyi* ATCC 14126 reacted with all four *Vibrio* spp. as well as against all the *V. harveyi* strains and these were also recognized by MAb H4 and H6. However, MAb H5 recognized 13.5 – 14 kDa bands on Western blot that were not present in the SDS PAGE for the different

Vibrio. More strains of *V. harveyi* from different origins together with non – *Vibrio* species needed to be tested, but it was realized that additional MAbs against *V. harveyi* were necessary to specifically detect all isolates of *V. harveyi* (Phianphak *et al*., 2005).

Plate and dipstick enzyme-linked immunosorbent assays (ELISA) were developed for the rapid detection of *Vibrio harveyi* from penaeid shrimp and water. The ELISA, which incorporated a polyclonal antiserum produced in a female New Zealand white rabbit, detected 10^5 cells of *V. harveyi*/ml. Also, the systems detected *V. harveyi* in water from Chinese shrimp hatcheries. The systems permitted the recognition of a wide range of *V. harveyi* isolates, but not those of other taxa. Western blot analysis of bacterial outer membrane proteins (OMP) indicated that epitope was recognized, with many immunoreactive bands in common between isolates of *V. harveyi* (Robertson *et al*., 1998).

1.3. Evolution of vibrios

Variety of events including mutations, chromosomal rearrangements, loss of genes by deletion, gene acquisitions through duplication or lateral transfer are the driving forces for evolution and diversification of bacteria (Makino *et al*., 2003, Hacker *et al*., 2003).These factors allow the best adaptive response of the cell within its natural environment (Coenye *et al*., 2005), also help in tracing bacterial genomes and reconstruction of evolutionary relationships. Mobile genetic elements and lateral or horizontal gene transfer are efficient mechanisms to introduce new phenotypes into bacterial genome (Kurland *et al*., 2003). Gene duplication involves mechanistic antecedent of gene innovation, leading to genetic novelty, facilitating adaptation to changing environments and exploiting new niches (Hooper and Berg, 2003). Gene duplication and consequent functional divergence are considered as important evolutionary steps, leading to adaptive radiation and broadening the phenotypes.

An important feature of *Vibrio* genome is the presence of superintegrons. Integrons are natural cloning and expression systems that constitute transferable elements responsible for evolution mainly of multidrug resistance (Rowe-Magnus *et al*., 2002a, b). Integrase (*intI*) mediates the recombination between a proximal primary recombination site (*attI*) and a target recombination sequence, called the *attC* site (59bps), found associated with a single open reading frame, organized as a circular site termed the gene cassette. Insertion of gene cassette at the *attI* site drives the expression of the encoded proteins. A comparison of the superintegrons of *V.cholerae* and *V.parahaemolyticus* revealed that there is substantial difference between the two gene cassettes (Makino *et al*., 2003), suggesting that the superintegrons are highly diverse between *Vibrio* species. Chromosomal superintegrons of *Vibrio* might be a genetic source leading to the evolution of resistance to clinically relevant antibiotics through integron-mediated recombinant (Rowe-Magnus *et al*., 2003). Comparative analysis of the integron integrases, shows that they clearly group together and form a specific clade (Rowe-Magnus *et al*., 2003). Also all integron Integrase contain a stretch of species specific 16 amino acids located between the conserved patches of tyrosine recombinase family (Messier and Roy, 2001; Nield *et al*., 2001). Integrons are ancient structure steering evolution by species-specific clustering of the superintegron genes among the bacterial population. This mechanism is seen in vibrios, as systems of gene cluster enabling bacterial adaptation and is termed as *Vibrio* radiation. Comparison of the gene cassette contents between different *Vibrio* species indicates that majority of the cassettes are unique to the host species (Rowe-Magnus *et al*., 2003). Extensive polymorphism is observed among closely related isolates, suggesting plasticity for these structures and their microevolution through massive Integrase-mediated gene acquisition or loss and cassette rearrangement. Comparison of the nucleotide sequence of vibrios shows that the Integrative and conjugative elements (ICEs) contain conserved set of genes that mediate regulation, excision, integration and conjugative transfer

of the respective ICEs (Beaber *et al*., 2002a). The proficiency of partnership of integrons and mobile DNA elements is confirmed by marked differences in codon usage among cassettes within the same mobile integrons, indicating that the genes are of diverse origin.

Genomic islands are large DNA regions acquired by lateral gene transfer and inserted into the host chromosomes. The exact character can vary from one island to another, but usual features include insertion near to a tRNA gene, presence of insertion or prophage like elements, flanked by direct repeats and the presence of Integrase gene. Most genomic islands identified in vibrios include virulence related gene cluster, termed as *Vibrio* pathogenic island (VPI). VPI contains pathogenic islands (PAIs) which are regions of bacterial genome, between 10-200kb in length, having characteristic feature of transposable elements, insertional sequence, parts of phages but differing in G+C content and codon usage when compared to the remaining genome. Generation of PAIs often starts with the integration of plasmids, phages or conjugative transposons into specific target genes (tRNA genes), preferentially on the chromosomes (Kaper and Hacker, 1999). On integration into the bacterial genome, these inserted elements experience multiple genetic events, such as mutations, deletions and insertions of genes under specific selective pressure, before resulting in the formation of PAIs (Kaper and Hacker, 1999). Sequencing the PAI genome revealed that this region is widespread in the bacterial genome, offering evolutionary advantage. PAIs and superintegrons score over mutations in bacterial evolution, as entire gene clusters or operons are transferred and incorporated in the host genome, resulting in a dramatic change in the host behavior (Groisman and Ochman, 1996).

Plasmids are diverse in vibrios and are used in differentiation of strains within a species, by a technique called Plasmid profiling. *Vibrio* plasmids vary in size ranging between 0.8 to 290kb, even within a single serogroup of a specific species. The frequent identification of prophage

DNA in *Vibrio* chromosomes shows how widespread are plasmids in microbes, enhancing the chances of mobilizable plasmids along with their conjugative counterparts. On a smaller evolutionary scale, intraspecific and interspecific homologous recombination takes place between vibrios. The number of nucleotide substitutions caused by recombination versus point mutation is in the ratio 3:1, influencing the microevolution of *Vibrio* genome.

VHML (*V.harveyi* Myovirus like) infected *V.harveyi* are not able to hydrolyze L-glutamic acid 5-(4-nitroanilide), indicating a lack of operational glutamyltranspeptidases, whereas the uninfected strains could hydrolyze this compound. The variability in phenotypic profile indicates that VHML integrates into the host genome and causes changes in the phenotypic profile of the organism, causing the misidentification of *V.harveyi* isolates (Vidgen, 2006).

The evolution of virulence in mutualistic associations: Symbiosis among Vibrionaceae occurs with many marine host species, especially in vibrios colonizing crustacean (Bowser *et al*., 1981), mollusc (McFall-Ngai, 2002), or fish hosts (Schiewe *et al*., 1981; Wiik *et al*., 1989; Toranzo and Barja, 1990). Although a number of these pathogenic vibrios have common physiological attributes, it has always been a question of whether virulence or virulence factors (i.e., pathogencity islands) were common among the symbionts. Investigations assaying biochemical features (Lunder *et al*., 2000), iron sequestration (Tolmasky *et al*.,1985), and plasmid profiling (Sorum *et al*., 1990) grouped many of the pathogens together, according to their specific hosts that they infect. Although this may provide a "common ground" for all species studied, 5S and 16S rRNA molecular data provide evidence that most of these alliances are not robust (Wiik *et al*., 1995) and the pathogenic species of *Vibrio* are not monophyletic. This is probably due to the fact that most phenotypic characters are more likely to place species

or species groups according to the type of habitat and the abiotic factors that influence the phenotype of that particular species or strain (Cohan, 2002).

1.4. History of *V.harveyi*

V.harveyi was first described as species of *Acromonobacter* by Johnson and Shunk in 1936. Later on this bacterium was grouped along with other luminescent bacteria under the name *Beneckea harveyi*. In 1981, Baumann *et al*., abolished the names *Beneckea* and *Lucibacterium* and transferred it into *Vibrio* based on its characteristic shape. With the advent of large-scale prawn culture, *V.harveyi* got attention as a shrimp and prawn pathogen, particularly in tropical areas. *V.harveyi* is very closely related phenotypically and genotypically to *V.carchariae* that the latter strain is considered as a junior synonym of *V.harveyi* by Gauger and Gomez-Chiarri (2002). Great diversity of *V.harveyi* poses certain difficulties in the biochemical determination and identification of environmental vibrios. Identification and typing of *Vibrio* strains using genomic approaches and ribotyping are useful for taxonomic studies and identification to the subspecies level (Austin *et al*., 1995). The two central members of the *Vibrio* core group which are closely related include *Vibrio campbellii* and *Vibrio harveyi* which are known to thrive in similar environments and share a high degree of genetic and phenotypic similarity. *V. harveyi* strain CAIM 1792 provides important insights into the metabolic capability, pathogenicity and genetic plasticity of each and aid in adjusting the attribution of certain characteristics (e.g. bioluminescence, obligate organoheterotrophy) that have previously been used to define *V. harveyi* and *V. campbellii*.

Outbreaks of vibriosis have been reported worldwide, however, *V.harveyi* causes disease in a variety of aquatic organisms, including marine fish, bivalves and crustaceans. Infections in fish are mostly as opportunistic pathogen or through stress in captive environment than report of disease in

invertebrates. Most *V.harveyi* strains are not harmful to larvae of *P.monodon*; however, some strains are extremely pathogenic. Symptoms exhibited by *V.harveyi* on fishes include anorexia and darkening of the whole fish, along with appearance of local hemorrhagic ulcers on mouth or skin surface and focal necrotic lesion in the muscle or eye opacity. Lavilla-Pitogo *et al.* (1998) reported epizootic of luminescent, non-sucrose-fermenting *V.harveyi* in larvae of *P.monodon* in Philippines. Luminous vibriosis is the widely used term for mortality caused by *V.harveyi* in penaied prawns. *V.harveyi* enters the larval prawn through mouth and feeding apparatus, and usually found colonizing the oral cavity of the larvae (Lavilla-Pitogo *et al*., 1990). Infections caused by *V.harveyi* are usually septicemic, with the pathogenic agent being isolated from the hemolymph and hepatopancreas of infected animals (Liu *et al*., 1996 a, b). Increase in amounts of organic matter in ponds, tanks and use of contaminated equipment between ponds are the probable factors for *V.harveyi* spread. Aerosol transmitted contamination by *V.harveyi* of Marine algal cultures given as feed, Artemia cysts carrying *V.harveyi* or from cross contamination from workers hands or equipment are considered as other causes of vibriosis (Owens, 2006). The ability of *V.harveyi* to utilize a wide variety of organic compounds as carbon and energy source aids the survival of this specie when competing for scare nutrients present in the marine environments (Ramesh *et al*., 1989). Variation in environmental conditions of the susceptible host, particularly when raised under intensive cultures with cold temperatures, overcrowding and inadequate water circulation, facilitate the outbreak of the disease, thereby causing massive destruction to aquaculture industries.

1.5 Bacterial Adaptations

1.5.1. Biofilm formation

Majority of bacteria have the biofilm forming property, which involves the assemblages of bacteria on a surface encased by an

extracellular matrix, rather than as free-swimming entities (Costerton *et al*., 1978). Bacteria within the biofilm show increase in resistance and metabolic efficiency of the population, compared to their planktonic counterparts to variety of stresses, including UV, acidic conditions, dehydration, oxidative environment and antimicrobial agents (Jefferson, 2004). Biofilm-mediated attachments to abiotic and biotic surfaces are important for survival of *Vibrio* spp. Most vibrios show attachment to copepods, crustaceans, insects, plants and filamentous green algae using the property of biofilm formation (Hood and Winter, 1997; Bourne at al., 2006). The ability to attach to external and mucosal surfaces is an important virulence determinant of bacteria. Protozoan grazing is identified as one of the key biotic pressures faced by bacteria, which is overcome by the formation of microcolonies or flocs. *Vibrio* species may use marine animals as vehicles for survival when encountered with protozoan grazing pressure.

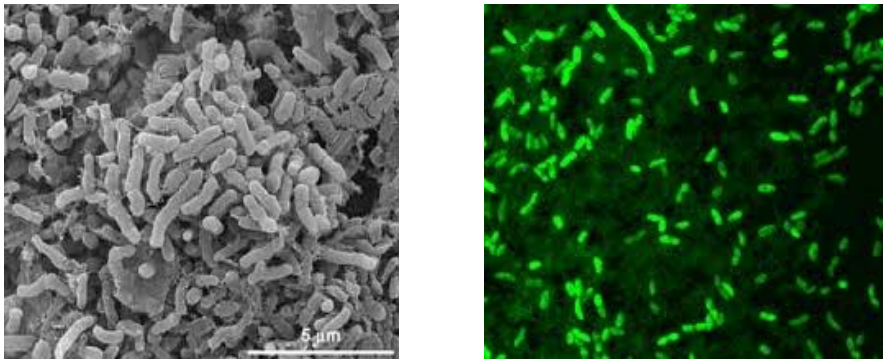


Fig 1.3: Bacterial assemblage for Biofilm formation (www.scoopweb.com)

In response to this pressure, bacterial communities develop inedible phenotypes, referred to as grazing-resistant varieties; this adaptation brings about profound changes in the structural and taxonomic position of the communities (Matz *et al*., 2002b). Protozoan grazing is considered as one of the selective forces in evolution of pathogens, as bacteria develop various virulence factors as adaptive measures to protect themselves against predation.

1.5.2. Capsule and EPS

The production of capsule and *Vibrio* exopolysaccharides (EPS) are of relevance during infection and resistance to environmental stresses (Costerton *et al*., 1978, 1981). The opaque or rugose cells are more resistant to infection compared to their translucent or smooth counterparts. The capsule in the rugose cells helps to evade phagocytosis and switch to the smooth stage for dispersal and colonization of new sites. Vibrios have the ability to switch from encapsulated to uncapsulated morphotypes based on the environmental niches they occupy. In addition to the genes necessary for the capsule and EPS production as response to varied environment, these genes are also involved in biofilm formation (Kierek and Watnick, 2003a). Elevated level of intracellular cytidine leads to increase in EPS production and thus biofilm formation. Quorum-sensing (QS) regulates biofilm formation and influences attachment to biotic surfaces in a number of *Vibrio* species (Hammer and Bassler, 2003). QS repression by HapR, flagellum-regulated repression of EPS and increased EPS regulation are seen in rugose morphotypes. The presence of multiple signaling pathways for regulating EPS and biofilm formation indicates that different pathways operate in diverse environments or selection of different strains occurs under certain conditions (Heithoff and Mahan, 2004). Vibrios are found to possess mannose-sensitive hemagglutinin (MSHA) pilus which enables their attachments to cellulose, but was not required for biofilm maturation. Vibrios have similar or overlapping mechanisms regulating attachment to chitin and other surfaces in seawater favoring bacterial colonization. Evolution of new phenotypic traits enhances the attachment and colonizing behavior, surreptitiously increasing the ability of the bacteria to invade host organisms.

1.5.3. Starvation adaptation mechanism

Vibrios exhibit an elaborate and highly developed starvation adaptation mechanism, by altering the gene expression as well as physiological changes for survival in unfavorable conditions (Kolter *et al*., 1993). Vibrios adapt to starvation stress by reducing its cellular volume, DNA and ribosomal content and the rate of protein synthesis (Ostling *et al*., 1993). First stage of starvation adaptation is governed by the accumulation of guanosine 3'-diphosphate 5'diphosphate (ppGpp), followed by the shutdown of macromolecular synthesis, increased rate of protein degradation and reorganization of cellular components (Cashel *et al*., 1996). Second stage is the decrease in ppGpp and increase in the macromolecular synthesis, followed by shifts in fatty acid composition of the membrane, degradation of reserve materials and activated resistance development towards a variety of stress (Wong and Wang, 2004). Third phase again involves the gradual decline in macromolecular synthesis and metabolic activities, such as endogenous respiration, modification to tolerate and survive in stressed environment until the emergence of favorable conditions. During starvation, specific proteins related to peptide chain elongation, protein folding, carbon metabolism and stress resistance exist in oxidized state, leading to the formation of aberrant proteins owing to microincorporation of aminoacids (Dukan and Nyström, 1999). Reduction of translation accuracy is caused by ribosomes which are starved for the cognate tRNAs, resulting in protein degradation in starved cells (Nyström, 2004). Starvation induced proteins (Sti) are synthesized in the initial starvation phase, as these proteins offer protection against external stress such as heat, osmotic stress and oxidation (Dukan and Nyström, 1999). Thus, making the starved cells resistant to a variety of stresses is termed as starvation induced cross protection (Jenkins *et al*., 1990).

Vibrios can tolerate carbon shortage for a month or longer, making use of the carbon stored in the inclusion bodies as reserve of glycerol or

poly 3- hydroxybutyrate. Carbon limitation and hike in cAMP levels stimulate protease activity in vibrios, mediating both detachment from surfaces and penetration into mucus layers during tissue colonization (Benitez-Nealson, 2000). Carbon starvation results in both energy and nutrient limitations, while nitrogen and phosphorus starvation do not cause cessation of growth. Bacteria still continue to grow, utilizing the intracellular reserve of nitrogenous polymers (Mason and Egil, 1993). Similarly, inorganic polyphosphates is essential for adaptation to stress and survival in stationary phase (Rao and Kornberg, 1996). Starvation induction is mediated by several regulators including σfactor, RpoS in many species (Lange and Hengge-Aronis, 1991). Bacteria have evolved complex mechanisms to cope up with the environment induced stress, characterized by changes in gene expression, physiology and morphology.

1.5.4. Viable but nonculturable response (VBNC)

Vibrio spp. during prolonged unfavorable conditions enter a stage where the cells become incapable of undergoing cellular division on the normal growth supporting media but remain metabolically active (Oliver, 1993; Rice et at., 2000). During environmental stress such as starvation, salinity variations, variations in visible light and/or temperature differences, bacteria enter the VBNC state (Lee and Ruby, 1995). VBNC cells have a thickened periplasmic space to resist heat, cold or desiccations. However, bacteria exhibiting loss of cultivability and reproducibility under stress conditions revert to normal state breaking the period of dormancy when the conditions become favorable. Stasis is caused by a variety of conditions that induce the expression of regulators involved in the prevention and repair of damages caused to cellular components. VBNC population exhibits a decrease in superoxide dismutase activity, resulting in an increase in oxidative damage and induction of stress regulons, such as those regulated by RpoS and RpoE.

1.5.5. Other adaptations:

Vibrios are well adapted to live in the gut of marine animals, establishing themselves in the hepatopancreas, hemolymph and digestive tract. Vibrios have developed mechanisms for tolerating low pH, secreted bile acids and anaerobic environments. Once inside the gut, vibrios colonize the gut of the host by overcoming and adapting itself to the host defense mechanisms, especially those preventing bacterial invasion and growth. High substrate affinity of vibrios suggests adaptation to growth under high-nutrient conditions occurring in host gut or in planktonic microenvironments. Respiratory activity under low-nutrient conditions in seawater mesocosms, indicates long term survival of vibrios in substrate limiting environments (Armada *et al*., 2003). Maintenance of high ribosomal content after shift from starvation stress enables a rapid growth in response to favorable conditions (Pernthaler *et al*., 2001). Chemotaxis towards chitin, sugar monomers, amino acids and response to limited concentration of carbon, indicates the ability of vibrios to exploit nutrient-rich microenvironments (Bassler *et al*., 1991; Larsen *et al*., 2004). ToxR and to a lesser extend ToxS enhance resistance of *Vibrio* to bile, and bile in the growth medium increases expression of OmpU, which helps vibrios to tolerate high bile concentration in the host (Wang *et al*., 2003).

1.6. Virulent Factors of vibrios

Vibrio spp. show great variation in terms of pathogenicity associated with host species, its developmental stage, bacterial dose, bacterial species and particular strains, and exposure time and stress (Lightner, 1996; Saulnier *et al*., 2000a; Aguirre-Guzmán *et al*., 2001).

1.6.1. Extracellular products

Different *Vibrio* extracellular products (ECP) have been identified and proposed as putative virulence factors in the species pathogenic to

shrimp (Liu *et al*., 1996, 1997; Lee *et al*., 1997a, 1999; Chen *et al*., 1999, Harris & Owens, 1999; Montero & Austin, 1999). A thermo-labile cytotoxic factor was detected in the ECP from *V. penaeicida*, which produces 100% mortality in juvenile *Litopenaeus stylirostris* (Goarant *et al*., 2000). Proteolytic enzymes, such as cysteine and serine proteases, metalloproteases, and hemolysins, have been isolated from *Vibrio harveyi*, *V. anguillarum*, and *V. alginolyticus* (Lee *et al*., 1997a; Harris & Owens, 1999). *V. harveyi* produces an extracellular 38 kDa protein with protease, phospholipase, and hemolytic activities for *Penaeus monodon* (Liu *et al*., 1997). Zinc metalloprotease Emp, secreated as a 48kDa proenzyme is implicated as a virulence factor in *V.anguillarum* (Staroscik *et al*., 2005). *V.harveyi* produces proteases, phospholipase, hemolysins or exotoxins important for pathogenicity (Liu *et al*., 1996). Bacterial haemolysin has been suggested as an important virulent factor of pathogenic vibrios (Chang *et al*., 1996). Therefore, haemolytic assay has been used in the differentiation of virulent strain among suspected pathogens (Chang *et al*., 1996).

An exoprotease has been purified from the extracellular product of *V.harveyi* 820514 by a combination of ammonium sulphate precipitation, hydrophobic interaction chromatography and anion exchange chromatography on fast protein liquid chromatography. Purified protease appears to be a cysteine protease by virtue of the inhibition of enzyme activity, iodoacetamide, iodoacetic acid, N-ethylamaleinide, p-chloro meruribenzoate. It is the first cysteine protease found in *Vibrio spp*. Cysteine protease is a major exotoxin lethal to *P.monodon*, interfering with hemostasis, leading to formation of unclottable hemolymph (Liu and Lee, 1999). A thermostable exotoxin of *V.harveyi* having proteolytic, hemolytic and cytolytic activity was recovered from diseased postlarvae of *Penaeus vannamei* (Montero and Austin, 1999). ECPs from *V.harveyi* VIB 645 containing caseinase, gelatinase, phospholipase, lipase and hemolysins with

high titre of hemolytic activity to salmonids erythrocytes were determined by Zhang and Austin (2000).

1.6.2. Adhesins and Outer membrane proteins

Animal-bacterial cell interactions are often maintained by the recognition of the sugars on the host cell membrane by bacterial surface proteins called Adhesin (Costerton *et al*., 1978, 1981). Many Gram –ve bacteria have mannose – recognizing adhesins and specificity of interaction is conferred by variations in the bacterial adhesins that corresponds to differences in the microenvironment of the mannose residue on the host receptor. The outer membrane proteins are encoded by O*mpU* functions as an adhesin. Outer membrane proteins (Omps) called porins participate in adhesion to host. Adherence of *V. cholerae* to a variety of cell lines *in vitro* and colonization of infant mice are inhibited by Fab fragment from anti-*OmpU* antibodies (Provenzano and Klose, 2000; Simonet *et al*., 2003).

1.6.3. Lipopolysaccharides

The most common bacterial inducer of animal cell death is bacterial LPS and specifically, the lipid–A portion of LPS, which is the most conserved component of the molecule (Nikaido, 1988; Nesper *et al*., 2000). LPS of bacteria inhibits further cell proliferation and induces cell death. The opportunistic pathogen, *Vibrio vulnificus* expresses lipopolysaccharide antigens on its outer membrane surface. Five O-antigen- specific MAb were used to detect distribution of the serotypes among *V. vulnificus* strains isolated from various settings. While a number of *V. vulnificus* strains were unrecognized by the five MAb, and some strains were recognized by more than one MAb, the application has proven useful in demonstrating O-antigen distribution in both clinical and environmental isolates (Zuppardo *et al*; 2001). Montero and Austin (1999) suggested that the LPS might constitute the lethal toxin of *V.harveyi* E_2 to penaeid shrimp.

1.6.4. Flagella as chemotactic and virulence agent

The two flagellar systems operate to propel bacteria under different circumstances. The polar flagella aids in swimming and lateral flagella in swarming are composed of multiple flagellin subunits, sheathed by a membrane and rotate by using energy derived from the sodium membrane potential. The presence of peritrichous flagella functional in viscous environments enables bacterium to move over and colonize surfaces (McCarter, 1999). Flagellar navigation brings about chemotaxis response in vibrios enabling them to move away from unfavourable environments, a response important for bacterial survival and colonization. Motility and chemotaxis have shown to play the role in virulence of *V.anguillarum* (Larsen and Boesen, 2001). Antigenicity of lateral flagella of different species differs from each other, except for the lateral flagella of *V. parahaemolyticus* and *V. alginolyticus* which share the same epitopes. Also two kinds of antigenic determinants or sites are present. One is an antigen on the surface of the intact flagella and the other located inside the flagella which becomes exposed when flagella are solubilized to flagellin monomers. Thus confirmed that *V. parahaemolyticus* is divided into three types HL1, HL2 and HL3 and they showed no cross reactivity with H-antigens of the serotype of *V. parahaemolyticus* and other strains. (Shinoda *et al*.,1976). Flagella consists of flagellinA, essential for virulence and the expression of *virB* and *virC* genes, responsible for production of major surface antigens, located on the outer sheath of flagellum, important for virulence (Norqvist and Wolf-Watz,1993). The chemotaxis genes (*che*) are differentially regulated within *Vibrio* spp. and mutation to this region results in different rotational biases and profound difference in colonization exhibited by the bacterium. Many pathogenic *Vibrio* species are attracted towards mucus, enabling their colonization in the intestinal mucus as seen in *V.alginolyticus* infection to fish (Bordas *et al*., 1998). The genome sequence of *Vibrio* species reveal a plethora of potential chemoreceptors found distributed on both the

chromosomes. The genes mainly identified include the methyl-accepting chemotaxis protein (MCP) genes, involved in sensing and responding to varied environmental signals (Gestwicki *et al*., 2000). Flagellar motors participate in signal transduction cascade, influencing the expression of cell surface polysaccharide, which mediates important function such as biofilm formation and host colonization (Watnick *et al*., 2001; Lauriano *et al*., 2004).

1.6.5. Type Three Secretion System

The type three section systems (TTS) enable many pathogenic Gram negative bacteria to directly inject eukaryotic cells using fibrous structures on bacterial surface called injectisomes. TTS forms an important part of the *Vibrio* pathogenic islands, mediating virulence. Structural components of TTS are highly conserved between different pathogenic species (Park *et al*., 2004). Bacteria using this mechanism share atleast 8 genes and many have over 20 components that are essential for proper functioning. Certain species can be artificially induced for TTS for substrate recognition, by growth at 37ºC in the absence of calcium, causing protein secretion into the media. The signals for protein secretion are located on the first 15 codons of the ORF. mRNA signals TTS export by coupling its translation with the secretion of encoded polypeptide. mRNAs are eventually relieved from folded structure for a productive interaction between charged ribosomes and TTS machine and the proteins are secreted across the bacterial envelope in a Co-translational manner. Henke and Bassler (2004) reported a functional TTSS in *V.harveyi* governed by TTSS genes which is homologues to those found in *V.alginolyticus* and *V.tubiashii*. Different vibrios use different kinds of natural targets for protein injection by TTSS, enabling a better understanding of the lifecycle of vibrios in natural environments.

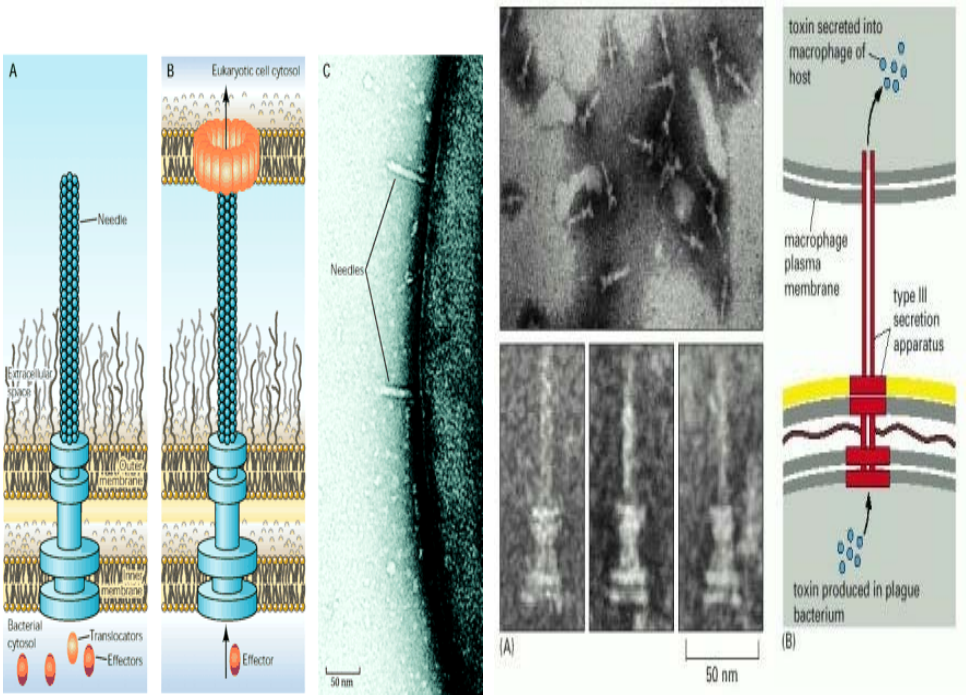


Fig: 1.4 Type three secretion system governed by *V.harveyi* injectisomes (physiologyonline.physiology.org/content/20/5/326F1.expansion.html)

1.6.6. Integron mediated resistance

Integrons are natural genetic engineering platforms that incorporate ORFs and convert them into functional genes, ensuring correct expression. All integrons are characterized by 3 key elements necessary to produce functionally effective exogenous genes: a) gene coding for an Integrase of tyrosine recombinase family (*intI*), b) a primary recombination site (*attI*), and c) a strong outward-oriented promoter (Pc). Integrons are able to capture one or more gene cassettes from the environment and incorporate them by using site- specific recombination. The integron Integrase only mobilizes the gene cassettes within the integrons. The role of integrons and gene cassettes in dissemination of multidrug resistance in Gram-negative bacteria is well established (Hall and Strokes, 1993). Based on the integrase gene sequences, at least eight different classes of integrons have been described in Gram-negative bacteria (Nield *et al.*, 2001). Class 1 integrons

are found associated with functional transposons such as Tn21 (Liebert *et al*., 1999) and Class 2 integrons inside Tn7 derivatives (Radstrom *et al*., 1994). Class 1 integrons found in clinical isolates mainly govern multidrug resistance; contribute to the spread of genetic determinants of antibiotic resistance by horizontal gene transfer, although not mobile elements themselves, they are frequently associated with plasmids and transposons (Fluit and Schmitz, 1999). Integrase gene of Class 1 integrons (IntI) code for site-specific recombinase responsible for cassette insertion (Collis *et al*., 1993) along with the attI site where the cassettes are integrated and a promoter (Pc) enables the transcription of the cassette-encoded genes, hence these two are suspected as the reservoirs of antimicrobial resistance genes within the microbial populations (Pai *et al*., 2003). The increasing incidence of integrons and other resistance determinants among veterinary microorganisms reduces therapeutic options for both human and animal diseases due to an increased prevalence of resistant zoonotic pathogens, which could subsequently cause human infections during processing and preparation procedures (Hopkins *et al*., 2005). Exchange of genes for resistance to antibiotics between bacteria in aquaculture environment and bacteria in terrestrial environment, including bacteria of animal and human pathogens has been shown by Schmidt *et al*., (2001). Many classes of antimicrobial agents, such as aminoglycosides, chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole have been reported as active antimicrobials (Zhao *et al*., 2001). Five different classes of mobile integrons are involved in the dissemination of antibiotic resistance genes. All five are physically linked to mobile DNA structures, either associated with insertion sequences, transposons and/or conjugative plasmids, serving as vehicles for intra-and inter- species transmission of genetic material. Class 1 integrons confer resistance to all β-lactams, aminoglycosides, chlroramphenicol, trimethoprim, streptothricin, rifampin, erythromycin and antiseptics of quaternary ammonium compounds (Rowe-Magnus and Manzel, 2002). Recruitment of exogenous genes is the most rapid

adaptation against antimicrobial compounds and the integron functions provides gene cassette system that are perfectly suited to face challenges of multiple antibiotic treatment regimens.

1.6.7. Transposon mediated resistance

Prevalence of highly virulent *V.harveyi* strains harbouring a transferable chloramphenicol-resistance determinant together with other extracellular virulence factors may hamper the production of penaeid shrimp larvae (Abhraham, 2006). The presence of the transposon Tn1721 carrying *tetA*, *tetR* genes and novel β-lactamases, antibiotic resistance determinants, makes them resist antibiotics. Antibiotic resistance can originate from gene mutations or by horizontal transfer between phylogenetically diverse bacteria. β-Lactamases, the enzymes that hydrolyze β-lactam antibiotics, are the main source of resistance to these drugs. Genes for β-lactamases may be found on chromosomes, plasmids, transposons, and integrons. TEM-1 β-lactamase gene is common among Gram-negative bacteria; it is one of the main causes of bacterial resistance to β-lactam antibiotics. The *blaTEM1* gene was detected in most of the isolates resistant to ampicillin and this gene is widespread in clinical as well as isolates from natural oligotrophic lake (Pontes *et al*., 2009). Integrative and Conjugative elements (IECs) are diverse class of mobile elements found integrated to the chromosomes of Gram +ve and –ve bacteria. ICEs encode conjugation systems that can transfer the excised DNA into a new host, where it integrates into the host chromosome by site specific recombination. Different ICEs integrate into a variety of sites and encode diverse recombination, conjugation and regulation systems. They also carry genes encoding a variety of functions including catabolic pathways, antibiotic resistances, nitrogen fixation and phage mediated resistance mechanism (van der Meer and Sentchilo, 2003).

1.6.8. Resistance mediated by Plasmids

Bacteria that contain antibiotic resistance plasmids have shown to exhibit higher rates of survival in aquatic environments. Genes that encode resistance are the resistance determinants present in the R factor, whose products inactivate the antibiotics or prevent the antibacterial drug from contacting its target within the cell. A conjugative R factor plasmid in a *V.harveyi* strain virulent to *P.monodon* was reported by Harris (1993). This R factor conferred resistance to erythromycin, streptomycin, kanamycin, sulfafurazole and cotimoxazole. Bacteriocins, another class of plasmid-derived proteins produced by bacteria, exhibit antimicrobial activity against sensitive or closely related bacterial species. McCall and Sizemore (1979) reported a bacteriocin-like substance in *V.harveyi*, which caused lethality by a plasmid and was termed as harveyicin. Apart from their variable distribution, *Vibrio* plasmids show considerable microheterogeneity and modification of expression levels of some siderophore biosynthesis genes (Di Lorenzo *et al*., 2003).

1.6.9. Bacteriophage mediated virulence

Phages thrive in bacterial population where they constantly transfer their genetic elements by horizontal gene transfer (Boyd *et al*., 2001). Lysogenic cycle exhibited by phages confers virulence to *V.harveyi*. A temperate phage in *V.harveyi* VH1039 isolated from tea brown gill syndrome in *P.monodon* was identified as lysogenic siphovirus (Pasharawipas *et al*., 1998). Oakey and Owens (2000) isolated a bacteriophage from a toxin-producing strain of *V.harveyi*, and termed it as VHML, which caused upregulation of certain bacterial extracellular proteins. VHML harbored by *V.harveyi* strains stimulate hemolysin production and excretion of proteins from cells and contributes to expression of virulence (Munro *et al*., 2003; Austin *et al*., 2003).

1.6.10. Quorum sensing

Quorum sensing is a process that allows bacteria to communicate using secreted chemical signaling molecules called **Auto inducers** (Nealson and Hastings, 1979; Miller and Bassler, 2001; Natrah *et al*., 2011, Ruwandeepika *et al*., 2011). Quorum sensing is important for the regulation of population density dependent cellular processes in bacteria, including the production of antibiotics, virulent factors, conjugation, transformation, swarming behavior and biofilm formation (Fuqua *et al*., 1994; Whitehead *et al*., 2001). This mechanism enables a group to express specific genes only at particular population densities, but becomes unproductive when undertaken by individual bacterium (Xavier and Bassler, 2003). Three distinct autoinducers have been identified. **LuxR/I-type** systems are preliminarily used by Gram-negative bacteria, in which the signaling molecule is an acyl-homoserine lactone (AHL), the peptide signaling systems used primilarly by Gram-positive bacteria is the ***lux*S/AI-2** signaling used for interspecies communication, and the **AI-3**/epinephrine/norepinephrine interkingdom signaling system. Quorum Sensing was first described in the regulation of bioluminescence in *V.fischeri* and *V.harveyi* (Nealson and Hastings, 1979; Henke and Bassler, 2004a, b, c). N-(β-Hydroxybutyryl) homoserine lactone is an autoinducer molecule of *V.harveyi*, which enables bacteria to monitor its own population and regulate virulence gene expression (Milton *et al*., 1997, 2006). AI-2 is found to be produced by a large number of bacterial species, including *V.harveyi* which interacts with luminescence operon, composed of *lux*CDABEGH genes by the phosphorylation of regulatory protein *luxO* (Bassler *et al*., 1997).

1.7. Treatment measures

1.7.1. Antibiotic usage and its drawbacks

Treatment with antibiotics and chemotherapeutics continues to be an unavoidable control measure in aquaculture industry, unless an alternative

replaces this traditional measure to control microbial agents in the culture systems. One of the most frequently used procedures to avoid the incorporation of undesirable bacteria is by antibiotic administration in the water or via live feed like *Artemia* (Brown, 1989; Touraki *et al*., 1999). Antibiotics are also used in animal production system at sub-therapeutic level to boost food conservation. Teuber (1999) stated that the problem with drug resistance in human medicine will not be solved if there is a constant influx of resistant genes into human microflora via food chain. Feed with antimicrobial additives increases animal production and are beneficial on economic basis, but from a long term perspective their frequent use is questioned, as it is a matter of concern related to environment protection, animal welfare, and health.

Unconsumed feed, faeces etc., containing antibiotics reach sediment at the bottom of the rearing tanks, exerting selective pressure, altering composition of the sediment micro flora and promoting the overgrowth of antibiotic-resistant bacteria (Kim *et al*., 2004 a, b). Disposal of antibiotics into the surrounding aquaculture sites has enhanced the number of antibiotic resistant bacteria, harbouring new and previously uncharacterized resistant determinants (Miranda *et al*., 2002, 2003). The determinants of antibiotic resistance have the potential of being transmitted by horizontal gene transfer to bacteria of the terrestrial environment, including human and animal pathogens (Rhodes *et al*., 2000). A strong association between the presence of integron and multiple antibiotic resistance (MAR) phenotype has been observed (Leversteinvan-Hall *et al*., 2002).The development of multidrug resistant bacteria carrying the virulent-resistant genes is a serious threat to aquatic organisms, and is of concern with regards to the development of resistance to human pathogens. Residues of most commonly used antibiotics, such as erythromycin, oxytetracycline and chloramphenicol are found in shrimp meat which may cause health hazards in human on long term consumption (Bourne *et al*., 2006). Virulent microbes re-enter the

aquatic systems, establish biofilms on water pipes, air lines or in the animal gut, leading to clogging of the systems (Bourne *et al*., 2006). When the resistant microbes establish themselves in the host body, there exist high chances of exchange of genetic information, especially the transfer of r-plasmids, enabling their resistance to further dosage of antibiotics (Bourne *et al*., 2006).

The ever increasing concern over the potential harm to aquaculture systems is by the effluent discharge into receiving water bodies, bioaccumulation of harmful chemicals, contamination by aquatic products, which elevates human risks associated with storage and handling of these chemicals. Certain control measures and regulations to be followed by the producers are presented by FAO, 1995 in the "Code of conduct for Responsible Fisheries" to regulate the use of chemical inputs in aquaculture which has hazardous impact on human health and environment. Increase in the number of resistant varieties has resulted in the banning the use of certain antibiotics in aquaculture systems, necessitating the management strategies using immunostimulants, vaccines, probiotics, and phage therapy.

1.7.2. Probiotics as potential prophylactics

Chemicals including antimicrobial drugs, pesticides and disinfectants have been conventionally used to control diseases (Gomez-Gil *et al*., 2000, Dahiya *et* al., 2010). Abuse of these chemicals has brought forward development of environment-friendly aquaculture to resolve the problem and to develop sustainable aquaculture, and research on probiotics for aquatic animal health has been augmented (Gatesoupe, 1999, Castex *et al*., 2008). Probiotics are viable bacteria that beneficially influence the host by improving its intestinal microbial balance (Wang and Xu, 2006, Vine *et al*., 2006). The addition of antagonistic bacteria to water results in *vivo* disease reduction and /or reduction in the number of pathogenic bacteria in the culture systems (Moriarty, 1997, 1998, Gram *et al*., 1999). Bacteria

occurring in aquatic ecosystems may have the ability to inhibit the growth of other microorganisms by producing antimicrobial substances. Addition of probiotics into culture ponds: 1) enhances decomposition of organic matter, 2) reduces nitrogen and phosphorus concentrations, 3) leads to greater availability of dissolved oxygen, 4) reduction of blue-green algae (Boyd *et al*., 1984), 5) controls the level of ammonia, nitrite and hydrogen sulphide (Carmignani and Bennett, 1977), 6) lowers the incidence of disease and offers greater survival (Nogami and Maeda, 1992), 7) production of inhibitory compounds (Chythanya *et al*., 2002), 8) competition for chemicals and available energy, 9) becomes a source of macro and micronutrients (Verschuere *et al*., 2000a, b), 10) enhances competition to adhesion sites (Garcia *et al*., 1997), 11) enhances immune response (Rengpipat *et al*., 1998, 2000, 2003), 12) improve water quality and interaction with phytoplankton, and 13) increases enzymatic contribution to digestion and better shrimp and fish production (Tovar *et al*., 2002).

The range of probiotics examined for use in aquaculture encompasses Gram positive and negative bacteria, bacteriophages, yeast and unicellular algae (Iriano and Austin, 2002a, b). Generally, probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals. The identification of potential probionts has, however, expanded over the years to include species such as *A.hydrophila*, *A.media*, *B.circulans*, *B.subtilis*, Carnobacterium, *Clostridium butyricum*, Photosynthetic bacteria, *Saccharomycese boulardi*, *S.cerevisiae*, Streptococcus, *V.alginolyticus*, and *V.fluvialis* (Vijayan *et al*., 2006, Zhou *et al*., 2006, Kumar *et al*., 2006). Various *Lactobacillus* spp., *Bacillus* spp. (Aly *et al*., 2008b), *Carnobacterium* spp., *Aeromonas* spp. (Irianto and Austin, 2002b), *Micrococcus* spp. (Jayaprakash *et al*., 2005), *Pseudomonas* spp. (Vijayan *et al*., 2006; Holstrom *et al*., 2003a), *Vibrio* spp. (Austin *et al*., 1995, Balacazar *et al*., 2007), yeast (Gatesoupe, 1999) and mixed cultures (Wang and Xu, 2006), etc in protecting fish and shellfishes from pathogens.

Several studies on probiotics have been conducted during the last decades; however, the methodological and ethical limitations of animal studies make it difficult to understand the mechanisms of probiotic action, thereby revealing partial explanations. Nevertheless, some possible benefits linked to the administration of probiotics have already been suggested as: 1) competitive exclusion of pathogenic bacteria; 2) source of nutrients and enzymatic contribution to digestion; 3) direct uptake of dissolved organic material mediated by the bacteria; 4) enhancement of immune response against pathogenic microorganisms; 5) antiviral effects and 6) influence on water quality (Moriarty, 1998; Gomez-Gil, 2000; Balcazar *et al*., 2006).

Screening of antagonism in environmental bacteria against pathogens by *in vitro* plate assay has been widely carried out (Verschuere *et al*., 2000 a, b). However, selection based on properties such as adhesion, colonization to intestine, skin and other surfaces and growth parameters such as competition for nutrients, replication rate, production of antimicrobial substances, adaptation to the acidic environment of the gastrointestinal tract etc. has created importance in recent years (Vine *et al*., 2004b). The hypothesis that preemptive colonization of the intestine and other portals of entry of pathogens by autochthonous bacteria with or without antagonism but with better adhesion, colonization and growth characteristics compared to pathogens can prevent pathogen invasions and improve survival (Hjelm *et al*., 2004 a, b, Vine *et al*., 2004a).

Currently, the four common methods employed to screen for inhibitory substances *in vitro* include; the double layer method, well diffusion method, cross-streak method and disc diffusion method. The principle behind all these methods is that a bacterium (the producer) produces an extracellular substance which is inhibitory to itself or another bacterial strain (the indicator). The inhibitory activity is displayed by growth inhibition of the indicator in the medium (Kesarcodi-Watson *et al*., 2008). Two major pitfalls of *in vitro* antagonism based selection of potential

probionts are: 1) the other modes of probiotic activity such as immunostimulation, digestive enzyme production, competition for attachment sites or nutritional requirements, etc, need to be evaluated as the environmental conditions are widely different from that carried out on an agar plate in the laboratory, and 2) *in vitro* antagonism of a pathogen by a probiotic strain need not necessarily confer *in vivo* protection to the cultured animals. The property expressed *in vitro* may not be elicited under *in vivo* conditions. Gram *et al*., (2001) found that *P.fluorescence* strain AH2 was inhibitory to *A.salmonicida* pathogenic to salmon *in vitro*. However, no protective effect was found when transferring the same probiont to an *in vivo* challenge experiment. The methods to select probiotic bacteria for use in aquaculture include: 1) collection of background information (probiotics should not be pathogenic to the desired host, acceptable by host through ingestion, potential colonization and replication, reach the site of action within the host, preferably should not carry virulence resistant or antibiotic resistant genes), 2) acquisition of potential probiotics, 3) evaluation of the ability of potential probiotics to out-compete pathogenic strains, 4) assessment of the pathogenicity of the potential probiotics, 5) evaluation of the effect of the potential probiotics in host, and 6) economic cost/benefit analysis (Gomez-Gil., 2000). The putative probiotics can be added to the host or to its ambient environment through several ways: a) addition to the artificial diet, b) addition to the culture water, c) bathing, and d) addition via live feed (Austin *et al*., 1995, Gomez-Gil,1998).

1.7.3. Immunostimulants

Short-term immunity is offered by vaccination or immunostimulation, due to the non-specific immune response of crustaceans. Immunostimulants are considered as an attractive alternative prophylactic measure to control microbial infections and stress reduction in shrimp (Logothetis and Austin, 1996). Immunostimulants are agents which stimulate the non-specific immune mechanisms on their own or specific

immune mechanisms when coupled with an antigen. They activate the immune system of animals imparting resistance to infections caused by viruses, bacteria, fungi and parasites. Certain immunostimulants may act on animal cell membranes, making the surfaces more conductive to antigen uptake, while others can mimic animal's natural products, hence recognized as self by the host system. Wide range of substances such as microbial derivates, plant or animal extracts, vitamins, hormones and synthetic chemicals have been reported to have immunostimulatory effects. Many synthetic polymers with repeated subunits, such as muramyl dipeptide, polynucleotides, polyadenylic polyuridylic acid, etc. have immunostimulatory effect on animals. Increase in growth and better survival in penaeid post-larvae were observed prior to the administration of *Vibrio* bacterins in the hatchery systems (Vici *et al*., 2000). Complete Freund's adjuvant was the first immunostimulant used in animals to elevate the immune response. However, now FCA is used in conjugation with injection of bacterins. β-1,3-1,6- glucan (yeast cell wall extract) (Song and Sung, 1994) induces non-specific disease resistance to tiger shrimp especially against pathogenic vibrios, enhancing stress tolerance induced during hatching, transport and ammonia accumulation, suggesting the immunostimulatory effect of glucan (Song *et al*., 1994). 1,3-β-D glucans incorporated into diet of brooder enhance the functional status of macrophages and neutrophils, modify immunosuppression and resistance to challenge with Gram –ve bacteria, enhance haemocyte- phagocyte activity, cell adhesion and superoxide anion production, and activate polyphenoloxidase in haemolymph (Scholz *et al*., 1999). Vitamin-C is a popular immunostimulant added to diet of certain animals that have impaired antibody response as it enhances phagocytic engulfment of the pathogen and improves the immune mechanism. Immunostimulants and adjuvants can be administered before, with or after vaccines to amplify the specific immune response by elevating circulating antibody titers and number of plaque forming cells. In case where disease outbreaks are cyclical

and can be predicted, losses maybe reduced by activating the non-specific defense mechanisms and the immunostimulats maybe used in anticipation of events to prevent huge losses due to disease out break.

1.7.4. Vaccines

Adams *et al*., (1991) have suggested the use of biological control methods such as vaccine and immunostimulants to prevent disease outbreaks and achieve sustainable production. During the last two decades, vaccination is carried out as a preventive method against various bacterial pathogens, leading to a lowered use of antibiotics dramatically (Sommerset *et al*., 2005). Though there is no specific memory in shrimps, a partial specificity in immune response was observed in the case of vaccine treated shrimps. However, vaccines composed of inactivated *Vibrio* species are reported to protect shrimps from vibriosis and to improve growth and survival of vaccinated shrimps. Li *et al*., (2010) observed that the outer membrane protein (OmpK) can be used as an ideal vaccine against vibriosis caused to Orange-spotted grouper (*Epinephelus coioides*). Pereira *et al*. (2009) observed that cultivable penaied shrimps can be protected against vibriosis, using formalin- killed *V.harveyi* vaccine. Maximum relative percentage survival at 1% vaccine concentration exposed for 5hrs, showed that vaccination is highly significant and enhances the resistance of shrimp post larvae to vibriosis. Genetically engineered subunit and DNA vaccines are being used increasingly in veterinary vaccine development. Vaccines absorbed to, held within or conjugated to particles or large molecules may aid uptake and efficacy of vaccines. Vaccines maybe coated to latex beads and bentonite or placed in lipososmes or mixed with light oils and administered, leading to increase vaccine uptake, when the vaccines are given tropically. Conjugation with haptens or small antigenic molecules to larger carrier molecules may also help immunogenicity of some vaccine, especially dealing with subunit, recombinant or synthetic vaccines that are expensive and difficult to prepare. Ergosan and Vibrimax vaccines showed

significant enhancement in survival rate and promoted health status of *V.harveyi* and WSSV challenged juvenile stages of shrimp during the period of culture (Heidarieh, 2010). AquaVac™ Vibromax™ is a multivalent vaccine for shrimp that enhances resistance against a multiplicity of *Vibrio* species including *V.anguillarum* biotype I and II, *V.parahaemolyticus*, *V.harveyi* and *V.vulnificus*. AquaVac™ Ergosan™ is an algine based immunomodulator extracted from marine algae. The active ingredients, including algines and polysaccharides, are known to strengthen the full range of natural defense systems in fish. It is completely a natural product and as such is an accepted feed ingredient. A divalent vaccine containing formalinized cells and ECP of *V.alginolyticus* was developed by Morinigo *et al*., (2002). A divalent vaccine prepared with formalinized whole cells and extracellular product of *Solea senegalensis* (Kaup), against *Vibrio harveyi* and *Photobacterium damselae* subsp. *Piscicida* has been attempted (Arijo *et al*., 2005). Addition of sodium alginate in diet of white shrimp (*Litopenaeus vannamei*), cleared the pathogen *V.alginolyticus* and elevated immune parameters namely enhanced phagocytic index, phenoloxidase activity, respiratory burst and superoxide dismutase activity, but decreased glutathione peroxidase activity (Cheng *et al*., 2005).

1.7.5. Phage therapy

Phages are abundant in marine ecosystems; comprising about 10^4 to 10^7 phage particle/ml. Temperate phages are also present in large numbers as lysogenic phages found in marine bacteria. Despite the extensive research carried out to control bacterial diseases in fish and shellfishes, still there exists a significant loss to farmers and potentially on wild stocks (Austin and Austin, 1999). One alternative control strategy that has received limited attention for aquaculture is the use of phage therapy; a concept first developed in 1918 by D'Herelle (Douglas, 1975). High specificity to target bacterial populations, effectiveness against multidrug resistant pathogens, spontaneous mutation of phages aiding rapid response to phage resistant

mutants, low production cost without any known side effects in comparison to antibiotics have boosted up the use of phage as therapeutics. Phage therapy has been explored with members of *Escherichia*, *Staphylococcus*, *Salmonella*, *Klebsiella*, *Proteus* and *Pseudomonas* for localized and systemic infections caused by *V.vulnificus*. *Siphoviridae* and *Myoviridae* phages are found specifically infect *V.harveyi*. Wu and Chao (1987) have described phage therapy against milkfish vibriosis. However, there are problems associated with phages as therapeutic agents, especially as phages are effective agents in the transfer of virulence factors or toxin genes (McGrath *et al*., 2004). There are also other phage-associated toxins, of which the CTX cholera toxin (Davis *et al*., 2000b), botulinum toxin (Brussow *et al*., 2004), shiga-toxin (Strauch *et al*., 2004) and dipththeria toxin (Brussow *et al*., 2004) are well known. The extreme specificity of phages renders them ideal candidates for applications designed to increase food safety during the production process. Moreover, phages or phage derived proteins can also be used to detect the presence of unwanted pathogens in food or the production environments, which allows quick and specific identification of viable cells (Hagens *et al*., 2007). Two important concerns need to be addressed: Are the effects of phages harmless upon consumption, and how can phage resistance is dealt with?

Phage typing is a popular tool to differentiate bacterial isolates, and is used in epidemiological studies with the aim of identifying and characterizing outbreak-associated strains. Although more sophisticated systems for differentiation are available, such as ribotyping, random amplified polymorphic DNA-PCR fingerprinting, or pulsed field gel electrophoresis of enzyme-digested DNA, the variable sensitivity to a set of bacteriophages (phage typing) remains a useful method because of its speed, relative simplicity, and cost-effectiveness. Various phage typing schemes exist for all common food-borne pathogens such as *Salmonella*,

Campylobacter, *E. coli*, and *Listeria* (Majtanova and Majtan, 2006; Hopkins *et al*. 2004).

1.7.6. Quorum Sensing (QS) Inhibition

Since the appearance of antibiotic resistant bacteria has become universal, there is an increasing need for novel strategies to control infectious diseases like vibriosis. Biofilm forming bacteria have developed mechanisms to tolerate conventional antimicrobial treatments. The inactivation of the QS mechanism by the process called quorum quenching has resulted in the decrease of the pathogenicity caused by the luminescent vibrios. Cinnamaldehyde and its derivatives reduce virulence in vibrios by decreasing the DNA-binding activity of QS response regulator LuxR (Gilles *et al*., 2008). QS inhibitors affect the starvation and reduce virulence in several *Vibrio* species interfering with LuxPQ (Gilles *et al*., 2009). *Delisea pulchra*, a temperate marine macro red algae found in the Australian coast is capable of producing biologically active compounds (brominated furanone) with a broad range of antifouling and antimicrobial activity, especially inhibiting luminescence and toxin production in *V.harveyi*. This algae contains (5Z)-4-Bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone which inhibits swarming motility and biofilm formation in *Bacillus subtilis* and *E.coli* (Ren *et al*., 2002). Extracts of *D.pulchra* have been found to reduce the growth rate of *S.aureus* and *S.epidermidis*, and inhibit the swarming of *P.mirabilis* (Gram *et al*., 1996). *Bacillus thuringiensis*, *B.cereus* and *B.mycoides* were tested for AHL-inactivating enzymes. Exudates of pea seedlings inhibit QS in *Chromobacterium violaceum* but were found to activate QS in bacteria such as *Pseudomonas* and *Serratia*. The use of green water containing *Chlorella* during Tilapia culture (*Oreochromis*) has been suggested for minimizing *V.harveyi* (Fredson *et al*., 2006). The ability of the green water grow-out culture of *P.monodon* to prevent outbreaks of luminious vibriosis was investigated by screening associated isolates of bacteria, fungi, phytoplankton, fish skin mucous for anti-*Vibrio* metabolites

(Gilda *et al*., 2005). Natural furanone blocks QS regulated gene expression in *V.harveyi* by decreasing the DNA-binding activity of the QS transcriptional regulator LUXRvh and not by interacting with the receptor signal molecules. As furanones block all the 3 channels of *V.harveyi* QS transduction cascade, it is not necessary to develop different furanone compounds to protect the hosts. Furanones posses no or very small selective pressure on the bacteria, hence chances development of resistance are lesser than conventional antibiotics, thus making these antipathogenic compounds an attractive sustainable biocontrol strategy (Defoirdth, *et al*., 2007, 2008; Tinh, 2007).

1.8. Diagnostics for shellfish health management

Effective disease management of finfish and shellfish requires sensitive, accurate and rapid diagnosis without sacrificing the animals. The successful implementation of the diagnostic methods solely depends on the stage of disease progression at which the method is being used and the results are being interpreted. The effective control and treatment of diseases of aquatic animals require access to diagnostic tests that are rapid, reliable and highly sensitive. In many cases, post-mortem necropsy and histopathology have been the primary methods for the diagnosis of fish and shellfish diseases. Direct culture of pathogens is also widely used; however, these methods are time-consuming. Current diagnostic methods are categorized into 3 levels; **Level-1** includes farm or production site information and records on health management. **Level-2** uses specialised techniques such as microscopy, histopathology and antibody based diagnostic method. **Level-3** includes advanced techniques such as PCR based methods; multiplex testing using the Bio-Plex Protein Array System, ribotyping, and micro-array technology are bringing a new dimension to aquatic animal health control.

Histopathology provides information on host-pathogen interactions at structural and functional levels, detected using light microscope as signs

of cloudy swelling, hydropic degeneration. Tissue necrosis, enteritis, fibrous encapsulation, nodule formation, xenomas etc are some of the common histopathological changes, based on which the pathogenic mechanisms of microbes, functional status of target organs, severity of a disease, cause of mortality and possible aetiology can be determined. However, these methods often lack specificity and many pathogens are difficult to detect when present in low numbers or when there are no clinical signs of disease. Histopathology being a non-specific diagnostic tool has certain limitations, but the advantages of using histopathology for aquatic animal health diagnostics and management overweigh its limitations.

Immunological techniques such as ELISA or dot-blot, agglutination (slide/latex); fluorescent antibody test (FAT/IFAT) (Adams 2004) are excellent diagnostic tools for pathogenic detection due to the specificity of antibody -antigen binding. Initially polyclonal antibodies (PAbs) were used for detection, however, serious drawback of cross reactivity; availability in limited amounts and requirements of animals at various stage of antibody production have made this technique unpopular. Meanwhile, monoclonal antibodies (MAbs) overcome these limitations, hence are used as an effective immunological tool at different stages of disease detection. ELISA, one of the solid-phase enzyme immunoassay (EIA), is developed by application of the same antibody overlay principles used for the detection of antigens *in situ*. More sensitive ELISA detection system may be obtained by incorporating flourogenic substrates, alkaline phosphatase or beta galactosidase. Dot immunobinding assay first developed by Hawkes *et al*. (1982) using nitrocellulose is claimed equally or more sensitive than ELISA. Other diagnostic tools used include Immunoblotting, in which proteins are transferred from a gel after electrophoretic separation on to nitrocellulose membrane developed by Towbin *et al*. (1979) and Latex agglutination assay that detects antigen in a sample using antibody bound to a bead or other visible material. The main disadvantage associated with

Latex agglutination assay is that it is less sensitive than PCR and micro debris present along with the antigen are likely to affect the precipitation of latex particles leading to non-specific adsorption (Hu *et al*., 2010). Also, unbalanced amounts of either antibody or antigen can give false-negative results. In addition, many bacteria have common or related antigens and some antiserums may react with those bacteria, which have similar antigens. Another limiting factor is the size of the particles of the antigen, which must be opaque and large enough to cause turbidity and visible sediment. Immunofluorescence and Immunohistochemistry techniques employing antigen, labelled antibody or fluorescent dyes (flourescein iso-thio-cyanate, rhodamine iso- thio - cyanate, etc) have gained important application. The main advantages of these tests are sensitivity and rapidity, but due to danger of cross-reactions, additional test are required for confirmation. A significant problem with most fluorescence techniques is photobleaching. Loss of activity caused by photobleaching can be controlled by reducing the intensity or time-span of light exposure, by increasing the concentration of fluorophores, or by employing more robust fluorophores that are less prone to bleaching (e.g. Alexa Fluors or DyLight Fluors).

Advanced detection technique such as PCR is largely qualitative and certainly more valuable diagnostic tool than mere qualitative detection method, for revealing the severity of infection in culture ponds. Many molecular techniques are potentially faster or more sensitive than traditionally used methods such as culture, serology and histology. Molecular methods can circumvent problems inherent in study of organisms for which no *in vitro* culture medium or methods are available, and have the potential to greatly increase sensitivity of detection (Lightner, 2005). Many techniques are available to detect or exploit such genetic variations that denote subspecies or strains and can also assist in detecting the pathogens that are present in low numbers and can be used to differentiate antigenically similar pathogens. Various Competitive PCR methods have

been developed for *in vivo* determination of even low levels of shrimp infectivity. DNA based techniques include various PCR targeted to specific conserved sequence of interest. RT-PCR, nested, real time, reverse cross blot PCR (rcb-PCR) and RT-PCR enzyme hybridisation assay (Cunningham, 2004) and multiplex PCR are being used to identify the pathogenic organisms at, above or below species level, allowing the diagnosis of infections in which the causative organisms are not easily cultured or are uncultivable. Real time PCR is more advantageous than traditional PCR as it involves both amplification and quantification of PCR product which is determined by FRET (Fluorescent Resonance Energy Transfer) using probes- Quencher and Reporter.

Ribotyping techniques are used in detection and identification of highly conserved bacterial ribosomal operons encoding for 16S and/or 23S rRNA genes by hybridizing with labeled probes (Thompson *et al*., 2004). The technique has been developed based on the principle that all bacteria carry three operons which are highly conserved and are therefore useful for ribotyping. For the construction of oilgonucleotide probes for hybridization, particular rRNA sequences that are species or group specific are used. An added advantage of ribotyping is its usefulness in differentiating bacterial strains into different serotypes. Hence, the probe DNA sequence used must be very specific for the virulent gene/factor associated with the pathogenecity.

DNA probe technology identifies a microorganism by probing its genetic composition, using variety of haptens such as biotin or digoxygenin and detection by antibody binding coupled to fluorescent, chemiluminescent or colorimetric detection methods. The use of probes in *in-situ* hybridization, applied to tissue sections or imprints, provides means to examine the location of pathogens within tissues and cells. Such methods have great advantages in applications in large-scale diagnosis of certain pathogens. Present advancement is the use of DNA and or antibody based Microarray

systems, which enable multiple pathogens to be screened and detected at one stretch on the array with the detectable signals. Plasmid profiling is another technique to type disease-causing aquatic vibrios (Le Chevalier *et al*., 2003).

1.9. Existence of Beneficial forms

All aquatic organisms are exposed to a varied microflora inhabiting the aquatic ecosystem, having an easy access to host surfaces. Complex and highly evolved mechanisms aid in the interrelationship between aquatic organisms and their indigenous microflora including pathogens. Bacteria present in the intestine may either be beneficial to aquatic organism, in terms of nutritional value they impart (Campbell and Buswell, 1983) or in the prevention of colonization of gut by the host specific pathogenic bacteria (Westerdahl *et al*., 1991).

1.9.1. Bacterial Communication

Certain beneficial forms of vibrios exist amidst the numerous pathogenic forms. Communication between bacteria and their hosts is an essential component of both beneficial symbiosis and pathogenic associations. Recognition of specific-cell surface receptor molecules and favorable adaptation to host internal environment favors bacterial colonization for normal growth, development, and function (Bassler *et al*., 1993, 1994, 1997). Cell – cell communication by diffusible extracellular molecules or signals is evident in bioluminescent bacteria commonly found associated with marine animal tissues. These molecules enable antipredatory defense, defensive camouflage strategy and cryoprotection at lower temperatures to the host (Henke and Bassler, 2004a). Bacteria induce the host to secrete lipopolysaccharides (LPS), which trigger developmental response. Beneficial symbiotic *V.fischeri*, turns down the expression of the peroxidase gene in tissues but turns up the expression of this gene in tissues (specifically gills) when it acts as a pathogen (Winans and Bassler, 2002).

1.9.2. Fermentative vibrios

Vibrionaceae exhibits two different fermentative patterns: mixed-acid fermentation and 2, 3-butanediol fermentation, which are distinguished by Voges-Prosker (VP) and Methyl red (MR) tests. Microbes with mixed fermentative mode are MR -postitve and VP- negative, while the other exhibits a reverse pattern. Mixed acid fermentors produce acetic, lactic and succinic acids along with ethanol, CO_2 and H_2 while butanediol fermentors produce less amount of acids, instead produce butanediol, ethanol, CO_2 and H_2 as the main products. Vibrios are ubiquitous in marine sediments, causing decomposition of organic matter via fermentative pathways, leading to the formation of small organic molecules, such as lactate, butyrate, propionate, acetate, formate, CO_2 and H_2, which serve as main substrates for sulfate reduction and partly for methane formation.

1.9.3. Chitin Degradation

Chitin, a (1→4)-β linked homopolymer of N-acetyl-D-glucosamine, is a widely found structural polysaccharide produced by various marine organisms, especially as an important element of crustacean exoskeleton. Chitin degradation is an important attribute of marine microbes, via complex pathways, including sensing, attaching, transporting and catabolism of natural chitin (Meibom *et al*., 2004). When attached to zooplanktons and algal cells, vibrios can mediate degradation of highly polymeric substances, acting as important contributors to recycling of particulate matter. Partial hydrolysis of complex polymers occurs extracellularly prior to transport into periplasmic space. Chitinase activity is the most important enzymatic activity in marine environment, as annually about 10^{11} tons of chitin is produced in marine systems in the form of zooplankton exoskelektons. The occurrence of vibrios in the gut of marine fauna, suggest the commensal role of vibrios causing the decomposition of organic matter. Great diversity of chitin structures present in the

environment necessitates bacteria to produce different forms of chitinase (Svitil *et al*., 1997, 1998). Presence of Chitinase aids the invasion of pathogen and provides nutrients directly in the form of amino acids or by direct exposure to other host tissues for enzymatic degradation. Chitinase consists of a group of hydrolytic enzymes capable of breaking polymeric chitin to chitin oligosaccharides, diacetylchitobiose and N-acetylglucosamine, which are controlled by the expression of two genes (Thompson *et al*., 2001). Specific attachment of *V.harveyi* to chitin is mediated by two peptides present in the outer membrane of the cells. Initial attachment is mediated by a 53kDa peptide and the other 150kDa peptide is induced by chitin for time-dependent attachment to the surface and cause pathogenicity to host animals (Montgomery and Kirchman, 1993, 1994). Analysis of gene sequence in vibrios shows that Chitinase genes only partially follows 16S rRNA gene phylogeny, suggesting that the deviation in phylogeny may be the result of lateral gene transfer.

1.9.4. Degradation of Polycyclic Aromatic Hydrocarbons (PAH)

Phenanthrene, a PAH present in coal tar and petroleum, formed as by product of petroleum refinery are degraded by many *Vibrio* species (Geiselbrecht *et al*., 1996), suggesting that *Vibrio* species function as effective biodegraders in aquatic environments. Extracellular hydrolysis of complex polymers suggests an important cross-feeding mechanism in microbial communities (Riemann and Azam, 2002). Shifts in dominant and active forms of bacteria may strongly influence the pattern of polymer hydrolysis and cycling of dissolved organic matter in the aquatic systems. Geiselbercht *et al*. (1996) isolated polycyclic aromatic hydrocarbon degrading marine bacteria from Puget Sound sediments and phenotypically analysed them.

1.9.5. Mucinase Production

Vibrios produce mucinase, a metalloprotease, which allows the bacteria to overcome the mucus barrier that covers the gastrointestinal epithelium. This mechanism is particularly exhibited by *V.cholerae* (Colwell, 2004).

1.9.6. Tetrodotoxin (TTX) production

Many *Vibrio* species, particularly *V.alginolyticus*, has been associated with TTX production and this toxin has been transmitted to puffer fish and other TTX containing organisms which initially were unable to produce the toxin but might have acquired the trait via the food chain (Lee *et al*., 2000). *V.harveyi* is capable of producing marine toxins, such as tetrodoxin and anyhdro-tetrotoxins (Simidu *et al*., 1987). TTX binds to nerve cell sodium channels in myelinated and nonmyelinated nerves, hence has found widespread application as a research reagent in neurobiology, as pain killers, management of pain associated with withdrawal from herion and other opioid drugs. Saxitoxin and TTX when mixed in small quantities have anaesthetic property (Simidu *et al*., 1987).

1.9.7. Siderophore production

Siderophore-mediated iron transport system causes increase in virulence of some bacterial pathogens (Griffiths, 1987). Iron acquisition mechanism in pathogenic bacteria is limited to strong binding capacity of this element to high-affinity iron-binding proteins of animal body fluids. Many bacteria have complex system to transport iron into the cell in the form of siderophores, coupled with iron-repressible outer membrane receptors for siderpohore/iron complex (Aznar *et al*., 1989). Siderophores, low molecular weight Fe (III)-specific ligand function in receptor-dependent iron transport and act as virulence factors in animal and plant diseases. An increase in virulence of some pathogens is observed, when the host animals

were fed or injected with soluble iron. Siderophore are reported as major virulence determinant in *A.salmonicida*, *V.anguillarium*, *V.cholerae* and *A.hydrophila*. Siderophore activity in *V.harveyi* is linked to pathogenicity in vertebrates but not in invertebrates, mainly because of tight binding of iron by high-affinity iron-binding proteins such as transferrin and lactoferrin in serum and secretions (Crosa, 1989). Invertebrates seem to lack iron-binding compounds such as lactoferrin and transferrin (Owens *et al*., 1996).

1.9.8. Bioactive compound production

Marine bacteria are known to produce brominated compounds and bromine has an effect on production of antibiotics (Marwick *et al*., 1999). Several *Vibrio* species were isolated from marine sponge *Dysidea* sp. that synthesized cytotoxic and antibacterial tetrabromodiphenyl ethers (Marwick *et al*., 1999). Other bioactive compound isolated from *Vibrio* include anticyanobacterial compound beta-cyanoalanine (Yoshikawa *et al*., 2000), that could prevent algal blooms.

1.9.9. Enzyme production

Vibrio spp. produce a wide variety of extracellular proteases, including detergent–resistant alkaline serine exoprotease. Vibrios produce collagenase, important for various industrial and commercial applications including dispersion of cells in tissue culture. Vibrios are known to produce neutral protease called vimelysin. *Vibrio* proteases are responsible for breakdown of feather waste (Sangali and Brandelli, 2000).

1.9.10. Bioluminescence as a Reporter System

Bioluminescent bacteria are used to develop biosensors and as diagnostic devices for medicine, aquaculture and environmental monitoring. *lux* genes responsible for bioluminescence are cloned into a gene sequence or operon which turns functional when stimulated by a defined

environmental feature. In case of toluene degradation, the enzymes are activated in the presence of toluene. When *lux* genes are inserted into a toluene operon, the engineered bacterium glows yellow-green in the presence of toluene and reports for its degradation (Applegate *et al.*, 1997). Also the lux system is responsible for monitoring and biodegradation of naphthalene (Burlage *et al.*, 1990), monitoring alginate production and many other compounds (Applegate *et al.*, 1997).

1.9.11. Role in Nutrient Cycling

Bacteria and protists play a major role in recycling of organic matter released from primary producers to supply regenerated nutrients, acting as sink for carbon lost during respiratory loss as CO_2. Through heterotrophic growth in organic substrates, vibrios contribute to nutrient cycling within the diverse habitat they occupy. Members of the family Vibrionaceae are involved in both uptake and mineralization of carbon, nitrogen and phosphorus and vibrios exhibit a population turnover and disproportionately contribute to ecosystem nutrient cycling. The extent to which vibrios cause nutrient recycling is a product of their abundance and activity.

Vibrios consume a wide array of carbon substrates and degrade them through extracellular digestion. Vibrios engage in both respiratory and fermentative metabolisms and transform organic carbon into cell materials and waste products. During aerobic and anaerobic respirations, 30 to 50% of organic matter is utilized for biomass formation. However, during fermentation, large amounts of metabolic end products are excreted. Organic acids, alcohols and H_2 formed as metabolic end products in some species, stimulate anaerobic food chains. Vibrios produce volatile organic compounds, such as acetone, during metabolism of leucine (Nemecek-Marshall *et al.*, 1999).

Nitrogen cycling involves series of microbial transformation stages, including: a) fixation of dinitrogen to organic nitrogen (N); b) dissimilatory

reduction of nitrate to produce nitrite or ammonia; c) nitrification of ammonia to nitrite or nitrate; and d) ammonification of organic nitrogen to ammonia (Herbert, 1999). However, vibrios participate in the transformation process except for nitrification. Nitrogen-fixing bacteria fix atmospheric nitrogen and have a profound effect on net community production by input of new nitrogen to nutrient- limited ecosystems. Nitrogen fixation is mediated by vibrios using the cytoplasmic nitrogenase enzyme complex (Coyer *et al*., 1996). Occurrence of nitrate assimilation genes (*nasA*) in vibrios is correlated with ability to grow on nitrate as sole nitrogen source (Allen *et al*., 2001). Many facultative aerobic bacteria can replace oxygen with nitrate as terminal electron acceptor via dissimilatory nitrate reduction. Several alternating electron acceptors, such as nitrate, fumerate and trimethylamine N-oxide, support anaerobic respiratory growth of vibrios (Proctor and Gunsalus, 2000). The dissimilatory reduction of nitrate to ammonia, carried out by marine vibrios is about 80% of overall nitrate consumption in marine sediments (Bonin, 1996). Reminerilization of nitrogenous compounds such as nucleic acids, proteins and polyamino-sugars to simple carbon compounds and ammonia is a vital mechanism in nutrient recycling via microbial loop. Nutrient status and C:N ratio in the environment determine whether ammonia is incorporated into the microbial biomass or excreted into the environment. Mechanism of microbial consumption of polymeric nitrogenous compounds as both carbon and nitrogen sources involves extracellular hydrolysis of nitrogenous polymers to simpler subunits followed by the uptake of the monomers.

Vibrios have a number of extracellular enzymes that participate in degradation of phosphorus-containing macromolecules, also playing a role in the recycling of organic phosphorus into inorganic forms available for primary production. Inorganic phosphorus and polyphosphate ions, found in the dissolved marine phosphorus pools, can be directly utilized by microbes and phytoplanktons. The soluble non-reactive phosphorus pools, containing

less macromolecular fractions such as monophosphate esters, nucleic acids and phosphonates are degraded extracellularly before utilization (Benitez-Nelson, 2000). Phosphate-generating exoenzymes are important for recycling organic phosphorus including alkaline phosphatase, phosphodiesterase and 5'nucleotidases (Hoppe, 2003). Alkaline phosphatase cleaves inorganic phosphate of phosphorylated compounds under neutral or alkaline conditions of the marine ecosystem (Roy *et al*., 1982). 5' Nucleotidases degrade 5' nucleotides to inorganic phosphate and a base prior to its transport into the cytoplasm for subsequent metabolism. Hydrolysis of soluble nonreactive phosphorus by 5' Nucleotidases, supplies as much as half the phosphate required by planktons in coastal California waters (Benitez-Nelson, 2000). 3'5'cyclic nucleotide phosphodiesters enable the metabolism of extracellular cyclic nucleotides such as cAMP. Such periplasmic enzymatic activity of vibrios in mineralizing organic compounds to inorganic compounds and carbon substrates for growth helps enrich local environments with dissolved pools of nutrients that can be utilized by several producer communities.

1.10. Vibriosis

In shrimp or prawn larval rearing systems and grow outs, vibriosis has been designated as systemic bacterial infection caused by several species of *Vibrio*, such as *V. harveyi*, *V. parahaemolyticus, V. alginolyticus,* etc. (Singh *et al*., 1989, Lavilla Pitago *et al*., 1990, Karunasagar *et al*., 1994, Abraham and Manley, 1995). Among them *V. harveyi* has been designated as a potential pathogen in penaeids and V. *alginolyticus* in both penaeids and non-penaeids.

1.11. Significance of the present Study

In shrimp culture, vibrios are still the most important bacterial pathogen responsible for much of the losses. In penaeids, vibrios are known to be the pathogen causing systemic infections and necrotic appendages.The

present study focuses on vibrios especially *Vibrio harveyi* isolated from shrimp (*P. monodon*) larval production systems from both east and west coasts during times of mortality. A comprehensive approach has been made to work out their systematics through numerical taxonomy, confirm their identity through 16S rRNA gene sequence analysis and RAPD profiling to determine diversity and to segregate the virulent from non virulent isolates based on the presence of virulent genes as well as their phenotypic expression**.** The information gathered help develop a simple scheme of identification based on phenotypic characters, and to segregate the virulent from non-virulent strains of *V. harveyi*. The study also reveals the heterogeneity within *V. harveyi* clade.

Objectives

1. Phenotypic characterization and Numerical Taxonomy of vibrios
2. Genotypic characterization of vibrios based on RAPD profiling and analysis of housekeeping genes
3. Phenotypic expression of virulence- *in vitro* assays
4. Genotypic characterization using virulent and luminescent gene markers
5. Pathogenicity of *Vibrio harveyi* in animal model.

CHAPTER-2

Phenotypic characterization and numerical taxonomy of vibrios

2.1. Introduction

2.1.1. History of vibrios

The first *Vibrio* species discovered was *V. cholerae* in 1854 by the Italian physician Filippo Pacini while studying outbreaks of this disease in Florence. Nearly 30 years latter, Robert Koch obtained pure cultures of *V. cholerae*, as little bent resembling a comma or a spiral, highly motile and swarms on gelatin plates and concluded that this organism was indeed the causative agent of cholera (Brock, 1999). In 1893 Koch and his team examining the outbreak of cholera in Hamburg, Germany realized that vibrios were ubiquitous in aquatic environments and that many "forms" of vibrios were non-pathogenic to humans (Brock, 1999).

The family Vibrionaceae comprises species ubiquitously distributed, with majority of bacterial populations occupying aquatic habitats and in association with eukaryotes (Bang *et al*., 1978). Members of Vibrionaceae included species pathogenic to humans (Arias *et al*., 1997a, b), as part of the normal microbiota as well as primary or secondary pathogens of fish (Alsina and Blanch, 1994a). Associations established by vibrios range from mutualistic, e.g., *Vibrio fischeri*-bobtail squid (Baumann *et al*., 1973, 1983) to pathogenic, e.g., *V. cholerae*-humans (Breed *et al*., 1957). Vibrios are Gram-negative, non-sporulating rods, usually the cells are 1µm in width and 2-3 µm in length and motile by atleast one polar flagellum, mesophilic, chemoorganotrophic and have a facultatively fermentative metabolism (Alsina and Blanch, 1994b). They are generally able to grow on marine agar and on the selective medium viz., thiosulfate-citrate bile salt-sucrose agar (TCBS agar) and are mostly oxidase positive. Most vibrios do not grow at

4ºC and in media with high salinity. They are capable of utilizing D-glucose, D-fructose, maltose, dextrin, glycogen, D-trehalose, N-acetyl-D-glucoseamine, methyl pyruvate, L-asparagine, aconitate, L-proline or inosine as the sole carbon source. Most vibrios reduce nitrate, produce acetoin, and are susceptible to the vibriostatic compound 0/129. Many vibrios cannot utilize N-acetyl-D-galactosamine, L-erythritol, *m*-inositol, xylitol, α-hydroxy butyric acid, D-saccharic acid, D,L-carnitine and phenyl ethylamine as sole carbon source. Most vibrios showed leucine arylamidase, acid and alkaline phosphatase activity, but not urease, tryptophane deaminase, α-mannosidase, α-fucosidase and β-glucuronidase activity. However, there are vibrios which show exceptional variations in these phenotypic traits (Baumann *et al*., 1984). Fatty acid profiling showed that most abundant fatty acids in vibrios are 16:1ω7*c* and/or 15 iso 2-OH, 16:0, 18: 1ω7*c*, 14:0 and 16:0 iso which corresponds to >70% of all fatty acids in most species. Phenotypic features of vibrios has a preponderant role in classification, but as new species are been described, the heterogeneity amidst the species has also widened, demanding modern approaches incorporating molecular elements for precise identification and taxa allocation.

2.1.2. Taxonomy of Vibrio

Vibrios belong to the *Gammaproteobacteria* according to 16S rRNA gene sequence analysis. This family is in continuous change, comprising the genera *Vibrio* sensu stricto, *Listonella*, and *Photobacterium* (Austin *et al*., 1995, 1996; Heidelberg, 2002 a, b; Castro, *et al*., 2002). Other genera included in the *Vibrionaceae* by various authors are *Allomonas* (Austin *et al*., 1999), *Salinivibrio* (Farmer and Hickman-Brenner 1992) and *Enhydrobacter* (Baumann and Baumann 1977). Although *Aeromonas*, *Plesiomonas* and *Shewanella* were previously included in this family, their taxonomic current status has been recently changed (Garrity and Holt, 2001).

According to Bergey's Manual of Systematic Bacteriology (1983), there are eight genera within the current family Vibrionaceae: *Vibrio* (65spp.), *Allomonas* (1 sp.), *Catenococcus* (1sp.), *Enterovibrio* (2spp.), *Grimontia* (1sp.), *Listonella* (2spp.), *Photobacterium* (8spp.) and *Salinivibrio* (1sp.). The genera *Allomonas* (Kalina *et al*., 1984) and *Enhydrobacter* (Staley *et al*., 1987) were tentatively allocated to the family Vibrionaceae based on phenotypic characteristics, but it is now known that *Allomonas* belongs to *Vibrio* and *Enhydrobacter* to *Moraxella* (Thompson *et al*., 2003a). Several novel species of *Vibrio* isolated mainly from the aquatic environment and marine organisms have been identified in the last few years, including species related to *Vibrio tubiashii* (i.e. *Vibrio brasiliensis*, *Vibrio coralliilyticus*, *Vibrio neptunius*, and *Vibrio xuii*) (Ben- Haim *et al*., 2003; Thompson *et al*., 2003b); species related to *Vibrio splendidus* (i.e. *Vibrio tasmaniensis*, *Vibrio kanaloae*, *Vibrio pomeroyi* and *Vibrio chagasii*) (Thompson *et al*., 2003a); species related to *Vibrio halioticoli* (i.e. '*Vibrio ezurae*', '*Vibrio gallicus*' and *Vibrio superstes)* (Hayashi *et al*., 2003; Sawabe *et al*., 2007); species related to *V. harveyi* (i.e. *Vibrio rotiferianus)* (Gomez-Gil *et al*., 2003a) and species related to *Vibrio furnissii*, i.e. *Vibrio pacinii* (Gomez-Gil *et al*., 2003b).

The number of species belonging to the genus *Vibrio* has increased with the descriptions of new species including *V.scophthalmi* (Cerda´-Cuellar *et al*., 1997), *V.diabolicus* (Raguenes *et al*., 1997), *V.pectenicida* (Lambert *et al*., 1998), *V.halioticoli* (Sawabe *et al*., 1998), *V.rumoiensis* (Yumoto *et al*., 1999), *V. viscosus* and *V.wodanis* (Lunder *et al*., 2000), *V.aerogenes* (Shieh *et al*., 2000), *V. cyclotrophicus*, *V.lentus* (Macia´n *et al*., 2001a), *V. agarivorans* (Macia´n *et al*., 2001b) and *V.calviensis* (Denner *et al*., 2002), *V.hispanicus* (Gomez-Gil., 2004b). The high variability found among the species, which are much related phenotypically, makes the identification of new isolates difficult. For instance, *V. anguillarum*-related organisms (Bryant *et al*., 1986a, b; Toranzo and Barja 1990) present

difficulties because of their great diversity, which lead to definitions of new phenotypes within the same species (Ortigosa *et al*., 1994; Montes *et al*., 1999).

The taxonomic group Vibrionaceae is extremely diverse. As there is most likely a low number of known species within this group, new species descriptions should be expected during the coming years, particularly in regards to marine ecosystems. This rapid increase in the number of classified *Vibrio* spp., as well as discrepancies over the use of certain tests, is making routine species identification an increasingly complex endeavor. A practical set of biochemical keys for the routine identification of *Vibrio* spp. was developed by Alsina and Blanch (1994a, b), which are proved useful for identifying species for both environmental and clinical purposes, and were widely used in numerous studies (Martı´nez-Picado *et al*., 1996; Montes *et al*., 1999; Oxley *et al*., 2002; Hjelm *et al*., 2004; Maugeri *et al*., 2000; Baffone *et al*., 2006). They serve as an ideal method for rapid, routine biochemical identification in which a large number of isolates are involved, particularly in environmental studies. However, the great abundance of vibrios in aquatic environments, the high diversity detected among vibrionaceae, the increasing number of environmental studies, and the availability of molecular methods for analyzing microbial diversity in the environment have all proven to be determinant factors in the quest to define the large number of new species identified in recent years (Croci *et al*., 2007). Biochemical keys have facilitated the identification of 46 phena (*Vibrio* spp., *Photobacterium* spp., *Plesiomonas* spp. and others). However, these keys should be updated if they are to be of continued practical use for the routine identification of species in this genus.

2.1.3. Numerical Taxonomy of *V.harveyi*

Vibrio harveyi, marine Gram-negative luminous organism requiring sodium chloride for its growth was originally named as *Achromobacter*

harveyi (after Harveyi, a pioneer in the systematic study of bioluminescence; (Johnson and Shunk, 1936). Later, it has been named as *Lucibacterium harveyi*, and *Beneckea harveyi,* it is currently taxonomically positioned as *V. harveyi* (Farmer and Janda, 2005). Phenotypically *V. harveyi* is highly heterogeneous and therefore extremely difficult to identify using conventional bacteriological tests or kits relying upon biochemical reactions (Vandenberghe *et al*., 2003). 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, 1994, Macia´n *et al*., 1996, Ortigosa *et al*., 1994). However, variations in results of some species have been reported, making their identification difficult (Pujalte *et al*., 1992). The phenotypic and genotypic studies, including 16S rDNA sequencing (Gauger and Gomez-Chiarri, 2002), showed that *V. harveyi* belongs to the core species of the genus *Vibrio* (Dorsch *et al*., 1992) and that DNA: DNA hybridization (Baumann *et al*., 1984), 16S and 23S rDNA sequences and amplified fragment length polymorphism fingerprinting (Dorsch *et al*., 1992) determined that *V. harveyi* is closely related to *V. campbellii* and *V. alginolyticus*. The phenotypic heterogeneity is further confounded by evidence that *V. harveyi* contain mobile genetic elements such as plasmids and bacteriophages (Harris and Owens, 1999) and some of which govern phenotypic characteristics (Munro *et al*., 2003).

Fatty acids methyl ester (FAME) profiling is generally very useful as a chemotaxonomic marker, and apparently for the differentiation of various species of Vibrionaceae. The similarity of FAME profiles among the different species examined were strikingly similar, and hence concluded that this technique could be used as an additional phenotypic feature (Lambert *et al*., 1983). Biolog has been one of the most widely used phenotypic

techniques for the identification of Vibrionaceae in the last decade (Klingler *et al*., 1992, Vandenberghe *et al*., 2003). 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). But non-motile *Vibrio* species, e.g., the *V. halioticoli* group, have been detected (Sawabe *et al*., 2003), suggesting that the presence of flagella is not an essential diagnostic feature. Likewise, oxidase-negative *V. metschnikovii* and *V. gazogenes* strains have been documented (Alsina, and Blanch, 1994 a,b).This suggests that a method for differentiating and clustering the strains with ease requires further studies.

List of vibrios (Thompson *et al*., 2005)

1. *Vibrio aerogenes* LMG 19650T Seagrass bed in Nanwan bay (Taiwan) Sediment
2. *V. aestuarianus* LMG 7909T Oregon (United States) Oyster
3. *V. agarivorans* LMG 21449T Valencia (Spain) Seawater
4. *V. alginolyticus* LMG 4409T Japan Spoiled horse mackerel (*Trachurus trachurus*)
5. *V. anguillarum* LMG 4437T Norway Diseased cod (*Gadus morhua*)
6. *V. brasiliensis* LMG 20546T LCMM Floriano´polis (Brazil), 1999 Bivalve larvae (*Nodipecten nodosus*)
7. *V. calviensis* LMG 21294T Bay of Calvi (Mediterranean), France Seawater
8. *V. campbellii* LMG 11216T Hawaii (United States) Seawater
9. *V. chagasii* LMG 21353T AARS Austevoll (Norway), 1997 Gut of turbot larvae (*Scophthalmus maximus*)
10. *V. cholerae* LMG 21698T Asia Clinical
11. *V. cincinnatiensis* LMG 7891T Ohio (United States) Human blood and cerebrospinal fluid
12. *V. coralliilyticus* LMG 20984T Indian Ocean near Zanzibar, 1999 Diseased *Pocillopora damicornis*
13. *V. crassostreae* LMG 22240T IFREMER La tremblade (France) Hemolymph of diseased reared oysters (*Crassostera gigas*)
14. *V. cyclitrophicus* LMG 21359T Washington (United States) Creosote-contaminated sediment
15. *V. diabolicus* LMG 19805T East Pacific Rise, 1991 Dorsal integument of polychaete (*Alvinella pompejana*)
16. *V. diazotrophicus* LMG 7893T Nova Scotia (Canada) Sea urchin (*Strongylocentrotus*)

17. *V. ezurae* LMG 19970T Kanagawa (Japan), 1999 Gut of abalone (*Haliotis diversicolor supertexta*)
18. *V. fischeri* LMG 4414T Massachusetts (United States), 1933 Dead squid
19. *V. fluvialis* LMG 7894T Bangladesh Human feces
20. *V. fortis* LMG 21557T Ecuador, 1996 *Litopenaeus vannamei* larvae
21. *V. furnissii* LMG 7910T Japan Human feces
22. *V. gallicus* LMG 21330T Brest (France), 2001 French abalone *Haliotis tuberculata*
23. *V. gazogenes* LMG 19540T Massachusetts (United States) Mud from saltmarsh
24. *V. halioticoli* LMG 18542T Kumaishi (Japan); 1991 Gut of abalone (*Haliotis discus hanai*)
25. *V. harveyi* LMG 4044T Massachusettes (United States), 1935 Dead amphipod (*Talorchestia* sp.)
26. *V. hepatarius* LMG 20362T CENAIM (Ecuador), 2000 Digestive gland of white shrimp (*Litopenaeus vannamei*)
27. *V. hispanicus* LMG 13240T Barcelona (Spain), 1990 Culture water
28. *V. ichthyoenteri* LMG 19664T Hiroshima (Japan) Gut of diseased Japanase flounder (*Paralichtys olivaceus*)
29. *V. kanaloaei* LMG 20539T IFREMER (France), 1998 Diseased oyster larvae (*Ostrea edulis*)
30. *V. lentus* LMG 21034T Mediterranean coast, Valencia (Spain) Oysters
31. *V. logei* LMG 19806T United States Gut of Arctic scallop
32. *V. mediterranei* LMG 11258T Valencia (Spain) Coastal seawater
33. *V. metschnikovii* LMG 11664T Asia Diseased fowl
34. *V. mimicus* LMG 7896T North Carolina (United States) Infected human ear
35. *V. mytili* LMG 19157T Valencia (Spain) Bivalve (*Mytilus edulis*)
36. *V. natriegens* LMG 10935T Sapelo Island (United States) Salt marsh mud
37. *V. navarrensis* LMG 15976T Villa Franca Navarra (Spain), 1982 Sewage
38. *V. neonatus* LMG 19972T Kanagawa (Japan), 1999 Gut of abalone (*Haliotis discus discus*)
39. *V. neptunius* LMG 20536T LCMM Floriano´polis (Brazil), 1998 Bivalve larvae (*Nodipecten nodosus*)
40. *V. nereis* LMG 3895T Hawaii (United States) Seawater
41. *V. nigripulchritudo* LMG 3896T Hawaii (United States) Seawater
42. *V. ordalii* LMG 13544T Washington (United States), 1973 Diseased coho salmon (*Oncorhynchus rhoddurus*)
43. *V. orientalis* LMG 7897T Yellow Sea (China) Seawater
44. *V. pacinii* LMG 19999T Dahua (China), 1996 Healthy shrimp larvae (*Penacus chinensis*)
45. *V. parahaemolyticus* LMG 2850T Japan Diseased human

46. *V. pectenicida* LMG 19642T Brittany (France), 1991 Diseased bivalve larvae (*Pecten maximus*)
47. *V. pelagius* LMG 3897T Hawaii (United States) Seawater
48. *V. penaeicida* LMG 19663T Kagoshima (Japan) Diseased kuruma prawn (*Penaeus japonicus*)
49. *V. pomeroyi* LMG 20537T LCMM Floriano´polis (Brazil), 1998 Healthy bivalve larvae (*Nodipecten nodosus*)
50. *V. proteolyticus* LMG 3772T United States Intestine of isopod (*Limnoria tipunctala*)
51. *V. rotiferianus* LMG 21460T ARC Gent (Belgium), 1999 Rotifer in recirculation system (*Brachionus plicatilis*)
52. *V. ruber* LMG 21676T Keelung (Taiwan) Seawater
53. *V. rumoiensis* LMG 20038T Japan Drain pool of a fish-processing plant
54. *V. salmonicida* LMG 14010T Norway Diseased Atlantic salmon (*Salmo salar*)
55. *V. scophthalmi* LMG 19158T Spain Turbot juvenile (*Scophthalmus maximus*)
56. *V. splendidus* LMG 19031T North Sea Marine fish
57. *V. superstes* LMG 21323T Australian Coast Gut of abalone (*Haliotis laevigata* and *H. rubra*)
58. *V. tapetis* LMG 19706T Landeda (France) Clam (*Tapes philippinarum*)
59. *V. tasmanienis* LMG 20012T MPL (Tasmania) Atlantic salmon (*Salmo salar*)
60. *V. tubiashii* LMG 10936T Milford, Conn. (United States) Hard clam (*Mercenaria mercenaria*)
61. *V. vulnificus* LMG 13545T U.S.A. Human wound infection
62. *V. wodanis* LMG 21011T Norway, 1988 Salmon with winter ulcer (*Salmo salar*)
63. *V. xuii* LMG 21346T

2.2 Materials and Methods

2.2.1. Purification and stocking of cultures

One hundred forty seven isolates of vibrios recovered from shrimp hatcheries of East and West coasts of India during mass larval mortalities, maintained at the National Centre for Aquatic Animal Health were revived, checked for purity and stored in different conditions, such as slant culture and stab culture overlayed with liquid paraffin. Three sets of the isolates were stocked at -80°C by adding 200µl 60%glycerol. Preliminary phenotypic characterization of all the wild strains was carried out employing the dichotomous key of Alsina and Blanch (1994a, b), and were identified

as *V.harveyi, V.parahaemolyticus*, *V.alginolyticus*, *V.fluvialis, V.cholerae*, *V.mediterraneii, V.proteolyticus, V.nereis, V.vulnificus,* and *V.splendidus*). Based on this identification corresponding type strains were purchased from BCCM/LMG (Belgian Co-ordinated Collection of Micro-organisms, Belgium) and MTCC (IMTECH, Chandigargh, India) for further phenotypic characterization and numerical taxonomy.

Table-2.1: Details of the isolates and its source

Code	Sample Type	Condition	Stage	Tank Details	Hatchery	Location
V1-V14	Rearing Water	Mass Mortality	PL-10	NA	Kakinada	Andhra Pradesh
V15-V35	PL	Moribund	PL-10	NA	Kakinada	Andhra Pradesh
V36	PL	Normal	PL-5	L-9	Kakinada	Andhra Pradesh
V37-V42	Water	Drain Out	NA	NA	Kakinada	Andhra Pradesh
V43	Mysis	Normal	M-1	L-15	Kakinada	Andhra Pradesh
V44-V53	Nauplii	Mass Mortality	N to Zoea	Quarantine Tank	Kakinada	Andhra Pradesh
V54-V61	Raw Seawater	Intake	NA	NA	Kakinada	Andhra Pradesh
V62-V64	Rearing Water	Normal	NA	Crab Tank	Kakinada	Andhra Pradesh
V65	Crab Carapace	Normal	NA	Crab Tank	Kakinada	Andhra Pradesh
V66-V72	Beach Sand	NA	NA	NA	Kakinada	Andhra Pradesh
V73-V87	PL	Mass Mortality	NA	NA	Azhikode	Kerala
V88-V 92	PL	Mass Mortality	NA	NA	Kodungallore	Kerala
V93-V125	PL	Mass Mortality	NA	NA	Azhikode	Kerala
V126-V133	PL	Necrotic	NA	NA	Trichur	Kerala
V134-V147	PL	Mass Mortality	NA	NA	Kollam	Kerala

Table-2.2: Details of the Type strains

Code	Acc No:	Strain	Isolated from
V148	LMG 4409	*V.alginolyticus*	Spoiled horse mackerel causing food poisoning, Japan
V149	MTCC 4439	*V.alginolyticus*	
V150	MTCC 3906	*V. cholerae*	Clinical specimen- human cholerae epidemic-1960, India
V151	LMG 11654	*V.fluvialis*	Human faeces
V152	LMG 4044	*V.harveyi*	Dead amphipod (*Talorchestia sp.*), Woods Hole, Masaachusetts, United States.
V153	LMG 11258	*V.mediterranei*	Coastal marine plankton, Valencia, Spain.
V154	LMG 3895	*V.nereis*	Seawater enriched with propoanol, Oahu Hawaii, United States.
V155	LMG 2850	*V.parahaemolyticus*	patients suffering from "Shirashu" food poisoning, Japan.
V156	LMG 3772	*V.proteolyticus*	Intestine of wood-boring isopod (*Limnoria tripuncata*) intestine, United States.
V157	LMG 19031	*V. splendidus*	Marine fish, NCIMB
V158	LMG 13545	*V.vulnificus*	Human blood, United States.

2.2.2. Phenotypic characterization

All the isolates were screened by using the following tests: Gram stain, luminescence by observation in dark, oxidase activity, glucose fermentation, motility and resistance to O/129 and an array of biochemical test on Biolog plates for determining their phenotypic profile.

2.2.2.1. Motility assay

a) Soft agar method

Motility was tested by soft agar method in ZoBell's Marine Agar 2216E having the following composition.

Ingredients	Amount
Yeast extract	0.1g
Peptone	0.5g
Ferric phosphate	0.01g
15ppt seawater	100mL
Agar	3g
pH	7.2 ±0.1

Molten medium was poured into tubes in 3ml aliquots and autoclaved at 15lbs for 15min. Stab inoculated the medium with the cultures and incubated at 28 ± 0.5°C for 24 to 48hr. Rhizoidal growth from the line of inoculation towards the peripheral area was considered as the sign of motility. A thick growth along the line of inoculation was considered negative.

b) Hanging drop method

The organisms were grown in ZoBell's 2216e broth of the above composition. A loop full of the 18 to 24-hr-old culture was placed at the centre of the coverslip. Vaseline was spotted at the corners of the coverslip to facilitated adherence of the coverslip to the slide. The cavity slide was kept over the drop in such a way that the drop should come within the cavity. The whole preparation was inverted quickly so that the drop of the culture was seen hanging from the coverslip. The slide was placed under oil immersion objective and observed for actual displacement of cells that could very well be differentiated from Brownian movement (Cowan and Steel, 1965).

2.2.2.2. Flagellar Staining (Rhodes, 1959)

Silver deposition staining method (Fontana and modified by Rhodes, 1959) was employed. With a pipette 2ml of 15ppt sterilized seawater was added to a young actively growing (18hr old) slope culture and gently agitated. The tubes were incubated in an incubator at 28±0.5°C for 30 mins and a large loopful of the culture suspension was removed from the tube and placed at one end of the slide until the drop spreads on the slide. The slide is then air dried at room temperature, followed by flooding the slide with Reagent-A and incubated for 2 min, washed with distilled water and air dried. Stained with Reagent-B till a brownish colour develops, air dried the slides and observed under oil immersion microscope.

Reagent-A

Tannic acid	-	5gms
$FeCl_3$	-	1.5gms
NaOH	-	1% solution- 1ml
Formalin- 15% solution	-	2ml
Distilled water	-	98ml

Reagent-B

Silver nitrate	-	2gms
Distilled water	-	100ml

2.2.2.3. Oxidation Fermentation reaction (Cowan and Steel, 1965)

This characteristic is usually determined by inoculating the organisms into deep agar medium supplemented with 1% glucose in the culture tubes (Collins *et al.*, 2004). MOF medium (Himedia Laboratories, Bombay) (22gms) was employed to which 15g agar was added to 1000ml of distilled water and autoclaved at 15lbs for 15min. Prior to cooling the autoclaved basal medium1% D-glucose or dextrose was added aseptically and transferred the sterile medium into 4ml aliquots aseptically into sterile tubes and autoclaved at 10lbs for 10min and converted to slants with a long butt. The cultures were stabbed and streaked and with an inoculation needle after solidification of agar and incubated at 28±0.5°C.

Acidic changes at or near the surface indicated that the substrate was oxidized by the organism, whereas the development of uniform acidity

throughout the tube showed that the organism was facultatively anaerobic. The results were recorded as follows:

O- Oxidation (yellow colouration in the butt)
F- Fermentation (yellow colouration throughout the tube)
FG- Fermentation with gas production
Alk / N – alkaline reaction (pink or purple colouration in the slant and no reaction in the butt)

2.2.2.4. Hydrogen sulphide production on TSI medium (Cowan and Steel, 1965)

Many bacteria produce hydrogen sulphide from organic sulphur compounds in the medium. There are numerous tests for the detection of H_2S production and these vary widely in their sensitivity (Cowan and Steel, 1965). TSI is a multipurpose medium containing the sugars sucrose, lactose and glucose along with phenol red as the indicator. If an organism ferments any of these sugars, or any combination of them, the medium will turn to yellow because of acidic pH caused by end products of fermentation. TSI agar medium (Himedia Laboratories, Bombay) supplemented with 1.5% agar was heated to dissolve the solids in water, mixed and transferred into tubes as 5-6 ml aliquots. Sterilized at 10 lbs for 15 min and cooled to form slopes with deep butts. Stabbed and streaked the tubes, incubated at 28 ± 0.5^0C for 24 to 48 hrs. Any of the following reactions could be noticed.

Observation	**Inference**
Yellow colouration of the slope	Oxidative reaction
Pink or purple colouration of the slope	Alkaline reaction
Yellow colouration throughout the tube or in the butt	Fermentative reaction
Blackening of the butt	Hydrogen sulphide production
Split or gas bubble in the butt	Gas production

2.2.2.5. Kovac's Oxidase test (Cytochrome oxidase activity) (Kovacs' 1956)

This test is used to find out whether the culture is capable of producing Cytochrome Oxidase enzyme. The detection of Cytochrome Oxidase

activity is used as a differentiating test mainly for the aerobic and facultatively anaerobic groups of Gram negative bacteria. Oxidase enzyme is believed to oxidize phenol, amines etc. indirectly by bringing about the oxidation of cytC. It then oxidizes the phenols or the amines. The test solution (tetramethyl-p-phenylene diamine dihydrochloride) is oxidized to indophenol, a blue coloured compound with which the activity is detected.

According to the methods recommended by Kovacs' (1956), the organisms were freshly grown on ZoBell's - Marine Agar 2216E. A platinum loop was used to pick the growth and made a compact smear on a filter paper moistened with 2-3 drops of a 1% solution of tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive result was recorded when the smear turned bluish-violet within 10 seconds, indicating the formation of indophenol.

2.2.2.6. Sensitivity to vibriostat compound O/129 (2,4-diamino-6,7-di-iso propyl pteridine phosphate) Shewan *et al*., (1954)

The sensitivity of vibrios to the vibriostat agent O/129 has long been recognized by Shewan *et al* (1954). This compound is very effectively used for differentiating *Vibrio* and *Photobacterium* from *Aeromonas* and *Leucibactrium*. *Vibrio* and *photobacterium* are sensitive to the vibriostat compound while *Aeromonas* and *Leucibacterum* are resistant.

Antibiotic assay filter paper disc of 6mm diameter (Whatman No.1) were prepared aseptically to contain 150µg/ml of the compound. The discs were stored in refrigerator (4^0C) and used as required (Furniss *et al*., 1978). The ZoBell's- Marine Agar plates were swabbed with the suspension of the test bacterial organism to get a confluent growth and the discs were placed on it with an appropriate spacing. The cultures that were sensitive to the pteridine compound developed a clearing zone around the disc.

2.2.2.7. Catalase test (Collins *et al*., 2004)

The principle of this test is that when organisms containing catalase are mixed with hydrogen peroxide (H_2O_2), gaseous oxygen is liberated.

The test organisms are grown on a slope of ZoBell's agar. A thick smear of the organism was made from a 24 hr culture on a clean slide and a drop of hydrogen peroxide is placed on it. Immediate formation of gas bubbles indicated the liberation of oxygen and positive catalase test (Collins *et al*., 2004).

2.2.2.8. Production of Indole (Cowan and Steel, 1965)

Certain bacteria produce indole by decomposition of tryptophan, which is present in tryptone broth. This liberated indole reacts with Kovacs' reagent to produce red colour at the top of the medium (Cowan and Steel, 1965).

Composition of the test medium.

Ingredients	Amount
Tryptone	1.5g
NaCl	5.0g
pH	7.5±0.3
Distilled water	1000ml

The medium was dispensed as 3 ml aliquots into tubes and autoclaved at 15 lbs for 15min. The isolates were inoculated and incubated for 48 hrs, after incubation, 0.5 ml of Kovacs' reagent was added to each tube.

Preparation of Kovac's reagent

ρ - dimethyl amino benzaldehyde	5g
Amyl alcohol	75ml
Con.HCl	25ml

2.2.2.9. Methyl Red and Voges-Proskauer tests

These tests are normally carried out with cultures grown in glucose-phosphate peptone water, which has the following composition.

Ingredients	Amount
Glucose	5.0g
K_2HPO_4	5.0g
Peptone	5.0g
Distilled water	1000ml

The medium was dispensed as 5 ml aliquots in small culture tubes and autoclaved at 10 lbs for 10min. The inoculated tubes were then incubated for 7 days until good growth was obtained.

Methyl Red test (Collins *et al*., 2004)

This test detects the production of sufficient acid during the fermentation of glucose and the maintenance of conditions such that the pH of an old culture is sustained below a value of about 4.5. A few drops of methyl red indicator were added to the culture and a resultant definite red colour was considered positive. Shades intermediate between yellow and red were considered as doubtful positive results. The indicator was prepared by dissolving 0.1g methyl red in 300 ml 95% ethyl alcohol, which was then diluted to 500 ml with distilled water (Collins *et al*., 2004).

Voges-Proskauer test (Acetoin production) (Collins *et al*., 2004)

Some organisms, after producing acids from glucose, are capable of converting acids to acetylmethyl carbinol or 2, 3-butanediol, which are neutral substances. Aeration in the presence of alkali then converts the products to diacetyl, which in turn reacts with the peptone constituents producing a pink colouration. An aliquot of 1ml of this medium was taken and transferred to a sterile tube. Then 0.6 ml of 5% solution of alpha naphtol in absolute ethanol was added followed by 0.2 ml of 40% KOH, and mixed well. A positive reaction was indicated by the development of a pink colour in 2-5 min, becoming crimson in 30 min with intermittent shaking of the tube to ensure maximum aeration (Collins *et al*., 2004).

2.2.2.10. Production of Urease Christensen (1946)

Urease catalyses the following reaction:

$H_2NCO\ N\ H_2 + H_2\ O \longrightarrow 2NH_3 + CO_2$

This test is used to determine the production of the enzyme urease by microorganisms, whereby the urea is hydrolyzed to form ammonia, which is highly alkaline. In a medium used for determination of urease activity, urea and a pH indicator were incorporated. A positive result is shown by a rise in the pH value resulting from the hydrolysis of urea and a respective change in the colour of the indicator used.

The medium devised by Christensen (1946) has the following composition.

Ingredients	Quantity
Peptone	1.0g
NaCl	5.0g
Glucose	1.0g
KH_2PO_4	2.0g
Phenol red (0.2% solution)	5ml
Agar	20.0g
pH	7.2±0.2
Distilled water	995ml

Yeast extract (0.1%) was also added for organisms requiring growth factors. The medium was prepared in bottles, sterilized and cooled to about 55^0C. A 20% solution of urea previously sterilized by filtration was then added to give a final concentration of 2% urea in the molten medium. The completed medium was dispensed into tubes and converted to slants. A control without urea was also included.

The tests and the controls were inoculated and incubated for 24 hrs at 28 ± 0.5^0C. Urease activity caused the yellow indicator to change to red.

2.2.2.11. Citrate utilization (Collins *et al*., 2004)

This test demonstrates the ability of the microbes to utilize the test compound citrate as a sole source of carbon and energy. Utilization of citrate and growth in citrate agar results in an alkaline reaction, which

changes the colour of the medium, provided. In this medium (Simmon's citrate agar), bromothymol blue indicator was used which changed from green to bright blue on utilization of citrate (Collins *et al.*, 2004). The composition of Simmon's citrate agar medium has the following composition.

Ingredients	Quantity
Sodium citrate	0.2g
$MgSO_4$. $7H_2O$	0.02g
NaCl	15.0g
$(NH_4)_2HPO_4$	1.0g
K_2HPO_2	1.0g
Bromothymol blue	0.02g
Agar	20.0g
pH	6.9
Distilled water	1000ml

Simmon's citrate agar medium was prepared in the form of slants in tubes. The slants were inoculated by streaking over the surface with a loopful of culture and incubated for 3-4 days. Colour change from green to bright blue indicated that the culture was positive.

2.2.2.12. Nitrate reduction test (Holt *et al.*, 1994)

This is a test for the presence of enzyme nitrate reductase which causes the reduction of nitrate, in the presence of a suitable electron donor, to nitrite which can be tested by an appropriate colorimetric reagent. Autoclaved at 15 lbs for 15min in 5 ml aliquots, the tubes were inoculated with the test culture and incubated at 28±0.4^{0}C for 48 hrs (Holt *et al.*, 1994).

Composition of nutrient broth

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
KNO_3	1.0g
pH	7.5
Distilled water	1000ml

Preparation of reagents:

Solution A

Sulphanilic acid	:	1.0g
5N (glacial acetic acid)	:	100 ml

Solution B

Dimethyl α – naphthylamine	:	0.6g
5N (glacial acetic acid)	:	100 ml

The presence of nitrite could be determined by adding to 5 ml of the culture 0.5 ml of reagent A, followed by 0.5 ml of reagent B. The development of a red colour indicated that the nitrate had been reduced to nitrite.

2.2.2.13. Aminoacid decarboxylation tests (Moller, 1955)

The aminoacid decarboxylase test demonstrates the bacterial decarboxylation of lysine, arginine, and ornithine, and these tests are of particular use in identifying members of *Enterobacteriaceae* (Moller, 1955).

These tests are based on the ability of some bacteria to decarboxylate an aminoacid to corresponding amine with the liberation of CO_2. The production of these decarboxylases is induced by a low pH and, as a result of their action; the pH rises to neutrality or above. This is achieved by cultivating the test organisms in a fermentable carbohydrate medium. The lysine and ornithine reactions are truly decarboxylase tests, but the arginine reaction is recognized now as a dihydrolase test.

10g of the L-aminoacid (L (+) Lysine dihydrochloride, or L (+)-Ornithine monohydrochloride, or 20g of the DL form, was incorporated in Falkow's medium (modified from Falkow, 1958), containing the following composition.

Ingredients	Quantity
Peptone	5.0g
NaCl Yeast extract	5.0g 3.0g
Glucose	1.0g
Bromocresol purple (0.2% solution)	10 ml
Distilled water	990ml

The solids were dissolved in distilled water and pH adjusted; added the indicator solution. Sterilized the medium at 15 lbs for 15min, cooled and amino acids were added. Readjusted the pH if required, dispensed in 2 ml aliquots into sterile tubes and overlaid with liquid paraffin, followed by sterilization at 10 lbs for 10min.

An inoculum from a culture of the test organisms on a solid medium was introduced with a straight inoculating wire through the paraffin. Various controls included a tube containing only the basal medium was also inoculated and examined daily for 4 days. As a result of the bacterial fermentation of the glucose in the medium, the indicator turned yellow. The control tube without the aminoacid remained yellow; but a subsequent change to violet or purple in the tests indicated that alkaline degradation products were produced in the course of decarboxylation of the particular aminoacid.

Arginine Dihydrolases (Thornley, 1960)

The ability of certain organisms to produce an alkaline reaction in arginine containing medium under relatively anaerobic conditions has been used by Thornley (1960) to differentiate between certain Gram negative bacteria, especially *Pseudomonas* spp. The alkaline reaction is thought to be due to the production of ornithine, CO_2 and NH_3 from arginine. Thornley's medium has the following composition.

Ingredients	Quantity
Peptone	1g
NaCl	5g
K_2HPO_4	0.3g
Agar	3g
L(+)-arginine hydrochloride	10g
pH	7.2
Distilled water	1000ml

The solids were dissolved in distilled water and pH adjusted; phenol red was added as the indicator solution. Medium was sterilized at 15 lbs for 15 min and aminoacids added and readjusted the pH to 6.5 if required. Dispensed in 2 ml aliquotes into test tubes and overlaid with liquid paraffin and sterilized at 10 lbs for 10min. The test organisms were stab inoculated into the medium through the liquid paraffin layer. Color changes were recorded after incubation at 28 ± 0.5° C for upto 7 days, the color change from yellowish orange to red is considered as positive.

2.2.2.14. ONPG (β-galactosidase) test (Collins *et al*., 2004)

The β-galactosidase (ONPG) test, determines the presence of the enzyme β-galactosidase by utilizing o-nitrophenyl- β-D-galactopyranoside, to differentiate late lactose fermenting organisms (Collins *et al*., 2004).

Medium

O-nitrophenyl- β-D-galactopyranoside :0.6 g

pH 7.5

Distilled water 100 ml

The solution was sterilized by filtration through 0.22µm filter. To 3 parts of 1% peptone in seawater (pH 7.5) 1 part of the above medium was added aseptically and isolates were inoculated and incubated for 24hrs at 28±0.4^{0}C. Yellow colour indicated positive reaction.

2.2.2.15. Gluconate test (Collins *et al*., 2004)

The ability of an organism to oxidize gluconates to a non-reducing compound 2-keto-gluconate, which subsequently accumulates in the medium, can be tested with a suitable reagent (Collins *et al*., 2004).

Composition of the medium

Ingredients	Quantity
Peptone	1.5 g
Yeast extract	1.0 g
NaCl	5g
Dipotassium hydrogen phosphate (K_2HPO_4)	1.0 g
Potassium gluconate	40.0 g
Distilled water	1000ml
pH	7.0

This medium was distributed in 10ml aliquots in screw-capped tubes and autoclave at 15 lbs for 15min.

Benedict's qualitative reagent

Sodium Citrate, 173g and anhydrous Sodium carbonate, 100g were added to 800 ml distilled water and dissolved by heating. $CuSO_4.5\ H_2O$ (17.4 g in 100 ml of distilled water) was added slowly, with gentle stirring, when cooled, made up to 1000ml with 15ppt seawater.

Method

An aliquot of 1ml of the medium was aseptically added into clean, sterile tubes and inoculated with the isolates and incubated at 28^0C for 48hrs. Following incubation, 1 ml Benedict's reagent was added and placed the tube in a boiling water bath for 10 min and observed for the production of a coloured precipitate of cuprous oxide. The test result was read as:

Green to orange precipitate : Positive

Blue colour of the reagent unchanged : Negative

2.2.2.16. Acid and gas production from sugars (Collins *et al*., 2004)

Fermentation of carbohydrates can be demonstrated by the production of acid or acid and gas (CO_2 and/ or H_2) in liquid medium in test tubes. Hugh and Leifsons' basal medium was used for this purpose (Collins *et al*., 2004).

Hugh and Leifsons' medium has the following composition,

Ingredients	Quantity
Peptone	2.0g
NaCl	5.0g
K_2HPO_4	0.3g
Phenol red (1% aqueous solution)	30 ml
pH	7.3±0.2
Distilled water	970ml

The carbohydrates were added to a final concentration of 0.1% (w/v). Acid production was readily observed by incorporating into the medium an appropriate pH indicator such as phenol red. The basal medium was first autoclaved at 15 lbs for 15min along with plugged tubes. All the carbohydrates were added to the sterile basal medium to a final concentration of 0.1% (w/v). The medium was dispensed into the sterile tubes aseptically and was autoclaved at 10 lbs for 10min.

The tubes were inoculated with an inoculation needle and incubated at 28±0.5^{0}C for 3 days and the results recorded. The production of acid induced a change in the phenol red indicator, which changed from pink to yellow under acidic condition. The following carbohydrates (sugars and sugar alcohols) were used for the production of acid.

Polyhydric alcohols	Adonitol, mannitol, sorbitol, myo-inositol
Pentoses	Arabinose, xylose, rhamnose
Hexoses	Glucose, fructose, mannose, galactose
Disaccharides	Sucrose, maltose, lactose, trehalose, cellobiose
Trisaccharides	Raffinose
Polysaccharides	Starch, inulin

2.2.2.17. Sodium chloride tolerance test

Growth at different concentrations of NaCl upto 10% (w/v) was tested in 1% tryptone broth at pH 7.3±0.3 containing varying amounts of analytical grade NaCl. The medium containing 0, 3, 6, 8 and 10% NaCl was dispensed in 3 ml aliquots into tubes, sterilized at 15 lbs for 15min and inoculated with a 24hr culture. Growth was detected visually by observing turbidity.

2.2.2.18. Utilization of sugars using GN2 Biolog plates

Utilization of sugars (95) as single carbon source was determined using GN2 Biolog Microplates – Biolog catalog # 1101 (GN system, Biolog, Hayward, CA, USA) based on reduction of tetrazolium in response to the process of metabolism rather than producing acid as the byproduct. Colonies of 18hr old culture were added to the GN2 inoculation fluid- Biolog catalog # 72101 and the OD was adjusted to the turbidity of the GN2 standard coloration. A 150 µl aliquot of the bacterial culture dissolved in the inoculation fluid was added to each of the 96 wells of a GN2 Biolog plate and incubated overnight at 28 ºC (Chang- Ping Yu and Yue-Hwa Yu, 2000). Plates were scored colorimetrically at 570nm for the utilization of carbon source present in each well determined by purple coloration due to the reduction of tetrazolium dye in the positive wells with a score of 1 for utilization 0 for no utilization.

2.2.3. Clustering based on unweighted average linkage

Based on the phenotypic characterization of pure cultures, data matrix was generated by coding the results obtained from the tests as '1' for positive, '0' for negative, and '9' for doubtful results. The data matrix prepared in 'Excel' spreadsheets (Microsoft Office package) was converted to proprietary matrix files by the program NTedit, Version 1.1b (Applied Biostatics Inc) and rectangular data matrix generated was analyzed. Similarities were calculated by the simple matching coefficient using statistical module, sequential agglomerative hierarchical nested cluster method (SAHN) and clustering was achieved based on unweighted pair-group method with arithematic means (UPGMA) Sneath and Sokal (1973) employing the software Numerical

Taxanomy and Multivariate Analysis System (NTSYS pc 2.0) Version 2.02i (Applied Biostatics Inc) (Rohlf, 1998). Dendrogram of the isolates was constructed using the results of biochemical characterization. Jaccard's distance, coefficient and percentage similarity of the clusters were obtained from the dendrogram of phenotypic profile (Wallwork, 1976). Also using the dendrogram, a dichotomous key was constructed for clustering the isolates of vibrios obtained from the east and west coast of India. The error probability value and mean variance value for each phena was analysed following the method of Sneath and Johnson (1972).

Formulas employed:

A) Jaccard's distance $d_{ij} = 1- (p/ p+q+r) = q+r/ p+q+r+s$

Where p= Number of variables positive

q= Number of variables positive for 1 and negative for other

r= Number of variables negative for 1 and positive for other

B) Jaccard's Coefficient = 1- jaccard's distance

C) Expected Mean variance $E (S') = S (2p-1)^2 + 2p (1-p)$

Where S= similarity coefficient, p= probability at 0.05p.

D) Standard Error $SE (S') = \sqrt{2p (1-p)/n} \times \sqrt{1-2p (1-p)}$

Where p= probability at 0.05p, n= number of tests

E) Average Probable value $= \frac{[S'-2p (1-p)]}{(2p-1)^2}$

F) Test of Variance $S_i^2 = d/2t$

Where d= Number of strains giving diverging result, t= Total number of tests

2.2.4. Reproducibility Assessment

Reproducibility of each phenotypic character tested aids in determining the probability of the isolates to be grouped as the same strain (Butler *et al*., 1975). Hence the bacterial isolates from each phena were

randomly selected along the reference strains and repeated the 135 tests to determine the significance of reproducibility of each phenotypic character by employing chi-square test (Tables- 2.5 and 2.6).

2.2.5. Validation of Dichotomous Key

Validation of the constructed dichotomous key was carried out with the randomly selected isolates from each phenon and the reference strains to the set of phenotypic characters enlisted in the dichotomous key. The validation was statistically analyzed employing chi-square test of significance.

2.3. Results

2.3.1. Characterization of bacterial isolates

The isolates (158nos) could be analyzed based on phenotypic characters employing UPGMA yielding **17 Phenon** defined at a Jaccards coefficient range of 0.55 to 0.988 (Table-4). The final data matrix contained information on 135 unit characters, giving a co-phenetic correlation coefficient (r) at 0.80. A dendrogram (Fig. 2. 1) representing the phena was constructed using similarity coefficient (NYSYS pc 2.0). Seven phena did not group with any of the type strains, however, exhibited closeness to the neighboring clusters which were integrated with the type strains at 100%S, and hence could be reasonably identified. Dendrogram analysis showed that, three type strains, viz., V. *nereis* (V154) (LMG 3895), *V. proteolyticus* (V156) (LMG 3772) and *V. splendidus* (V157) (LMG 19031) occupied individual positions without joining to any of the environmental isolates studied.

All the isolates (158 operational taxonomic units) were assigned to genus *Vibrio* based on Alsina and Blanch (1994 a, b) and were grouped into 3 core groups. Under Core group-1 with phenon1 to 6, the **Phenon-1** contained majority of the isolates (83nos) obtained from a single hatchery along Kakinada coast, Andhra Pradesh, during an incident of mass mortality

of post larvae and mysis. Phenon-1 characterized by their luminescence differed from its counterpart phena 2&3 diverging at 97 and 94%S respectively. **Phenon-1** varied from other two by giving positive reaction to utilization of β- Methyl- D-glucoside, L- Analylglycine, Glycyl L- glutamic acid and D-Serine. While **Phenon-2** could be differentiated from the isolates in phena1&3 by having negative results for Voges–Proskauer (VP) test and utilization, of L-aspargine, L-aspartic acid and Glycyl-L-aspartic acid and was exceptionally positive to utilization of α-Clycodextrin. Strikingly, (V152) the type stain of *V.harveyi* (LMG 4044) did not join with the isolates in phena1&2, instead was grouped along with **Phenon-3** at 100%S consisted of 5 bacterial isolates obtained from Kodungallore hatchery, Kerala. Phenon-3 varied from phena 1&2 in negative result for luminescence, growth at 10% NaCl, Aesculin hydrolysis, D-Galactosidase, Melibiose, D-Trehalose, D-Cellobiose and Gentiobiose utilization. This phenon was identified as *V. harveyi* since the type stain (V152) (LMG 4044) joined with the cluster. Since, phenon 3 joined with the phena 1 & 2 at 95%S, they were also designated as *V. harveyi*.

Phenon-4 was identified as *V.parahaemolyticus*, as V155 *(*LMG 2850) type strain of *V.parahaemolyticus* joined with the environmental isolates at 100%S. This phenon was correlated at 95%S to the isolates of *V.harveyi*. **Phenon -5** had 5 isolates clustered at 100%S joining with the type strain *V. mediterranei* (LMG 11258) (V 153). **Phenon -6** had only 3 isolates without any type strains integrated. However, Phenon 5 and 6 could join together at 97% S and thereby phenon-6 could be identified as *V. mediterranei.* The isolates clustered under the phena 4, 5 & 6 belonged to the group of 32 isolates which were obtained from a shrimp hatchery at Azhikode, Kerala, during mass mortality of larvae. The isolates of phenon5 were positive and the isolates of phenon 6 were negative to VP test, utilization of Succinic acid methylester, D-Gluconic acid, D-L- lactate, Glycyl- L- glutamic acid, D-Galactose, D-mannose, L-Rhamnose and

Pyruvic acid methylester, and caused marginal differences among the isolates into phena 5& 6. These results suggested that the isolates of *V.parahaemolyticus* were more closely related to those of *V.mediterranei* than to those of *V.harveyi*. As evidenced, the isolates of *V.harveyi* formed majority of the core group and the other phenon were correlated to it at 0.94r, this core group could be considered as *V.harveyi* core group.

The remaining 23 isolates from a hatchery at Azhikode, Kerala were grouped under the second and third core groups. The second core group included phena 7 to 15 which were subdivided into 2 groups A&B, correlated at 0.85r. The second core group was subdivided into A&B sub groups, which joined at 84%S. **Group-A** consisted of phena 7 to 12 and **Group-B** of 13 to 15.

Group –A, consisted of two bacterial isolates belonged to **Phenon-7** exhibited 97%S to **Phenon-8** which contained the lone type strain, *V. proteolyticus* (V156) (LMG 3772). Variation shown by LMG 3772 was mainly for the reaction to TSI (K/K), negative result for ONPG, utilization of L-Arabinose, Uronic acid, Bromosuccinic acid, Hydroxyl-L-proline, L-Pyroglutamic acid, 2-Amino ethanol and Inosine. Positive results were obtained for lipase, utilization of D-glucosomic acid, D-gluconic acid, Propionic acid, D-saccharic acid, Succinic acid, L-Alaninamide, L-Phenylalanine and Sebacic acid. **Phenon-9** with 8 isolates obtained from necrotic post larvae from a shrimp hatchery in Ollur, Kerala, were identified as *V.alginolyticus* having the type strains V148 (LMG 4409) & V149 (MTCC 4439), joining with the clusters. This phenon gave a correlation of 0.91 & 0.86r to phena7&8 and phena10&11 respectively, thus evidenced that the isolates of *V.alginolyticus* showed a great degree of closeness to members of *V.proteolyticus* than to the isolates which belonged to *V.nereis.*

Phenon-10 incorporated 10 bacterial isolates from two hatcheries in Kerala (Azhikode and Kollam) isolated during an incident of mass mortality

of post larvae could not be identified as no type strain joined with it, but it showed 98.8%S to **Phenon-11** containing (V154) type strain of *V.nereis* (LMG 3895). Phenon-11 differed from the former by giving negative reactions for Tween 80, utilization of D-Cellobiose, m-Inositiol, D-Psicose, cis- Aconitic acid, D-Glucosominic acid, β- Hydroxybutyric acid, D,L-Lactic acid and Quinic acid. Positive reactions were given by the type strain of *V.nereis* for the utilization of D-Mannose, L-Arabinose, Gentiobiose, D-Galacturonic acid, α- Ketoglutatric acid and α- Hydroxybutyric acid. Phena10&11 gave a correlation to phenon-12 at 0.93r, suggesting that the isolates clustered into these three were interrelated. **Phenon-12** with one isolate joined with (V151) type strain of *V.fluvialis (*LMG 11654) at 100%S. Since the majority of the isolates clustered into this group were either formed of isolates of *V.alginolyticus* or exhibited a close similarity to *V.alginolyticus*, the members could be grouped under *V.alginolyticus* sub group.

Group B was comprised of Phenon 13 to 15 of the second core group. **Phenon-13** with 12 bacterial isolates obtained from a hatchery at Azhikode during a mass morality of post larvae were clustered with the type strains of *V. cholerae* (MTCC 3906), V150, which correlated with phenon14 at 0.91r and to *V.alginolyticus* sub group at 0.85r. **Phenon-14** had 7 isolates obtained from a hatchery at Kollam, Kerala during an incident of mass mortality of larvae, which integrated with the type strain *V. vulnificus* (LMG 13545) at 100%S. The isolate V140, obtained from the same source was individually placed as **Phenon-15** in the dendrogram, showed 98.8% similarity to phenon-14. These 2 clusters varied by exhibiting positive results to utilization of D & L- Alanine, D-L-lactate, Succinic acid, Bromosuccinic acid, L-Proline, α-D-glucose, D-Trehalose, Succinic acid Methylester, Formic acid, D-Glucosominic acid, L-Serine, L-Threonine. Negative reactions were obtained for Indole, ONPG, and utilization of D-Galactose, D-Glucoronic acid, D-Saccharic acid, Succinamic acid, D-

Glucuronic acid, L-Leucine, Hydroxyl-L-proline, D-Mannitol, D-Mellibiose, Xylitol, D-Serine, D-L Carnitine, γ-Amino butyic acid, Inosine, Uridine and L-Phenylalanine. Majority of the isolates in this group were formed of isolates of *V. cholerae* or exhibited closeness to *V. cholerae*, hence this core group has been recognized as *V. cholerae* core group.

The third core group at 100%S contained two bacterial isolates from a hatchery at Azhikode, during mass mortality of post larvae forming **Phenon- 16. Phenon-17** contained the type strain *V. splendidus* V157 (LMG 19031) which joined with Phenon 16 at 97%S. The difference in property shown by these two interrelated phena were mainly for the reaction to MOF (oxidative), negative results for Indole, Nitrate reduction, Aesculine hydrolysis, Utilization of Tween 80, N-acetyl D-Glucoseamine, Adonitol, L-Arabinose, D-Galactose, m-Inositol, L-Rhamnose, Pyruvic acid methylester, acetic acid, cis-Aconitic acid, Citric acid, D-Gluconic acid, D-Glucosaminic acid, Sebacic acid, Succinic acid, L-Alaniamide, D-Alanine, L-Pyroglutamic acid, Uronic acid, D,L- α- glycerol. Positive results were shown for the utilization of Dextrin, D-Cellobiose, Maltose, D-Trehalose, Succinic acid methylester and D-L-Lactic acid. The third core group, termed as the *V. splendidus,* consisted of phenon16 &17, exhibited a correlation of 0.80r to the *V. cholerae* core group.

A dichotomous key was constructed based on the phenotypic traits of the isolates for identification of vibrios associated with shrimp hatchery systems (Fig.2.2).

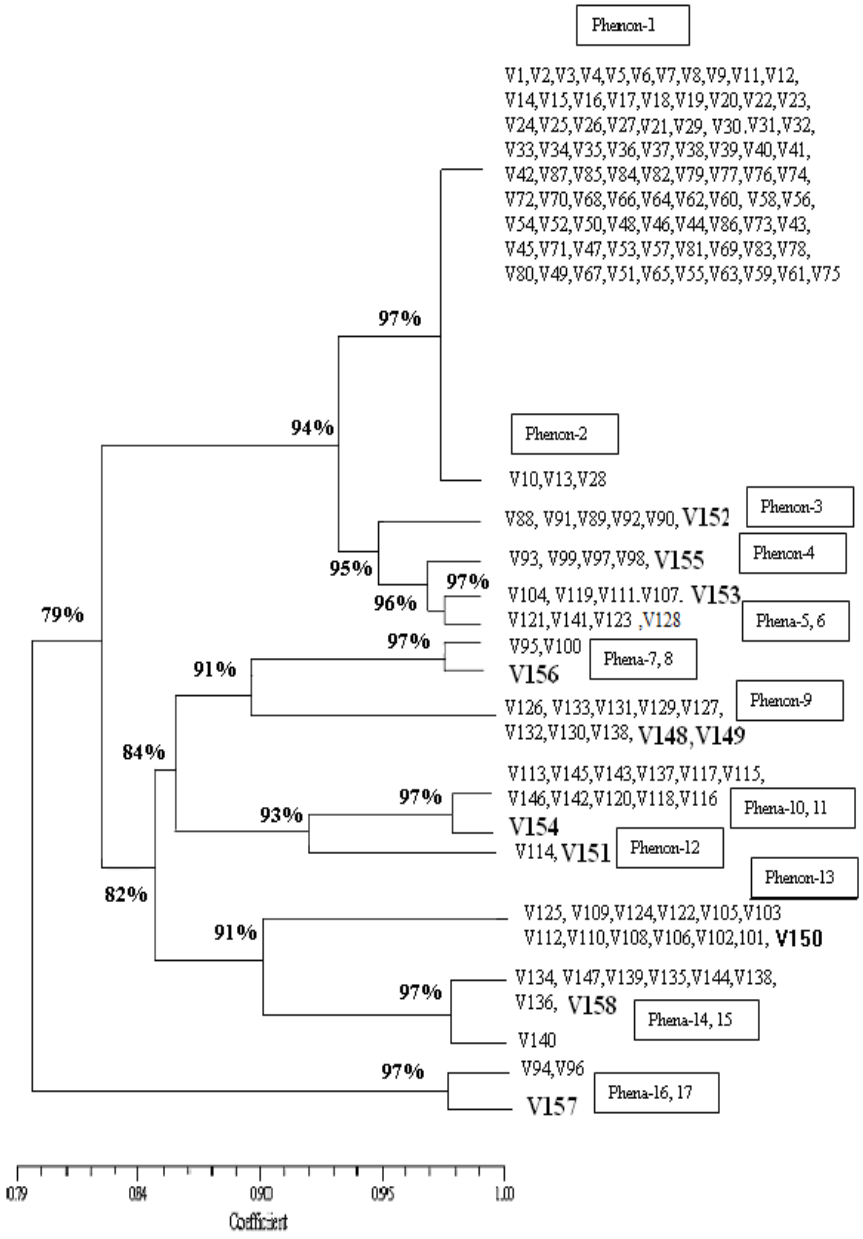


Fig 2.1: Dendrogram based on phenotypic characters of the bacterial isolates. V152- *V.harveyi* (LMG 4044), V155- *V. parahaemolyticus* (LMG 2850), V153- *V.mediterranei* (LMG 11258), V156- *V.proteolyticus* (LMG 3772), V148- *V.alginolyticus* (LMG 4409) & V149- *V.alginolyticus* (MTCC 4439), V154- *V.nereis* (LMG 3895), V151- *V.fluvialis* (LMG 11654), V150- *V. cholerae* (MTCC 3906), V158- *V.vulnificus* (LMG 13545), V157- *V. splendidus* (LMG 19031)

Table 2.3: Results of phenotypic characterization of the bacterial isolates

Phenotypic characteristics	+VES	-VES	Phenotypic characteristics	+VES	-VES
Luminescence	87	71	α-Cyclodextrin	44	114
Gram –ve rods	158	0	Dextrin	133	25
Motility	158	0	Glycogen	155	3
Flagella	158	0	Tween 40	155	3
Growth in TCBS	158	0	Tween 80	158	0
MOF	157	1	N-AcetylD Glucosamine	7	151
TSI	157	1	N-Acetyl-β- D Mannosamine	158	0
Catalase	158	0	Adonitol	18	140
Kovacs Oxidase	158	0	L-Arabinose	5	153
Indole	158	0	D-Arabitol	22	136
MR	158	0	D-Cellobiose	129	29
VP	56	102	i-Erythritol	5	153
Citrate	155	3	D-Fructose	156	2
ONPG	130	28	L-Fucose	15	143
Nitrate Reduction	158	0	D-Galactose	63	95
Growth in 0% NaCl	26	132	Gentiobiose	106	52
Growth in 3% NaCl	158	0	α-D-Glucose	155	3
Growth in 6% NaCl	158	0	m-Inositol	30	128
Growth in 8% NaCl	145	13	α-D-Lactose	33	125
Growth in 10% NaCl	102	56	Lactulose	20	138
Arginine	17	141	Maltose	153	5
Lysine	118	40	D-Mannitol	155	3
Ornithine	118	40	D-Mannose	128	30
Amylase	158	0	D-Melibiose	15	143
Chitinase	158	0	β-Methyl- D-Glucoside	118	40
Dnase	158	0	D-Psicose	31	127
Gelatinase	158	0	D-Raffinose	2	156
A-Hemolyase	0	158	L-Rhamnose	13	145
B-Hemolyase	0	158	D-Sorbitol	20	138
Γ-Hemolyase	158	0	Sucrose	54	104
Lecithinase	158	0	D-Trehalose	150	8
Lipase	101	57	Turanose	23	135
Aesculin	125	33	Xylitol	2	156
Elastin	13	145	Pyruvatic Acid Methyl Ester	69	89
Inulin	0	158	Succinic Acid Mono-methyl Ester	75	83
Gluconate	0	158	Acetic Acid	88	70
Tartarate	158	0	cis-aconitic acid	61	97
O/129 (150µg/disc)	158	0	Citric acid	37	121
Self pelleting	158	0	Formic acid	9	149
Precipitation after boiling	158	0	D-Galactonic acid Lactone	5	153

D-Galacturonic acid	17	141	L-Glutamic Acid	157	1
D-Gluconic acid	154	4	Glycyl- L-Aspartic Acid	145	13
D-Glucosaminic acid	27	131	Glycyl- L-Glutamic Acid	145	13
D-Glucoronic acid	89	69	L-Histidine	52	106
A-HydroxybutyricAcid	19	139	Hydroxy-L-Proline	30	128
B-HydroxybutyricAcid	17	141	L-Leucine	5	153
Γ-HydroxybutyricAcid	12	146	L-Ornithine	7	151
p-Hydroxy-Phenylacetic Acid	2	156	L-Phenylalanine	6	152
Itaconic acid	2	156	L-Proline	147	11
A-KetobutyricAcid	3	155	L-Pyroglutamic acid	30	128
A-KetoglutaricAcid	21	137	D-Serine	96	62
A-KetovalericAcid	5	153	L-Serine	123	35
D,L-Lactic Acid	154	4	L-Threonine	131	27
Malonic acid	17	141	D,L Carnitine	2	156
Propionic acid	39	119	γ-AminobutyricAcid	29	129
Quinic acid	3	155	Uronic acid	26	132
D-saccharic acid	14	144	Inosine	156	2
Sebacic acid	3	155	Uridine	140	18
Succinic Acid	143	15	Thymidine	126	32
Bromosuccinic acid	132	26	Phenyethyl amine	2	156
Succinamic Acid	11	147	Putrescine	27	131
Glucuronamide	7	151	2-Aminoethanol	10	148
L-Alaninamide	51	107	2,3-Butanediol	1	157
D-Alanine	110	48	Glycerol	158	0
L-Alanine	127	31	D,L α-D-Glycerol	138	20
L-Alanyl- Glycine	135	23	α, D-Glucose- 6-Phosphate	155	3
L-Asparagine	153	5	D glucose 6-Phosphate	158	0
L-Aspartic acid	156	2			

Table 2.4: Results of Correlation and percentage similarity of the bacterial isolates

Clusters	P	q+r	Jaccards Distance	Jaccards coefficient	% Similarity
1&2	66	9	0.12	0.88	88
2&3	59	16	0.213	0.787	78.7
3&4	63	19	0.231	0.769	76.9
4&5	69	17	0.198	0.802	80.2
5&6	84	1	0.012	0.988	98.8
6 &7	72	44	0.038	0.61	61
7&8	93	12	0.114	0.886	88.6
8&9	70	45	0.39	0.61	61
9&10	66	23	0.26	0.74	74
10&11	80	1	0.012	0.988	98.8
11&12	68	53	0.44	0.68	68
12&13	82	41	0.33	0.67	67
13&14	70	33	0.32	0.68	68
14&15	72	15	0.172	0.828	82.8
15 & 16	56	46	0.45	0.55	55
16&17	73	29	0.207	0.793	79.3

Expected Mean variance E (S') = S $(2p-1)^2$ + 2p (1-p)

Where S= similarity coefficient, p= probability at 0.05p.

- Cluster 1&2, 5&6, 7&8, 14 &15, 16&17 = 0.97 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)
  - = 0.97 $(0.1\ -1)^2$ + 0.1 (0.95)
  - = 0.97x 0.81 + 0.095
  - = 0.7857 + 0.095 = **0.8807**
- Cluster 2&3= 0.94 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)
  - = 0.94 $(0.1\ -1)^2$ + 0.1 (0.95)
  - = 0.94x 0.81 + 0.095
  - = 0.7614+ 0.095 = **0.8564**
- Cluster 3&4= 0.95 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)
  - = 0.95 $(0.1\ -1)^2$ + 0.1 (0.95)
  - = 0.95x 0.81 + 0.095
  - = 0.7695+ 0.095 = **0.8645**
- Cluster 4&5= 0.96 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)
  - = 0.96 $(0.1\ -1)^2$ + 0.1 (0.95)

- = 0.96x 0.81 + 0.095
- = 0.7776 + 0.095 = **0.8726**

❖ Cluster 8&9, 13 & 14 = 0.91 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)

- = 0.91 $(0.1\ -1)^2$ + 0.1 (0.95)
- = 0.91x 0.81 + 0.095
- = 0.7371 + 0.095 = **0.8321**

❖ Cluster 9&10= 0.85 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)

- = 0.85$(0.1\ -1)^2$ + 0.1 (0.95)
- = 0.85x 0.81 + 0.095
- = 0.6885 + 0.095 = **0.7835**

❖ Cluster 11 &12= 0.93 $(2x\ 0.05-1)^2$ + 2x 0.05 (1- 0.05)

- = 0.93 $(0.1\ -1)^2$ + 0.1 (0.95)
- = 0.93x 0.81 + 0.095
- = 0.7533 + 0.095 = **0.8483**

C) Standard Error SE (S') = $\sqrt{2p\ (1-p)/n}$ x $\sqrt{1-2p\ (1-p)}$

Where p= probability at 0.05p, n= number of tests

For n= 135, p= 0.0217

SE (S') = $\sqrt{2x\ 0.0217\ (1-0.0217)/135}$ x $\sqrt{1-2x\ 0.0217\ (1-0.0217)}$

= $\sqrt{0.0434\ (0.9783)/135}$ x $\sqrt{1-0.0434\ (0.9783)}$

= $\sqrt{0.0424/135}$ x $\sqrt{1-0.0424}$

= $\sqrt{3.145}$ x $\sqrt{0.95755}$

= 1.773 x 0.9785 = **1.735**

D) Average Probable value $= \dfrac{[S'-2p\ (1-p)]}{(2p-1)^2}$

$= \dfrac{[1.735-\ 2x\ 0.0217\ (1-0.0217)]}{(2x0.0217-1)^2}$

$= \dfrac{[1.735-\ 0.0434\ (0.9783)]}{(0.0434-1)^2}$

$= \dfrac{1.69255}{0.915}$ **= 1.85**

E) Test of Variance $S_i^2 = d/2t$

Where d= Number of strains giving diverging result, t= Total number if tests

d= 106, t= 135

S_i^2= 106/ 2x135= 106/ 270= **0.3926**

Average Mean variance = 1.85 ± 0.3926

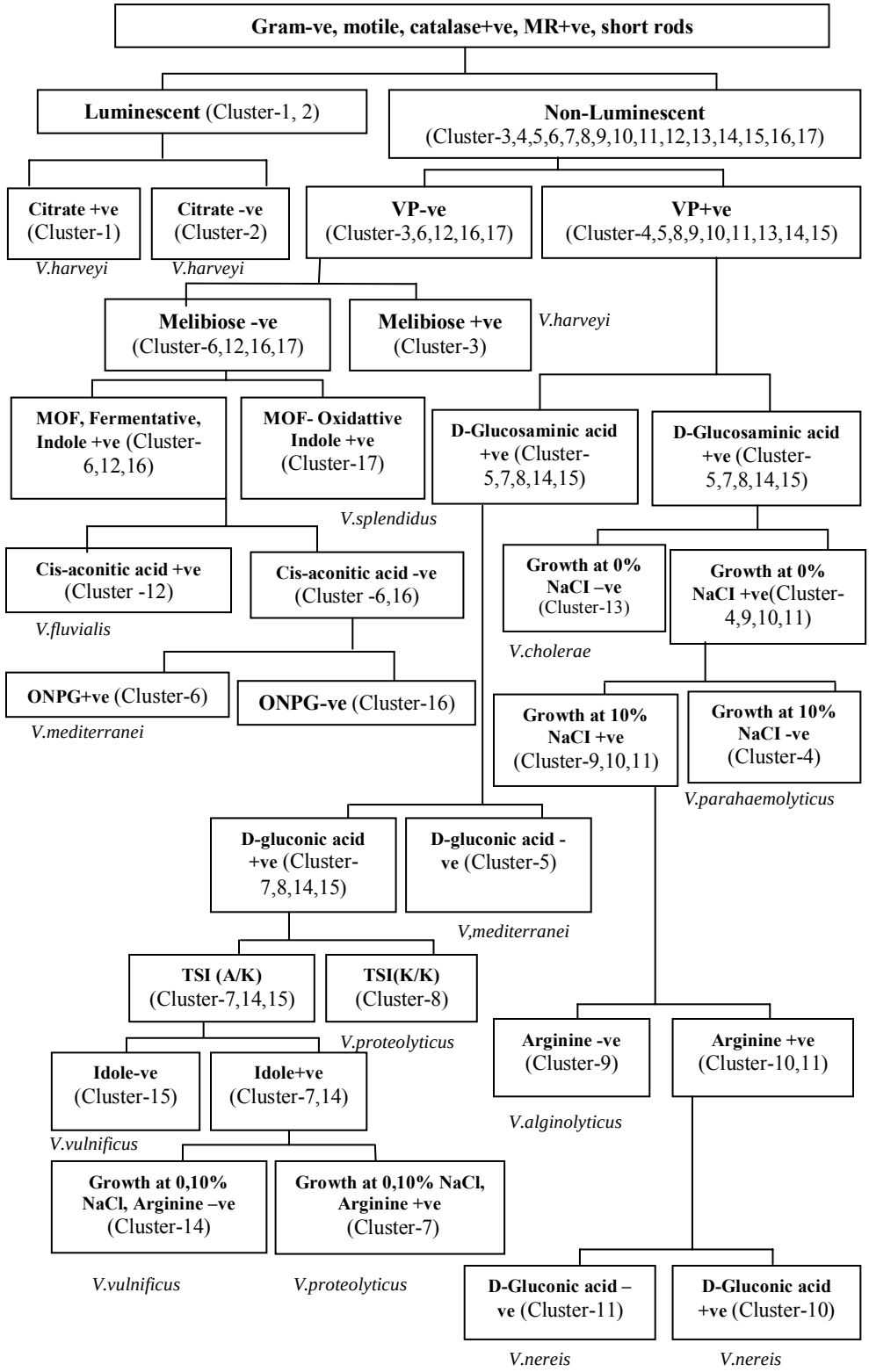


Fig 2.2: Dichotomous key for identification of vibrios isolated from shrimp hatcheries along the East-West coasts of India

2.3.2. Reproducibility assessment:

The mean reproducibility value for each phenon was analysed as an erroneous value of 1.85 ± 0.3926%, at a probability value $p \geq 0.05$, an acceptable value according to Sneath and Johnson, (1972). The significance of reproducibility of each phenotypic character (test) exhibited by the randomly selected bacterial isolates was found to be acceptable at 0.01 probability. The disagreement of reproducibility assessed for 4455 individual tests was obtained as 158 (3.546%), indicating that the reproducibility value was significantly below 10% and in accordance with the observations of Sneath, (1974). Chi-square test of independence between reproducibility and each phenotypic character tested with the randomly selected bacterial isolates from each phena was significant at 0.001 probability. The variations in response to metabolic fingerprinting obtained by inoculating the broth of each isolates into the Biolog GN2 plates was observed to be responsible for the divergence of the isolates and their clustering into different phena.

Table 2.5a: Chi square test for determining the degree of reproducibility by the isolates selected from each phena for the phenotypic characters

Sl.No	Isolates	Code	Expected (E)	Observed (O)	O-E	$(O-E)^2$	$(O-E)^2/E$
1	V3	V3	75	74	-1	1	0.0135
2	V21	V21	80	70	-10	100	1.4285
3	V36	V36	81	76	-5	25	0.3289
4	V45	V45	81	76	-5	25	0.3289
5	V54	V54	81	76	-5	25	0.3289
6	V57	V57	81	76	-5	25	0.3289
7	V64	V64	81	81	0	0	0
8	V71	V71	77	76	-1	1	0.0131
9	V76	V76	72	72	0	0	0
10	V81	V81	73	73	0	0	0
11	V88	V88	66	66	0	0	0
12	Vhl	V152	96	91	-5	25	0.2747
13	vpa6	V97	71	65	-6	36	0.5538
14	Vpal	V155	73	71	-2	4	0.0563
15	vm15	V104	83	80	-3	9	0.1125
16	vm27	V141	85	83	-2	4	0.0481
17	Vml	V153	82	78	-4	16	0.2051
18	vpr4	V95	104	97	-7	49	0.5051

19	Vprl	V156	104	95	-9	81	0.8526
20	va3	V128	76	69	-7	49	0.7101
21	Val	V148	82	76	-6	36	0.4736
22	Vam	V149	82	76	-6	36	0.4736
23	vn24	V113	84	80	-4	16	0.2
24	Vnl	V154	82	78	-4	16	0.2051
25	vf26	V114	109	102	-7	49	0.4803
26	Vfl	V151	108	101	-7	49	0.4851
27	vc12	V102	98	92	-6	36	0.3913
28	Vcm	V150	80	78	-2	4	0.0512
29	vv9	V135	80	72	-8	64	0.8888
30	vv23	V140	81	72	-9	81	1.125
31	Vvl	V158	80	77	-3	9	0.1168
32	vsp3	V94	83	72	-11	121	1.6805
33	Vspl	V157	85	75	-10	100	1.1764
			2756	**2596**	-160	1092	**13.8381**

Chi- Square: 2x2 Contingency table

	Observed	**Expected**	**Row total**	
Present	2343	2501	4844	t1
Absent	2112	1954	4066	t2
Column total	**4455**	**4455**	**8910**	t3
O x t1	21580020.00	E x t1	21580020.00	
O x t1/ t3	2422	E x t1/ t3	2422	
O x t2	18114030	E x t2	18114030	
O x t2/ t3	2033	E x t2/ t3	2033	
Calculation of Expected Frequency (E)				
	Observed	**Expected**	Total	
Present	2422	2422	4844	
Absent	2033	2033	4066	
	4455	4455	8910	
Calculation of difference between observed and expected values				
	Observed	**Expected**		
Present	79	-79		
Absent	-79	79		
Significance is set at 0.05				
Calculation of chi- square value				
Groups	**(O-E)- 0.05**	**[(O-E)- 0.05]²**	**[(O-E)-0.05]²/E**	
1	78.95	6233.1	2.5735	
2	-79.05	6248.9	3.0737	
3	-79.05	6248.9	2.5800	
4	78.95	6233.1	3.0659	
			11.2932	
No: of rows-1		No: of columns-1		
2-1=1		2-1=1		
At Degree of freedom =1				
Probability		0.05	0.01	0.001
Table value		3.84	6.64	10.83
Calculated value		11.29	11.29	11.29
Difference b/w calculated & table value		**7.45**	**4.65**	**0.46**

Table 2.5 b: Chi square test for determining the degree of reproducibility of all 135 phenotypic characters by the isolates

Sl. No	Phenotypic characters	Expected (E)	Observed (O)	O-E	$(O-E)^2$	$(O-E)^2/E$
P1	**Luminescence**	10	10	0	0	0
P2	**Gram -ve rods**	33	33	0	0	0
P3	**Motility**	33	33	0	0	0
P4	**Flagella**	33	33	0	0	0
P5	**Growth in TCBS**	33	33	0	0	0
P6	**MOF**	32	32	0	0	0
P7	**TSI**	32	32	0	0	0
P8	**Catalase**	33	33	0	0	0
P9	**Kovacs Oxidase**	33	33	0	0	0
P10	**Indole**	33	33	0	0	0
P11	**MR**	33	33	0	0	0
P12	**VP**	17	17	0	0	0
P13	**Citrate**	32	32	0	0	0
P14	**ONPG**	25	25	0	0	0
P15	**Nitrate Reduction**	33	33	0	0	0
P16	**Growth in 0% NaCl**	8	8	0	0	0
P17	**Growth in 3% NaCl**	33	33	0	0	0
P18	**Growth in 6% NaCl**	33	33	0	0	0
P19	**Growth in 8% NaCl**	29	29	0	0	0
P20	**Growth in 10% NaCl**	16	16	0	0	0
P21	**Arginine**	8	8	0	0	0
P22	**Lysine**	23	23	0	0	0
P23	**Ornithine**	23	23	0	0	0
P24	**Amylase**	33	33	0	0	0
P25	**Chitinase**	33	33	0	0	0
P26	**Dnase**	33	33	0	0	0
P27	**Gelatinase**	31	31	0	0	0
P28	**α-Hemolyase**	0	0	0	0	0
P29	**β-Hemolyase**	2	2	0	0	0
P30	**γ-Hemolyase**	33	33	0	0	0
P31	**Lecithinase**	31	31	0	0	0
P32	**Lipase**	17	17	0	0	0
P33	**Aesculin**	24	24	0	0	0
P34	**Elastin**	2	2	0	0	0
P35	**Inulin**	2	2	0	0	0
P36	**Gluconate**	2	2	0	0	0
P37	**Tartarate**	33	33	0	0	0
P38	**O/129 (150µg/disc)**	32	32	0	0	0
P39	**Self pelleting**	33	32	-1	1	0.0303
P40	**Precipitation after boiling**	23	23	0	0	0
P41	**α-Cyclodextrin**	18	12	-6	36	2
P42	**Dextrin**	30	23	-7	49	1.6333
P43	**Glycogen**	31	30	-1	1	0.0322
P44	**Tween 40**	33	29	-4	16	0.4848
P45	**Tween 80**	16	16	0	1	0.0625
P46	**N-AcetylD Glucosamine**	22	22	0	0	0

P47	**N-Acetyl-β- D Mannosamine**	16	13	-3	16	1
P48	**Adonitol**	4	4	0	0	0
P49	**L-Arabinose**	8	7	-1	1	0.125
P50	**D-Arabitol**	15	15	0	0	0
P51	**D-Cellobiose**	12	11	-1	4	0.3333
P52	**i-Erythritol**	23	23	0	0	0
P53	**D-Fructose**	13	13	0	1	0.0769
P54	**L-Fucose**	15	10	-5	25	1.6666
P55	**D-Galactose**	13	11	-2	4	0.3076
P56	**Gentiobiose**	31	28	-3	16	0.5161
P57	**α-D-Glucose**	16	13	-3	16	1
P58	**m-Inositol**	9	9	0	0	0
P59	**α-D-Lactose**	7	7	0	0	0
P60	**Lactulose**	21	20	-1	1	0.0476
P61	**Maltose**	31	31	0	1	0.0322
P62	**D-Mannitol**	27	26	-1	4	0.1481
P63	**D-Mannose**	13	13	0	1	0.0769
P64	**D-Melibiose**	15	14	-1	1	0.0666
P65	**β-Methyl- D-Glucoside**	22	16	-6	36	1.6363
P66	**D-Psicose**	2	2	0	0	0
P67	**D-Raffinose**	7	5	-2	4	0.5714
P68	**L-Rhamnose**	7	7	0	0	0
P69	**D-Sorbitol**	14	13	-1	1	0.0714
P70	**Sucrose**	22	20	-2	4	0.1818
P71	**D-Trehalose**	15	14	-1	4	0.2666
P72	**Turanose**	3	2	-1	1	0.3333
P73	**Xylitol**	18	16	-2	4	0.2222
P74	**Pyruvatic Acid Methyl Ester**	22	18	-4	16	0.7272
P75	**Succinic Acid Mono-methyl Ester**	20	16	-4	16	0.8
P76	**Acetic Acid**	18	14	-4	25	1.3888
P77	**cis-aconitic acid**	15	13	-2	4	0.2666
P78	**Citric acid**	7	7	0	0	0
P79	**Formic acid**	3	3	0	0	0
P80	**D-Galactonic acid Lactone**	6	5	-1	1	0.1666
P81	**D-Galacturonic acid**	20	19	-1	1	0.05
P82	**D-Gluconic acid**	16	16	0	1	0.0625
P83	**D-Glucosaminic acid**	5	5	0	0	0
P84	**D-Glucoronic acid**	16	14	-2	9	0.5625
P85	**α-HydroxybutyricAcid**	5	4	-1	1	0.2
P86	**β-HydroxybutyricAcid**	4	3	-1	1	0.25
P87	**γ-HydroxybutyricAcid**	1	1	0	0	0
P88	**p-Hydroxy-Phenylacetic Acid**	2	0	-2	4	2
P89	**Itaconic acid**	3	3	0	0	0
P90	**α-KetobutyricAcid**	6	6	0	0	0
P91	**α-KetoglutaricAcid**	5	3	-2	4	0.8
P92	**α-KetovalericAcid**	20	18	-2	4	0.2
P93	**D,L-Lactic Acid**	15	13	-2	9	0.6
P94	**Malonic acid**	10	7	-3	9	0.9

P95	**Propionic acid**	2	0	-2	4	2
P96	**Quinic acid**	3	3	0	0	0
P97	**D-saccharic acid**	2	2	0	0	0
P98	**Sebacic acid**	21	21	0	0	0
P99	**Succinic Acid**	28	27	-1	4	0.1428
P100	**Bromosuccinic acid**	18	11	-7	64	3.5555
P101	**Succinamic Acid**	3	3	0	0	0
P102	**Glucuronamide**	7	7	0	0	0
P103	**L-Alaninamide**	23	19	-4	16	0.6956
P104	**D-Alanine**	26	26	0	0	0
P105	**L-Alanine**	28	26	-2	9	0.3214
P106	**L-Alanyl- Glycine**	29	29	0	0	0
P107	**L-Asparagine**	33	32	-1	4	0.1212
P108	**L-Aspartic acid**	32	32	0	1	0.0312
P109	**L-Glutamic Acid**	30	25	-5	36	1.2
P110	**Glycyl- L-Aspartic Acid**	31	24	-7	64	2.0645
P111	**Glycyl- L-Glutamic Acid**	24	18	-6	49	2.0416
P112	**L-Histidine**	11	11	0	0	0
P113	**Hydroxy-L-Proline**	4	4	0	0	0
P114	**L-Leucine**	5	5	0	0	0
P115	**L-Ornithine**	6	5	-1	1	0.1666
P116	**L-Phenylalanine**	21	21	0	0	0
P117	**L-Proline**	16	15		4	0.25
P118	**L-Pyroglutamic acid**	11	9	-2	4	0.3636
P119	**D-Serine**	29	29	0	0	0
P120	**L-Serine**	25	22	-3	9	0.36
P121	**L-Threonine**	10	9	-1	4	0.4
P122	**D,L Carnitine**	7	5	-2	4	0.5714
P123	**γ-AminobutyricAcid**	6	5	-1	1	0.1666
P124	**Uronic acid**	22	14	-8	64	2.9090
P125	**Inosine**	33	29	-4	25	0.7575
P126	**Uridine**	31	29	-2	9	0.2903
P127	**Thymidine**	9	8	-1	4	0.4444
P128	**Phenyethyl amine**	10	5	-5	25	2.5
P129	**Putrescine**	3	0	-3	9	3
P130	**2-Aminoethanol**	1	1	0	0	0
P131	**2,3-Butanediol**	22	22	0	0	0
P132	**Glycerol**	31	31	0	1	0.0322
P133	**D,L α-D-Glycerol**	32	30	-2	9	0.2812
P134	**α, D-Glucose- 6-Phosphate**	33	32	-1	4	0.1212
P135	**D glucose 6-Phosphate**	33	33	0	1	0.0303
		2501	2343	-157	783	**46.7173**

Chi-square at 0.05 and degree of freedom 30 = 43.77, therefore for df 32 at 0.05p= 46.69
Calculated Chi-sqaure value = 46.72
Difference between the Calculated and Table chi-square value = 0.03
Since the calculated value is less than the table value, the reproducibility can be accepted at or above 0.05 probability .

Since the calculated value is greater than the table value, the selected isolates gave a significant association between the tests and reproducibility.

Variations in the reproducibility were observed for the utilization of carbon sources such as Dextrin, L-Fucose, β-Methyl-D-Glucoside, Bromosuccinic acid, Glycyl-L-Aspartic acid, Glycyl-L- glutamic acid and Uronic acid.

2.3.3. Validation of the constructed dichotomous key

To determine validity of the constructed dichotomous key, isolates randomly selected from each phenon along with the type strains (33Nos) were subjected to the phenotypic characterization. The isolates replicated the results when subjected to the set of phenotypic characters according to the dichotomous key. This result suggested that the dichotomous key constructed from the present study could be put into use for routine identification of vibrios. Analysis of the phenotypic profile shows that the most profuse *Vibrio* species inhabiting the East and West Indian coast associated with shrimp mortality were *V. harveyi, V.nereis, V. alginolyticus*, V.*cholerae* and *V. mediterraneii*.

Table 2.6: Chi square test for validation of the dichotomous key of the representative isolates of each phenon

Sl.No	Isolates	Expected (E)	Observed (O)	O-E	$(O-E)^2$	$(O-E)^2/E$
1	V3	8	8	0	0	0
2	V21	8	7	-1	1	0.125
3	V36	8	8	0	0	0
4	V45	8	8	0	0	0
5	V54	8	8	0	0	0
6	V57	8	8	0	0	0
7	V64	8	9	1	1	0.125
8	V71	8	8	0	0	0
9	V76	8	8	0	0	0
10	V81	8	8	0	0	0
11	V88	9	7	-2	4	0.4444

12	V152	9	9	0	0	0
13	V97	7	6	-1	1	0.1428
14	V155	7	7	0	0	0
15	V104	8	7	-1	1	0.125
16	V141	7	6	-1	1	0.1428
17	V153	8	7	-1	1	0.125
18	V95	10	9	-1	1	0.1
19	V156	8	8	0	0	0
20	V128	7	7	0	0	0
21	V148	7	7	0	0	0
22	V149	7	7	0	0	0
23	V113	10	10	0	0	0
24	V154	9	9	0	0	0
25	V114	9	9	0	0	0
26	V151	9	9	0	0	0
27	V102	12	11	-1	1	0.0833
28	V150	12	10	-2	4	0.3333
29	V135	9	7	-2	4	0.4444
30	V140	9	8	-1	1	0.1111
31	V158	9	7	-2	4	0.4444
32	V94	7	7	0	0	0
33	V157	8	8	0	0	0
		277	262	-15	25	**2.7468**

Table value for Chi square at 0.05 and degree of freedom df 32 at 0.05p= 46.69

Observed Chi square value = 2.747

Difference between the Table and observed Chi-square value = 43.94

The Calculated value is very less than the table value, hence there is no variation in the reproducibility and the key for identification can be accepted

Chi- Square: 2x2 Contingency table

	Initial	**Reproducibility**	**Row total**
Tests	**277**	**262**	**539**
isolates	**277**	**265**	**542**
Column total	554	527	1081
initial x t1	298606	Reproducibility x t1	284053
initial x t1/ t3	276.23	Reproducibility x t1/ t3	262.77
initialx t2	300268	Reproducibility x t2	285634
Initialx t2/ t3	277.77	Reproducibility x t2/ t3	264.23
Calculation of Expected Frequency (E)			
	Initial	**Reproducibility**	**Total**
Tests	276.23	262.77	539
isolates	277.77	264.23	542
	554	527	1081

Significance is set at 0.05			
Calculation of chi- square value			
Groups	**(O-E)- 0.05**	**[(O-E)- 0.05]²**	**[(O-E)- 0.05]²/E**
1	0.72	0.52	0.001882489
2	-0.82	0.67	0.002412068
3	-0.82	0.67	0.002549758
4	0.72	0.52	0.001967982
			0.008812298

Degree of freedom	
No: of rows-1	No: of columns-1
2-1=1	2-1=1
Degree of freedom =1	
Expected chi-square value from table at 0.05 level = 3.84	
Calculated chi-square value = 0.0088	

Differences between the calculated and the table value =3.831

The Calculated value is less than the table value

Therefore, there is no significant difference in the reproducibility assessment among the selected strains subjected to different phenotypic characters.

Since the calculated value is greater than the table value, there is significant association between the strains and reproducibility is obtained on subjecting the strains to 135 biochemical tests.

2.4. Discussion

Numerical Taxonomy uses quantitative methods to estimate phenetic similarity, examine character correlations, and group OTUs; and "aims to develop methods that are objective, explicit, and repeatable" (Sneath and Sokal, 1973). The best classifications are based on the largest number of characters, with all characters afforded equal weightage. Classifications are based on quantitative measures of overall (phenetic) similarity or distance between taxa (called OTUs = operational taxonomic units); and patterns of character correlation are used to i) recognize distinct taxa; and ii) draw systematic inferences, giving assumptions about evolutionary pathways and

mechanisms. A similarity or distance value gives a quantitative comparison of two species; showing the resemblance between two objects, usually on a scale from 1 to 0. A branching diagram that linked entities by estimates of overall similarity was constructed using UPGMA (Unweighted pair group method with arithmetic averages) cluster analysis to determine the degrees of overall phenetic similarity of taxa from which phylogenetic relationships could be inferred (Sneath and Sokal, 1973).

An extensive phenotypic characterization of 158 isolates of *Vibrio* and analysis of numerical taxonomy using UPGMA yielded 17 phena which clustered into 3 core groups. The characteristic features exhibited by the isolates in each phena were compared with the characters originally described by Alsina, Noguerola and Blanch and any variation from the early results discussed. The phenotypic profiles of the isolates grouped into phena 5 & 6 were similar to those previously described by Noguerola and Blanch, (2008) for *V. mediterranei*, except for the ONPG test. Analysis of these results suggested that the isolates of *V. parahaemolyticus* were more related to those of *V. mediterraneii* at 97%S than to *V. harveyi* at 95%S. As the isolates of *V. harveyi* formed majority in core group-1 and the other phena were correlated to it at 94%S, this group could be considered as the *V. harveyi* core group.

The isolates clustered into Phenon-7&8 were the isolates and type strain of *V.proteolyticus*, giving variable results to TSI and ONPG test, this result is similar to that seen in The Bergey's Manual of Systematic Bacteriology, 2nd Edition (Baumann and Schubert, 1983), suggesting that the isolates of *V.proteolyticus* can be either positive or negative to these two tests. The isolates in Phenon-9, 12 and 13 showed 100%S to the type strains of *V.alginolyticus*, *V.fluvialis* and *V.cholerae* respectively, suggesting that the isolates were members of the same strain. The isolates in Phenon- 10&11 are the isolates and type strain of *V.nereis* related at 93% varying mainly to the utilization of carbon source carried out in Biolog

plates. According to Baumann and Schubert, (1983), isolates of *V.nereis* can be either positive or negative to Tween-80 used for determining lipolysis; similar results were in this study, with the wild isolates being positive and the type strain negative. Buchrieser (1995) isolated many strains of *V. vulnificus* from a single organism, suggesting that the heterogeneity among the strains of *V. vulnificus* is immense. Of the three biotypes of *V. vulnificus*; biotypes B1 and B3 were positive for indole, while biotype B2 was negative (Noguerola and Blanch 2008). The isolate V140 was indole negative, which varied from the closely related isolates of **Phenon-14** in indole reaction (indole positive), inferring that the isolate in **Phenon-15** resembled *V. vulnificus* biotype B2. Noguerola and Blanch (2008) observed that *V. splendidus* exhibited variability to indole test, but the isolates of *V. splendidus* (**Phena -16 & 17)** used in this study were all indole positive.

The correct identification of environmental isolates is still in discussion as they show biochemical variabity (Pujalte *et al*., 1993; Ortigosa *et al*., 1994), hence an accurate identification key based on biochemical test is required. An important feature of the dichotomous key developed in this study was that antibiotic sensitivity tests were not used at any point as the criteria for identification, instead relied exclusively on biochemical characterization. Presently employed dichomotous keys are proposed by Alsina and Blanch (1994a, b) and Noguerola and Blanch 2008 for identification of vibrios, in which sensitivity to antibiotic was included. Avoiding antibiotic sensitivity test for identification is significant due to the fact that multidrug resistance varieties may lead to erroneous results. Nevertheless, the key is comparable to the dichotomous key developed by (Noguerola and Blanch 2008), for the identification of *Vibrio.* A prominent difference between these two keys is in the use of antibiotic sensitivity as the criterion for identification by (Noguerola and Blanch 2008). Other than that, except for the disparity towards indole and ONPG test, the isolates analyzed exhibited identical results to 35 tests out of 45 considered by

Noguerola and Blanch (2008). To ensure correct identification based on the dichotomous key, validation and reproducibility of the phenotypic characters were carried out. Dichotomous keys are defined as the practical and routine identification scheme of bacterial species based on phenotypic characterization, and not as the main criteria for taxonomical studies or systematics.

Variation in the reproducibility was observed for the utilization of carbon sources such as Dextrin, L-Fucose, β-Methyl-D-Glucoside, Bromosuccinic acid, Glycyl-L-Aspartic acid, Glycyl-L- glutamic acid and Uronic acid carried out in Biolog GN2 plates. Biolog GN2 plates have been originally developed for clinical isolates; however, their application for environmental isolates is well documented (Johnsen *et al*., 1996, Truu *et al*., 1999). Phenotypic identification of vibrios using Biolog plates showed that different *Vibrio* species clustered within the same Biolog group and certain strains which were misidentified as *V.harveyi* based on Biolog metabolic fingerpritinting were later on correctly identified as *V.campbellii, V.rotiferianus* or other new species (Gomez-Gil *et al*., 2004b).

Kühn *et al*. (1991) calculated the similarity between strains based on correlation coefficient, thereby the isolates presenting correlation coefficient higher than 0.975 were assigned to the same biochemical type. In this study, the isolates which exhibited a correlation coefficient of 1 were assigned as representatives of the same phenon. The correlation coefficient values were used to determine the interrelatedness among the neighboring phena. Majority of the phenon exhibited interrelatedness at correlation coefficient greater than 0.90r, except for phenon 9&10, which correlated at 0.85r, suggesting that the isolates clustered into these two phena diverged very much compared to the isolates in the other phena.

The criteria for construction of dichotomous key were discriminatory power (high probability of a positive or negative result), ease

of application, reduction in total test number and inclusion of commercial kits used for routine and rapid identification of the environmental isolates (Maugeri *et al*., 2004, Baffone *et al*., 2006). The tests included in the dichotomous key possessed high discriminatory powers, with differentiation thresholds of ≥90% as sure positives and those of ≤10% as negatives. The differentiation threshold at 90% allowed us in identifying, and most efficiently discriminating the isolates of genus *Vibrio* into various phena.

The isolates replicated the results when subjected to the set of phenotypic characters according to the dichotomous key. This is suggestive of the application of the dichotomous key constructed from the present study for use in the routine identification of vibrios from shrimp larval rearing systems especially in the east and west coasts of India. Analysis of the phenotypic profile showed that the dominant species of *Vibrio* associated with mass mortality of larvae of penaeids was *V. harveyi* in both the coasts of India. This species was very highly prevalent in a hatchery at, Kakinada, Andhra Pradesh, causing mass larval mortality. The other species were *V. mediterranei (n=11), V. nereis (n=6) and V. cholerae (n=11)* isolated from a hatchery at Azhikode, Kerala, *V. alginolyticus (n=8)* and *V. vulnificus* (n=7) were the ones isolated from a hatchery at Ollur and Kollam, Kerala respectively during mass mortality of larvae. This is the first ever accomplished comprehensive study of the numerical taxonomy of *Vibrio* associated with larval mortality in shrimp hatcheries. Through this work the isolates could be identified to species level. Another impact of this study was the easiness with which the representative isolates from each phenon could be segregated for investigating their pathogenicity.

2.5. Conclusion

Through this work, employing numerical taxonomy, the species of *Vibrio* associated with shrimp hatcheries in the east and west coasts of India could be identified; besides this, a dichotomous key was also developed for their easy identification in field laboratories. The dichotomous key proposed

by us is comparatively better to the identification scheme of vibrios available till date. Highlight of the key is that only 13 biochemical tests are used as the identification criteria. These tests, when developed into a kit, can be used for identification of *Vibrio* associated with shrimp hatcheries very easily in limited time. This key does not use any antibiotic as an identification test, ruling out the problem of misidentification resulted by antibiotic resistant strain.

CHAPTER-3

Genotypic characterization of vibrios

3.1. Introduction

3.1.1. Genotypic characters of vibrios

Traditionally, both detection and identification of marine vibrios have been depended on their growth on thiosulphate citrate bile salt sucrose (TCBS) selective medium and subsequent characterization by biochemical tests (Diggles *et al*., 2000). Taxonomy of *Vibrio* spp. is in the process of revision due to the increasing data obtained with molecular techniques, where different genes are examined or where whole genome is inspected. Since 1980s, the genus *Vibrio* has been subjected to an extensive taxonomic revision, with species of vibrios distributed into five phylogenetic robust clades corresponding to the genera *Vibrio*, *Photobacterium*, *Salinivibrio*, *Enterovibrio*, and *Grimontia* based on 16S rRNA gene sequences (Azam, 2001; Bang, 1978; Banin, 2001).Various DNA based methods have also been used to identify and type these organisms, including: pulsed-field gel electrophoresis, amplified fragment length polymorphism fingerprinting and enterobacterial repetitive intergenic consensus sequence (ERIC) PCR (Jiang *et al*., 2000 a, b; Rivera *et al*., 1995), fluorescent *in situ* hybridization (Hernandez and Olmos, 2006). Variations in the length and sequence of the 16S–23S intergenic spacer regions (IGSs) of rRNA (rrn) operons have been used to design species-specific PCR primers and/or probes for bacterial identification (Kong *et al*., 2002).

3.1.2. 16S rRNA

The ability to differentiate subtypes is important for the recognition of disease outbreaks, the determination of sources of infection, the detection of particularly virulent strains, host distribution and geographical origin of possible variants of a specific pathogen (Olive and Bean, 1999; Soll, 2000). The available molecular methods used for subtyping differ widely in their

ability to differentiate among strains (Soll, 2000). It is observed that the 16S rRNA is unable to resolve closely related species (Nagpal *et al*., 1998), such as the ones clustered in the *Vibrio* core group, namely *V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, *V. campbellii*, *V. natriegens* and the newly described *V. rotiferianus* (Gomez-Gil *et al*., 2003 a). The comparison of 16S rRNA gene sequences has been recognized as an invaluable tool for confirming bacterial species identity but not for differentiating among strains, since the sequence shows limited intraspecific variations (Drancourt *et al*., 2000). Nucleotide sequences, primers and probes of 16S rRNA gene have been determined for *Vibrio* species, which are not useful for clustering of the species of this genus.

Special emphasis has been paid to sequencing of the 16S rRNA gene, although other genes, such as those for 23S rRNA, 16S–23S intergenic spacer region (IGS), or the *gyrB* gene, have been employed for taxonomic positioning of various strains (Dorsch *et al*., 1992; Venkateswaran *et al*., 1998). The 23S rRNA gene and the 16S–23S intergenic spacer contains regions where the sequences vary significantly, hence these regions are more useful in phylogeny than the 16S gene. Comparison of the nucleotide sequence of 16S–23S intergenic spacer region of *V.cholerae*, indicated strain-to-strain variation and that the spacer region is effective for differentiation at intraspecific level (Heidelberg *et al*., 2000). The identification of vibrios isolated from the aquaculture environment has been imprecise and is labour-intensive, requiring many biochemical and/or physiological tests (Vandenberghe *et al*., 2003). Several highly powerful molecular tools, e.g. amplified fragment length polymorphism (AFLP), (Rademaker *et al*., 2000; Gurtler and Mayall, 2001) and repetitive extragenic palindromic elements polymerase chain reaction (repPCR), have become readily available for the identification of bacteria, including vibrios (Thompson *et al*., 2001; Sawabe *et al*., 2003).

3.1.3. DNA-DNA Hybridization

DNA-DNA hybridization remains the "gold" standard for species delineation having at least 70% DNA-DNA similarity under stringent conditions (Stackebrandt *et al*., 2002). DNA-DNA hybridization experiments carried out in microplates in which DNA is non-covalently adsorbed and subsequently hybridized with photobiotin-labeled probe DNA (Willems *et al*., 2001) are much faster than the classic DNA-DNA hybridization techniques (e.g., initial renaturation, hydroxyapatite, and S1 nuclease). This technique can be performed in quadruplicate and with reciprocal reactions simultaneously, and has high correlation with classic techniques (Goris, 1998).

3.1.4. AFLP

Amplified fragment length polymorphism (AFLP) indices varies in the whole genome of different strains, hence considered as useful information in tracing short and long term evolution of bacterial isolates (Lan and Reeves, 2002). AFLP technique involves the digestion of total genomic DNA with two restriction enzymes, subsequent ligation of the restriction fragments with the halfsite-specific adaptors to all restriction enzymes; selective amplification of these fragments with two PCR primers that have corresponding adaptor and restriction site sequences at their target site, followed by the electrophoretic separation of the PCR products on polyacrylamide gels with selective detection of fragments that contain fluorescent labeled primer and computer-assisted numerical analysis of the banding patterns (Huys and Swings, 1999). AFLP fingerprinting has been carried out for isolates of *V.alginolyticus*, *V.cholerae*, *V.harveyi*, *V.vulnificus* and *P.damselae* (Vandenberghe *et al*., 1999; Lan and Reeves, 2002; Arias *et al*., 1997 a,b; Thyssen at al., 2000). Thompson *et al*. (2001) carried out AFLP of vibrios using HindIII/TaqI as the RE combination, obtaining 102±24 bands with mean reproducibility at 91±3%. The analysis of the banding patterns revealed that *V.trachuri* and *V.shilonii* were highly related to *V.harveyi* and *V.mediterranei*

respectively, indicating that they were synonyms. AFLP is a reliable fingerprinting identification and classification tool for vibrios. However, the variations in AFLP indices in the whole genome, including regions of unknown functions such as those coded by the pseudogenes or mobile genetic elements, limits its use as a phylogenetic marker.

3.1.5. RAPD

Molecular approaches that interrogate the whole genome appears to be a way forward to highlight what may be only minimal differences between strains. RAPD-DNA fingerprinting method has been widely used in the development of molecular diagnostic techniques for bacteria, because it allows a comparative analysis of genomes between different isolates of the same species by employing distinct molecular markers (Sudeesh *et al*., 2002). *V.harveyi, V.alginolyticus* and *V. parahaemolyticus* are characterized by RAPD-PCR techniques revealing greater genetic diversity among the three species. RAPD-PCR is a means of rapidly detecting polymorphisms for genetic mapping and strain identification (Welsh and McClelland, 1990). The method applies PCR with a single short oligonucleotide primer, randomly amplifying short fragments of genomic DNA, which are size-fractionated by agarose gel electrophoresis. The method has considerable appeal because it is generally faster and less expensive than any previous method for detecting DNA sequence variation. The fact that RAPDs survey numerous loci in the genome makes the method particularly attractive for analysis of genetic distance and phylogeny reconstruction. The amplification of a fragment depends strictly on the exact match between the oligonucleotide primer and a site on the genomic DNA, thus if one DNA sample amplifies a particular band and another DNA sample does not, it is assumed that a single nucleotide substitution in a primer site accounts for the difference (Clark and Lanigan, 1993). The recent advent of standardized and optimized reagents has greatly improved the accessibility and reproducibility of the RAPD method (Hyytiä *et al*., 1999). These improvements led to an increase in the number of successful applications of

the RAPD method for differentiating strains of marine bacterial pathogens (Romalde *et al*., 1999; 2002, Magariños *et al*., 2000; Ravelo *et al*., 2003).

RAPD analysis has several advantages including relative shorter time required to complete the analysis after standardization, prior knowledge of the organisms genome is not necessary, availability of series of primers for analysis, minimal operational cost, requirement of relatively small amount (approx 20ng) of high molecular weight DNA and simpler protocol allows strain differentiation based on the differences in nucleotide sequences in the entire genome (Gopalakrishnan and Mohindra, 2002). This technique has become increasingly important for discriminating strains of food-borne pathogens and to trace the routes of transmission and implementation of suitable control measure based on the results. Therefore, RAPD can be considered as powerful tool for the identification of strain variation or for population studies. Also this method is simple, fast and specific, that it could be very useful for typing and differentiating environmental vibrios, which are relatively difficult to identify using other techniques (Sudeesh *et al*., 2002). It has been shown that the rDNA sequence similarities between *Vibrio* spp. are so high that 16S rDNA genes cannot be used for differentiation at the species level (Kita-Tsukamoto *et al*., 1993). For this reason, screening with different gene-specific oligonucleotides is to be developed for *V. harveyi*.

3.1.6. Multi Locus Sequence Typing

In 1998, Maiden and co-workers proposed MLST a modified version of multilocus enzyme electrophoresis (MLEE) for studying the population biology and epidemiology of *Neisseria meningitides*. Essential genes are conserved in bacteria and other organisms but the sequences may vary, making them useful candidates for phylogenetic analysis. In addition, the genera within vibrios are defined on the basis of their shared sequence similarities in different loci. Species within the genus *Vibrio* share at least

85% gene sequence similarity in *recA*, *rpoA*, and *pyrH* (Thompson *et al*., 2005). DDH technique is time-consuming and can be performed in relatively few laboratories and, more importantly, the DDH data are not cumulative in online databases. Clearly, a reliable and straightforward alternative is the use of MLSA. MLSA based on the *recA*, *rpoA*, and *pyrH* genes of species form discrete clusters showed that the species have a cutoff level of 94% gene sequence similarity (Thompson *et al*., 2004). However, some groups of species, e.g., the *Vibrio splendidus* and *Vibrio harveyi* show variations on the basis of *recA*, *gyrB*, and *gapA* based MLSA. Thus, it is very important to evaluate additional genetic markers that can distinguish closely related species of vibrios. Some studies suggest that recombination might have occurred between different sister species, such as between *V. cholerae* and *V. mimicus* and between *V. harveyi* and *V. campbellii*, but it is not clear how prevalent and widespread this process is when all groups of vibrio species are analyzed simultaneously.

Accurate identification of vibrios at the family and genus levels is obtained by 16S rRNA gene sequencing, whereas identification at the species and strain levels requires the application of genomic analysis, including DNA-DNA hybridization, repetitive extragenic palindromic PCR (rep PCR), and amplified fragment length polymorphism (AFLP) analysis (Thompson *et al*., 2004). These techniques are essential for reliable species identification, because several vibrios have nearly identical 16S rRNA sequences and similar phenotypic features. The sequencing of housekeeping genes is emerging as an alternative to overcome this problem and may improve the current pragmatic definition of bacterial species (Fig. 3.1) (Sawbae *et al*., 2007). Different loci, e.g., 23S rRNA, *gapA*, *gyrB*, *hsp60*, and *recA* (Thompson *et al*., 2005; Gomez-Gil *et al*., 2004; Le Roux *et al*., 2004) have been used for phylogenetic studies and the identification of *Vibrionaceae* species. So far these genes (except for *recA*) have only been examined in a very limited number of species and strains. Alternative phylogenetic markers should fulfill several criteria, as put forward by

Zeigler (2003): (i) the genes must be widely distributed among genomes, (ii) the genes must be present as a 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 to 2,250 nucleotides [nt]), and (iv) the sequences must predict whole genome relationships with acceptable precision and accuracy that correlate well with the 16S rRNA data and with whole genome similarities measured by DNA-DNA hybridization. A combination of *in silico* analyses and recent experimental studies of different bacteria, including *Bacillus*, *Proteobacteria*, lactic acid bacteria, *Mycobacterium*, and *Mycoplasma*, suggested that the RNA polymerase alpha subunit gene *rpoA*, *recA*, and the uridylate kinase gene (*pyrH*) fulfill these requisites and could therefore be used for identification purposes (Gevers *et al*., 2004; Thompson *et al*., 2005; Zeigler, 2003).

V. harveyi and *V. campbellii* are genetically related species with a DNA-DNA similarity value of 69% and a 16S rRNA similarity higher than 97% (Gomez-Gil *et al*., 2004). Also, *V. harveyi* and *V. carchariae* were synonymous, with *V.harveyi* having precedence as the senior synonym (Pedersen *et al*. 1998; Gauger and Gomez-Chiarri 2002). Information obtained from multilocus sequence analysis is, therefore, essential for the accurate and reliable identification of *Vibrio* species.

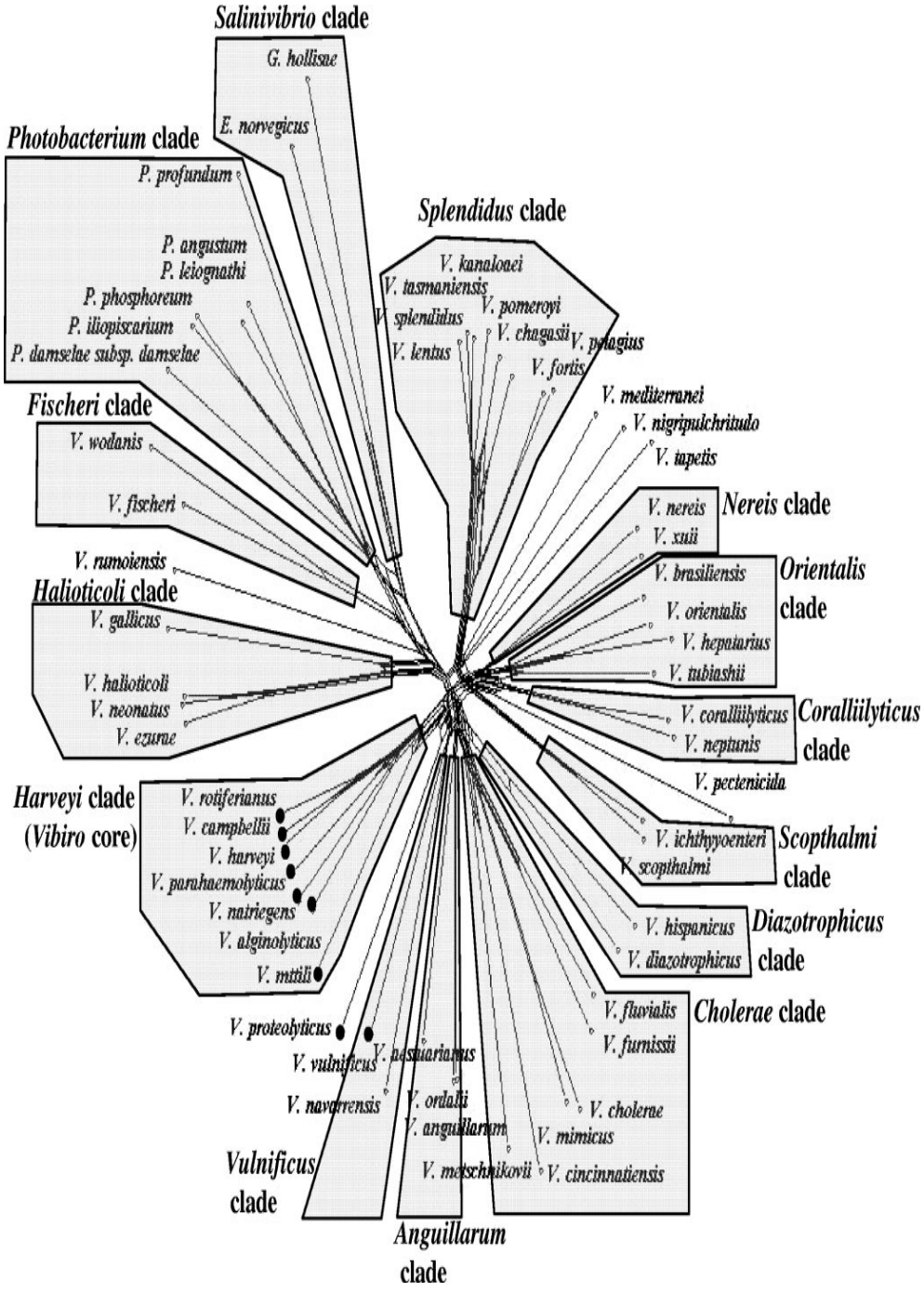


Fig:3.1 Concatenated split network tree based on nine gene loci-*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *topA*, and 16S rRNA gene sequences from 58 taxa were concatenated and reconstructed using the SplitsTree4 program with all nodes supported by 100 bootstrap replications.(Sawbae *et al*., 2007)

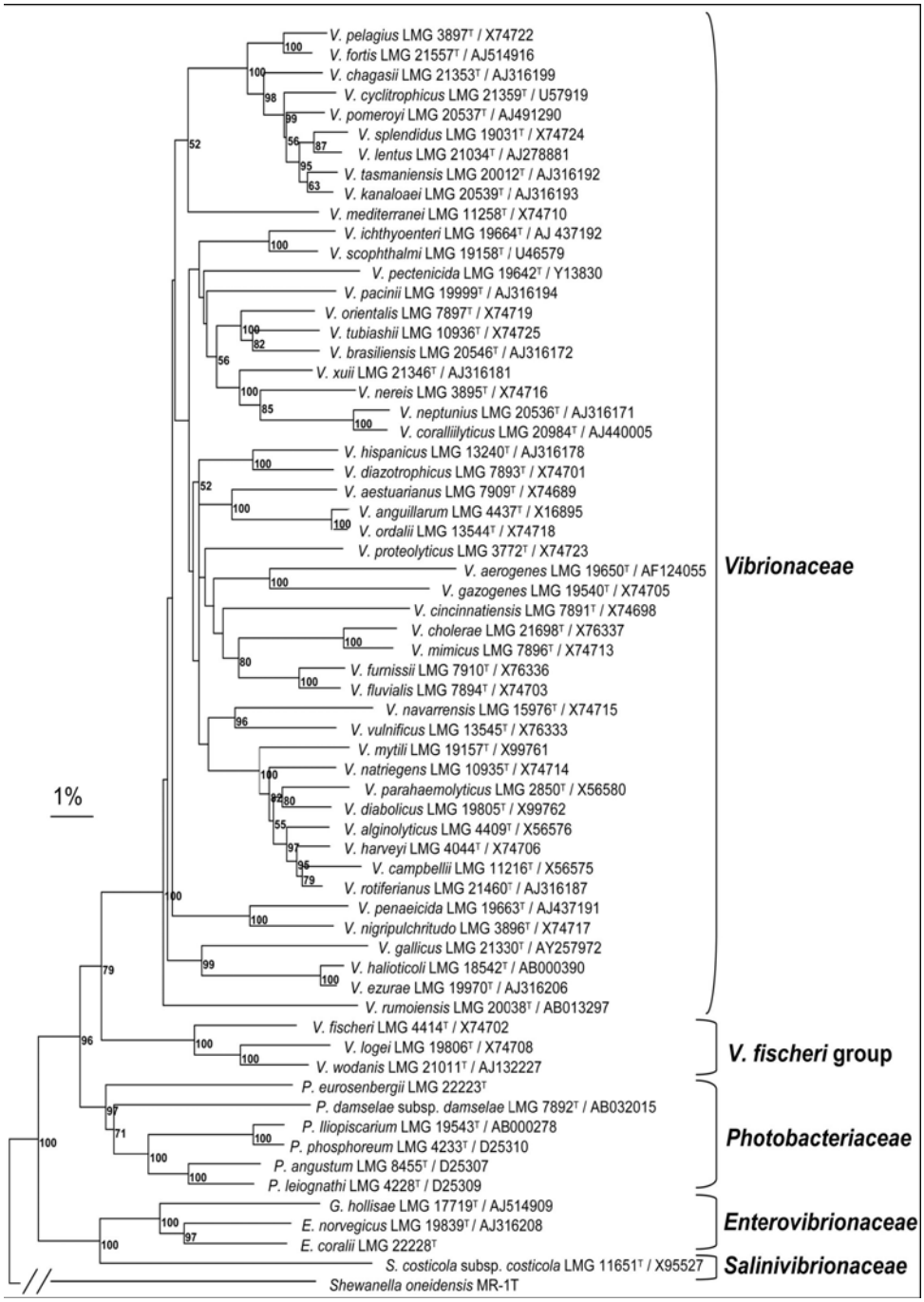


Fig: 3.2 Phylogenetic tree based on the neighbor-joining method, using the 16S rRNA, *recA* and *rpoA* concatenated gene sequences from the type strains of each species belonging to different families of vibrios. Felsenstein, (1985) method was employed for Bootstrap percentages after 1,000 simulations are shown. Bar, 1% estimated sequence divergence (Thompson *et al*., 2005).

3.1.7. Role of Housekeeping Genes in phylogenetic Analysis

Various housekeeping genes in particular, the *recA* gene essential for genetic recombination are used to demonstrate the divergence among interrelated *Vibrio* species and it had more discriminatory power than 16S rRNA gene in phylogenetic analysis of *Vibrionaceae* (Fig. 3.2) (Thompson *et al*., 2004). A 600bp sequence of *hsp60* gene analyzed among 15 *Vibrio* species showed an identity of 71 to 82%, suggesting that this gene could also be a useful phylogenetic marker. The various genes involved in DNA replication are also well conserved, such as the *gyrB* gene coding for DNA gyraseB subunit. Based on phenotypic and 16S rRNA analysis, >99% similarity was shown by the strains of *V.alginolyticus* and *V.parahaemolyticus*, whereas the *gyrB* sequence established 86.8% identity between the two strains. Comparison of the *gyrB* sequence was useful for phylogenetic analysis of *V.splendidus* and its related species (Le Roux *et al*., 2004) and also PCR targeted to this gene for the identification of *V.hollisae* has been developed (Vuddhakul *et al*., 2000b).

Other molecular chronometers include the *sodA* gene coding for superoxide dismutase catalyzing dismutation of the superoxide radical to H_2O_2 and O_2. This is conserved in prokaryotes and eukaryotes and is useful for identification of *Vibrionaceae,* and the gene targeted PCR is used for the identification of *V.parahaemolyticus* (Shyu and Lin, 1999). The *lux* genes of various luminescent bacteria, especially the *luxA* gene coding for luciferase showed 99% identity among *V.choleare* and 77% identity between *V.cholerae* and *V.harveyi* (Palmer and Colwell, 1991). The *fur* gene coding for a regulator of an iron uptake system is detected in many bacterial species (Colquhoun and Sorum, 2002). Although genetic differentiation of *V.harveyi* from related species is not easy (Gomez-Gil *et al*., 2004; Oakey *et al*., 2003), PCR based analysis of these chronometers from various *Vibrio* species shows that these genes can be effectively used for phylogenetic analysis of genus *Vibrio*.

Rationale of the study is to analyse the amplicons of eight genes (i.e., *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *topA*, and 16S rRNA), to determine the genotypic heterogeneity among the isolates.

3.2. Materials and Methods

3.2.1. DNA extraction using DNAZol (Invitrogen) from 158 isolates:

A single colony of the isolate was inoculated into LB broth and incubated for 18hrs. An aliquot of 2ml of the overnight grown culture was taken in a 2ml MCT and centrifuged at 10000xg for 10min at 4 °C. The supernatant decanted and the pellets were re-suspended in 1ml TNE (Tris-NaCl- EDTA buffer, pH-7.5).The above process was repeated twice. The pellets were re-suspended in 50µl TNE and added 20 µl (10mg/ml) Proteinase K, mixed gently and incubated at 37°C for 1hr. An aliquot of 1ml DNAZol was added and mixed thoroughly using cut-tips until the mass dissolved completely. The homogenate was centrifuged at 12,000xg for 10min at 4°C and transferred the supernatant into a fresh 1.5ml MCT. An aliquot of 0.5ml 100% ice cold ethanol was added, mixed by gentle inversion and incubated at room temperature for 5-10mins, or kept in -20°C for 30min. The homogenate was centrifuged at 12,000xg for 10 min at 4°C to form pellet. The supernatant (100% ethanol) was drained off and washed the pellet twice with 1ml 75% ethanol. The pellets were air-dried and dissolved in 200µl 8mM NaOH and 20µl Hepes buffer and incubated at 4°C. The DNA concentration was determined by running on 0.8 % agarose and the extracted DNA were stored at -20 ^{0}C in aliquots using 5mM Tris Cl (pH 8) until use.

3.2.2. Construction of Randomly amplified polymorphic DNA (RAPD) profile using a set of Operon primers

Standardization of RAPD-PCR was carried out with aid of a set of 20 RAPD primers using 11 type strains (Table-3.1) and one isolate from each cluster obtained upon phenotypic grouping. The primers screened were

obtained from Operon Technologies, USA. The 20 primers used for standardization and thereby for RAPD fingerprinting are as follows:

Table-3.1: Details of the Operon primers used

Sl.No	Primer	Primer Code	Primer Sequence	Concentration (pmoles/ml)
1	OPA-03	NP111	AGTCAGCCAC	5194
2	OPA-04	NP112	AATCGGGCTC	5090
3	OPA-05	NP113	AGGGGTCTTG	5194
4	OPA-06	NP114	GGTCCCTGAC	5743
5	OPA-07	NP115	GAAACGGGTG	4627
6	OPA-08	NP116	GTGACGTAGG	4894
7	OPD-05	NP117	TGAGCGGACA	4801
8	OPD-06	NP118	ACCTGAACGG	4990
9	OPD-08	NP119	GTGTGCCCCA	5743
10	OPD-11	NP120	AGCGCCATTG	5302
11	OPD-15	NP121	CATCCGTGCT	5919
12	OPD-16	NP122	AGGGCGTAAG	4627
13	OPD-20	NP123	ACCCGGTCAC	5616
14	OPAC-10	NP124	AGCAGCGAGG	4685
15	OPAH-01	NP125	TCCGCAACCA	5415
16	OPAH-02	NP126	CACTTCCGCT	4685
17	OPAH-03	NP127	GGTTACTGCC	5656
18	OPAH-04	NP128	CTCCCCAGAC	5876
19	OPAH-05	NP129	TTGCAGGCAG	5090
20	OPAH-O6	NP130	GTAAGCCCCT	5533

From the 20 primers screened, 7 primers (OPA-3, OPA-4, OPA-5, OPA-7, OPAC-10, OPD-16 and OPD-20) were selected based on the resolution of the distinct detectable bands. These 7 primers were used to construct the RAPD profile of the 158 isolates. The reaction mixture for

RAPD-PCR consisted of 1.0 µl Taq polymerase, 2.5 µl10x Buffer, 2.0 µl dNTP mix, 0.5 µl Mg Cl_2, 1.5 µl Primer, 1 µl Template DNA and 16.5 µl MilliQ. Amplifications were performed on a thermal cycler, which was programmed for an initial denaturation cycle of 95°C for 4mins, followed by 45 cycles of denaturation at 94 ºC for 1min, annealing at 36 °C for 1min and primer extension at 72 °C for 2mins. The program also included a final primer extension step at 72 °C for 10mins. The amplified products were analysed on 1.5% agarose gel electrophoresis carried out at a constant current of 60mA. Images of agarose gels were analyzed by manually transforming the scored DNA fragments obtained into binary data matrix by scoring as presence (1) or absence (0) for each isolate and comparing with the distinct bands at equivalent sites obtained by running 1kb and 100bp markers. Clustering and dendrogram construction by each bacterial isolate upon amplification using the chosen 7 primers based on similarity coefficient was carried out with the software NTSYS pc version 2.0. Further, population wise delineation of the 158 isolates with the seven selected primers was carried out using the software PopGene32. Accordingly, the isolates were grouped into 17 populations (clusters) as constructed examining the phenotypic characters (by way of numerical taxonomy) were processed using the software PopGene32. The amplicons represented as bands for each of the isolates were scored as binary data matrix and population wise analysis of correlation was carried out. Percentage similarity between each population was represented as dendrogram.

3.2.3. Amplification of Housekeeping genes:

The representative isolates (35 Nos. including the type stains) were selected from the dendrogram constructed based on phenotypic characterization. Genomic DNA was extracted using DNAzol method as described above in 3.3.1. The extracted DNA was stored at -20 ºC in aliquots using 5mM Tris Cl (pH 8) until use. The genomic DNA of the 35

isolates was amplified for the already reported housekeeping gene markers (Table-3.2). Following amplification, the banding pattern was analyzed by running on 1% agarose gel. Subsequent to agarose gel electrophoresis, the molecular weight of the bands was analysed using Quantity1 software. The bands were scored as 0 or 1 for absence and presence respectively to the corresponding positions of 100bp maker. The scored data was processed in the PopGene32 software and a dendrogram was constructed.

Table- 3.2: Details of Housekeeping Genes

Gene	***PRIMER SEQUENCE***
gapA (glyceraldehydes-3-phosphodehydrogenase)	AACTCACGGTCGCTTTCAAC CGTTGTCGTACCAAGATAC
ftsZ (Cell division protein)	GCTGTTGAACACATGGTACG GCACCAGCAAGATCGATATC
topA (topoisomerase I)	GAGATCATCGGTGGTGATG GAAGGACGAATCGCTTCGTG
mreB (rod shaping protein gene B-subunit)	ACTTCGTGGCATGTTTTC CCGTGCATATCGATCATTTC
gyrB (gyraseB)	GAAGTTATCATGACGGTACTTC CCTTTACGACGAGTCATTTC
pyrH (uridylate kinase)	GATCGTATGGCTCAAGAAG TAGGCATTTTGTGGTCACG
recA (recombinaseA)	GTCTACCAATGGGTCGTATC GCCATTGTAGCTGTACCAAG
16S rRNA	GAGTTTGATCCTGGCTCA ACGGCTACCTTGTTACGACTT

16S rRNA gene sequence analysis

This was carried out to confirm the identity of the representative isolates of the phena generated by numerical taxonomy (Fig. 2.1). PCR products of representative isolates (25 nos) were sequenced (SciGenom Labs Pvt Ltd, Cochin, India) and the 16SrRNA gene sequences obtained were matched with the (http://www.ncbi.nih.gov/sites/entrez?db=nuccore) Genbank database using the BLAST search algorithm (Altschul *et al*., 1990). These sequences were deposited with GenBank to obtain accession numbers.

3.3. Results

3.3.1. RAPD profiling

All 158 isolates were subjected to RAPD-PCR with the 7 selected Operon primers and scored (1) for the presence and (0) for absence of distinct bands at equivalent sites obtained by running 1kb and 100bp markers. The scores obtained with each primer were processed to determine the divergence pattern of the isolates using NTSYS pc. 2.0. Amplification of the isolates with the 7 selected primers gave 13, 11, 15, 14, 12, 13 and 14 loci respectively. All the loci obtained with the selected 7 primers were analysed, yielding 27, 26, 46, 48, 41, 44 and 44 clusters respectively (Figs-3.3 to 3.16). The clusters obtained with each primer where compared with the source of isolation and represented in Table-3.3. Dendrograms obtained suggests that there existed a wide heterogenicity among the isolates of vibrios, exhibiting a correlation coefficient ≥0.62r (62%S). Highest extend of heterogeneity was exhibited by isolates of *V.harveyi*, followed by *V.vulnificus*, *V.mediterranei*, *V.alginolyticus*, *V.cholerae* and *V.nereis*. Since, the isolates were widely diverging; analysis of banding pattern of each phena with all the 7 selected primers in total was carried out to determine if there was any relation between banding pattern and the source of isolation. The banding pattern shown by each bacterial phenon (population) upon amplification, the total loci were processed using PopGene32 (Fig. 3.17). Dendrogram obtained showed that all the isolates grouped into 8 Clusters and were interrelated at ≥76%. Phenon1 & 2 having the isolates of *V.harveyi* was correlated at 99.1%, suggesting a high degree of homogeneity; hence these isolates in these two phena could be members of the same species. These two phena joined with the isolates of *V.mediterranei* in phena 3 & 4 at 91.6%S and to the 5th phenon of *V.harveyi* at 89.1%S. At 84.5%S this cluster (Cluster-1) joined with Cluster-2 having the isolates grouped to phena 6, 7, and 8. Phena 7 and 8 comprising isolates of *V.vulnificus* at 97.4%S, joined with isolates of *V.fluvialis* of phenon6 at 86%S. The first cluster of *V.harveyi* showed 84.2%S, 82%S, 81%S and

79.8%S to the phena representing the isolates of *V.nereis* in Clusters 3 & 4, *V.parahaemolyticus* in Cluster-5 and *V.splendidus* in Cluster-6 respectively. At 86.8%S the isolate and type strain of *V.proteolyticus* (Cluster-7), grouped under phena 14 and 15 were correlated. At 80.6%S isolates of *V.alginolyticus* (phenon16) and of *V.cholerae* (phenon17) in cluster-8 were related and this cluster showed 76%S to the first cluster of *V.harveyi*.

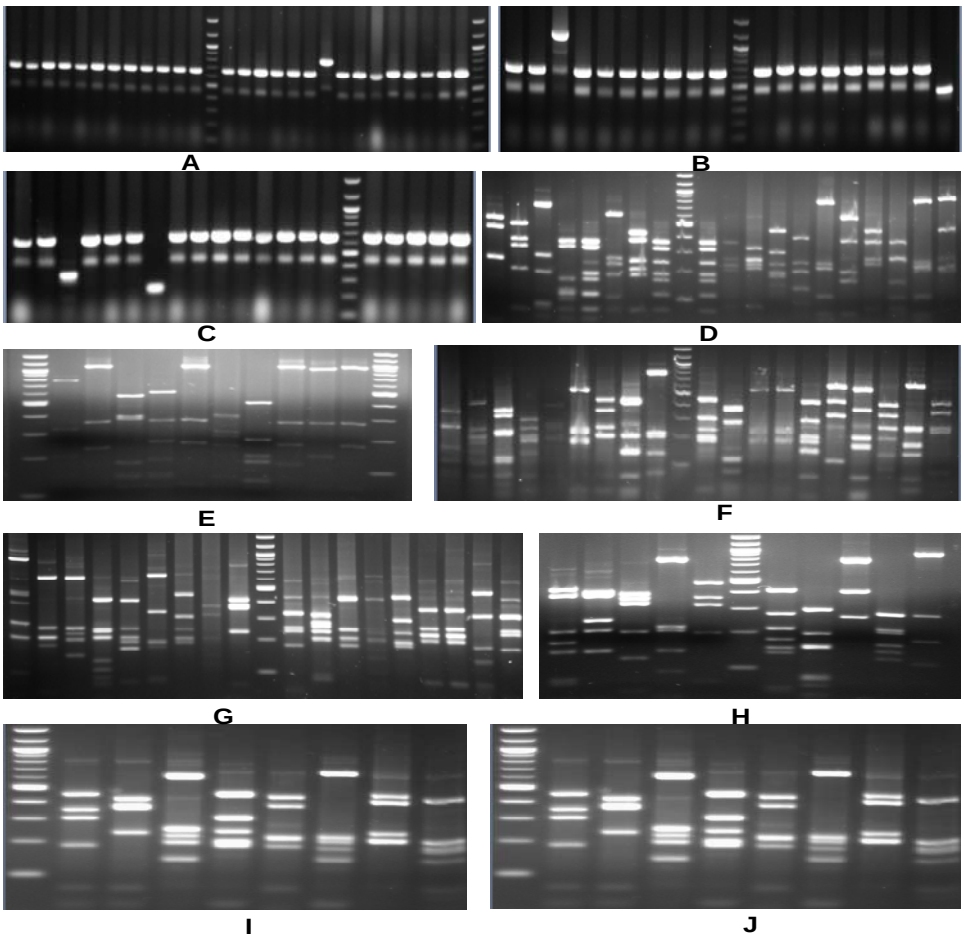


Fig: 3.3 Amplicons obtained using the primer OPA-3

A- V1 to V12, 1Kb Marker, V13 to V28, 1Kb Marker
B- V29 to V38, 1Kb Marker, V39 to V47
C- V48 to V62, 1Kb Marker, V63 to V67
D- V68 to V75, 1Kb Marker, V76 to V86
E- 100bp Marker, V87 to V96, 100bp Marker
F- V97 to V104, 1Kb Marker, V105 to V114
G- V115 to V123, 1Kb Marker, V124 to V132
H- V133 to V137, 100bp Marker, V138 to V142
I- 100bp Marker, V143 to V150
J- 100bp Marker, V151 to V158

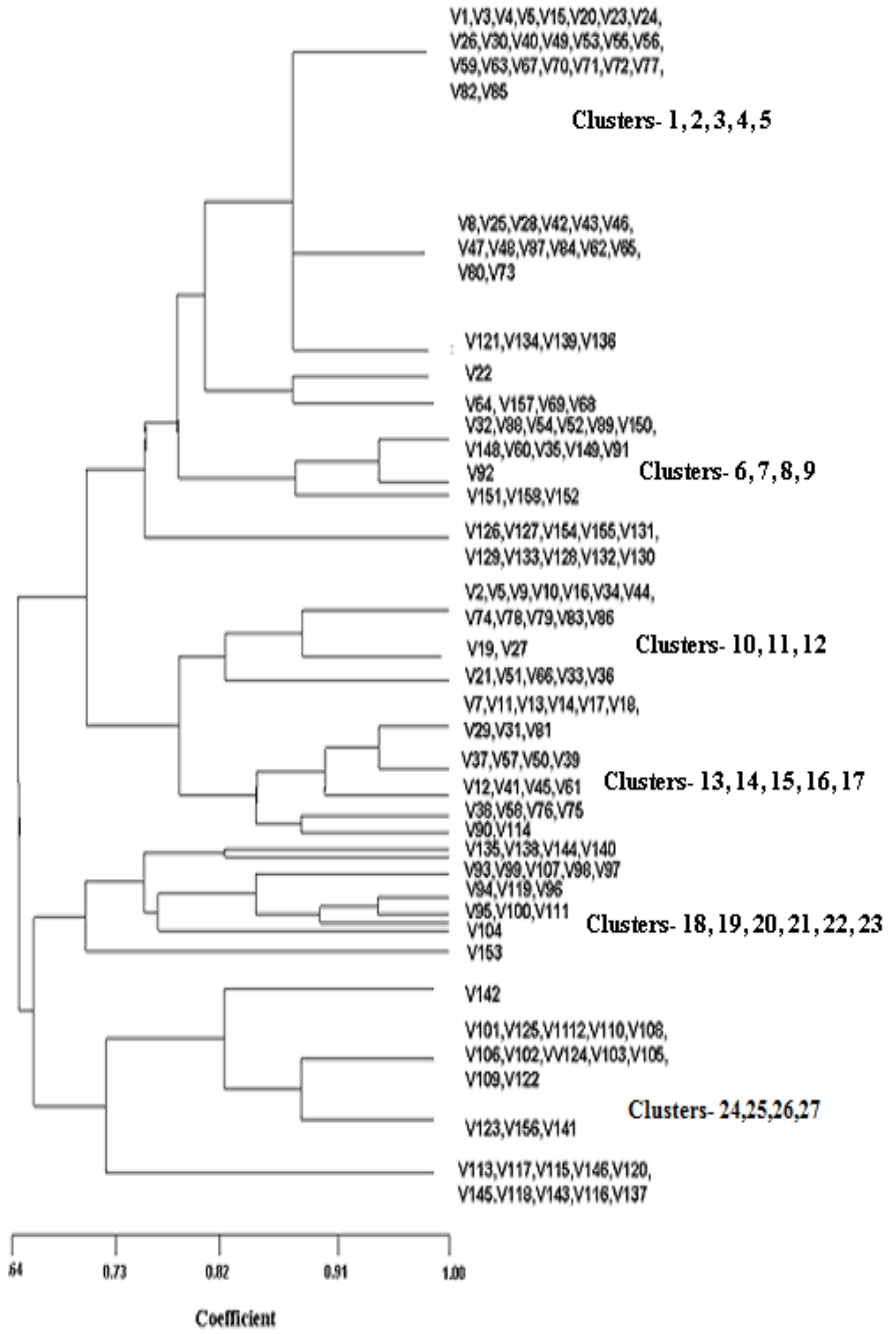


Fig: 3.4 Dendrogram of the *Vibrio* spp. based on RAPD profile using the primer OPA-3

Clusters- 1, 2, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18 - Isolates of *V. harveyi* ; Clusters- 19, 20 - Isolates of *V. parahaemolyticus;* Clusters- 3, 21, 22, 23, 24, 27 - Isolates of *V.mediterranei* ;Clusters- 23, 27- Isolates of *V.proteolyticus;* Clusters- 9- Isolates of *V.alginolyticus* ; Clusters- 9, 24, 25, 28- Isolates of *V.nereis* ; Clusters- 8, 18- Isolates of *V.fluvialis;* Clusters- 5, 26- Isolates of *V. cholerae* ; Clusters- 3, 8, 19- Isolates of *V.vulnificus;* Clusters-5, 20 - Isolates of *V. splendidus*

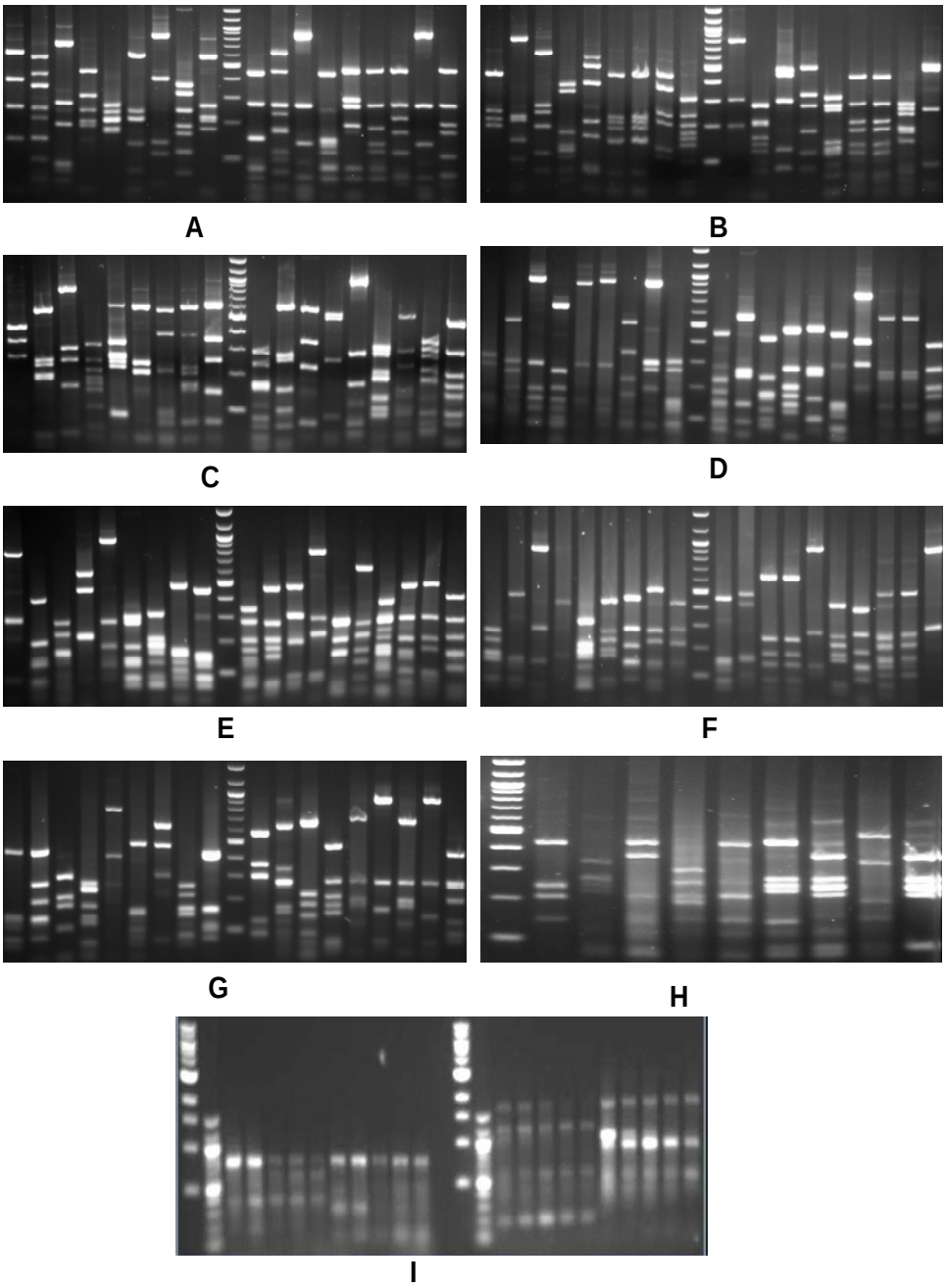


Fig: 3.5 Amplicons obtained using the primer OPA-4

A- V1 to V9, 1Kb Marker, V10 to V19
B- V20 to V28, 1Kb Marker, V29 to V37
C- V38 to V46, 1Kb Marker, V47 to V55
D- V56 to V64, 1Kb Marker, V65 to V74
E- V75 to V83, 1Kb Marker, V84 to V93
F- V94 to V102, 1Kb Marker, V103 to V111
G- V112 to V120, 1Kb Marker, V121 to V129
H- 100bp Marker, V130 to V137
I- 1Kb Marker,100bp Marker, V138 to V147, 1Kb Marker,100bp Marker, V148 to V158

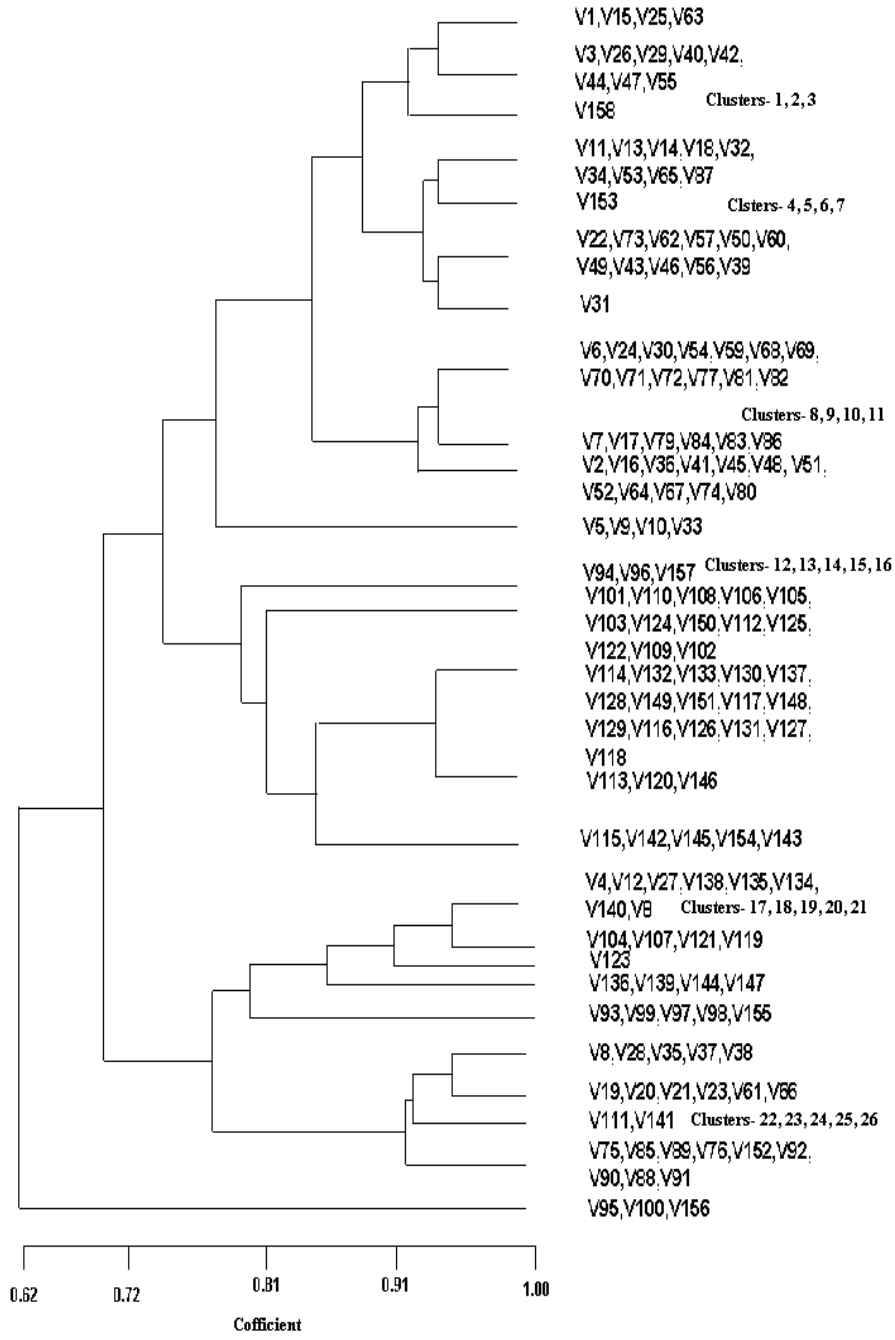


Fig: 3.6 Dendrogram of the *Vibrio* spp. based on RAPD profile using the primer OPA-4

Clusters- 1, 2, 4, 6, 7, 8, 9, 10, 11, 17, 22, 23, 25- Isolates of *V. harveyi* ; Clusters- 21 - Isolates of *V. parahaemolyticus* ; Clusters- 18, 19, 24 - Isolates of *V.mediterranei*; Clusters- 23 - Isolates of *V.proteolyticus;* Clusters- 14 - Isolates of *V.alginolyticus* ; Clusters- 15, 16 - Isolates of *V.nereis*; Clusters- 14 - Isolates of *V.fluvialis;* Clusters- 13- Isolates of *V. cholera*; Clusters- 3, 17, 20 - Isolates of *V.vulnificus;* Clusters-12- Isolates of *V. splendidus*

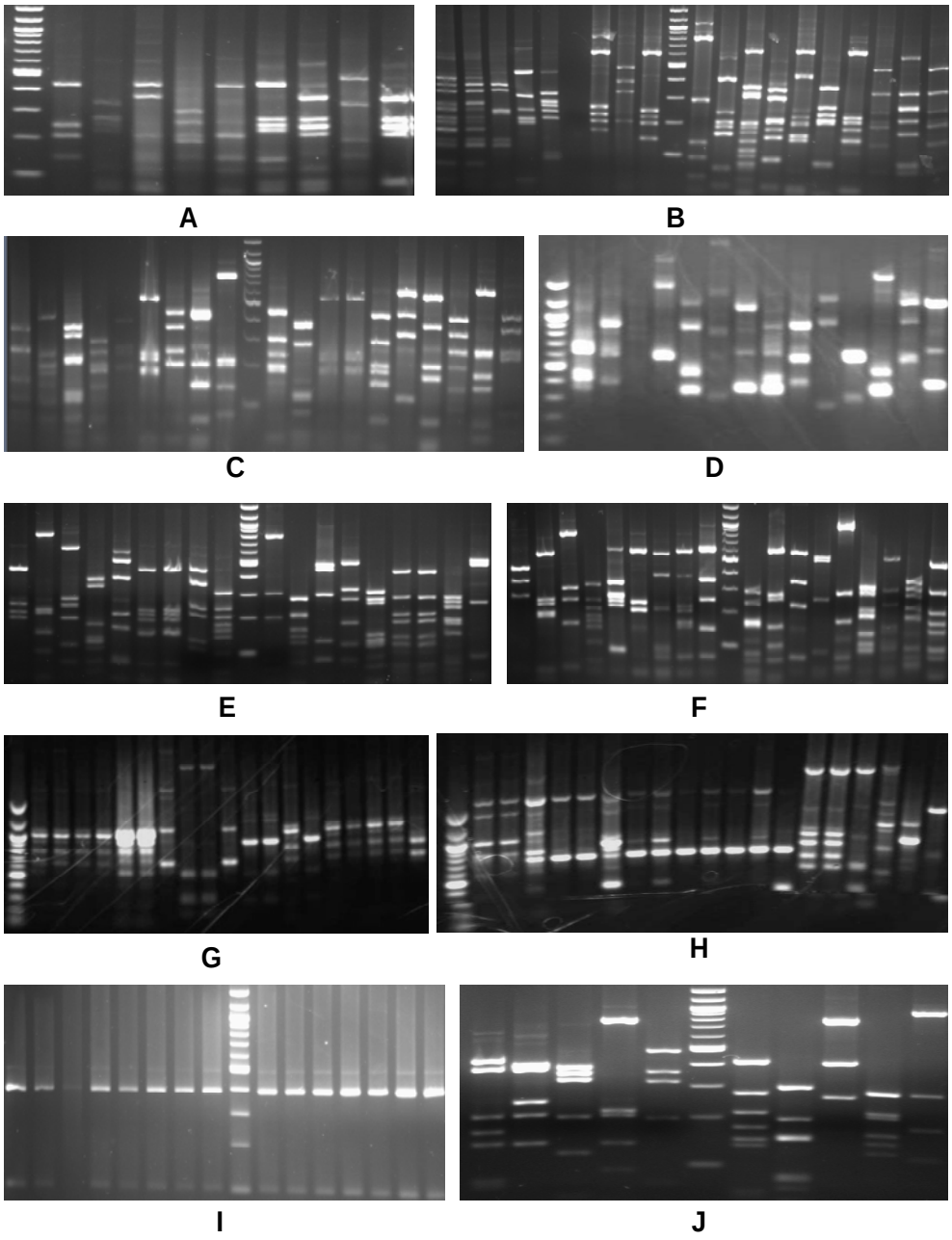


Fig: 3.7 Amplicons obtained using the primer OPA-5

A- 100bp Marker, V1 to V9
B- V10 to V17, 1Kb Marker, V18 to V27
C- V28 to V36, 1Kb Marker, V37 to V46
D- 1Kb Marker, V47 to V60
E- V61 to V69, 1Kb Marker, V70 to V78
F- V79 to V87, 1Kb Marker, V88 to V96
G- 100bp Marker, V97 to V115
H- 100bp Marker, V116 to V134
I- V135 to V141, 1Kb Marker, V142-V148
J- V149 to V153, 100bp Marker, V154 to V158

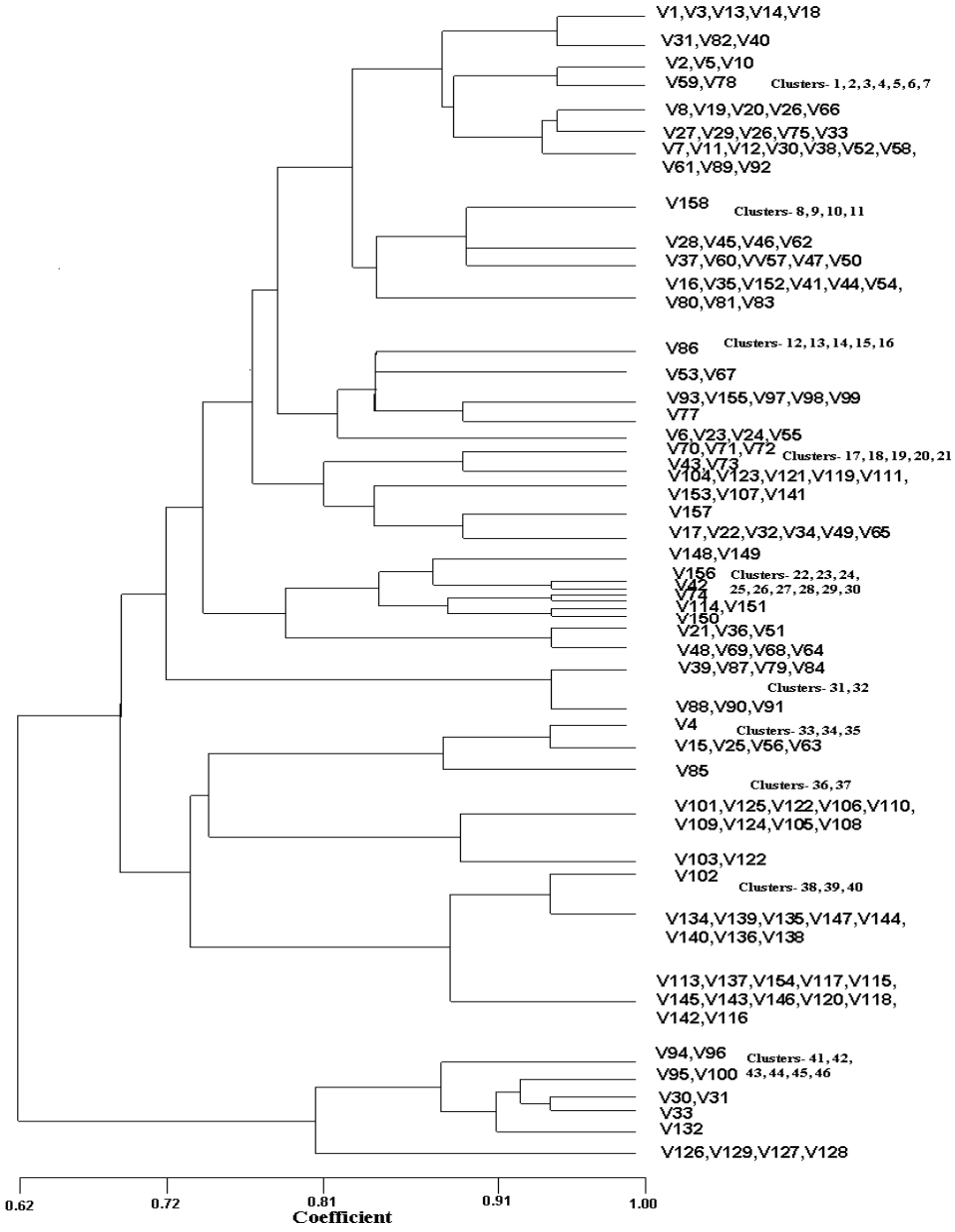


Fig: 3.8 Dendrogram of the *Vibrio* spp. based on RAPD profile using the primer OPA-5

Clusters- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18 , 19, 22, 25, 26, 29, 30, 31, 32, 33, 34, 35, 43, 44- Isolates of *V. harveyi* ; Clusters- 14- Isolates of *V. parahaemolyticus*; Clusters- 14, 20 - Isolates of *V.mediterranei*; Clusters- 24, 42 - Isolates of *V.proteolyticus;* Clusters- 23, 45, 46 - Isolates of *V.alginolyticus*; Clusters- 40- Isolates of *V.nereis*; Clusters- 27- Isolates of *V.fluvialis;* Clusters- 28, 36, 37, 38- Isolates of *V. cholerae*;; Clusters- 8, 39- Isolates of *V.vulnificus;* Clusters- 21, 41 - Isolates of *V. splendidus*

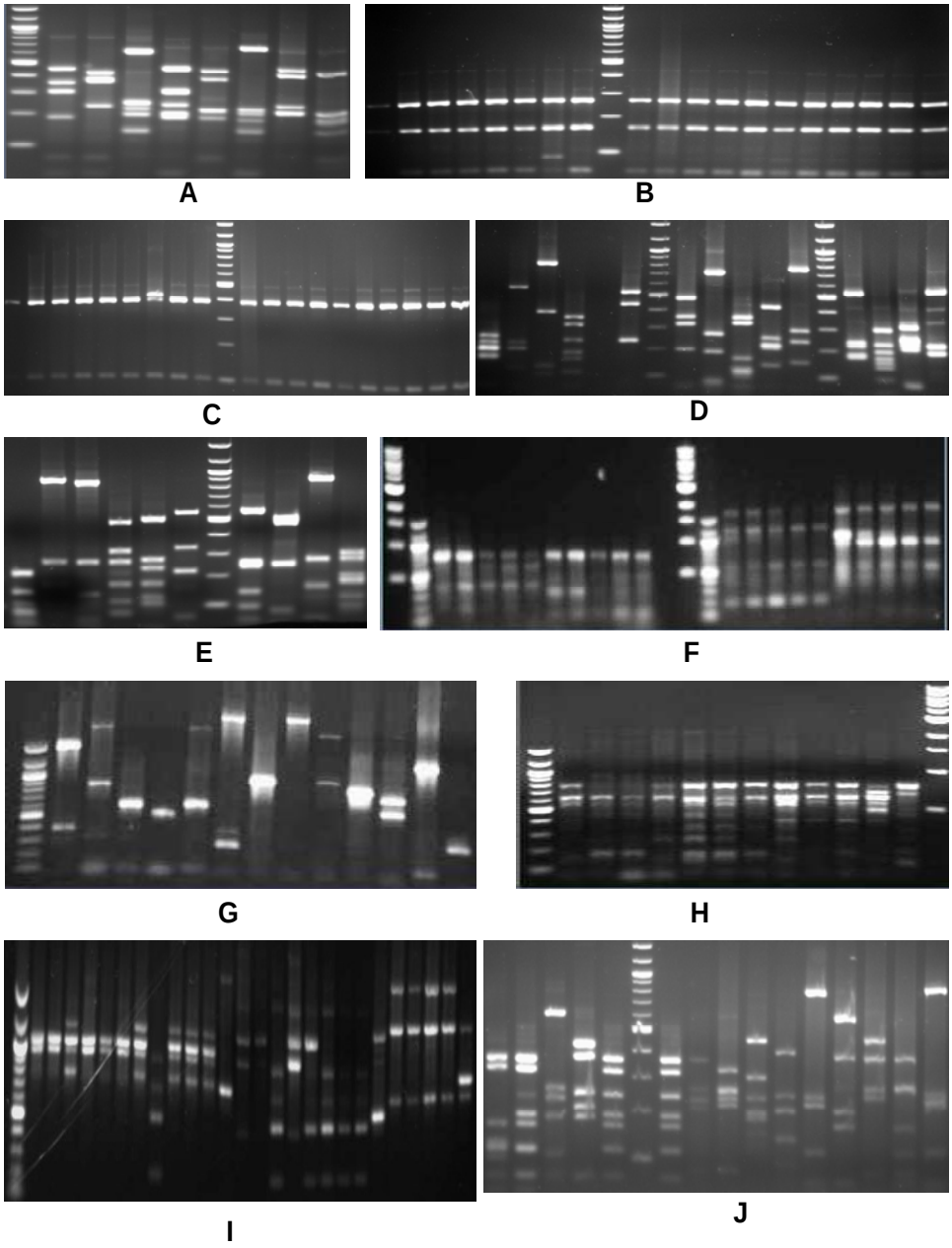


Fig: 3.9 Amplicons obtained using the primer OPA-7

A- 1Kb Marker, V1 to V8
B- V9 to V16, 1Kb Marker, V17 to V27
C- V28 to V36, 1Kb Marker, V37 to V46
D- V47 to V51 1Kb Marker, V52 to V56, 1Kb Marker, V57 to V60
E- V61 to V66, 1Kb Marker, V67 to V70
F- 1Kb Marker, 100bp Marker, V71 to V81, 1Kb Marker, 100bp Marker, V82 to V91
G- 100bp Marker, V92 to V104
H- 100bp Marker, V105 to V116
I- 1Kb Marker, V117 to V142
J- V143 to V148, 1Kb Marker, V149 to V158

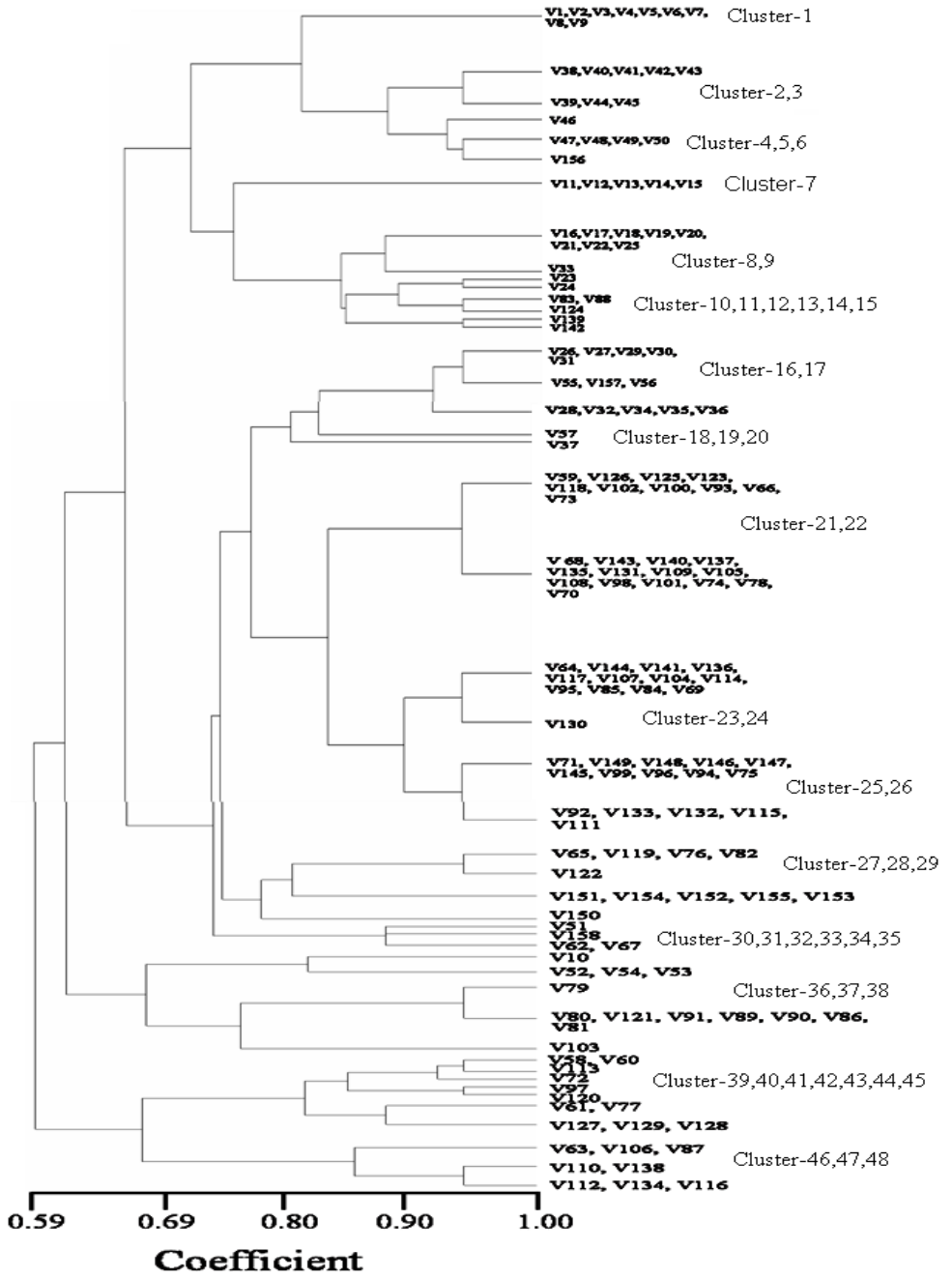


Fig: 3.10 Dendrogram of the *Vibrio* spp. based on RAPD profile using the primer OPA- 7

Clusters- 12, 13, 14, 15, 21, 22, 23, 24, 25, 27, 30, 33, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48- Isolates of *V. harveyi*; Clusters- 5, 31 - Isolates of *V. parahaemolyticus*; Clusters- 6, 29, 32- Isolates of *V.mediterranei;* Clusters- 35- Isolates of *V.proteolyticus;* Clusters- 1, 26, 34- Isolates of *V.alginolyticus*; Clusters- 9, 18, 20, 33- Isolates of *V.nereis*; Clusters- 11, 16- Isolates of *V.fluvialis;* Clusters- 7, 8, 10, 28 - Isolates of *V. cholerae*; Clusters- 2, 3, 4- Isolates of *V.vulnificus;* Clusters-17, 19 Isolates of *V. splendidus*

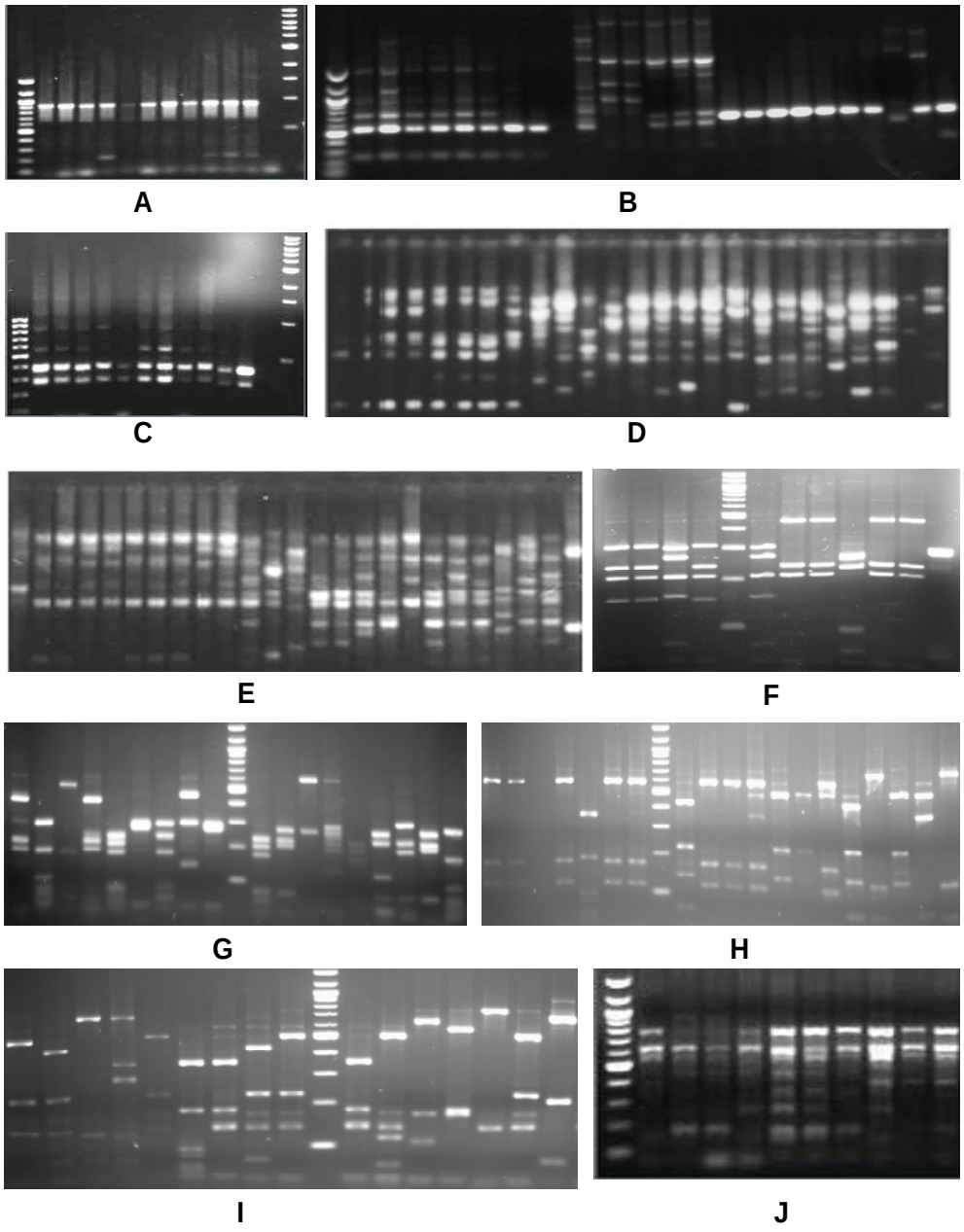


Fig: 3.11 Amplicons obtained using the primer OPAC-10

A- 100bp Marker, V1 to V12, 1Kb Marker
B- 100bp Marker,V13 to V24
C- 100bp Marker,V25 to V35, 1Kb Marker
D- V36 to V60
E- V61 to V85
F- V86 to V89, 1Kb Marker, V90 to V96
G- V97 to V105, 1Kb Marker, V106 to V114
H- V115 to V120, 1Kb Marker, V121 to V132
I- V133 to V141, 100bp Marker, V142 to V148
J- 100bp Marker, V149 to V158

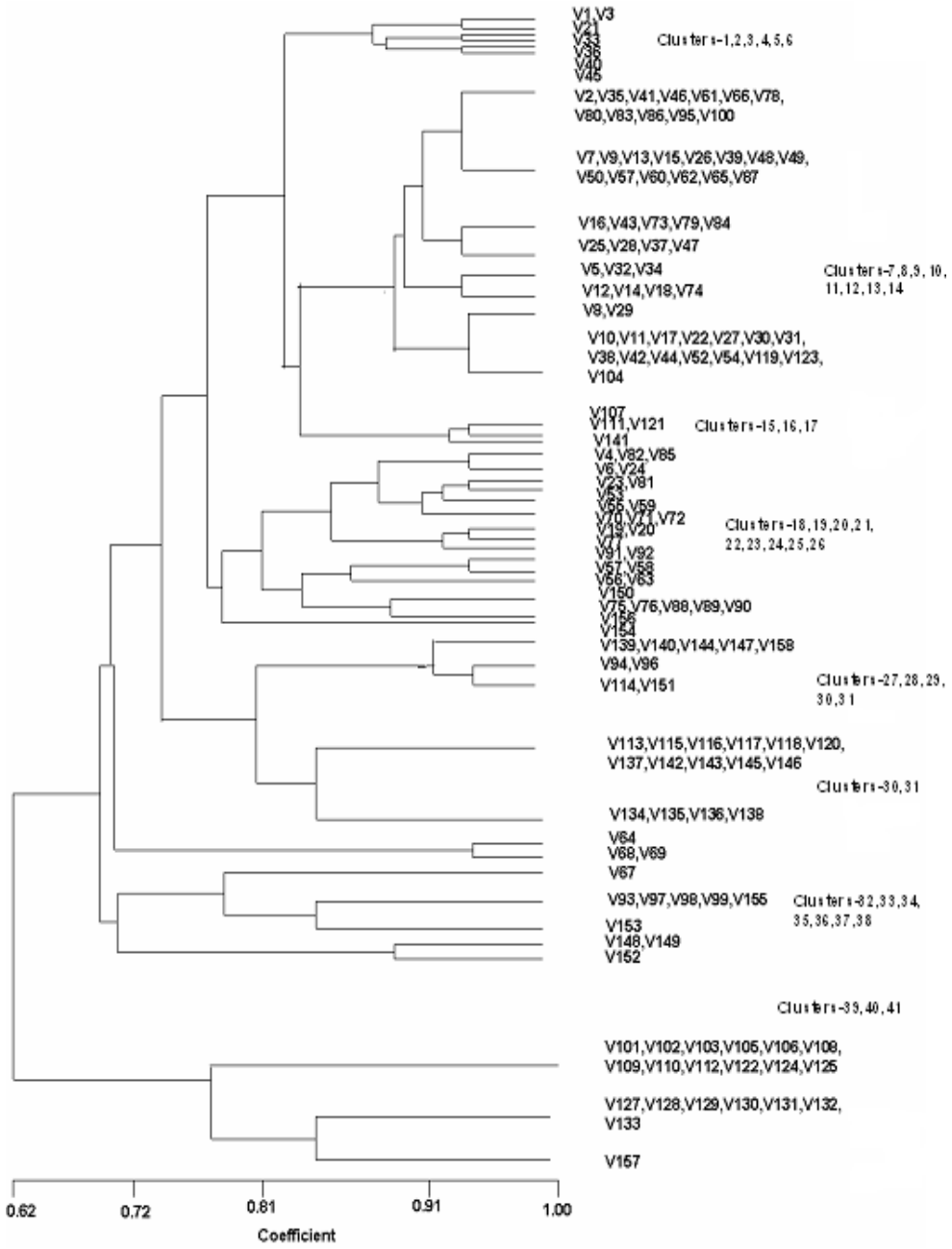


Fig: 3.12 Dendrogram of the *Vibrio* spp. based on RAPD profile using the primer OPAC-10

Clusters- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 34, 35, 36, 40- Isolates of *V. harveyi*; Clusters- 37 - Isolates of *V. parahaemolyticus*; Clusters- 11, 12, 13, 14, 38 - Isolates of *V.mediterranei*; Clusters – 7, 27- Isolates of *V.proteolyticus;* Clusters- 39, 42- Isolates of *V.alginolyticus*; Clusters- 28, 32 - Isolates of *V.nereis;* Clusters- 31- Isolates of *V.fluvialis;* Clusters-25, 41 - Isolates of *V. cholerae*; Clusters- 29, 33- Isolates of *V.vulnificus;* Clusters-20, 40 - Isolates of *V. splendidus*

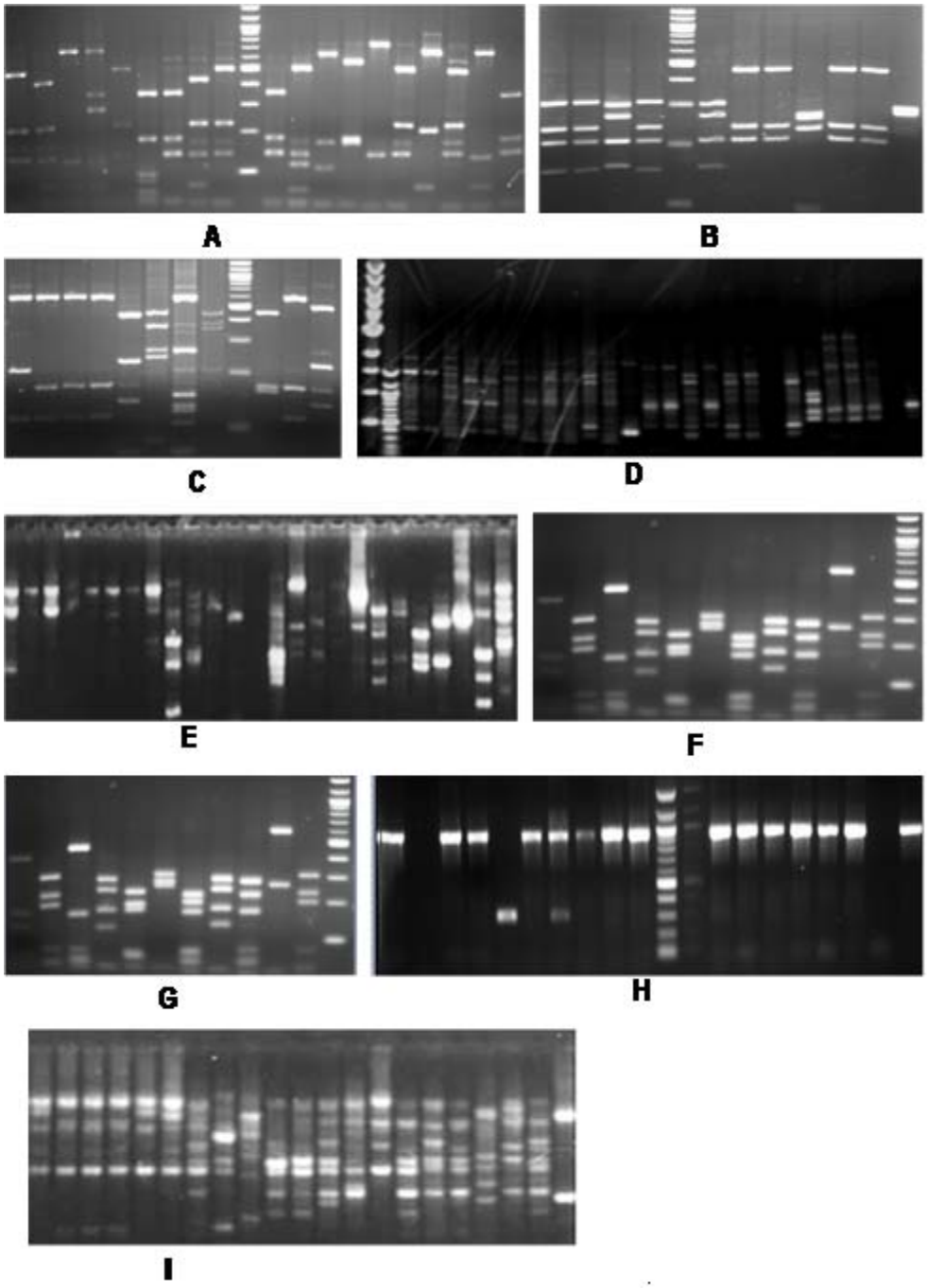


Fig: 3.13 Amplicons obtained using the primer OPD-16

A- V1 to V9, 1Kb Marker, V10-V19
B- V20- V23, 100bp Marker, V24 to V30
C- V31 to V38 100bp Marker,V39 to V41
D- 1Kb Marker, 100bp Marker, V42 to V65
E- V66 to V89, 100bp Marker
F- V90 to V100, 1Kb Marker
G- V101 to V111, 1Kb Marker
H- V121 to V130, 1Kb Marker, V131 to V139
I- V140 to V145, 100bp Marker, V147 to V158100bp Marker

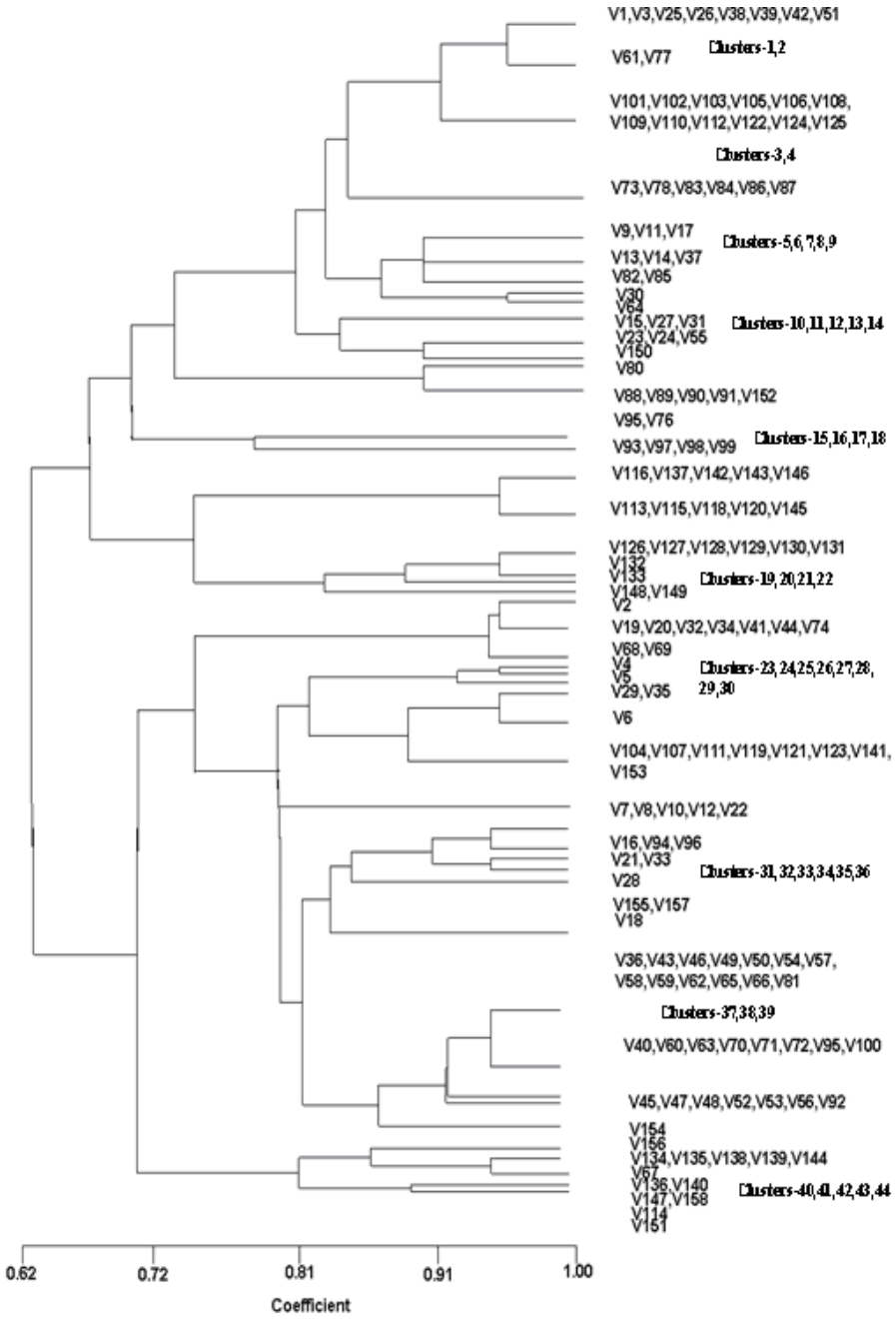


Fig: 3.14 Dendrogram of the *Vibrio* spp. based on RAPD profile using the OPD-16

Clusters- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 27, 28, 35- Isolates of *V. harveyi* ; Clusters- 31 - Isolates of *V. parahaemolyticus* ; Clusters- 18, 30, 35, 36 - Isolates of *V.mediterranei;* Clusters- 34- Isolates of *V.proteolyticus;* Clusters- 40, 14, 425, 43- Isolates of *V.alginolyticus*; Clusters- 30, 39- Isolates of *V.nereis*; Clusters- 29- Isolates of *V.fluvialis;* Clusters- 44- Isolates of *V. cholerae*; Clusters- 30, 32, 33, 37, 38- Isolates of *V.vulnificus;* Clusters-20, 26 - Isolates of *V. splendidus*

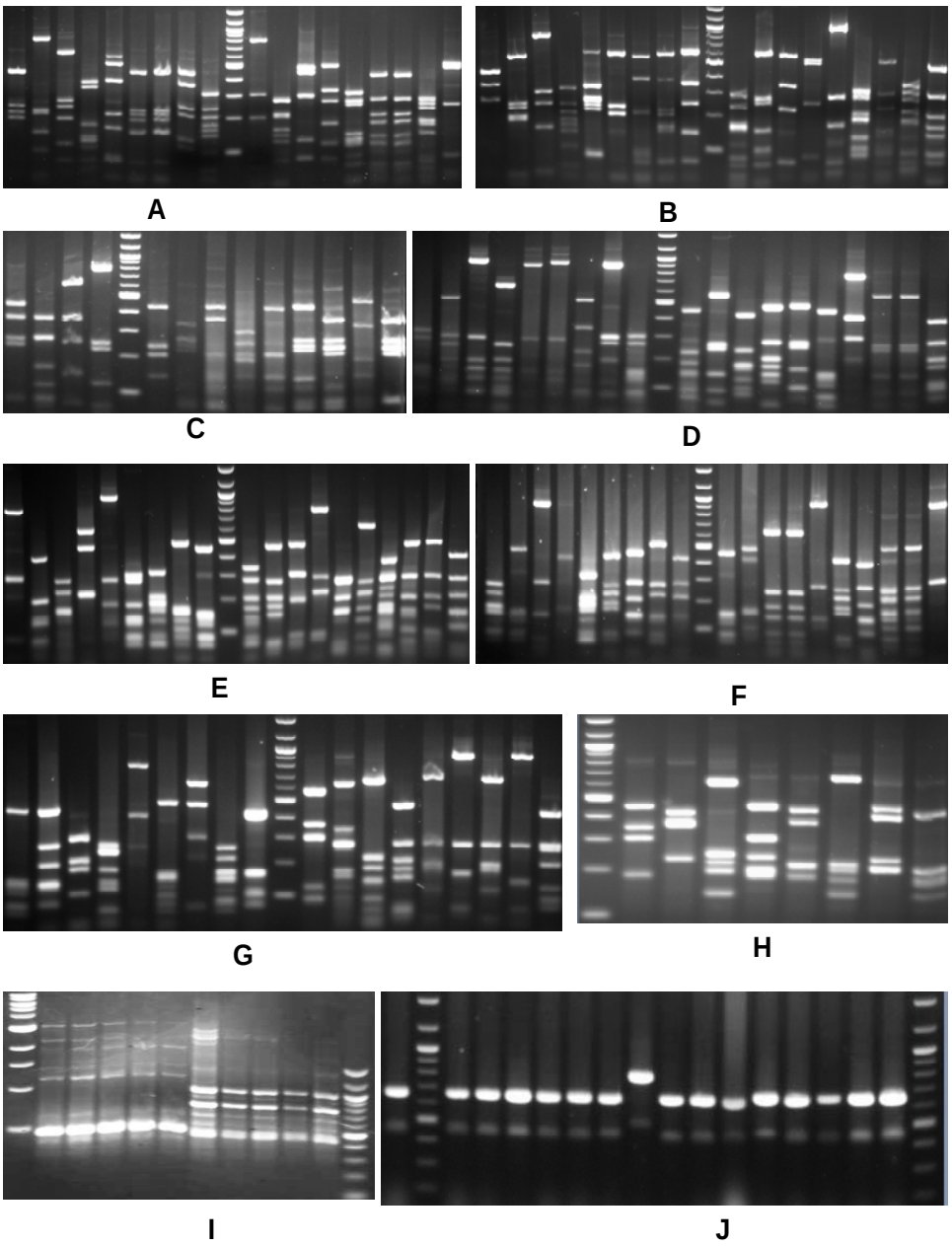


Fig: 3.15 Amplicons obtained using the primer OPD-20

A- V1 to V9, 1Kb Marker, V10-V18
B- V19- V27, 1Kb Marker, V28 to V36
C- V37 to V40, 1Kb Marker,V41 to V49
D- V50 to V58, 1Kb Marker, V59 to V68
E- V69 to V77, 1Kb Marker, V78 to V87
F- V88 to V96, 1Kb Marker, V96 to V106
G- V107 to V115, 1Kb Marker, V116 to V124
H- 100bp Marker, V125 to V132
I- 1Kb Marker, V133 to V142, 100bp Marker
J- V143 to V158, 1Kb Marker

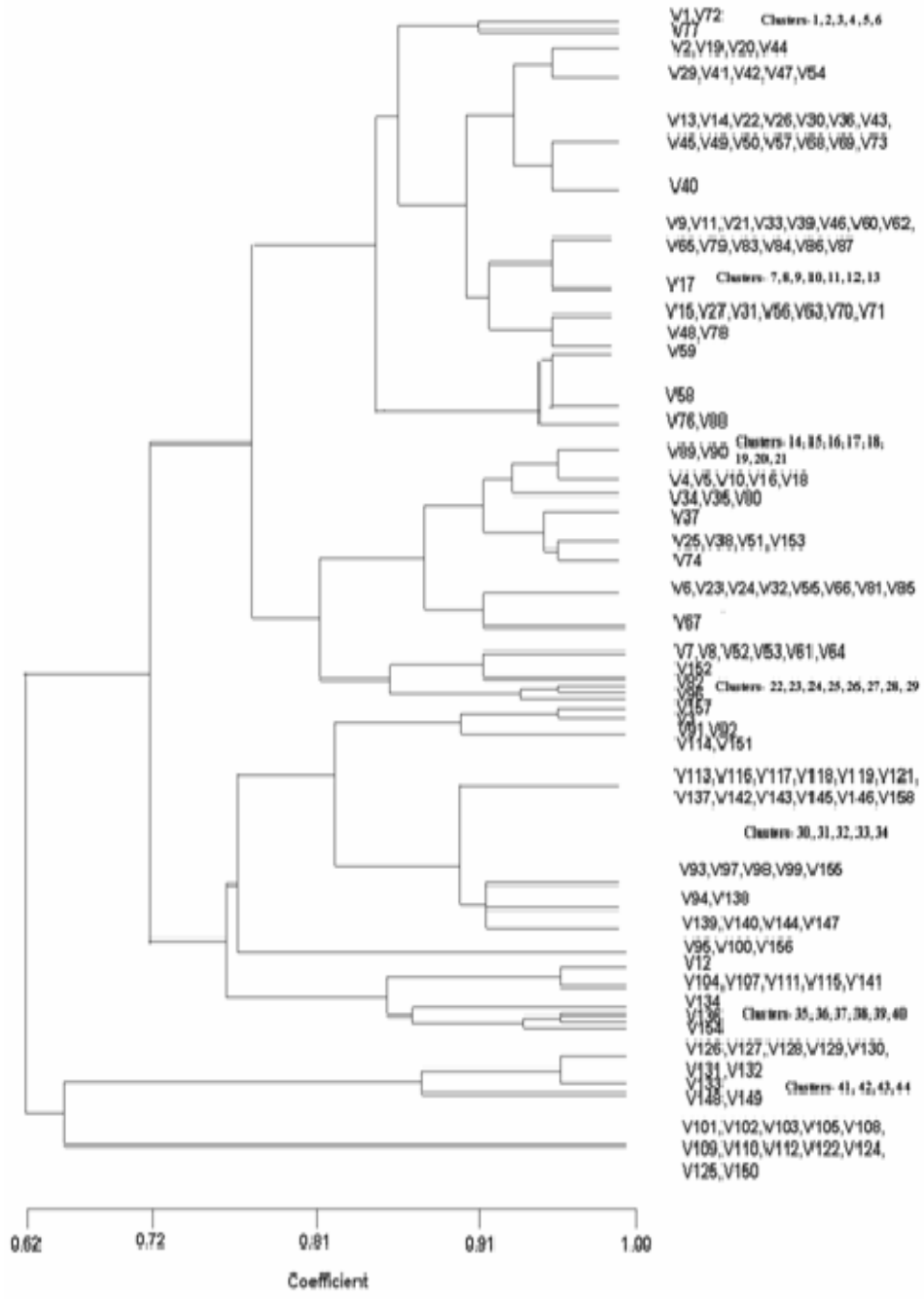


Fig: 3.16 Dendrogram of the *Vibrio* spp. based on RAPD profile using the OPD-20

Clusters- 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 27, 28, 35- Isolates of *V. harveyi*; Clusters- 31- Isolates of *V. parahaemolyticus*; Clusters- 18, 35 - Isolates of *V.mediterranei;* Clusters- 34- Isolates of *V.proteolyticus;* Clusters- 32, 33, 40, 41, 42, 43- Isolates of *V.alginolyticus*;Clusters- 30, 35, 39- Isolates of *V.nereis*; Clusters- 29- Isolates of *V.fluvialis;* Clusters- 44- Isolates of *V. cholerae*; Clusters- 30, 33, 36, 37, 38- Isolates of *V.vulnificus;* Clusters-26, 32- Isolates of *V. splendidus*

Table-3.3: Cluster analysis based on phenotypic characterization and RAPD profiling, correlated with the source where from the strains were isolated

Phenon	OPA-3	OPA-4	OPA-5	OPA-7	OPAC-10	OPD- 16	OPD-20	SOURCE
P1 *V.harveyi*	1,2,4,5,6,10, 11,12,13,14, 15,16,17	1,2,4,6,7,8,9, 10,11,17,22, 23,25	1,2,3,4,5, 6,7,9,10,1 1,12,13,1 5,16,17,1 8,19,22,2 5,26,29,3 0,31,33,3 4,35,43, 44	12,13,14, 15,21,22, 23,34,25, 27,33,36, 37,38,39, 40,41,42, 43,44,45, 46,47,48	1,3,4,5,6,7,8,9, 10,11,15,16,17, 18,19,20,21,22, 23,24,26,34,35, 36	1,2,3,4,5,6,7, 8,9,10,11,12, 13,14,15,16, 17,18,19,20, 21,22,23,24, 27,35	1,2,,4,5,6, 7,8,9,10,1 1,12,13,15 ,16,17,18, 19,20,21,2 2,24,27,35	Mass Mortality & moribund, PL, zoea,mysis, nauplii from Kakinada, Andhra Pradesh
P2 *V.harveyi*	1,10,12, 13	11,23	3,5,7	22	2,8,11	5,7,15	7,15	Mass Mortality & moribund, PL, Kakinada, Andhra Pradesh
P3 *V.harveyi*	6,7,8,18	25	7,11,32	25,26,30	23,26,40	13,14,28	13,14,23, 25,28	PL, mass mortality, Kodungallore, Kerala & LMG 4044- dead amphipod (*Talorchestia sp.*), Woods Hole, Masaachusetts, United States.
P4 *V.parahaemolyticus*	19, 20	21	14	,31	37	31	31	PL, mass mortality, Azhikode, Kerala & LMG 2850- patients suffering from "Shirashu" food poisoning, Japan.
P5 *V.mediterraneii*	21,22,23,24	5,18	20	29,32	11,12,13,38	18,36	18,35	Post Larvae, mass mortality,Azhikode, Kerala & LMG 11258- Coastal

								marine plankton, Valencia, Spain
P6 *V.mediterraneii*	3,27	18,24,19	14	6	14	30,35	35	Post Larvae, mass mortality, Azhikode, Kerala
P7 *V.proteolyticus*	23	26	42	35	7	34	34	Post Larvae, mass mortality, Azhikode, Kerala
P8 *V.proteolyticus*	27	26	24	35	27	34	34	LMG3772- intestine of wood-boring isopod (*Limnoria tripuncata*) intestine, United States.
P9 *V.alginolyticus*	6, 9	14	23,45,46	1,26,34	39,42	40,41,42,43	32,33,40, 41,42,43	Post Larvae, necrotic, Trichur, Kerala, LMG 4409- spoiled horse mackerel causing food poisoning, Japan, MTCC 4439
P10 *V.nereis*	24,25, 28	15,16	40	9,18,33	32	30	30,35	Post Larvae, mass mortality, Azhikode, Kerala
P11 *V.nereis*	9	16	40	20	28	39	39	LMG3895- seawater enriched with propoanol, Oahu Hawaii, United States.
P12 *V.fluvialis*	8,18	14	27	11,16	31	29	29	Post Larvae, mass mortality, Azhikode, Kerala, LMG 11654- human faeces

P13 *V. cholerae*	26,5	13	28,36,37, 38	7,8,10,28	25,41	44	44	Post Larvae, necrotic, Trichur, Kerala, MTCC3906- clinical specimen- human cholerae epidemic- 1960, India
P14 *V.vulnificus*	3,8,19	3,17,20	8,39	2,3,4	29,33	30,32,33, 37,38	30,33,36, 37,38	Post Larvae, mass mortality, prawn larval hatchery, Kollam, Kerala, LMG13545- human blood, United States
P15 *V.vulnificus*	19	17	39	3	29	33	33	Post Larvae, mass mortality, prawn larval hatchery, Kollam, Kerala
P16 *V. splendidus*	20	12	41	17	20	25,32	32	Post Larvae, mass mortality, Azhikode, Kerala

Comparisons of the clusters of the isolates obtained based on both phenotypic and genotypic characters showed that there exists a further divergence of the isolates formerly grouped together as a single cluster based on the phenotypic characters alone.

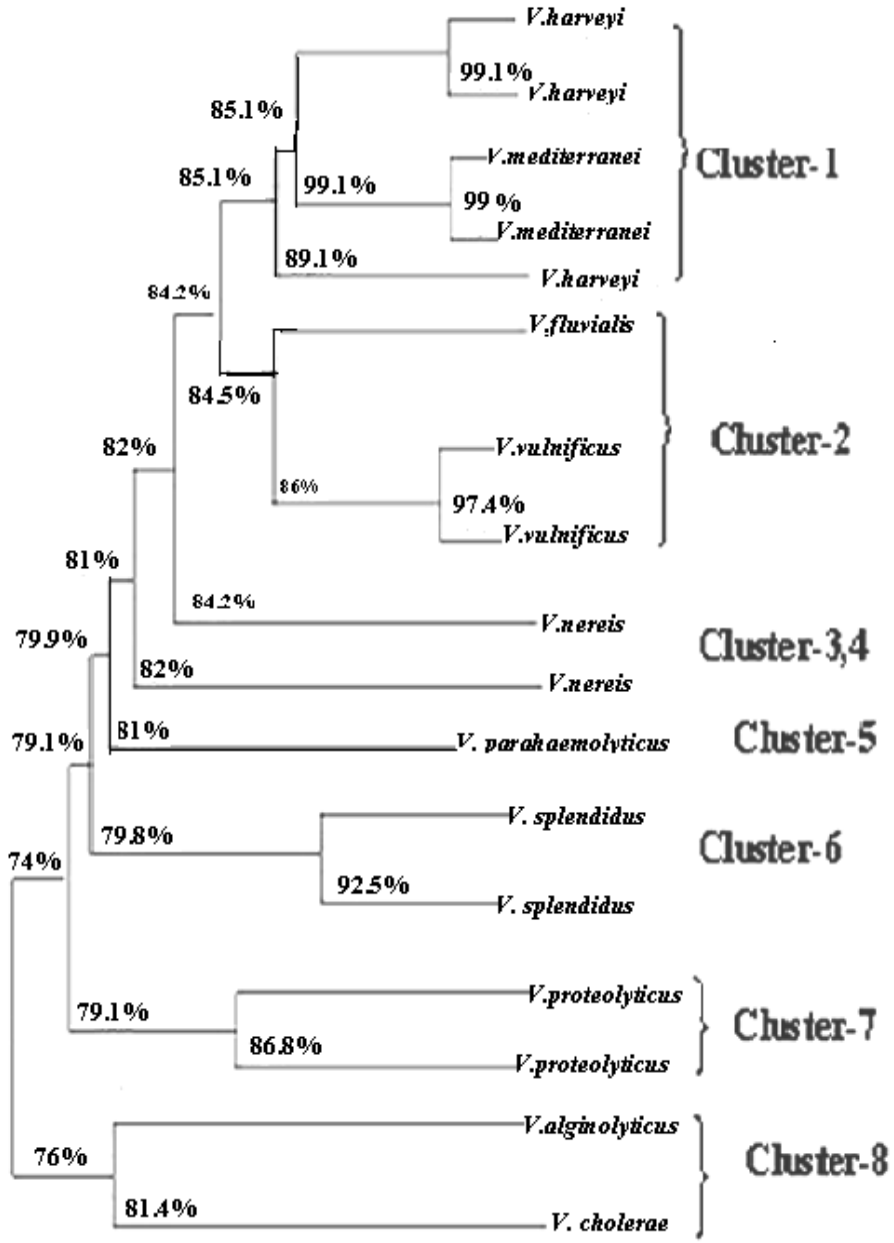


Fig-3.17 Construction of RAPD profile based on the population clusters obtained on phenotypic characterization of the 17 phena with all 7 Operon primers

3.3.2. Banding pattern analysis of housekeeping genes

Thirty five representative isolates when amplified with 8 housekeeping genes and processed in NTSYSpc software yielded 8 dendrograms. The clusters obtained with each primer and the similarities between interrelated clusters are described below.

***ftsZ*:**

Amplification of the 35 isolates with *ftsZ* gene primer yielded 10 clusters ≥ 92%S. Cluster-1 consisted of the lone isolate of *V.harveyi* (V3), exhibited ≥ 92%S to 8 isolates of *V.harveyi* grouped together as cluster-2 at 100%S. Cluster-2 also exhibited ≥ 92%S to cluster-3 which contained one isolate of *V.harveyi* (V11), one isolate of *V.vulnificus* (V34) and the type strain of one isolate of *V.harveyi*. Cluster-3 exhibited ≥ 92%S to cluster-4 containing isolates of *V.alginolyticus* and *V.proteolyticus* sharing 100%S. Cluster-4 exhibited ≥ 92%S to cluster-5 containing isolate and type strain of *V.cholerae*. Cluster-5 exhibited ≥ 92%S to cluster-6 containing type strains of *V.fluvialis* and one isolate each of *V.nereis* and *V.parahaemolyticus*, sharing 100%S. Cluster-6 exhibited ≥ 92%S to cluster-7 containing a lone type strain of *V.nereis*. Cluster-8 at 100%S consisted of isolates of *V.splendidus*, exhibited ≥ 92%S to cluster-7. Cluster-8 which exhibited ≥ 92%S to cluster-9 contained isolates of *V.fluvialis*, *V.mediterranei*, *V.parahaemolyticus*, *V.nereis* and *V.vulnificus* sharing 100%S between them. Cluster-10 containing lone isolate of *V.harveyi*, which remained as an outgroup, joining with the other 34 isolates ≥ 64%S.

***gapA*:**

Amplification of the 35 isolates with *gapA* gene primer yielded 7 clusters ≥ 93%S. Cluster-1 containing the isolates of *V.harveyi* joined at 93%S to cluster-2 containing isolates of *V.harveyi* and one isolate of *V.alginolyticus* (V13). Cluster-2 exhibited 93%S to the lone isolate (type strain of *V.harveyi*) in cluster-3. Cluster-3 exhibited 93%S to cluster-4

containing type strain of *V.alginolyticus* (V14). Cluster-4 exhibited 93%S to cluster-5 containing type strain of *V.alginolyticus* (V15), isolates of *V.cholerae*, *V.fluvialis*, *V.mediterranei*, *V.proteolyticus* and *V.splendidus*, related at 100%S. Cluster-5 exhibited 93%S to cluster-6 containing type strains of *V.cholerae* and *V.mediterranei* sharing 100%S to isolates of *V.nereis* and *V.parahaemolyticus*. Cluster-6 exhibited 93%S to cluster-7 containing type strain of *V.parahaemolyticus* which shared 100%S with the type strain of *V.vulnificus*.

***topA*:**

Amplification of the 35 isolates with *topA* gene primer yielded 10 clusters at ≥ 91%S. The isolates of *V.harveyi* in clusters1 & 2 were related at 95%S to each other and to cluster-3 containing isolates of *V.alginolyticus*, *V.cholerae*, *V.fluvialis*, *V.mediterranei*, *V.nereis*, *V.parahaemolyticus* and *V.proteolyticus*. Cluster-3 was related to cluster-4 at 95% S, where as cluster-4 with the isolate of *V.splendidus* shareed 95%S to cluster-5 *V.splendidus*. Cluster-5 exhibited 95%S to isolates of *V.harveyi* in cluster-6 containing one isolate each of *V.harveyi*, *V.fluvialis*, *V.mediterrane*i, isolates of *V.cholerae* and *V.vulnificus* and type strains of *V.alginolyticus* and *V.parahaemolyticus*. Cluster-6 showed 95%S to cluster-7 containing isolate of *V.harveyi*, which in turn exhibited 95%S to lone isolate of *V.harveyi* in cluster-8. Clusters-7&8 joined at 92%S to isolates in clusters-1 to 6. Clusters- 9 &10 contained isolates of *V.harveyi* interrelated at 95%S, these two clusters were related with the isolates in other clusters at ≥91%S.

***recA*:**

All the 35 isolates showed wide range of heterogeneity, but are interrelated ≥90%S. Cluster1 consisted of the isolate of *V.harveyi* (V3) exhibiting >96%S to type strain of *V.nereis* in Cluster-2. Cluster-3&4 grouped with it the remaining isolates of *V.harveyi* at >96%S. Cluster-5 contained the isolates of *V.mediterranei*, *V.neries*, *V.vulnificus* and type strain of *V.splendidus*, sharing 100%S, also exhibiting >96%S to a lone

isolate of *V.vulnificus* in cluster-6. Isolates of *V.nereis* in cluster-7 exhibited 100%S to isolate of *V.proteolyticus* and >93%S to type strain of *V.parahaemolyticus* (Cluster-8). Cluster-8 >93%S joins to isolates in cluster-9 which included strains of *V.alginolyticus*, *V.cholerae*, *V.fluvialis*, *V.splendidus* and type strain of *V.proteolyticus*. Cluster-10 & 11 are formed up of the isolate of *V.alginolyticus* showing >96%S and joined with the above six clusters at >91%S. The lone isolate of *V.parahaemolyticus* (V27) in cluster-12 joined with the above seven clusters at 90%S.

***merB*:**

Thirty five isolates amplified exhibited ≥90%S and were represented as 7 clusters. Cluster-1 consisted of one isolate of *V.harveyi* (V1) which exhibits 98%S to other isolates of *V.harveyi* in cluster-2 sharing 100%S between them. Clusters 1& 2 joined with the other clusters ≥93%S. Cluster-3 was consisted of the type strain and six isolates of *V.harveyi* joined with cluster-4 which contained isolates of *V.alginolyticus*, *V.cholerae*, *V.fluvialis*, *V.mediterranei*, *V.nereis*, *V.parahaemolyticus* and *V.proteolyticus* at 94%S. Cluster-4 joined with cluster-5 at 95%S containing isolates of *V.splendidus* and *V.vulnificus*. Cluster-5 joined with cluster-6 at 95%S, in which is grouped the isolate of *V.alginolyticus*, which shared 100%S to the type strains of *V.proteolyticus* and *V.vulnificus*. Cluster-7 contained the type strain of *V.parahaemolyticus* (V28), joining with the isolates in the above six clusters ≥90%S.

***gyrB*:**

35 isolates amplified were grouped into 4 clusters, which were inter-related ≥93%S. Cluster-1 at 100%S contained isolates of *V.harveyi*, *V.splendidus*, *V.vulnificus* and type strain of *V.nereis*, joining with cluster-2 at 93%S. Cluster-2 contained one isolate of *V.harveyi* (V2) which shares 100%S to type strains of *V.parahaemolyticus* and *V.proteolyticus*. At 93%S cluster-3 containing isolates of *V.alginolyticus*, *V.cholerae*, *V.fluvialis*, *V.mediterranei* and *V.nereis* joined with cluster-2. Cluster-3 exhibited

93%S to cluster-4 containing isolates and type strain of *V.harveyi*, isolate of *V.alginolyticus* and *V.nereis* and type strain of *V.vulnificus*.

***pyrH*:**

Thirty five isolates were grouped into 4 main clusters inter-related at ≥93%S. Cluster-1 at 100%S contained isolates and type strain of *V.harveyi*, type strain of *V.mediterranei* and isolates of *V.alginolyticus* and *V.nereis*. This cluster joined with cluster-2 at 93%S, which contained isolates of *V.harveyi*, type strain of *V.fluvialis*, isolates of *V.mediterranei* and *V.vulnificus* related at 100%S. Cluster-2 joined with cluster-3 at 93%S, containing isolates of *V.alginolyticus* and *V.cholerae*. Cluster-3 also exhibited 93%S to cluster-4 containing isolates of *V.parahaemolyticus*, *V.proteolyticus*, *V.splendidus*, *V.vulnificus* and type strain of *V.nereis*.

16S rRNA:

Thirty five isolates were grouped into 9 clusters inter-related at ≥95%S. Cluster-1 has a lone isolate of *V.harveyi* exhibiting 95%S to cluster-2 having four isolates of *V.harveyi* sharing 100%S. Cluster-2 showed 95%S to cluster-3 having two isolates of *V.harveyi*, which exhibited 95%S to three isolates of *V.harveyi* in cluster-4. Cluster-4 exhibits 95%S to cluster-5, containing one isolate and type strain of *V.harveyi*. Cluster-5 at 95%S showed similarity to cluster-6 containing type strain of isolates of *V.alginolyticus* and *V.fluvialis*, which exhibited 95%S to cluster-7. Cluster-7 at 95%S was related to cluster-8 which had the isolates of *V.mediterranei*, *V.vulnificus* and *V.nereis*. This cluster joined with cluster-9 at 95%S, which contained isolates of *V.nereis V.parahaemolyticus*, *V.proteolyticus*, *V.splendidus* and *V.vulnificus*, inter-related at 100%S.

The 16SrRNA sequences of the 25 selected vibrio isolates from the clusters obtained on phenotypic characterization were compared with the GenBank database using the BLAST algorithm. The wild isolates of vibrios which were phenotypically characterized and clustered exhibited 95 to 100% similarity (Table 3.5) to *vibrio* strains deposited in the GenBank database and were assigned with accession numbers (Appendix-1). On

comparing the results of phenotypic and genotypic characterisation, all the representative isolated identified through numerical taxonomy could be confirmed of their identity based on 16S rRNA sequence analysis. However, isolates of *V.harveyi* also shared 95%S to 100%S to NCBI deposits of *V.rotiferanus* (LMG21460), and *V.natriegenes* (ATCC 14048). Similarly the isolate of *V.parahaemolyticus* (MCCB 133) showed 95%S to NCBI deposits of *V.parahaemolyticus* (ATCC 17802) and 96%S to *V.natriegenes* (ATCC 14048).The isolate of *V.alginolyticus* (MCCB 112) which was deposited with Genbank as *Vibrio* sp., shared 96%S with NCBI deposits of *V.natriegenes* (ATCC 14048) and 97%S to *V.alginolyticus* NCBI deposits (ATCC 17749).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

Fig: 3.18 Amplicons from 35 isolates of vibrios using *ftsZ* gene primer

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

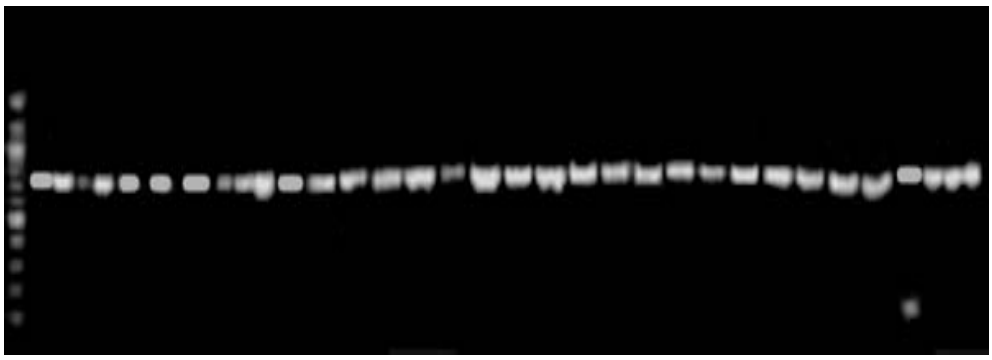


Fig: 3.19 Amplicons from 35 isolates of vibrios using *gapA* gene primer

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

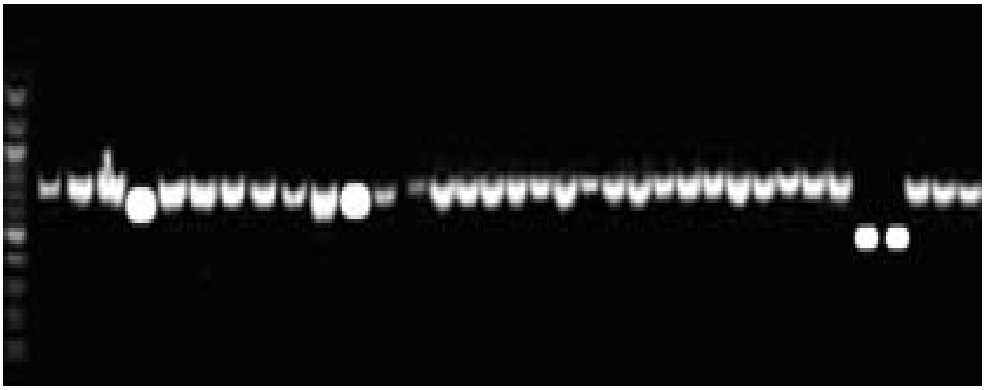


Fig: 3.20 Amplicons from 35 isolates of vibrios using *topA* gene primer

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

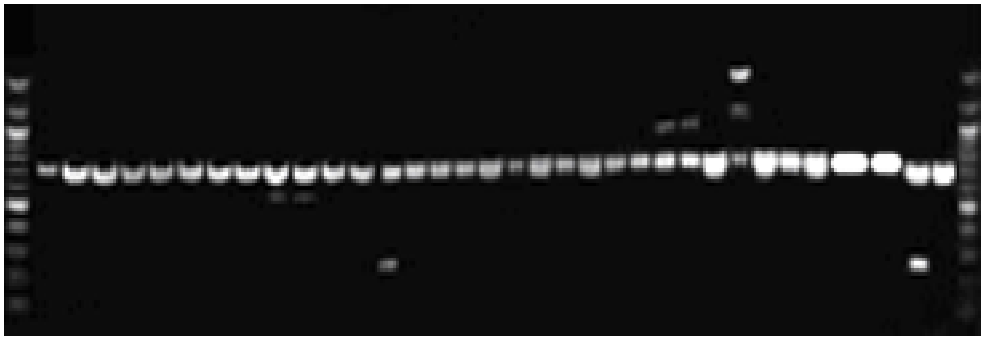


Fig: 3.21 Amplicons from 35 isolates of vibrios using *recA* gene primer

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

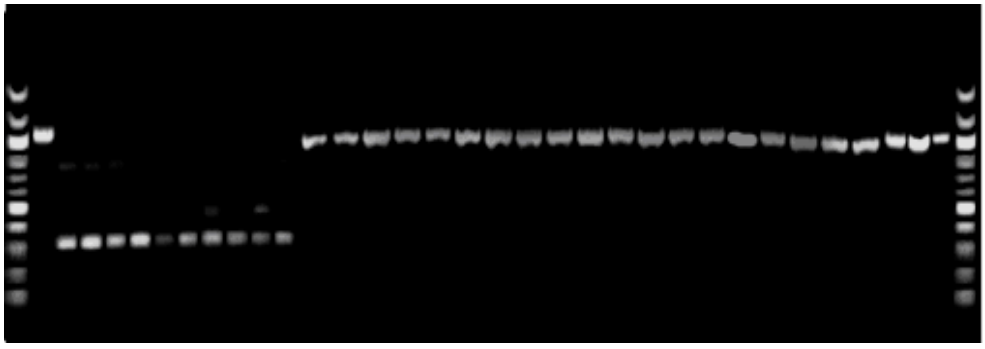


Fig: 3.22 Amplicons from 35 isolates of vibrios using *merB* gene primer

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

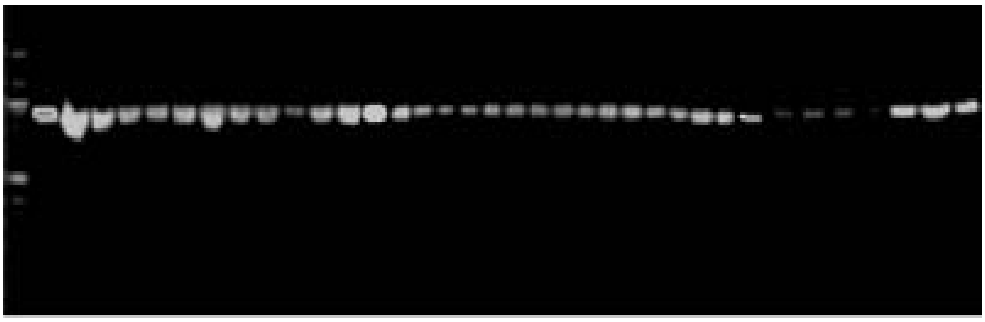


Fig: 3.23 Amplicons from 35 isolates of vibrios using *gyrB* gene primer

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

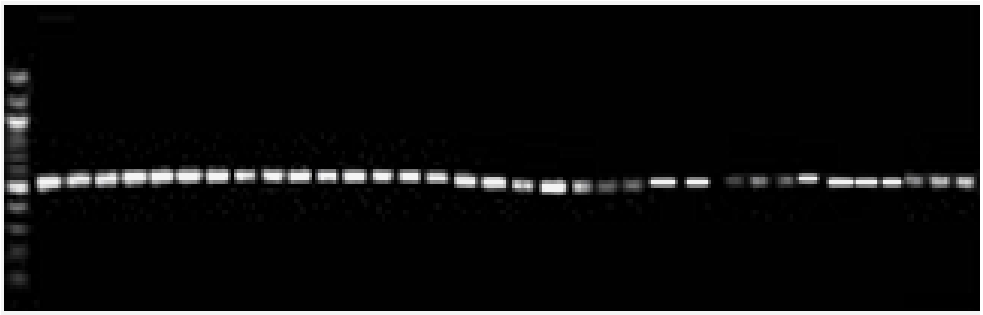


Fig: 3.24 Amplicons from 35 isolates of vibrios using *pyrH* gene primer

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

Fig: 3.25 Amplicons from 35 isolates of vibrios using 16SrRNA gene primer

Lane1-1kb Marker, Lane2Vh3, Lane3-Vh28, Lane4-Vh36, Lane5-Vh45, Lane6-Vh54, Lane7- Vh57, Lane8-Vh64, Lane9-Vh71, Lane10-Vh76, Lane11-Vh81, Lane12-Vh88, Lane13-VhL (LMG 4044), Lane14-Va3, Lane15-VaL(LMG 4409), Lane16-VaM(MTCC 4439), Lane17-Vc12, Lane18-Vc35, Lane19-VcM(MTCC 3906), Lane20-Vf26, Lane21-VfL(LMG 11654), Lane22-Vm18, Lane23-Vm26, Lane24-VmL(LMG 11258), Lane25-Vn30, Lane26-Vn32, Lane27-VnL(LMG 3895), Lane28-Vpa6, Lane29-VpaL(LMG 2850), Lane30-Vpr4, Lane31-VprL(LMG 3772), Lane32-Vsp3, Lane33-VspL (LMG 19031), Lane34-Vv9, Lane35- Vv23, Lane36-VvL (LMG 13545)

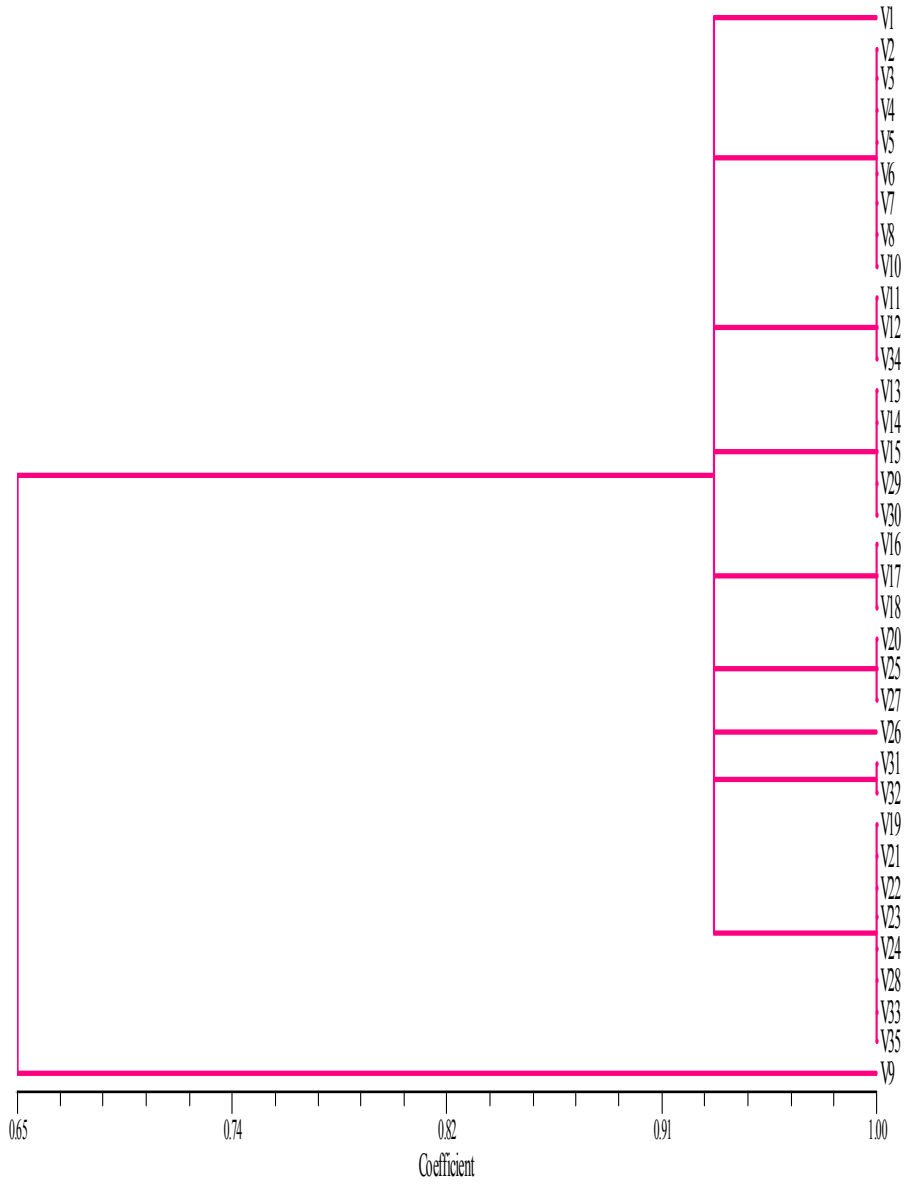


Fig: 3.26 Relatedness of the isolates based on amplification with *ftsZ* gene primer

Isolates V1 to V12 - (*V.harveyi*- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (*V.alginolyticus*- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (*V.cholerae*- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (*V.fluvialis*- Vf26, VfL(LMG 11654))
V21 to V23- (*V.mediterranei*- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (*V.nereis*-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (*V.parahaemolyticus*- Vpa6, VpaL(LMG 2850)),
V29 to V30- (*V.proteolyticus*-Vpr4, VprL(LMG 3772)),
V31 to V32- (V,splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (*V.vulnificus*-Vv9, Vv23, VvL (LMG 13545))

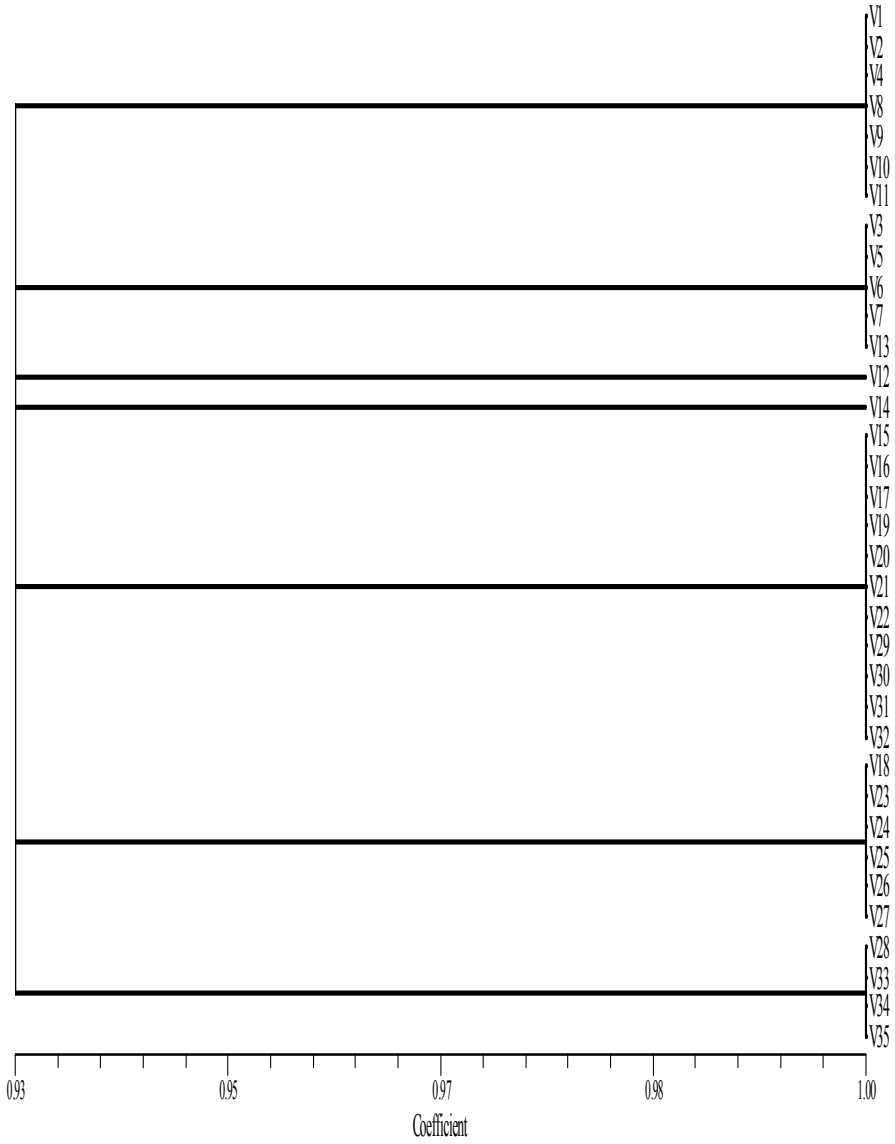


Fig: 3.27 Relatedness of the isolates based on amplification with *gapA* gene primer

Isolates V1 to V12 - (*V.harveyi*- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (*V.alginolyticus*- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (*V.cholerae*- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (*V.fluvialis*- Vf26, VfL(LMG 11654))
V21 to V23- (*V.mediterranei*- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (*V.nereis*-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (*V.parahaemolyticus*- Vpa6, VpaL(LMG 2850)),
V29 to V30- (*V.proteolyticus*-Vpr4, VprL(LMG 3772)),
V31 to V32- (V,splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (*V.vulnificus*-Vv9, Vv23, VvL (LMG 13545))

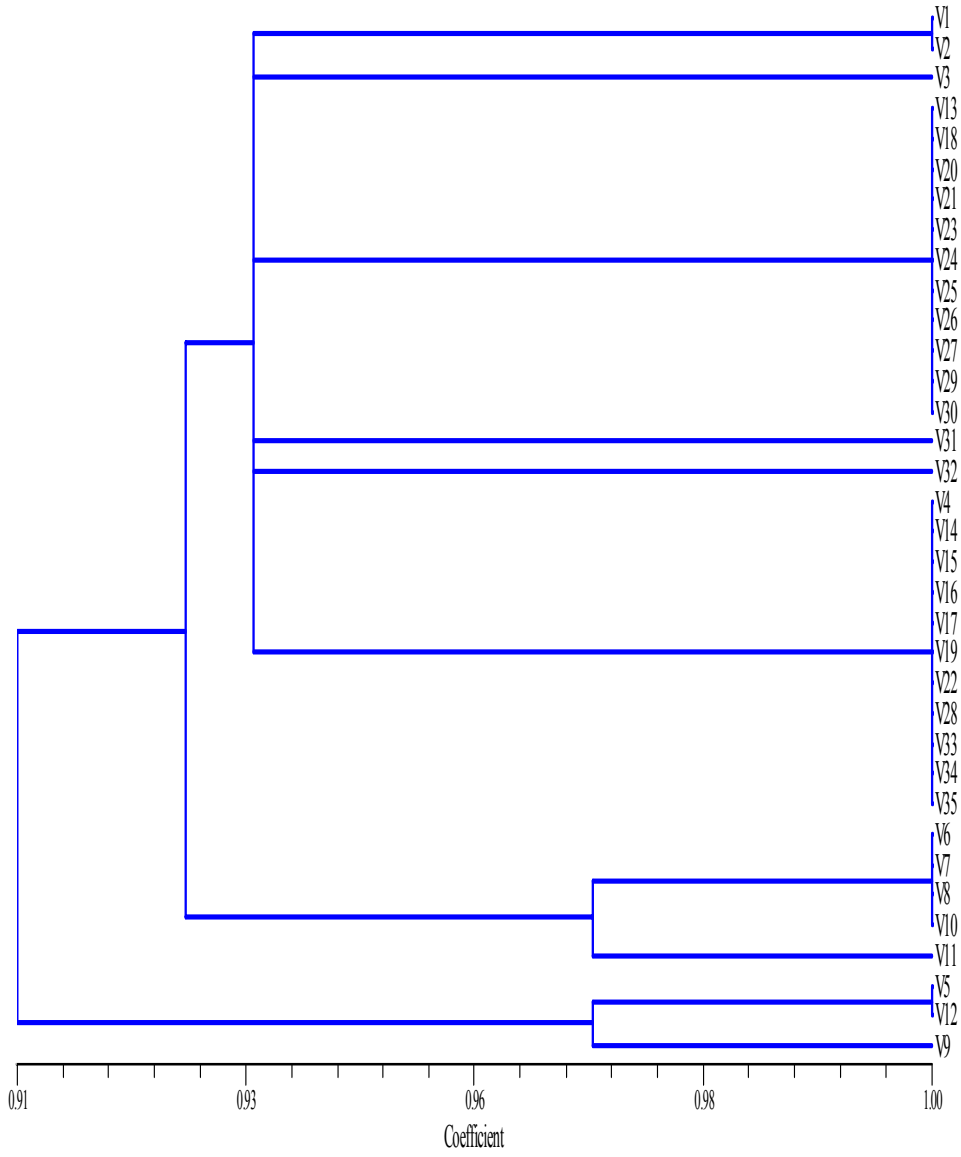


Fig: 3.28 Relatedness of the isolates based on amplification with *topA* gene primer

Isolates V1 to V12 - (*V.harveyi*- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (*V.alginolyticus*- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (*V.cholerae*- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (*V.fluvialis*- Vf26, VfL(LMG 11654))
V21 to V23- (*V.mediterranei*- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (*V.nereis*-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (*V.parahaemolyticus*- Vpa6, VpaL(LMG 2850)),
V29 to V30- (*V.proteolyticus*-Vpr4, VprL(LMG 3772)),
V31 to V32- (V,splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (*V.vulnificus*-Vv9, Vv23, VvL (LMG 13545))

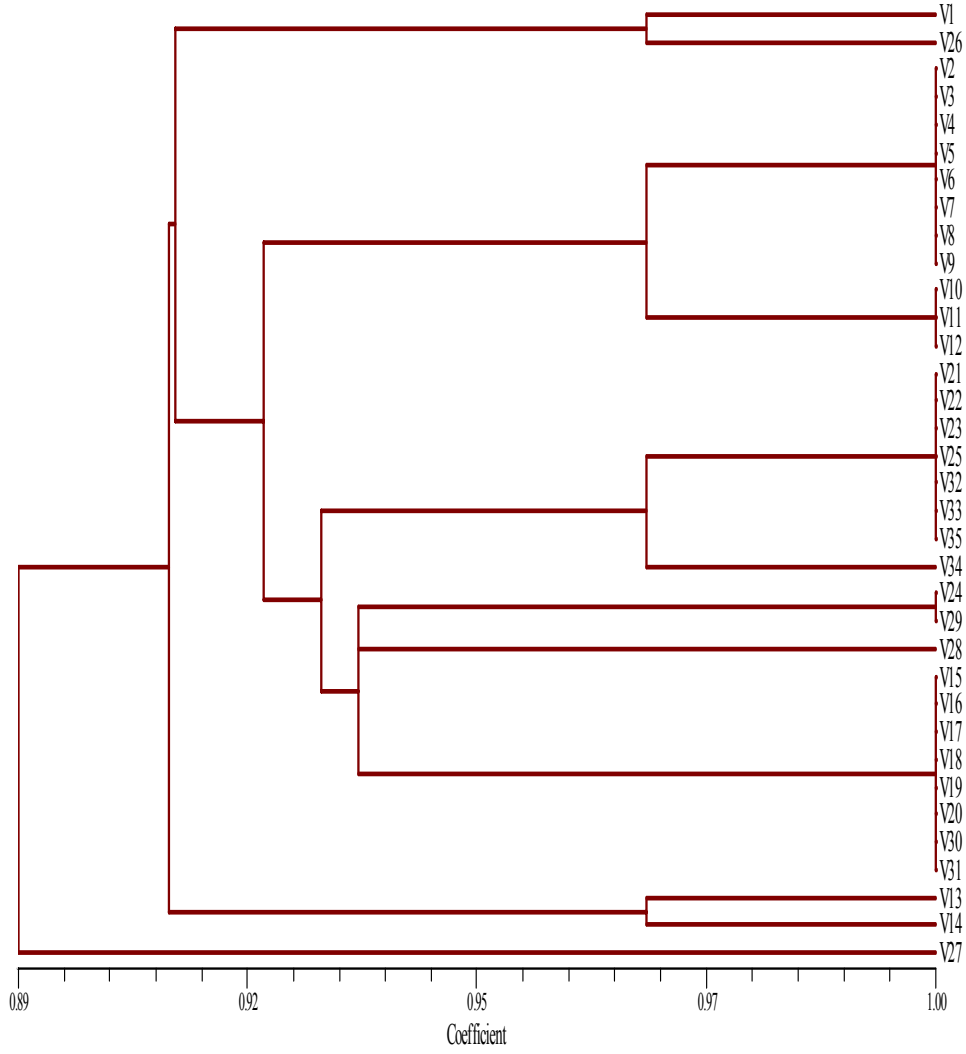


Fig: 3.29 Relatedness of the isolates based on amplification with *recA* gene primer

Isolates V1 to V12 - (*V.harveyi*- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (*V.alginolyticus*- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (*V.cholerae*- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (*V.fluvialis*- Vf26, VfL(LMG 11654))
V21 to V23- (*V.mediterranei*- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (*V.nereis*-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (*V.parahaemolyticus*- Vpa6, VpaL(LMG 2850)),
V29 to V30- (*V.proteolyticus*-Vpr4, VprL(LMG 3772)),
V31 to V32- (V,splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (*V.vulnificus*-Vv9, Vv23, VvL (LMG 13545))

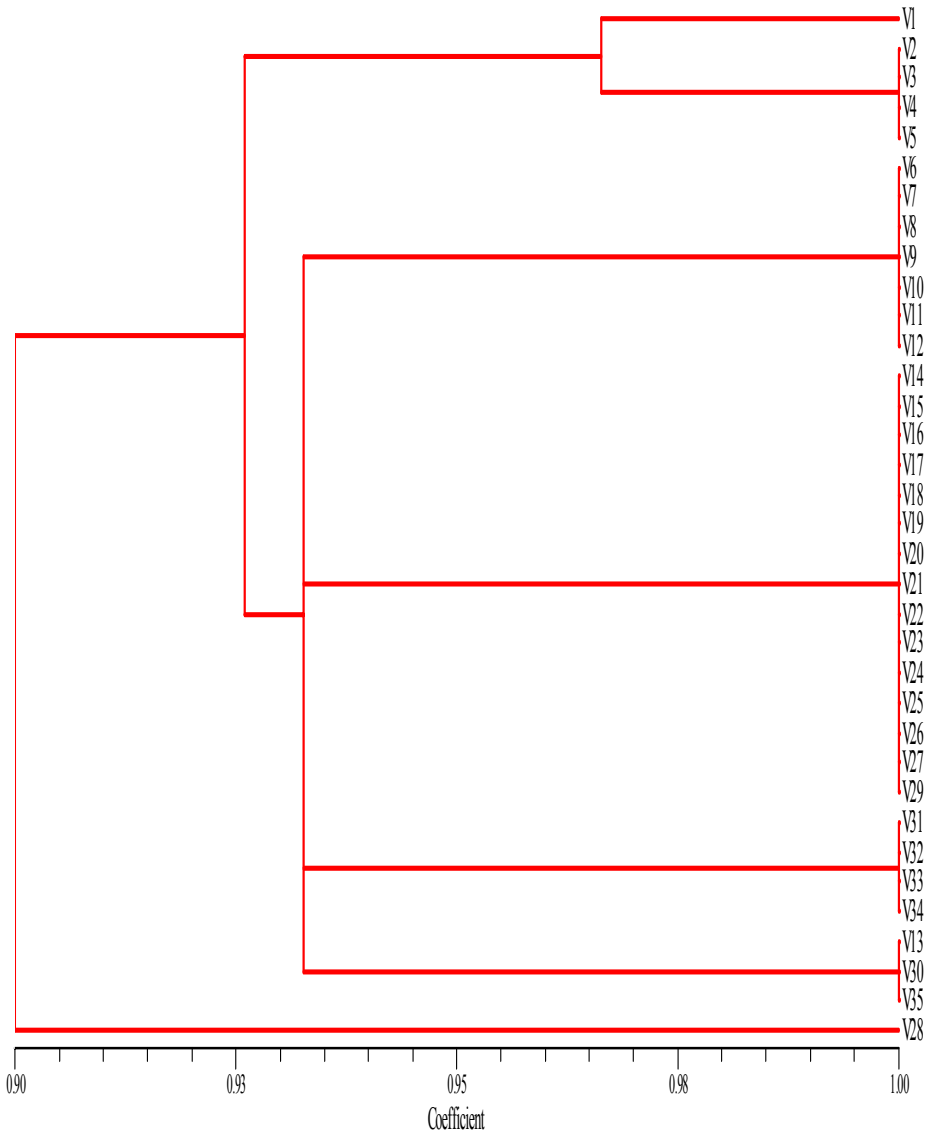


Fig: 3.30 Relatedness of the isolates based on amplification with *merB* gene primer

Isolates V1 to V12 - (*V.harveyi*- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (*V.alginolyticus*- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (*V.cholerae*- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (*V.fluvialis*- Vf26, VfL(LMG 11654))
V21 to V23- (*V.mediterranei*- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (*V.nereis*-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (*V.parahaemolyticus*- Vpa6, VpaL(LMG 2850)),
V29 to V30- (*V.proteolyticus*-Vpr4, VprL(LMG 3772)),
V31 to V32- (V,splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (*V.vulnificus*-Vv9, Vv23, VvL (LMG 13545))

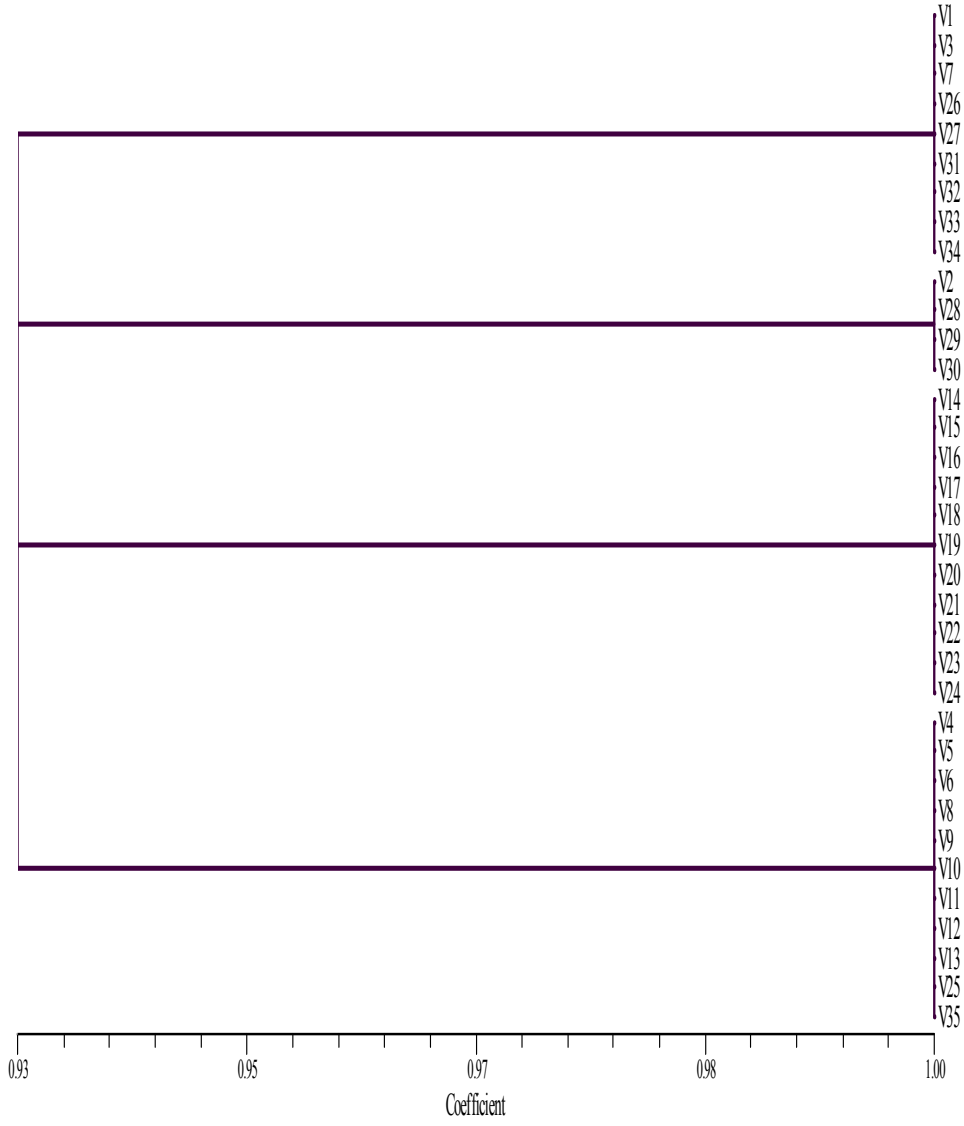


Fig: 3.31 Relatedness of the isolates based on amplification with *gyrB* gene primer

Isolates V1 to V12 - (*V.harveyi*- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (*V.alginolyticus*- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (*V.cholerae*- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (*V.fluvialis*- Vf26, VfL(LMG 11654))
V21 to V23- (*V.mediterranei*- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (*V.nereis*-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (*V.parahaemolyticus*- Vpa6, VpaL(LMG 2850)),
V29 to V30- (*V.proteolyticus*-Vpr4, VprL(LMG 3772)),
V31 to V32- (V,splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (*V.vulnificus*-Vv9, Vv23, VvL (LMG 13545))

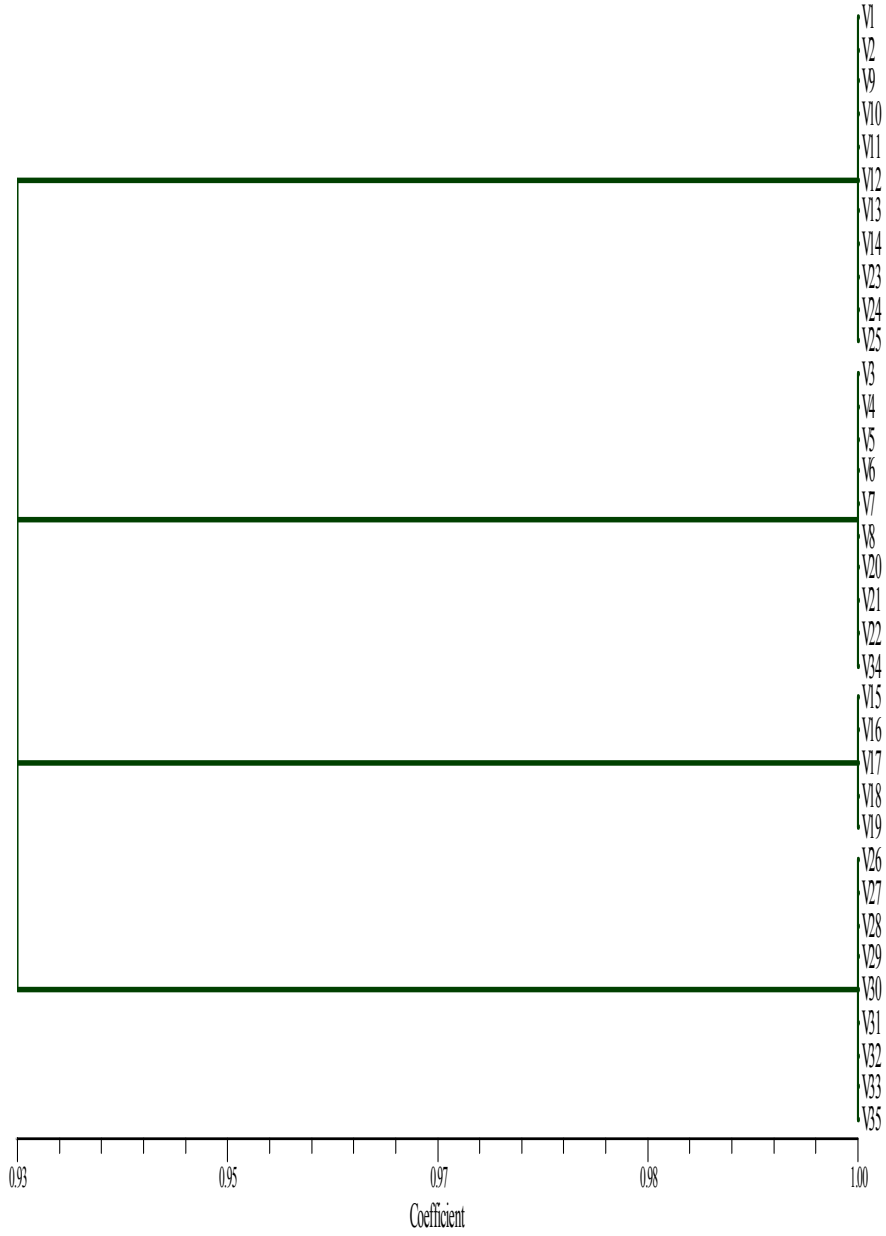


Fig: 3.32 Relatedness of the isolates based on amplification with *pyrH* gene primer

Isolates V1 to V12 - (*V.harveyi*- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (*V.alginolyticus*- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (*V.cholerae*- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (*V.fluvialis*- Vf26, VfL(LMG 11654))
V21 to V23- (*V.mediterranei*- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (*V.nereis*-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (*V.parahaemolyticus*- Vpa6, VpaL(LMG 2850)),
V29 to V30- (*V.proteolyticus*-Vpr4, VprL(LMG 3772)),
V31 to V32- (V,splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (*V.vulnificus*-Vv9, Vv23, VvL (LMG 13545))

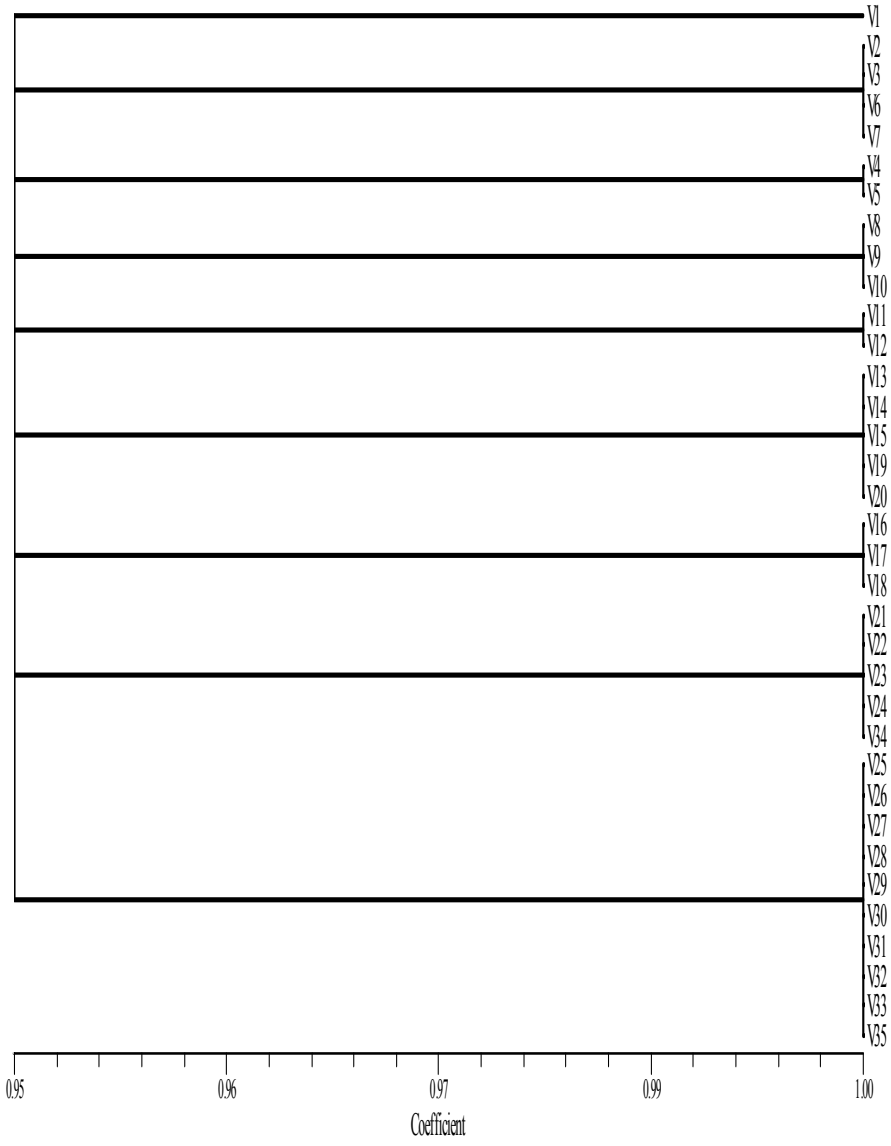


Fig: 3.33 Relatedness of the isolates based on amplification with *16S* gene primer

Isolates V1 to V12 - (*V.harveyi*- Vh3, Vh28, Vh36, Vh45, Vh54, Vh57, Vh64, Vh71, Vh76, Vh81, Vh88, VhL (LMG 4044)),
V13 to V15- (*V.alginolyticus*- Va3, VaL(LMG 4409), VaM(MTCC 4439)),
V16 to V18- (*V.cholerae*- Vc12,Vc35, VcM (MTCC 3906)),
V19 to V20- (*V.fluvialis*- Vf26, VfL(LMG 11654))
V21 to V23- (*V.mediterranei*- Vm18, Vm26, VmL(LMG 11258)),
V24 to V26- (*V.nereis*-Vn30, Vn32, VnL(LMG 3895))
V27 to V28- (*V.parahaemolyticus*- Vpa6, VpaL(LMG 2850)),
V29 to V30- (*V.proteolyticus*-Vpr4, VprL(LMG 3772)),
V31 to V32- (V,splendidus-Vsp3, VspL (LMG 19031)),
V33 to V35- (*V.vulnificus*-Vv9, Vv23, VvL (LMG 13545))

Analysis of the isolates based on amplification of all 8 housekeeping genes:

The dendrogram obtained with the combination of all 8 housekeeping genes showed that the representative isolates were grouped into three core groups. Core group1 was with isolates of *V.harveyi* belonging to Phena 1 & 2 correlated at 89%S (Fig- 3.34 . These 2 phena showed 82.2%S to isolates of *V.parahaemolyticus* (phenon 3) and 79%S to phenon 17 having the isolate and type strain of *V.harveyi*. The second core group consisted of the isolates of *V.mediterranei* (phenon 4), joining to the type strain of *V.nereis* at 92.9%S. This cluster showed 92.3%S to isolates of *V.cholerae* (phenon 6), 91.2%S to isolates of *V.nereis* (phenon 7), 90.8%S to isolates of *V.mediterranei* (phenon 8) and to *V.fluvialis* (phenon 9) grouped at 94.6%S. The third core group consisted of the isolates of *V.proteolyticus* (phenon 10) joined with the isolates of *V.alginolyticus* (phenon 11) at 98.2%S and to the type strain of *V.proteolyticus* (phenon 12) at 94.7%S. This cluster joined with the isolate and type strain of *V.splendidus* (phena13 & 14) at 88.5%S. 100%S existed between the isolate and type strain of *V.vulnificus*; this group showed 83.1%S to core groups 2 & 3. At 80.36%S third core group exhibited similarity to the first core group. Core group 2 was related with third core group at 84.5%S.

Table: 3.4 Molecular weight of the amplicons given by 35 isolates of vibrios with the 8 housekeeping gene primers

Isolates	Code	*ftsZ*	*gapA*	*topA*	*recA*	*merB*	*gyrB*	*pyrH*	16SrRNA
Vh3	V1	700	700	1070.76	1037.14	1322.49, 1124.82, 961.26	883.84	526.74	1635.17
Vh28	V2	669.84	709.41	1058.63	974	415.16	837.07	545.35	1713.66
Vh36	V3	660.08	821.22	694.88	961.26	422.95	883.84	550.11	1719.2
Vh45	V4	674.77	700	700	974	434.91	915.94	554.9	1795.91
Vh54	V5	674.77	821.22	1095.44, 527.75	980.44	443.07	932.17	559.74	1762.55
Vh57	V6	655.25	837.5	987.68	980.44	451.39	932.17	554.9	1729.8
Vh64	V7	669.84	837.5	987.68	980.44	455.61	891.88	559.74	1705.91
Vh71	V8	669.84	700	987.68	974	455.61	932.17	550.11	1829.91
Vh76	V9	660.08	700	1000	974	459.86	924.02	545.35	1812.83
Vh81	V10	650.46	709.41	981.58	974, 800	464.16	948.68	535.96	1847.15
Vh88	V11	636.29	709.41	969.49, 560.8	974	464.16	915.94	531.33	1882.11
VhL	V12	622.34	694.88	1000	974	469.49	907.94	522.18	1882.11
Va3	V13	0	848.53	757.2	556.71, 185.56	1040.43	944.85	522.18	1917.74
VaL	V14	0	930.68	711.08	573.77	1065.47	983.92	508.76	1917.74
VaM	V15	0	953.24	716.69	582.04	1082.55	983.92	491.49	1935.81
Vc12	V16	869.36	962.41	722.34	600	1082.55	983.92	462.83	1972.45
Vc35	V17	863.35	967.03	745.4	600	1091.11	983.92	443.39	1972.45
VcM	V18	863.35	1009.16	757.2	628.4	1073.95	983.92	421.14	1954.04
Vf26	V19	939.64	967.03	728.04	623.57	1082.55	975.98	406.93	1935.81
VfL	V20	953.24	981.03	769.19	648.07	1073.95	975.98	552.74	1935.81
Vm18	V21	900	981.03	751.28	638.16	1082.55	960.29	552.74	1527.81
Vm36	V22	917.41	990.47	745.4	668.37	1073.95	968.1	568.06	1506.26
VmL	V23	939.64	1018.4	769.19	684	1065.47	960.29	530.04	1517
Vn30	V24	921.81	1000	769.19	948.68, 689.29	1091.11	968.1	533.92	1517
Vn32	V25	962.41	1056.22	775.26	992.5, 704.69	1065.47	929.65	545.73	1495.6
VnL	V26	811.6	1046.64	769.19	684	1091.11	851.47	604.89	1485.01
Vpa6	V27	962.24	1027.73	775.26	1470.92, 1124.35, 782.61	1091.11	887.61	600	1485.01
VpaL	V28	930.68	781.37	709.41	1297.53	1376.49, 936.27	845.28	604.89	1474.5
Vpr4	V29	0	995.22	769.19	714.16	1057.06	845.28	609.82	1464.07
VprL	V30	0	962.41	751.28	709.41	1000	845.28	609.82	1474.5
Vsp3	V31	574.21	957.81	4667.17	642.53	991.26	858.58	609.82	1433.2
VspL	V32	594.56	953.42	432.07	642.53	991.26	865.35	609.82	1443.41
Vv9	V33	911.93	769.19	728.04	684.74	992.5	865.35	600	1499.75
Vv23	V34	631.64	763.17	716.69	668.37, 255.08	977.68	865.35	594.56	1508.35
VvL	V35	936.27	787.53	711.08	678.75	1024.61	900	589.16	1433.2

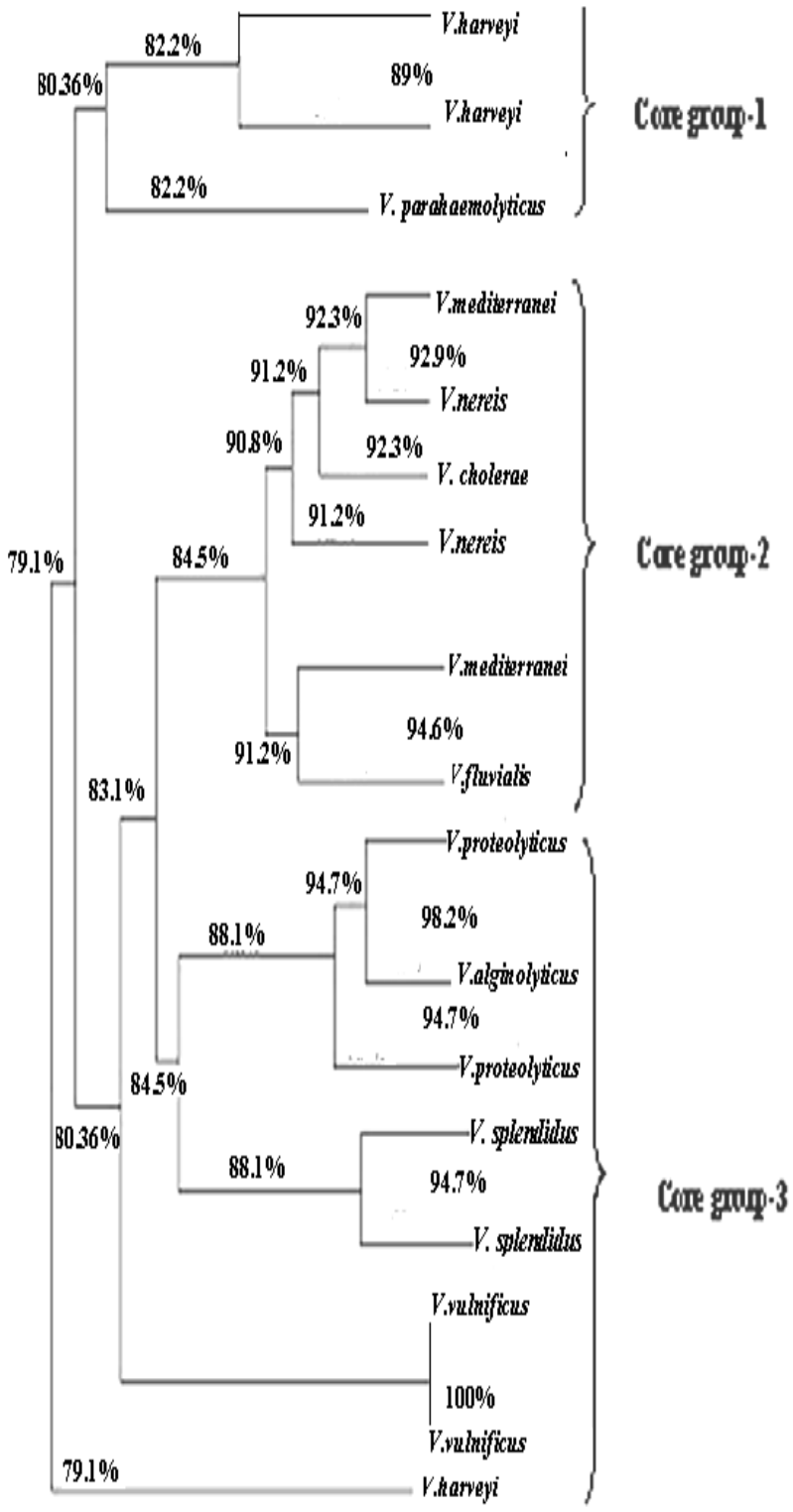


Fig: 3.34 Analysis of Housekeeping genes using Popgene of the 17 Phena obtained

Table-3.5: Identification based on Phenotypic and Genotypic characterization of the isolates which represented each phenon

Sl No	Isolates	Phenon	Identification based on Phenotypic characterization	Identification based on Genotypic (16S rRNA sequencing) characterization	GenBank Accession Number	Percentage Similarity
V1	MCCB 111	Phenon-1	*V.harveyi*	*V.harveyi*	EU404191	98
V2	MCCB 170	Phenon-2	*V.harveyi*	*V.harveyi*	KC291496	100
V3	MCCB 171	Phenon-1	*V.harveyi*	*V.harveyi*	KC291497	98
V4	MCCB 172	Phenon-1	*V.harveyi*	*V.harveyi*	KC291498	98
V5	MCCB 173	Phenon-1	*V.harveyi*	*V.harveyi*	KC291499	98
V6	MCCB 174	Phenon-1	*V.harveyi*	*V.harveyi* *V.rotiferianus*	KC291500	98 96
V7	MCCB 175	Phenon-1	*V.harveyi*	*V.harveyi*	KC747735	95
V8	MCCB 176	Phenon-1	*V.harveyi*	*V.harveyi*	KC747734	100
V9	MCCB 177	Phenon-1	*V.harveyi*	*V.harveyi*	KC747736	99
V10	MCCB 178	Phenon-1	*V.harveyi*	*V.harveyi* *V.rotiferianes* *V.natriegenes*	KC747737	99 99 99
V11	MCCB 179	Phenon-3	*V.harveyi*	*V.harveyi*	KC747738	99
V12	MCCB 112	Phenon-9	*V.alginolyticus*	*Vibrio sp.* *V.natriegenes* *V.alginolyticus*	EU402969	96 97 96
V13	MCCB 169	Phenon-9	*V.alginolyticus*	*V.alginolyticus*	KC291501	98
V14	MCCB 129	Phenon-13	*V.cholerae*	*V.cholerae*	KC291502	97
V15	MCCB 162	Phenon-13	*V.cholerae*	*V.cholerae*	KC747739	96
V16	MCCB 130	Phenon-12	*V.fluvialis*	*V.fluvialis*	KC291503	99
V17	MCCB 131	Phenon-5	*V.mediterranei*	*V.mediterranei*	KC747742	97
V18	MCCB 164	Phenon-6	*V.mediterranei*	*V.mediterranei*	KC747743	97
V19	MCCB 132	Phenon-10	*V.nereis*	*V.nereis*	KC291504	96
V20	MCCB 165	Phenon-10	*V.nereis*	*V.nereis*	KC291505	98
V21	MCCB 133	Phenon-4	*V.parahaemolyticus*	*V.parahaemolyticus* *V.natriegenes*	KC747740	95 96
V22	MCCB 134	Phenon-7	*V.proteolyticus*	*V.proteolyticus*	KC291506	98
V23	MCCB 135	Phenon-16	*V.splendidus*	*V.splendidus*	KC291507	97
V24	MCCB 136	Phenon-14	*V.vulnificus*	*V.vulnificus*	KC747741	95
V25	MCCB 163	Phenon-15	*V.vulnificus*	*V.vulnificus*	KC291508	98

3.4. Discussion:

The current taxonomy of vibrios is based mainly on genomic data, as this approach establishes highly informative measure of intra and interspecific genomic relatedness between strains; enabling reproducible and stable classification frame. Application of various techniques, including RAPD profiling, House Keeping gene profiling and 16S rRNA gene sequence analysis indicated the occurrence of several species within the family Vibrionaceae.

RAPD-PCR, using arbitrary primers to detect polymorphism has been used in discrimination of microbes both at inter and intrapecies level. RAPD-PCR and pathogenicity testing of *P.monodon* revealed that non-luminescent, sucrose fermenting biotypes of *V.harveyi* could be important aeitologial agents of vibriosis (Alavandi *et al*., 2006). Bramha Chari *et al*. (2006) demonstrated the rapid detection of marine luminous and non-luminous *V. harveyi* isolates for molecular epidemiology. Main drawback of using RAPD fingerprinting for subtyping of microbial populations is the reproducibility of the same banding pattern. However, in this study of the 20 selected Operon primers, 7 primers exhibited distinct and reproducible banding pattern ranging from 100-4500 base pair. Similar results were observed by Somarny *et al*. (2002) with RAPD-PCR of 25 isolates of two different *Vibrio* species (*V. cholerae* and *V. harveyi*) with 20 different primers and observed that 14 oligonucleotide primers yielded clear and reproducible bands corresponding to amplified products ranging in size from 250 – 6,000 nucleotide base pairs. Somarny *et al*. (2002) suggested that *V. harveyi* isolates could be grouped into one cluster, whereas *V. cholerae* isolates were grouped into another clusters on the analysis of dendrogram produced from RAPD fingerprint analysis. However, in the present study, the isolates of *V.harveyi* which initially were grouped into three distinct phena based on phenotypic characterization, exhibited further genetic diversification into many clusters upon amplification with each of the 7 selected primers. Maiti *et al*., (2009) showed that diversity existed among *V. harveyi* isolates

based on the analysis of RAPD profiles obtained with primers CRA25 and PM3 individually. Cluster analysis carried out by Maiti *et al*. (2009) based on combined similarity matrix grouped all strains into 15 clusters, indicating a genetically heterogeneous group of *V. harveyi* to be prevalent along the Indian coast. Similar observations were obtained with the selected 7 Operon primers and the results were in agreement with previous studies reporting the presence of a large number of heterogenic genotypes within *V. harveyi* (Hernandez and Olmos, 2004; Alavandi *et al*., 2006).

The study conducted by Somary *et al*. (2002) showed that one amplicon of size 800 bp was shared by almost all *V. harveyi* isolates and with PM-3, two bands of sizes 700 bp and 850 bp were common to nearly all strains. Pujalte *et al*. (2003) reported that most of the *V. harveyi* isolates in their study amplified a common band of 800bp when subjected to RAPD-PCR using Primer M13. Similar results were obtained from the present study, where 600, 400 and 200bp bands were found to be shared by most of the *Vibrio* isolates which were subjected to fingerprinting with 7 selected primers. The sharing of common bands indicated the presence of a highly conserved genomic region in diverse *Vibrio* strains. This assumes significance as amplification of common fragments by RAPD-PCR with a particular primer has been shown to be useful in genetic amplifications and hybridization assays for diagnostic purpose (Dalla *et al*., 2002). Further, these highly conserved fragments could be ideal for identifying strains that are atypical or which may be difficult to identify by phenotypic tests.

To determine whether correlation existed between the RAPD type and source of the isolates, the data with all the 7 primers corresponding to the loci obtained were processed using PopGene software. Interestingly, vibrios isolated from post larvae with necrosis from Trichur, Kerala, identified as *V.alginolyticus* and *V.cholerae* on the basis of phenotypic characterization were grouped together exhibiting 80.6%S., hence clustered together into the same core group (*V.cholerae* core group). Also the isolates

obtained from Azhikode, Kerala, during mass mortality of post larvae were closely related, especially the isolates of *V.parahaemolyticus*, *V.splendidus* and *V.proteolyticus*, suggesting that there existed a relation between the RAPD pattern and source of isolation. Bramhachari *et al*. (2006) analysed several samples isolated from the same location and found that the isolates shared similar RAPD pattern, but no correlation was obtained between a given RAPD type and the geographical location or the source of the isolates. In this study isolates belonging to *V.mediteraneii* were grouped into the cluster of *V. harveyi* and isolates of *V.fluvialis* into *V.vulnificus*. Though the isolates were obtained from different sources, they exhibited high relatedness, above 91%, 86% and 84.5%S respectively, suggesting that the isolates had some genes in common that remained conserved. These results suggested that the isolates analysed had unique bands representing in the fingerprinting pattern which could be used in the recognition of genera and species. This technique is simple and rapid and could also be useful in molecular epidemiology for tracing the route of infection and for implementing suitable control measures for the pathogen (Maiti *et al*., 2009). Further studies are required to clearly establish an association between particular RAPD pattern to virulence and disease, which could have important implication in the discrimination of pathogenic strains from the non-pathogenic forms.

Examination of various genomic loci is more stable in species discrimination rather than the analysis of the 16S rRNA which screenes only 5 to 10% of the total bacterial genomic content. Garg *et al*. (2003) analysed sequence of *dnaE*, *lap*, *recA*, *pgm*, *gyrB*, *cat*, *chi*, *rstR* and *gmd* genes and concluded that homologous recombination may have occurred; leading to cohesion of the species. In this study based on the analysis of the amplicons size obtained using the eight housekeeping genes, the 35 isolates of vibrios could be clustered into three core- groups at ≥79%S. The banding pattern exhibited by all eight housekeeping genes was distinct enabling the

clustering of the isolates except for the gene *ftsZ*. The primer of *ftsZ* gene, which coded for cell division protein failed to amplify for isolates and type strains of *V.alginolyticus* and *V.proteolyticus*, suggesting that *ftsZ* was not a good phylogenetic marker. However, the MLSA carried out by Sawbae *et al*. (2007) on 78 isolates, showed that *ftsZ* gene which was selected as one of the housekeeping genes enabled effective clustering of the isolates and >85% sequence homogeneity using ClustalX program. According to Thompson *et al*., (2005), the genus *Vibrio* is heterogeneous and polyphyletic, with *V.fischeri*, *V. logei*, and *V. wodanis* grouping closer to genus *Photobacterium*. Also *V. halioticoli*, *V. harveyi*, *V.splendidus*, and *V. tubiashii*-related species form groups within the genus *Vibrio*. Similar results were obtained from this study with the isolates of *V.parahaemolytics* grouped along with *V.harveyi* core group, the isolates of *V.mediterraneii*, *V.nereis*, *V.cholerae* and *V.fluvials* were clustered together as core group-2 (*V.cholerae* core group), although the isolates were obtained from different sources. Also the isolates of *V.proteolytics*, *V.alginolyticus*, *V.splendidus* and *V.vulnificus* were grouped together as the core group 3. Interestingly the 5 isolates of *V.harveyi* obtained from Kodungallor, Kerala and the type strain of *V.harveyi* (LMG 4044) diverged widely from the other *V.harveyi* isolates obtained from Kakinada, Andhra Pradesh. Similarly the isolates of *V.alginolyticus* which occupied a major position in *V.cholerae* core group based on phenotypic characterisation and RADP fingerprinting, occupied a position away from *V.cholerae* group and was clustered along with the isolates grouped into core group 3.

Analysis of 16S rRNA sequence of 25 wild isolates of vibrios suggests that heterogeneity exist at the inter and intra species level, especially considering the isolates of *V.harveyi*, *V.parahaemolyticus* and *V.alginolyticus*, which could be the result of horizontal gene transfer or plasmid exchange or the high degree of mobility of *Vibrio* genetic elements suggesting the possibility of conflicting histories (Thompson et al. 2004a, b,

Thompson et al. 2005, Thompson et al. 2007, Thompson et al. 2009). These three isolates could be clustered phenotypically and identified genotypically at ≥95% as *V.harveyi*, *V.parahaemolyticus* and *V.alginolyticus*, hence we consider the wild isolates clustered into these three phena as members of *V.harveyi*, *V.parahaemolyticus* and *V.alginolyticus*. Further studies are needed to determine the exact cause/s of inter-relatedness of isolates, enabling their clustering under respective clade or core group.

Swabae *et al*. (2007) estimated a process of recombination existing based on the rate of amino acid substitutions in housekeeping protein genes which resulted in radiation of different sister species. High correlation between pair wise similarity of *rpoA*, *atpA*, *recA* and 16S rRNA, which are in agreement with polyphasic taxonomic studies, suggests that these genes may be used as an alternative phylogenetic identification markers. Thompson *et al*., (2005) differentiated families of Vibrionaceae, Photobacteriaceae, Enterovibrionaceae and Salinivibrionaceae on the basis of each genetic locus of the housekeeping genes, with each species clearly forming separate clusters with 98, 94, and 94% *rpoA*, *recA*, and *pyrH* gene sequence similarity respectively. Further studies are needs to be carried out on sequence analysis of the amplicons obtained to determine the exact cause for divergence of the isolates from their respective clade or core group.

Sequence of vibrio genome and their phylogenetic comparison suggested that consistent phylogenies for each chromosome, gene organization and phylogeny of the respective origins confirmed their shared history (Kirkup *et al*., 2010). The gene content of a conserved region is useful to infer phylogeny and chromosome specific genes and provide an estimate of the history of the whole chromosome. MLSA schemes devise include analysis of a numbers of genes, rather than examining a single gene for estimating the phylogenic relatedness. Hence separate MLSA schemes are not required for determining the interrelatedness between species. These

genes have potential primer sequences that are hypothetically capable of creating phylogenetic trees with the highest resolution and consistent signal.

From the present study we confirm phenotypic characterization as an important tool for the identification of the wild isolates of vibrios. Identification of the isolates using 16S rRNA gene alone which screens only 5 to 10% of the total bacterial genomic content (Thompson et al. 2004a, b, Thompson et al. 2005, Thompson et al. 2007, Thompson et al. 2009), without studying the phenotypic profile of the wild strains may lead to erroneous identification, hence a detailed investigation of the phenotypic profile of the isolates is a prerequisite for identifying wild strains rather than completely depending on genotypic characterization such as analysis of 16S rRNA gene. Analysis of the amplicon size obtained is in accordance with the study of Thompson *et al*. (2005), suggesting that *recA*, *topA*, and *pyrH* genetic loci could be used for species variations.

3.5. Conclusion

The isolates of vibrios studied diverged widely from the ones which were grouped together as a cluster, based on phenotypic characterisation, suggesting the presence of a large number of heterogenic genotypes within the isolates. However, the presence of conserved regions suggests that the isolates shared the same phylogenic lineage. The RAPD profile suggested that the isolates analyzed having unique bands could be used in the recognition of genera and species. A detailed investigation of the phenotypic profile of the isolates is important for identifying wild strains rather than completely depending on genotypic characterization such as analysis of 16S rRNA gene alone. Similarity at ≥95% with the isolates deposited in GenBank database was exhibited by 25 wild isolates of vibrios based on 16S rRNA gene sequence analysis. Of the eight different housekeeping gene markers only *pyrH recA*, *topA*, and genes could be used as powerful markers for the identification of vibrios.

CHAPTER-4

Phenotypic characterization of virulence - *In vitro* assays

4.1. Introduction

4.1.1. Bacterial pathogens of aquatic organisms

Aquatic ecosystems harbour a pool of macro and micro organisms performing a pivotal role in the nutrient cycling and influencing the health of the associated organisms. Imbalance in the aquatic systems is augmented by unstable, stressed environment and the emergence of pathogens (Thompson *et al*., 2004). There has been the growing concern about environmental mismanagement in aquaculture which culminates in serious economic loss mainly due to disease outbreak, ultimately resulting in shortage of aquaculture products. Stress caused by poor water quality, makes fishes and shellfishes susceptible to less virulent pathogens. The lifespan of most intensive culture systems seldom exceeds 5-10 years due to anthropogenic pollutants and emerging diseases. The cultured marine shrimps are subjected to bacterial toxins in three different ways (Thompson *et al*., 2004). First, microbes can produce a toxin in food, sediment, water or detritus which is then ingested (Harris and Owens, 1999). Second, microbes colonize a wound or mucosal surface, even in the intestinal tract or on the gills (Takahashi *et al*., 1998) and cuticle. Third, exotoxins produced by them may either act locally or enter the bloodstream and attack susceptible organs or tissues (Takahashi *et al*., 1998).

4.1.2. Vibrios and virulence

Symptoms of disease in shrimp caused by strains of vibrios include darkened pigment, eye damage, exophthalmia, sluggish behavior, twirling, spiral or erratic movement and lethargy. Virulence and infectivity depend on both microbial properties and environmental factors (Austin and Zhang, 2006).

The mode of normal microbial infection in shrimp/fish consists of three basic steps (1) microbe enters the host, (2) within the host tissues the microbe deploys iron-sequestering systems like siderophore mechanism, to steal iron from the host and (3) the microbe eventually damages the host by means of extracellular products such as hemolysins and proteases (Lee *et al*., 1997a).

V.harveyi, the causative agent of luminous vibriosis, is recognized as a primary pathogen of penaied shrimp throughout Asia and Latin America (Lightner and Redman, 1998; Lavilla Pitogo *et al*., 1998). *V.harveyi* is the major etiological agent characterized by significant histopathological changes in the hepatopancreas of shrimps affected by luminous vibriosis (Lavilla Pitogo *et al*., 1998). Despite the role of *V.harveyi* as a serious pathogen of marine animals, its pathogenic mechanisms have yet to be fully elucidated.

4.1.2.1. Extracellular products (ECP)

Extracellular products (ECP) have been considered to be important determinants of virulence in *V. harveyi*. The pathogenic role and virulence mechanism of exotoxins are studied (Harris and Owens, 1999). Exotoxins are produced by a variety of Gram +ve and –ve bacteria, which are secreted into the culture medium or temporarily stored in the cytoplasm or periplasm and released by bacterial lysis (Salyers and White, 1994). Many bacterial exotoxins affect the extracellular matrix or nuclear membrane of target host cells. Others might alter the protein metabolism by interfering with some elements of the host-cell structure (Casadevall and Pirofski, 2000; Schmitt *et al*., 2000). Some exotoxins cause either enzymatic hydrolysis or pore formation that can disrupt the selective ion flux through the plasma membrane or interact with elongation factors and rRNA. ECPs such as chitinase, hemolysins, cysteine, serine and metalloproteases have been isolated from cell-free culture supernatants (CFS) of *V. harveyi*, *V. anguillarum*, *V.alginolyticus, A.salmonicida* and other species (Cipriano *et*

al., 1981; Liu *et al*., 1996, 1997; Svitil *et al*., 1997; Harris and Owen, 1999). Proteolytic exotoxins like metalloproteases affect several penaeid shrimp species with pathogenic similarity based on biochemical descriptions (Fukasawa *et al*., 1988, a, b), but they are poorly represented in terms of nucleotide or amino acid sequences.

V.harveyi secretes protease (a type of exotoxins as a virulent factor for pathogenesis) to hydrolyse the peptide bond in proteins and therefore breaks the proteins down to their constituent monomers. The production of cysteine protease is pathogenic for invertebrates like shrimps. Nakayama *et al*. (2005, 2006b) suggest that there will be direct relation between protease production and luminescence signals. Saeed (1995) studied the association of *V. harveyi* with mortalities, and noted that ECP was toxic to brown spotted grouper. Furthermore, Liu *et al*. (1996) studied the pathogenicity of strains, recovered from diseased tiger prawn (*Penaeus monodon*) and determined that virulence occurred with both live bacteria and ECP. Both the live virulent bacteria and their ECP exhibited stronger proteolytic (caseinase), phospholipase and haemolytic activities than those of non-virulent reference strains. These results indicated that there were differences between isolates of *V. h*arveyi in terms of pathogenicity, and revealed that proteases, phospholipase, haemolysins and/or other exotoxins might well exert significant roles in the pathogenicity of *V. harveyi* in the tiger prawn. When *Artemia franciscana* nauplii were inoculated with different *V. harveyi* isolates, there were significant correlations between naupliar mortality and production of proteases, phospholipases or siderophores, but not between mortality and lipase or gelatinase production, hydrophobicity or haemolytic activity of the isolate (Soto-Rodriguez *et al*., 2003a, b).

4.1.2.2. Outer Membrane Proteins

The outer membrane of Gram –ve bacteria is distinguished by a unique component such as lipopolysaccharide (LPS) and a unique set of proteins (Nikaido, 2003). The two polysaccharides common to Gram –ve

bacteria are mucopeptide and lipopolysaccharide and synthesis of these compounds occurs by membrane bound enzymes. The outer membrane of enteric bacteria such as *E.coli*, *Salmenella typhi*, *Proteus mirabilis* etc., constitutes a permeability barrier for hydrophilic substances greater than 550-650D (Nakae and Nikaido, 1975). Exclusion limit by the vesicles is due to certain proteins named porins (Nakae, 1976a, b) which vary from strain to strain having polypeptides of 35,000- 40,000 D. Changes in the cell envelop (**env**) antigens are of potential significance for protective immunity than changes in cytosolic or periplasmic proteins, resulting from the adaptation to the intestinal environment (Jonson *et al*., 1989). Adherence and colonization are likely to be the multifactorical processes involving motility of vibrios, chemotactic events and trapping in the mucus gel, establishing intimate association with the intestinal mucosa and finally the inability to do so resulting in a reduction of virulence (Schrank and Verwey, 1976).

4.1.2.3. Pathogenic Islands

Pathogenic Islands (PAIs) accommodate large cluster of genes contributing to virulence and are widely distributed in pathogenic strains, but not in the non pathogenic ones. PAIs encode genes for hemolysins, toxins, enzymes, membrane proteins and type three secretion system (TTSS) (Hueck, 1998). PAIs not only mediate pathogenicity but can include other aspects such as antibiotic resistance, symbiosis, metabolism, degradation and secretion thereby increases bacterial fitness to certain environment (Hueck, 1998).

4.1.2.4. Quorum Sensing

Variety of quorum sensing (QS) and their complex multichannel networks regulate the switch converting a non-pathogenic strain to a pathogenic one, enabling the survival of organisms in different niches (Bassler *et al*., 1997). Presence of multiple signal system protects the organisms from similar autoinducers (AIs) and signal degradation by other organisms and provides

effective response to varied environmental conditions. QS systems control the behaviors such as biofilm formation, symbiosis and regulates starvation adaptation, resistance to oxidative stress and virulence factor expression (McDougald *et al*., 2001,2003). The LuxR (QS regulator) homologue SmcR positively regulates metalloprotease expression and negatively regulates hemolysin expression (Shao and Hor, 2001).

In this study, *in vitro* assays were performed as they establish an indirect correlation with the virulence of the isolated stains of vibrios. Various biochemical characters of the *Vibrio* isolates along with the analysis of their virulence factors such as hydrolytic potentials of various extracellular products and surface characteristics were studied to determine the relationship between these factors and the virulence established. Analysis of the hydrolytic potentials and antibiotic sensitivity of the 158 isolates of vibrios, enabled us in selecting the most pathogenic/virulent and antibiotic resistant strains.

4.2. Materials and Methods

4.2.1. Detection of hydrolytic potential

4.2.1.1. Aesculin Activity

The ability of microorganisms to hydrolyze the glycoside to aesculitin and glucose can be investigated by incorporating 0.1% Aesculin. ZoBell's 2216E agar with the following composition was used as the basal medium for demonstrating aesculin production.

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
Aesculin	1.0g
Agar	20.0g
pH	7.3±0.2
Distilled Water	1000ml

The test medium was prepared according to the above composition, autoclaved at 15 lbs for 15min and poured into plates. The plates containing the medium was spot inoculated with the test organism, and the plates were incubated until good growth was obtained at 28^0C for 24-72 hrs. Ferric citrate was added to the medium at a concentration of 0.05%. A positive reaction was shown by the development of a brownish black colour produced by aesculitin in combination with the iron (Jefferies *et al*., 1957).

4.2.1.2. Amylase Activity

ZoBell's 2216E agar with the following composition was used as the basal medium for demonstrating amylase production.

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
Soluble starch	5.0g
Agar	20.0g
Distilled Water	1000ml
pH	7.3±0.2

The test medium was prepared according to the above composition, autoclaved at 15 lbs for 15min and poured into plates. The plates containing the medium was spot inoculated with the test organism, and the plates were incubated until good growth was obtained at 28^0C for 24-72 hrs. The tubes were flooded with Gram's iodine solution prepared.

Ingredients	Quantity
Iodine	1.0g
KI	2.0g
Distilled water	100 ml

Amylase producing or starch utilizing organisms showed a halo zone around and beneath them. The colour of the zones depended on the

degree of hydrolysis of the starch, when it was hydrolyzed to the stage of dextrin, then the zones were reddish brown, and when the breakdown had gone further, they turned colourless (Jefferies *et al*., 1957).

4.2.1.3. Chitinase Activity

Chitinolytic bacteria hydrolyze chitin to N-acetyl-D-glucosamine. This hydrolysis can be easily tested by incorporation of colloidal chitin in a suitable basal medium (Holding and Collee, 1971). Purified colloidal chitin (Lingappa and Lockwood, 1961) was made by treating crude chitin alternatively with cold concentrated HCl, filtered through glass wool, precipitated in distilled water and washed several times in distilled water until neutral. This colloidal chitin was then added to the nutrient basal medium having the following composition per litre:

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
NaCl	15.0g
Colloidal chitin	5.0 (or 5% v/v)
Agar	20.0g
pH	7.5±0.3
Distilled water	1000ml

The medium was sterilized at 15 lbs for 15min and poured into plates. The test cultures were spot inoculated and incubated for 7 days at 28^0C. Hydrolysis of chitin was represented by a halozone around the colonies.

4.2.1.4. DNAase activity

A plate test for the demonstration of bacterial decomposition of nucleic acid was described by Jeffries *et al*. (1957). DNA is readily soluble in water. Fresh solution of DNA substrate (0.2%) was added to a liquid nutrient agar basal medium having the following composition.

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
DNA free acids	2.0g
Agar	20.0g
pH	7.2±0.2
Distilled water	1000ml

The DNA containing medium was sterilized at 15 lbs for 15min and poured onto plates. The test organisms were heavily seeded to produce a confluent growth on the plate. After incubation for 1-2 days, the plates were flooded with 1N HCl. DNAase activity resulted in the production of clear zones surrounded by turbidity produced by the precipitation of the unaffected substrate.

4.2.1.5. Elastin activity

ZoBell's 2216E agar with the following composition was used as basal medium for demonstrating elastase activity.

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
Elastin Congo Red	1.0g
Agar	20.0g
pH	7.3±0.2
Distilled water	1000ml

The test medium was prepared according to the above composition, autoclaved at 15 lbs for 15min and poured into plates. The plates containing the medium was spot inoculated with the test organism, and the plates were incubated until good growth was obtained at 28^0C for 24-48 hrs. The positive isolates developed an orange coloured zone around the area of growth.

4.2.1.6. Gelatinase activity

When proteolytic organisms are grown on a plate of nutrient medium, into which gelatin (2%) is incorporated, zones of gelatinase activities around the colonies are demonstrated. The plates are flooded with acid mercuric chloride solution, which reacts with gelatin in the medium to produce opacity, where the gelatin has been hydrolyzed and the medium remain clear (Frazier, 1926).

The medium has the following composition,

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
Gelatin	20.0g
Agar	20.0g
pH	7.3±0.2
Distilled water	1000ml

The prepared medium was autoclaved at 15 lbs for 15 min and poured into plates. The test organisms were spot inoculated and the plates incubated at 28^0C for 24-72 hrs.

Gelatinase production was tested by flooding the plates with mercuric chloride solution of the following composition:

$HgCl_2$ – 5.0g

Con. HCl – 20 ml

Distilled water – 100 ml

4.2.1.7. Haemolytic Assay

4.2.1.7. a) Haemolytic Assay on human blood agar

Hemolytic activity was determined on ZoBell's 2216E agar plates containing 5% (vol/vol) human blood (Swift *et al.*, 1999). Hemolytic activities of the strains were categorized as alpha, beta or gamma based on

the lytic zones produced. ZoBell's agar with the following composition was used as the basal medium.

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	15.0g
Aesculin	1.0g
Agar	20.0g
Distilled water	1000ml
pH	7.3±0.2

The test medium was prepared according to the above composition, autoclaved at 15 lbs for 15min and poured into plates. The plates containing the medium was spot inoculated with the test organism, and the plates were incubated until good growth was obtained at 28^0C for 24-72 hrs.

The test medium was prepared according to the above composition, autoclaved at 15 lbs for 15min and poured into plates. The plates containing the medium was spot inoculated with the test organism, and the plates were incubated until good growth was obtained at 28^0C for 24-72 hrs.

4.2.1.7. b) Haemolytic assay on prawn blood agar

Haemolytic activity was carried out using the 12 isolates of *V.harveyi* by a modified method of Chang (1996). Haemolymph required for the assay was drawn from wild caught adult (30± 10gms) *M .rosenbergii* using sterile capillary tubes of 2mm outer diameter and inner 1mm diameter. Haemolymph was collected aseptically with the capillary tube from the area beneath the rostrum spine after disinfection with sodium hypochlorite (200ppm), by allowing the solution to flow through the area for 2min. Followed by washing the area with 70% ethanol and sterilized distilled water; it was wiped dry with sterile absorbent cotton swab. To prevent clotting of haemolymph, citrate-EDTA containing 0.1M glucose,

30mM Tris Sodium Citrate, 26mM citric acid and 10mM EDTA dissolved in distilled water was used. pH of the buffer was adjusted to 4.6 and osmolarity to 350mOsm (by adding NaCl) and sterilized at 10lbs for 10 mins. Capillary tubes were rinsed with the buffer before haemolymph collection. An aliquot of 1ml collected haemolymph was transferred to a sterile tube containing 0.2ml citrate- EDTA buffer and stained by adding 150µl of 2% (w/v) Rose Bengal (dissolved in citrate-EDTA buffer) gently allowing complete mixing. ZoBell's 2216E agar medium was prepared and autoclaved, and cooled to room temperature, prior to pouring in to plates, 1ml of the stained haemolymph preparation was added to 15ml basal medium, mixed gently and poured into plates. Isolates were spot inoculated on to the prawn-blood agar plates and incubated at 28º C for 3 to 7 days. Plates were observed for clearing zone around growth and lysis of haemocytes.

4.2.1.8. Lecithinase activity

Bacterial phospholipases (lecithinases) decompose phospholipid complexes that occur as emulsifying agents in serum and egg yolk (Holding and Collee, 1971). The enzymatic activity breaks the emulsion and liberates free fats so that turbidity is produced.

The test organisms were cultured on ZoBell's agar medium having the following composition per litre:

Ingredients	Quantity
Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	20.0g
Agar	20.0g
Distilled water	1000ml
pH	7.2±0.2

An aliquot of 4% sterile fresh egg yolk emulsion (Himedia) was added to the sterile basal medium at 55^0C just before the plates were poured. The test organisms were spot inoculated heavily and incubated at 27^0C for 24-48 hrs. Phospholipase production was characterized by a zone of turbidity in the medium surrounding each colony.

4.2.1.9. Lipase activity

Tributyrin or glyceryl tributyrate is commonly used for studying lipolytic activities.

Composition of Tributyrin agar medium

Ingredients	Quantity
Peptone	5.0
Beef extract	5.0
Yeast extract	1.0
NaCl	15.0
Tributyrin	10.0
Agar	20.0
Distilled water	1000ml
pH	7.2±0.2

Tributyrin was first mechanically blended into the nutrient broth to form a stable emulsion, agar added and sterilized at 121^0C for 15min. Plates were poured while mixing well each time. Test organisms were spot inoculated and the plates were incubated at 28^0C for 3-4 days. A positive result was indicated by zone of clearing around the colonies of lipolytic organisms, where the tributyrin was hydrolyzed (Rhodes, 1959).

4.2.1.10. X-gal assay for luminescence detection

Production of luminescence by the luminescent bacteria was detected by "Agar spot assay" using X-gal as the substrate in the basal medium. ZoBell's agar medium was prepared in 15ppt seawater, autoclaved at 15 lbs for 15mins. The medium was cooled and added 200 µl/ 50ml of X-gal dissolved in dimethylformamide to get a stock concentration of

20mg/ml. The medium was poured into plates and dried the plates for 24hrs at 28ºC. X-gal assay was performed with 12 isolates of *V.harveyi* and the type strain of *A.hydrophila* (ATCC 7966) which was selected as the negative control. All isolates were spot-inoculated on the plates and incubated for 24-48hrs at 28ºC. Positive strains developed blue-green colored colonies and negative strains were colorless.

4.2.2. Siderophore Production

Arnow's assay was used to determine the catechol type siderophore production of the 12 isolates of *V.harveyi*, employing Fiss minimal media (Aznar *et al*., 1989).

Composition of Fiss Minimal Medium

Ingredients	Quantity
KH_2PO_4	5.03g
L-Asparagine	5.03 g
Glucose	5.0 g
Mg SO_4	50mg
$ZnCl_2$	500µg
Milli Q Water	1000ml

- Low Iron concentration- Fe SO_4 – 139 µg/L
- High Iron concentration- Fe SO_4 – 5.56 mg/L

Preparation

Fiss minimal medium without adding Fe SO_4, L- asparagine and glucose was prepared, divided into two portions of 500ml, added the different concentrations of Fe SO_4 and autoclaved at 15lbs for 15 min. Cooled the medium and added L- asparagine and glucose, filter sterilized using 0.22µm membrane filter (Millipore) and autoclaved at 10 lbs for 10min. Transferred 20ml of the medium into autoclaved conical flask. Harvested bacterial cells by centrifugation at 8000xg for 10min washed with PBS and repeated the centrifugation to remove all media ingredients. Inoculated 500 µl of the isolate and incubated for 24 hrs in a shaker at 28ºC. After incubation, centrifuged the medium at 8000xg for 10 mins and collected the supernatant. To 1ml of the supernatant, 1ml 0.5M HCl was

added, followed by addition of 1ml Nitite- Molybdate reagent (10gms Sodium Nitrite and 10gms Sodium Molybdate dissolved in 100ml MilliQ). Three different controls were maintained:

1. Without Fe SO_4 and without inoculum
2. Without inoculum and with low Fe SO_4
3. Without inoculum and with high Fe SO_4

Positive cultures turned yellow due to reaction with nitrous acid and control remained colourless. Then added 1M NaOH, positive cultures turned orange-red to pink in the presence of excess NaOH and the control remained colourless. The intensity of colour determined the amount of catechol present. The medium was kept undisturbed for 5mins at 28ºC for the full development of colour which was measured at 500nm.

4.2.3. Suicide Phenomenon

Certain bacteria, when inoculated into nutrient broth containing glucose, produce enough acid to reduce pH and minimize growth (Namdari and Cabelli, 1989, 1990). In fact, glucose suppresses the tricarboxylic acid cycle, which results in acetate accumulation and bacterial cell death. This self-killing activity, known as suicide phenomenon, was inversely correlated with virulence and enteropathogenicity (Namdari and Bottone, 1988). In this test, 12 isolates of *V.harveyi* were grown in ZoBell's broth containing 0.5% glucose. The non-suicide strains maintained the uniform turbidity throughout the medium, but the suicidal strains remained as pellets at the bottom.

4.2.4. Autoagglutination & Precipitation after boiling

The auto agglutination test for self pelleting (SP+) and precipitation after boiling (PAB+) was carried out as described by Janda *et al*., (1987). Autoagglutination (AAG) activity is known to be a marker of virulence in several Gram-negative bacterial pathogens, including *Vibrio cholerae, Bordetella pertussis, Neisseria gonorrhoeae, Yersinia* and *Aeromonas* species. Strong auto-agglutinating property of *A. salmonicida* was found to

correspond with virulence and the presence of A-layer on the surface of the organism.

Individual bacterial isolates were evaluated for their ability to auto agglutinate (AA) in brain heart infusion broth (BHIB) as follows: A loopful of each isolate was inoculated into 6ml of filter sterilized BHIB and incubated at 28^0C for 18 hrs in static culture. At the end of the incubation period, cultures were observed for evidence of self-pelleting (SP), which was indicated by the virtual absence of growth in the broth phase and the appearance of a large aggregate of organisms as a button in the butt of the tube. Strains displaying such a tendency were designated $SP^{++.}$ After this initial determination, BHIB cultures were vortexed for 30s to suspend growth and then split into two equal fractions (3ml). One aliquot was held at room temperature for 1h, while the other was placed in a boiling water bath for the same period of time. After incubation, boiled tubes were allowed to cool for 10 min and then compared with unheated controls. Strains which exhibited a reduction in the turbidity were considered positive for precipitate after boiling (PAB^+) while the unheated controls were not turbid.

The relative degree of precipitation (RDP) was calculated by measuring the absorbance of each culture (A_{540}) in a spectrophotometer according to the following formula:

RDP = A_{540} (untreated) – A_{540} (heated)

Three auto aggregation phenotypes were found and defined as follows. **Strongly auto aggregating Agg** strains showed a high auto aggregation percentage aggregating immediately, forming a precipitate and resulting in a clear solution. **Non-auto aggregating Agg** strains were unable to auto aggregate (auto aggregation percentage r10%) and produced constant turbidity. **Mixed Agg (±)** strains showed an auto aggregation percentage of around 50% and their suspension showed both a precipitate and constant turbidity.

4.2.5. Biofilm formation

Biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primary polysaccharide material (Davies *et al*., 1998). Biofilm forming microbes elicit specific mechanisms for initial attachment to surface, development of community structure and ecosystem. Biofilm associated cells can be differentiated from their suspended counterparts by generation of extracellular polymeric substance matrix (Rodney, 2002). Cell surface hydrophobicity, presence of fimbriae and flagella and production of extracellular substance like proteolytic enzymes, all influence the rate and extend of microbial cell attachment.

Determination of Biofilm formation

Biofilm assay was carried out using the modified protocol of O'Toole and Kolter (1998). All the 12 isolates selected based on phenotypic characterization were grown in LB broth and incubated overnight at 28ºC. Prior to incubation the OD of the samples was measured at 600nm, which formed the initial OD. The samples were diluted 1:100 using fresh broth and transferred to sterile 96 well flat bottom tissue culture plates and incubated at 28ºC for 24hrs. After incubation the wells were rinsed with autoclaved MilliQ water. Associated biofilm was then stained with 1% crystal violet for 15min. Excess stain was washed off slowly with PBS (pH- 7.4) and the biofilm attached to the plates was quantified by solubilising the stained biofilm with 200µl 95% ethanol and absorbance read at 570nm.

4.2.6. Surface Hydrophobicity

The physicochemical properties of the bacterial surface, especially hydrophobicity plays a vital role in mediating its adherence to surfaces of a variety of materials including animal tissues (Magnusson *et al*., 1980). Fimbriae contribute to cell surface hydrophobicity, as it contains high proportion of hydrophobic aminoacid residues, which helps in overcoming the initial electrostatic repulsion barrier that exists between the cell and

substratum (Corpe, 1980). Most of the proteolytic enzymes have a role in adherence, determined by microbial adhesion to hydrocarbons, MATH or bacterial adhesion to hydrocarbon test, BATH (Lee and Yii, 1996).

Cell surface hydrophobicity of bacteria was evaluated by their adherence to xylene, a hydrophobic solution, used in determining MATH or BATH (Rosenberg *et al*., 1980, 1984, 1986). The overnight incubated bacterial cultures were harvested, washed twice with PBS (phosphate buffered saline) and resuspended in PBS (pH 7.4) to obtain 0.1OD at $Abs_{600nm.}$ Aliquots of 1.2 ml samples were then placed in duplicate in test tubes and aliquots of 0.5 ml p-xylene were added. Following 10 min pre-incubation at 30^{o}C, the tubes were vortexed for 1 min and allowed to stand at room temperature for 20 min; then the lower aqueous phase was removed and the OD measured at Abs_{600nm} (OD2). The results were expressed as the percentage decrease in absorbance (Abs_{600}) of the lower aqueous phase compared with Abs_{600} of the initial cell suspension. Per cent hydrophobicity was determined using the formula:

$$\% \text{ Hydrophobicity} = \frac{OD_1 - OD_2}{OD_1} \times 100.$$

4.2.7. Adherence Assay

Eleven representative isolates of *V. harveyi*, type strain of *V.harveyi* (LMG 4044), *Bacillus* MCCB101, *Arthrobacter* MCCB104 and *V.cholerae* MTCC 3906 were examined for their adherence to HEp-2 cells following the method of Snoussi *et al* (2008a) with certain modifications. The HEp-2 cells were grown in Eagle's MEM (Himedia) with 2mM glutamine, 1.5g/l sodium bicarbonate with 10% fetal bovine serum to a semi-confluent layer in a 24-well culture plate (Greiner Bio-One). Bacterial isolates were washed twice in PBS (pH- 7.4), the pellets were resuspended in PBS and the OD of the isolates adjusted to 1 ($\approx 10^{9}$ CFU/mL) at Abs_{600nm} in serum free MEM and a double dilution was carried out. The wells with semi-confluent layer of HEp-2 cell were washed with PBS (pH- 7.4) twice to remove unattached

cells. An aliquot of 100 µl (10^7 CFU/mL) of bacterial suspension was added to the wells containing adhered semi-confluent layer of HEp-2 cells and incubated at 37°C for 2 hrs. The monolayers were washed three times with PBS to remove nonadherent bacteria, fixed with 70% ice cold methanol, and stained with 10% Giemsa for 20 min. The adherence patterns were examined under microscope (Olympus DSS- Imaagetech, Singapore Ltd). The adhering bacteria on Hep-2 cells were also determined by DAPI staining and observed under microscope. Adhesion index was assayed as: NA = non adhesive (0–10 bacteria/cells); W = weak adhesion (10–20 bacteria/cells); M = medium adhesion (20–50 bacteria/cells); S = strong adhesion (50–100 bacteria/cells).

4.2.8. Cytotoxicity assay

The cytotoxic activity was detected by examining the effects of supernatants of the 12 *V.harveyi* isolates on the monolayer of HEp-2 (Human laryngeal epithelial cell line) cells. For this purpose, colonies from 18-24 hr old cultures were inoculated into 5 ml of ZoBell's broth in a 100 ml Erlenmeyer flask. These were incubated at 30^0C and at 230 rpm on an incubator shaker for 18 hrs and centrifuged at 6000x g for 20 min at 4^0C. The supernatants were transferred to fresh tubes and filtered through 0.22 µm pore size syringe filter (Millipore). Sterility of each preparation was confirmed by streaking onto ZoBell's agar plates and incubating at 30^0C for 24 to 48 hrs.

HEp-2 cells were maintained in Eagle's minimum essential medium (EMEM, HiMedia) with 10% faetal bovine serum (FBS, HiMedia) (Mosmann, 1983). The toxicity of cell-free preparations was assayed in 96-well tissue culture plates. In each well, cells were grown to confluent monolayers. In a 96 chamber deep well plate, serial five-fold dilutions of each cell free supernantant (CFS) were prepared in the same medium in which cells were grown. The medium from the cell culture plate was drained off and 100 µl of each dilution of each CFS was added into the wells of the cell culture plates. For each assay,

a control consisting of Hep-2 with MEM and Hep-2 in filter sterilized uninoculated broth was run in hexaplicate along with the samples. The plates were incubated at 37^0C in 5% CO_2 and examined six hourly in an inverted microscope and scored for characteristic cytopathic effect (CPE). The $TCID_{50}$ (the dilution at which 50% of the wells lost viability) for each isolate (for cytotoxicity assay) was calculated based on Spearman's method and ratio of cytotoxicity were calculated with the following formulae:

To estimate the 50% end point **($TCID_{50}$)** =

$$\text{Highest dilution giving 100\% CPE} + \tfrac{1}{2} - \left[\frac{\text{Total number of test units showing CPE}}{\text{Number of test units per dilution}}\right]$$

MTT assay

The cytotoxic potential of the cell free supernatant was determined following incubation of exponentially growing cells using the MTT assay. This method is based on the reduction of the tetrazolium salt, methylthiazolyldiphenyl-tetrazolium bromide (MTT) into a crystalline blue formazan product by the cellular oxidoreductases of viable cells (Mosmann, 1983). An aliquot of 50 µl solution of MTT (5 mg/ml) (Sigma) was added to each well and incubated for a period of 5 hrs. Following incubation the medium was aspirated and MTT-formazan crystals formed were dissolved in 200µl dimethylsulfoxide (DMSO). Absorbance was recorded immediately at 570nm in microplate reader (TECAN Infinite Tm, Austria).

The $TCID_{50}$ (the dilution at which 50% of the cells lost viability) of each isolate (for cytotoxicity assay) was also calculated based on PROBIT analysis. Percentage mortality was converted into probit value obtained from David Finney's table (Finney, 1952; Finney and Stevens, 1948), which was plotted against concentration.

Finney's table converts % mortality to probits (short for probability unit), determines probits corresponding to the % responded. For example, for a 17% response, the corresponding probit would be 4.05. Additionally, for a 50% response (LC50), the corresponding probit would be 5.00.

Transformation of percentages to probits

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
—	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

4.2.9. Antibiotic susceptibility test

Antibiotic use in aquaculture has been chiefly prophylactic and seldom therapeutic (Cabello, 2006) which results in large quantities of antibiotics reaching the environment where the bacteria present in the sites are exposed to them at sub-lethal levels (Le & Munekage, 2004). Susceptibility to 81 selected readymade antibiotic discs from HiMedia Laboratories, India, was tested on ZoBell's agar plates by the disc diffusion method of Baur *et al*., 1966. The plates were incubated at 28±1ºC for 18 hrs and the clearing zone formed around the discs was recorded using Hi Antibiotic Zone Scale (Himedia). Multiple Antibiotic Resistance (MAR) indexing of bacteria is a useful method to distinguish various sources of anthropogenic influence to identify regions of high antibiotic contamination (Krumperman, 1983, Alekshun *et al*., 2007). MAR indexing allows the study of associations of antibiotic usage and bacterial resistance to them (Parveen *et al*., 2006), therefore it would be useful to examine this phenomenon in shrimp hatchery settings. The MAR index of the isolates based on the source of isolation was calculated as per Krumperman (1983). The MAR index (number of antibiotics to which the isolate was resistant / total number of antibiotics tested) was determined for each isolate. Also the MAR index for each source (aggregate antibiotic resistance score of all isolates from the sample / number of antibiotics × number of isolates from the sample) was also determined (Krumperman, 1983).

4.3. Results

4.3.1. Phenotypic expression of virulence - *In vitro* assays

All the 158 isolates were positive for hydrolytic assays such as amylase, gelatinase, DNA-ase, chitinase, lecitinase and γ-hemolysin on human blood agar medium (Fig. 4.1). Of the 158 isolates examined, 125, 13 and 101 isolates were positive for aesculin, elastase, and lipase hydrolysis respectively. All the 11 isolates and the type stain of *V.harveyi* showed haemocyte lysis and a zone of clearance on prawn blood agar. Also they were negative for catechol type siderophores, as no growth was observed in Fiss minimal medium supplemented with L-asparagine and glucose. These 12 isolates developed bluish-green colonies on X-gal plates while the type strain of *A.hydrophila* produced no colouration.

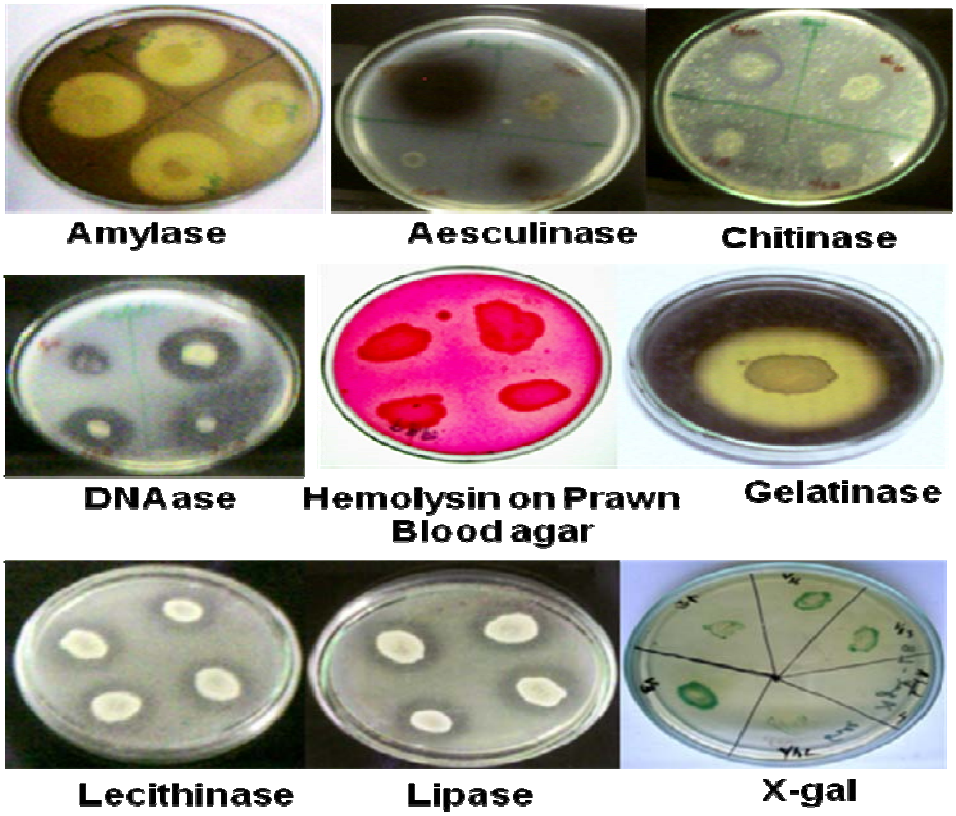


Fig 4.1: Hydrolytic potential shown by the isolates of Vibrios

4.3.2. Agglutination and Precipitation

Thick pellets were observed for all the isolates of *V.harveyi* at the bottom of the tubes upon 24hr incubation, indicating that the isolates were positively suicidal. The isolates were positive for auto agglutination test, self pelleting (SP+) and precipitation after boiling (PAB+) (Table- 4.1).

Table 4.1: Details of isolates showing agglutination and precipitation before and after boiling

Sl.No	Code	SP^+/SP^-	$PAB^{+/}PAB^-$	Unheated (A_{540})	Heated (A_{540})	RDP
1	V1	+	+	1.403	0.639	0.764
2	V2	+	+	1.211	0.737	0.474
3	V3	+	+	1.25	0.856	0.394
4	V4	+	+	1.22	0.755	0.465
5	V5	+	+	1.269	0.6958	0.574
6	V6	+	+	1.447	0.488	0.959
7	V7	+	+	1.281	0.766	0.515
8	V8	+	+	1.348	0.872	0.476
9	V9	+	+	1.067	0.726	0.341
10	V10	+	+	1.248	0.529	0.719
11	V11	+	+	1.54	0.538	1.002
12	V12	+	+	1.11	0.359	0.751
13	V13	+	+	1.395	0.368	1.027
14	V14	+	+	1.591	0.542	1.049
15	V15	+	+	1.317	0.617	0.7
16	V16	+	+	1.314	0.807	0.507
17	V17	+	+	1.444	0.446	0.998
18	V18	+	+	1.115	0.396	0.719
19	V19	+	+	1.122	0.753	0.369
20	V20	+	+	1.521	0.289	1.232
21	V21	+	+	0.932	0.56	0.372
22	V22	+	+	1.612	0.52	1.092
23	V23	+	+	0.965	0.661	0.304
24	V24	+	+	1.2	0.807	0.393
25	V25	+	+	1.299	0.833	0.466

26	V26	+	+	1.281	0.468	0.813
27	V27	+	+	1.434	0.849	0.585
28	V28	+	+	1.271	0.782	0.489
29	V29	+	+	1.227	0.792	0.435
30	V30	+	+	1.378	0.653	0.752
31	V31	+	+	1.363	0.743	0.62
32	V32	+	+	1.208	0.623	0.585
33	V33	+	+	1	0.707	0.293
34	V34	+	+	1.083	0.499	0.584
35	V35	+	+	1.368	0.68	0.668
36	V36	+	+	1.077	0.608	0.469
37	V37	+	+	1.056	0.806	0.25
38	V38	+	+	1.308	0.784	0.524
39	V39	+	+	1.38	0.88	0.5
40	V40	+	+	1.42	0.916	0.504
41	V41	+	+	1.224	0.762	0.462
42	V42	+	+	1.399	0.751	0.648
43	V43	+	+	0.903	0.653	0.25
44	V44	+	+	1.354	0.696	0.658
45	V45	+	+	1.414	0.689	0.725
46	V46	+	+	1.397	0.407	0.99
47	V47	+	+	1.495	1.042	0.453
48	V48	+	+	1.238	1.025	0.213
49	V49	+	+	1.495	0.817	0.678
50	V50	+	+	1.229	0.496	0.733
51	V51	+	+	1.394	0.547	0.847
52	V52	+	+	1.335	0.537	0.798
53	V53	+	+	1.51	0.542	0.968
54	V54	+	+	1.569	0.407	0.733
55	V55	+	+	1.323	0.299	1.024
56	V56	+	+	1.181	0.435	0.746
57	V57	+	+	1.509	0.38	1.129
58	V58	+	+	1. 151	0.624	0.527
59	V59	+	+	1.149	0.42	0.729
60	V60	+	+	1.573	0.508	1.065
61	V61	+	+	1.28	0.51	0.77

62	V62	+	+	1.256	0.447	0.805
63	V63	+	+	1.438	0.525	0.913
64	V64	+	+	1.476	0.795	0.681
65	V65	+	+	1.337	0.489	0.846
66	V66	+	+	0.708	0.516	0.196
67	V67	+	+	1.559	0.928	0.631
68	V68	+	+	1.676	0.606	1.07
69	V69	+	+	1.644	0.521	1.123
70	V70	+	+	1.509	0.405	1.104
71	V71	+	+	1.297	0.655	0.642
72	V72	+	+	1.327	0.632	0.695
73	V73	+	+	1.492	1.135	0.363
74	V74	+	+	1.448	0.504	0.944
75	V75	+	+	1.124	0.647	0.477
76	V76	+	+	1.407	0.491	0.916
77	V77	+	+	1.338	0.556	0.782
78	V78	+	+	1.453	0.674	0.779
79	V79	+	+	1.462	0.55	0.912
80	V80	+	+	1.476	0.538	0.936
81	V81	+	+	0.932	0.196	0.736
82	V82	+	+	1.476	0.579	0.897
83	V83	+	+	1.71	0.6	1.11
84	V84	+	+	1.384	0.699	0.685
85	V85	+	+	1.443	0.729	0.714
86	V86	+	+	0.867	0.672	0.195
87	V87	+	+	1.49	1.095	0.395
88	V88	+	+	1.269	0.6958	0.574
89	V89	+	+	1.338	0.556	0.782
90	V90	+	+	1.384	0.699	0.685
91	V91	+	+	1.327	0.632	0.695
92	V92	+	+	1.297	0.655	0.642
93	V93	+	+	1.28	0.51	0.77
94	V94	+	+	1.256	0.447	0.805
95	V95	+	+	1.229	0.496	0.733
96	V96	+	+	1.394	0.547	0.847
97	V97	+	+	1.238	1.025	0.213

98	V98	+	+	1.229	0.496	0.733
99	V99	+	+	1.2	0.807	0.393
100	V100	+	+	1.299	0.833	0.466
101	V101	+	+	1.281	0.468	0.813
102	V102	+	+	1.271	0.782	0.489
103	V103	+	+	1.227	0.792	0.435
104	V104	+	+	1.378	0.653	0.752
105	V105	+	+	1.281	0.468	0.813
106	V106	+	+	1.256	0.447	0.805
107	V107	+	+	1.229	0.496	0.733
108	V108	+	+	1.269	0.6958	0.574
109	V109	+	+	1.299	0.833	0.466
110	V110	+	+	1.281	0.468	0.813
111	V111	+	+	1.378	0.653	0.752
112	V112	+	+	1.363	0.743	0.62
113	V113	+	+	1.394	0.547	0.847
114	V114	+	+	1.335	0.537	0.798
115	V115	+	+	1.327	0.632	0.695
116	V116	+	+	1.337	0.489	0.846
117	V117	+	+	1.308	0.784	0.524
118	V118	+	+	1.38	0.88	0.5
119	V119	+	+	1.384	0.699	0.685
120	V120	+	+	1.443	0.729	0.714
121	V121	+	+	1.269	0.6958	0.574
122	V122	+	+	1.338	0.556	0.782
123	V123	+	+	1.384	0.699	0.685
124	V124	+	+	1.327	0.632	0.695
125	V125	+	+	1.297	0.655	0.642
126	V126	+	+	1.28	0.51	0.77
127	V127	+	+	1.256	0.447	0.805
128	V128	+	+	1.229	0.496	0.733
129	V129	+	+	1.394	0.547	0.847
130	V130	+	+	1.238	1.025	0.213
131	V131	+	+	1.229	0.496	0.733
132	V132	+	+	1.229	0.496	0.733
133	V133	+	+	1.394	0.547	0.847

134	V134	+	+	1.335	0.537	0.798
135	V135	+	+	1.317	0.617	0.7
136	V136	+	+	1.314	0.807	0.507
137	V137	+	+	1.378	0.653	0.752
138	V138	+	+	1.363	0.743	0.62
139	V139	+	+	1.354	0.696	0.658
140	V140	+	+	1.414	0.689	0.725
141	V141	+	+	1.397	0.407	0.99
142	V142	+	+	1.495	1.042	0.453
143	V143	+	+	1.238	1.025	0.213
144	V144	+	+	1.495	0.817	0.678
145	V145	+	+	1.229	0.496	0.733
146	V146	+	+	1.394	0.547	0.847
147	V147	+	+	1.335	0.537	0.798
148	V148	+	+	1.407	0.491	0.916
149	V149	+	+	1.338	0.556	0.782
150	V150	+	+	1.397	0.407	0.99
151	V151	+	+	1.394	0.547	0.847
152	V152	+	+	1.378	0.653	0.752
153	V153	+	+	1.363	0.743	0.62
154	V154	+	+	1.335	0.537	0.798
155	V155	+	+	1.354	0.696	0.658
156	V156	+	+	1.297	0.655	0.642
157	V157	+	+	1.28	0.51	0.77
158	V158	+	+	1.256	0.447	0.805

SP – Self pellitizing, PAB – Precipitation after boiling, RDP - relative degree of precipitation

Analysis of the hydrolytic potentials of 158 isolates revealed that maximum clearance zone was shown by the isolates of *V.harveyi*. Hence 11 representative isolates with maximum activity, along with the type strain of *V.harveyi*, VHL(LMG 4044) were used for further studies to segregate the most virulent strain/s.

4.3.3. Biofilm formation, Cell surface Hydrophobicity and Adherence

Table: 4.2 Determination of Biofilm formation by the isolates of *V.harveyi*

Sl No.	Isolates	Initial OD	OD1	OD2	OD3	MEAN	SD
1	V3	1.067	2.96665	2.6739	2.5987	2.746417	0.158726
2	V28	0.577	1.0989	0.9143	0.8607	0.957967	0.102029
3	V36	0.9143	1.3554	1.2679	1.2017	1.275	0.062948
4	V45	1.059	2.5469	2.5432	2.4846	2.5249	0.028536
5	V54	1.005	1.9447	1.9453	1.9278	1.939267	0.008112
6	V57	1.014	2.3857	2.3716	2.3383	2.3652	0.019873
7	V64	1.012	2.1236	2.098	1.9995	2.0737	0.053498
8	V71	0.997	1.8982	1.8708	1.8869	1.8853	0.011243
9	V76	1.01	1.9829	1.9712	1.9653	1.973133	0.007314
10	V83	0.947	1.8158	1.8052	1.8034	1.808133	0.005471
11	V88	0.831	1.1791	1.1618	1.1608	1.167233	0.008401
12	VHL	0.942	1.5601	1.5244	1.5002	1.528233	0.024604

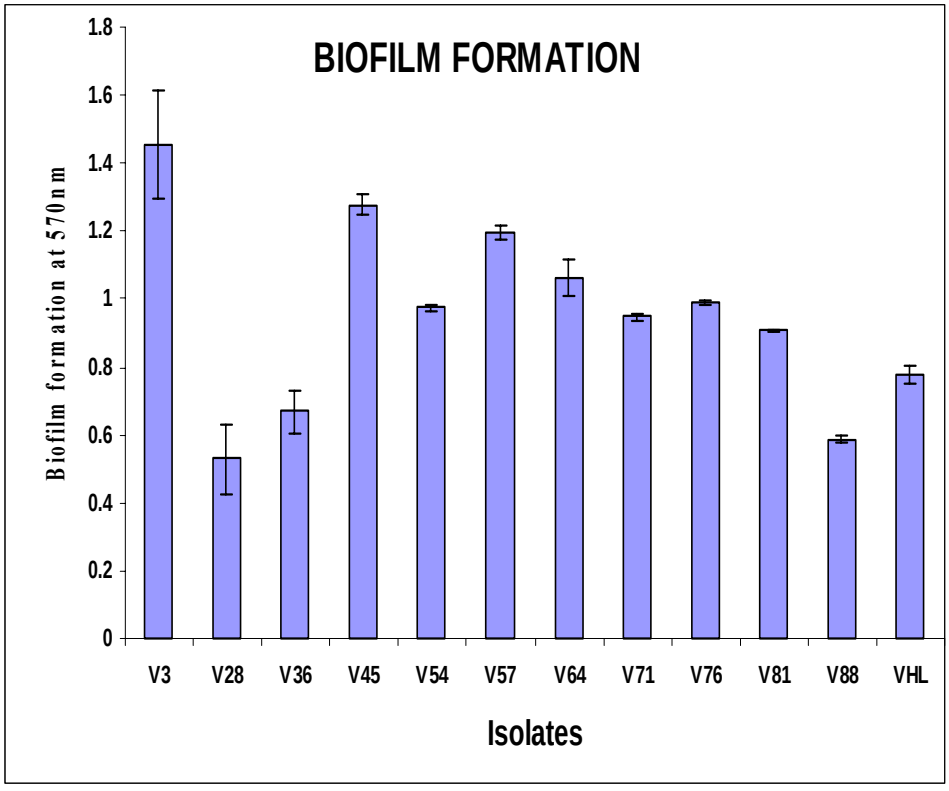


Fig.4.2: Absorbance at 570nm as a measure of biofilm formation by the 12 representative isolates of *V.harveyi*.

The results of Biofilm formation suggested that all isolates of *V.harveyi* exhibited cell - cell communication, resulting in the formation of Biofilms. Among the 12 isolates of *V.harveyi* subjected to Biofilm formation assay, isolates V28, V36 and V88 expressed the least Biofilm forming capacity, which could be correlated with their poor Quorum Sensing ability and low initial cell count.

Table 4.3: Determination of Cell Surface Hydrophobicity by the isolates of *V.harveyi* (V3 to V88, VhL) *V.cholerae* (VcM), *Arthrobacter* and *Bacillus*

Isolates	A	B	Mean	SD
V3	53.9	53.9	53.9	72.0624
V28	53.6	57.7	55.65	73.7113
V36	71.5	50.9	61.2	75.3678
V45	41	46.4	43.7	76.7415
V54	40	36.1	38.05	79.052
V57	75.7	93.8	84.75	81.805
V64	32.9	38.3	35.6	80.9351
V71	72.3	58.7	65.5	83.8428
V76	44.4	52.8	48.6	83.7229
V83	28	24.3	26.15	84.5238
V88	32.5	21.2	26.85	87.2821
VhL	31	18.1	24.55	88.9279
VcM	-5.9	21.4	7.75	87.7238
Arthrobacter	-216	-226	-221	96.077
Bacillus	-22	-36.5	-29.25	7.25

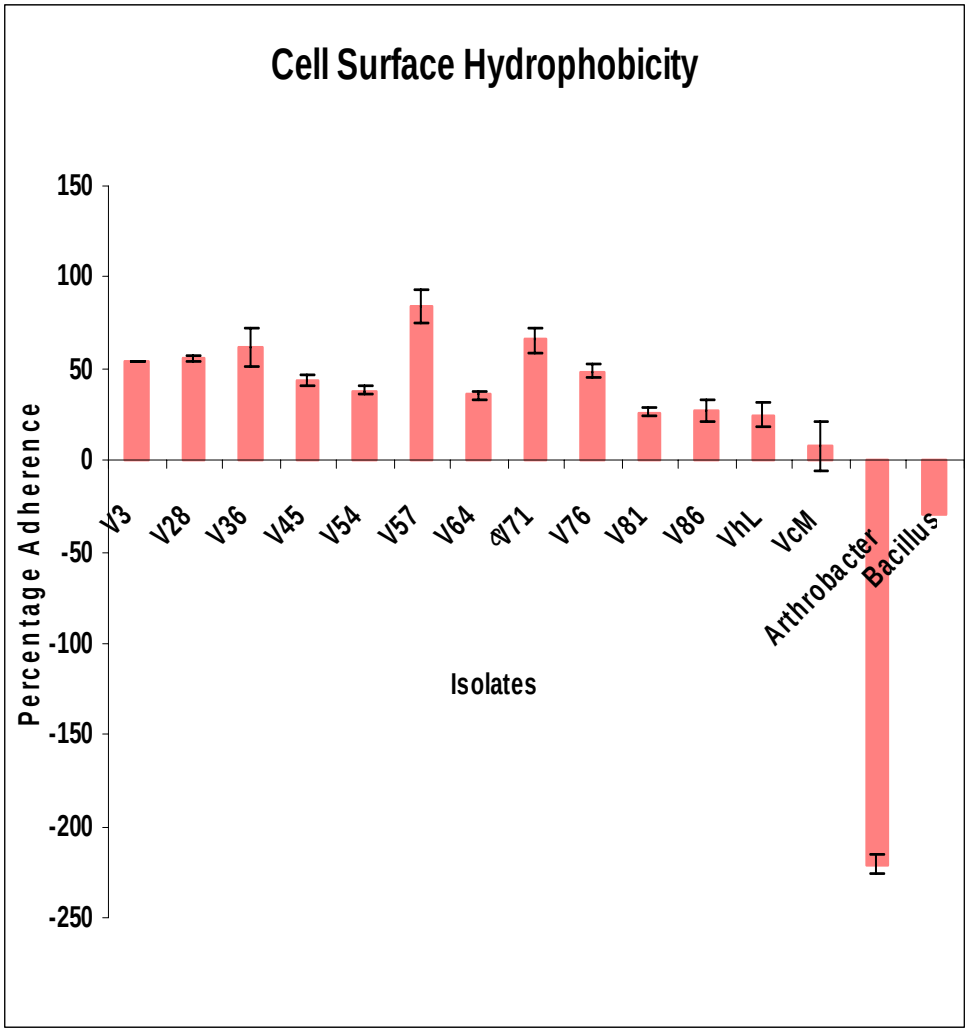


Fig. 4.3: Cell Surface Hydrophobicity exhibited by the isolates

Per cent hydrophobicity values less than 20 were considered as weakly hydrophobic. The study showed that the isolates *Bacillus* MCCB101, *Arthrobacter* MCCB104 and *V.cholerae* type strain VcM-(MTCC 3906) were weakly hydrophobic. Of the 12 isolates of *V. harveyi* V3, V28, V36, V57, V71 were strongly hydrophobic and the remaining isolates and the type strain of *V.harveyi* were moderately hydrophobic.

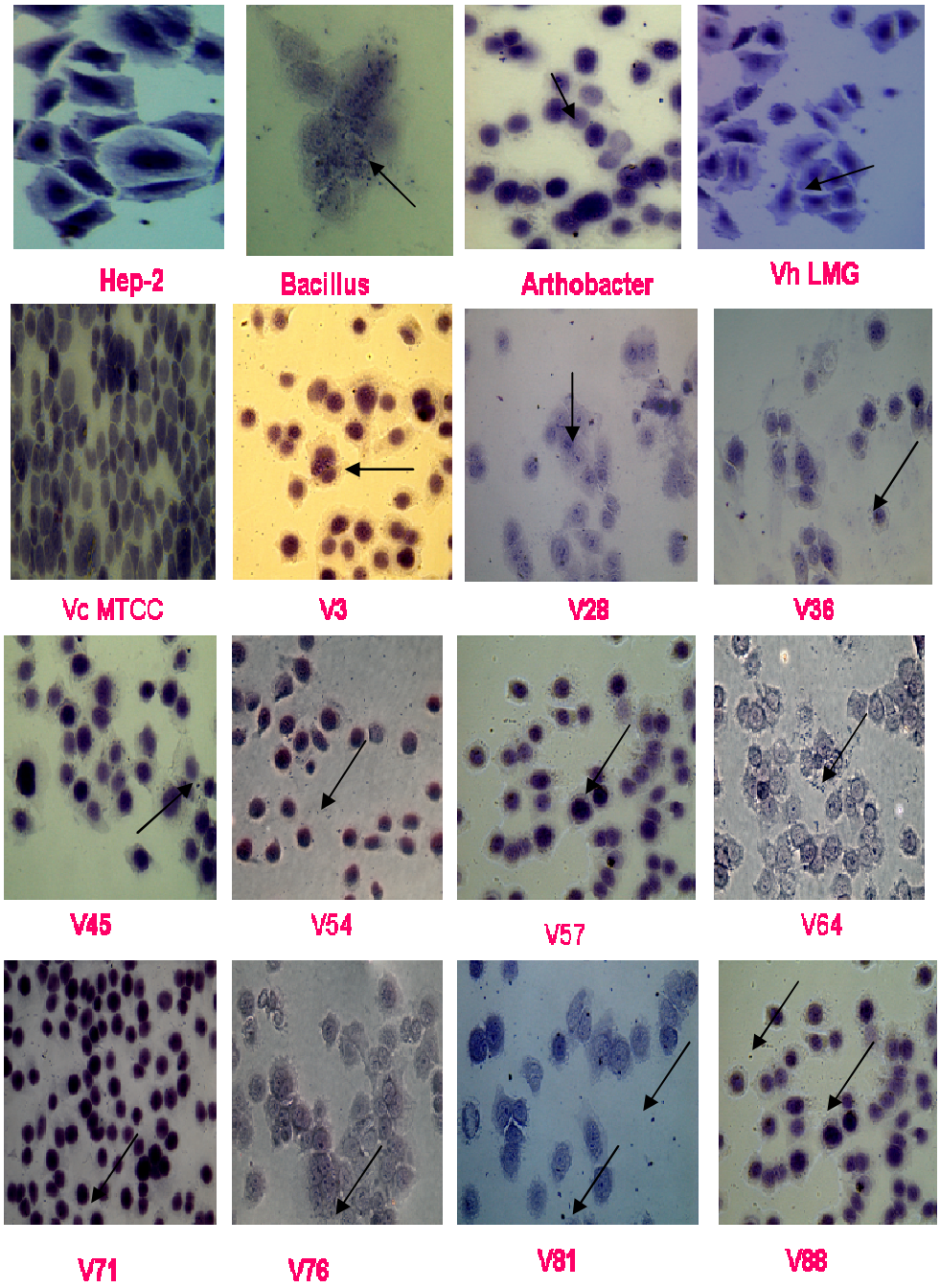


Fig. 4.4: Adherence pattern exhibited by the isolates on Hep-2 cell line determined using Giemsa staining

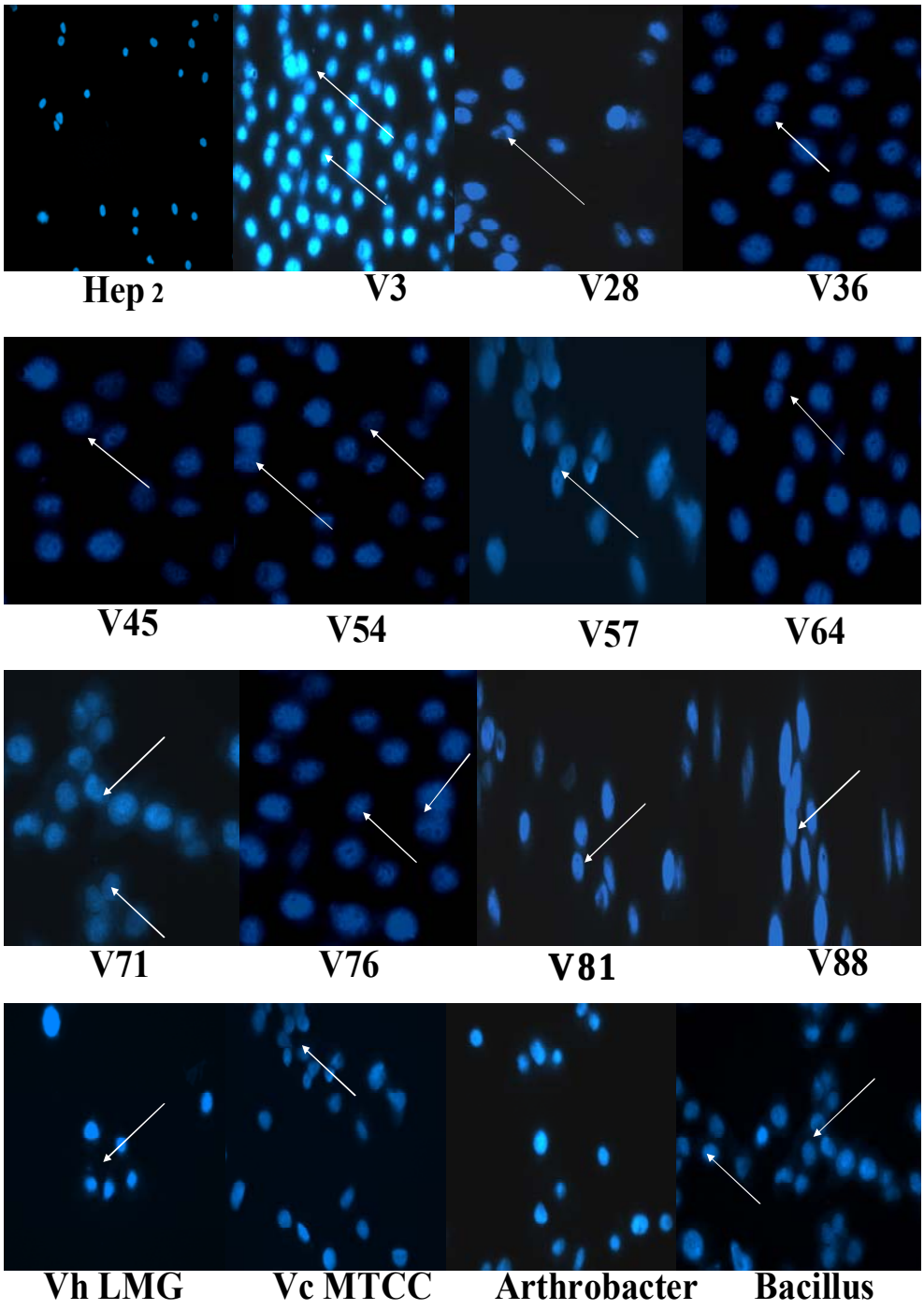


Fig. 4.5: Adherence pattern exhibited by the isolates on HEp-2 **cell line determined by DAPI staining**

The rate of adherence to the semi confluent layer of Hep-2 cells could be clearly visible for *Bacillus*, as the bacterial cells were larger in size when compared to Vibrios. Among Vibrios an evident adherence pattern was shown by the isolates V3, V45, V88, V54 and V57 in comparison with the other isolates of *V.harveyi*. Least level of adherence was exhibited by *Arthrobacter* MCCB104, *V.cholerae* strain MTCC 3906, suggesting that the adhering vibrios might have an effectual role in causing pathogenicity.

4.3.4. Cytotoxicity study on Hep-2 Cells

Table-4.4: Result of cytotoxicity mediated mortality expressed as $TCID_{50}$ and mean probit values by isolates of *V.harveyi* on Hep-2 cells

Isolates	$TCID_{50}$/ml	Mean Probit Value
V3	$10^{6.17}$	8.98
V28	$10^{4.67}$	9.28
V36	$10^{5.5}$	10.20
V45	10^{6}	7.28
V54	$10^{5.5}$	6.34
V57	$10^{5.3}$	14.13
V64	$10^{5.67}$	5.93
V71	$10^{5.17}$	10.92
V76	$10^{5.8}$	8.10
V81	10^{6}	4.36
V88	10^{6}	4.48
VhL	$10^{6.17}$	4.09

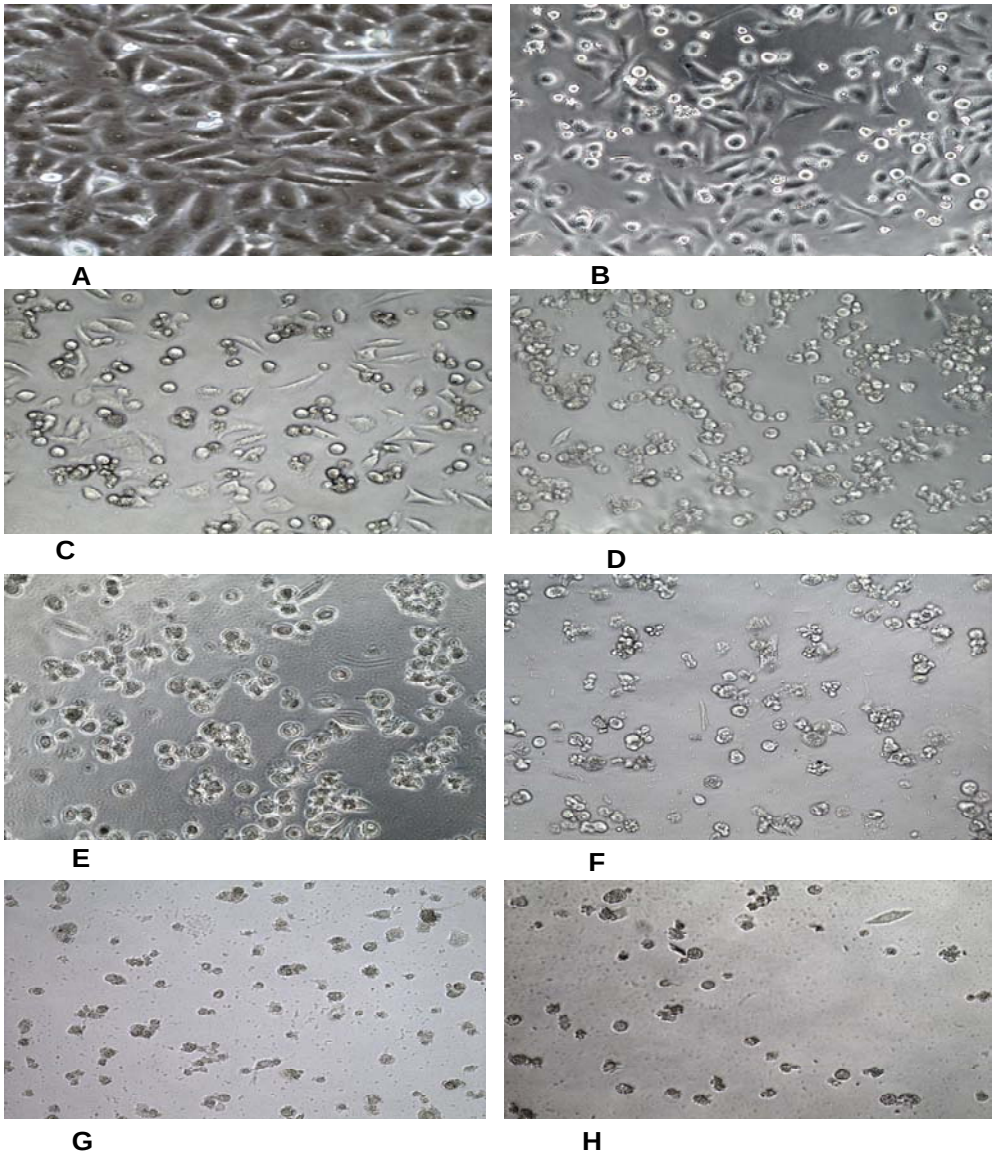


Fig. 4.6: Cytotoxicity caused by the cell free supernatant of *V.harveyi* on HEp-2 **cells, showing rounding, granulation and cell burst. A-Monolayer of Hep-2 cells, B to H cytopathic changes on Hep-2 cells.**

Table-4.5: Result of cytotoxicity mediated mortality by isolates of *V.harveyi* on Hep-2 cells determined as % mortality using David Finley's table

Concentration	V3	V28	V36	V45	V54	V57	V64	V71	V76	V81	V88	VHL
0	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33
1:05	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33
1:25	7.33	5.52	6.41	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33	7.33
1:125	7.33	4.75	5.52	6.28	6.08	5.55	6.15	6.15	5.18	6.15	6.23	7.33
1:625	5.18	3.66	4.48	5	3.92	3.92	4.45	4.45	2.67	4.45	4.82	5
1:3125	3.28	0	3.52	3.72	0	0	0	0	0	0	0	0
Total	37.78	28.59	34.59	36.99	31.99	31.36	32.59	32.59	29.84	32.59	33.04	34.32

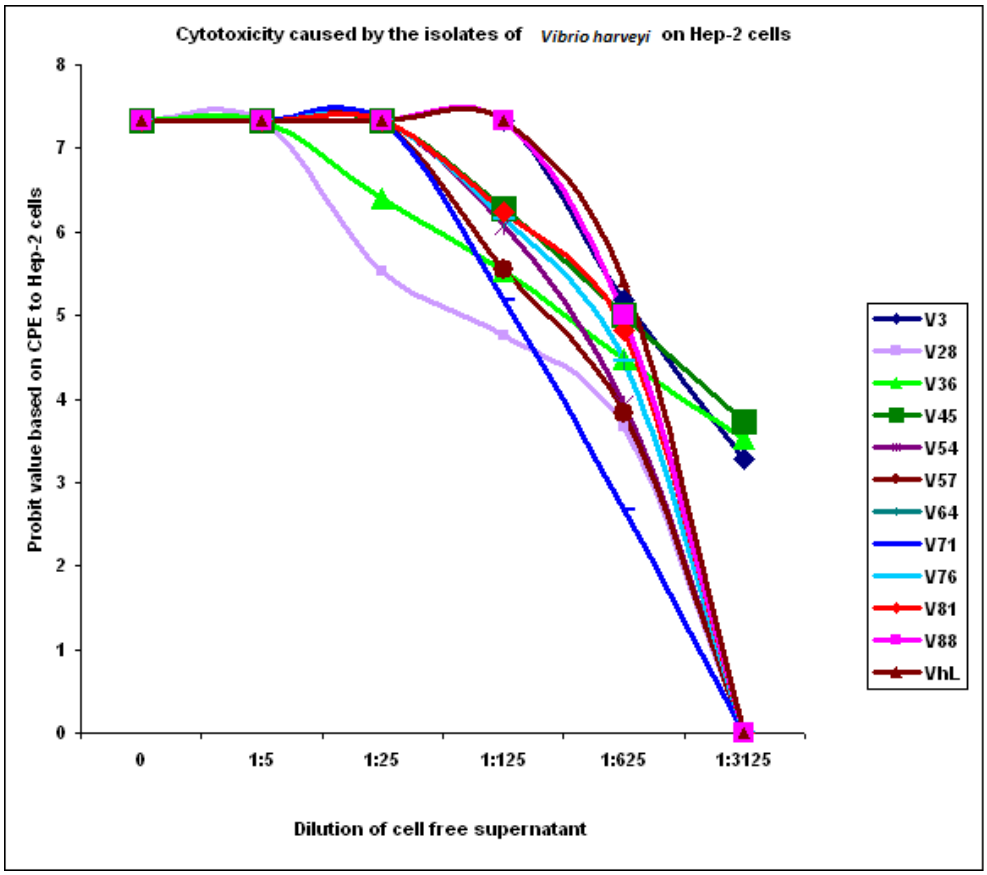


Fig. 4.7: Result of cytotoxicity mediated mortality by isolates of *V.harveyi* on Hep-2 cells determined as % mortality using David Finley's table

Isolates	Mean Biofilm Formation	Mean CSH	Mean Probit Value
V3	2.75	53.9	8.98
V28	0.95	55.65	9.26
V36	1.28	61.2	10.2
V45	2.52	43.7	7.28
V54	1.94	38.05	6.34
V57	2.37	84.75	14.13
V64	2.07	35.6	5.93
V71	1.89	65.5	10.92
V76	1.97	48.6	8.1
V81	1.81	26.15	4.36
V88	1.17	26.85	4.48
VhL	1.53	24.55	4.09

Table 4.6: Correlation between Biofilm formation, CSH and Probit values exhibited by the isolates of *V.harveyi*

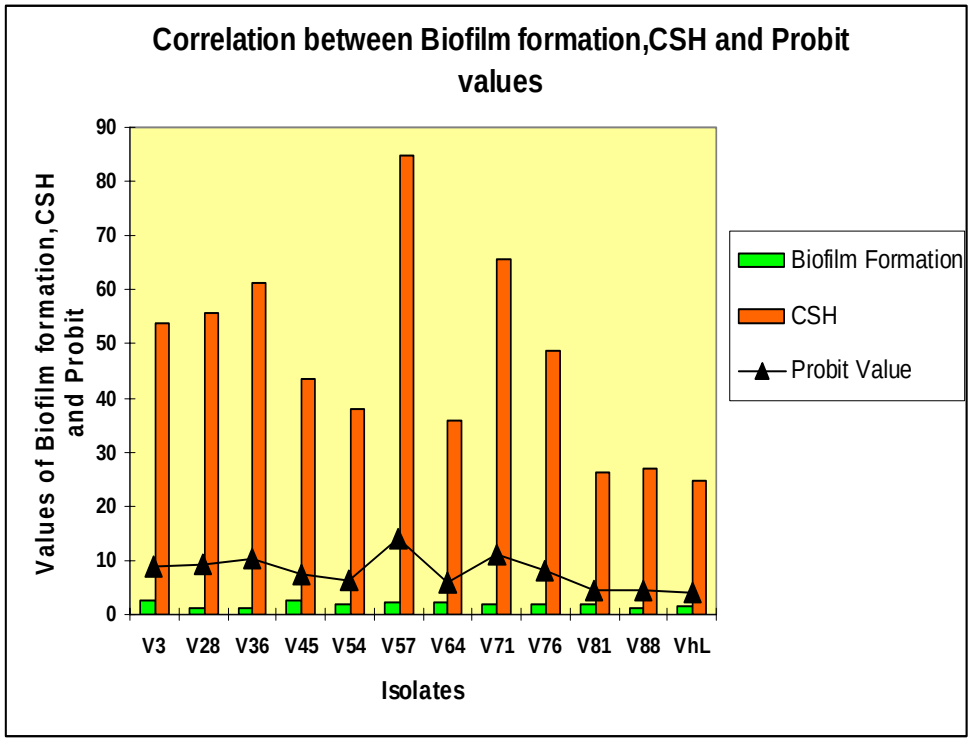


Fig. 4.8: Pearson's correlation of the isolates of *V.harveyi*

Correlation was determined between biofilm formation, cell surface hydrophobicity and mean probit value by Karl Pearson's method. Moderately positive correlation (0.2447) exists between Biofilm formation and CSH and a moderately positive correlation (0.2447) exists between Biofilm formation and mean probit value. Also a perfect positive correlation exists between CSH and mean probit value. The critical values of the correlation coefficient at degree of freedom between columns are very much acceptable at 0.05, 0.01 & 0.001 probabilities.

Table 4.7: Pearson's correlation between Biofilm formation, CSH and Probit values exhibited by the isolates of *V.harveyi*

Degree of Freedom	0.05	0.01	0.001	Observed values
Between biofilm formation & CSH (df = 2)	0.95	0.99	0.999	0.2447
Between biofilm formation & mean probit value (df = 2)	0.95	0.99	0.999	0.2447
Between CSH & mean probit value (df = 2)	0.95	0.99	0.999	1

4.3.5. Antibiotic susceptibility test:

MAR index values of all the isolates were above 0.2, the lowest (0.24) being with those of MPLA - Non-luminescent (Table-4.9). The sensitivity of the isolates to the different classes of antibiotics showed that the isolates were mostly sensitivite to Lincosamide, Peptides (Glycopeptides and Polypeptides), β-lactams, Steroids and Tetracyclines (Fig-4.8 a, b).

Table-4.8(a): Sensitivity of the isolates to different antibiotic class

Antibiotic Class	Number of isolates Sensitive	Number of isolates Resistant	Antibiotic Class	Number of isolates Sensitive	Number of isolates Resistant
β-lactams			Bacitracin 8 units	62	96
Amoxycillin 10mcg	47	111	**Tetracyclines**		
Ampicillin 10mcg	48	110	Chlorotetracycline 30mcg	102	56
Methicillin 5mcg	52	106	Doxycycline hydrochloride 10mcg	51	107
Oxacillin 1mcg	46	112	Minocyclin 30mcg	70	88
Pipemidic Acid 20mcg	50	108	Oxytetracycline 30mcg	62	96
Pencillin G 10 units	42	116	Tetracycline 10mcg	57	101
Carbenicillin 100mcg	95	63	**Chloromphenicol**		

Cefachlor 30mcg	124	34	Chloramphenicol 10mcg	116	42
Cefadroxil 30mcg	73	85	**Rifamycins**		
Cefalexin 30mcg	72	86	Rifampicin 2mcg	114	44
Cefaloridine 10mcg	34	124	**Lincosamides**		
Cefalothine 30mcg	85	73	Clindamycin 10mcg	20	138
Cefamandole 30mcg	138	20	Lincomycin 2mcg	59	99
Cefaperazone 75mcg	100	58	**Steroids**		
Cefaradine 25mcg	67	91	Fusidic Acid 10mcg	50	108
Cefazolin 30mcg	76	82	**Nitrofurans**		
Cefoxitin 30mcg	123	35	Furaxone 100mcg	130	28
Ceftazidime 30mcg	113	45	Furazolidone 50mcg	95	63
Ceftizoxime 30mcg	144	14	Nitrofurazone 100mcg	141	17
Ceftriaxone 10mcg	140	18	**Heterocyclic compounds**		
Cefuroxime 30mcg	120	38	Methanamine Mandalate 3mcg	124	34
Cephotaxime 10mcg	136	22	**Sulfonamides**		
Cloxacillin 1mcg	12	146	Sulfadiazine 100mcg	21	137
Imipenem10mcg	125	33	Sulfafurazole 300mcg	92	66
Ticarcillin 75mcg	50	108	Sulfamethizole 300mcg	36	122
Glycopeptides			Sulfamethoxy-pyridazine 300mcg	37	121
Vancomycin 5mcg	6	152	Sulfaphenazole 200mcg	85	73
Aminoglycosides			Trimethoprim 5mcg	122	36
Spectinomycin 100mcg	80	78	Triple Sulphas 300mcg	91	67
Amikacin 10mcg	142	16	**Quinolones**		
Framycetin 100mcg	129	29	Ciprofloxacin 1mcg	135	23
Gentamycin 10mcg	145	13	**Fluoroquinolones**		
Kanamycin 30mcg	141	17	Enrofloxacin 5mcg	88	70
Neomycin 30mcg	139	19	Floxidine 20mcg	90	68

Netillin 10mcg	151	7	Lomefloxacin 10mcg	64	94
Streptomycin 10mcg	105	53	Pipemidic Acid 20mcg	50	108
Tobramycin 10mcg	139	19	Nalidixic Acid 30mcg	55	103
Macrolides			Nitroxoline 30mcg	113	45
Azithromycin15mcg	106	52	Norfloxacin 10mcg	127	31
Clarithromycin 15mcg	74	84	Ofloxacin 2mcg	130	28
Erythromycin 10mcg	42	116	Pefloxacin 5mcg	84	74
Tylosine 15mcg	71	87	Sparfloxacin 5mcg	63	95
Oleandomycin 15mcg	40	118	**Aminocoumarins**		
Roxithromycin 30mcg	91	67	Novobiocin 30mcg	126	32
Spiramycin 30mcg	42	116	**Metronidazole**		
Nitrofurantoin			Metronidazole 4mcg	131	27
Nitrofurantoin 100mcg	143	15	**Fosfomycin**		
Polypeptides			Fosfomycin 50mcg	114	44
Polymyxin B 50 units	64	94			
Colistin 10mcg	55	103			

Table-4.8(b): Response of the isolates to different antibiotic class

Antibiotic Class	Total number of isolates sensitive to the antibiotic class	Mean Sensitivity to antibiotic class	Total number of isolates resistant to the antibiotic class	Mean Resistance to antibiotic class
β-lactams	2112	162.46	1838	141.38
Glycopeptides	6	6	152	152
Polypeptide	181	60.33	293	97.67
Aminoglycosides	1171	130.11	251	27.89
Macrolides	466	66.57	640	91.43
Tetracyclines	342	68.4	606	89.6
Chloromphenicol	116	116	42	42
Rifamycins	114	114	44	44
Lincosamides	79	79	237	237
Steroids	50	50	108	108
Nitrofurans	366	122	108	36

Heterocyclic compounds	124	124	34	34
Sulfonamides	484	69.14	622	88.86
Quinolones	135	135	23	23
Fluoroquinolones	864	86.4	716	71.6
Aminocoumarins	126	126	32	32
Nitrofurantoin	143	143	15	15
Metronidazole	131	131	27	27
Polypeptides	119	59.5	197	98.5
Fosfomycin	114	114	44	44

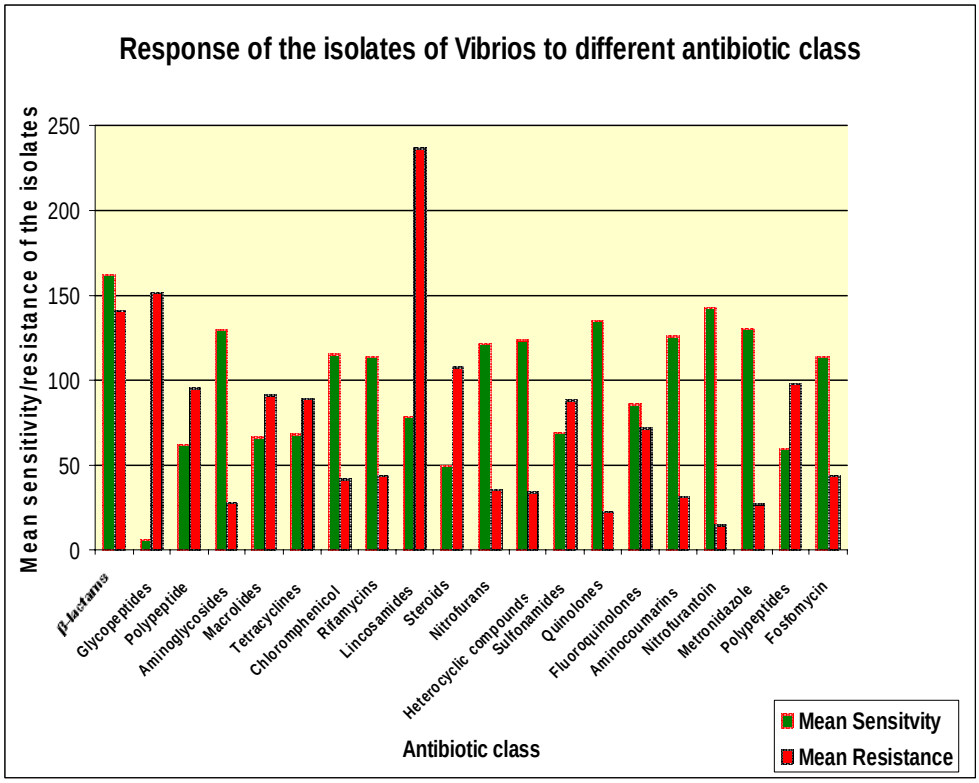


Fig. 4.9: Isolates of vibrios showing sensitivity to different antibiotic class

Table 4.9: Multiple Antibiotic Resistance (MAR) Index of the isolates

Strain	Sensitive	Resistant	MAR Index	V50	26	55	0.6790
V1	17	64	0.7901	V51	18	63	0.7777
V2	11	70	0.8642	V52	25	56	0.6913
V3	18	63	0.7778	V53	26	55	0.6790
V4	45	36	0.4444	V54	45	36	0.4444
V5	43	38	0.4691	V55	46	35	0.4320
V6	44	37	0.4568	V56	28	53	0.6543
V7	39	42	0.5185	V57	23	58	0.7160
V8	45	36	0.4444	V58	33	48	0.5925
V9	44	37	0.4568	V59	32	49	0.6049
V10	45	36	0.4444	V60	37	44	0.5432
V11	36	45	0.5556	V61	22	59	0.7283
V12	45	36	0.4444	V62	26	55	0.6790
V13	49	32	0.3951	V63	27	54	0.6667
V14	42	39	0.4815	V64	32	49	0.6049
V15	43	38	0.4691	V65	21	60	0.7407
V16	44	37	0.4568	V66	24	57	0.7037
V17	41	40	0.4938	V67	23	58	0.7160
V18	56	25	0.3086	V68	22	59	0.7284
V19	47	34	0.4198	V69	26	55	0.6790
V20	37	44	0.5432	V70	22	59	0.7284
V21	51	30	0.3704	V71	28	53	0.6543
V22	41	40	0.4938	V72	32	49	0.6049
V23	50	31	0.3827	V73	43	38	0.4691
V24	43	38	0.4691	V74	29	52	0.6420
V25	52	29	0.3580	V75	36	45	0.5556
V26	42	39	0.4815	V76	33	48	0.5926
V27	33	48	0.5926	V77	55	26	0.3210
V28	34	47	0.5802	V78	26	55	0.6790
V29	35	46	0.5679	V79	32	49	0.6049
V30	32	49	0.6049	V80	30	51	0.6296
V31	43	38	0.4691	V81	26	55	0.6790
V32	47	34	0.4198	V82	27	54	0.6667
V33	56	25	0.3086	V83	35	46	0.5679
V34	43	38	0.4691	V84	32	49	0.6049
V35	33	48	0.5926	V85	42	39	0.4815
V36	24	57	0.7037	V86	18	63	0.7778
V37	25	56	0.6914	V87	29	52	0.6420
V38	37	44	0.5432	V88	52	29	0.3580
V39	34	47	0.5802	V89	45	36	0.4444
V40	35	46	0.5679	V90	59	22	0.2716
V41	32	49	0.6049	V91	70	11	0.1358
V42	38	43	0.5309	V92	44	37	0.4568
V43	22	59	0.7284	V93	44	37	0.4568
V44	30	51	0.6296	V94	48	33	0.4074

V45	31	50	0.6173	V95	61	20	0.2469
V46	35	46	0.5679	V96	61	20	0.2469
V47	29	52	0.6420	V97	26	55	0.6790
V48	33	48	0.5926	V98	32	49	0.6049
V49	39	42	0.5185	V99	67	14	0.1728
V100	60	21	0.2593	V130	50	31	0.3827
V101	68	13	0.1605	V131	50	31	0.3827
V102	67	14	0.1728	V132	49	32	0.3951
V103	69	12	0.1481	V133	50	31	0.3827
V104	62	19	0.2346	V134	60	21	0.2593
V105	67	14	0.1728	V135	57	24	0.2963
V106	63	18	0.2222	V136	66	15	0.1852
V107	75	6	0.0741	V137	26	55	0.6790
V108	66	15	0.1852	V138	32	49	0.6049
V109	72	9	0.1111	V139	30	51	0.6296
V110	60	21	0.2593	V140	26	55	0.6790
V111	72	9	0.1111	V141	27	54	0.6667
V112	47	34	0.4198	V142	35	46	0.5679
V113	68	13	0.1605	V143	32	49	0.6049
V114	51	30	0.3704	V144	42	39	0.4815
V115	71	10	0.1235	V145	18	63	0.7778
V116	67	14	0.1728	V146	29	52	0.6420
V117	65	16	0.1975	V147	28	53	0.6543
V118	70	11	0.1358	V148	23	58	0.7160
V119	69	12	0.1481	V149	33	48	0.5926
V120	58	23	0.2840	V150	32	49	0.6049
V121	71	10	0.1235	V151	37	44	0.5432
V122	61	20	0.2469	V152	22	59	0.7284
V123	71	10	0.1235	V153	26	55	0.6790
V124	53	28	0.3457	V154	27	54	0.6667
V125	63	18	0.2222	V155	32	49	0.6049
V126	46	35	0.4321	V156	21	60	0.7407
V127	50	31	0.3827	V157	24	57	0.7037
V128	53	28	0.3457	V158	23	58	0.7160
V129	52	29	0.3580				

Table. 4.10: Multiple Antibiotic Resistance (MAR) Index of vibrios isolated from various *Penaeus monodon* larval rearing hatcheries

Source	No. of isolates	Strains	MAR Index for each source
MPLW	14	V1-V14	0.54
MPLS	21	V15-V35	0.47
MNL	10	V44-V53	0.64
PLN, MysN	2	V36, V43	0.72
HDO	6	V37-V42	0.59
RSW-I	8	V54-V61	0.59
RW-C, CC	4	V62-V65	0.67
BS	7	V66-V72	0.69
MPLA (Lumniscent)	15	V73-V87	0.5
MPLQ	5	V88-V92	0.33
MPLA (Non-Lumniscent)	33	V93-V125	0.24
NPL	8	V126-V133	0.38
MPLK	14	V134-V147	0.33

- MPLW: Water from postlarval tank where mortality occurred
- MPLS: Larvae from postlarval tank- Santir where mortality occurred
- MNL: Nauplii which failed to metamorphose to protozoea due to luminescent bacteria
- PLN: Post larvae which completed the larval cycle
- MysN: Mysid larvae which completed the larval cycle
- HDO: Water from hatchery drain out, RSW-I: Intake seawater before treatment
- RW-C: Water from crab maintenance tanks
- CC: Crab carapace
- BS: Sand around intake point on the beach
- MPLA(Lumniscent): Larvae from postlarval tank- Azhikode where mortality occurred
- MPLQ: Larvae from postlarval tank- Queen's hatchery where mortality occurred
- MPLA (Non-Lumniscent): Larvae from postlarval tank- Azhikode where mortality occurred
- NPL : Necrotic postlarvae
- MPLK: Larvae from postlarval tank- Kollam where mortality occurred

4.4. Discussion

4.4.1. Phenotypic expression of virulence by the isolates:

All 158 isolates when subjected to the analysis of their phenotypic expression of virulence revealed that all the isolates were able to produce the tested hydrolytic enzymes except for aesculin, elastase, and lipase. Most *V.harveyi* produce hydrolytic enzymes such as gelatinase, amylase, lipase and chitinase associated with virulence (Austin and Zhang, 2006). Extracellular products such as chitinases, hemolysins, alkaline proteases, cysteine proteases, alkaline metalchelator-sensitive proteases, serine proteases and metalloproteases have been isolated from cell-free culture supernatants (CFS) of *V. harveyi*, *V. anguillarum*, *V. alginolyticus* (Harris and Owens, 1999) and other species. Leung and Stevenson (1988) suggested that extra cellular proteases aid the microbe to overcome the initial host defense mechanism. Different extracellular products (ECP) with toxic effects on shrimp have been identified and characterized from a variety of *Vibrio* species and strains isolated from marine organisms and also from the environment (Harris and Owens, 1999). *V.harveyi* secretes protease to hydrolyse the peptide bond in proteins to their constituent monomers, indicating a direct relationship between protease production and luminescence signals (Nakayama *et al*., 2005, 2006). Overall the present study suggests that all the representative isolates and the type strain of *V. harveyi* do have the basic requirements to infect as pathogens to shrimp. Besides, they autoagglutinate, self palletize and precipitate after boiling, suggesting their virulence. Janda *et al*. (1987) observed an association between autoagglutination and self pelletization with pathogenicity expressed by mesophilic aeromonads, thereby considered these characters as virulence markers. Also they were negative for catechol type siderophores, since no growth was observed in Fiss minimal media supplemented with L-asparagine and glucose. This result confirmed as that observed by Owens *et al*., (1996) with isolates of *V.harveyi*. These 12 isolates developed bluish-green colonies on X-gal plates while the type strain of *A.hydrophila* which

was negative for luminescence had no colouration. Thick pellets were observed for all the isolates of *V.harveyi* at the bottom of the tubes after 24hr incubation, indicating that the isolates were positively suicidal.

Ability to form biofilm is an advantage to *V. harveyi* which was highest in the isolate V3 and lowest in V28, and in between for other isolates, suggesting the varying levels of virulence by mediating Quorum Signals effectively. Colwell *et al*. (2002) observed that the biofilm of *V.cholerae* on phtyplanktons and zooplanktons was predominant in cholerae epidemic. Also eel pathogen *V.vulnificus* serovar E has been reported to form biofilm on the epidermal cells of eels (Marco-Noales *et al*., 2001). All the isolates of *V.harveyi* were either strongly or moderately hydrophobic when compared to the controls such as *Bacillus* MCCB101 and *Arthrobacter* MCCB104 which were hydrophilic and *V.cholerae* strain MTCC 3906 weakly hydrophobic. These results suggested a clear distinction between the virulent *V. harveyi* and non virulent control isolates. The physicochemical properties of the bacterial surface, especially hydrophobicity plays an important role in their adherence to a variety of surfaces, thereby they are important in the process of pathogenesis (Magnusson *et al*., 1980). The ability to utilize wide range of substrates as hydrocarbon source by *Vibrio sp*. exhibiting an effective hydrolytic potential, is an important adaptive mechanism (de Carvalho and Fernandez, 2010). High cell surface hydrophobicity (CSH) is considered as an added advantage in colonization of mucosal surfaces, biofilm formation and adhesion to epithelial cells by bacteria (Scoaris *et al*., 2008). *V.harveyi* isolates were cytotoxic on HEp-2 cell line exhibiting CPE revealed by rounding, shrinkage of cytoplasm and dislodgement of cells, showing that the cell free supernatant harboured toxins which played an active role in pathogenesis. The most important virulent property of haemolysin and enterotoxin involve exhibition of cytotoxicity *in vitro* (Ghatak *et al*., 2006). Adherence is an important factor of pathogenicity which is mediated by

nonspecific hydrophobicity and specific interaction of the bacterial cell surface receptors with the receptors on the host epithelial cells (Duguid and Old, 1980). Baffone *et al*., (2005) reported that the strains of halophilic vibrios adhere weakly or moderately by human colon carcinoma (CaCo-2 and HEp-2 cells. The wild isolates of *V. harveyi* such as V3, V45, V88, V54 and V57 exhibited high level of adherence pattern besides the control isolate of *Bacillus* MCCB101. It has to be pointed out that the same *Vibrio* isolates were exhibiting high biofilm forming ability and also suggesting that the adhering vibrios might have an effectual role in causing pathogenicity. All the isolates of *V.harveyi* exhibited positive correlation between biofilm formation, CSH and cytotoxicity by PROBIT analysis, confirming that these three properties have a role in adherence, colonisiation and extend of pathogenicity. Significant correlations were obtained by Soto-Rodriguez *et al*. (2003) between naupliar mortality and production of proteases, phospholipases or siderophores, but not between mortality and lipase production, gelatinase production, hydrophobicity or hemolytic activity. The results suggest that the virulence of the strains tested was more related to the production of particular exoenzymes than to the measured colonization factors. The physicochemical properties of the bacterial surface especially hydrophobicity plays a vital role in mediating its adherence to the surface of a variety of materials including animal tissues (Magnusson *et al*., 1980). Hydrophobic interaction provides driving force for host-parasite interaction through displacement of water and formation of adhesive bonds (Lachica and Zink, 1984). Hydrophobic interactions tend to increase with an increasing non-polar nature of microbial cell, the substratum or surfaces involved. Fimbriae contribute to cell surface hydrophobicity, as it contains high proportion of hydrophobic aminoacid residues, which helps in overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum (Corpe, 1980).

4.4.2. Antibiotic susceptibility test:

Susceptibility of 158 isolates of vibrios to 81 selected antibiotics showed that the sensitivity varied with the strains. The MAR index above 0.2 suggested that the isolates were originated from high-risk source of antibiotic contamination. The development of multi-drug resistant vibrios posed an additional threat of antimicrobial resistance to be acquired by human pathogenic bacteria in the environment (Holmstrom *et al*., 2003b). Evidence to this effect was obtained during an outbreak of cholera in Latin America in 1992, where antibiotic resistance in *V. cholerae* was linked with antibiotic resistant bacteria offered by overuse of antibiotics in Ecuadorian shrimp farms (Angulo *et al*., 2004). Norfloxacin, oxytetracycline, enrofloxacin, ciprofloxacin, chloramphenicol, erythromycin, furazolidone, nifurpirinol, oxolinicacid, ormetoprim, rifampicin, trimethoprim and various sulfonamides are commonly used drugs in aquaculture (Graslund and Bengtsson, 2001; Holmstrom *et al*., 2003b). The exceptionally high resistance to the antibiotics, showed the futility of their application in controlling infections caused by vibrios in *P. monodon* larval rearing systems. When the relationship between the isolates of different sources or seasons or regions was analysed based on their resistance profile, it was observed that the isolates were highly heterogenous in terms of the resistance to varied antibiotic classes. Quinolones/ fluroquinolones and their derivatives such as oxolonic acid and enrofloxacin have been reported as a potent chemotherapeutants against *V. harveyi* (Roque & Gomez-Gil, 2003); in this study also the sensitivity of the isolates to these antibiotics was extremely high. Also it is shown that vibrios are most susceptible to aminoglycosides group of antibiotics and use of the antibiotics such as tetracylines, macrolides, β-lactams and some quinolones in aquaculture settings is futile. The isolates of vibrios in this study are resistant to Lincosamide, Peptides (Glycopeptides and Polypeptides), β-lactams, Steroids and Tetracycline class of antibiotics. β-Lactams are among the most frequently used antimicrobials, and resistance to this class of agents is

often mediated by the genes for TEM-1β-lactamases which is commonly found on chromosomes, plasmids, transposons, and integrons of the Gram-negative bacteria (Pontes *et al*., 2009). Also, the antibiotic residue in the harvested shrimp meat prevents its entry into international market (Pakshirajan, 2002).

4.4.3. Hydrolytic property, auto agglutination and Precipitation Potential:

All the 158 isolates utilized for this study were positive for hydrolytic assays such as amylase, gelatinase, DNA-ase, chitinase, lecitinase, γ-hemolysin on human blood agar medium, and for auto agglutination test for self pelleting (SP+) and precipitation after boiling (PAB+). Of the 12 isolates, V3 and V45 possessed high degree of biofilm forming ability, which might enable these isolates to mediate the Quorum Signals effectively. Furthermore, *V. harveyi* V3, V28, V36, V57 and V71 were strongly hydrophobic and the remaining isolates and the type strain of *V.harveyi* were moderately hydrophobic. The reference isolates such as *Bacillus* MCCB101, *Arthrobacter* MCCB104 and *V.cholerae* strain MTCC 3906 were weakly hydrophobic. The rate of adherence to the semi confluent layer of Hep-2 cells could be clearly visible for *Bacillus*, as they were larger in size when compared to vibrios. Among vibrios an evident of adherence pattern was shown by the isolates V3, V45, V88, V54 and V57 in comparison with the other isolates of *V.harveyi*, *Arthrobacter* MCCB104 and *V.cholerae* strain MTCC 3906, suggesting that the adhering vibrios might have an effectual role in causing pathogenicity. Major cytopathic effects on Hep-2 cells included rounding, granulation and cell burst. The highest CPE was exhibited by isolates V3, V45, type strain of *V.harveyi* (LMG 4044 designated as VhL), V36 and V88.

4.5. Conclusion

Based on the observations it is understood that, the isolates of *V.harveyi* exhibited all the phenotypic traits responsible for causing pathogenesis. The isolates of *V.harveyi* revealed an evident adherence pattern in comparison with *Arthrobacter* MCCB104 and *V.cholerae* MTCC 3906. Moreover the isolates of *V.harveyi* showed effective adherence, hydrophobicity, biofilm and colonizing properties and hence they may be more effective in eliciting pathogenicity. Among the selected isolates of *V.harveyi,* the ones marked as V3, V36, V45 and V88 showed highest the phenotypic characters responsible for pathogenicity. However, further studies are warranted to ascertain which of the 12 isolates of *V.harveyi* are most virulent, moderately virulent and least virulent.

CHAPTER-5

Genotypic characterization and Pathogenicity of *Vibrio harveyi*

5.1. Introduction

5.1.1. Virulence factors expressed by microorganisms

Information on virulent factors and protective antigens expressed by microorganisms during the infectious process is of central importance for understanding the pathogenicity of the microbe and immunity of the host in molecular terms. It is well understood that bacteria can alter their metabolism rapidly in response to environmental changes and that they may exist in a variety of physiological states that can be quite different from one another. Environmental chemical factors such as various nutrients, ions, trace metals, and vitamins, as well as physical factors such as temperature, oxygen tension, growth stage, etc., have been found to influence the expression of bacterial virulent factors.

Most investigations of the bacterial virulence have been carried out with organisms grown *in vitro* under conditions that may differ substantially from the *in vivo* milieu. In general, little is known about the alterations that occur in pathogenic bacteria as they adapt to and multiply in the environment and also found in host tissues during infection (Johnson *et al*., 1989). Variations in biotic or abiotic parameters such as water temperature, salinity, management practices, pathogens, factors of host-sensitiveness to infection such as physiological states, age, moulting or and genetics, and the failure of antibiotics and non-ingested medicated food to reach the target infected tissues results in numerous shrimp diseases (Takahashi *et al*., 1985).

5.1.2. Pathogenicity and virulence of *Vibrios*

Vibrios are amongst the most important bacterial pathogens of aquatic organisms causing hemorrhagic septicemia, necrotic appendages, gill obstruction and mass larval mortality. Many *Vibrio* species are ubiquitous in aquaculture settings associated with all cultured species (fish, mollusks and crustaceans). Vibrios are richly isolated from shrimps with diseases such as 'Red Disease Syndrome', 'Luminescent vibriosis', 'Bolitas negricans', 'Summer Syndrome', 'Penaeid bacterial septicemia', 'Red Leg Disease', 'Shell disease', 'Brown spot disease', 'Black spot disease', 'Burned spot disease', and 'Rust disease'. Vibrios identified from diseased and healthy *P. monodon* samples are *V. aestuarianus*, *V. alginolyticus*, *V. anguillarum*, *V. campbelli*, *V. cholerae*, *V. costicola*, *V. damsela*, *V. fischeri*, *V. fluvialis*, *V. furnissii*, *V. haloplanktis*, *V. harveyi*, *V. hollisae*, *V. ichtyoenterii*, *V. logei*, *V. mediterranei*, *V. metschnikovii*, *V. natriegens*, *V. nigripulchritudo*, *V. parahaemolyticus*, *V. pelagius*, *V. penaeicida*, *V. protelyticus*, *V. splendidus*, *V. tubiashii* and *V. vulnificus.* ECPs like hemolysins, variety of proteases, hydrolytic enzymes, toxR, TCP, VPI, Ctxφ, lysogenic phages, etc., regulate virulence of vibrios.

Pathogenesis has been investigated in shrimps employing different infection methods, such as immersion, intramuscular or sinus injection and oral intubations (Grisez *et al.,* 1996). Pathogenesis varies greatly and is a complex process affected by many variables, including host, species of *Vibrio*, developmental stage, physiological conditions, environmental stress, dose, time and infection method. Mechanism of pathogenicity induced by *Vibrio* infections is still unclear and complex and also related to several factors including cytotoxins, enterotoxins, adhesive factors and lytic enzymes (Ottaviani *et al*., 2001). The ability to adhere to the host epithelial cells is recognized as the first step of infection in several *Vibrio* spp. (Alam *et al*., 1996) and also as an auxillary virulence associated factor (Baffone *et al*., 2005).

5.1.3. Virulent genes of *Vibrio harveyi*

Vibrios harbour diverse genomes as revealed by different genomic techniques, including amplified fragment length polymorphism (AFLP), Multilocus Sequence Typing, repetitive extragenic palindrome polymerase chain reaction (rep-PCR), ribotyping and whole genome analysis. Several PCR methods targeting the genes responsible for pathogenesis have been developed to identify the virulent *V. harveyi* isolates. However, strains other than *V. harveyi* have been reported to give false-positive results due to the fact that majority of the complementary sequences have been shared by a variety of organisms belonging to the same core group. ToxR, hemolysin genes etc are present in most vibrios including *V.parahaemolyticus*, *V.fischeri*, *V.vulnificus*, *V.alginolyticus*, *V.hollisae*, *V.mimicus, V.fluvialis* and *V.anguillarum,* sharing 60 to 85% similarity with the organisms in *Vibrio* core group.

5.1.3.1. *toxR* and *toxS* gene cluster

Several factors are known or suspected for pathogenicity including various extracelluar proteins, metalloproteases, cell-bound hemagglutinins and pilus important for colonization and coregulation of toxin (Taylor *et al*., 1987). ToxR (32KDa transmembrane protein) is identified as the "master switch" regulating the expression of atleast 17 distinct genes. The gene **toxR** encodes for the transmembrane transcriptional regulator. ToxR plays a role in coordinate regulation of virulent gene expression as well as in the transcription of genes encoding for Outer membrane porins such as OmpT, OmpU, TCP and other genes involved in colonizing (Taylor *et al*., 1987; Miller *et al*., 1987; Miller and Mekalanos, 1988). This gene is found to be present in most vibrios including *V.parahaemolyticus*, *V.fischeri*, *V.vulnificus*, *V. alginolyticus*, *V.hollisae*, *V.mimicus, V.fluvialis*, *V.anguillarum* and *Photobacterium* spp (Lin *et al*., 1993; Reich and Schoolnik, 1994; Welch and Bartlett, 1998; Lee *et al*., 2000; Osorio and Klose, 2000; Okuda *et al*., 2001). The *toxR* gene codes for the regulators

that stimulate virulence expression in *V.cholerae*, *V.parahaemolyticus*, *V.vulnificus* and *V.harveyi*. The *toxR* gene, controlling the expression of outer membrane protein (OMP) is widely distributed in the family Vibrionaceae, including both pathogenic and nonpathogenic species, suggesting that this gene is involved in adaptation to environmental changes (Okuda *et al*., 2001). Nucleotide sequence identity among vibrios is relatively low and the universal distribution of this gene widely among vibrios makes *toxR* useful for species specific PCR identification. Highest homology with *V.harveyi* toxR fragment was observed with *V. parahaemolyticus*, sharing 68% identical nucleotides. Intestinal colonization is believed to be mediated by colonization factors expressed by vibrios, the best characterized of which is the **toxin-coregulated pilus (TCP)** (Taylor *et al*., 1987). Expression of *TCP* is coordinated by the *toxR regulon*. Molecular mechanism of *TCP* biosynthesis involves many of the genes present in the *tcp* gene cluster (Kaufman *et al*., 1993, Ogierman *et al*., 1993). OMP's expression is regulated by the *toxR* and *tox*S genetic loci in conjugation with environmental signals. This locus also influences the expression of bacterial virulent factors (various toxins and extracellular protease), fimbrial and other surface antigens of importance for adherence and colonization and also the microbial penetration into or across host epithelial layers (Beachey, 1981; Torres *et al*., 2005).

5.1.3.2. Protease

Marine vibrios have been recognized as producers of several commercially important enzymes such as L-aparaginase, L-glutaminase, protease, α-amylase and chitinase. Luminous *V.harveyi* produces proteinaceous exotoxins (**T1 and T2**) in cell free supernatant (CFS) of culture broth (Harris and Owens, 1999).Virulence in *V. harveyi* has been attributed by the production of an extra cellular protein referred to as toxin T1 with a molecular mass of approximately 100 kDa (Harris and Owens, 1999). The extra cellular protein is produced during the mid exponential

phase of growth and has sequence similarity to virulence-associated proteins in *Salmonella*, *Shigella*, and *Bacillus* species. *V. harveyi* stain 820514 isolated from diseased *P.monodon* produced a highly toxic ECP (LD_{50} 1.2 µg protein/g body weight) containing protease, phospholipase and hemolysin (Lee *et al*., 1999a, b). Shrimps injected with the toxic protease had haemolymph of abnormal color that would not clot (Lee *et al*., 1997, Chen *et al*., 1999). Production of enzymatic activities and enterotoxin could be influenced by environmental factors, including salinity and temperature (Kelly, 1982; Kaysner *et al*., 1987). Lower dosages of ECP are capable of killing animals when held at higher temperature.

a) Hemolysin:

Hemolysin, a toxin from *Vibrio* spp is an important virulent factor in the pathogenic processes of many organisms, causing hemorrhagic septicemia and diarrhoea. It can lyse erythrocytes and a variety of other cells including mast cells, neutrophils, and polymorphonuclear cells as well as it enhances virulence by causing tissue damage. In cardiac cells, hemolysin depolarizes extra cellular Na^+ on the cell membrane, affecting conductive and entry of Na^+. Molecular epidemiological studies revealed a strong correlation between the possession of particular hemolysin genes and the ability to cause disease, supporting the fact that these genes are important virulent genes (Zhang *et al*., 2001; Zhang and Austin, 2005; Conejero and Hedreyda, 2004). V*ibrio harveyi* hemolysin with hemolytic activity towards fish erythrocytes was found to contain three closely related hemolysin genes designated as *vhhA*, *vhhB* and *vhhC*. The ORFs of *vhhA* and *vhhB* are 1,254 nucleotides long and are predicted to encode identical polypeptides of 418 amino acids with a deduced molecular mass of 47.3 kDa. The nucleotide sequences of *vhhA* and *vhhB* are 98.8% identical and differ at only 15 nucleotide positions. *V.harveyi* VHH protein shows extensive homology (85.6%) with the *V.parahaemolyticus* TL (thermolabile) protein which confer thermolabile hemolytic activity (Shinoda *et al*., 1991, Taniguchi, *et*

al., 1986). Also *Vhh* exhibits sequence homology with genes encoding for hemolysin in *V.cholerae* non O1 (64.3% identity), strong homology to the lecithinases of *V. mimicus* (65.3% identity) (Kang *et al*., 1998) and *V.hollisae* (Yamasaki *et al*., 1991). In many vibrios, the hemolysin-producing genes (*tdh/trh*) are located close to the *Ure* gene that codes for urease (Kaysner *et al*., 1987; Osawa *et al*., 1996). It is apparent that identification of the hemolysin-producing genes using multiplex PCR, along with the positive reaction for urease, could be used as an indicator of potentially virulent strains of this pathogen in shellfish and shrimp.

b) Cysteine, Serine and Metallo Protease:

Cysteine protease hampers the coagulation of haemolymph which plays an essential defense role concerning prevention of both the loss of haemolymph through breaks in the exoskeleton and dissemination of bacteria throughout the host body. Proteolytic enzymes, such as cysteine, serine and metalloproteases have been isolated from *V.harveyi*, *V.anguillarum*, and *V. alginolyticus* (Lee *et al*., 1996, 1997; Harris and Owens, 1999). A 38kDa cysteine protease was found to neutralize the clotting ability of normal prawn haemolymph, facilitating the propagation of *V.harveyi* in *P.monodon*, though the definite mechanism responsible for the *in vivo* inactivation of clotting ability by bacteria remains unproven (Lee *et al*., 1995).

ATP-dependent clp-serine protease activates cleavage of peptides in various proteins by hydrolyzing ATP. Serine protease has a chymotrypsin-like activity, causing the degradation of misfolding proteins. Three extracellular alkaline metal–chelator-sensitive proteases produced by *V. harveyi* isolated from seawater and a 22 kDa extracellular cysteine protease produced by an isolate from diseased tiger prawn have been purified (Fukasawa *et al*., 1988a, b; Liu *et al*., 1997).

c) Chitinolytic Activity:

Chitinolytic activity is fundamental to lesion progression, and microbial proteases and lipase may also be involved in exoskeletal breakdown, particularly in the initial stages of shell disease (Ramaiah *et al*., 2000). Two enzymes are usually required for chitin degradation: a chitinase giving the disaccharide N, N'-diacetylchitobiose $(GlcNAc)_2$ and a "chitobias" which activates cleavage of $(GlcNAc)_{2\ to}$ GlcNAc (N,N'acetylchitobiose). Pathogenicity of chitinolytic isolates capable of causing shell disease can be considered on two levels:

1. Their ability to contribute to exoskeletal breakdown by the expression of chitinase activity (External pathogenicity).
2. By penetrating the cuticle and causing damage to host tissues and to overwhelm the cellular and humoral defences of the host (Internal pathogenicity).

Vibrios play an important role in chitin degradation and the genes involved are conserved among many *Vibrio* species. The chitobiase gene of *V.vulnificus* and *V.harveyi* are closely related than to those of *V.parahaemolyticus*. BLAST analysis showed that 578 base nucleotide sequence of *V.vulnificus*, *V. parahaemolyticus* and *V.fluvialis* were similar.

5.1.3.3. Type Three Secretion System

The type three section system (TTS) enables many pathogenic Gram –ve bacteria to directly infect pathogenic eukaryotic cells using fibrous structures on bacterial surface called injectisomes. TTS forms an important part of the *Vibrio* pathogenic islands, mediating virulence. TTS has two distinct subunits, the secretion machine and the injection device. The specific protein secretion machine allows establishment of disease in the host by directing several different toxins either into extracellular milieu or into cytosol of host cells. The injection device subset antihost factors mainly polypeptides into cytoplasm of the host immune cells or damage the

epithelial tissues. Genes required for the synthesis and assembly of TTS machines are clustered, and transfer of such gene cluster is thought to transform otherwise non-pathogenic species into virulent forms. TTS operon consists of translocation proteins coded by *vopD*, *vopB*, *vscY*, *vscX*, *vscO*, *vscP*, *vscQ*, *vscR*, *vscS*, *vscT* and *vscU*. TTS regulator consist of *vcrG*, *vcrR*, *vcrD, vscN* and *vopN* genes coding for low calcium response protein, ATP synthase of TTS and outer membrane protein of TTS. TTS effectors have shown to affect multiple host cell functions by altering and activating various intracellular cascades.

5.1.4. Quorum Sensing and Luminescent genes of *Vibrio harveyi*

Vibrios exhibit complex cooperative behaviour like conjugal plasmid transfer, biofilm formation and virulence. Many of these behavioural traits are regulated by QS mechanism (Bassler *et al*., 2004a). QS is a gene regulation mechanism in which bacteria coordinates the expression of certain genes in response to the presence or absence of small signal molecules (Autoinducers), discovered in the regulation of bioluminescence in *V.fischeri* and *V.harveyi* (Nealson and Hastings, 1978, 1979). The types of signals, receptors and mechanisms of signal transduction and target outputs of each QS system reflects the unique biology carried out by a particular bacterial species. Two proteins, LuxI (autoinducer synthase) produces the AHL autoinducer Homoserine lactone and LuxR the cytoplasmic auto inducer receptor/ DNA binding transcriptional activator (Defoirdt *et al*., 2008) bind to the receptor and activate the transcription of certain genes including those responsible for the synthesis of the inducer itself. With exponential bacterial growth, the concentration of inducer in the surroundings increases, activating the synthesis of more inducer molecules. This forms a positive feed back loop as the concentration of the inducer in the surroundings keep increasing. Once a threshold concentration is attained, activation of the receptor triggers the signal transduction cascade to switch on specific genes in the bacterial

cells, leading to a coordinated population response (Stevens *et al*., 1994). This system is predominantly used for intraspecies communication as extreme specificity exists between the LuxR proteins and their cognate AHL signals.

Quorum Sensing is a complex system in which one strain may encode multiple autoinducers and may have interspecies signals that can sense the population of other bacteria. QS enables bacteria to co-ordinate and respond quickly as a single entity to environmental changes, availability of nutrients, other microbes or toxins. QS is important for pathogens to mediate virulence to escape the immune response of the host and establish a successful infection. QS provides explanation for why some disease causing virulence factors are not expressed during the early stages of encounter with the host. However, QS becomes unproductive when undertaken by individual bacterium (de Kievit and Iglewski, 2000). Three distinct autoinducers have been identified; **LuxR/I-type** systems are used by Gram-negative bacteria, with acyl-homoserine lactone (AHL) as the signaling molecule. Gram-positive bacteria use the peptide signaling systems, ***luxS*/AI-2** for interspecies communication and **AI-3**/epinephrine/norepinephrine for interkingdom signaling system.

V.harveyi has two AI systems, AI-1 (hydroxybutanoyl-L homoserine lactones) for species-specific QS and AI-2 (furanone-related compound furanosyl borate diester) for non-specific QS (Bassler *et al*., 1993). Luminescence in *V.harveyi* is controlled by Quorum Sensing mechanism via signaling molecules synthesized and excreted by the cell, which accumulate in the media and are sensed by the cells, resulting in induction of light. Bioluminescent reactions in *V.harveyi* are catalyzed by **luciferase**, which activates *luxCDABEGH* operon which contains the critical genes required for light emission, *lux AB* encoding the α and β subunits of luciferase and *luxCDE* encoding the fatty acid reductase subunits (Engebrecht and Silverman, 1984, Meighen, 1994). The role of the *lux GH* genes is still

unknown, although the gene products are implicated in the metabolism and or synthesis of flavins. (Swartzman, 1990; Meighen, 1991, 1994).

AI-2 is produced by LuxS enzyme, involved in the metabolism of SAM (S-ribosylhomocysteine) and DPD (4.5-dihydroxy-2, 3-pentanedione) (Bassler *et al*., 1993, 1994). The two autoinducers interact with their respective transmembrane two-component sensor kinases viz *LuxN* and *LuxQ* (Bassler *et al*., 1993, 1994). *V.harveyi* is composed of a soluble periplasmic AI2 binding *LuxP* (periplasmic protein receptor of AI-2) which complexes with LuxQ and phosphorelates cascade resulting in density dependent activation of the lux operon. At low concentrations of autoinducers in the early stages of cellular growth, *LuxN* and *LuxQ* sensors undergo an autophosphorylation on a histidine residue followed by an intramolecular transfer of the phosphoryl group to an aspartate residue. The two signals are then integrated by transfer of the phosphoryl group to *LuxO* (Bassler *et al*., 1994) via a small phosphoryl protein, *LuxU* (Freeman and Bassler, 1999) which also receives phosphorylation signals both from *LuxQ* and *LuxN* via parallel homoserine lactone based QS circuit. It phosphorylates the aspartate residue of the response regulator *LuxO*. *Phospho-Lux*O (activated form of *Lux O*), together with σ54 (Lilley and Bassler., 2000), activates the expression of small regulatory RNAs (sRNAs). The complexes of these sRNAs and sRNA chaperone protein Hfq destabilize the mRNA of the QS master regulator *LuxR*, resulting in indirect repression of the lux operon transcription (Lenz *et al*., 2004). Thus destabilized LuxR (transcriptional activator) causes the phosphorylation and repression of the luciferase operon, blocking the induction of luminescence at the early stages of cellular growth (Swartzman *et al*., 1992, 1993). As the cell grows and autoinducers accumulate in the media, *LuxN* and *LuxQ* sense their cognate AIs, inhibiting their kinase and dephosphorylation of *P-LuxO*, resulting in its activation and the expression of the lux operon (Freeman and Bassler, 1999a, b, Freeman, 2000).

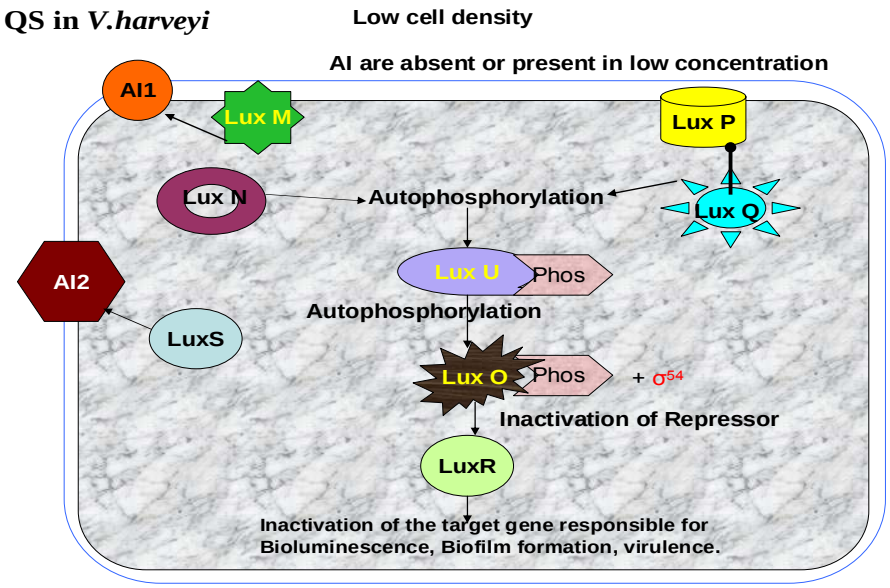


Fig: 5.1 Quorum sensing exhibited by *V.harveyi* at low cell densities (Self designed figure)

In some pathogenic Gram –ve and +ve bacteria, **Furanosyl borate diester** synthesis is controlled by *luxS* gene (Chen *et al*., 2002; Miller and Bassler, 2001). Manefield *et al*. (1999, 2000) studied that halogenated furanose acts by displacing AHL from its receptor protein (*LuxR* or *LuxR* homologue) which, thus inhibiting transcriptional activation of genes encoding the QS phenotype and production of extracellular toxin, which appears in the supernatant of *V.harveyi* cultures concurrently with the expression of luminescence. The induction of luminescence requires cAMP and cAMP receptor protein, which activate the transcription of *luxR* (Dunlap and Greenberg, 1985, 1988; Dunlap and Ray, 1989). Moreover the function of *LuxR* protein depends on sufficient concentrations of diffusible acylhomoserine lactone signal. (Fuqua *et al*., 1996; Greenberg, 1997).

A brief drop in the concentration of either AIs activates the kinase activity in one sensor and a very little Lux O-P will accumulate. The inclusion of LuxU in the cascade system prevents a brief drop in the concentration of one or both AIs. The genes underlying QS are distributed

in a discontinuous manner among bacterial species (Surette *et al*. , 1999; Miller and Bassler, 2001) suggesting that they have been a subject to loss or horizontal transfer. The presence of AI2 in a number of marine *Vibrio* species suggest that this family of signal –dependent genes may be more conserved and widespread than AHL class of signaling genes (Mc Dougald *et al*., 2003). The AIs and its biosynthetic pathways are the same among all bacterial species that posses *luxS*, hence proposed that this system could be used in the interspecies communication. (Mok *et al*. 2003). *V.parahaemolyticus* produces an AI1- like activity, indicating that *V.harveyi* system1 is highly specific, while several bacterial species produce AI2-like activity, indicating that the system2 is less specific. The function of the higher sensitive, higher specific system1 is to monitor the environment for *V.harveyi* while the function of the lower sensitive and lower specific system2 is to monitor the environment for other species of bacteria (Bassler *et al*., 1997).

5.1.5. Bacteriophages mediated virulence

Bacteriophages can also mediate toxicity to *V.harveyi* in *P.monodon* by the transfer of a toxin gene or a gene controlling toxin production (Ruangpan *et al*., 1999, Cheetham and Katz, 1995). The bacteriophage VHML *(V.harveyi* myovirus like) originated from a toxin producing strain of *V.harveyi*, has a potential toxin gene on the VHML genome. The toxin gene shows DNA sequence similar to the reported active site of the ADP-ribosylating group of toxins. ADP-RT's include toxins from other bacteria reported previously to be a result of lysogenic bacteriophage conversion. The phage with the toxin genes causes infection to *V. harveyi* host cells, integration of the phage genome into the hosts' chromosome and subsequent production of the putative toxin, thereby conferring virulence to *V.harveyi*. VHS1 a Siphoviridae-like phage of *V.harveyi* enhances virulence of *V. harveyi* for the black tiger shrimp by 100 times or more (Khemayan *et al*., 2006). Increased virulence of *V.harveyi* lysogenized by VHS1 could result

either from phage induced production of host bacterial toxins or from toxins of phage genome origin. In support of the concept of mobile elements, Ruangpan *et al.* (1999) reported that gross signs of brown gills and high shrimp mortality arose from combined intramuscular injection of VH1039 and a bacteriophage partner, while injection of either partner alone caused no mortality.

5. 1.6. Beneficial forms of vibrios

Certain beneficial forms of vibrios exist amidst the numerous pathogenic forms. The maintenance of homeostasis is an essential cellular process that is mediated by sensory and regulatory proteins whose activities control various gene expression and enzymatic activities. Communication between bacteria and their hosts is an essential component of both beneficial symbiotic and pathogenic associations. Recognition of specific-cell surface receptor molecules and favorable adaptation to host internal environment favours bacterial colonization for normal growth, development, and function. Cell to cell communication by diffusible extracellular molecules or signals is evident in bioluminescent bacteria, commonly found associated with marine animal tissues, as members of the enteric consortia, as opportunistic pathogens, enabling antipredatory defense and defensive camouflage strategy to the host. Bacteria induce the host to secrete lipopolysaccharides (LPS), which triggers developmental response. Beneficial symbiotic vibrios, turns down the expression of the peroxidase gene in tissues but upregulates the expression of this gene in tissues (specifically gills) when it acts as a pathogen. Vibrios harbouring the external surface of marine zooplanktons have extensive chitinolytic activity. They play a significant role in the mineralization of chitin in the aquatic systems by utilizing it as both carbon and nitrogen source (Montgomery and Kirchman, 1993). Although vibrios as a whole are considered pathogenic to shrimps, the recent literature indicates that all are not pathogenic, suggesting that there are benevolent vibrios also. Therefore a foolproof diagnostic

system that could enable the differentiation of pathogens from non-pathogenic forms is essential.

As the sequences of the virulence genes are known, it would be possible to standardize PCR detection and further sequencing of the genes of *V.harveyi*. During the course of events, it would be possible to evaluate the presence of virulence factors other than that reported till date. The amplicons obtained using protease gene primers like haemolysin, cysteine, serine and zinc metalloproteases, suggest that the isolates are pathogenic; hence in the present work the pathogenicity assay of the 12 isolates of *V.harveyi* on *Artemia* nauplii and post larvae of *P.monodon* was studied to find out whether there is any variation in the extend of pathogenicity caused.

5.2. Materials and Methods

5.2.1. Amplification of genes

The representative isolates (12 including the type strain of *V.harveyi*-LMG 4044) were segregated from the phylogenetic tree constructed based on phenotypic characterization and Numerical Taxonomy. Genomic DNA was extracted using DNAzol method. The integrity of DNA samples was estimated by visualizing samples on a 1% agarose gel stained with ethidium bromide and electrophoresing at 110 V for 60–90 mins. The extracted DNA was stored at -20 ^{0}C in aliquots using 5mM Tris Cl (pH 8) until use. The genomic DNA of the 12 isolates was amplified for the already reported virulent and luminescent gene markers (Table-5.1). For PCR , the reaction mixture of total volume of 25µl was prepared containing 1.5µl bacterial genomic DNA (50 ng), amplified with 2.0µl of 10X Thermopol buffer (New England Biolabs), 1.5µl of 0.5U Taq DNA Polymerase (New England Biolabs), 2µl of 10pmol each of the forward and reverse primers, 2.0 µl dNTP mix and 14µl of MilliQ. Amplifications were carried out in a thermocycler (Master Cycler, Eppendorf) programmed for an initial denaturation 1 × 95 °C for 5 min followed by 35 × (95 °C for 1min, annealing temperatures of respective primers for 1 min, 72 °C for 1min) and final primer extension step

1 × 72 °C for 10 min. The amplified products were separated on 1% agarose gel electrophoresis, carried out at a constant current of 400 mA, 110V. Images of agarose gels were analyzed by manually transforming the scored DNA fragments obtained into binary data matrix- by scoring as presence (1) or absence (0) for each isolate and represented in Table-5.2. Clustering and dendrogram construction based on similarity coefficient were carried out with the software NTSYS pc version 2.0.

Samples which varied in amplicons size from the reported ones were selected for sequencing. The amplified products were separated on 1% agarose gel, purified using PCR clean up kit (Sigma) and single pass sequencing was carried out at Xcelris Labs Ltd, Ahmedabad, India. All sequences obtained were matched with the database in Genbank using the BLAST algorithm and processed using the softwares Bioedit, ClustalW and Mega 4.

Table 5.1 Primers of the virulent and quorum sensing gene markers

Sl No	Genes	Primer Sequence	Bps	Tm
1	**Vhh1 (Vh-beta-Lactamase gene)** AF217649 Teo *et al*.,2000	**NP161F: 5' CGAGTGCAACGTACGCC3'** **NP161R: 5' CGAGCGCTAAATAGTCTTGT 3'**	**1148**	**51° C**
2	**ToX R** AY247418 Conejero and Hedreyda2003	**NP162F: 5'ACTCAAGCCTTACTCAAGCGATTTCCA 3'** **NP162R: 5'TGACTTCGACTGGTGAAGACTCAGCA 3'**	**969, & 578**	**58° C**
3	**RpoS**	**NP167F: 5'AGTGGTTATGGCCAACAAAAGGGAGA 3'** **NP167R: 5'AGCAAGTCTTATGGTCTAGCGGTTGCT3'**	**1787**	**58° C**
4	**FlaB** EU240945 Bai *et al*.,2008	**NP164F: 5' AACGTATCAGCGATGACC 3'** **NP164R: 5' TTGAAACGGTTCTGGAAT3'**	**923**	**58° C**

5	**Zinc Metalloprotease** Bai *et al*.,2008	**NP179F: 5' AAATCATTCCAAATCGGTGC 3'** **NP179R: 5' TCTTTGATTCGGCTCTTA3'**	**611**	**60° C**
6	**clp serine protease** Bai *et al*.,2008	**NP175F : 5'TACCAAGAAAAAAACGCAATGTCGCC 3'** **NP175R: 5'GTGGCTCAACACTGCATCCACAAT 3'**	**578**	**60° C**
7	**Cysteine protease** Bai *et al*.,2008	**NP176F: 5'ATTCATGACCAACGTATTCTGATCT 3'** **NP176R: 5'TATTCCCATTCGATTGTCGCTGGTGGCTTA 3'**	**1554**	**60° C**
8	**Type Two Secretion** Bai *et al*.,2008	**NP177F: 5'TGGAAGTATTCCAGTACTACCCATGGCT 3'** **NP177R: 5'TACACTCCTAGAATCGACGTAAAGTACCAG 3'**	**869**	**60° C**
9	**Lux M** VIBLUXLMN Bassler *et al*., 1993	**NP226F: 5' CTCGCTGTCGGTAACAG 3'** **NP169R: 5' CCTTCGCATCGATAGCTC3'**	**282**	**51° C**
10	**Lux N** VIBLUXLMN Bassler *et al*., 1993	**NP170F: 5' CTGTGTACTCACTGTTTATC 3'** **NP170R: 5' GTCTAATTCGCGTTCTCCA 3'**	**2048**	**51° C**
11	**Lux L** VIBLUXLMN Bassler *et al*., 1993	**NP169F: 5' CTGTGTACTCACTGTTTATC 3'** **NP226R: 5' GTCTAATTCGCGTTCTCCA 3'**	**393**	**51° C**
12	**LuxA** EU201035 O'Grady and Wimpee	**NP168F: 5'ATTCCGTTTTGGTATTTGTCGCGGTT 3'** **NP168R: 5'AACAAATATTGTCAATACCCGTCGCA 3'**	**683**	**51° C**
13	**LuxP** U07069 Bassler *et al*., 1994	**NP277-F: 5' TGAAGAAAGCGTTACTATTTTCCCT 3'** **NP277-R: 5'ATTATCTGAATATCTAAATGCGCGCTT 3'**	**1097**	**51° C**
14	**LuxO** L2622	**NP275F: 5'ACGAAGACGAGCGTGTTCTTGTGAC 3'** **NP275 R :**	**2559**	**57.5° C**

	Bassler *et al*., 1994	**5'AATACGTCCGTATTCATACGTTTTGTTTTT 3'**		
15	**Lux U** L2622 Bassler *et al*., 1994	**NP276F: 5'TGCAAAACGTATTGCGTAATATCGT 3'** **NP276R: 5'TCCAAGAACGGTAGGCGTCACGAGT 3'**	**639**	**54.3° C**
16	**LuxR** DQ108980 Miyamoto & Meighen	**NP274F: 5'AGAATTCACGAATACGTTCCTG3'** **NP274R: 5'GCCTAGTACGAGGTCTCTTGCAATTGAGTCC-3'**	**420**	**62° C**
17	**LuxS** AF120098 Surette *et al*., 1999	**NP279F: 5'TGCCTTTATTAGACAGCTTTACCGTAGA 3'** **NP279R: 5'TAGTCGATGCGTAGCTCTCTCAGCA**	**519**	**55.8° C**
18	**LuxD** J03950 Miyamoto *et al*., 1988	**NP280F: 5'AGGAAAATTACAATGAATAATCAATGCAAG 3'** **NP280R: 5'TAAGCCATTTCTGGCGTACGGCTT 3'**	**936**	**59.2° C**
19	**OmpK**	**NP287F: 5'TGCGTAAATCACTTTTAGCTCTTAGCC 3'** **NP287R: 5'AGAACTTGTAAGTTACTGCGATGTAGTGAC 3'**	**812**	**60° C**
20	**VhhP** FJ025787 Sun *et al*., 2009	**NP288F: 5' TGGATGTAAATGAGTTTGG3'** **NP288R: 5'CGTTACGATTATTTGATAG3'**	**588**	**50° C**
21	**ToxS** EU240944 Bai *et al*.,2008	**NP272 F: 5'ACTGGCGGACAAAATAACCAGCTGA 3'** **NP272R: 5'ACAGTACCGTAGAACCGTGATTCAAGCTAG 3'**	**640**	**50° C**
22	**Hly**	**NP562F :5'CGATTGGGAATGGGCAGAAAATC3'** **NP562R :5'TTTGAGAAGTGTCCCAAGTGTCCCAAGAACCAGC3'**	**360**	**57° C**
23	**FlaC** EU240947 Bai *et al*.,2008	**NP282F: 5'ATCATTCCAAATCGGTGCGGACTCA 3'** **NP282R : 5'TTTGATTCGGCTCTTAGACGCGT**	**578**	**60° C**

		TA 3'		
24	**VcrR** AY524044 Henke and Bassler .2004	**NP284F:** **5'TACTTTTTTCTCTTTTTAAGTGGG CGGT 3'** **NP284R:** **5'TGGAATGCCTTCTCACTGAGTCT CTAGT 3'**	**413**	**57.2° C**
25	**VScN** AY524044 Henke and Bassler .2004	**NP285F:** **5'AACGACTTCTCATACATCACCGA AAC 3'** **NP285R:** **5'CATAAGCCTGCAAGCCCACGC 3'**	**1322**	**58° C**
26	**VopD** AY524044 Henke and Bassler .2004	**NP283F:** **5'GCGAAGCTTACTGGACGCGCTGA CCTTTAC-3'** **NP283R:** **5'GCGCTCGAGTACCGTAGGGATA GAGGC-3'**	**1004**	**58° C**
27	**VopN** AY524044 Henke and Bassler .2004	**NP284F:** **5'AGTAACTTGATAAGCCATAGTTT GCCTGCT 3'** **NP284R:** **5'ACTATCAATAGCCAAATTTTAAC GGGCA 3'**	**893**	**58° C**
28	**Chi A**	**NP565F:5'TAATGCTAGATGAACTTG AAGCAGAAACA3'** **NP565R:5'ATTAGCTCACCAGTCGAA CGGTTCCA3'**	**600**	**60° C**

5.2.2. Determination of the protein profile of *V.harveyi* by SDS-PAGE

The proteins of the isolate of *Vibrio harveyi* (V3) were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS –PAGE) following the method of Laemmli (1970) using 4% stacking gel and 15 % resolving gel at a constant current of 12mA. After electrophoresis, gels were stained with 0.025 % Coomassie brilliant blue stain R-250 and then destained in a solution of 5% methanol and 7% acetic acid and visualized in a gel doc system. Molecular weight for the protein band obtained was determined by comparing with the molecular weight standards from Bangalore Genei. The size of the bands obtained was compared with those of references.

5.2.3. Pathogenicity assay of *V.harveyi* on Gnotobiotic *Artemia* nauplii

Preparation of Gnotobiotic *Artemia* nauplii*:* Gnotobiotic *Artemia nauplii* were reared according to the methodology proposed by Sorgeloos *et al* (1977). About 2g of *Artemia* cyst was immersed in tap water for 10min and kept in continuous suspension by aeration through sterilized cartridge filters. All equipments used for rearing gnotobiotic artemia were previously sterilized. The *Artemia* cysts were disinfected with 20% sodium hypochlorite solution for 1 hour. The cysts were sieved using 112µm sieve and washed thoroughly to remove sodium hypochlorite solution. The cysts were kept for hatching in sterile 28ppt seawater with adequate aeration using air sparger and the required temperature was provided by 60W bulb. As the chorion dissolved, a gradual colour change was observed in the cyst from dark brown via white to orange. When the colour changed to orange the decapsulated cysts were filtered immediately on a 100µm sieve. The decapsulated cysts were thoroughly washed with filtered and autoclaved sea water to remove the residual chlorine. *Artemia* nauplii hatched out over night were collected in a 120µm sieve and washed thoroughly with filtered autoclaved seawater.

Disinfection of the nauplii: After the nauplii have hatched out from the cyst, it was treated with 2ml of Penicillin- Streptomycin added to 1L sterile 28ppt seawater and then incubated for 6hrs. Presence of antibiotic residue was evaluated by homogenizing the nauplii prior to washing with 15ppt sterile seawater. Overnight incubated *V.harveyi* was swabbed onto ZoBell's 2216E agar plate and autoclaved Whatmann's filter paper disc impregnated with 20µl of macerated nauplii suspension and was incubated for 24hrs. After 24hrs, the zone of clearance was determined.

Mortality assay: The remaining nauplii were washed with sterile 28ppt seawater and 300 nauplii each were introduced into 15ml sterile 28ppt seawater with 0.01% peptone in test tubes, followed by inoculating 10^7

cfu/ml of 12 selected isolates. Uninoculated controls were also maintained and incubated the tubes in a shaking water bath for 24hrs at 35ºC and at100rpm. After 6hrs of incubation, the dead nauplii, settled at the bottom of the tubes were collected and counted. The live and moribund nauplii were washed with sterile 28ppt seawater, macerated, serially diluted and 100µL were spread plated onto ZoBell's 2216e agar and TCBS plates and incubated for 24hrs. After 24hrs incubation, the colonies formed in both the plates were counted.

5.2.4. Pathogenicity assay of *V.harveyi* using *P. monodon* Post larvae

To evaluate the pathogenicity of the isolates of *V.harveyi*, post larvae (PL-5) of *P.monodon* were obtained from Royal Plaza hatchey, Kodungallur, Thrissur. The PL were allowed to acclimatize in 25L tanks, fed with gnotobiotic *Artemia* and provided with aeration and illumination. PL were also disinfected with 2ml Penicillin- Streptomycin into 1L sterile 28ppt seawater and incubated for 24hrs. Presence of antibiotic residue was evaluated by homogenizing the PL after washing with 15ppt sterile seawater. Overnight incubated *V.harveyi* broth culture was swabbed onto ZoBell's 2216e agar plate and autoclaved Whatmann's filter paper disc impregnated with 20µl of macerated PL suspension was placed and incubated for 24hrs. After 24hrs, the zone of clearance was determined.

Mortality assay: Disinfected PL were washed, counted and 30nos were added into 1L autoclaved seawater taken into 3L capacity round bottom plastic containers with proper aeration and illumination (Fig. 5.2). PL in all the containers were fed uniformly with freshly hatched gnotobiotic *Artemia* nauplii (6-8 nauplii/mL). PL was challenged with bacterial suspension of 10^9cfu/ml to a final concentration in the rearing bottles. Unchallenged control was also maintained. Larvae were assessed for mortality to determine the cumulative mortality at the end of the experiment. After 72hrs, the moribund larvae were collected, washed, homogenized and were

serially diluted. The dilutions 10^{-4}, 10^{-6} and 10^{-8} were spread plated onto ZoBell's 2216e agar and TCBS plates and incubated for 24hrs. After 24hrs incubation, the colonies formed in both the plates were counted.

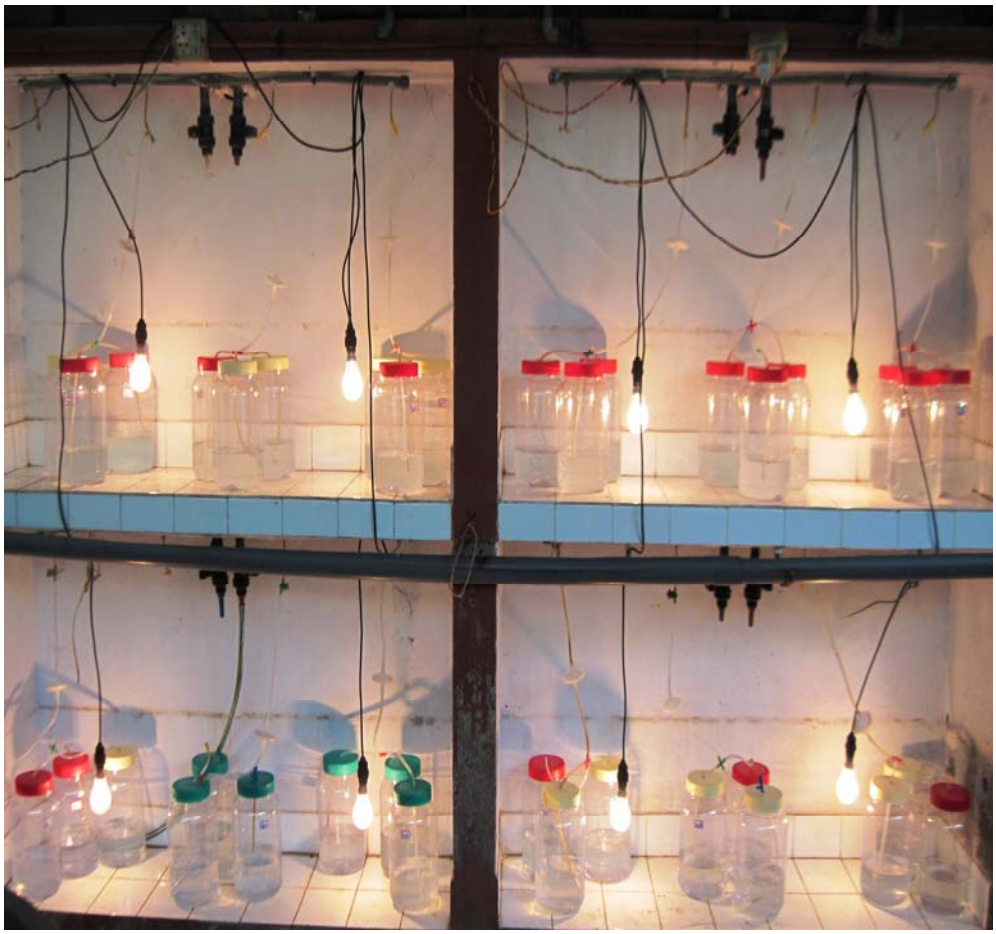


Fig: 5.2 Bioassay set up for determining the pathogenicity to post larvae (PL-5) of *P.monodon* upon challenge with the 12 isolates of *V.harveyi*

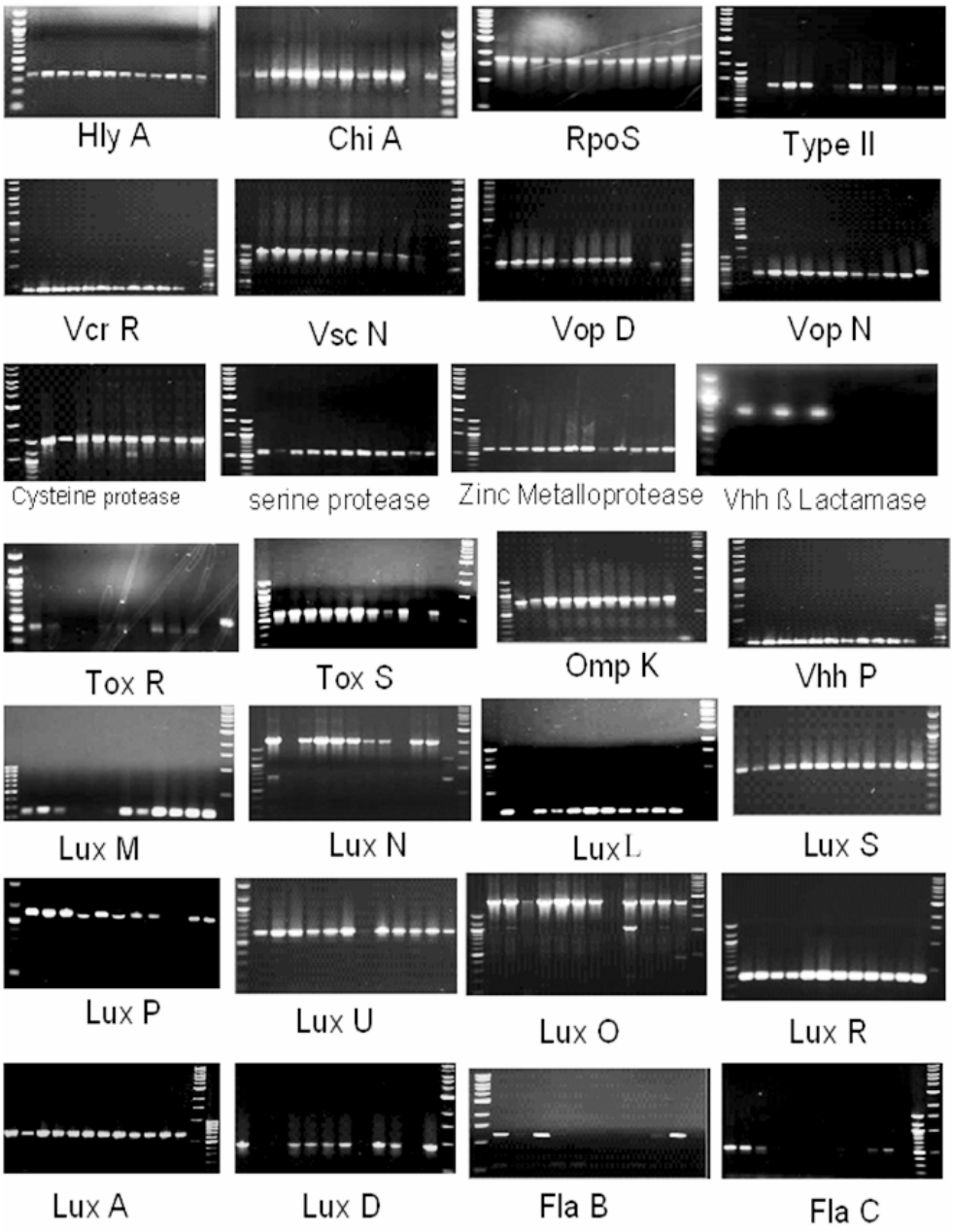
Monitoring water quality parameters: To confirm that pathogenicity to PL was caused by *V.harveyi* infection and not by the accumulation of ammonia and nitrite in the experimental bottles, water quality parameters like ammonia and nitrite were recorded at every 24hrs interval for 72hrs. No water exchange was made during the period of the experiment.

5.3. Results:

5.3.1. Banding pattern of the gene markers

Majority of the virulent and luminescent genes investigated could be amplified using the genomic DNA of the 12 isolates of *V. harveyi*. Among them *V. harveyi* V3 was positive for all the 28 genes amplified, followed by V45 (26 amplicons), V88 and by V57 (25 amplicons), V36, V54 and by V76 (24 amplicons) V28 and V71 (23 amplicons) and by V81 (22amplicons). From the type strain *V.harveyi* (LMG 4044) 17 amplicons alone could be obtained (Fig- 5.2 and Tables- 5.2), suggesting that *V.harveyi* V3 was the most potent pathogen out of the 12 representative isolates whereas V81 along with *V.harveyi* (LMG 4044) were the least potent ones.

Dendrogram constructed based on the amplicons obtained for the 28 selected gene markers revealed that all the wild isolates of *V.harveyi* shared homogeneity and were related to each other ≥78%. The isolates of *V.harveyi* V3 and V45 were closely placed in the dendrogram, suggesting that these isolates have the maximum genetic relatedness. The type strain of *V.harveyi* LMG 4044 was placed away from the wild strains, but joined to the latter at 60%S. This variation was mainly because of the type strain of *V.harveyi* failed to give amplicons for 11 out of the 28 genes analysed.



Lane 1- *V.harveyi* (V3), Lane 2- *V.harveyi* (V28), Lane 3- *V.harveyi* (V36), Lane 4- *V.harveyi* (V45), Lane 5- *V.harveyi* (V54), Lane 6- *V.harveyi* (V57), Lane7 - *V.harveyi* (V64), Lane 8- *V.harveyi* (V71), Lane 9- *V.harveyi* (V76), Lane 10- *V.harveyi* (V81), Lane 11- *V.harveyi* (V88), Lane 12- *V.harveyi* (VhL- LMG 4044).

Fig 5.3 Amplicons obtained with virulent and quorum sensing gene markers with the representative isolates and type strain of *Vibrio harveyi*.

Table: 5.2 Scoring the amplification obtained with Virulent and Quorum sensing gene primers for the isolates of *V.harveyi*

Sl No	Virulent genes	V3	V28	V36	V45	V54	V57	V64	V71	V76	V81	V88	VhL
1	HlyA	1	1	1	1	1	1	1	1	1	1	1	1
2	ChiA	1	1	1	1	1	1	1	1	1	1	0	1
3	RpoS	1	1	1	1	1	1	1	1	1	1	1	1
4	Type II secretion	1	0	1	1	1	1	1	0	1	0	1	1
5	VcrR	1	1	1	1	1	1	1	1	1	1	1	0
6	VscN	1	1	1	1	1	1	1	1	1	1	0	0
7	VopD	1	1	1	1	1	1	1	1	1	0	1	0
8	VopN	1	1	1	1	1	1	1	1	1	1	1	1
9	Cysteine Protease	1	1	1	1	1	1	0	1	1	1	1	1
10	Clp Serine protease	1	1	1	1	1	0	1	1	1	1	1	1
11	Zinc Metalloprotease	1	1	1	1	0	0	1	1	1	1	1	1
12	Vhh- βlactamase	1	0	0	0	0	1	0	0	0	0	1	0
13	ToxR	1	1	0	0	1	1	0	1	1	1	1	1
14	ToxS	1	1	1	1	1	1	1	1	1	0	1	0
15	OmpK	1	1	1	1	1	1	1	1	1	1	1	0
16	VhhP	1	1	1	1	1	1	1	1	1	1	1	0
17	LuxM	1	0	1	1	1	1	1	1	1	1	1	1
18	LuxN	1	0	1	1	1	1	1	1	0	1	1	0
19	LuxL	1	1	1	1	1	1	1	1	1	1	1	0
20	LuxS	1	1	1	1	1	1	1	1	1	1	1	1
21	LuxP	1	1	1	1	1	1	1	1	0	0	1	1
22	LuxU	1	1	1	1	1	1	0	1	1	1	1	1
23	LuxO	1	1	0	1	1	1	1	0	1	1	1	1
24	LuxR	1	1	1	1	1	1	1	1	1	1	1	1
25	LuxA	1	1	1	1	1	1	1	1	1	1	1	1
26	LuxD	1	0	0	1	1	1	1	0	1	1	0	1
27	FlaB	1	1	1	1	0	1	1	1	1	1	1	0
28	FlaC	1	1	1	1	0	0	0	0	0	0	1	0
	TOTAL	28	23	24	26	24	25	23	23	24	22	25	17

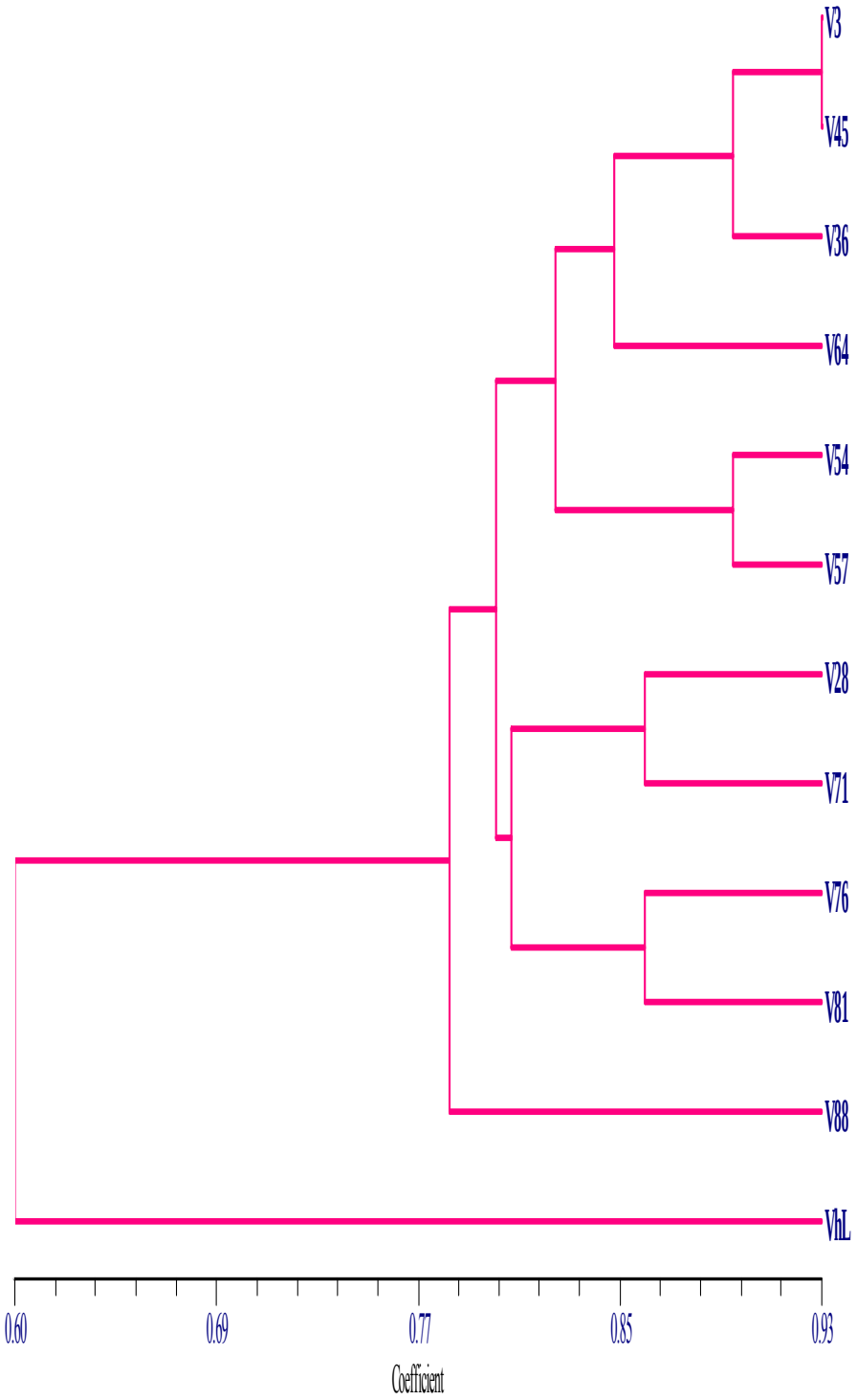


Fig: 5.4 Dendrogram of the isolates constructed based on the amplification to the selected gene markers

5.3.2. Sequence analysis of the virulent and luminescent genes

The amplicons obtained using the respective primers were sequenced and BLAST analysed and the evolutionary history of the strain was inferred using the UPGMA method (Sneath and Sokar, 1993). Phylogenetic analyses were conducted in MEGA4 and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was determined (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The results of BLAST Analysis are given in Table-5.3.

Table: 5.3 Details of BLAST Analysis of the gene markers selected based on the variations in the amplicons size already reported.

Sl. No:	Primer code	Identity to GenBank deposits
1	NP170F/R (Lux N)	a) 88%S to *V.harveyi* ATCC BAA 1116, Chromosome1, complete sequence (CP000789.1) b) 88%S to *V.harveyi LuxL,M,N* (L13940.1) c) 89%S to *V.campbellii LuxN* (FM212935.1) d) 88%S to *V.campbellii LuxN* (FM212936.1)
2	NP275F/R (LuxO)	a) 98%S to *V.harveyi* ATCC BAA 1116, Chromosome1, complete sequence (CP000789.1) b) 98%S to *V.harveyi* repressor protein (LuxO) (L26221.2) c) 83%S to Vibrio sp. Ex 25 Chromosome1 (LuxO) (CP001805.1) d) 76%S to *Photobacterium profundum* SS9 (LuxO) (CR378666.1) e) 84%S to *V.alginolyticus* MVP01 repressor protein of LuxO (DQ499603.1)
3	NP175F/R (Clp Serine	a) 97%S to *V.harveyi* strain ATCC BAA-1116 chromosome I,

	Protease)	(CP000789.1) b) 92%S to Vibrio sp. Ex25 chromosome 1(CP001805.1) c) 92%S to *Vibrio parahaemolyticus* RIMD 2210633 DNA, chromosome 1 (BA000031.2) d) 85%S to *Vibrio vulnificus* chromosome I YJ016 DNA (BA000037.2), CMCP6 (AE016795.3), MO6-24/O (CP002469.1) e) 84% S *Vibrio splendidus* LGP32 chromosome 1 (FM954972.2) f) 84% S *Vibrio furnissii* NCTC 11218 chromosome 1 (CP002377.1) g) 83%S *Vibrio cholerae* MJ-1236 chromosome 1 (CP001485.1)
4	NP179F/R (Zinc Metalloprotease)	a) 97%S to *Vibrio harveyi* ATCC BAA-1116 chromosome I (CP000789.1) b) 91%S to *Vibrio parahaemolyticus* RIMD 2210633 DNA, chromosome 1 (BA000031.2) & Vibrio sp. Ex25 chromosome 1 (CP001805.1) c) 85%S to *Vibrio vulnificus* YJ016 DNA, chromosome I (BA000037.2), *Vibrio vulnificus* CMCP6 chromosome I (AE016795.3) &*Vibrio vulnificus* MO6-24/O chromosome I (CP002469.1) d) 84%S to *Vibrio splendidus* LGP32 chromosome 1 (FM954972.2) e) 83%S to Vibrio cholerae MJ-1236 chromosome 1 (CP001485.1) & *Vibrio cholerae* O1 biovar eltor str. N16961 chromosome I (AE003852.1)
5	NP176F/R (cysteine protease)	a) 98%S to *V.harveyi* ATCC BAA 1116, Chromosome1, complete sequence (CP000789.1) b) 89%S to Vibrio sp. Ex 25 Chromosome1, complete sequence (CP001805.1) c) 90%S to *V.parahaemolyticus* RIMD 2210633 DNA Chromosome1

		(BA000031.2) d) 92%S to *V.vulnificus* YJ016 DNA Chromosome1 (BA000037.2) e) 91%S to *V.vulnificus* CMCP 6 Chromosome1 (AE016795.3) f) 91%S to *V.vulnificus* MO6-24/0 Chromosome1 (CP002469.1) f) 84%S to *V.splendidus* LGP32 Chromosome1 (FM954972.2)
6	NP283F/R (VopD)	a) 97%S to *V.harveyi* ATCC BAA 1116, Chromosome1, complete sequence (CP000789.1) , hypothetical/ putative protein region b) 97%S to *V.harveyi* type three secretion locus (AY524044.1)
7	NP284F/R (VopN)	a) 98%S to *V.harveyi* ATCC BAA 1116, Chromosome1, complete sequence (CP000789.1) , hypothetical/ putative protein region b) 98%S to *V.harveyi* type three secretion locus (AY524044.1) c) 95%S to *Photobacterium damselae* subsp. piscicida clone pPD27 type III secretion system gene (AY647223.1) d) 79%S to V.parahaemolyticus RIMD 2210633 DNA, chromosome1 (BA000031.2)
8	NP285F/R (VcrR)	a) 98%S to *Vibrio harveyi* ATCC BAA-1116 chromosome I (CP000789.1) b) 98%S to *Vibrio harveyi* type III secretion locus (AY524044.1) c) 76%S to Vibrio sp. Ex25 chromosome 1 (CP001805.1)
9	NP286 (VscN)	a) 96%S to *Vibrio harveyi* ATCC BAA-1116 chromosome I (CP000789.1), b) *Vibrio harveyi* type III secretion locus (AY524944.1) and c) *Photobacterium damselae subsp. piscicida* clone pPD27 type III secretion system gene cluster (AY647223.1)
10	NP562F/R (HlyA)	a) 98%S to *Vibrio campbellii* vcamhly gene for hemolysin, (AB271112.1),

		(AB271111.1), (AB271110.1), (AB271109.1), (CP000790.1) b) 97%S to *Vibrio harveyi* strain VH34 hemolysin gene (EU827170.1) c) 97%S to *Vibrio campbellii* strain VIB 285 VHH/TLH hemolysin gene (DQ663484.1) d) 97%S to *Vibrio campbellii* CAIM 519T hemolysin gene (vch) (DQ434995.1) e) 97% S to *Vibrio campbellii* strain NBRC 15631 hemolysin (vch) gene (DQ356918.1)
11	NP288 (VhhP2)	97%S to *Vibrio harveyi* ATCC BAA-1116 chromosome I (CP000789.1)
12	NP162 (toxR)	a) 93%S to *Vibrio harveyi* ATCC BAA-1116 chromosome I (CP000789.1) b) 88%S to *Vibrio parahaemolyticus* RIMD 2210633 DNA, chromosome 1 (BA000031.2) c) 79%S to *Vibrio vulnificus* YJ016 DNA, chromosome I (BA000037.2) d) 78%S to *Vibrio vulnificus* CMCP6 chromosome I (AE016795.3) and *Vibrio vulnificus* MO6-24/O chromosome I (CP002469.1)
13	NP272F/R (ToxS)	a) 100%S to *V.harveyi* ATCC BAA 1116, Chromosome1, complete sequence (CP000789.1) b) 87%S to *V.harveyi* VIB400, ToxS sequence (EU240943.1)
14	NP287F/R (OmpK)	a) 97%S to *V.harveyi* strain EiGR021101 outer membrane protein K (ompK) gene (GU318328.1) b) 96%S to *Vibrio harveyi* strain NB1014 outer membrane protein (ompk) gene (DQ279076.1) c) 95%S to *Vibrio harveyi* FJXUE2 outer membrane protein K (ompK) gene (GU318329.1) d) 92%S to *Vibrio harveyi* outer membrane protein precursor (ompK) gene (AY332563.1) e) 91%S to *V.harveyi Vibrio harveyi*

		strain EsHS020801 outer membrane protein K (ompK) gene (GU318336.1), *Vibrio harveyi* strain SpGY020601 outer membrane protein K (ompK) gene (DQ279075.1) e) 91%S to *Vibrio fluvialis* strain 1.1533 OmpK (ompK) gene (FJ462705.1) f) 91%S to *Vibrio parahaemolyticus* strain STO11 outer membrane protein OmpK gene (FJ394376.1)
15	NP177F/R (type three secretion loci)	93%S to *V.harveyi* ATCC BAA 1116, Chromosome1, complete sequence (CP000789.1), hypothetical/ putative protein region

(Appendix – 2: Nucleotide and Protein sequences)

5.3.3. Determination of proteins of *V.harveyi* (V3)

The outer membrane of Gram-negative pathogenic bacteria has an important role in the interaction between bacteria with hosts in adherence, uptake of nutrients from the host, and altering the host defense mechanisms (Ningqiu *et al*., 2008). From this study the isolate of V. harveyi (V3) was identified as the most potent pathogenic strain, and protein profile of this isolate was carried out to determine the presence of outer membrane proteins. The SDS – PAGE of the proteins extracted from *V. harveyi* yielded bands having the molecular weights 29,000 KDa, 35,000 KDa, 38,000 KDa, 43,000 KDa, 47,000 KDa and 52,000KDa. These bands were compared with those in literature stating the molecular weight of the outer membrane proteins of *V.harveyi*.

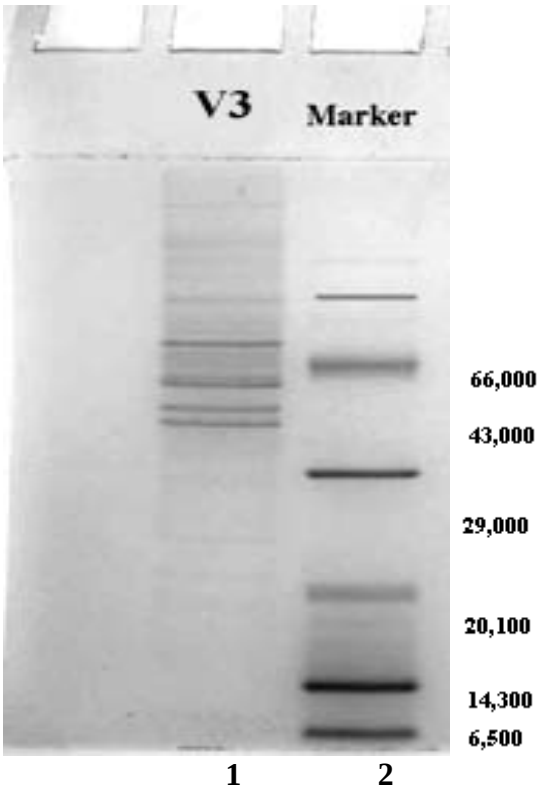


Fig: 5.5 SDS PAGE of *Vibrio harveyi* (V3) Lane1- The protein profile of *Vibrio harveyi* isolate V3. Lane 2- Molecular mass marker.

5.3.4. Pathogenicity of *V.harveyi* on gnotobiotic *Artemia* nauplii

The test of infectivity of *V.harveyi* carried out on Gnotobiotic *Artemia* nauplii showed that the type stain, *V.harveyi* LMG 4044, was associated with the lowest mortality of 17.8% (Fig- 5.6). On plating the macerated larvae suspension at the lowest count on ZoBell's 2216e and TCBS plates was obtained (Table-5.4). A positive correlation (0.8379r) was obtained between ZB+TCBS plate counts and per cent *Artemia* nauplii mortality, caused by the 12 isolates of *V.harveyi*. The isolate V3 on the other hand was associated with the highest cumulative mortality of 51.25% of *Artemia* nauplii, followed by the isolates V45 (50.25%) and V88 (45.4%) respectively. The other isolates were associated with the mortality of *Artemia* nauplii between 17.8 and 45.4%. The 12 isolates of *V.harveyi* exhibited a moderately positive correlation of 0.757r existed between per

cent *Artemia* nauplii mortality and number of amplicons of virulent and luminescent genes markers (Table-5.5 and Fig-5.7).

Table: 5.4 Mortality rate of *Artemia* nauplii challenged with 10^7 cfu/ml of *V.harveyi* and its relation to plate count

Isolates	ZB plate count (10^6 cfu/ml)	TCBS plate count (10^6 cfu/ml)	*Artemia* mortality%
V3	12.5	9.3	51.125
V28	3.6	4.2	18.0
V36	6.45	6.45	39.625
V45	9.15	9.55	50.25
V54	7.2	6.7	37.125
V57	7.35	5.9	40.625
V64	6.5	5.33	35.375
V71	3.02	2.6	19.0
V76	6.7	6.1	26.0
V81	6.55	6.5	20.875
V88	7.15	5.4	45.375
VhL	1.9	1.65	17.75

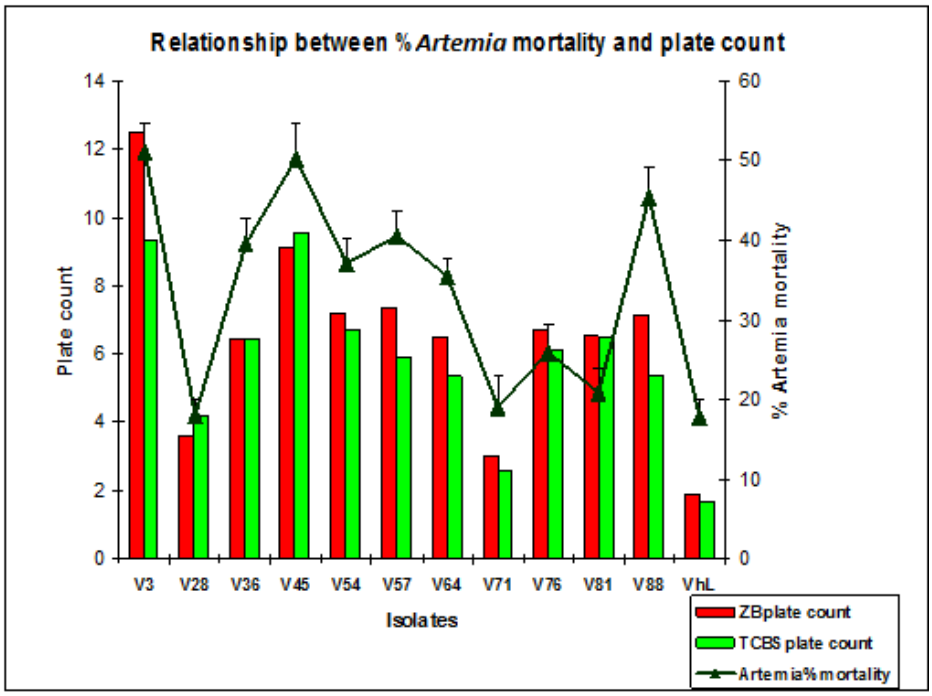


Fig: 5.6 Mortality rate of *Artemia* nauplii challenged with 10^7 cfu/ml of *V.harveyi* and its relation to plate count

Table: 5.5 Mortality of *Artemia* nauplii challenged with 10^7 cfu/ml of *V.harveyi* and its relation to plate count and number of amplicons obtained

Isolates	ZB+TCBS plate count (10^6 cfu/ml)	% mortality	Number of Amplicons
V3	21.8	51.125	28
V28	7.8	18	23
V36	12.9	39.625	24
V45	18.7	50.25	26
V54	13.9	37.125	24
V57	13.25	40.625	25
V64	11.83	35.375	23
V71	5.62	19	23
V76	12.8	26	24
V81	13.05	20.875	22
V88	12.55	45.375	24
VhL	3.55	17.75	17

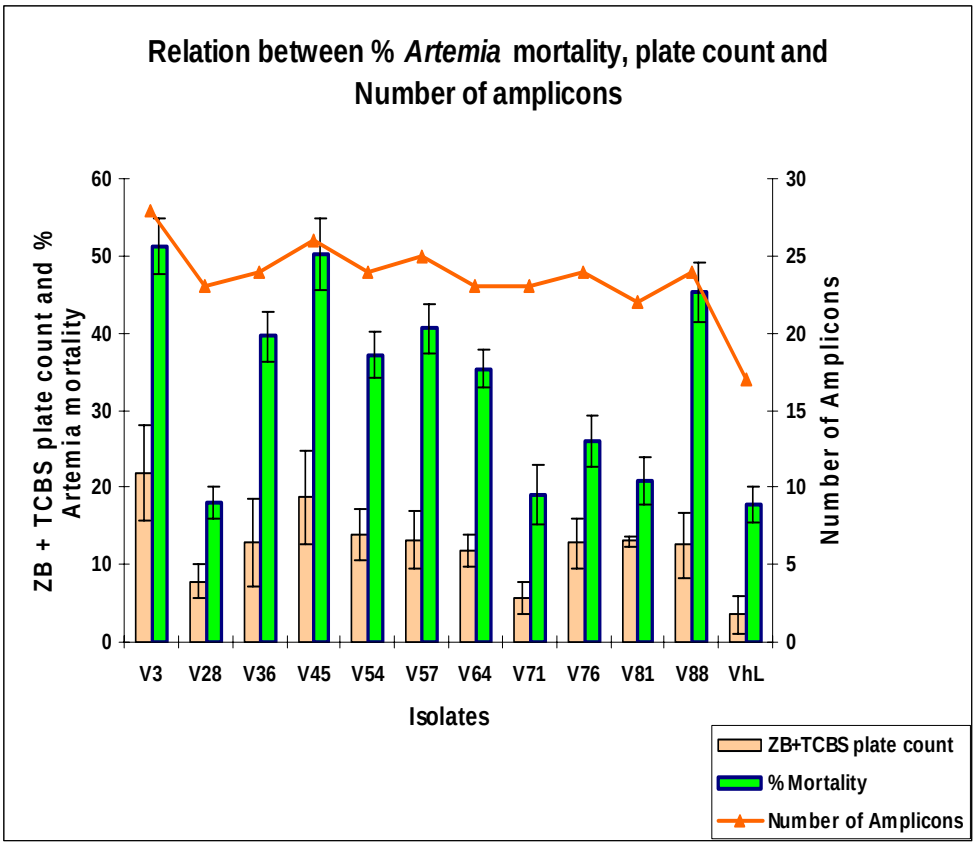


Fig: 5.7 Relationship between percentage *Artemia* mortality, plate count and number of amplicons exhibited by the isolates of *V.harveyi*

Correlation was determined between Plate count, % Mortality and Number of amplicons by Karl Pearson's method. A positive correlation of (0.8379), (0.8544) and (0.7575) existed between all the three parameters tested. The critical values of the correlation coefficient at degree of freedom between columns were very much acceptable at 0.05, 0.01 and 0.001 probabilities (Table- 5.6). Analysis of correlation between the isolates, given as challenge to gnotobiotic *Artemia* nauplii showed that a perfect positive correlation existed between all the isolates.

Table: 5.6 Karl Pearson's Correlation between mortality to *Artemia* nauplii, plate count and number of amplicons

Degree of Freedom	0.05	0.01	0.001	Observed values
Between columns (df = 2) Plate count and % Mortality	0.95	0.99	0.999	0.8379
Between columns (df = 2) Plate count and Number of amplicons	0.95	0.99	0.999	0.8544
Between columns (df = 2) % Mortality and Number of amplicons	0.95	0.99	0.999	0.7575

5.3.5. Pathogenicity assay of *V.harveyi* on Post larvae

Test of infectivity of *V.harveyi* on *P. monodon* post-larvae showed that isolates V3 and V88 were associated with 100% mortality, followed by V76 with 38.6% and the type strain *V. harveyi* LMG 4044 with 27.6% mortality, while all other wild isolates were associated with low level or no mortality at all, comparable almost to the uninoculated controls (Table 5.7 and Fig. 5.8).

Table: 5.7 Mortality rate of post-larvae challenged with 10^9 cfu/ml of *V.harveyi* and its relation to plate count

Isolates	ZB count (10^6 cfu/ml)	TCBS count (10^6 cfu/ml)	Mortality%
V3	31.33	18.66	100
V28	8.63	7.33	12.22
V36	8.63	2	15.53
V45	23.66	19.33	7.77
V54	7.53	4.66	0
V57	17	4.66	0
V64	5.33	4.33	0
V71	20.66	11	24.43
V76	12	11	38.66
V81	9	3.5	0
V88	41.33	30.66	100
VhL	7.66	12	27.63
Control	0.33	0.33	0

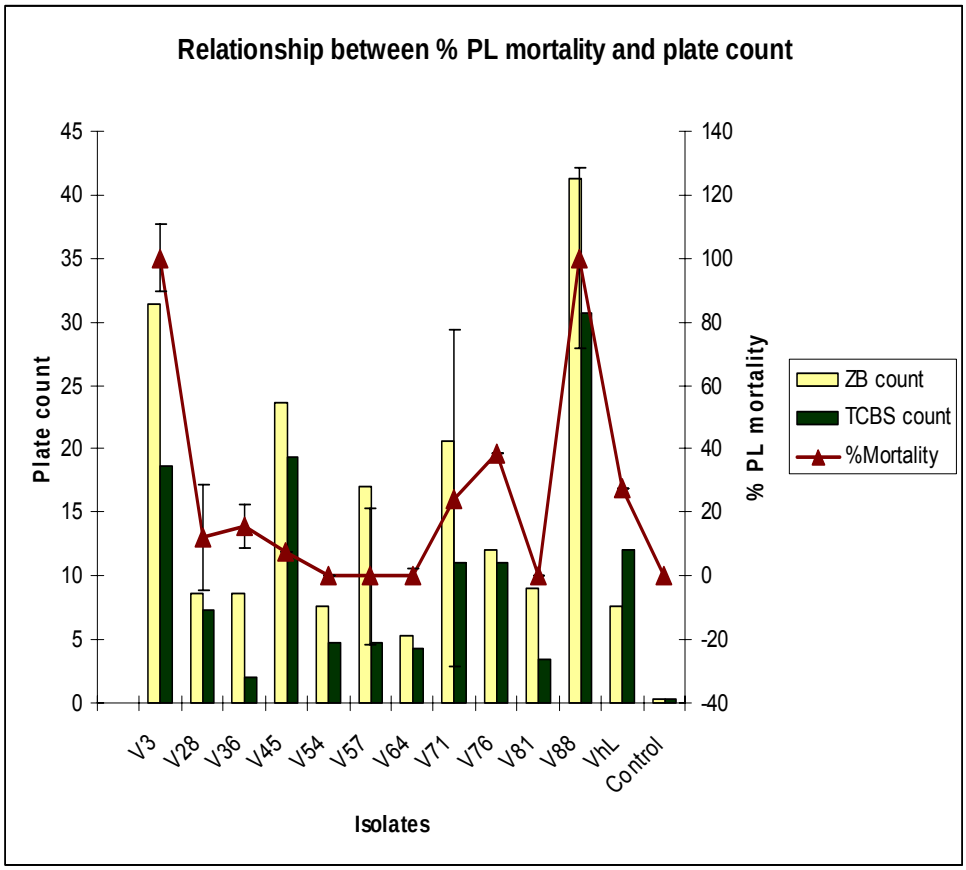


Fig: 5.8 Mortality rate of Postlarvae challenged with 10^9 cfu/ml of *V.harveyi* and its relation to plate count

Table: 5.8 Karl Pearson's Correlation between mortality to PL, plate count and number of amplicons

Degree of Freedom	0.05	0.01	0.001	Observed values
(df = 2) Between ZB Plate count and TCBS plate count	0.95	0.99	0.999	0.906
(df = 2) Between ZB Plate count and % PL Mortality	0.95	0.99	0.999	0.814
(df =2) Between TCBS plate count and % PL Mortality	0.95	0.99	0.999	0.812

Table: 5.9 Mortality rate of Postlarvae challenged with 10^9 cfu/ml of *V.harveyi* and its relation to plate count and number of amplicons obtained

Isolates	ZB+TCBS plate count (10^6 cfu/ml)	%Mortality	Number of Amplicons
V3	50	100	28
V28	15.97	12.22	23
V36	10.63	15.53	24
V45	43	7.77	26
V54	12.19	0	24
V57	21.67	0	25
V64	9.67	0	23
V71	31.67	24.43	23
V76	23	38.66	24
V81	12.5	0	22
V88	72	100	24
VhL	19.67	27.63	17
Control	0.67	0	0

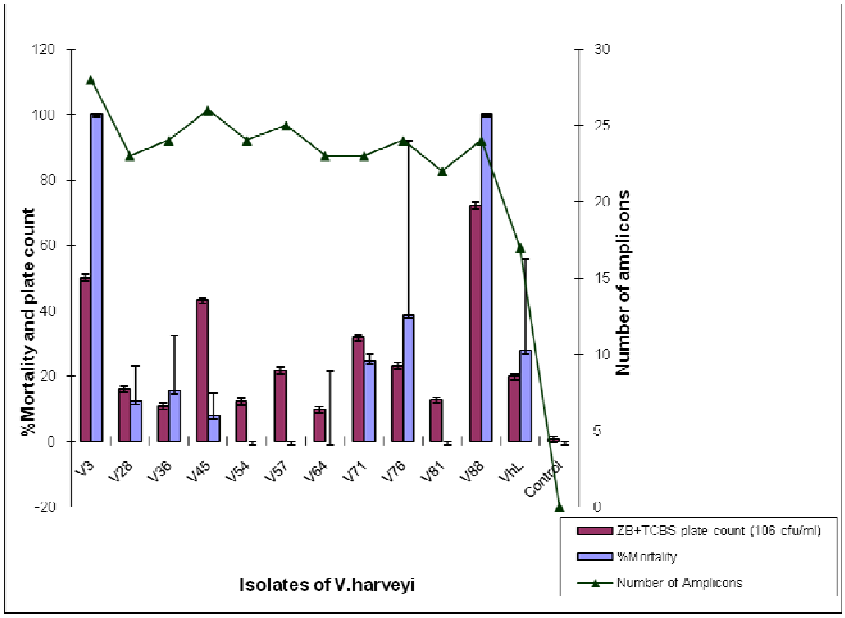


Fig: 5.9 Relationship between percentage *P. monodon* postlarval mortality, plate count and number of amplicons exhibited by the isolates of *V.harveyi*

Correlation coefficients determined by Karl Pearson's method at degree of freedom 2 between columns are very much acceptable at 0.05, 0.01 and 0.001 probabilities. A positive correlation (0.906r) was obtained between ZB and TCBS plate count. In the same way a positive correlation (0.814r) was obtained between ZoBell's plate count and % postlarval mortality (Table- 5.8). A positive correlation was obtained (0.812) between TCBS plate count and % Pl mortality. A low positive correlation of 0.477r was obtained between plate count and number of amplicons still a lower positive correlation of 0.306 was obtained between % mortality of PL and the number of amplicons of the pathogens (Table 5.9, 5.10 and Fig- 5.9)

Table: 5.10 Karl Pearson's Correlation between plate count and number of amplicons and % mortality of PL and number of amplicons

Degree of Freedom	0.05	0.01	0.001	Observed values
(df = 2) Between Plate count and % Mortality	0.95	0.99	0.999	0.814
(df = 2) Between Plate count and Number of amplicons	0.95	0.99	0.999	0.477
(df =2) Between % Mortality and Number of amplicons	0.95	0.99	0.999	0.306

5.3.6. Correlation of pathogenicity of the isolates to postlarvae with water quality

The water quality parameters like ammonia and nitrite were recorded at every 24hrs interval for 72hrs, to evaluate the change of water quality over a period of time and to confirm validity of the hypothesis that the bacterial challenge was responsible for the mortality caused (Table-5.11 and Fig- 5.10). Since no water exchange was provided during the period of the experiment, it was likely that the accumulation of ammonia and nitrite might have caused stress to the postlarvae creating a favourable environment for bacterial invasion and expression of pathogenicity. The results obtained suggested that the concentration of nitrite accumulated was very low, but ammonia was found to increase with time. pH stood within a range 7.5 to 8 and temperature at 30± 1 ºC. Hence a simple correlation coefficient was worked out between total ammonia and nitrite accumulated and % PL mortality, to determine whether ammonia and nitrite accumulated in the containers had any role in causing mortality to the postlarvae.

Table: 5.11 Nitrite and Ammonia accumulation in the containers with time progression

	Nitrite				Ammonia			
Isolates	**0hr**	**24hrs**	**48hrs**	**72hrs**	**0hr**	**24hrs**	**48hrs**	**72hrs**
V3	0	0.041	0.063	0.004	0.33	1.07	6.871	13.91
V28	0	0.049	0.042	0.013	0.438	1.592	7.679	14.94
V36	0	0.013	0.053	0.027	0.419	1.025	5.666	13.51
V45	0	0.018	0.063	0.026	0.259	1.128	8.353	13.45
V54	0	0.02	0.085	0.033	0.22	1.378	15.2	16.41
V57	0	0.033	0.048	0.045	0.37	1.262	7.83	10.22
V64	0	0.025	0.053	0.017	0.433	1.057	6.484	15.19
V71	0	0.039	0.068	0.023	0.385	1.244	4.122	14.38
V76	0	0.03	0.09	0.047	0.42	0.929	8.61	9.777
V81	0	0.006	0.05	0.051	0.31	0.829	12.9	11.85
V88	0	0.012	0.067	0.172	0.212	0.683	10.97	11.58
VhL	0	0.008	0.049	0.043	0.258	0.638	11.62	12.7
Control	0	0.025	0.007	0.024	0.033	0.342	1.161	0.747

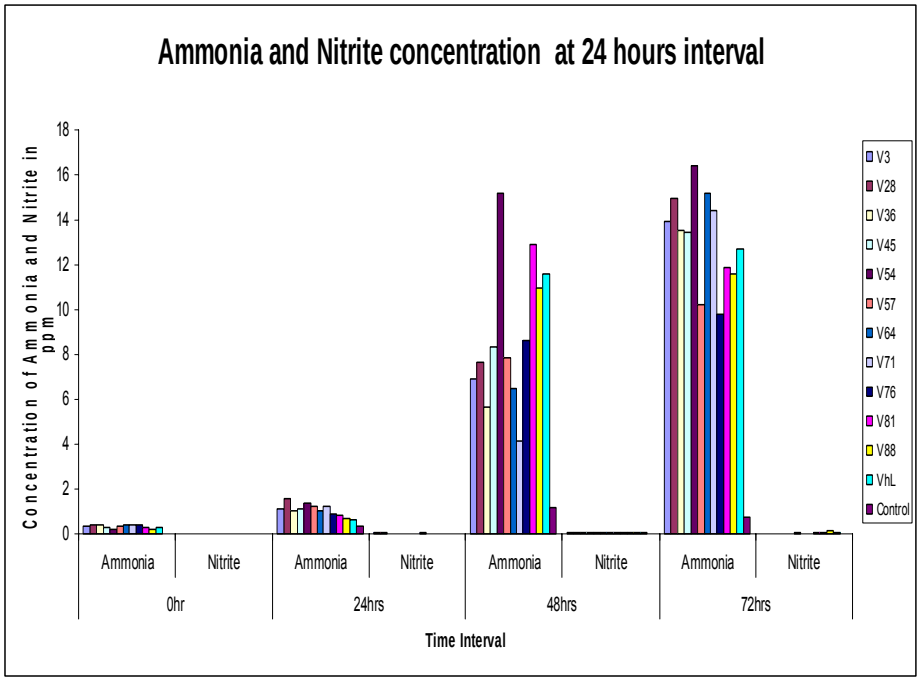


Fig: 5.10 Nitrite and ammonia accumulation in the containers with time progression

Table: 5.12 Relation between plate count, percentage PL mortality and final ammonia and nitrite accumulated at 72hr

Isolates	ZB+ TCBS plate count	% PL Mortality	Total Ammonia(ppm)	Nitrite (ppm)
V3	50	100	13.91	0.004
V28	15.97	12.22	14.94	0.013
V36	10.63	15.53	13.51	0.027
V45	43	7.77	13.45	0.026
V54	12.19	0	16.41	0.033
V57	21.67	0	10.22	0.045
V64	9.67	0	15.19	0.017
V71	31.67	24.43	14.38	0.023
V76	23	38.66	9.777	0.047
V81	12.5	0	11.85	0.051
V88	72	100	11.58	0.172
VhL	19.67	27.63	12.7	0.043
Control	0.67	0	0.747	0.024

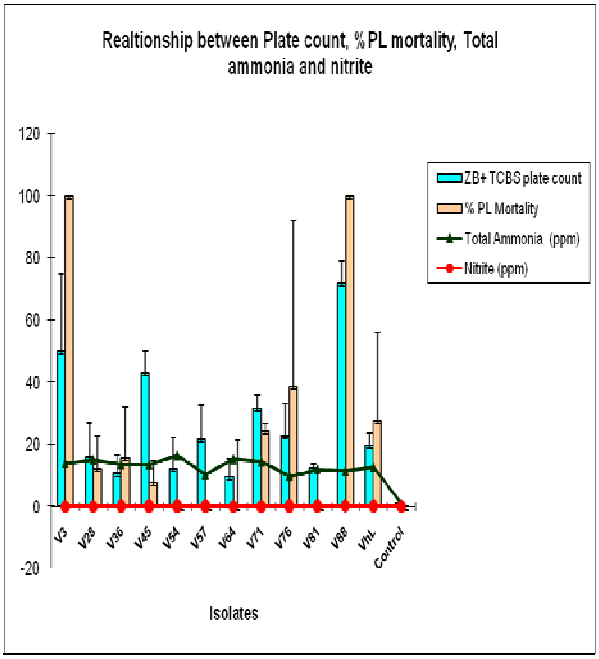


Fig: 5.11 Relation between plate count, percent PL mortality and final ammonia and nitrite accumulated at 72hr

Table: 5.13 Karl Pearson's Correlation between plate count percent PL mortality and final ammonia and nitrite accumulated at 72hr

Degree of Freedom	0.05	0.01	0.001	Observed values
b/w columns (df = 3) ZB and TCBS Plate count and % PL Mortality	0.878	0.959	0.991	0.833
b/w columns (df = 3) ZB and TCBS Plate count and 72hr Ammonia concentration	0.878	0.959	0.991	0.213
b/w columns (df = 3) % PL Mortality and 72hr Ammonia concentration	0.878	0.959	0.991	0.086
b/w columns (df = 3) ZB and TCBS Plate count and 72hr Nitrite concentration	0.878	0.959	0.991	0.605
b/w columns (df = 3) % PL Mortality and 72hr Nitrite concentration	0.878	0.959	0.991	0.495
b/w columns (df =3) 72hr Ammonia concentration and 72hr Nitrite concentration	0.878	0.959	0.991	-0.117

The critical values of the correlation coefficient at degree of freedom between columns are very much acceptable at 0.05, 0.01 and 0.001 probabilities. High positive correlation (0.833r) existed between ZB and TCBS plate count and % PL mortality. Meanwhile a weak positive correlation existed between % PL mortality and 72hr Ammonia concentration (0.086r) and a moderate positive correlation (0.495r) existed between 72hr Nitrite concentration and % mortality of PL. It has been noticed that the nitrite accumulation varied between 0.004 and 0.172ppm which is well below the toxic level to PL (Table- 5.12, 5.13, Fig- 5.11).

5.3.7. Correlation of pathogenicity with Amplification of the gene markers

Table: 5.14 Relation between mortality of *Artemia* and postlarvae challenged with *V.harveyi* with number of amplicons obtained

Isolates	% Artemia Mortality	%PL Mortality	Number of Amplicons
V3	51.25	100	28
V28	18	12.22	23
V36	39.625	15.53	24
V45	50.25	7.77	26
V54	37.1	0	24
V57	40.62	0	25
V64	35.4	0	23
V71	19	24.43	23
V76	26	38.66	24
V81	20.9	0	22
V88	45.4	100	25
VhL	17.8	27.63	17

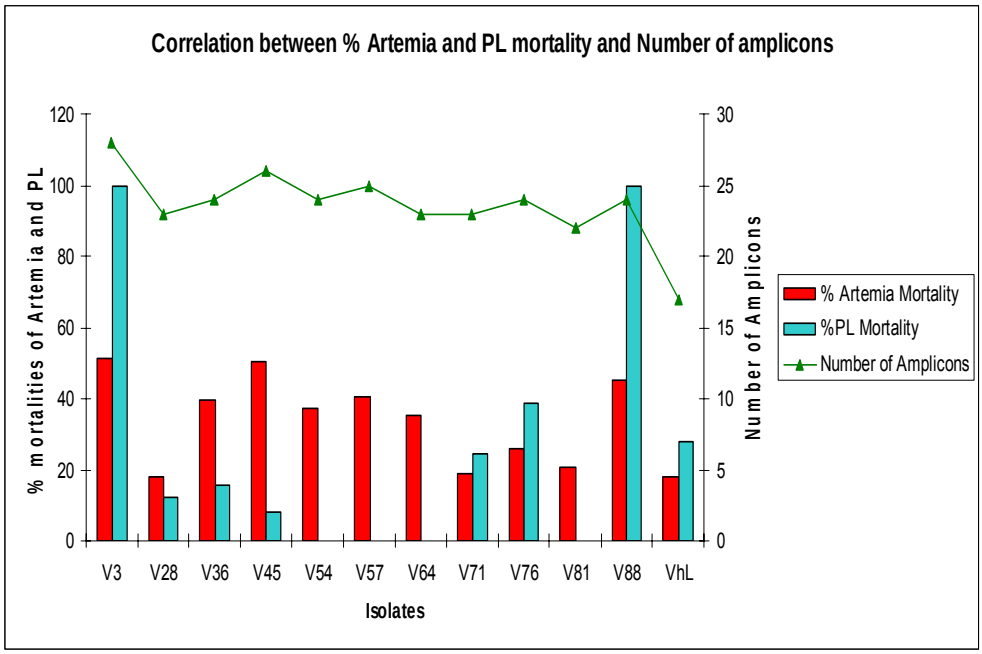


Fig:5.12 Relation between mortality of *Artemia* and post larvae challenged with *V.harveyi* with number of amplicons obtained

Correlation coefficients determined by Karl Pearson's method at degree of freedom 2 between columns are very much acceptable at 0.05, 0.01 & 0.001 probabilities. A moderate positive correlation of 0.386r was obtained between the % mortality of *Artemia* and PL when challenged with the isolates. In the same way a perfect positive correlation (0.757r) existed between % *Artemia* mortality and Number of Amplicons. Meanwhile a low positive correlation of 0.309r existed between the % PL mortality and Number of Amplicons of the isolates (Table- 5.14, 5.15, Fig- 5.12).

Table: 5.15 Karl Pearson's Correlation between %mortality to *Artemia* nauplii, % PL mortality and number of amplicons

Degree of Freedom	0.05	0.01	0.001	Observed values
(df = 2) Between % Artemia mortality and % PL mortality	0.95	0.99	0.999	0.386
(df = 2) Between % Artemia mortality and Number of Amplicons	0.95	0.99	0.999	0.757
(df = 2) Between % PL mortality and Number of Amplicons	0.95	0.99	0.999	0.309

5.4. Discussion

5.4.1. Genotypic characterization:

All 12 isolates of *V. harveyi* contributed positive amplicons for *hlyA*, *rpoS*, *vopN*, *luxS*, *luxR* and *luxA* genes, suggesting that these genes are conserved within the species. However, the presence of *hlyA* gene in all the isolates suggested their capability to cause haemolysis in animals under stressed conditions. This is supported by the lysis of haemocytes in prawn blood agar by all the isolates of *V. harveyi*. Presence of *luxS*, lux*R* and lux*A* genes in all the isolates suggested that the isolates mediated strong cell to cell communication by diffusible extracellular molecules or signals (auto inducers), enabling quorum sensing and indirectly favouring virulence expression (Bassler *et al*., 1997). Majority of the isolates except V54, V57 and V64 gave positive amplification to protease genes, suggesting that the presence of protease genes is linked with luminescence and virulence. The isolate V3 gave positive amplicons for all the 28 genes studied while the type strain of *V.harveyi* (LMG 4044) showed only 17 positive amplicons. Based on these observations, the isolate *V. harveyi* V3 has been ranked first as the most potent pathogen among the 11 representative isolates and the type strain. Assessing by the same pattern the isolate of *V. harveyi* V81 with 22 amplicons could be considered as the least pathogenic one and all other isolates positioned themselves in between.

Sequence of the amplicons obtained with the forward and reverse primers of LuxN,O, Clp-Serine Protease, Zinc Metalloprotease, Cysteine Protease, Type3-secretion genes, Type4-secretion genes (VopN and VcrR), toxR, toxS and Vhhp2 genes revealed that the *V.harveyi* isolate (V3) MCCB111 shared 93 to 100% similarity to complete sequence of *V.harveyi* ATCC BAA 1116, Chromosome1(CP000789.1). The sequences obtained using the primers NP177F/R (type three secretion loci) showed identity (93%) with only 1 GenBank deposit, while the primers NP272F/R (*ToxS*) and NP283F/R (*VopD* of type three secretion loci) showed identity to 2

GenBank deposits at 98%. The identity with limited number of GenBank deposits suggested the uniqueness of the isolate MCCB111 in terms of sequence analysis with *TTS*, *ToxS* and *VopD* gene primers; hence it deserves to be placed as a novel candidate in GenBank. The sequences obtained with the primers NP176F/R (cysteine protease), NP275F/R (LuxO) and NP284F/R (VopN of type three secretion loci) showed identity to isolates of *Vibrio* and *Photobacterium* species at ≥76%. Kita-Tsukamoto *et al*. (1993) stated that there is a high degree of genetic similarity across taxa. From this study it was found that the cysteine protease, *LuxO* and *VopN* genes were distributed among the members of family Vibrionaceae, without being restricted to *V.harveyi* or its core group alone, suggesting the cause of homogeneity and interspecies relatedness.

5.4.2. Analysis of proteins by SDS-PAGE

The SDS – PAGE of the proteins obtained from *V. harveyi* (V3) yielded bands corresponding to the molecular weights of the marker at 29kDa, 35kDa, 38kDa, 43kDa, 47kDa and 52kDa respectively. Similar results were obtained by Abdallah *et al*. (2009), according to whom the bands formed at 21KDa, 27KDa, 35KDa, 38 KDa, 43 KDa, 47 KDa and 52KDa were typical of the outer membrane proteins and flagellin. A 38-kDa OmpU protein of *Vibrio cholerae* is positively regulated by *toxR*, which also regulates critical virulence factors such as cholera toxin and the toxin-coregulated pilus colonization factor. This OmpU was found to play a role in the adhesion of *V.cholerae* to mammalian cells (Sperandio *et al*., 1995). The protein profile of the isolate of *V. harveyi* (V3) studies yielded bands of 38kDa and 29kDa which could be the outer membrane protein of the isolate and amplification of the *Omp*K gene with the primer for *Omp*K yielded a product of 812bps. According to Zhang *et al* (2007), OmpK or its homologs of molecular masses 25–29 kDa are widely distributed within *Vibrio* and *Photobacterium.* The outer membrane of Gram-negative pathogenic bacteria has an important role in the interaction between bacteria with hosts

in adherence, uptake of nutrients from the host, and subverting host defense mechanisms (Ningqiu *et al*., 2008). From this study, the isolate *V. harveyi* (V3) was identified as the most potent pathogenic strain which is capable of adhering effectively to the host cell, colonize and cause mortality which might be mediated by the activation of the *Omp*K gene. However, further works needs to be carried out to confirm that the *Omp*K gene of this virulent isolate is expressed or upregulated.

5.4.3. Pathogenicity assay of *V.harveyi*

Statistical analysis of the correlation between percentage mortality of *Artemia* nauplii and the number of amplicons in each mortality associated isolate revealed that a moderately positive correlation existed between them. Among them the isolate V3 which was positive for all the 28 virulent and luminescent marker genes was associated with the highest per cent mortality in *Artemia* nauplii. While the type strain *V.harveyi* (LMG 4044) from which 17 marker genes could be amplified was associated with the lowest mortality. Among the wild isolates V28 and V71, with which 23 marker gene amplicons could be obtained, were associated with 18 and 19% mortality, behaving similar to the type strain *V.harveyi* (LMG 4044). Meanwhile the other wild isolates with which 24-26 marker gene amplicons could be obtained were associated with moderate level of mortality (35-50%). Accordingly, based on the challenge on *Artemia* nauplii the 12 isolates could be differentiated into three groups as the one associated with highest mortality, the ones with lowest mortality comparable to that in the controls and the ones with moderate mortality. Also the reisolation of *V.harveyi* from moribund nauplii on to ZoBell's 2216E agar and TCBS agar plates suggested that the mortality of the nauplii was mediated by infection caused by the respective isolates. The type stain of *V.harveyi* LMG 4044, showed the lowest plate count and lowest mortality rate of the nauplii.

The challenge experiments carried out on PL-5 showed that the isolate V3 was again the most potent pathogen causing 100% mortality

within 72hrs of challenge. Meanwhile the isolate V88 also could cause same level of mortality. However, no amplification with the marker genes such as chiA, *VscN* and *luxD* could be obtained. The type strain *V.harveyi* (LMG 4044) could be placed next in terms of mortality caused (28%). In all such cases luminescent colonies could be reisolated from the moribund larvae. These observations suggested that the presence of all the 28 marker genes analyzed here were not the sole factors responsible for the expression of virulence. This fact was further proved by the number of amplicons (24, 25, 22) obtained from the isolates V54, V57, V81, which exhibited no mortality at all after 72hrs of challenge, resembling the unchallenged control. The common factor exhibited by these three isolates was the lack of amplification of *flaC* genes suggesting that the absence of this gene might be a factor which made them non-pathogenic. The absence of *vhhβ*, *toxR*, type-two secretion system, protease genes (cysteine, serine and zinc metalloprotease) and lux genes (LuxO, N, D, P, U) also determine the extent of pathogenicity caused to *P. monodon* PL. It could be hypothesized that absence of these genes might be determining the extent of virulence of the wild *V. harveyi* isolates. Although from the isolate V57, 25 marker genes could be amplified; it was not capable of causing mortality to PL, as it was observed that two major groups of proteases genes were absent. Also the isolate V28 from which 23 amplicons could be obtained, but could not get amplified 3 lux genes apart from *vhhβ* and type - two secretion system genes caused only 8% mortality, indicating that the absence of these genes may not be affecting virulence caused. The type strain of *V.harveyi* (LMG 4044) with which no amplification of 11 marker gene could be obtained (3 genes of the type three secretion system, *toxS*, *ompK*, $vhhP_2$, 2 lux genes and 2 flagellar genes) (Table-5.2) was still capable of causing 28% mortality in *P.monodon* PL. Absence of these 11 genes suggested that they failed to play a major role in expression of pathogenecity. However, their presence might complement the virulence expression. Ruwandeepik *et al.* (2010) suggested that *V.harveyi* showed high variance in virulence to *Artemia*, although they

contained typical virulence genes. However, some isolates that caused high mortality did not show the presence of any atypical virulence gene, whereas some isolates that caused low mortality were found to be positive for the atypical genes. Similar results as described by Ruwandeepik *et al* (2010) were obtained from the present study indicating that the presence of the typical genes did not seem to make a difference to the virulence. The results showed highest positive correlation between the number of amplicons and % *Artemia* mortality, confirming that the presence of various genes markers analysed have a role in mediating pathogenicity in *Artemia*. However, a moderate correlation existed between the number of amplicons and % PL mortality, which supported the view that mere presence of the genes studied was not sufficient for eliciting pathogenicity and that the activation and expression of the virulent genes confer infectivity and thereby mortality. According to Ruwandeepik *et al* (2010), the presence or absence of a virulence factor is not critical for virulence of the isolate because the bacteria produce many different virulent factors and hence it could not be proved that the presence of which specific virulent factor is the key factor associated with virulence to the host. However, it does not exclude the possibility that these virulent factors may be essential for virulence towards different host. To understand the key regulator of *V.harveyi* mediating virulence in *P.monodon*, an extensive study on the expression of pathogenicity at molecular level is required.

Pathogenicity varies greatly and is a complex process influenced by several variables, including host, the species of pathogen, developmental stage of larvae, their physiological conditions, environmental stress, dosage of the pathogen, duration and infection method (de la Pena *et al*., 1993, 1995; Karunasagar *et al*., (1994); Lee *et al*., 1996). From this study it is concluded that among *V. harveyi* there exist isolates 1) which can cause total mortality of larvae 2) which are unable to cause any mortality at all and

3) which position themselves in between. Therefore, it is unscientific to declare entire *V. harveyi* clade as pathogenic to shrimp larvae.

High ammonia concentration was found in the experimental bottles with PL while in the unchallenged control bottles, ammonia was present in negligible concentration. The high ammonia concentration in the challenged flask might be due to the degradation of dead *Artemia* nauplii, which was not consumed by the post-larvae and the degradation of faecal matter of post larvae as well as the excretion of ammonia by the post- larvae. In the control tanks ammonia got generated only from the postlarvae as the *Artemia* larvae were consumed by them. Statistical analysis revealed that the high concentration of ammonia was not the causative factor for mortality. However, it might have served as a predisposing factor for *Vibrio* invasion because 100% mortality was shown by a very few isolates and there recorded situations where no mortality could be registered. This suggests that under stressful conditions a non pathogen shall remain non invasive and non pathogenic while a virulent strain shall cause mortality.

There exist a high degree of genetic similarity for several genes across taxa; therefore, the specificity of the detection method can be compromised (Kita-Tsukamoto *et al*., 1993, Ruimy *et al*., 1994) using bacterium-specific genes (e.g., virulence loci) as targets for multiplex PCR amplification to permit more specific detection as well as subspecies and strain differentiation (Gonza´lez *et al*., 2004). Based on this study *vhhβ*, *toxR*, type- two secretion and protease (cysteine, serine and zinc metalloprotease) gene markers can be used for developing multiplex PCR as diagnostics enabling the segregation of potent pathogenic strains from their non-pathogenic counterparts.

5.5. Conclusion

The 12 representative isolates of *V.harveyi* could be differentiated into three groups as the one associated with highest mortality, the ones with lowest mortality comparable to that in the controls and the ones with moderate mortality. Therefore it is unscientific to declare the entire *V. harveyi* clade as pathogenic to shrimp larvae. Also the mere presence of the virulent genes will not elicit 100% mortality and on the other hand the expression of these genes, variations in the environmental conditions and induction of stress to the host animal, make them susceptible to pathogenic invasions.

CHAPTER-6

Conclusion and scope of future research

Aquaculture has become the fastest growing food producing segment and is one among the major contributors to National economic development, global food supply and nutritional security. The need of the hour is the development of new approaches to control diseases, which are cost-effective, ecologically sustainable, industrially durable and safe to administer. The continuing decline of marine fisheries and the increased demand for sea food by consumers have created a gap between demand and supply. To meet the demand up to 85%, shrimp/prawn production has to be stepped up to intensive cultivation practices typified by ultra high stocking densities and feed loading .Under such practices, as much as 40% of pond water have to be exchanged every few days to remove toxic waste metabolites. Discharge of nutrient-enriched waste water and bottom sediments from prawn pond in to adjacent coastal waters has frequently resulted in eutrophication, oxygen depletion, spread of diseases to wild population and "genetic pollution" as a result of farmed marine species mixing with wild stocks (Cognetti *et al*., 2006). Effective management of water quality in prawn pond is critical pre-requisite not only for maximizing the productivity but also for mitigating the adverse impact of discharging.

Several approaches have been proposed as alternatives to chemotherapy to increase aquaculture production, including improved animal husbandry practices, improvement in the nutritional quality of feed, the use of 'microbially matured' rearing water colonized by non-pathogenic bacteria (Skjermo *et al*., 1997), disinfection of fish eggs, biocontrol using autochthonous microbes to repress the growth if pathogens in rearing environment (Nogami and Maeda, 1992), treatment with UV, use of nonspecific immunostimulants or vaccination (Anderson, 1992), phage

therapy and probiotic bacteria to exclude or inhibit pathogens (Gatesoupe, 1999).

Vibrios are found in broad ranges of environment and are able to persist because of their ability to survive cycles of feast and famine. Starvation adaptive pathway protects vibrios against number of stresses and prepares themselves for subsequent overgrowth under favorable conditions. Biofilm formation to protect them from protozoan grazing, regulation of virulence, host colonization etc is the various strategic measure adapted by vibrios for their survival to varied environmental conditions. Vibrios harboring the external surface of marine zooplanktons have extensive Chitinolytic activity. They play a significant role in the mineralization of chitin in the aquatic systems by utilizing it as both carbon and nitrogen source. Proteinaceous bacteriocin-like inhibitory substance (BLIS) produced by *V.harveyi* inhibits *V.fischeri*, *V.gazogenes, V.parahaemolyticus* and *V. alginolyticus* as pathogens of shrimp, clam, seabreams etc.

Other agents responsible for Virulence

The capability of bacteriophages for the movement of genetic material amongst bacteria constitutes one type of vehicle for transferring important virulent factors (Payne *et al*., 2004). Virulence of *V. harveyi* may be controlled by quorum sensing in so far as it has been confirmed that this regulates type III secretion (Henke and Bassler, 2004a, b). The capability of the pathogen to attach to chitin by means of a specific protein-mediated mechanism may be of significance for the adhesion, colonization and subsequent infection of the host (Montgomery and Kirchman 1993, 1994). Interestingly, it has been suggested that the ability of the bacteria to bind iron could be an important virulence factors (Owens *et al*., 1996). Moreover, the persistence and survival of *V. harveyi* in shrimp hatcheries have been attributed to its ability to form biofilms with resistance to disinfectants and antibiotics (Karunasagar *et al*., 1994).

Management measures adapted for disease control

- Checking the pathogen entry into culture system through seed, feed, water and carriers.
- Stocking disease free and healthy hatchery seeds in well prepared ponds.
- Stocking density should be limited to 30,000/ ha water spread area.
- Consistent maintenance of optimal water parameters helps avoid stress factors throughout the crop.
- Adopting bio-secured systems including closed, reduced water exchange or increased water re-use and other bio-secure practices.
- Adopting better management practices such as disinfecting water, brood screening, seed screening, rinsing of eggs and nauplii of shrimps with clear water, significantly reduces water borne infections.
- Monitoring shrimp health conditions through rapid diagnostic techniques and adjustment of feed quality according to growth and days of culture.
- Preventing the use of antibiotics and pesticides during culture period.
- Avoiding the feed with trash fish or other by-products to cultured animals.
- Avoiding discharged water from ponds affected or suspected to be affected by pathogens into natural environment.
- Above strategies together with crop holiday, crop rotation, reservoir system and good management practices help in better management of disease control.

Role of Molecular tools in identification of vibrios

The principles of polyphasic taxonomy and the advent of new techniques such as DNA-DNA hybridization, nucleotide composition, measurements of amino acid sequence differences, screening of phenotypic characters, including various carbohydrates, proteins, lipids, aminoacids, and alcohols as source of carbon and/or energy, enzyme activity, salinity and temperature tolerance, luminescence, antibiograms and morphological features have proved as a firm basis for the current taxonomy of *Vibrio*.

The identification of *Vibrio* species requires the application of genomic analyses, including Amplified Fragment Length Polymorphism (AFLP), repetitive extragenic palindromic elements PCR (rep-PCR) and 16S rRNA gene sequencing (Thompson *et al*., 2001). *recA* has been suggested as a potential marker to unravel phylogenetic relationships among the higher taxonomic ranks such as families, classes and phyla because of its ubiquity and house-keeping function in bacteria (Ludwig and Klenk, 2001; Zeigler, 2003). Several highly powerful molecular tools, such as AFLP, FAFLP (fluorescent amplified fragment length polymorphism), IGS (intergenic spacer region), rep-PCR (Gurtler and Mayall, 2001), have become readily available for the identification of bacteria, including vibrios (Thompson *et al*., 2001; Sawabe *et al*., 2003). The phenotypic and genetic heterogeneity and the presence of mobile genetic elements in *V. harveyi* mean that species-specific marker common to all isolates would be extremely difficult, if not impossible, to locate.

Practical applications of molecular identification techniques are limited mostly to medically important strains. This reflects the need for rapid, easy and reliable identification systems for both clinical laboratories and aquaculture industries. The virulence genes appear to function as important candidates for identification of species and also for the differentiation of the pathogenic strain from its non-pathogenic counterparts.

Selection of suitable target genes and standardizing their detection conditions are the key criteria for development of sophisticated molecular identification systems like multiplex and real-time PCR.

Significance of the present Study

The present study focuses on vibrios especially *Vibrio harveyi* isolated from shrimp (*P. monodon*) larval production systems from both east and west coasts during times of mortality. A comprehensive approach has been made to work out their systematics through numerical taxonomy and group them based on RAPD profiling and to segregate the virulent from non- virulent isolates based on the presence of virulent genes as well as their phenotypic expression**.** The information gathered has helped to develop a simple scheme of identification based on phenotypic characters and segregate the virulent from non virulent strains of *V. harveyi*.

The subject matter in the thesis has been divided with the following heads:

- Numerical Taxonomy of vibrios based on un-weighted average linkage.
- Construction of its RAPD profile and analysis of amplicons of the house keeping genes in the *Vibrio* isolates (selected from Numerical taxonomy based on phenotypic characters).
- Detection of virulent and luminescent gene markers in the isolates of *V.harveyi* selected from the clusters obtained from Numerical taxonomy, based on phenotypic characters and RAPD fingerprinting.
- Evaluation of the extent of pathogenicity by determining the relation between phenotypic expressions based on *in vitro* assays.
- Determination of the correlation between the amplicons obtained using virulent and luminescent genes and the pathogenicity expressed in animal models.

Overall achievements of this work are summarized as given below:

- *Vibrio* spp. isolated from shrimp (*P. monodon*) larval production systems of both east and west coasts during times of mortality, and the type strains from BCCM/LMG (Belgium) and MTCC (IMTECH, Chandigargh, India) were subjected to phenotypic characterization and subsequent numerical taxonomy.
- Numerical taxonomy of 158 isolates was carried out (employing UPGMA method) by analyzing 135 phenotypic characters or operational taxonomic units (OTUs), based on which a dendrogram was constructed using the software NTSYS p.c. **17 Phena** defined at a Jaccards coefficient range of 0.55 to 0.988. The reproducibility of the unit characters was validated at a probability value $p \leq 0.05$ using Chi-square test.
- A dichotomous key was constructed based on the phenotypic traits of the isolates for identification of vibrios associated with shrimp hatchery systems. Based on the dichotomous key developed only 9 biochemical tests or phenotypical characters are sufficient enough for the identification of vibrios from shrimp larval rearing systems especially in the east and west coasts of India.
- Identification of the isolates based on sensitivity to antibiotics was employed in the dichotomous key of Alsina and Blanch (1994a, b). However, the key developed from this study for the identification of vibrios from shrimp larval rearing systems especially in the east and west coasts of India, does not employ antibiotic sensitivity. Several multidrug resistant forms are present in the environment especially in aquaculture systems where antibiotics are constantly being used as a control measure against bacterial pathogens. In this context, development of a dichotomous key with exclusion of antibiotic screening prevents error and/ or misidentification of the

environmental isolates, which are very likely to carry the antibiotic resistance gene transferred via R-plasmid.

- The isolates reproduced the results when subjected to the set of phenotypic characters according to the dichotomous key, suggesting that the present study could be useful in the routine identification of vibrios from shrimp larval rearing systems especially in the east and west coasts of India.
- Genotypic characterization of vibrios isolated from shrimp larval rearing systems in the east and west coasts of India was carried out employing molecular tools such as RAPD (Random Amplified Polymorphic DNA) and MLBPA (Multi-locus basepair analysis) using the primers for housekeeping genes.
- RAPD fingerprinting was carried out using 7 selected Operon primers which exhibited distinct and reproducible banding pattern ranging from 100- 4500 base pair and the results of amplification were scored and dendrograms were constructed using the softwares NTSYS p.c and PopGene 32.
- The interrelation between each isolates was examined based on similarity coefficient. The results suggested that the isolates of vibrio investigated diverged widely from the isolates which were grouped together as a cluster based on phenotypic characterisation. Divergence pattern exhibited by the isolates was highly different when analysed with each of the 7 different primers individually. This result suggests that a large number of heterogenic genotypes within the isolates of vibrios do exist.
- Considering the vibrio isolates obtained from similar sources as a population and analyzing the banding pattern obtained with all the 7 primers in total and processing the data of the 92 loci in PopGene32

software revealed that the isolates were grouped into 8 Clusters and were interrelated at ≥76%.

- Amplicons of 600, 400 and 200bp were found to be shared by most of the *Vibrio* isolates which were subjected to fingerprinting with 7 selected primers. The sharing of common bands indicated the presence of a highly conserved genomic region in diverse *Vibrio* strains. The presence of conserved region suggested that the isolates shared the same phylogenic lineage. This assumes significance as amplification of common fragments by RAPD-PCR with a particular primer has been shown to be useful in genetic amplifications and hybridization assays for diagnostic purpose.
- The dendrogram constructed exhibited a correlation between a given RAPD type and the geographical location or the source of the isolates. In this study isolates belonging to *V.mediteraneii* were grouped into the cluster of *V. harveyi, V.fluvialis* and *V.vulnificus*. Though the isolates were obtained from different sources, they exhibited high relatedness above 91% 86%S and 84.5%S respectively, suggesting that the isolates obtained from the same geographical area have a few genes in common which remains conserved, while the other genes have been acquired by the isolates through horizontal gene transfer or mutations.
- The representative isolates of vibrios (35 Nos. including the type stains) were selected from the dendrogram constructed based on phenotypic characterization were amplified with already reported housekeeping gene markers (*gapA*, *ftsZ*, *topA*, *mreB*, *gyrB*, *pyrH*, *recA* and *16S rRNA*).
- The results obtained with all the 8 different housekeeping gene primers were interpreted using the software PopGene 32. Analysis of house keeping genes showed that the representative isolates were grouped into three core groups, interrelated ≥ 79%S.

- From this study, it was found that *recA*, *topA*, and *pyrH* genes among the 8 different housekeeping genes could be used as powerful markers for the identification of vibrios. As these three genes have potential sequences that are capable of creating phylogenetic trees with the highest resolution and consistent signal, and hence could be used for species discrimination.
- Genotypic analysis of 25 wild isolates of vibrios suggests that these isolates shared similarity at ≥95% with the isolates deposited in GenBank database. Identification of wild strains without studying their phenotypic profile of the may lead to erroneous identification, hence a detailed investigation of the phenotypic profile of the isolates is a prerequisite for identifying wild strains rather than completely depending on genotypic characterization such as analysis of 16S rRNA gene.
- The extent of virulence exhibited by the isolates of vibrios could be analysed by various *in vitro* assays including determining the hydrolytic potential, auto-agglutinating, self-pelleting, biofilm formation, cell surface hydrophobicity, adherence and cytotoxicity.
- All 158 isolates were positive for hydrolytic assays such as amylase, gelatinase, DNA-ase, chitinase, lecitinase, γ-hemolysin on human blood agar medium, and for auto agglutination test for self pelleting (SP+) and precipitation after boiling (PAB+). Of the 158 isolates examined, 125, 13 and 101 isolates were positive for aesculin hydrolysis, elastase, and lipase production respectively.
- Biofilm measurements at 570nm showed that of the 12 representative isolates, V3 and V45 possessed high degree of biofilm forming ability.
- *V. harveyi* V3, V28, V36, V57 and V71 were strongly hydrophobic and the remaining isolates and also the type strain of *V.harveyi* were

moderately hydrophobic and hence were more effective in eliciting pathogenicity in comparison with the reference isolates, *Bacillus* MCCB101, *Arthrobacter*MCCB104 and *V.cholerae* MTCC 3906 which were weakly hydrophobic.

- *V.harveyi* isolates were cytotoxic on HEp-2 cell line exhibiting CPE revealed by rounding, shrinkage of cytoplasm and dislodgement of cells which showed that the cell free supernatant harbored toxins which played an active role in pathogenesis.
- The isolates were also subjected to antibiotic susceptibility test using 81 different antibiotics and the MAR index was calculated. MAR index values above 0.2, suggested that majority of the isolates have originated from areas susceptible to constant antibiotics use.
- The sensitivity of the isolates to the different classes of antibiotics showed that the isolates were mostly resistant to Lincosamide, Peptides (Glycopeptides and Polypeptides), β-lactams, Steroids and Tetracycline class of antibiotics. Hence these antibiotics should not be used in aquaculture settings as a prophylactic measure targeting elimination of the pathogenic *Vibrio* population in shrimp hatchery systems.
- The extent of virulence exhibited by the selected 12 isolates of *Vibrio harveyi*, analysed based on the amplicons obtained with already reported virulent and luminescent gene markers was worked out.
- All 12 isolates of *V. harveyi* gave positive amplicons for *hlyA*, *rpoS*, *vopN*, *luxS*, *luxR* and *luxA* genes, suggesting that these genes were conserved within the species. However, the presence of *hlyA* gene in all the isolates suggested their capability to cause haemolysis in animals under stressed conditions.

- Presence of *luxS*, lux*R* and lux*A* genes in all the isolates suggested that the isolates mediated strong cell to cell communication by diffusible extracellular molecules or signals (auto inducers); enabling quorum sensing and indirectly favoring virulence expression. Also, majority of the isolates of *V.harveyi* except V54, V57 and V64 gave positive amplification to protease genes, suggesting that the presence of protease genes was linked with luminescence and virulence.
- The isolate V3 gave positive amplicons for all the 28 genes investigated while the type strain of *V.harveyi* (LMG 4044) showed only 17 positive amplicons and the isolate of *V. harveyi* V81 with 22 amplicons. Based on these observations, the isolate *V. harveyi* V3 (MCCB 111) could be ranked first as the most potent pathogen among the 11 representative isolates, while the type strain *V.harveyi* (LMG 4044) and isolate of *V. harveyi* V81 could be considered as the least potent ones .
- Pathogenicity assay of the 12 isolates *V.harveyi* was carried out on animal models such as gnotobiotic *Artemia* nauplii and post larvae of *Penaeus monodon*.
- The challenge study conducted on gnotobiotic *Artemia* nauplii with the 12 isolates of *V.harveyi* revealed that V3 was the most potent pathogen out of the 12 representative isolates and V81 along with *V.harveyi* (LMG 4044) were the least potent ones.
- The challenge experiments carried out on PL-5 showed that the isolates of *V.harveyi* V3 and V88 were the most potent pathogens amongst the 12 isolates of *V.harveyi* causing 100% mortality. VhL (LMG 4044) exhibited 27.6% mortality, while all other isolates exhibits very low or no mortality, behaving similar to that of the control.

- The 12 isolates of *V.harveyi* could be differentiated into three groups as the one associated with highest mortality, the ones with lowest mortality comparable to that in the controls and the ones with moderate mortality. Therefore it is unscientific to declare the entire V. *harveyi* clade as pathogenic to shrimp larvae.
- The type strain of *V.harveyi* (LMG 4044) gave no amplification for 11 marker gene but was still capable of causing 28% mortality in *P.monodon* PL. This result suggests that the 11 genes which were absent failed to play a major role in expression of pathogenecity. However, their presence might complement the virulence expression.
- The presence or absence of a single virulence factor is not critical for virulence of the isolate because the bacteria produce many different virulence factors. Hence, it could not be proved that the presence of specific virulence factor was the key factor associated with virulence to the host. However, it does not exclude the possibility that these virulence factors may be essential for virulence towards different host.
- From this study it can be concluded that the mere presence of the virulent genes will not elicit 100% mortality and that the expression of these genes, variation in the environmental conditions and induction of stress to the host animal, make them susceptible to pathogenic invasions.

Scope of future works

From the present study an excellent foundation on the characteristics of the 147 wild strains of vibrios isolated from larval rearing system could be obtained. Also the extent of pathogenicity expressed by the strains could be categorised into three different levels as highly, moderately potent and non pathogenic forms. This point to the fact that the entire *V. harveyi* clade is not pathogenic to shrimp larvae and the beneficial forms needs to be

retained as the natural flora for the proper functioning of the aquaculture settings.

- The difference in the banding pattern within *V.harveyi* isolates when subjected to RAPD fingerprinting and the divergence of the isolates which were clustered together based on phenotypic characterization is an area that can be taken up for further research. By sequencing the bands which are not conserved and comparing the sequences with the database will provide a better idea regarding the causes for heterogeneity and variation in virulence among *V.harveyi* isolates.

- Future works focuses on the expression of virulence and the factors responsible for the activation of the virulent factor or factors that remains suppressed in normal conditions and are expressed during adverse conditions needs to be carried out. Hence expression studies using mRNA is required which will provide further information on the virulent genes that are expressed during pathogenesis.

- For preventing large scale mortality caused in the larval production systems, the pathogenic forms should be clearly differentiated from the non-pathogenic forms at the earliest. Further works can be carried out for screening the pathogenic ones by developing a multiplex PCR as a diagnostic tool enabling the segregation of potent pathogenic strains from their non-pathogenic counterparts.

- The mechanism by which mobile elements and *V.harveyi* phages mediated virulence in a non-pathogenic *V.harveyi* strain is a vital area for further study.

- *V.harveyi* (V3) is found to be the most potent strain in this study. Hence by using this isolate as antigen, monoclonal antibody can be developed. These MAbs can be fused with fluorescent dyes and can be used for immunofluorescence and immunohistochemical studies. Based on histopathological and immunochemical methods the presence or

absence of *V.harveyi* isolates at the site of infection such as in the gills or hepatopancrease could be determined. A quantitative assay to enumerate *V. harveyi* in water and in larval body can be formulated.

The ban on the use of antibiotics in aquaculture settings, due to the development of multiple drug resistance strains necessitates the development of alternative methods to control vibriosis. In this context research has to be focused on evaluation of putative probiotics and vibriopahge therapy. These two methods are sustainable and will improve shrimp productivity without resorting to any antibiotic treatment.

- Another mechanism that can be targeted towards suppression of the virulence expression of *V.harveyi* without any impact on the bacterial growth is by disrupting the QS mechanism at different check points. Recently researchers are keen on identifying the checkpoints targeting inhibition at three different levels such as 1. Signal generation, 2. AHL signal dissemination and 3. Signal receptor.

Precisely this work opens up new avenues of research to further examine the genetic heterogeneity of *V.harveyi*, explore the conditions at which pathogenicity is expressed by them and development of multiplex PCR for the detection and segregation of virulent from non virulent isolates. Various alternative methods for preventing vibriosis in lieu of antibiotic are other strategies for sustainable shrimp larval production technologies.

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* References not referred to in original.

APPENDIX-1

16S rRNA Sequences deposited with GenBank

LOCUS KC291496 1475 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio harveyi* strain MCCB 170 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291496
ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 gcctaacaca tgcaagtcga gcggaaacga gttatctgaa ccttcgggga acgataacgg
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1441 cacgggtcac accatgggag tgggctgcaa aagaa

LOCUS KC291497 1451 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio harveyi* strain MCCB 171 16S ribosomal RNA gene, partial equence.
ACCESSION KC291497

ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
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ORIGIN

1 gcctaacacg ctaatgcaag tcgagcggaa acgagttatc tgaaccttcg gggaacgata
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LOCUS KC291498 1478 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio harveyi* strain MCCB 172 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291498
ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

ORIGIN

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LOCUS KC291499 1464 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio harveyi* strain MCCB 173 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291499
ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
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ORIGIN

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721 agatactgac actcagatgc gaaagcgtgg ggagcaaaca ggggattaga taccctggta
781 gtccacgccg taaaacgatg tctacttgga ggttgtggcc ttgagccgtg gctttcggag
841 ctaacgcgtt aagtagaccg cctggggagt acggtcgcaa gattaaaact caaatgaatt
901 gacgggggcc cgcacaagcg gtggagcatg tggtttaatt cgatgcaacg cgaagaacct
961 tacctactct tgacatccag agaactttcc agagatggat tggtgccttc gggaactctg
1021 agacaggtgc tgcatggctg tcgtcagctc gtgttgtgaa atgttgggtt aagtcccgca
1081 acgagcgcaa cccttatcct tgtttgccag cacttcgggt cgggaactcc agggagactg
1141 ccggtgataa accggaggaa ggtggggacg acgtcaagtc atcatggccc tacgagtag
1201 ggctacacac gtgctacaat ggcgcataca gagggcggcc acttgcgagagtgagcgaa
1261 tcccaaaaag tgcgtcgtag tccggatcgg agtctgcaac tcgactccgt gaagtcggaa
1321 tcgctagtaa tcgtggatca gaatgccacg gtgaatacgt tcccgggcct tgtacacacc
1381 gcccgtcaca ccatgggagt gggctgcaaa agaagtaggt agtttaacct tcgggaggac
1441 gcttaccact tgtggcgcat acag

LOCUS KC291500 1442 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio harveyi* strain MCCB 174 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291500
ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 aacgataacg gcgtcgagcg gcggacgggt gagtaatgcc taggaaattg ccctgatgtg
61 ggggataacc attggaaacg atggctaata ccgcataata cctacgggtc aaagaggggg
121 actcttaccg ggtacctctc cgcgtactca ggatatgcct aggtgggatt agctagttgg
181 tgaggtaatg gctcaccaag gcgacgatcc ctagctggtc tcgagaggat gatcagccac
241 actggaactg agacacggtc cagactccta cgggaggcag ccagtgggga atattgcaca
301 atgggcgcaa gcctgatgca gccatgccgc gtgtgtggaa gaaggccttc gggttgtaaa
361 gcactttcag tcgtgaggaa ggtagtgtag ttaatagcct gcattatttg acgttagcga
421 cagaagaagc accggctaac tccgtgccag cagccgcggt aatacggagggggtgcgagc
481 gttaatcgga attactgggc gtaaagcgca tgcaggtggt ttgttaagtc agatgtgaaa
541 gcccggggct caacctcgga atagcatttg aaactggcag actagagtac tgtagagggg
601 ggtagaattt tcaggtgtag cggtgaaatg cgtagagatc tgaaggaata ccggtggcga
661 aggcggcccc ctggacagat actgacactc agatgcgaaa gcgtggggagcaaacagga
721 ttagataccc tggtagtcca cgccgtaaaa cgatgtctac ttggaggttg tggccttgag

781 ccgtggcttt cgggagctaa cgcgttaagt agaccgcctg gggagtacgg tcgcaagatt
841 aaaactcaaa tgaattgacg gggcccgca caagcggtgg agcatgtggt tttaattcga
901 tgcaacgcga agaaccttac ctactcttga catccagaga actttccaga gatggattgg
961 tgccttcggg aactctgaga caggtgctgc atggctgtcg tcagctcgtg ttgtgaaatg
1021 ttgggttaag tcccgcaacg agcgcaaccc ttatccttgt ttgccagcac ttcgggtcgg
1081 gaactccagg gagactgccg gtgataaacc gaggaaggtggggacgacgtcaagtcatc
1141 atggcccttа cgagtagggc tacacacgtg ctacaatggc gcatacagag gcggccaac
1201 ttgcgagagt gagcgaatcc caaaaagtgc gtcgtagtcc ggatcggagt ctgcaactcg
1261 actccgtgtt aagtcggaat cgctagtaat cgtggatcag atgatgccac ggtgaataac
1321 gttcccgggc cttgtacaca ccgcccgtca agtcaccatg ggagtggtac gacctgcaaa
1381 agaagtaggt agtttaacct tcgggaggat cagcttcgac cgacttcgga atcgctagta
1441 at

LOCUS KC291501 1465 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio alginolyticus* strain MCCB 169 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291501
ORGANISM *Vibrio alginolyticus* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN
1 acgctggcgg caggcctaac acatgcaagt cgagcggaaa cgagttatct gaaccttcgg
61 ggaatgataa cggcgtcgag cggcggacgg gtgagtaatg cctaggaaat tgccctgatg
121 tgggggataa ccattggaaa cgatggctaa taccgcatga tgcctacggg ccaaagaggg
181 ggaccttcgg gcctctcgcg tcaggatatg cctaggtggg attagctagt tggtgaggta
241 agggctcacc aaggcgacga tccctagctg gtctgagagg atgatcagcc acactggaac
301 tgagacacgg tccagactcc tacgggaggc agcagtgggg aatattgcac atgggcgca
361 agcctgatgc agccatgccg cgtgtgtgaa gaaggccttc gggttgtaaa gcactttcag
421 tcgtgaggaa ggtagtgtag ttaatagctg cattatttga cgttagcgac agaagaagca
481 ccggctaact ccgtgccagc agccgcggta atacggaggg tgcgagcgtt aatcggaatt
541 actgggcgta aagcgcatgc aggtggtttg ttaagtcaga tgtgaaagcc cggggctcaa
601 cctcggaata gcatttgaaa ctggcagact agagtactgt agaggggggt agaatttcag
661 gtgtagcggt gaaatgcgta gagatctgaa ggaataccgg tggcgaaggcggccccctgg
721 acagatactg acactcagat gcgaaagcgt ggggagcaaa caggattaga taccctggta
781 gtccacgccg taaacgatgt ctacttggag gttgtggcct tgagccgtgg ctttcggagc
841 taacgcgtta agtagaccgc ctggggagta cggtcgcaag attaaaactc aaatgaattg
901 acgggggccc gcacaagcgg tggagcatgt ggtttaattc gatgcaacgc gaagaacctt
961 acctactcct gacatccaga gaactttcca gagatggatt ggtgccttcg ggaactctga
1021 gacaggtgct gcatggctgt cgtcagctcg tgttgtgaaa tgttgggtta agtcccgcaa
1081 cgagcgcaac ccttatcctt gtttgccagc gagtaatgtc gggaactcca gggagactgc
1141 cggtgataaa ccggaggaag gtggggacga cgtcaagtca tcatggccct acgagtagg

1201 gctacacacg tgctacaatg gcgcatacgg agggcggccaacttgcgagagtgagcgaat
1261 cccaaaaagt gcgtcgtagt ccggattgga gtctgcaact cgactccatg aagtcggaat
1321 cgctagtaat cgtggatcag aatgcaacgg tgaatacgtt cccgggcctt gtacacaccg
1381 cccgtcacac catgggagtg ggctgcaaaa gaagtaggta gttaagccttcgggggggacg
1441 cttaccactt tgtggttcat gactg

LOCUS KC291502 1486 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio cholerae* strain MCCB 162 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291502
ORGANISM *Vibrio cholerae* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 tggcggacag gcctaacaca tgtcaagtcc tgaggcgagc agattccaca gcaggacact
61 tgttccttgg tgtggcgagc ggcggacggg tgagtaatgc ctgggaacgt attgcccggt
121 agaggggggat aaccattgga aacgatggct aataccgcat aacctcgcaa gagcaaagca
181 ggggaccttc tggagcctat gcagactaac cggatatgcc caggtgtgga ttacgacgta
241 cgttggtgag gtaagggctc caccaaggcg acgtatccct agctggtctg agaggatgat
301 cagccacact ggaactgaga cacgtgtcca gactcctacg ggaggcagcc gtggggaat
361 gacttgcaca atgggcgcaa gcctgatgca gccatgccgc gtgtatgaag aaggccttcg
421 ggttgtaaag tactttcagt agggaggaag gtggttaagc taatacctta atcatttgac
481 gttacctaca gaagaagcac cggctaactc cgtgccagca gccgcggtaa tacggagggt
541 gcaagcgtta atcggaatta ctgggcgtaa agcgcatgca ggtggtttgt taagtcagat
601 gtgaaagccc tgggctcaac ctaggaatcg catttgaaac tgacaagcta gagtactgta
661 gaggggggta gaatttcagg tgtagcggtg aaatgcgtag agatctgaag gaataccggt
721 ggcgaaggcg gccccctgga cagatactga actcagatgcgaaagcgtggggagcaaac
781 aggattagat accctggtag tccacgccgt aaacgatgtc tacttggagg ttgtgaccta
841 gagtcgtggc tttcggagct aacgcgttaa gtagaccgcc tggggagtac ggtcgcaaga
901 ttaaaactca aatgaattga cgggggcccg cacaagcggt ggagcatgtg gtttaattcg
961 atgcaacgcg aagaacctta cctactcttg acatccagag agatctagcg agacgctgga
1021 gtgcctcggg gagctctgag acaggtgctg catgctgtcg tcagctcgtg ttgtgaaatg
1081 ttgggttaag tcccgcaacg agcgcaaccc ttatccttgt ttgccagcac ggaatgttgg
1141 gaactccagt gagactgcgg gtgataaacc ggaggaaggt ggggacgacgcaagtcatc
1201 atggcccttа cgagtagggc tacacaccgt gctacaatgg cgtatacaga gggcagcgat
1261 tccgcgaggt ggagcgaatc tcacaagaga tacgtcgtga gtccggattg gagtctgcaa
1321 ctcgactcca tgaagtcagg aatcgctagt aatcgcaaat cagaatgttg cggtgaatac
1381 gttcccgggc cttgtacaca ccgcccgtca caccatggga gtgggctgca aagaagcag
1441 gtagtttaac cttcgggagg acgcttgcca cttttgtggt ccatga

LOCUS KC291503 1443 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio fluvialis* strain MCCB 130 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291503
ORGANISM *Vibrio fluvialis* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 gcagctgaga agctgtacgt agagtgcaag tctgagcgcg cagcgacaac attgaacctt
61 cgggggattt gttgggcggc gagcggcgga cgggtgagta atgcctggga aattgccctg
121 atgtgggggа taaccattgg aaacgatggc taataccgca tgatagcttc ggctcaaaga
181 gggggacctt cgggcctctc gcgtcaggat atgcccaggt gggattagct agttggtgag
241 gtaagggctc accaaggcga cgatccctag ctggtctgag aggatgatca gccacactgg
301 aactgagaca cggtccagac tcctacggga ggcagcagtg gggaatattg acaatgggc
361 gcaagcctga tgcagccatg ccgcgtgtat gaagaaggcc ttcgggttgt aaagtacttt
421 cagcagtgag gaaggaggta tcgttaatag cggtatcttt tgacgttagc tgcagaagaa
481 gcaccggcta actccgtgcc agcagccgcg gtaatacgga gggtgcgagc ttaatcgga
541 attactgggc gtaaagcgca tgcaggtggt ttgttaagtc agatgtgaaa gcccggggct
601 caacctcgga attgcatttg aaactggcag gctagagtac tgtagagggg ggtagaattt
661 caggtgtagc ggtgaaatgc gtagagatct gaaggaatac cggtggcgaa gcggccccc
721 tggacagata ctgacactca gatgcgaaag cgtggggagc aaacaggatt agataccctg
781 gtagtccacg ccgtaaacga tgtctacttg gaggttgtgg ccttgagccg tggctttcgg
841 agctaacgcg ttaagtagac cgcctgggga gtacggtcgc aagattaaaa ctcaaatgaa
901 ttgacggggg cccgcacaag cggtggagca tgtggtttaa ttcgatgcaa ccgcgaagaa
961 ccttacctac tcttgacatc cagagaactt agcagagatg ctttggtgcc ttcgggaact
1021 ctgagaacag gtgctgcatg gctgtcgtca gctcgtgttg ggaaatgttg ggttaagtcc
1081 cgcaacgagc gcaaccttat ccttgtttgc cagcgagtaa tgtcgggaac tccagggaga
1141 cgtgccggtg ataaaccgga ggaaggtggg gacgacgtca agtcatcatg cccttacga
1201 gtagggctac acacgtgcta tcaatggcgc atacagaggg cggccaagtt tgcgaaagtt
1261 gagcgaatcc caaaaagtgc gtcgtagtcc ggattggagt ctgcaactcg actccatgaa
1321 gtcggaatcg ctagtaatcg tgaatcagaa tgtcacggtg aatacgttcc cgggccttgt
1381 acacaccgcc cgtcacacca tgggagtggg ctgcaaaaaa agcaggtagt ttaaccttcg
1441 gga

LOCUS KC291504 1480 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio nereis* strain MCCB 132 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291504
ORGANISM *Vibrio nereis* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.

TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 tgaacgctgg cggcaggcct aacacatgct tgaagtcgag cggaaacgag gtatctgaac
61 cttcggggta acgataacgg cgtcgagcgg cggacgggtg agtaatgcct gggaacttgc
121 cctgatgtgg gggataacca ttggaaacga tggctaatac cgcataatag cttcggctca
181 aagaggggga ccttcgggcc tctcgcgtca ggatatgccc aggtgggatt agctagttgg
241 tgaggtaaaa ggctcaccaa ggcaacgatc cctagctggt ctgagatcag ccgtctcact
301 ggaactgaga ggatgatcag ccacactgga actgagacac ggtccagact ctacgggag
361 gcagcagtgg ggaatattgc acaatgggcg caagcctgat gcagccatgc cgcgtgtatg
421 aagaaggcct tcgggttgta aagtactttc agcagtgagg aaggtggttg cgttaatagc
481 ggtattaatt tgacgttagc tgcagaagaa gcaccggcta actccgtgcc agcagccgcg
541 gtaatacgga gggtgcgagc gttaatcgga attactgggc gtaaagcgca tgcaggtggt
601 gtgttaagtc agatgtgaaa gcccggggct caacctcggg aagtagacat ttgaaactgg
661 cacactagag tacttgtaga ggggggtaga atttcaggtg tagcggtgaa atgcgtagag
721 atctgaagga ataccagtgg cgaaggcggc cccctggaca gatactgaca ctcagatgcg
781 aaagcgtggg gagcaaacag gattagatac cctggtagtc cacgccgtaa acgatgtcta
841 cttggaggtt gtgggcttga gccgtggctt tcggagctaa cgcgttaagt agaccgcctg
901 ggggagtacg gtcgcaagat taaaactcaa atgaattgac gggggcccgc caagcggtg
961 gagcatgtgg tttaattcga tgcaacgcga agaaccttac ctacctcttg acatccagag
1021 aatctttcca gagatggatt ggtgccttcg ggaactctga gacaggtgct gcatggctgt
1081 cgtcagctcg tgttgtgaaa tgttgggtta agtcccgcaa cgagcgcaac ccttatcctt
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1261 ggcgcataca gaggcgcggc caaccttgcg aaagtgagcg aatcccaaaaagtgcgtcg
1321 tgagtccgga ttggagtctg caactcgact ccatgaagtc ggaatcgcta gtaatcgtgg
1381 atctgaatgc cacggtggta aggttgcgtt cccgggcctt gtacacaccg cccgtcgcac
1441 catgggagtg ggcctacgcg agcatctttc gcactactgc

LOCUS KC291505 1487 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio nereis* strain MCCB 165 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291505
ORGANISM *Vibrio nereis* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 tcatggctca gattgaacgc tggcggcagg cctaacacat gcaagtcgag cggaaacgag
61 ttatctgaac cttcgggtga aacgataacg gcgtcgagcg gcggacgggt gagtaatgcc

121 tgggaaattg ccctgatgtg ggggataacc attggaaacg atggctaata ccgcataata
181 gcttcggctc aaagaggggg accttcgggc ctctcgcgct caggatatgc ccaggtggga
241 ttagctagtt ggtgaggtaa aggctcacca aggcaacgat ccctagctgg tctgagagga
301 tgatcagcca cactggaact gagacacggt ccagactcct acgggaggcagcagtgggga
361 atattgcaca atgggcgcaa gcctgatgca gccatgccgc gtgtatgaag aaggccttcg
421 ggttgtaaag tactttcagc agtgaggaag gtggttgtcg ttaatagcgg tattaatttg
481 acgttagctg cagaagaagc accggctaac tccgtgccag cagccgcggtaatacggagg
541 gtgcgagcgt taatcggaat tactgggcgt aaagcgcatg caggtggtgt gttaagtcag
601 atgtgaaagc ccggggctca acctcggaat agcatttgaa actggcacac tagagtactt
661 gtagaggggg gtagaatttc aggtgtagcg gtgaaatgcg tagagatctg aaggaatacc
721 agtggcgaag gcggcccccc ggacagatac tgacactcag atgcgaaagc tggggagca
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901 agattaaaac tcaaatgaat tgacggggcc cgcacaagcg gtggagcatg tggtttaatt
961 cgatgcaacc gcgaagaacc ttacctactc ttgacatcca agagaacttt ccagagatgg
1021 attgagtgcc ttcgggaact ctgagacaag gtgctgcatg gctgtcgtcc agctcggtgt
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1141 taatggtggg aactccaggg agactgccgg tgataaaccg aggaaggtggggacgacgt
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1321 gtctgcaact cgactccatg aagtcggaat tcgctagtaa tcgtggatca gaatgccacg
1381 gtgaatacgt tcccgggcct tgtacacacc gcccgtctca ccatgggagt gggctgaacg
1441 tgaaacaacc acctcaagtc gtaacaaggt agccgtacgc tagcatc

LOCUS **KC291506** 1477 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio proteolyticus* strain MCCB 134 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291506
ORGANISM *Vibrio proteolyticus* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN
1 attgaacgct ggcggcaggc ctaacacatg caagtcgagc ggaaacgaga tatctgaacc
61 ttcggggaac gatatcggcg tcgagcggcg gacgggtgag taatgcctgg gaaattgccc
121 tgatgtgggg gataaccatt ggaaacgatg gctaataccg cataatagct tcggctcaaa
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241 tgaggtaagg gctcaccaag gcgacgatcc ctagctggtc tgagaggatg atcagccaca
301 ctggaactga gacacggtcc agactcctac gggaggcagc atggggaata ttgcacaatg
361 ggcgcaagcc tgatgcagcc atgccgcgtg tgtgtgaaga aggcctcggg ttgtaaagca
421 ctttcagtcg tgaggaaggt agtgtattta atagatgcat tatttgacgt tagcgacaga
481 agaagcaccg gcttccgtgc cagtgcagcc gcggtaatac ggagggtgcc gagcgttaat

541 cggaattact gggcgtaaag cgcatgcagg tggtgtgtta agtcagatgt gaaagcccgg
601 ggctcaacct cggaatagca tttgaaactg gcagactaga gtactgtaga gggggggta
661 gaatttcagg tgtagcggtg aaatgcgtag agatctgaag aataccggtg gcgaaggcgg
721 ccccctggac agatactgac actcagatgc gaaagcgtgg ggagcaaaca ggattagata
781 ccctggtagt ccacgccgta aacgatgtct acttggaggt tgtggccttg agccgtggct
841 ttcggagcta acgcgttaag tagaccgcct ggggagtacg gtcgcaagat taaaactcaa
901 atgaggggc ccgcacaagc ggtggagcat gtggtttaat tcgatgcaac gcgaacctta
961 cctactcttg acatccagag aactttccag agatggattg gtgccttcgg gaactctgag
1021 acaggtgctg catggctgtc gtcagctcgt gttgtgaaat gttgggttaa gtcccgcaac
1081 gagcgcaacc cttatccttg tttgccagca cgtaatggtg ggaactccag ggagactgcc
1141 ggtgataaac cggaggaagg tggggacgac gtcaagtcat catggccctt cgagtaggg
1201 ctacacacgt gctacaatgg cgcatacaga gggcggccaa cttgcgaaag gagcgaatc
1261 ccaaaagtgc gtcgtagtcc ggattggagt ctgcaactcg actccatgaa gtcggaatcg
1321 ctagtaatcg tggatcagaa tgccacggtg aatacgttcc cgggccttgt acacaccgcc
1381 cgtcacacca tgggagtggg ctgcaaatag aattgggcta gtttaacctt cgggaagtcg
1441 aacaagcact ttgtggttca tgactggcga gcaggca

LOCUS KC291507 1332 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio splendidus* strain MCCB 135 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291507
ORGANISM *Vibrio splendidus* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN
1 ggcggacggg tgagtaatgc ctaggaaatt gccttgatgt gggggataac cattggaaac
61 gatggctaat accgcataat gcctacgggc caaagagggg gaccttcggg cctctcgcgt
121 caagatatgc ctaggtggga ttagctagtt ggtgaggtaa tggctcacca aggcgacgat
181 ccctagctgg tctgagagga tgatcagcca cactggaact gagacacggt ccagactcct
241 acgggaggca gcagtgggga atattgcaca atgggcgaaa gcctgatgca ccatgccgc
301 gtgtatgaag aaggccttcg ggttgtaaag tactttcagt tgtgaggaag ggggtgtcgt
361 taatagcggc atctcttgac gttagcaaca gaagaagcac cggctaactc cgtgccagca
421 gccgcggtaa tacggagggt gtcgagcgtt aatcggaatt tactgggcgt aaagcggcat
481 gcaggtggtt agattaagtc cgatgtgaaa gccccgggct caacctggga atggcatttg
541 aaacttggtc agactagagt actgtagagg gggggtagaa tttcaggtgt aagcggtgaa
601 atgcgtagag atctgaagga ataccggtgg cgaaggcggc cccctggaca atactgaca
661 ctcagatgcg aaaggcgtgg ggagcaaaca ggattagata ccctggtagt ccacgccgta
721 aacgatgtct acttggaggt tgtggccttg agccgtggct ttcgggagct aacgcgttaa
781 gtagaccgcc tggggagtac ggtcgcaaga ttaaaactca aatgaattga cgggggcccg
841 cacaagcggt ggagcatgtg gtttaattcg atgcaacgcg aagaacctta cctactcttg
901 acatccagag aagccagcag gagacgcagg tgtgccttcg ggagctctga acaggtgct

961 gcatggctgt cgtcagctcg tgttgtgaaa tgttgggtta agtcccgcaa cgagcgcaac
1021 ccttatcctt gtttgccagc gagtaatgtc gggaactcca gggagactgc cggtgataaa
1081 ccggaggaag gtggggacga cgtcaagtca tcatggccct tacgagtagg ctacacacg
1141 tgctacaatg gcgcatacag agggcggcga acttgcgaga gtgagcgaat ccaaaaagt
1201 gcgtcgtagt ccggattgga gtctgcaact cgactccatg aagtcggaat cgctagtaat
1261 cgtagatcag aatgctacgg tgaatacgtt cccgggcctt gtacacaccg cccgtcacac
1321 catgggagtg gg

LOCUS KC291508 1420 bp DNA linear BCT 31-DEC-2012
DEFINITION *Vibrio vulnificus* strain MCCB 163 16S ribosomal RNA gene, partial sequence.
ACCESSION KC291508
ORGANISM *Vibrio vulnificus* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN
1 gagaaacttg tttcatcggg tggcgagcgg cggacgggtg agtaatgcct gggaaattgc
61 cctgatgtgg gggataacca ttggaaacga tggctaatac cgcatgatgc ctacgggcca
121 aagaggggga ccttcgggcc tctcgcgtca ggatatgccc aggtgggatt agctagttgg
181 tgaggtaagg gctcaccaag gcgacgatcc ctagctggtc tgagaggatg atcagccaca
241 ctggaactga gacacggtcc agactcctac gggaggcagc agtggggaat attgcacaat
301 gggcgcaagc ctgatgcagc catgccgcgt gtgtgaagaa ggccttcggg ttgtaaagca
361 ctttcagttg tgaggaaggt ggtgtcgtta atagcggcat catttgacgt tagcaacaga
421 agaagcaccg gctaactccg tgccagcagc cgcggtaata cggagggtgc agcgttaat
481 cggaattact gggcgtaaag cgcatgcagg tggtttgtta agtcagatgt gaaagcccgg
541 ggctcaacct cggaactgca tttgaaactg gcagactaga gtactgtaga gggggggtaga
601 atttcaggtg tagcggtgaa atgcgtagag atctgaagga ataccggtgg cgaaggcggc
661 cccctggaca gatactgaca ctcagatgcg aaagcgtggg gagcaaacag gattagatac
721 cctggtagtc cacgctgtaa acgatgtcta cttggaggtt gtggccttga gccgtggctt
781 tcggagctaa cgcgttaagt agaccgcctg gggagtacgg tcgcaagatt aaaactcaaa
841 tgaattgacg gggcccgca caagcggtgg agcatgtggt ttaattcgat gcaacggcgg
901 aagaatcctt acctactctt tgacatccag agaatgccta gcggagaacg caggtagtgc
961 cttcgggaac tcttgagaac aggtgctgca tggcttgtcg gtcagctcgt gtttgtgaaa
1021 tgttgggtta agtcccgcaa cgagcgcaac ccttatcctt gtttgccagc gagtaatgtc
1081 gggaactcca gggagactgc cggtgataaa cggaggaaggtggggacgacgtcaagtca
1141 tcatggccct tacgagtagg gctacacacg tgctacaatg gcgcatacag gggcggcca
1201 acttgcgaaa gtgagcgaat cccaaaaagt gcgtcgtagt ccggattgga gtctgcaact
1261 cgactccatg aagtcggaat cgctagtaat cgtggatcag aatgccaggt gaatacgttc
1321 ccgggccttg tacacaccgc cgtcacacca tgggagtggg ctgcaaaaga gtgggtagt
1381 ttaaccttcg ggaggacgct caccactttg tggttcatga

LOCUS KC747734 1477 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio harveyi* strain MCCB 176 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747734
ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

```
   1 ctggctcaga ttgaacgctg gcggcaggcc taacacatgc aagtcgagcg gaaacgagtt
  61 atctgaacct tcggggaacg ataacggcgt cgagcggcgg acgggtgagt aatgcctagg
 121 aaattgccct gatgtggggg ataaccattg gaaacgatgg ctaataccgc ataacgccta
 181 cgggccaaag agggggacct tcgggcctct cgcgtcagga tatgcctagg tgggattagc
 241 tagttggtga ggtaagggct caccaaggcg acgatcccta gctggtctga gaggatgatc
 301 agccacactg gaactgagac acggtccaga ctcctacggg aggcagcagt ggggaatatt
 361 gcacaatggg cgcaagcctg atgcagccat gccgcgtgtg tgaagaaggc cttcgggttg
 421 taaagcactt tcagtcgtga ggaaggtagt gtagttaata gctgcattat ttgacgttag
 481 cgacagaaga agcaccggct aactccgtgc cagcagccgc ggtaatacgg agggtgcgag
 541 cgttaatcgg aattactggg cgtaaagcgc atgcaggtgg tttgttaagt cagatgtgaa
 601 agcccggggc tcaacctcgg aattgcattt gaaactggca gactagagta ctgtagaggg
 661 gggtagaatt tcaggtgtag cggtgaaatg cgtagagatc tgaaggaata ccggtggaga
 721 aggcggcccc ctggacagat actgacactc agatgcgaaa gcgtgggagc aaacaggatt
 781 agataccctg gtagtccacg ccgtaaacga tgtctacttg gaggttgtgg ccttgagccg
 841 tggctttcgg agccaacgcg ttaagtagac cgcctgggga gtacggtcgc aagattaaaa
 901 ctcaaatgaa ttgacggggg cccgcacaag cggtggagca tgtggtttaa ttcgatgcaa
 961 cgcgaagaac cttacctact cttgacatcc agagaacttt ccagagatgg attggtgcct
1021 tcgggaactc tgagacaggt gctgcacggc tgtcgtcagc tcgtgttgtg aaatgttggg
1081 ttaagtcccg caacgagcgc aacccttatc cttgtttgcc agcgagtaat gtcgggaact
1141 ccagggagac tgccggtgat aaaccggagg aaggtgggga cgacgtcaag ccatcatggc
1201 ccttacgagt agggctacac acgtgctaca atggcgcata cagagggcgg ccaacttgcg
1261 agagtgagcg aatcccaaaa agtgcgtcgt agtccggacc ggagtctgca actcgactcc
1321 gtgaagtcgg aatcgctagt aatcgtggat cagaatgcca cggtgaatac gttcccgggc
1381 cttgtacaca ccgcccgtca caccatggga gtgggctgca aaagaagtag gtagtttaac
1441 cttcgggagg acgcttacca ctttgtggtt catgact
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LOCUS KC747735 1467 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio harveyi* strain MCCB 175 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747735
ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 gcctaacaca tgcaagtcga gcggaaacga gttatctgaa ccttcgggga acgataacgg
61 cgtcgagcgg cggacgggtg agtaatgcct aggaaattgc cctgatgtgg gggataacca
121 ttggaaacga tggctaatac cgcataatac ctwcgggtca aagaggggga cgcctacggg
181 ccaaagaggg gggaccttcg ggcctctcgc gtactcagga tatgcctagg tgggattagc
241 ctagttggtg aggtaatggc tcaccaaggc gacagatccc tagctggtct cgagaggatg
301 atcacgccac actggaactg agacacggtc cagactccta cgggaggcag cagtggggaa
361 tattgcacaa tgggcgcgca agcctgatgc acgccatgcc ggcgctgtgt ggaaggaaga
421 aggccttcgg gttgtaaagc actttcaggt cgtgaggaag gtagtgtagt taatagcctg
481 ccattatttg acgtatgcga cagaagaagc accggctaac tccgtgccag cagccgcggt
541 aatacggagg ggtggcgagc gttaatcgga attactgggc gtaaagcgca tgcaggtggt
601 ttgttaagtc agatgtgaaa gcccggggct caacctcgga attgcatttg aaaactggca
661 gactaggagt actgtagagg ggggtagaat tttcaggtgt agcggtgaaa tgcgtagaga
721 tctgaaggaa taccggtggc gaaggcggcc cccctggaca gatactgaca ctcagatgcg
781 aaaagcgtgg ggagcaaaca ggattagata ccctggtagt ccacgccgta aaacgatgtc
841 tacttggagg ttgtggcctt gagccgtggc tttcgggagc taacgcgtta agtagaccgc
901 ctggggagta cggtcgcaag attaaaactc aaatgaattg acgggggccc gcacaagcgg
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1021 agaactttcc agagatggat tggtgccttc gggaactctg agacaggtgc tgcatggctg
1081 tcgtcagctc gtgttgtgaa atgttgggtt aagtcccgca acgagcgcaa cccttatcct
1141 tgtttgccag cgagtaatgt cgggaaactc cagggagact gccggtgata aaccggagga
1201 aggtggggac gacgtcaagt catcatggcc cttacgagta gggctacaca cgtgctacaa
1261 tggcgcatac agagggcggc caacttgcga gagtgagcga atcccaaaaa gtgcgtcgta
1321 gtccggatcg gaggtctgca actcgactcc gtgaagtcgg aatcgctagt aatcgtggat
1381 cagaatgcca cggtgaatac gttcccgggc cttgtacatc accgcccgtc acaccatggg
1441 agtgggctgc aaaagaagta ggtagtt

LOCUS KC747736 1398 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio harveyi* strain MCCB 177 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747736 ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN
1 gagttatctg aaccttcggg gaacgataac ggcgtcgagc ggcggacggg tgagtaatgc
61 ctaggaaatt gccctgatgt gggggataac cattggaaac gatggctaat accgcataat
121 gcctacgggc caaagagggg gaccttcggg cctctcgcgt caggatatgc ctaggtggga
181 ttagctagtt ggtgaggtaa gggctcacca aggcgacgat ccctagctgg tctgagagga
241 tgatcagcca cactggaact gagacacggt ccagactcct acgggcaggc agcagtgggg
301 aatattgcac aatgggcgca agcctgatgc agccatgccg cgtgtgtgaa gaaggccttc
361 gggttgtaaa gcactttcag tcgtgaggaa ggtagtgtag ttaatagctg cattatttga
421 cgttagcgga cagaagaagc accggctaac tccgtgccag cagccgcggt aatacggagg
481 gtgcgagcgt taatcggaat tactgggcgt aaagcgcatg caggtggttt gttaagtcag
541 atgtgaaagc ccggggctca acctcggaat tgcatttgaa actggcagac tagagtactg
601 tagaggggggg tagaatttca ggtgtagcgg tgaaatgcgt agagatctga aggaataccg
661 gtggcgaagg cggccccctg gacagatact gacactcaga tgcgaaagcg tggggagcaa
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781 ttgagccgtg gctttcggag ctaacgcgtt aagtagaccg cctggggagt acggtcgcaa
841 gattaaaact caaatgaaat tgacgggggc ccgcacaagc ggtggagcat gtggtttaat

901 tcgatgcaac gcgaagaacc ttacctactc ttgacatcca gagaactttc cagagatgga
961 ttggtgcctt cgggaactct gagacaggtg ctgcatggct gtcgtcagct cgtgttgtga
1021 aatgttgggt taagtcccgc aacgagcgca acccttatcc ttgtttgcca gcgagtaatg
1081 tcgggactcc agggagactg ccggtgataa accggaggaa ggtggggacg acgtcaagtc
1141 atcatggccc ttacgaagta gggctacaca cgtgctacaa tggcgcatac agagggcggc
1201 caacttgcga gagtgagcga attcccaaaa agtgcgtcgt agtccggatc ggagtctgca
1261 aactcgactc cgtgaagtcg gaatcgctag taatcgtgga tcagaatgcc acggtgaata
1321 cgttcccggg cctttgtaca caccgcccgt cacaccatgg gagtgggctg aaaagaaagt
1381 agggtagttt aaccttcg

LOCUS KC747737 1400 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio harveyi* strain MCCB 178 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747737
ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN
1 aaattggcca atgaagttga aaaagcgtgg gggacaaacc gggtttattt cccctggtta
61 ttccaccccc ttaaaggaat ttttctgtgg aggttggggc ccttgaacct gggttttgga
121 gttaacggcg ttaaatagac gccctgggggg agtacgttgg aagattaaaa ctaaatgaat
181 gtcggggccc gcacaagcgg tggagcattt gtttaattgg atcaacgggg aagaacatta
241 ctatttttga ttcccagaga acttccagag atggattggt gccttcggga actctgagac
301 aggtgctgca tggctgtcgt cagctcgtgt tgtgaaatgt tgggttaagt cccgcaacga
361 gcgcaaccct tatccttgtt tgccagcgag taatgtcggg aactccaggg agactgccgg
421 tgataaaccg gaggaaggtg gggacgacgt caagtcatca tggcccttac gagtagggct
481 acacacgtgc tacaatggcg catacagagg gcggccaact tgcgagagtg agcgaatccc
541 aaaaagtgcg tcgtagtccg gatcggagtc tgcaactcga ctccgtgaag tcggaatcgc
601 tagtaatcgt ggatcagaat gccacggtga atacgttccc gggccttgta cacaccgccc
661 gtcacaccat gggagtgggc tgcaaataga agtaggcaac gataacggcg tcgagcggcg
721 gacgggtgag taatgcctag gaaattgccc tgatgtgggg gataaccatt ggaaacgatg
781 gctaataccg cataatgcct acgggccaaa gaggggggacc ttcgggcctc tcgcgtcagg
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1141 cggtaatacg gagggtgcga gcgttaatcg gaattactgg gcgtaaagcg catgcaggtg
1201 gtttgttaag tcagatgtga aagcccgggg ctcaacctcg gaattgcatt tgaaactggc
1261 agactagagt actgtagagg ggggtagaat ttcaggtgta gcggtgaaat gcgtagagat
1321 ctgaaggaat acccggtggc gaaggcggcc ccctggacag atactgacac tcagatgcga
1381 aagcgtgggg agcaaaccag

LOCUS KC747738 1419 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio harveyi* strain MCCB 179 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747738

ORGANISM *Vibrio harveyi* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 gcctaacacg ctaatgcaag tcgagcggaa acgagttatc tgaaccttcg gggaacgata
61 acggcgtcga gcggcggacg ggtgagtaat gcctaggaaa ttgccctgat gtgggggata
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181 ggcctctcgc gtcaggatat gcctaggtgg gattagctag ttggtgaggt aagggctcac
241 caaggcgacg atccctagct ggtctgagag gatgatcagc cacactggaa ctgagacacg
301 gtccagactc ctacgggcag gcagcagtgg ggaatattgc acaatgggcg caagcctgat
361 gcagccatgc cgcgtgtgtg aagaaggcct tcgggttgta aagcactttc agtcgtgagg
421 aaggtagtgt agttaatagc tgcattattt gacgttagcg gacagaagaa gcaccggcta
481 actccgtgcc agcagccgcg gtaatacgga gggtgcgagc gttaatcgga attactgggc
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601 attgcatttg aaactggcag actagagtac tgtagagggg ggtagaattt caggtgtagc
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1021 tgctgcatgg ctgtcgtcag ctcgtgttgt gaaatgttgg gttaagtccc gcaacgagcg
1081 caacccttat ccttgtttgc cagcgagtaa tgtcgggact ccagggagac tgccggtgat
1141 aaaccggagg aaggtgggga cgacgtcaag tcatcatggc ccttacgaag tagggctaca
1201 cacgtgctac aatggcgcat acagagggcg gccaacttgc gagagtgagc gaattcccaa
1261 aaagtgcgtc gtagtccgga tcggagtctg caaactcgac tccgtgaagt cggaatcgct
1321 agtaatcgtg gatcagaatg ccacggtgaa tacgttcccg ggcctttgta cacaccgccc
1381 gtcacaccat gggagtgggc tgaaaagaaa gtagggtag

LOCUS KC747739 1382 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio cholerae* strain MCCB 129 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747739
ORGANISM *Vibrio cholerae* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 gacgggtgag taatgcctgg gaaattgccc ggtagagggg gataaccatt ggaaacgatg
61 gctaataccg cataacctcg caagagcaaa gcaggggacc ttcgggcctt gcgctaccgg
121 atatgcccag gtgggattag ctagttggtg aggtaagggc tcaccaaggc gacgatccct
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241 gaggcagcag tggggaatat tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt
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361 accttaatca tttgacgtta cctacagaag aagcaccggc taactccgtg ccagcagccg
421 cggtaatacg gagggtgcaa gcgttaatcg gaattactgg gcgtaaagcg catgcaggtg
481 gtttgttaag tcagatgtga aagccctggg ctcaacctag gaatcgcatt tgaaactgac
541 aagctagagt actgtagagg ggggtagaat ttcaggtgta gcggtgaaat gcgtagagat
601 ctgaaggaat accggtggcg aaggcggccc cctggacaga tactgacact cagatgcgaa
661 agcgtgggga gcaaacagga ttagataccc tggtagtcca cgccgtaaac gatgtctact
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781 gagtacggtc gcaagattaa aactcaatga attgacgggg cccgcacaag cggtggagca
841 tgtggtttaa ttcgatgcaa cgcgaagaac cttacctact cttgacatcc agagaatcta
901 gcggagaccg ctggagtgcc ttcgggagct tctgagaaag gtgctgcatg gctttcgtcc
961 gctcggtgtt gtgtaatgtt gggttaagtc ccgcaacgag cgcaaccctt atccttgttg
1021 gccagcacgt ttatggtggg aactccaggg agactgccgg tggtataacc gaaggaaggt
1081 ggggacgacg tcaggtctat catggccctt acgagtaggg ctacacacgg tgctacaatg
1141 gcgtatacag agcgcatcga ttaccgcgaa ggtggagcga atctcacaag gaacgtcgta
1201 gtccggatcg gagtcggcca ctcgactccg tagggtctga atcgctagtc ctcgcaagtc
1261 agaaggttgc ggcctgaaca cgttctctgg gccttgaaca cagcttaggt caatccgtgc
1321 ctgtggactg ctaacctcac acacatagct tccaccttcg ggactaaccg gtgaggcgca
1381 at

LOCUS **KC747740** 1441 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio parahaemolyticus* strain MCCB 133 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747740
ORGANISM *Vibrio parahaemolyticus* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN
1 acgataacgg cgtcgagcgg ccgtggacgg ggtgagtaat gcctaggaaa ttgccctgat
61 gtgggggata accattggaa acgatggcta ataccgcatg atgcctacgg gccaaagagg
121 gggaccttcg ggcctctcgc gtcaggatat gcctaggtgg gattagctag ttggtgaggt
181 aagggctcac caaggcgacg atccctagct ggtctgagag gatgatcagc cacactggaa
241 ctgagacacg gtccagactc ctacgggagg cagcagtggg gaatattgca caatgggcgc
301 aagcctgatg cagccatgcc gcgtgtgtga agaaggcctt cgggttgtaa agcactttca
361 gtcgtgagga aggcagtgta gttaatagct gcattagttt gacgttagcg acagaagaag
421 caccggctaa ctccgtgcca gcagccgcgg taatacggga gggggcgagc gttaatcgga
481 attactgggc gtaaagcgca tgcaggtggt ttgttaagtc agatgtgaaa gcccggggct
541 caacctcgga attgcatttg aaactggcag actagagtac tgtagagggg ggtagaattt
601 caggtgtagc ggtgaaatgc gtagagatct gaaggaatac cggtggcgaa ggcggccccc
661 tggacagata ctgacactca gatgcgaaag cggtggggag caaacaggga ttagataccc
721 tggtagtcca cgccgtaaac gatgtctact tggaggttgt ggccttgagc cgtggctttc
781 ggagctaacg cgttaagtag accgcctggg agagtacgtc gcaagattaa aactcaaatg
841 aattgacggg ggcccgcaca agcggtggag catggggctt aattcgatgc aacgcgaaga
901 accttaccta ctccttgact tccagagaac tttccagaga atgattggtg ccttcgggaa
961 ctctgagaca ggtgctgcat gcctgccgtc agctcgagtt gtgaaaatgt tgggctaggt
1021 cccgcaacga gggcaaccct tatccttgtt tgccagcgag tattgccggg aactctaggg
1081 aaactgcctg gtgataaacc atggaggaag gtggggtacg ccatgcagtc actatggccc

1141 ttacgagtag ggctacacac gtgctacaat ggcgcataca gagggcggca aacttgcgaa
1201 aatgagcgaa atcccaaaag tgcgtcgtag tccggattgg agtttgcaac tcgcactcca
1261 tgaagtcgaa tcgctagtaa tcgtggatca gaatgccacg gtgaatacgt tcccgggcct
1321 tgtgacacac cgtcagtcac accatgggag tgggctgcaa aagaagtagg tagtttaacc
1381 ttcaccttac cacttgccac tttgtggttc atgactgggt agaagtcgta acaaggtaac
1441 c

LOCUS KC747741 1381 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio vulnificus* strain MCCB 136 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747741
ORGANISM *Vibrio vulnificus* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN
1 gagcggcgga cgggtgagta atgcctggga aattgccctg atgtgggggа taaccattgg
61 aaacgatggc taataccgca tgatgcctac gggccaaaga gggggacctt cgggcctctc
121 gcgtcaggat atgcccaggt gggattagct agttggtgag gtaagggctc accaaggcga
181 cgatccctag ctggtctgag aggatgatca gccacactgg aactgagaca cggtccagac
241 tcctacggga ggcagcagtg gggaatattg cacaatgggc gcaagcctga tgcagccatg
301 ccgcgtgtgt gaagaaggcc ttcgggttgt aaagcacttt cagttgtgag gaaggtggtg
361 tcgttaatag cggcatcatt tgacgttagc aacagaagaa gcaccggcta actccgtgcc
421 agcagccgcg gtaatacgga gggtgcgagc gttaatcgga attactgggc gtaaagcgca
481 tgcaggtggt ttgttaagtc agatgtgaaa gcccggggct caacctcgga actgcatttg
541 aaactggcag actagagtac tgtagagggg ggtagaattt caggtgtagc ggtgaaatgc
601 gtagagatct gaaggaatac cggtggcgaa ggcggccccc tggacagata ctgacactca
661 gatgcgaaag cgtggggagc aaacaggatt agataccctg gtagtccacg ctgtaaacga
721 tgtctacttg gaggttgtgg ccttgagccg tggctttcgg agctaacgcg ttaagtagac
781 cgcctgggga gtacggtcgc aagattaaaa ctcaaatgaa ttgacggggg cccgcacaag
841 cggtggagca tgtggtttaa ttcgatgcaa cggcggaaga atccttacct actctttgac
901 atccagagaa tgcctagcgg agaacgcagg tagtgccttc gggaactctt gagaacaggt
961 gctgcatggc ttgtcggtca gctcgtgttt gtgaaatgtt gggttaagtc ccgcaacgag
1021 cgcaaccctt atccttgttt gccagcgagt aatgtcggga actccaggga gactgccggt
1081 gataaaccgg aggaaggtgg ggacgacgtc aagtcatcat ggcccttacg agtagggcta
1141 cacacgtgct acaatggcgc atacagaggg cggccaactt gcgaaagtga gcgaatccca
1201 aaaagtgcgt cgtagtccgg attggagtct gcaactcgac tccatgaagt cggaatcgct
1261 agtaatcgtg gatcagaatg ccaggtgaat acgttcccgg gccttgtaca caccgccgtc
1321 acaccatggg agtgggctgc aaaagaagtg ggtagtttaa ccttcgggag gacgctcacc
1381 a

LOCUS KC747742 1410 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio mediterranei* strain MCCB 131 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747742
ORGANISM *Vibrio mediterranei* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)

AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India
ORIGIN

1 tgaagagttt gatcatagct cagattgaac gatggcctca ggcctaacgt atgcaagtcg
61 agcggaaact tgttaactca cccttcgggt aacgttaacg gcgtcgagcg gcgtacgggt
121 gagtaatgcc tgggaaattg ccctgatgtg ggggataacc attggaaacg atggctaata
181 ccgcatgatg cctacgggcc aaagaggggg accttcgggc ctctcgcgtc aggatatgcc
241 caggtgggat tagctagttg gtgaggtaag ggctcaccaa ggcgacgatc cctagctggt
301 ctgagaggat gatcagccac actggaactg agacacggtc cagactccta cgggaggcag
361 cagtggggaa tattgcacaa tgggcgcaag cctgatgcag ccatgccgcg tgtgtgaaga
421 aggccttcgg gttgtaaagc actttcagtt gtgaggaagg tgggtacgtt aatagcggca
481 tcatttgacg ttagcaacag aagaagcacc ggctaactcc gtgccagcag ccgcggtaat
541 acggagggtg cgagcgttaa tcggaattac tgggcgtaaa gcgcatgcag gtggtttgtt
601 aagtcagatg tgaaagcccg gggctcaacc tcggaactgc atttgaaact ggcagactag
661 agtactgtag agggggggtag aatttcaggt gtagcggtga aatgcgtaga gatctgaagg
721 aataccggtg gcgaaggcgg ccccctggac agatactgac actcagatgc gaaagcgtgg
781 ggagcaaaca ggattagata ccctggtagt ccacgctgta aacgatgtct acttggaggt
841 tgtggccttg agccgtggct ttcggagcta acgcgttaag tagaccgcct ggggagtacg
901 gtcgcaagat taaaactcaa atgaattgac gggggcccgc acaagcggtg gagcatgtgg
961 tttaattcga tgcaacgcga agaaccttac ttactttga catccagaga agctagcgga
1021 gacgctggta gtgccttcgg gacctctgag acaggtgctg catggctgtc gtcagctcgt
1081 gttgtgaaat gttgggttaa gtcccgcaac gagcgccacc cttatctttg tttgccagcg
1141 agtaatgtcg tgaactccag ggagtctgcc ggtgataaac cggaggaagg tggggacgac
1201 ctcaagtcat catggcactt acgagtaggg ctacacacgt gtttcaatgg ttcatactgg
1261 gggccgccat cttgcgaagg tgagcgaatc ccaataagtg cgtcgtagtc cggattggag
1321 tctgcaactc gactccatga agtcggaatc gctagtaatc gtagatcaga atgctacggt
1381 gaatacgttc ccgggccttg tacacaccgc

LOCUS KC747743 1397 bp DNA linear BCT 07-MAR-2013
DEFINITION *Vibrio mediterranei* strain MCCB 164 16S ribosomal RNA gene, partial sequence.
ACCESSION KC747743
ORGANISM *Vibrio mediterranei* Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio.
REFERENCE 1 (bases 1 to 1477)
AUTHORS Sreelakshmi,N.B., Pai,S.S. and Bright Singh,I.S.
TITLE Phenotypic and genotypic characterization of Vibrio isolated from shrimp hatcheries along the Indian coast
Submitted (22-FEB-2013) National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Cochin, Kerala 682016, India

ORIGIN

1 agtagctcag attgaacgat ggcctcaggc ctaacgtatg caagtcgagc ggaaacttgt
61 taactcaccc ttcgggtaac gttaacggcg tcgagcggcg tacgggtgag taatgcctgg
121 gaaattgccc tgatgtgggg gataaccatt ggaaacgatg gctaataccg catgatgcct
181 acgggccaaa gaggggggacc ttcgggcctc tcgcgtcagg atatgcccag gtgggattag
241 ctagttggtg aggtaagggc tcaccaaggc gacgatccct agctggtctg agaggatgat
301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat

361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt gtgaagaagg ccttcgggtt
421 gtaaagcact ttcagttgtg aggaaggtgg gtacgttaat agcggcatca tttgacgtta
481 gcaacagaag aagcaccggc taactccgtg ccagcagccg cggtaatacg gagggtgcga
541 gcgttaatcg gaattactgg gcgtaaagcg catgcaggtg gtttgttaag tcagatgtga
601 aagcccgggg ctcaacctcg gaactgcatt tgaaactggc agactagagt actgtagagg
661 ggggtagaat ttcaggtgta gcggtgaaat gcgtagagat ctgaaggaat accggtggcg
721 aaggcggccc cctggacaga tactgacact cagatgcgaa agcgtgggga gcaaacagga
781 ttagataccc tggtagtcca cgctgtaaac gatgtctact tggaggttgt ggccttgagc
841 cgtggctttc ggagctaacg cgttaagtag accgcctggg gagtacggtc gcaagattaa
901 aactcaaatg aattgacggg ggcccgcaca agcggtggag catgtggttt aattcgatgc
961 aacgcgaaga accttactta cttttgacat ccagagaagc tagcggagac gctggtgtgc
1021 cttcgggacc tctgagacag gtgctgcatg gctgtcgtca gctcgtgttg tgaaatgttg
1081 ggttaagtcc cgcaacgagc gccaccctta tctttgtttg ccagcgagta atgtcgtgaa
1141 ctccagggag tctgccggtg ataaaccgga ggaaggtggg gacgacctca agtcatcatg
1201 gcacttacga gtagggctac acacgtgtgt tcaatggttc atactggggg ccgccatctt
1261 gcgaaggtga gcgaatccca ataagtgcgt cgtagtccgg attggagtct gcaactcgac
1321 tccatgaagt cggaatcgct agtaatcgta gatcagaatg ctacggtgaa tacgttcccg
1381 ggccttgtac acaccgc

APPENDIX-2

Nucelotide and Protein sequence obtained of the isolate of *V.harveyi* (V3) with the 15 primers

1. LuxN (NP 170F/R) 1710bps

cagcagctaattgctagtactggacccccccctcatgctaggtcgcgatgcgaacaagcattagtaagctgag
tctaactaagaccaactactgatcgccggtatcttattgttcatgctttcgacggcnatattcggggacgcatga
catagtttatgggagatttctcgttgacttggttaccaccagccttgtcgattagtgaaatgctctttgttggttatg
cactgctgacctcccgcttctacagcgtaaagtacattgcttacctcgctcttatcgcgctgcaggtctgagcc
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gcgcccttattggtattacttgcccacctttttgtataataagggggccgatatgcatcgggcttgataatggcga
cgctttctggtcagtacagcaaatcctgtcccttgaagaggattttcagctttcttttgntgncgcaagaaagtc
gtctgggtaagctgctgcaaattcccaatgacaaactacgcctcgtaaccagtaactacaacgaaacctttttac
gaagagtacctttcatcaaaccgctcggtgttagtgtttgatgaactctctgagagctcgaatacatgtatcggc
aaagcgctcatgaagcgctgtatgacaaatgagctcaacacaccgctttggttatgccgctgtatgggcaag
gtaatcggtacgcacttattggttctccccacagagcacacccaatgtctcgacgaggaatctcgcagtcaac
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agttcagttgcaaattgaagcattgaaacagcatattgagaaccatgcgccggtgatcagatcaaactagata
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tccctgttgtcccgaatgcaccaaaagcagccacattacgcacgccttacttcaacgattggaagcaaaataa
acgaagtaatgaacataaggtcgcgtccaacgtacaaatgaataaccaagcaccatcagtgctcatcgtcga
ngataaagaggtgcaacgtgcactggttcagatgtatgtgaaccaacttggcgttaacagcttacaggcaaa
catcgg

QQLIASTGPPLMLGRDANKH**AESN*DQLLIAGILLFMLSTAIFGDA*HSL
WEISR*LGYHQPCRLVKCSLLVMHC*PPASTA*STLLTSLLSRCRSEPFSFCL
*APYLSHLRTGIGGLKPPLSAPLLVLLAHLLYNKGADMHRA**WRRFLVST
ANPVP*RGFSAFFXXRKKVVWVSCCKFPMTNYAS*PVTTTKPFTKSTFHQT
ARC*CLMNSLRARIHVSAKRS*SAV*QMSSTHRFGYAAVWAR*SVRTYWF
SPQSTPNVSTRNLAVNPATRVQVPLSGSPYRQVECVTLLLKFSCKLKH*NSI
LRTMRR*SDQTRYENGKPQFSAVANSSISFARSE*QLARARTYRYDLDS*SR
RPSCQPLRF*K*ENHRKDSSATAH*FCGKTQ*DLI*LCHFQSDS*RNLLFVY*
IADRQTEISTKTGPYENTLILXDTGPGIDETISHKIFDDFFSYQKSGGSG*VW
GTASV*CVLFRQN*V*VLTW*IHRISVVLPCCPECTKSSHITHALLQRLEAK*
TK**T*GRVQRTNE*PSTISAHRRX*RGATCTGSDVCEPTWR*QLTGKHR

2. LuxO (NP275F/R) 1940bps

aaaccgaatggcagaagacttaaccgaatacctgcacgagcacgatgtgaaagttcgttaccttcactcagactttgac
acggttgagcgtgtagaaatcattcgcgaccttcgtttgggtgaatttgatgtgttggttgggatcaacttattgcgagaag
gtctcgatatgccggaggtgtcgctggtggcaatcttggatgcagataaagaaggcttcttgcgttcagagcgttcattg
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gaaagcgatggacgaaactgatcgtcgtcgagagaagcaaaaagcgtataacgaagagatgggtattgagcctcaa
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KPNGRRLNRIPARARCESSLPSLRL*HG*ACRNHSRPSFG*I*CVGWDQLIAR
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VDEESDGRN*SSSREAKSV*RRDGY*ASSLEAKY*RHYGVGGYHQIEASA*
Y*ASAAIESGRAFSDLRGHVSTTAGEGDQSFGSGDVPTRSRS*I*IGGPET*R
D*KAARSVYREQLILL*A*FHCHI*CPSDYVYDHSSGMSRAHICRQKRSQ*A
VGSIGSFCEHVVH*QRQLAR*PEGVKKYTAWFVP*FNLLISVYFV*NTHSLT
MIICKCKA*CDYYLKHNELRLTYPNHVSRPFTCHGG*CDPQSNQIKE*S*QP
W*PKLPRLHRQ*PNDAAGLPHH*LGSEQ*SEYFHHG*KWYG*RSVCRSDSR
SK*TW**AVYRHQLCGNPERPY*K*AVWSRKRCVYWCCE*PTRCGRAC*W
RHVVP**AL*NGSGSSN*AIALYPNGDIPKSRFF*NEERRCALCVCN*PRPLE
RSARRPFP*RLVLPFVRDSFAPSTAA*AW*RRY*NCILAAWLYVA*GRQEFR
PFRTRRD*KIQQLRMAG*RSPVAKRIA*YRGTEQWQRDHAGYVTATTESA
CCAPIGSKIY*T*HYDGVRYYAALDD

3. Clp SP (NP 175F/R) 865bps

ccttgcctgacacgctagcgcagtgattggttgccaacttcccgcggcgaacgctcttacgacatttactcacgtctgctt
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tctgaaaatccagataaagatattttcctatacatcaactcaccaggcggcagcgtaacagcaggtatgtctatctacgac
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PCLTR*RSDWLPTSRGERSYDIYSRLLKERVIFLTGQVEDHMANLVVAQLL
FLESENPDKDIFLYINSPGGSVTAGMSIYDTMQFIKPNVSTVCMGQACSMG
AFLLAGGAPGKRYVLPNSRVMIHQPLGGFQGQASDIQIHAQEILTIKQKLN
NLLAEHTGQPLEVIERDTDRDNFMSADQAVEYGIVDAVLSHHTVFPPAGP
ADMKFSIVWAHHFRGGRKFRLISCFSSAWTAGISGPGN*ISKPGPGTP*GLG
GSILGIFSRNIYSASARASLGDGRLYQEKNAMS

4. Zn MP (NP 179F/R) 1120bps

cgcaagtatttgacgcgctagtgccatggtggttgacaaacttcccgcggcgaacgctcttacgacatttactcacgtct
gcttaaagagcgcgtgatctttttaactggtcaagtggaagaccacatggcaaaccttgtcgtggctcaactacttttcctа
gaatctgaaaatccagataaagatattttcctatacatcaactcaccaggcggcagcgtaacagcaggtatgtctatctac
gacaccatgcagttcatcaagccaaatgtaagcacagtatgtatgggtcaagcttgctctatgggtgcgttcctgcttgca
ggtggtgcgccaggtaagcgttacgttctgcctaattcacgtgtgatgatccaccaaccactaggtggcttccaaggtca
agcttctgatattcaaattcatgctcaggaaatcctgaccatcaagcaaaagctaaacaacctattagcagaacatactgg
ccagcctcttgaagtgattgagcgcgatacggatcgtgacaacttcatgtcggcagatcaggcagtaaaatacggtatt
gtggatgcagttttgagcccacataccgttttctcttggctggacctgcgacatgaagttgtcagatcctatggctaatcctt
cagaggctggcagtagtttcgcgataggtgtttactctgcttgatggtcaggatttccgagcatgaagttgaatataagct
gacttggagcccctggtgcttggtggtataccacgtgaatagccaagtagcttacgggcgccggcacactgggggggg
tggcaccaaccgtgtccttttgtggtggaaagagtgacggtacaaaaggaaaacataccgctaccccgcccgttgaag
agatccccacggaaaaatttacgtttgtgttaatccccccgcgtgttcgcccacccagaggggggtttcaaaatataaga
tttttgattttgaaaattctgccccgggacacccggggcttcaaccctgttgtaaaccacctttttggccgacccttcggcc
agcctccgaaagtatttggcgaaatttttttttcacacccttttttagctacccagaaaaaaacgcaatgtcg

RKYLTR*CHGG*QTSRGERSYDIYSRLLKERVIFLTGQVEDHMANLVVAQL
LFLESENPDKDIFLYINSPGGSVTAGMSIYDTMQFIKPNVSTVCMGQACSM
GAFLLAGGAPGKRYVLPNSRVMIHQPLGGFQGQASDIQIHAQEILTIKQKL
NNLLAEHTGQPLEVIERDTDRDNFMSADQAVKYGIVDAVLSPHTVFSWLD
LRHEVVRSYG*SFRGWQ*FRDRCLLCLMVRISEHEVEYKLTWSPWCLVVY
HVNSQVAYGRRHTGGVAPTVSFCGGKSDGTKGKHTATPPVEEIPTEKFTFV
LIPPRVRPPRGGFQNIRFLILKILPRDTRGFNPVVNHLFWPTLRPASESIWRN
FFFHTLF*LPRKKRNV

5. Cysteine Protease (NP 176F/R) 980bps

gggtttcagtacggtcagctagtagcgcgccgtgtacgtgaaatcggtgtttactgtgaactgtggagctgggacgttga
agaagcggatattcgtgaattcaatccagacggtatcatcctatctggtggtccagaaagcgtaacggaagataactctc
cacgcgctcctcaatacgtatttgactctggtgtgccagtactaggtgtatgttacggcatgcaaactatggctgagcagc
taggtggtaaagttgcgggttctactgaacgtgaattcggctacgcacaagttaaagtttctggtgaatctgcactgtttaa
agatcttgagctaactcaagatgtgtggatgagtcacggtgacaaagtagtagaaattcctgctgacttcgtgaaagttg
gtgagacagatacatgtccgtacgctgcgatggcgaacgaagagaagaagtactacggcgttcagttccacccagaa
gtaacgcacacgaaaggcggcctacaaatgctagagaacttcgttcttggcgtatgtggctgtgagcgtctatggacttc
tgaatctatcatcgaagacgcggtcgctcgtattaaagagcaagtgggtgacgacgaagttatcctaggtctatctggtg

gtgttgattcatcggtagtagcgatgctggttcaccgtgcaatcggcgacaagctaacgtgtgtattcgtagataacggtc
ttcttcgtttaaacgaaggtcagcaagtaatggatatgtttggcgacaagtttggcctaaacatcattaaagttgatgctga
agatcgcttcctaaaagcacttgaaggcaagtcggatccagaagagaagcgtaagacaatcggtcacgtattcgtaga
cgtatttgatgaagagtctaagaagctgaaaaacgcgaaatggctagctcaaggtacgatttacccagacgttatcgaat
ctgctgcatctaagactgg

GFQYGQLVARRVREIGVYCELWSWDVEEADIREFNPDGIILSGGPESVTED
NSPRAPQYVFDSGVPVLGVCYGMQTMAEQLGGKVAGSTEREFGYAQVKV
SGESALFKDLELTQDVWMSHGDKVVEIPADFVKVGETDTCPYAAMANEE
KKYYGVQFHPEVTHTKGGLQMLENFVLGVCGCERLWTSESIIEDAVARIK
EQVGDDEVILGLSGGVDSSVVAMLVHRAIGDKLTCVFVDNGLLRLNEGQQ
VMDMFGDKFGLNIIKVDAEDRFLKALEGKSDPEEKRKTIGHVFVDVFDEES
KKLKNAKWLAQGTIYPDVIESAASKT

6. VopD (NP 283F/R) 951bps

aaccttaaatagtgcaaacatctcagacggaggaacgtctgagactaaaaccgaagctgctgctatccgtaccagcaat
gatagtgcagtaacgggtgcgaagaactaccaattagatgggccaaaagcgcctgcgattggcgatcaagcgcgtgt
ggtagaaaagctgatgagcgcgttggctccaaccgtaaatctattaatgcaaaccacagaaaaagcactgaatggtga
gcaagtggttaagtcaccatcggataccatttcgcaatcactgtctctgattactctgctttaccaagtgtctaagctaagc
cgtgagcaacaggtcctgcaacgtgaaattgccgttgaagcaaacgttgcaagcatcaagagccaagcggaagagtt
gaacaactctgctaaagcgatgatcgcgatggcggtagtatcaggcgtattggctggtgtgactgctattatcggtgccg
tgggttctttcaaagcgggtaaagaaatcaaggcagaagtcgcgggcaacaatgtattgaagacgcaaaaagcaggc
tttgatcaggttgaagagttaatgggcaatactaacttgtcgaaaactcagcaagatcaagtgagacgtgctcatgcgttc
gcaaaagatagcattactgacacgacaaatactttggctagtggtggccgcaagttcgacaagatgatgggcgcaaac
caatcgaagaacgcggtactgcagggacttggccaaatggcgaattcagctgcaaatgtagagcaaacgaaagctca
agctcgaagcaaagaggatgaggtattagcaactcgtgctcaagcagacaaacagagagccgatgaaaacattggct
tccaagaaagtttgctaaaagagctgcgtgagcttttccgctctatcgctgatagtcaaaaccaagcatggcgtgccccctt
tacggtt

NLK*CKHLRRRNV*D*NRSCCYPYQQ**CSNGCEELPIRWAKSACDWRSSA
CGRKADERVGSNRKSINANHRKSTEW*ASG*VTIGYHFAITVSDYSALPSV*
AKP*ATGPAT*NCR*SKRCKHQEPSGRVEQLC*SDDRDGGSIRRIGWCDCY
YRCRGFFQSG*RNQGRSRGQQCIEDAKSRL*SG*RVNGQY*LVENSARSSE
TCSCVRKR*HY*HDKYFG*WWPQVRQDDGRKPIEERGTAGTWPNGEFSCK
CRANESSSSKQRG*GISNSCSSRQTESR*KHWLPRKFAKRAA*AFPLYR**S
KPSMACPFTV

7. VopN (NP 284F/R) 876bps

ttcgaagtaccttgagtcttcccgcacgggagcctcggtcaatggtaattatcgaggtgaaactgtccgcgtacacaatg
caactcagtcgttgtttgatgcgatggaagagctgacggcgttaggttcagaaaaagcagaaaaagacctcacgaaac
gcaaagtgaaagacggcagcattcgcgtgaatgaagcgcatgagctggtttctgattacctaagaaaagtgcctgatct
tgagaaaaaccaaaagatcaaagacctcgctaccaaaatggcaagcggcaacttatcaacgattgctcagttacaggc
gtacctcaacggattctctgaagagaagagccatcagtacctcgcgctgcaagcagtgaaaaagttccttggcgccaat
ccagaaagtaagaacttattggcgctgatagaccaagccattctgacctttgagcagagcccggactcttgggctcaga
ttgatactgaaattcgtgtttcaagcttcgcggatgaatacagccaagagcaaggctttagcagtttgcaccaattgcgtg
gtttctatcgcgacacggtacatagctaccaaggtttaggctcagcatacaaggatgtggttgagcgttttggtgcaaaa
gaggtctccacggcggtcgattttatgttgcagggcatgagcgcagatttgagtgttcaaggtagcaatattgactccgtt

aagcttcagcttctgatgtccgatatgcaaaaacttaagacgttgaatacgcttcaagaccaagttagtaacctctaccag
atgttcaaacctcagcaggcaaactatggctatctaaaaataccaaattttaacgggcaacaaatttgacgcgccaattc

FEVP*VFPHGSLGQW*LSR*NCPRTQCNSVVV*CDGRADGVRFRKSRKRPH
ETQSERRQHSRE*SA*AGF*LPKKSA*S*EKPKDQRPRYQNGKRQLINDCSV
TGVPQRIL*REEPSVPRAASSEKVPWRQSRK*ELIGADRPSHSDL*AEPGLLG
SD*Y*NSCFKLRG*IQPRARL*QFAPIAWFLSRHGT*LPRFRLSIQGCG*AFW
CKRGLHGGRFYVAGHERRFECSR*QY*LR*ASASDVRYAKT*DVEYASRPS
**PLPDVQTSAGKLWLSKNTKF*RATNLTRQF

8. VcrR (NP 285F/R) 412bps

tcgtgtgctgcgcagagtactttcttcttgttcatcatggctcgcgccgcaggctcgtaagtagaattgtgctaatcgctgg
ctgctcatgttgctacctcgcaatacatcaacgttgccacgaatagcccagtcgagcgggtacaaacggatggcatgtt
ctgcgagcataaataacgcggagaaagggttgcttagaccttggtttgggctgattcttcgcagtaatacgatgatgattt
cgccctcttcgacacgaaagaccagctccatcccatcacattcataacgatgaccaagtaagatggtgcttttctgccaa
aagtagggcgtcacttcgatgcctgacgccactagagactcagtgagaaggcattccaattacttttttctctttttaagtg
ggcggtaa

SCAAQSTFFLFIMARAAGS*VELC*SLAAHVATSQYINVATNSPVERVQTD
GMFCEHK*RGERVA*TLVWADSSQ*YDDDFALFDTKDQLHPITFITMTK*D
GAFLPKVGRHFDA*RH*RLSEKAFQLLFSLFKWAV

9. VscN (NP 286F/R) 484bps

gaatggcgatccgccaacacgacgaggctatccgccttctgtttttgacgcactaccaaagctgatgggacgtgcaggt
caatcagacaaaggctccatcaccgcgctgtataccgtacttgtagaaggtgacgatatgactgagccggtggccgat
gagacccgttcgatactcgatggtcacatcattctttctcgcaaactggcggcgatgaaccactacccagccattgatgt
gcttcgttcagccagccgtgtgatgaatcaaatcgtcgataaaactcatcaagcgtccgcggctcatatgcgtgaaatgc
tggccaaatatgaagaagtcgagctgctgataaaaattggtgagtaccaacacggcgccgacagtcgcgcggatatg
gccatcgcacagggagatgatatccgagcgttcttacgccaaggcactgtaacgagatacgatcgcgaccactggttc
ttgctggtccaa

EWRSANTTRLSAFCF*RTTKADGTCRSIRQRLHHRAVYRTCRR*RYD*AGG
R*DPFDTRWSHHSFSQTGGDEPLPSH*CASFSQPCDESNRR*NSSSVRGSYA
*NAGQI*RSRAADKNW*VPTRRRQSRGYGHRTGR*YPSVLTPRHCNEIRSR
PLVLAGP

10. HlyA (NP 562F/R) 358bps

gtaagtcctggtcttcgtcggctattggtggacgcactctcgtttaaaaacatgttctataccaatacatcacaaagcgtga
tcaagcagcgctgtgaacaaacactcgatcttgcgaacgaaaatgcggacattacctacttcgctgccgataaccgttg
gtcatataaccactcgatttggagtaatgatcctgtgatgcagccagatcaaatcaacaaggtcgtggcattgggtgaca
gcttgtcggataccgggaacatctttaacgcatcacaatggcgattcccgaatccaaacagctggttcttgggaacttctc
aaaatcgattgggaagggcagaaaatccagatggca

VSPGLRRLLVDALSFKNMFYTNTSQSVIKQRCEQTLDLANENADITYFAAD
NRWSYNHSIWSNDPVMQPDQINKVVALGDSLSDTGNIFNASQWRFPNPNS
WFLGTSQNRLGRAENPDG

11. VhhP2 (NP 288F/R) 272bps

tttcctgacctggccccccggcaccgaatggagtgattggtaacccctacaacatagggcaagggaattcgggttgtat
tgccgtatggcaagccctacttggtgacgaatattccctatcaaataatcgtaacgattggatgtaaatgagtttggctacc
ctttaggaacaaacaaaggcaaccgaaatggagtgattggtaacccctacaacatagggcaagggaattcgggttgta
tgccgtatgcttgccctaaagccgtgttatact

FPDLAPRHRME*LVTPTT*GKGIRVVLPYGKPYLVTNIPYQIIVTIGCK*VWL
PFRNKQRQPKWSDW*PLQHRAREFGLYAVCLP*SRVI

12. toxR (NP 162F/R) 850bps

tgctgttgatctttttaaacttggccaagtggaacacctcattggcaaagcttgtcgtgggtaactacttttctaaaatctg
gaaaccaaataaatatttgttcctatacatcagtcaagaggcggcagggtaataacggtaatctatcttcaacaccttggn
ttcattaagccaaatggaacaccaatttttgggtccaatttggttcatgagggcgtgtgggcttgggggggggccccag
ggtgacattaacttttggccaatttcctgggaagaaaaacctacctctaaggtgctttaatggttagcttccgtattcaattta
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gcaacgaacaatctctgtcgggagagncgcaagaaaataaagtttggggatgtcgtggtgatcccccgaataccgtat
tctactgcctgatctgccgacatgaagttgtcacgatccgtatcgcgctcaatcacttcaagaggctggccagtatgttct
gctaataggttgtttagcttctgcttgatggtcaggatttctgagcatgaatttgaatatcaaagcttgaccttggaagcnct
agtggttggtggatcatcaccgtgaaattagcgaagtaacgcttactggcgcacacgtgcaagggaacaccactaaag
aagctgacgcatcaaaacggggttacattattaaaaaacccctatttgtttccagaatttagaaatgtagtttagaacgcca
aggtagtcataaattttccacttcccagtaaaaag

CC*SFLNLAKWNTSLAKLVVGNYFF*NLETK*IFVPIHQSRGGRVITVIYLQ
HLGFIKPNGTPIFGSNLVHEGVWAWGGAPG*H*LLANFLGRKTYL*GALM
VSFRIQFIVFESLPSLGLLTY*PITGQPVSMVEPV*QRTISVGRXARK*SLGMS
W*SPEYRILLPDLPT*SCHDPYRAQSLQEAGQYVLLIGCLASA*WSGFLSMN
LNIKA*PWKX*WLVDHHREISEVTLTGAHVQGNTTKEADASKRGYIIKKPL
FVSRI*KCSLERQGSHKFSTSQ*K

13. toxS (NP 272F/R) 645bps

cacggatagcgatcattcatacgagaatgtcacgctgcgtattttcgctggtcgtaatgacccaaccgacatctgcaact
aagggaggctgaaatgaagattaaaatagcatctgcggttttggccgtatccatccttttagtggctggctatattgggg
cagtgacctaaaagtggaacaggttctgacttcaaacgaatggcagtcaaccatggtgacactcatcaccgatactctg
ccagacgatacagttggcccactacgcaaagttaatgttgagtcgaatgtgaagtacttacctaacggtgactacattcgt
gtcgcgaacattcgattgtttgctcaaggctcaaatactgaatcaaccatcaatatctctgagaaaggtcgttgggaagta
agtgataactacttgctggtttcgccttctgaattcaaagatatctcagcatcgcaatcaaaagacttctctgagtcacagct
tcgtctcatcacacaaatctttaagttggatgcagaacaaagtcgccgtattgatgtagtcaacgaaaaaacactactact
gactagcttgaatcacggttcacggggtcctggtaatataactggcgggaaaaataaccagctgattctgaactatatc

HG*RSFIRECHAAYFRWS**PNRHLQLREAEMKIKIASAVLAVSILFSGWLY
WGSDLKVEQVLTSNEWQSTMVTLITDTLPDDTVGPLRKVNVESNVKYLPN
GDYIRVANIRLFAQGSNTESTINISEKGRWEVSDNYLLVSPSEFKDISASQSK
DFSESQLRLITQIFKLDAEQSRRIDVVNEKTLLLTSLNHGSRGPGNITGGKN
NQLILNYI

14. OmpK (NP 287F/R) 807bps

ataggtctaatagtctacctcgtgctatgtttgctgatctctcagacggcgacatccacaagaacgatatacaagtggatg
caattcaacctaatgggtgcatttgacgaacttccaggtgagtcttcacacgactacctagaaatggaattcggcggccg
ctctggtatcttcgatctttacggttacgttgacgtattcaacctagcgacagataaaggtagcgacaaagctggcgcac
ctaaaatcttcatgaagttcgctccacgtatgtctctagacggcctaactggtaaagacctatctttcggtccagttcaaga
gctttacgttgcaactctatttgagtgggacggtactgattacaaaacaaacccattctcagtaaacaaccaaaaagttgg
tctaggttctgacgtaatggttccatggttgggcaaaatcggcctaaacctatacggtacttaccaaggtaacaacaaag
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acatcgattaccaattcggtatgaaagatgagtactctactgctagcagcggtggtgcaatgttcaacggtatctactggc
actctgaccgcttcgcagttggttacggcctgaaaggttacaaagacgtttacggtatcaaagatactgacggcttcaaa
tctactggcttcggtcactacatcgcagtaactaaacaagttttatagtgcgtaaaattccttttagctcttagcctt

IGLIVYLVLCLLISQTATSTRTIYKWMQFNLMGAFDELPGESSHDYLEMEFG
GRSGIFDLYGYVDVFNLATDKGSDKAGAPKIFMKFAPRMSLDGLTGKDLS
FGPVQELYVATLFEWDGTDYKTNPFSVNNQKVGLGSDVMVPWLGKIGLN
LYGTYQGNNKDWNGFQISTNWFKPFYFFENGSFISYQGYIDYQFGMKDEY
STASSGGAMFNGIYWHSDRFAVGYGLKGYKDVYGIKDTDGFKSTGFGHYI
AVTKQVL*CVKFLLALSL

15. Type two Secretion (NP 177F/R) 870bps

aatttttcggtttgattgtgggtagtttcctcaacgtggttatttaccgtttacccaagatcatggaactagaatggcgacgtg
agtgcgctgaatccttccctgaatacaaaatcgaaccaccaaaagaaaaactgacattaagcgtaccacggtcatcttgt
cagcaatgtggcacgcaaattcgtattcgtgacaatatcccagtgattagttggctgcttctgcgcggtaaatgccacaa
ctgccaatctcctatcagcgtacgctaccctcttatcgaactgcttactgccttttgttcgggttttatcgcttttcacttcggtt
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gctatggtgactttaaactccttgcggcattgggcgcttggctgggttggcaatctctaccgatgatcattttgctctcttcg
gtggtcggcgtcatctttggtcttatccaactgcgcttgcaaaaacaaggtatcgagaaagcctttccttttcggcccttacc
ttgcgattgctggttgggtgagtctaatttggggccatcaaatcctcaactggtactttacgtcgattctaaggagtgtttgg
aagtattccagtactacccatggctattcgttgtgtttgccac

NFSV*LWVVSSTWLFTVYPRSWN*NGDVSALNPSLNTKSNHQKKN*H*AY
HGHLVSNVARKFVFVTISQ*LVGCFCAVNATTANLLSAYATLLSNCLLPFV
RVLSLFTSVLATSLSRWFSLPLY*LLPRLSTSILCCCQTN*LYH*CGQVSHLL
LLVSVLSAYKILSLVQWRATCVFGVFTGCLNF*QAKKAWAMVTLNSLRH
WALGWVGNLYR*SFCSLRWSASSLVLSNCACKNKVSRKPFLSALTLRLLV
G*V*FGAIKSSTGTLRRF*GVFGSIPVLPMAIRCVCH