CHARACTERIZATION AND PATHOGENICITY OF *VIBRIO CHOLERAE* AND *VIBRIO VULNIFICUS* FROM MARINE ENVIRONMENTS

Thesis submitted to

Cochin University of Science and Technology

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Under the Faculty of Science

By

ALPHONSA VIJAYA JOSEPH
Reg. No. 3372

Under the Guidance of
Dr. Sarita G. Bhat

MICROBIAL GENETICS LABORATORY
DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN - 682 022, KERALA, INDIA.

OCTOBER 2013

Characterization and Pathogenicity of *Vibrio cholerae* and *Vibrio vulnificus* from Marine environments

Ph.D. Thesis under the Faculty of Sciences

By

Alphonsa Vijaya Joseph
Research Scholar
Department of Biotechnology
Cochin University of Science and Technology
Kochi – 682 022
Email: vijayamanoj@gmail.com

Supervising Guide

Dr. Sarita G. Bhat
Associate Professor and Head
Department of Biotechnology
Cochin University of Science and Technology
Kochi – 682 022
Email: saritagbhat@gmail.com

Department of Biotechnology
Cochin University of Science and Technology
Kochi – 682 022

October 2013

Declaration

I hereby declare that the thesis entitled **"Characterization and Pathogenicity of *Vibrio cholerae* and *Vibrio vulnificus* from Marine Environments"** is the authentic record of research work carried out by me for my doctoral degree, under the supervision and guidance of Dr. Sarita G. Bhat, Associate Professor and Head, Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has previously formed the basis for the award of any degree, diploma, associateship or other similar titles or recognition.

Cochin - 682022
23/10/ 2013

AlphonsaVijaya Joseph

DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN - 682 022, KERALA, INDIA.
Ph: 0484 – 257667 | Email: saritagbhat@gmail..com | Fax: 91-484-2576267, 2577595

Dr. Sarita G. Bhat
Associate Professor & Head

Date:.....

Certificate

This is to certify that the thesis entitled "**Characterization and Pathogenicity of *Vibrio cholerae* and *Vibrio vulnificus* from Marine Environments**" is a record of bonafide research work done by Mrs. Alphonsa Vijaya Joseph under my supervision and guidance.

The thesis is the outcome of her original work and has not formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title and is worth submitting for the award of the degree of Doctor of Philosophy under the Faculty of Sciences of Cochin University of Science and Technology.

Dr. Sarita G. Bhat
Supervising Guide

Acknowledgement

At this moment of accomplishment of my thesis work, it is a pleasant task to acknowledge and appreciate all those who have contributed in many ways to the success of this study and made it an unforgettable experience for me. I look up with gratitude to God Almighty for the wisdom, health and strength bestowed on me to undertake this research work and enable its completion.

I express my profound sense of reverence and gratitude to my research guide and mentor, Dr.Sarita G. Bhat, who instilled in me the vision, encouragement and advice necessary for this doctoral program. She was always available with a constant stream of helpful information, wise and informed alternatives and patient, tolerant and generous backing. It is indeed an honour to be doing my doctoral program under her guidance, using her research expertise. The support and friendship extended by her at a personal level will always be cherished.

My special thanks to Dr. Padma Nambisan, for her valuable suggestions and advice as my doctoral committee member. The counsel rendered by Prof. Dr. C.S. Paulose, Prof.Dr. M. Chandrasekharan and Dr. Elyas K.K. are gratefully acknowledged.

I express my sincere thanks to the Management of St. Teresa's College, Ernakulam, Rev. Sr. Francis Ann (Former Manager), Rev. Sr. Tessa (Provincial and Manager) and Rev. Sr. Christabelle (Former Principal) for their support and permission at different stages of my Faculty Improvement Programme.

I gratefully acknowledge the unflagging encouragement received from my colleagues at the Department of Botany, St. Teresa's College. The former faculty members were always a source of inspiration, caring and concerned of my endeavours. My special appreciation and thanks are extended to the present faculty members, Dr. Elsam Joseph, Dr. Lizzy Mathew, Dr. Romilly Margaret Mendez, Dr. Liza Jacob and Dr. Sheela D. who always instilled in me the confidence to explore my research interests, have shared their knowledge and experiences and have provided a caring

and hilarious environment which have contributed immensely in accomplishing my academic goal. A special thanks to Smt. Neena Unnikrishnan who substituted my leave vacancy at college. I deeply miss Preetha Terence, my student turned colleague who always would affirm "Miss, You can!" I express my deep sense of gratitude to my friends and colleagues at St. Teresa's College whose encouragement and prayers have helped me throughout these years to accomplish this task.

I am thankful to Dr. Jayalekshmi S., Department of Physics, CUSAT and Smt. Sajimol Augutine, Department of Physics, St. Teresa's College, for the collaborative work on biocompatible ZnS nanoparticle as a stain for DNA which culminated in the filing of a patent.

I am deeply indebted to the research scholars at the Microbial Genetics Laboratory, Department of Biotechnology, CUSAT, for providing the most congenial and stimulating environment, so essential for me. This thesis has been kept on track and been seen through to completion with the help and support of the entire team in the lab. My sincere appreciation and thanks to: Mr.Siju M. Varghese, for being a mentor with critical questions and constructive ideas to improve my work, for helping me out with the instruments and the molecular work and moreover being an intimate friend in need; Dr. Jeena Augustine, a fabulous advisor who introduced me to the world of the nematode, C. elegans; Dr. Raghul Subin S., for always being a source of invaluable advice for shaping my work, especially with the Bioinformatic Tools, very generous with his time for me and his understanding and personal attention which were instrumental in guiding my work; Mrs. Helvin Vincent, my student turned tutor, an ardent supporter, who helped me with mathematical calculations, made sophisticated instruments easy to handle and acquainted me with the cloning experiments; Mrs. Smitha S., who lead me into the world of Microbiology, training me to make perfect cotton plugs in the first instance and was there throughout with her personal and scholarly interactions; Mrs. Linda Louis, who helped me cope with the qualifying examination, providing valuable and practical suggestions amidst the entertainment with her huge reportier of anecdotes and stories; Mrs. Harisree P. Nair, who has

always been dear to me with a bunch of enthusiasm and optimism, dedicating selfless perseverance and assistance throughout my work, be it cloning experiments, sequence submissions or processing of data; Ms. Mridula for all the loving support and care; Mr. Noble Kurian and Ms. Lakshmi for the affection and friendship showered on me. Words fail as I thank this crew of the lab- without their companionship, laughter, joy and care, my tenure in the department would have been uneventful. This department has made me proud of my academic roots and I hope to pass on the research values and dreams that I've imbibed from this glorious environment.

My special thanks to the members of the Immunotechnology Lab, Dr. Manzur Ali P.P., Mrs. Sapna K., Mrs. RekhaMol K.R. and Dr. Abraham Mathew. They were always ready with a helping hand for any technical problems. Sharing the lunch table and discussions during lunch are memorable moments that will be cherished.

I also extend my gratitude to Mr. Rameshkumar, Mrs. Roseline Alex, Mrs. Manjula P., Dr. Jikku Jose, Mrs. Jasmine Koshy, Mr. Cikesh P.C., Mrs. Bindhya, Mrs. Sudha Hariharan and all the research scholars of the Neuroscience laboratory for their friendship, help and co-operation.

I thank all the administerial staff of the department for their prompt support and help.

I remember with gratitude all my close relatives, though numerous to name, who have been supporting me mentally and spiritually, bearing my frustrations, cheering me on and celebrating my accomplishments.

Each stage of my work was a challenging activity and the completion would not have been possible without the love, support, patience and sacrifices of my husband, Manoj, who has been a true partner every step of my way and my daughters, Anna and Amie for putting up with my work- may be this thesis is a relief to them because it prevented me from giving their lives my undivided attention- Thank you.

AlphonsaVijaya Joseph

Contents

List of Tables

List of Figures

Abbreviations

%	Percentage
Ace	Accessory cholera enterotoxin
Acf	Accessory colonization factor
AFLP	Amplified Fragment Length Polymorphism
APW	Alkaline Peptone Water
BLAST	Basic Local Alignment Search Tool
bp	base pair
cAMP	cyclic Adenosine Monophosphate
CPS	Capsular Polysaccharide
CT	Cholera Toxin
CTAB	Cetyltrimethylammonium Bromide
Da	Dalton
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme Immunoassay
ERIC	Enterobacterial Repetitive Intergenic Consensus Sequences
EtBr	Ethidium Bromide
Fig.	Figure
FISH	Fluorescence *In Situ* Hybridization
g	Gram
HlyA	Haemolysin A
ICE	Integrating Conjugative Elements
IPTG	Isopropyl-β-D thiogalactoside
LB	Luria Bertani
LPS	Lipopolysaccharide
LSS	Loose Shell Syndrome
M	Molar
MAAC	Modified Arabinose-ammonium sulfate-cholate
MAR	Multiple Antibiotic Resistance
MEGA	Molecular Evolutionary Genetics Analysis
mg	milligram
min.	minute
mL	millilitre

MLSA	Multilocus Sequence Analysis
MLST	Multilocus Sequence Typing
MOF	Marine Oxidation Fermentation
MST	Microbial Source Tracking
NA	Nutrient agar
NCBI	National Center for Biotechnology Information
ng	nanogram
NGM	Nematode Growth Medium
O. D.	Optical density
^{o}C	Degree Celsius
Omp	Outer membrane proteins
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal Deoxyribonucleic acid
Rep	Repetitive extragenic palindromic
RFLP	Restriction Fragment Length Polymorphism
rpm	revolution per minute
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Real Time PCR
RTX	Repeat in toxin
SDS	Sodium dodecyl sulphate
sp.	species
TAE	Tris-Acetate-EDTA
TCBS	Thiosulphate-Citrate-Bile Salts-Sucrose
TCI	Thiosulphate-chloride-iodide
TCP	Toxin co-regulated Pilus
TDH	Thermostable Direct Hemolysins
TE	Tris-EDTA
TTG	Taurocholate Tellurite Gelatin
UPGMA	Unweighted pair group method with arithmetic average
UV-VIS	Ultraviolet-Visible
VBNC	viable but non-culturable
VHA	Vibrio harveyi Agar

VP	Voges–Proskauer
VPI	Vibrio Pathogenecity Island
WGD	White gut disease
WGST	Whole genome sequence typing
WHO	World Health Organization
Zot	Zonula occcludens toxin
µL	microliter
µM	micromolar
Φ	Phage

ABSTRACT

The genus *Vibrio*of the family Vibrionaceae are Gram negative, oxidase-positive, rod- or curved- rodshaped facultative anaerobes, widespread in marine and estuarine environments. *Vibrio* species are opportunistic human pathogens responsible for diarrhoeal disease, gastroenteritis, septicaemia and wound infections and are also pathogens of aquatic organisms, causing infections to crustaceans, bivalves and fishes. In the present study, marine environmental samples like seafood and water and sediment samples from aquafarms and mangroves were screened for the presence of *Vibrio* species. Of the134 isolates obtained from the various samples, 45 were segregated to the genus *Vibrio* on the basis of phenotypic characterization.like Gram staining, oxidase test, MoF test and salinity tolerance. Partial 16S rDNA sequence analysis was utilized for species level identification of the isolates and the strains were identified as *V. cholerae*(N=21), *V. vulnificus*(N=18), *V. parahaemolyticus*(N=3), *V. alginolyticus* (N=2) and *V. azureus* (N=1). The genetic relatedness and variations among the 45 *Vibrio* isolates were elucidated based on 16S rDNA sequences. Phenotypic characterization of the isolates was based on their response to 12 biochemical tests namely Voges-Proskauers's (VP test), arginine dihydrolase , tolerance to 3% NaCl test, ONPG test that detects β-galactosidase activity, and tests for utilization of citrate, ornithine, mannitol, arabinose, sucrose, glucose, salicin and cellobiose. The isolates exhibited diverse biochemical patterns, some specific for the species and others indicative of their environmental source.Antibiogram for the isolates was determined subsequent to testing their susceptibility to 12 antibiotics by the disc diffusion method. Varying degrees of resistance to gentamycin (2.22%), ampicillin(62.22%), nalidixic acid (4.44%), vancomycin (86.66), cefixime (17.77%), rifampicin (20%), tetracycline (42.22%) and chloramphenicol (2.22%) was exhibited. All the isolates were susceptible to streptomycin, co-trimoxazole, trimethoprim and azithromycin. Isolates from all the three marine environments exhibited multiple antibiotic resistance, with high MAR index value.

The molecular typing methods such as ERIC PCR and BOX PCR revealed intraspecies relatedness and genetic heterogeneity within the environmental isolatesof *V. cholerae* and *V. vulnificus*. The 21 strains of *V. cholerae*were serogroupedas non O1/ non O139 by screening for the presence O1*rfb* and O139 *rfb* marker genes by PCR.

The virulence/virulence associated genes namely *ctx*A, *ctx*B, *ace*, VPI, *hly*A, *omp*U, *rtx*A, *tox*R, *zot*, *nag*st, *tcp*A, *nin* and *nan*were screened in *V. cholerae* and *V. vulnificus*strains.The *V. vulnificus*strains were also screened for three species specific genes viz., *cps*, *vvh*and *viu*. In *V. cholerae* strains, the virulence associated genes like VPI, *hly*A, *rtx*A, *omp*U and *tox*R were confirmed by PCR. All the isolates, except for strain BTOS6, harbored at least one or a combination of the tested genes and *V. cholerae*strain BTPR5 isolated from prawn hosted the highest number of virulence associated genes. Among the *V. vulnificus*strains, only 3 virulence genes, VPI, *tox*R and *cps*, were confirmed out of the 16 tested and only 7 of the isolates had these genes in one or more combinations. Strain BTPS6 from aquafarm and strain BTVE4 from mangrove samples yielded positive amplification for the three genes.

The *tox*Rgene from 9 strains of *V. cholerae*and 3 strains of *V. vulnificus* were cloned and sequenced for phylogenetic analysis based on nucleotide and the amino acid sequences. Multiple sequence alignment of the nucleotide sequences and amino acid sequences of the environmental strains of *V. cholerae*revealed that the *tox*Rgene in the environmental strains are 100% homologous to themselves and to the *V. cholerae tox*R gene sequence available in the Genbank database. The 3 strains of *V. vulnificus* displayed high nucleotide and amino acid sequence similarity among themselves and to the sequences of *V. cholerae* and *V. harveyi* obtained from the GenBank database, but exhibited only 72% homology to the sequences of its close relative *V. vulnificus*.

Structure prediction of the ToxR protein of *Vibrio cholerae* strain BTMA5 was by PHYRE^2 software. The deduced amino acid sequence showed maximum

resemblance with the structure of DNA-binding domain of response regulator2 from *Escherichia coli* k-12 Template based homology modelling in PHYRE^2 successfully modelled the predicted protein and its secondary structure based on protein data bank (PDB) template c3zq7A.

The pathogenicity studies were performed using the nematode *Caenorhabditiselegans*as a model system. The assessment of pathogenicity of environmental strain of *V. cholerae*was conducted with *E. coli* strain OP50 as the food source in control plates, environmental *V. cholerae* strain BTOS6, negative for all tested virulence genes, to check for the suitability of *Vibrio* sp. as a food source for the nematode;*V. cholerae* Co 366 ElTor, a clinical pathogenic strain and *V. cholerae* strain BTPR5 from seafood (Prawn) and positive for the tested virulence genes like VPI, *hly*A, ompU,*rtx*A and *tox*R. It was found that *V. cholerae* strain BTOS6 could serve as a food source in place of *E. coli* strain OP50 but behavioral aberrations like sluggish movement and lawn avoidance and morphological abnormalities like pharyngeal and intestinal distensions and bagging were exhibited by the worms fed on *V. cholerae* Co 366 ElTor strain and environmental BTPR5 indicating their pathogenicity to the nematode.

Assessment of pathogenicity of the environmental strains of *V. vulnificus* was performed with *V. vulnificus* strain BTPS6 which tested positive for 3 virulence genes, namely, *cps*, *tox*Rand VPI, and *V. vulnificus* strain BTMM7 that did not possess any of the tested virulence genes. A reduction was observed in the life span of worms fed on environmental strain of *V. vulnificus*BTMM7 rather than on the ordinary laboratory food source, *E. coli* OP50. Behavioral abnormalities like sluggish movement, lawn avoidance and bagging were also observed in the worms fed with strain BTPS6, but the pharynx and the intestine were intact.

The presence of multi drug resistant environmental *Vibrio* strainsthat constitute a major reservoir of diverse virulence genes are to be dealt with caution as they play a decisive role in pathogenicity and horizontal gene transfer in the marine environments.

Chapter 1
INTRODUCTION

Marine ecosystems are highly complex and dynamic with enormous biodiversity. A surprising number of pathogens have been reported from marine environments and the probability of their transmission to humans and aquatic organisms is correlated to factors that affect their distribution. Both indigenous and introduced pathogens can be the cause of illness acquired from marine environments and their occurrence depends on ecology, source, and survival in these environments. Our current knowledge of the diversity and ecology of bacterial pathogens associated with marine environments stems from clinical accounts of marine-acquired illnesses, disease outbreaks of known aetiology in marine animals, and testing of marine environments for the presence of pathogen populations.

A large majority of known marine pathogens belong to the family Vibrionaceae, an important and ubiquitous group of bacteria in marine and estuarine environments. Characteristically they have a close association with aquatic animals either as symbionts, normal flora or as pathogens. The myriad types, the range of species and their diverse role in the marine environments make the Vibrionaceae an important, but challenging group of bacteria that need extensive and constant investigation.

The genus *Vibrio* of the family Vibrionaceae comprises of at least 74 distinct species. They are Gram negative, oxidase-positive, rod- or curved-rod shaped facultative anaerobes. These bacteria are halophilic, mesophilic

and chemoorganotrophic in nature and are widespread in marine and estuarine environments, very rarely found as freshwater inhabitants (Baumann *et al.,* 1984). Vibrios retain remarkable biodiversity in the marine environment, with species found in hydrothermal vents (Raguenes *et al.,* 1997), deep sea (Maruyama *et al.,* 2000), open water (Eilers *et al.,* 2000), estuaries (Barbieri *et al.,*1999) and marine sediments (Urakawa *et al.,* 2000; Raghul and Bhat, 2011). They are found in association with planktonic forms (Huq *et al.*, 2005), sponges (Thompson *et al.,* 2004) and corals (Rohwer *et al.,* 2001). They also occur commonly in marine and estuarine seafood like squid (Ford *et al.,* 1986), bivalve molluscs (Liu *et al.,* 2001; 2003), shrimp and prawn (Ruangpan and Kitao, 1991; Lemonnier *et al*., 2006; Sakai *et al.,* 2007) and finfish (Austin and Austin, 1993; Chatterjee and Haldar, 2012). Furthermore, environmental characteristics such as water temperature and salinity are known to influence the diversity of *Vibrio* spp. in the environment. They survive unfavourable environmental conditions by forming biofilms with which they can attach to algae, crustaceans or other marine organisms and utilize the nutrients released by these organisms (Visick, 2009, Yildiz and Visick, 2009) or enter a viable but non-culturable (VBNC) state, whereby they are metabolically active but do not form colonies on any media and are very resistant to environmental stress (Asakura*, et al.*, 2007; Oliver, 2005).

Vibrios are responsible for a number of clinical conditions such as cholera, gastroenteritis, septicaemia and wound infections (Thompson *et al.*, 2004; Jay *et al.*, 2005; Doyle and Beuchat, 2007).Twelve *Vibrio* species have been documented as potential food-borne disease agents in humans: *V. cholerae*, *V. parahaemolyticus, V. vulnificus, V.alginolyticus, V.furnissii, V.fluvialis, V.damsela, V.mimicus, V.hollisae, V.cincinnatiencis,*

V.harveyi and *V.metchnikovii* (Thompson and Swings, 2006; Adams and Moss, 2008). A few species are fish pathogens and some are involved in coral bleaching (Thompson *et al*., 2004).

Cholera, a life threatening disease that can spread rapidly as explosive epidemics from one region to another is characterized by profuse watery diarrhoea with flakes and mucus, dehydration and sometimes death, when adequate medical intervention is not instituted (Kaper *et al.*, 1995; Jay *et al.*, 2005; Talkington *et al.*, 2011). *Vibrio cholerae*, the etiological agent of cholera is wide spread in estuarine and marine waters around the world, where salinity is between 4 to 17% (Huq *et al.,* 2005). Infections by *V. cholerae* can lead to epidemics, pandemics or may be endemic in specific areas. Since its discovery in 1817 there have been seven cholera pandemics. The first six pandemics originated in the Indian Subcontinent with the Ganges Delta region serving as the main reservoir. The seventh pandemic started in 1961 in Indonesia and spread to other parts of the world (Kamal, 1974). The bacteria are carried around the world mostly by asymptomatic carriers and the most recent outbreak occurred in Haiti and it was linked to a clone in Nepal (Talkington *et al.,* 2011; Hendriksen *et al.*, 2011). In the aquatic environment, *V. cholerae* is found associated with a number of biotic and abiotic substrates where the pathogen can exist in high abundance, survive long periods of stress and, when conditions are right, cause human infection (Nair, 2008). The association of *V. cholerae* with aquatic substrates, with the capability of the bacteria to switch on survival strategies, such as biofilm formation (Huq *et al.*, 2008) and the viable but non-culturable (VBNC) state (Oliver, 2005; Alam *et al.*, 2007), explains the persistence and dissemination of the pathogen during inter epidemic periods.

V. vulnificus is regarded as an emerging pathogen and infection in humans was first reported in 1964 in the USA and in 1987 in Taiwan (Harwood *et al.,* 2004; Hsueh *et al.,* 2004). It is an opportunistic pathogen in the elderly or in immune-compromised individuals with impaired liver function, or underlying disease such as cirrhosis, diabetes mellitus or those on steroid therapy (Harwood *et al.,* 2004; Drake *et al.,* 2007). Infections are usually acquired through consumption of raw or improperly cooked shellfish or through contact with seawater (Hsueh *et al.,* 2004; Cazorla *et al.,* 2011). *V vulnificus* can also cause an infection of the skin when open wounds are exposed to warm seawater. It causes three important disease syndromes; septicaemia, necrotising wound infections and gastroenteritis with a mortality rate of 40 to 50% occurring one to two days after onset of the symptoms (Harwood *et al.,* 2004; Hsueh *et al.,* 2004; Cazorla *et al*., 2011). *V. vulnificus* is a common inhabitant of marine environments (Harwood *et al.,* 2004), being prevalent in seawater during summer when the temperatures are between 26 to 29°C (Hsueh *et al.* 2004). It occurs in environments with salinity of 0.5 to 2.5% (Harwood *et al.,* 2004), but their concentration was found to increase in oil polluted seawater (Tao *et al.,* 2011). They are also found associated with shellfish, sediments and planktons which are believed to act as reservoirs (Harwood *et al.,* 2004).

V. parahaemolyticus is associated with gastroenteritis manifested by profuse, watery diarrhoea free from blood and mucus, abdominal cramps, nausea, and vomiting. Outbreaks of *V. parahaemolyticus* food poisoning are associated with consumption of raw molluscs and cooked crustaceans in America and Europe, but in Japan, South East Asia, India and Africa raw fish is implicated as a vehicle for transmission (Jay *et al.,* 2005). *V. parahaemolyticus* is usually a member inhabiting coastal waters (Adams

and Moss, 2008), but has been recently isolated from fresh water fish (Noorlis *et al.,* 2011).

Vibrio alginolyticus is an opportunistic pathogen occasionally associated with cases of gastroenteritis and diarrhoea and causing systemic infections in immune-compromised individuals with severe burns, cancers or with a history of alcohol abuse (Oliver and Kaper, 1997). In healthy individuals *V.alginolyticus* is associated with extra intestinal infections such as wound or ear infections (Novotny *et al.,* 2004).

Recognition of environmental reservoirs, understanding the nature of bacterial interactions and their role in disease transmission represent an area of active research with significant implications in epidemiological studies. The long-term persistence of pathogens in the non-human environment (King *et al.*, 2008) proposes that environmental reservoirs play a significant role in affecting the evolution of pathogen virulence and reduces pathogen dependence on host-to-host contact for transmission and propagation, enabling it to evolve to higher virulence levels (Chun *et al.*, 2009).

The taxonomy of vibrios initially based on the classical methods of classification; identification and nomenclature where morphological features and biochemical reaction played an important role (Alsina and Blanch, 1994a; 1994b) has been replaced by the current trend based on the polyphasic approach that includes phenotypic and molecular methods (Thompson and Swings, 2006).

The16S rDNA PCR method not only increases the efficiency of bacterial identification due to its rapid and reproducible nature but is also

an efficient tool for phylogenetic studies and as a taxonomic marker (Thompson *et al.*, 2005). Various molecular techniques have also been used to type the strains belonging to the various *Vibrio* species including Amplified fragment length polymorphism(AFLP), Raandom amplified polymorphic DNA (RAPD), Restriction fragment length polymorphism (RFLP), Ribotyping, Repetitive extragenic palindromic (Rep) sequences, Pulsed field gel electrophoresis (PFGE), Enterobacterial repetitive intergenic consensus sequences (ERIC), BOX PCR, Multilocus sequence typing (MLST) and Whole genome sequence typing(WGST). Most of these techniques could also be used for the identification of specific *Vibrio* species, being important tools in epidemiological investigations and have been recommended for determining the source of virulent strains and their temporal and spatial distributions (Caburlotto *et al*., 2011; Cazorla *et al*., 2011; Hendriksen *et al*., 2011).

The extension of antibiotic resistance among *Vibrio* species is the most notable example of evolution that has been observed in this group over the past decades. Extensive use and misuse of antibiotics in medication, veterinary, agriculture and aquaculture have caused widespread antibiotic-resistant *Vibrio* strains in the environment. Antimicrobial drug resistance in *Vibrio* spp. can develop through mutation or through acquisition of resistance genes on mobile genetic elements, such as plasmids, transposons, integrons, and integrating conjugative elements (ICEs). The rapid emergence of multi-drug resistance in *V. cholerae* is a cause of great concern in public health perspective. Reports of drug-resistant *V. cholerae* strains are appearing with increasing frequency (Mukhopadhyay *et al*., 1998; Thungapathra *et al*., 2002; Jain *et al*., 2008; Roychowdhury *et al*., 2008; Ngandjio *et al*., 2009; Kumar *et al*., 2009). Antibiotic resistance

profiles have also been produced for environmental isolates of other *Vibrio* species (Manjusha *et al.,* 2005; Han *et al.,* 2007; Elhadhi, 2012). Emergence of resistance to multiple drugs is a serious clinical problem in the treatment and containment of the disease. Surveillance of multiple drug resistance of environmental strains of *Vibrio* is necessary to communicate the current status of resistance in a location, alert the clonal nature of the infectious agents and to spur the implementation of appropriate antibiotic and curative measures.

In *V. cholerae,* about 200 serogroups have been recognized of which only O1 and O139 can cause cholera (Albert, 1996). The others collectively called the non O1/ non O139 serogroups have not been associated with epidemics, nevertheless cases of gastroenteritis and extra-intestinal infections have been reported (Morris *et al*., 1990, Mukhopadhyay *et al*., 1995; Sharma *et al*., 1998; Dziejman *et al*., 2005). The pathogenesis of *V. cholerae* is a complex process involving the co-ordinated action of a number of virulence factors and the ability of *V. cholerae* strains to cause severe enteric infection depends on their virulence gene content.

The major virulence factors of *V. cholerae* include the cholera toxin (CT), which is responsible for the profuse diarrhoea and a colonization factor known as toxin co-regulated pilus (TCP). Apart from these, cholera pathogenesis depends on the synergistic effect of a number of other virulence-associated factors like the accessory cholera enterotoxin (Ace), cytotoxic haemolysin A (HlyA), zonula occcludens toxin (Zot), outer membrane proteins (Omp), heat stable enterotoxin (NagST), repeat in toxin (RTX) mannose-sensitive haemagglutinin (MSHA), neuraminidase (Nan) and accessory colonization factor (Acf) (Kaper *et al*., 1995; Lin *et al*., 1999; Thompson *et al*., 2004).

The genes encoding the cholera toxin (*ctx*AB*)* reside in the genome of a lysogenic filamentous phage, CTXΦ (Waldor and Mekalanos, 1996) and genes encoding the major colonization factor TCP, are part of a large cluster of genes referred to as the Vibrio pathogenecity islands (VPI) (Kovach *et al*., 1996; Faruque *et al*., 1998a).

The important virulence factors associated with the pathogenesis of *V. vulnificus* are its capsular polysaccharide (CPS), iron acquisition protein (VIU), flagellar hook protein, haemolysin (VVU), repeat in toxin (RtxA) and metalloprotease (Gulig *et al*., 2005). Other putative virulence factors include numerous extracellular enzymes like protease, mucinase, lipase, chondroitinase, hyaluronidase, DNase, esterase, and sulfatase (Desmond *et al*., 1984; Oliver *et al*., 1986).

The expression of virulence factors by pathogenic vibrios is co-ordinately regulated in response to specific external environmental stimuli that are received by integral membrane proteins that transduce cytoplasmic regulatory proteins to control gene expression. The ToxR regulon plays a central role in the coordinate regulation of the virulence properties in *V. cholerae* and encode products that function to promote intestinal colonization, toxin production and survival within the host (Miller *et al.*, 1987; Kovach *et al*., 1996).

1.1 Objectives of the Study

Environmental populations of *Vibrio* are characterized by heterogeneous distributions of multiple virulence factors, combinations of which regulate the epidemic potential (Faruque *et al.*, 1998b; Karaolis *et al.*, 1998; Chakraborty *et al.*, 2000).Transfer of virulence properties between different

species has been observed (Faruque *et al.*, 1998a; Boyd *et al.*, 2000) in cases where specific virulence factors are borne on mobile genetic elements. Thus, environmental interaction may confer enhanced pathogenicity on a subset of an environmental population.

Kerala constitutes approximately 10% of India's total coastline with its remarkable marine water resources and rich fertile coastal waters that offer immense scope for aquaculture activities like farming of shrimp, crabs, oysters, mussels and finfish. Aquaculture farm systems in mangrove areas are being developed more abundantly as this environment serves as a critical nursery to fisheries by creating shelters for spawning, hatching and feeding. Marine aquaculture settings and mangrove environments serve as foci or reservoirs for *Vibrio* strains that occur either as pathogens that are the cause of major diseases and low grade infections that erode productivity or as probionts providing beneficial effects. Kerala has experienced several cholera outbreaks (Radhakutty *et al.*, 1997; Bhanumathi *et al.*, 2002; John *et al.*, 2004; Geeta and Krishnakumar, 2005) caused by *V. cholerae* O1 El Tor strains and one case due to O139 strain (Bhanumathi *et al.*, 2002). The high population density of the state as well as its close proximity to aquatic systems makes Kerala, a suitable candidate for endemic cholera.

Moreover, reports on the incidence of CT and TCP among environmental *V. cholerae* El Tor strains from the coastal areas of Kerala (Kumar *et al.,* 2008) and *V. cholerae* O1 in tropical seafood in Cochin (Kumar and Lalitha, 2013) necessitates the screening for potential pathogenic vibrios from marine environments like seafood, aquafarms and mangroves. The marine environment may be a powerful incubator for new combinations of virulence properties due to the extremely large overall

population size of bacterial populations and efficient mixing timescales. These natural phenomena may be further enhanced by anthropogenic activities that introduce microbial species across geographical barriers (Ruiz *et al.,* 2000).The potential for outbreaks in natural or farmed marine environments needs to be recognized and approached with caution. Therefore, the present work was undertaken with the following specific objectives:

1) Phenotypic characterization, antibiotic profiling, molecular characterization and phylogenetic analysis of *Vibrio* species from marine environments

2) Molecular typing and detection of virulence associated genes in the environmental isolates of *V. cholerae* and *V. vulnificus*

3) Genetic analysis and comparison of toxin- regulatory gene *tox*R in the environmental isolates of *V. cholerae* and *V. vulnificus*

4) Pathogenicity studies with the nematode *Caenorhabditis elegans* as model organism

Chapter 2

REVIEW OF LITERATURE

2.1. Vibrionaceae

The family Vibrionaceae, first described by Veron (1965), resides within the Gammaproteobacteria with the members comprising one of the predominant bacterial groups in marine environments. They occur in coastal seawater samples, pelagic seawaters (Simidu *et al.,* 1982) and in highly eutrophicated areas (Simidu *et al.,* 1977). In marine environments, members of the family Vibrionaceae have a close association with aquatic animals either as symbionts, normal flora or as pathogens and are closely associated with many kinds of marine animals from plankton (Heidelberg *et al.,* 2002) to fishes (Arias *et al.,* 1995, Grisez *et al.,* 1997). To varying degrees, Vibrionaceae have an intimate relationship with all farmed marine species of aquatic animals like corals (Rosenberg and Loya, 2004), molluscs (Sawabe *et al.,* 2003), seagrass, sponges and shrimp (Gomez-Gil *et al.,*1998, Vandenberghe *et al.,*2003).Their relationship with luminous fish has been known since the last century (Nishiguchi *et al.,* 2004).

The ability of the group to occupy a wide diversity of habitats means that the Vibrionaceae have direct and indirect effects on aquaculture either as pathogens that are the cause of major disease, low grade infections that erode productivity or beneficial effects as probionts like some strains of *Vibrio alginolyticus (*Austin *et al.,* 1995) or *V. mediterranei* (Huys *et al.,* 2001).

The family Vibrionaceae currently comprises eight validly published genera: *Aliivibrio*, *Catenococcus*, *Enterovibrio*, *Grimontia*, *Listonella*, *Photobacterium*, *Salinivibrio*, and *Vibrio.* Currently some 98 species across the eight genera are recognised; most of the species occur in the genus *Vibrio*. Each genus has its own distinguishing morphological and physiological characteristics (Krieg and Holt, 1984). Phenotypic data used to define each genus are G/C content, the presence of sheathed polar flagella, requirement of sodium for growth, lipase activity, D-mannitol utilization, and sensitivity to vibriostatic compound 0/129 (Holt *et al.,*1994). The myriad of types, the range of species and their diverse role in aquaculture make the Vibrionaceae an important but challenging group of bacteria.

2.2. The Genus *Vibrio*

The first description of a *Vibrio* species took place in 1854, when the Italian physician Pacini discovered *V. cholerae*. The genus *Vibrio* comprises a diverse bacterial group consisting of at least 74 distinct species. They are gram-negative, oxidase-positive, rod- or curved rod-shaped facultative anaerobes. Generally, the organisms are halophilic, mesophilic and chemoorganotrophic in nature (Thompson *et al.,* 2004). Members of this group are widespread in aquatic habitats of various salinities. They are very common in marine and estuarine environments, and on the surfaces of marine plants and animals (Baumann *et al.,* 1984). They also occur naturally in the intestinal content of marine animals (Baumann *et al.,* 1984; Sakata, 1989). Some species are also found in freshwater habitats (Baumann *et al.,* 1984).

Two chromosomes are common to all *Vibrio* spp., one large and the other, a small, flexible chromosome (Okada *et al.*, 2005). High rates of

mutation, recombination and frequent horizontal gene transfer lead to high polymorphism and intraspecies variability of *Vibrio* spp. (Moreno *et al.*, 2002; Gonzalez-Escalona *et al.*, 2008; Hazen *et al.*, 2010). This high genome plasticity enables these bacteria to adapt to various niches and react rapidly to environmental changes.

2.2.1. Ecology of Vibrios

Vibrios retain remarkable biodiversity persisting in a variety of geographic locales as free living populations, or in association with eukaryotic hosts, including corals, molluscs, sponges and zooplankton (Blackwell and Oliver, 2008). Furthermore, environmental characteristics, such as water temperature and salinity, are also known to influence the diversity of *Vibrio* spp. in the environment (Beaz-Hidalgo *et al.,* 2010). *Vibrio* spp. survive unfavourable environmental conditions, by the formation of biofilms, by which they can attach to algae, crustaceans or other marine organisms, and utilize the nutrients released by these organisms (Huq *et al.*, 1983; Snoussi *et al.*, 2008; Visick, 2009; Yildiz and Visick, 2009). Another survival strategy of *Vibrio* spp. is to enter a viable but non-culturable (VBNC) state (Roszak & Colwell, 1987; Asakura*, et al.*, 2007) where they do not form colonies on any media, but are metabolically active and very resistant to environmental stress (Wong & Wang, 2004; Oliver, 2005).

Vibrio species are opportunistic pathogens that are widely distributed in marine aquaculture environment, causing infections to commercially important species of crustaceans (Sung *et al.,* 2001; Soto-Rodriguez *et al.,* 2010; Alagappan *et al.,* 2010; Ji *et al.,* 2011; Raissy *et al.,* 2011), bivalves (Tubiash *et al.,* 1970; Beaz-Hidalgo *et al.,* 2010) and fishes (Toranzo *et al.,* 2005; Won and Park, 2008; Pal and Das, 2010; Frans *et al.,* 2011). There

are 12 species that are routinely isolated from human clinical samples, and the diseases in which they are implicated include diarrheal disease, septicemia and wound infections (Daniels *et al.,* 2000).

Vibrio spp. are ubiquitous in the marine environment, with species found in hydrothermal vents (Raguenes *et al.,*1997), deep sea (Maruyama *et al.,*2000), open water (Eilers *et al.,*2000), estuaries (Barbieri *et al.,*1999) and marine sediments (Urakawa *et al.,*2000; Raghul and Bhat, 2011).*Vibrios* have also been found in rivers (Kenzaka *et al.,*1998), and *V. cholerae* is also known to inhabit freshwater environments (Baumann *et al.,*1980). One species, *V. navarrensis* has been reported in sewage outfalls (Urdaci *et al.,* 1991).

Ecologically and economically, the variety of parasitic and mutualistic relationships that the vibrios enter into with the aquatic flora and fauna are very relevant.

2.2.1.1. Planktons

The association of vibrios with planktonic organisms, especially copepods, has been suggested as an important component of *Vibrio* ecology (Sochard *et al.,* 1979; Huq *et al.,* 1983; 2005). Planktons represent organic rich microenvironments (Long and Azam, 2001; Grossart *et al.,* 2005) that can selectively enrich heterotrophic bacteria, including *Vibrios* (Huq *et al.,* 1983; Tamplin *et al.,* 1990; Lipp *et al.,* 2003; Long *et al.,* 2005). The production of an extracellular chitinase allows vibrios to utilize the chitinous exoskeletons of some plankton taxa as a source of carbon and nitrogen (Thompson *et al.,* 2004), hence presenting a competitive advantage when associated with plankton (Heidelberg *et al.,* 2002). Plankton colonized by pathogenic *Vibrio* species can potentially act as a

vehicle of disease transmission, as in the case of cholera (Huq and Colwell, 1996; Huq *et al.,* 2005).

The phytoplankton (diatoms and cyanobacteria) were shown to be significant correlates of *Vibrio* abundance. *V. cholerae* has been shown to attach to certain cyanobacteria and diatoms (Tamplin *et al.,* 1990; Salvesen *et al.,* 2000; Rehnstam-Holm *et al.,* 2010). However, the total vibrio community may respond differently to the presence of phytoplankton and the inverse relationship may reflect a release of nutrients that corresponds with the decay of a diatom or cyanobacterial bloom (Middelboe *et al.,* 1995; Riemann *et al.,* 2000). The decline of the bloom could then stimulate an increase in secondary production by heterotrophic bacteria, including *Vibrios* (Lancelot and Billen, 1984; Pomeroy and Wiebe, 2001).

Unculturable *Vibrio vulnificus* and *V. parahaemolyticus* were detected in plankton fractions by polymerase chain reaction amplification of DNA sequences for cytotoxin-haemolysin and thermolabile haemolysin respectively (Baffone *et al.,* 2006).

2.2.1.2. Sponges

Sponges maintain diverse symbiotic microbe populations that differ in composition from microbial populations of surrounding ambient waters (Webster and Blackall, 2008). *Vibrio* species exist in remote undersea environments in sponges inhabiting different depths and regions of benthic seas (Thompson *et al.,* 2004). The 16S rRNA gene sequences placed the majority of sponge-associated vibrios within the Harveyi clade (*Vibrio* core), which includes *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus, V. natriegens, V. rotiferanius* and *V. campbellii*, albeit precise species

determinations were largely intangible (Sawabe *et al.,* 2007; Hoffmann *et al.,* 2010).

2.2.1.3. Corals

Coral reefs are highly productive and very diverse ecosystems within coastal tropical environments, mainly in oligotrophic regions (Cortes, 2003). Corals harbour diverse bacterial communities and seem to exhibit some specificity within coral species (Rohwer *et al.,* 2001). Coral bleaching, i.e., the paling or the loss of color due to the disruption of symbiosis between the coral host and symbiotic Zooxanthellae, is one of the most serious diseases affecting corals worldwide (Rosenberg and Loya, 2004; Sutherland *et al.,* 2004).

V. shilonii (also known as *V. mediterranei*) and *V. coralliilyticus* have been proven to bleach corals, and their pathogenicity was shown to be temperature dependent. *V. shilonii* was identified as an etiological agent of the bleaching of *Oculina patagonica*, and the main disease steps, i.e., adhesion, penetration, and multiplication within the coral tissues have been described in detail (Banin *et al.,* 2000; Rosenberg & Ben-Haim, 2002; Cervino *et al.,* 2004). Within the coral tissues, most *V. shilonii* cells become viable but nonculturable (VBNC) but continue to be virulent. According to Sussman *et al.* (2003), the fire worm *Hermodice carunculata* is a winter reservoir and summer vector of *V. shilonii*. *V. coralliilyticus*, another temperature-dependent pathogen, was shown to cause patchy necrosis of tissues of *Pocillopora damicornis* when the coral was incubated at temperatures of 27°C or higher (Ben-Haim and Rosenberg, 2002; Ben-Haim *et al.,* 2003). *V. coralliilyticus* is also reported as the causative agent of white syndrome disease in other coral species (Bally and Garrabou,

2007; Sussman *et al.,* 2008; 2009; Vezzulli *et al.,* 2010).There are also rich commensal *Vibrio* populations associated with healthy corals (Arboleda and Reichardt, 2009; Kvennefors *et al.,* 2010) and the study on coral-mucus associated vibrios revealed that the integrons are particularly strong evolutionary hotspots in their genomes, with a very high flux of mobile genes coming in and going out that exceeds even that seen in more free-living vibrios (Koenig *et al.,*2011).

2.2.1.4. Biofilm Formation

The biofilm formation by *Vibrio* spp. on the exoskeletons of crustaceans and other marine organisms may in fact constitute a strategy to survive during starvation and/or other environmental stresses (Wai *et al.,* 1999). In biofilms these bacteria can use trapped and absorbed nutrients, resist antibiotics, and establish favourable partnerships with other bacteria or hosts (Thompson *et al.,* 2004; Snoussi*, et al.*, 2008; Visick, 2009; Yildiz and Visick, 2009). *Vibrio* moves along and attaches to surfaces with the aid of the flagellum and pili, which may act as adhesins. The synthesis of an exopolysaccharide is a prerequisite for biofilms and is believed to occur principally after attachment to solid surfaces and microcolony formation. Thus maturation from the planktonic state to the biofilm state requires a developmental alteration in flagellar, fimbrial, and exopolysaccharide synthesis, promoting the formation of the three dimensional architecture of mature biofilms (Kierek & Watnick, 2003; Fong *et al.,* 2010). Studies have also shown the antibiofilm activity of an exopolysaccharide A101 purified from culture supernatant of the marine bacterium *Vibrio* sp. QY101 not only inhibited biofilm formation, but also disrupted the established biofilms of some strains of bacteria (Jiang *et al.*,2011). The strong ability of

V. cholerae to form densely packed biofilms in the environment gives a survival advantage to this organism. Biofilm formation has been reported to facilitate the survival and transmission of *V. parahaemolyticus, V. alginolyticus* and *V. fischeri* in the environment (Wolfe *et al.,* 2004; Enos-Berlage *et al.,* 2005; Snoussi *et al.,*2008; Chavez-Dozal and Nishiguchi, 2011).

V. cholerae biomass in biofilms remains stable in the presence of surface-feeding protozoa whereas planktonic *V. cholerae* cells are rapidly eliminated which illustrates the importance of surface-associated growth as a protective niche for the environmental persistence of *V. cholerae* (Matz *et al.,* 2005).

2.2.2. Vibrios in Seafood

2.2.2.1. Squid

Vibrio sp. have been isolated from both normal and ulcerated mantles of squid (*Lolliguncula brevis*) (Ford *et al.,* 1986) and postmortem from hemolymph, abdominal fluid, and eyes of captive cuttlefish with myocarditis (Hulet *et al.,* 1979). Captive cephalopods are susceptible to disease and major health problems (Hanlon *et al.,* 1988; Scimeca and Oestman1995; 1996) due to their marine- adapted immune system and their thin, delicate, microvillar epidermal structure. Archived necropsy cases of *Sepia* spp. showed that mortality was commonly associated with bacterial cultures positive for *Vibrio alginolyticus,* a marine bacterium routinely found in coastal waters, sediment, and culture systems (Sangster and Smolowitz, 2003). Of the 53 cases in which bacterial cultures were taken, 33 were positive for *V. alginolyticus. V. parahaemolyticus* was detected by loop-mediated isothermal amplification from various seafood samples including

cuttlefish (Wang *et al.*,2011) and *V. cholera*e was reported from the squid samples obtained from wetmarket and supermarket in Malaysia (Vengadesh *et al.*,2012).

Symbiosis between sepiolid squids (Cephalopoda: Sepiolidae) and vibrio bacteria has become an ideal model to study environmentally transmitted symbioses between cooperative microbes and their eukaryotic hosts (Hanlon *et al.,* 1997; Nyholm and MacFall-Ngai, 2004). The symbiosis is established when environmentally transmitted, symbiosis-competent vibrios infect the light organs of newly hatched juvenile squid (McFall-Ngai and Ruby, 1998; Guerrero-Ferreira and Nishiguchi, 2007). Upon entrance into the light organ, the bacteria flourish in a relatively nutrient-rich, protected environment and in turn achieve high densities within the organ (Stabb, 2006). When a sufficiently high number of symbiont cells are achieved in the light organ, the bacteria become luminescent based on a communication mechanism known as quorum sensing (Miller and Bassler, 2001). Vibrio bacteria then induce a program of drastic morphological changes in the squid host, which helps to establish the symbiont population and completes development of the naïve light organ (Nishiguchi *et al.,* 2004). Development and timing of this vibrio population coincides with the nocturnal hunting behavior of the squid, in which the bacteriogenic luminescence is used in a predator/prey avoidance behavior known as counter-illumination (Jones and Nishiguchi, 2004; Zamborsky and Nishiguchi, 2011). *V. fischeri* and *V. logei* are apparently the only reported *Vibrio* species colonizing the light organs of squid (McFall-Ngai,1994; Fidopiastis *et al.*,1998; Ruby and Lee, 1998), but this seemingly specific partnership remains to be confirmed (Thompson *et al.*,2004).

2.2.2.2. Marine Bivalves

Vibrio species constitute an important percentage of the heterotrophic bacteria associated with marine bivalves, especially oysters, mussels and clams. Since the soft body of the oyster is consumed whole, either raw or lightly cooked, it is generally classified as a high-risk food (Desenclos *et al.,* 1991). They can accumulate large numbers of bacteria due to their filter-feeding habit, (Pruzzo *et al.*, 2005; Pujalte *et al.,* 2010) and *Vibrio* species are commonly isolated with 100-fold higher concentration in filter-feeding organisms than in the surroundingwater (Wright *et al.,* 1996).

V. parahaemolyticus, V. mimicus, V. alginolyticus and *V. vulnificus* present in molluscs, oysters and clams can be pathogenic and are linked with disease outbreaks of seafood associated gastroenteritis (McCoubrey, 1996; Daniels *et al.*,2000; Lozano-Leon *et al.*,2003; McLaughlin *et al.*,2005) and septicaemia (DePaola *et al.,* 2003) in humans.

Pathogenic *V. alginolyticus*, *V. parahaemolyticus* and *V. carchariae* have been isolated from diseased abalones with abscissions, ulceration in the mantle and general whitening (Huang *et al.*,2001; Liu *et al.*,2001; 2003; Nicolas *et al.,* 2002) causing mass mortalities in some cases (Nishimori *et al.*,1998; Cai *et al.,* 2006). The species *V. alginolyticus*, *V. tubiashii V. splendidus* and *V. anguillarum* were recognised as the main causal agents of the bacillary necrosis of oyster larvae characterized by bacterial colonization of the mantle, velum disruption, abnormal swimming, visceral atrophy, and lesions in the organs (Tubiash *et al.*,1970; Tubiash and Otto, 1986; Sugumar *et al.*,1998; Gay *et al.*,2004). These species along with *V. neptunius* and *V. pectenicida* have been reported as etiological agents of larval vibriosis in oysters and mussels (Nicolas *et al.,* 1996; Lambert *et al.,*

1998; Prado *et al.,* 2005). The classical *Vibrio* infections are Summer Mortality in juvenile oysters caused by *V. harveyi* (Garnier *et al.,* 2007; Allain *et al.,* 2009) and Brown Ring Disease in adult clams caused by *V. tapetis* (Borrego *et al.,*1996; Allam *et al.,*2006).

In bivalves, vibrio cells are killed by immune system components which involve both cell-mediated and humoral systems that operate in a coordinated way (Pruzzo *et al.*, 2005). The finding that some *Vibrio* strains are resistant to killing and are able to persist inside bivalves has suggested the possibility that these bacteria represent a bivalve-specific community (Murphree and Tamplin, 1995). However, the role of vibrios as regular components of bivalve microflora is yet to be defined, as bivalves may be a mere passive concentrator for the bacteria by their filtering activity (Vezzulli *et al.,* 2010).

2.2.2.3. Shrimp and Prawn

Vibrios are a perpetual component of the natural microflora of wild and cultured shrimp (Vanderzant *et al*., 1971; Sinderman, 1990; Ruangpan and Kitao, 1991) and tend to turn opportunistic pathogens when their natural defence mechanisms are suppressed (Brock and Lightner, 1990). Vibrios are etiological agents of vibriosis, a bacterial disease, responsible for the mass mortality of cultured shrimp worldwide (Lavilla-Pitogo *et al.,* 1990; 1998; Sakai *et al.,* 2007). Pathogenic strains of *V. parahaemolyticus*, *V. vulnificus* and *V. harveyi* have caused massive epidemics (Lightner, 1993; Jiravanichpaisal and Miyazaki, 1995; Lavilla-Pitogo, 1995). *Vibrio alginolyticus* (Liu *et al.,* 2004a; George *et al.,* 2005), *V. anguillarum* (Demircan and Candan, 2006), *V. cholera*e (Jayaprakash *et al.,* 2006), *V. fluvialis* (Ponnuraj *et al.,* 1995), *V. parahaemolyticus* (Abraham *et al.,*

1993), *V. mimicus* and *V. vulnificus* (Karunasagar *et al.*,1990), *V. damsela* (Aravindan & Kalavati, 1997), *V. harveyi* (Abraham and Manley, 1995) and *V. proteolyticus* CW8T2 (Verschuere *et al.,* 2000) have been isolated from larval shrimps.

Six species of *Vibrio*, *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, and *V. splendidus* were found to be associated with five types of diseases, i.e., tail necrosis, shell disease, red disease, loose shell syndrome (LSS), and white gut disease (WGD) in cultured shrimps from Andhra Pradesh and among these, LSS, WGD, and red disease caused mass mortalities (Jayasree *et al.,* 2006). Larval prawns are particularly susceptible to *V. harveyi* succumbing to luminescent bacterial disease (Lavilla-Pitogo *et al.,* 1998). *V. harveyi* was also reported as the causative agent of vibriosis in tiger prawn (*Penaeus monodon*) (Otta *et al.*,1999), kuruma prawn (*Penaeus japanicus*) (de la Pena *et al.*,1993; Liu *et al.*,1996; Chen *et al.*,2000) and pearl oyster (*Pinctada maxima*) (Lavilla-Pitogo *et al.,* 1990). A highly pathogenic *V. nigripulchritudo* was identified as the etiological agent for Summer Syndrome (Goarant *et al.*,2006a; b; Lemonnier *et al*., 2006) which resulted in mass mortality outbreaks in *Marsupenaeus japonicus* in Japan (Sakai *et al.,* 2007).

2.2.2.4. Fishes

The occurrence of *Vibrio* spp. as part of the normal microbiota and as primary or secondary pathogens as well as a contaminant of raw or under cooked seafood has been well established (Popovic *et al.,* 2010; Adebayo-Tayo *et al.,* 2011). *V. cholerae* non-O1 was isolated and identified from the gut of several individual fish (*Tilapia* sp.) from various freshwater bodies in northern Israel (Halpern *et al.*, 2008). Support for the finding that

V. cholerae survives in fish comes from the fact that some cholera outbreaks have been correlated with the consumption of seafood (Acosta *et al.*, 2001; Forssman *et al.*, 2007). *V. cholerae* was also recovered from faecal samples of apparently healthy Atlantic bottlenose dolphins (*Tursiops truncatus*) sampled in both Texas and Florida (Buck and McCarthy, 1994; Buck *et al.,* 2006). The species *V. parahaemolyticus* causes acute gastroenteritis and food poisoning in humans who consume raw or improperly cooked seafood (Hlady and Klontz, 1996; Pan *et al.,* 1997). Quintol and colleagues (2008) observed that the incidence of *V. parahaemolyticus* contamination ranged from 15 to 46.66% of fish samples collected from different fish markets of West Bengal, India and the same species was found to be prevalent at varying magnitude of 35-55% in fresh, marine and brackish water fish (Sanjeev and Stephen, 2012) from India.

2.2.2.4.1. Fish Pathogenesis

Vibriosis caused by infection by *Vibrio* sp., is one of the most prevalent diseases in fishes and other aquaculture-reared organisms and is widely responsible for mortality in aquaculture systems worldwide (Austin and Austin, 1993; Hjeltnes and Roberts, 1993; Chatterjee and Haldar, 2012).

Mortality caused by vibrios in reared fish is very common during early larval stages and can occur suddenly, leading sometimes to death of the entire population (Iwamoto *et al.,* 1995; Lambert *et al.,* 1998; Hansen and Olafsen, 1999; Diggles *et al.,* 2000; Olafsen, 2001). The mode of infection in fish consists of three basic steps (Larsen and Boesen, 2001; Larsen *et al.,* 2001): (i) the bacterium penetrates the host tissues by means of chemotactic motility (ii) within the host tissues the bacterium deploys iron-sequestering systems, e.g., siderophores, to "steal" iron from the host and (iii) the bacterium

eventually damages the fish by means of extracellular products, e.g., hemolysins and proteases.

2.2.2.4.2. Fish Pathogens

Vibrio species such as *V. vulnificus* biotype **2** (serovar E), V. *anguillarum,* V. *alginolyticus, V. salmonicida, V. damsela* and *V.carchariae* are pathogens of marine and fresh water fishes (Hjeltnes and Roberts, 1993). These species cause haemorrhagic septicaemia in marine fish and may lead to substantial mortalities in cultured populations (Wong and Leong, 1990).

V. vulnificus serovar E (formerly biotype 2) is a primary pathogen for eels (Tison *et al.,* 1982). As an eel pathogen, this serovar causes primary septicemia, that affects captured eels maintained in farms, occasionally causing high mortality rates resulting in economic losses (Biosca *et al.,* 1996; Dalsgard *et al.,* 1999).

V. anguillarum, the aetiological agent of classical vibriosis, possesses a wide distribution causing a typical haemorrhagic septicaemia in a wide variety of warm and cold water fish species of economic importance, including Pacific and Atlantic salmon (*Oncorhynchus* spp.), rainbow trout (*Oncorhynchus mykiss*), turbot, seabass, seabream, striped bass (*Morone saxatilis*), cod (*Gadus morhua*) and Japanese and European eel (*Anguilla japonica* and *Anguilla anguilla*) (Toranzo and Barja, 1993; Actis *et al.,* 1999; Frans *et al.,* 2011). *V. anguillarum* accounts for the mortality of turbot larvae (*Scophthalmus maximus*) culminating in the death of the fish (Grisez *et al.,* 1996) and is reported in Asian seabass (*Lates calcarifer*) (Kumaran *et al.,* 2010) and aquarium catfish (Rad and Shahsavani, 2010) samples.

V. alginolyticus is associated with vibriosis and have been isolated from outbreaks and mortalities in many marine fish species like silver sea bream in Hong Kong (Austin and Austin, 2007), sturgeon (*Acipenser baerii*) in Siberia (Costinar *et al.,* 2010), and in ornamental bird wrasse fish (*Gomphosus caeruleus*) in Egypt (El- Ghalil *et al.,* 2012).

V. salmonicida has been reported as the etiological agent of the Hitra disease or cold water vibriosis, characterized by severe anaemia and extensive haemorrhage that affects salmonids and cod cultured in Canada and Nordic countries of Europe (mainly Norway and UK) (Bruno, 1986; Egidius *et al.,* 1986).

V. damsela has been described as causing wound infections in bottlenose dolphins (Fujioka *et al.,*1988); damselfish (Love *et al.,*1981), yellowtail (Sakata *et al.,*1989), brown shark (Grimes *et al.,*1984) and in farmed turbot (Fouz *et al.,* 1992).

V. carchariae has been associated with gastroenteritis in marine fishes (Lee *et al.,* 2002) and was isolated from red drum (*Sciaenops ocellatus*) (Liu *et al.,* 2003) and farmed marine cobia fish (*Rachycentron canadum*) (Liu *et al.,* 2004b) with gastroenteritis syndrome.

2.3. Vibrios as Human Pathogens

Evidence from epidemiological and ecological studies has firmly established that some environmental strains of *Vibrio* sp. are etiologic agents of a wide range of diarrhoeal and systemic diseases in man. Perhaps the most provocative outcome has been recognition that the etiologic agent of cholera, *V. cholerae*, is commonly found as a natural resident of aquatic environments in cholera-free areas and that its presence is not necessarily

associated with faecal contamination or sporadic human infections (Rogers *et al.,* 1980; Feacham, 1981). Emergence of fulminating systemic infections associated with other *Vibrio* species in the environment is increasingly attracting more attention and these infections can range from self-limiting gastroenteritis and wound infections to severe necrotizing infections of soft tissues and fatal septicaemia in patients with underlying debilitation. In virtually every instance, infection can be associated with consumption of seafood or contact with natural aquatic environments (Janda *et al.,* 1988). Infection by human pathogenic vibrios is usually inadvertent and results through a complex series of ecological interactions before contact by the victim with aquatic environments, either by exposure or by consumption of seafood.

The genus *Vibrio* presently contains twelve species pathogenic to humans (Table 2.1). Those of prime medical concern are *V. cholerae, V. parahaemolyticus* and *V. vulnificus*. Other organisms implicated as opportunistic pathogens are *V. alginolyticus, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. mimicus, V. metschnikovii*, *V. cincinnatiensis* and *V. carchariae.*

2.3.1. *Vibrio cholerae*

Cholera is a highly infectious disease, caused by infection of the small intestine with *V. cholerae* O1 and O139 serotypes and is characterized by massive acute diarrhoea, vomiting, and dehydration. Death occurs in severe and untreated cases. The pathogenic strains include cholera toxin-producing strains which cause pandemic cholera; serogroups O1 (biotype Classical and ElTor) and O139 according to the LPS antigen. The bacterium uses the flagellum to reach the small intestine and attaches to mucosa cells by the

pili (coded by *tcp* genes; the toxin co-regulated pili). The cholera toxin, produced by the pathogen is engulfed by the mucosal cells which increases the cell cAMP levels leading to stimulation of intestinal secretions inducing neurotransmitters within the cells, followed by an increase in Cl-secretion. The ion channels normalizing the ion balance are blocked and large amounts of water flow into the lumen from the mucosa cells, causing massive diarrhoea. *V. cholerae* is often transmitted by water but fish or fish products in contact with contaminated water or faeces from infected persons also frequently serve as a source of infection (Colwell, 1996). India reported cholera cases and deaths to WHO regularly from 1997 to 2006 and over the 10-year period, the average number of cases reported annually was 3,631 (Kanungo *et al.,* 2010).

V. cholerae non O1/O139 serotypes are occasionally isolated from cases of diarrhoea (usually associated with consumption of shellfish) and have been isolated from a variety of extra-intestinal infections, including wounds, ear, sputum, urine, necrotizing fasciitis, skin and cerebrospinal fluid (Ko *et al*., 1998; Sharma *et al*., 1998; Morris and Acheson, 2003; Restrepo *et al*., 2006; Feghali and Adib, 2011). Some symptoms may be so severe as to mimic cholera (Hughes *et al.,* 1978; Piergentili *et al.,*1984). Cases of bacteraemia caused by non-O1 *V. cholerae* have been reported with a high rate of fatality especially with immune-compromised victims (Hughes *et al.,* 1978; Safrin *et al.,* 1988). While the great majority of these strains do not produce the cholera toxin, some strains may produce toxins yet unidentified. However, toxigenic non-O1/non-O139 strains carrying cholera toxin have been reported earlier from Calcutta, India (Sharma *et al*., 1998).

2.3.2. *Vibrio vulnificus*

V. vulnificus causes progressive infections with few diarrhoeal symptoms, making them unique amongst the pathogenic *Vibrios* (Blake *et al.,* 1980). The risk factors for severe *V. vulnificus* infections are chronic hepatic disease or immunodeficiency in patients (Ito *et al.,* 1999). Primary septicaemia or the gastrointestinal illness in human beings may follow after ingesting raw seafood, particularly of ocean filter feeders (e.g. oysters and clams) and fish infected by *V. vulnificus* (Morris and Acheson, 2003). For susceptible persons, septicaemia is associated with a mortality rate greater than 50% and may be characterized by fevers, chills, and skin lesions like blisters, swelling, and purpuras (Kumamoto *et al.*, 1998). Wound infections are seen after injury to the skin in marine environments or from the exposure of pre-existing wounds to seawater (Kumamoto *et al.,* 1998; Borenstein and Kerdel, 2003). Dieng and team (2001) described two cases of necrotising dermo-hypodermatitis due to *V. vulnificus* in fishermen after a penetrating scratch from a fish bone. More rarely, *V. vulnificus* has been associated with other clinical syndromes, including pneumonia (Kelly and Avery, 1980), osteomyelitis (Vartian and Septimus, 1990) spontaneous bacterial peritonitis (Holcombe, 1991), eye infections (DiGaetano *et al.,* 1989) and meningitis (Katz, 1988).

2.3.3. *Vibrio parahaemolyticus*

Seafood which is contaminated with *V. parahaemolyticus* and not cooked sufficiently may cause diarrhoeal disease (Mihajlovic *et al.,* 1982). Symptoms include mild diarrhoea with abdominal cramps and occasional bloody tinge, nausea, vomiting and fever. Hospitalization is rare, unless severe fluid loss has occurred, and the illness is generally self-limiting after

a few days (Huq *et al.,* 1979). A few extraintestinal infections have been reported, in particular, from wounds (Blake *et al.,* 1980) and a rare pneumonic form of *V. parahaemolyticus* infection has also been reported (Yu andUy-Yu, 1984).

2.3.4. *Vibrio alginolyticus*

V. alginolyticus often occurs as an opportunistic pathogen in mixed bacterial infections of extra-intestinal wounds in man (Bonner *et al.,* 1983). Most reports of wound infected with *V. alginolyticus* list mild cellulitis and varying amounts of seropurulent exudate as clinical features (Schmidt *et al.,* 1979). Most infections are self-limiting and opportunistic (Blake *et al.,* 1980; Wagner and Crichton, 1981). Curiously, the organism has a predisposition for hosts with ear disorders (Prociv, 1978).

2.3.5. *Vibrio fluvialis*

V. fluvialis is an important cause of cholera-like bloody diarrhoea and causes wound infection with primary septicaemia in immune compromised individuals (Huq *et al.,* 1985). There are also reports of food poisoning caused by this organism especially due to consumption of raw shellfish (Levine and Griffin, 1993) and the clinical indication of the disease includes mild to moderate dehydration, vomiting, fever, abdominal pain and diarrhoea. The pathogen has also been found in association with extra-intestinal infections (Yoshii *et al.,* 1987; Albert *et al.,* 1991).

2.3.6. *Vibrio damsela*

This organism is a marine pathogen associated with traumatic wound infections acquired in warm tropical and semi-tropical coastal areas (Kreger, 1984). In many instances of infection, the wounds were exposed to

warm seawater or brackish water at the time of injury. Typically, these would be lacerations of the foot or leg sustained while swimming or handling fish (Clarridge and Zighelboim-Daum, 1985).

2.3.7. *Vibrio furnissii*

Vibrio furnissii is considered to be a causative agent of acute gastroenteritis with symptoms including diarrhoea, abdominal cramps, nausea, and vomiting (Brenner *et al.,* 1983; Dalsgard *et al.,* 1997). The pathology of *V. furnissii* in gastroenteritis is potentially related to cytolysin and hemolysin production (Maghalhaes *et al.,* 1993). *V. furnissii* has been reported as an unusual cause of bacteremia and skin lesions after ingestion of seafood (Derber *et al.,* 2011).

2.3.8. *Vibrio hollisae*

V. hollisae has been associated with blood infections and cases of diarrhoea (Hickman *et al.,* 1982). This bacterium has been isolated from cases of gastroenteritis and septicaemia (Lowry *et al.,* 1986; Abbott and Janda, 1994) and was also reported to be a cause of bacteraemia developed by seafood consumption (Rank *et al.,* 1988).

2.3.9. *Vibrio mimicus*

V. mimicus has been isolated from a variety of clinical disorders associated with exposure to aquatic environments. Shandera and co-workers (1983) reviewed the clinical and epidemiological characteristics of infections associated with *V. mimicus*. The majority of isolates was from stool samples associated with gastroenteritis after consumption of raw oysters. A few ear infections arose after exposure to seawater.

2.3.10. *Vibrio metschnikovii*

The first published evidence of disease due to *V. metschnikovii* in humans was reported by Jean-Jacques and colleagues (1981) who isolated this organism from the blood and gall bladder of an elderly woman. There is little information on the ecology and pathogenicity of this organism other than reports of its isolation from freshwater environments (West *et al.,* 1983), estuaries, sewage and seafood and a few isolates from infections (Farmer *et al.*, 1988). Miyake and co-workers (1988) reported a clinical case of diarrhoea caused by *V. metschnikovii* and identified a cytolysin as a possible contributory virulence factor produced by this weakly pathogenic organism.

2.3.11. *Vibrio cincinnatiensis*

V. cincinnatiensis was ascribed to the pathogenic group of *Vibrios* by Brayton and co-workers (1986). In the single case reported, the organism was isolated from a case of septicaemia and meningitis in an elderly immune-competent man who had apparently not been recently exposed to seafood or saltwater (Bode *et al.,* 1986). Meningitis caused by pathogenic *Vibrios* is rare but often fatal (Hughes *et al.,* 1978). Interestingly, recovery in this case was uneventful.

2.3.12. *Vibrio carchariae*

The pathogenic *V. carchariae* was first isolated from a brown shark in captivity (Grimes *et al.,* 1984). This organism was later isolated and characterised from the wound of a girl who was attacked by a shark and had developed a series of deep parallel lacerations on her left leg with extensive damage to the gastrocnemius and soleus muscles. The pathogenicity was

attributed to the presence of this bacterium though there were no signs of extensive tissue invasion or systemic toxicity (Pavia *et al.,* 1989).

Table.2.1. Human pathogenic *Vibrio* species and their location in human clinical specimens

Species	Gastroenteritis	Wound	Ear	Septicaemia	Bacteremia	Lung	Meninges	Reference
V.cholerae O1/O139	P	R	-	-	-	-	-	Gaafar, 1970
V.cholerae Non O1/O139	P	P	R	R	R	-	R	MacRae *et al.*,1983
V. parahaemolyticus	P	P	R	-	R	R	R	Huq *et al.*,1979
V. vulnificus	P	P	-	P	R	R	R	Johnston *et al.*,1983
V. alginolyticus	-	P	R	-	R	R	-	English and Lindberg, 1977; Janda *et al.*,1986
V. fluvialis	P	-	-	-	-	-	-	Huq *et al.*,1985
V. damsela	-	P	-	-	-	-	-	Kreger, 1984; Clarridge and Zighelboim-Daum, 1985
V. furnissii	R	-	-	-	-	-	-	Brenner *et al.*,1983
V. hollisae	P	-	-	R	-	-	-	Morris *et al.*,1983
V. mimicus	P	R	R	-	-	-	-	Spira and Fedorka-Cray, 1983
V. metschinikovii	R	-	-	R	-	-	-	Linde *et al.,* 2004
V. cincinnatiensis	-	-	-	-	R	-	R	Brayton *et al.*,1986 ; Bode *et al.*,1986
V. carchariae	-	P	-	-	-	-	-	Pavia *et al.*,1989

P- Present; R- Rare; '-'Absent

2.4. Isolation of Vibrios

As the natural habitat of vibrios is the marine environment, sodium chloride is critical for the growth of vibrios and growth is favoured at a slightly alkaline pH in the range of 7.5-8.5. *Vibrio* spp. are facultatively anaerobic and do not, in general, have fastidious growth requirements, growing readily on peptone-based media as long as NaCl requirements are met. They grow well at temperatures between 15 and 30°C, depending on the strain under analysis (Thompson *et al.,* 2004).

Laboratory protocols for the isolation of vibrios from the environmental reservoirs or suspected clinical samples include both direct-plating and broth enrichment methods (McLaughlin, 1995). The most commonly used enrichment medium is alkaline peptone water (APW; 1% peptone, 1% NaCl, pH 8.4). Other broth media described for enrichment include Monsur's enrichment medium that contains trypticase, potassium tellurite, and sodium taurocholate (bile salts) and a modification of APW, in which potassium tellurite is added in concentrations of 1:100,000 to 1:200,000 (Rennels *et al.,* 1980).

Thiosulphate-citrate-bile salts-sucrose (TCBS) agar (Difco) is a selective medium commonly used to isolate members of the genus *Vibrio* from estuarine environments (West *et al.,* 1983; Pfeffer and Oliver, 2003). TCBS agar is commercially available and easy to prepare, requires no autoclaving, and is highly differential and selective. Sucrose fermenters produce yellow coloured colonies and non-fermenters produce green to blue-green colonies.

The use of thiosulphate-chloride-iodide (TCI) agar was described by Beazley and Palmer (1992). This medium employs potassium iodide

as the selective ingredient rather than the bile salts used in TCBS. Created primarily to isolate vibrios from gastrointestinal specimens, TCI included the ability to perform the oxidase test directly on the plate due to the lack of a fermentable sugar within this medium (Pfeffer and Oliver, 2003).

The Chromogenic Agar medium (CV1) uses the chromogenic technology for the isolation and detection of *V. parahaemolyticus, V. vulnificus, V. cholerae,* and *V. alginolyticus*, resulting in colonies which can be distinguished based on color development (mauve, dark blue, light blue, and white/colorless, respectively) (Nakashima *et al.,* 2007). Indeed, the ability to isolate and identify these four medically relevant pathogenic vibrios with one medium is highly advantageous.

A number of selective media have been formulated and developed for isolation and enumeration of *Vibrio* species. The Taurocholate Tellurite Gelatin (TTG) agar developed by Monsur (1961) for isolating *V. cholerae,* Cellobiose colistin Agar (CC) and modified Cellobiose polymyxin colistin (mCPC) agar for selective isolation of *V. cholerae* and *V. vulnificus* (Massad and Oliver, 1987), Vibrio vulnificus agar (VV) for isolation of *V. vulnificus* Biogroup 1(Brayton *et al.,*1983), Chromochecker Vibrio Agar-1(CVA-1) for *V. vulnificus* (Nakashima *et al.,*2007), Vibrio anguillarum agar (VAM) for presumptive isolation of *V. anguillarum* (Alsina *et al.,*1994c), Bromothymol Blue (BTB)-Teepol agar and modified arabinose-ammonium sulfate-cholate agar (MAAC) for *V. parahaemolyticus* (Honda *et al.,*1982), Vibrio harveyi Agar (VHA) for *V. harveyi* (Harris *et al.,*1996) and VAL agar for *V. alginolyticus* (Chang *et al.,* 2011) have proved to be effective in epidemiological and ecological studies.

2.5. Phenotypic Identification

Phenotypic traits used for the identification of *Vibrio* species are the Gram negative reaction, oxidase test where vibrios are always positive and the oxidation/fermentation (OF) test in which *Vibrio* species are facultatively fermentative. *Vibrio* species can be differentiated from one another and from *Aeromonas* species by the sensitivity test to the vibriostatic agent O/129 (Abbott and Janda, 1994). A practical set of biochemical keys for the routine identification of *Vibrio* spp. was developed (Alsina and Blanch 1994a; b) which proved useful for identifying species for both environmental and clinical purposes, and were widely used in numerous studies (Montes *et al.,* 1999; Oxley *et al.,*2002; Hjelm *et al.,*2004; Baffone *et al.,* 2006). They served as an ideal method for rapid, routine biochemical identification in which a large number of isolates was involved, particularly in environmental studies.

Commercial bacterial identification systems based on biochemical reactions are used in the vast majority of hospital-based clinical microbiology laboratories. O'Hara and co-workers (2003) evaluated six commercial systems (API 20E, Crystal E/NF, MicroScan Neg ID type 2, type 3, Vitek GNI and Vitek ID-GNB) for the ability to identify the 12 species of *Vibrio* found in clinical samples. They found that the accuracy of these identification systems ranged from 63.9% to 80.9% and at times even lead to erroneous identification. The characteristics shared by *Aeromonas* and *Vibrio* also result in classification of isolates in the wrong genus (Abbott and Janda, 1994).

Rapid identification of *V. cholerae* have been attained by detection of the lipopolysaccharide (LPS) antigen of *V. cholerae* O1 (Jesudason *et al.,*

1984; Andrade *et al.,* 1991) and O139 (Hasan *et al.,*1995; Agarwal *et al.,* 1995). A rapid, colorimetric immunodiagnostic kit, Cholera SMART, was used for direct detection of the presence of *V. cholerae* in clinical specimens and has demonstrated 95% sensitivity and 100% specificity for O1 strains (Hasan *et al.,* 1994) and 100% sensitivity and 97% specificity for O139 strains (Qadri *et al.,* 1995).

Tamplin and colleagues (1991) developed an enzyme immunoassay (EIA) and culture technique with monoclonal antibody FRBT37 that binds to a species-specific epitope of *V. vulnificus*, which aided identification of *V.vulnificus* in seawater, sediment and oysters. Compared to conventional method, immunological-based assays greatly eliminate the lengthy and labor-intensive cultural assays for *Vibrio* identification, but cross-reactions were distinguished owing to the nature of the test method, which limits the specific application of such assays (Parker and Lewis, 1995). The specificity of the monoclonal antibodies to develop rapid diagnostic tests for cholera O1 or O139 using colloidal gold particles and based on vertical-flow immune-chromatography principle were evaluated by Nato and team (2003) in clinical stool samples obtained from Madagascar and Bangladesh. The detection threshold with purified LPS was 10ng/mL for *V. cholerae* O1 and 50ng/mL for *V. cholerae* O139.

2.6. Genotypic Identification

An array of molecular techniques is gaining popularity for the identification of vibrios particularly with respect to pathogenic strains. Among DNA sequence based identification methods, analysis of 16S rRNA and other housekeeping gene sequences are the most popular and precise ones currently used to identify closely related *Vibrio* species. Among other

methods, ribotyping and PCR-based techniques, e.g., Amplified Fragment Length Polymorphism (AFLP), Fluorescence *In Situ* Hybridization (FISH), Random Amplified Polymorphic DNA (RAPD), Repetitive extragenic palindrome–PCR (rep-PCR), and Restriction Fragment Length Polymorphism (RFLP) have yielded the most valuable information and new insights into the identification of closely related bacterial strains.

2.6.1. PCR-based Detection and Identification

2.6.1.1. 16S rRNA Gene Based Identification

16S rRNA gene sequencing is considered by many authors to be a very reliable method for identification of bacteria including marine vibrios due to its rapid and reproducible nature (Gomez-Gil *et al.,* 2004; Harth *et al.,* 2007; Haldar *et al.,* 2011). This notion was supported by Petti and colleagues (2005) who showed that this method was able to correctly identify bacteria including pathogens that had been misidentified by traditional methods. The 16S rRNA gene (about 1,500 bp in length) consists of highly conserved regions and is present in almost all bacteria which may reveal deep-branching (e.g., classes, phyla) relationships, while variable regions may be demonstrated to be useful in discriminating species within the same genus. This feature has prompted researchers to use 16S rRNA both as a phylogenetic marker and as an identification tool (Wiik *et al.,* 1995).

There are also several problems associated with the use of the 16S rDNA for bacterial identification. No universally accepted criteria exist for the required level of homology to delineate isolates of the same species or genus. Some bacteria with different phenotypic characters may share up to 100% 16S rDNA sequences whereas less than 99% 16S rDNA sequence homology has been observed in bacteria belonging to the same species

(Harris and Hartley, 2003). In some species multiple heterogenous copies of the 16S rRNA gene operons exist (Case *et al.,* 2007; Pontes *et al.,* 2007). Some distantly related *Vibrio* species may share up to 99% 16S rDNA sequence homology (Shikongo- Nambabi, 2010). It is therefore recommended to combine the 16S rRNA gene sequence with other species specific morphological and physiological characteristics to correctly identify *Vibrio* species (Mienda, 2012). The 16S-23S Intergenic spacer (IGS) region nucleotide sequences complement the 16S rDNA in *Vibrio* species identification (Hoffmann *et al.,* 2010).

2.6.1.2. Identification Based on Housekeeping Genes

Keasler and Hall (1993) designed a multiplex PCR for detecting the cholera toxin (*ctx*A) gene in pathogenic and environmental *V. cholerae* O1 Classical and El Tor biotypes and for differentiating these biotypes through their differences in the toxin coregulated pilus (*tcp*A*)* genes. A multiplex PCR consisting of three primer pairs targeting the *rfb* (gene region specific for O1 and O139) and the cholera toxin (*ctx*A) gene was developed by Hoshino and colleagues (1998) and the *rfb* gene based PCR could detect up to 65 and 200 O1 and O139 cfu per assay in clinical samples respectively. A highly sensitive, specific and rapid method for detection of pathogenic *V. cholerae* in environmental water and drinking water sources was developed and evaluated by Theron and co-workers (2000) by means of a seminested *ctx*AB gene specific PCR. PCR targeting the *Omp*W (Outer membrane protein) gene was used in detection and identification of environmental *V. cholerae* strains (Nandi *et al.,* 2000; Le Roux *et al.,* 2004). Other approaches that have been followed include a multiplex Real Time PCR (RT- PCR) targeting four *V. cholerae* virulence genes (Gubala, 2006)

and a Real Time PCR targeting *V. cholerae* specific genes, i.e., repeat in toxin (*rtx*A), extracellular secretory protein (*eps*M), the toxic coregulated pilus A (*tcp*A) and *omp*W gene with a view to enabling detection of both toxigenic and non toxigenic strains (Gubala and Proll, 2006).

A number of genes have been used as targets to develop species specific PCR based detection for other pathogenic *Vibrio* species. In *V. parahaemolyticus*, these genes include the phosphatidyl serine synthetase gene (pR72H fragment) (Lee *et al.,* 1995; Robert-Pillot *et al.,* 2002), the gyrase B gene (Venkateswaran *et al.,* 1998), the species specific *tox*R gene (Kim *et al.,* 1999), the collagenase gene (Di Pinto *et al.,* 2006), the thermolabile direct haemolysin, *tlh* (Baffone *et al.,* 2006; Croci *et al.,* 2007), the thermostable direct haemolysin, *tdh* and the thermostable direct haemolysin related haemolysin, *trh* (Hervio-Heath *et al.,* 2002*;* Baffone *et al.,* 2006*)* and the metalloprotease gene (Luan *et al.,* 2007). The gyrase B (*gyr*B) gene of *V. parahaemolyticus* and of its close genetic relative *V. alginolyticus* was cloned and sequenced, and the primers (Vp-1 and Vp-2r) that amplified a 285bp fragment from the *V. parahaemolyticus gyr*B gene was used to detect *V. parahaemolyticus* by PCR (Venkateswaran *et al.,* 1998).

Di Pinto *et al.* (2006) used three oligonucleotide primer pairs specific for either *V. parahaemolyticus*, *V. cholerae* or *V. alginolyticus* collagenase gene, that also aided simultaneous detection of *V. parahaemolyticus* or *V. alginolyticus* that were present in alkaline peptone water (APW) enriched shellfish tissue homogenates. These primer pairs were recommended in discriminating between *V. alginolyticus* and *V. parahaemolyticus* (Montieri *et al.,* 2010).

Qian and colleagues (2008) cloned two proteins from *V. alginolyticus* (*Omp*K and *Omp*W), expressed them in *E. coli* and designed specific primers for these genes aiding rapid detection of *V. alginolyticus* in environmental samples. Dalmasso *et al.* (2009) designed a multiplex PER (Primer extension reaction) PCR protocol directed against *rpo*A gene to simultaneously detect and identify six human pathogenic *Vibrio* species (*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus* and *V. fluvialis*) in fishery products.

A multiplex PCR was designed against collagenase, *omp*K and *tox*R genes to differentiate virulent and avirulent strains of *V. alginolyticus* (Cai *et al.,* 2009; Shikongo-Nambabi, 2010). The test also improved the rapidity, sensitivity and specificity for *V. alginolyticus* detection and could detect up to 8.8×10^2 cfu. Genes targeted for the detection and identification of *V. vulnificus* included the gyrase B gene (Kumar *et al.,* 2006) and nested PCR directed to the haemolysin gene (*vvh*) (Lee *et al.,* 1998). Real Time PCR (RT-PCR) was developed to detect *V. vulnificus* in sea water and oyster tissue homogenates targeting the species specific *vvh* gene (Panicker *et al.,* 2004) and the TaqMan assay, a quantitative real-time PCR, was developed to target the *tox*R gene of *V. vulnificus* (Takahashi *et al.*,2005). Chakraborty and team (2006) developed a species specific PCR targeting the *tox*R gene of the less characterised human pathogen, *V. fluvialis* facilitating a successful differentiation of this pathogen from the closely related *Aeromonas* species.

Arias and associates (1995) developed a highly sensitive nested PCR specific for the 23S rDNA of *V. vulnificus*. Single locus *tox*R and hemolysin gene-targeted PCR were developed for the specific detection of

type strain *V. harveyi* (Conejero and Hedreyda, 2003) and a *tox*R-targeted PCR was reported for the detection of shrimp pathogenic Philippine *Vibrio* isolates (Ranoa and Hedreyda, 2005).

2.6.2. Identification Based on Multilocus Sequence Analysis (MLSA)

Multilocus sequence analysis (MLSA) is a promising taxonomic tool to identify and differentiate between closely related bacterial species. MLSA uses gene sequences from more than one locus, generally of housekeeping genes, that occurs as a single copy in the genome (Thompson *et al.,* 2005; Pontes *et al.,* 2007). It is required that these genes are long enough to give sufficient information and should be of the length that permits easy sequencing (Thompson *et al.,* 2005). The genetic loci that were initially used in MLSA for taxonomic studies of *Vibrio* species included the 16S rRNA gene, the RNA polymerase alpha subunit (*rpo*A), the recombinant repair protein (*rec*A) and the uridylate kinase gene (*pyr*H) (Thompson *et al.,* 2005). Thompson and associates (2007b) have also developed an MLSA scheme to distinguish *V. harveyi* from the closely related *V. campbellii* species using seven housekeeping genes; *rec*A, *top*A *(*topoisomerase I), *pyr*H, *fts*Z (a cell division protein), *gap*A (glyceraldehydes-3 phosphate dehydrogenase) and the *gyr*B DNA (gyrase B gene beta subunit). The resulting 3596 nucleotide long DNA gave a better resolution than when only three genes, *gyr*B, *rec*A and *gap*A were used. MLSA therefore acts as a buffer against mutations and horizontal gene transfer (HGT) problems associated with the 16S rDNA sequences (Thompson *et al.,* 2005). Whitehouse and team (2010) coupled Electrospray Ionization Mass Spectrometry to Multi Locus Sequence PCR (PCR/ESI-MS) using 8 primer pairs that target the whole Vibrionaceae family to simultaneously

detect, quantify and identify *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* species in sea water and fresh water in Georgia. The novel scheme is considered a robust and precise method to identify and discriminate between closely related *V. cholerae* from *V. mimicus*.

2.7. Antibiotic Resistance in Vibrios

Aquatic ecosystems have been proposed as reservoirs of antibiotic resistance (Biyela *et al.,* 2004) and resistant bacteria in coastal environments represent a serious biotic contamination and a means for the spread and evolution of resistance genes and their vectors (Young, 1993). These aspects have raised the concern about the potential threat of wide-spread antibiotic resistance to public health and mariculture industry and resistance mechanisms in natural marine environments.The progressive increase in antimicrobial resistance among *Vibrio* species is a serious impediment in the treatment and containment of the diseases caused by these pathogens. The resistance of *Vibrio* species to several antimicrobial drugs has been frequently reported in samples of clinical origin as well as of environmental origin in different endemic areas around the world.

Reports of drug-resistant *V. cholerae* strains are appearing with increasing frequency. Emergence of microbial resistance to multiple drugs is a serious clinical problem in the treatment and containment of the cholera-like diarrhoea. *V. cholerae* O139 isolates have been frequently reported to be resistant to various commonly used antibiotics, such as ampicillin, tetracycline, chloramphenicol, nalidixic acid, nitrofurantoin and to ciprofloxacin (Waldor *et al.,* 1996; Krishna *et al.,* 2006; Pan *et al.,* 2008). Increased resistance to newer fluoroquinolones, such as ciprofloxacin and

norfloxacin, among *V. cholerae* strains belonging to O1 serogroup has also been reported (Basu *et al.,* 2000).

In the last decade, the pattern of drug resistance among clinical *V. cholerae* strains have been studied extensively in India. The O1 El Tor strains obtained from 1997-2007 were resistant to furazolidone, ciprofloxacin, amoxicillin and cotrimoxazole (Chander *et al.,* 2009). Fluroquinolone resistance was exhibited in strains of O1, O139 and environmental non O1/ non O139 isolates from Hubli, India (Chandrasekhar *et al.,* 2008) and ciprofloxacin resistant O1 strains were reported by Kim and colleagues (2010) from Bangladesh. The O1 El Tor strain from Chennai exhibited multidrug resistance to cotrimoxazole, nalidixic acid, nitrofurantoin, spectinomycin, sulfamethoxazole–trimethoprim and streptomycin (Goel *et al.,* 2010). Environmental isolates of *V. cholerae* non O1/ non O139 strains from Kerala exhibited varying degrees of resistance to cefotaxime, nalidixic acid, streptomycin, tetracycline, trimethoprim, cotrimoxazole, furazolidone, neomycin, ofloxacin, ciprofloxacin, norfloxacin, spectinomycin, gentamicin and chloramphenicol (Manjusha *et al.,* 2005; Bernard, 2006; Jagadeeshan *et al.,* 2009).

Antibiotic resistance profiles have also been produced for environmental isolates of non-choleragenic *Vibrio* species. *V. vulnificus* isolates were reported to be resistant to gentamicin, ticarcillin, amikacin, augmentin and ampicillin (Zanetti*et al.,* 2001; Ottaviani *et al.,* 2001; Manjusha *et al.,* 2005; Elhadhi, 2012). However, norfloxacin, trimethoprim/sulfamethoxazole and tetracycline were susceptible towards the isolates.

Among the *V. parahaemolyticus* strains isolated from diverse environments, resistance was detected only to ampicillin and the isolates

were susceptible to cefotaxime, ciprofloxacin and tetracycline (Han *et al.*, 2007). Ottaviani and associates (2001) showed that *V. parahaemolyticus* were resistant to penicillin, carbenicillin, ampicillin, cephalothin, kanamycin and rifampicin and multi-drug resistant strains exhibiting resistance to five to eleven antibiotics with multiple antibiotic resistance index (MAR) ranging from 0.31 to 0.69, were isolated from cockles in Padang, Indonesia (Zulkifli *et al.*, 2009).

2.8. Molecular Typing of Vibrios

Molecular typing methods or fingerprinting methods, including Amplified Fragment Length Polymorphism PCR (AFLP), Random Fragment Length Polymorphism PCR (RFLP), Random Amplified Polymorphic DNA (RAPD), Repetitive Element Primed PCR (rep-PCR) , Repetitive Extragenic Palindromic-PCR (REP-PCR), Enterobacterial Repetitive Intergenic Consensus sequence PCR (ERIC-PCR), BOX-PCR (derived from the boxA element), Ribotyping, Pulsed Field Gel Electrophoresis (PFGE) and Whole Genome Sequence Typing (WGST) provide information at or below the subspecies level and are important tools in epidemiological investigations for determining the source of virulent strains and their temporal and spatial distributions (Tindall *et al.*, 2010).

2.8.1. Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a highly sensitive method for detecting polymorphisms in DNA using restriction enzymes that digest genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. The restriction fragments are then amplified using primers complementary to the adaptor sequence, the restriction site sequence and a few nucleotides inside the restriction site fragments. The

amplified fragments are separated on a gel, producing a specific AFLP pattern, that aid in the identification of genetic variation in strains and closely related species. This technique was used for molecular typing of various Vibrios (Thompson *et al.,* 2001), including *V. alginolyticus* (Ripabelli *et al.,* 2003), *V. cholerae* (Jiang *et al.,* 2000; Lan and Reeves, 2002; Mishra *et al.,* 2011), *V. harveyi* (Gomez-Gil *et al.,* 2004), *V. vulnificus* (Arias *et al.,* 1997a; b)), and *V. parahaemolyticus* (Thompson *et al.,* 2007a). Lan and Reeves (2002) examined 45 *V. cholerae* isolates from the seventh pandemic and partitioned these isolates into 38 AFLP profiles. They concluded that AFLP is the best tool for discriminating clones from the seventh pandemic and suggested the design of PCR primers which target particular AFLP bands that could be used for epidemiological analysis through multiplex PCR or microarray analyses. Arias and co-workers (1997a; b) examined 80 *V. vulnificus* strains and with AFLP analysis, were able to discriminate strains with identical ribotypes, thus concluding that AFLP is the most suitable and discriminatory tool for epidemiological studies of *V. vulnificus*.

2.8.2. Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism or RFLP is a technique that exploits variations in homologous DNA sequences. In RFLP analysis, the DNA sample is digested by restriction enzymes and the resulting restriction fragments are separated by gel electrophoresis. Saha and team (2006) developed a simple and rapid RFLP method based on the chromosomal *ori* sequence of *V. cholerae* and Chowdhury and his team (2010) developed an RFLP method targeting sections of the super integron region of the *V. cholerae* genome, both of which proved effective in delineating closely related biotypes of pathogenic *Vibrio* strains.

The PCR-RFLP technique consists of PCR amplification of certain genes, e.g., 16S rRNA, *gyrB*, and *rpoD*, and subsequent restriction of the PCR products with endonucleases to obtain band patterns (Urakawa *et al.,* 1997; 1999). According to Urakawa and colleagues (1997) who analysed the restriction patterns of the 16S rRNA of 35 species of Vibrionaceae, this technique is of practical application in the classification and identification of Vibrionaceae strains.

2.8.3. Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is an in vitro enzymatic reaction to specifically amplify a multiplicity of target sites in one or more nucleic acid molecules based on short synthetic oligonucleotides of arbitrary or semi-arbitrary primer sequences that produce a collection of amplified products of largely non-allelic nature (Williams *et al.,* 1990). The polymorphism within the set of DNA fragments thus generated, has wide application in discriminating microorganisms both at the interspecies and intraspecies level (Welsh and McClelland, 1993).This technique has been used in diversity studies of several *Vibrio* spp. (Shangkuan *et al*., 1997; Marhual *et al.*,2012; Sadok *et al.*,2013) and in *V. harveyi,* it has been used in differentiating pathogenic and non-pathogenic strains (Hernandez and Olmos, 2004; Alavandi *et al*., 2006; Maiti *et al.,* 2009).

2.8.4. REP PCR

The REP elements were first described as regulatory palindromic sequences within untranslated regions of operons with the ability to form stable stem-loop structures in transcribed RNA (Higgins *et al.,* 1982). This 38 bp REP consensus sequence contains six totally degenerate positions, including a 5 bp variable loop between each side of the conserved stem of

the palindrome (Stern *et al.,* 1984). These sequences have been associated with multiple functions including roles in transcription, termination, mRNA stability, and chromosomal domain organization *in vivo* (Versalovic, *et al.,* 1991). REP-PCR has been used for typing of *V. parahaemolyticus* (Maluping *et al.,* 2005) *V. tapetis* (Rodriguez *et al.,* 2006) *V. alginolyticus* and *V. vulnificus* isolated from diseased captive-bred seahorses (Balcazar *et al.,* 2010) and has proven to be a powerful tool for microbial ecology studies, environmental microbiology, molecular diagnostics, medical microbiology and epidemiological analyses.

2.8.5. Enterobacterial Repetitive Intergenic Consensus (ERIC) Sequences

Molecular typing based on ERIC pattern involves the application of oligonucleotide primers targeting families of short, highly conserved enterobacterial repetitive intergenic consensus (ERIC) sequences (Stern *et al.,* 1984; Hulton *et al.,* 1991). In ERIC-PCR, a band pattern is obtained by amplification of genomic DNA located between ERIC elements or between ERIC elements and other repetitive DNA sequences. ERIC sequences are present in species throughout the Enterobacteriaceae family (Versalovic *et al.,* 1991; Bachellier *et al.,* 1999). The use of appropriate outward-facing PCR primers directed at these repeated sequences generates multiple amplification products, which reflect distance polymorphisms between adjacent DNA repeats. These sequences are 126 bp long and appear to be restricted to transcribed regions of the genome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames (Rodriguez *et al.,* 2006). ERIC-PCR pattern has been chosen as a successful technique for intraspecies profiling of several bacteria, like *Bacillus anthracis* and *B. cereus* (Shangkuan

et al., 2000), *Salmonella enteritidis* (Cao *et al.,*2008), *Haemophilus* (Bruant *et al.,* 2003), *Enterobacter sakazakii* (Ye *et al.,* 2009), *Lactobacillus* (Stephenson *et al.,* 2009) and *Listeria monocytogenes* (Ruiz-Bolivar, 2011).

ERIC sequences are novel and highly conserved at the nucleotide sequence level, but their chromosomal locations differ between species (Hulton *et al.,* 1991). The ERIC sequence of *V. cholerae* is highly homologous with those found in Enterobacteriaceae species and is located near the hemolysin gene, apparently 'hitchhiking' with the hemolysin gene (Hulton *et al.,* 1991). It is speculated that a transpecific genetic exchange had affected a group of *E. coli* haemolysin genes and thus the ERIC sequence had 'hitchhiked' with the haemolysin gene (Hulton *et al.,* 1991).

Rivera and co-workers (1995) proposed the use of ERIC-PCR for typing diverse *Vibrio* species, including *V. mimicus*, *V. vulnificus*, *V. parahaemolyticus*, *V. campbellii*, *V. mediterranei*, and *V. alginolyticus*. This technique along with REP-PCR has been successfully employed in the discrimination of strains of *V. cholerae* (Rivera *et al.,* 1995), *V. vulnificus* (Radu *et al.,* 2000), *V. halioticoli* (Sawabe *et al.,* 2002), *V. parahaemolyticus* (Maluping *et al.,*2005) and *V. tapetis* (Rodriguez *et al.,*2006). The study on *V. cholerae* strains responsible for cholera outbreaks in India reported that heavy rainfall events could introduce different genotypes of *V. cholerae* in the affected area as evidenced by ERIC-PCR analysis (Goel and Jiang, 2011). The comparative analysis of the effectiveness of REP-PCR and ERIC for genetic analysis of the diversity of *V. cholerae* in edible ice from Jakarta revealed that the ERIC sequence is less complex and more discriminative than the REP sequence (Waturangi *et al.,* 2012).

2.8.6. BOX PCR

BOX elements, a class of repetitive sequences which were discovered in *Streptococcus pneumoniae* (Martin *et al.,* 1992) are naturally occurring, multi-copied, conserved, repetitive DNA sequences present in the genomes of most gram-negative and gram-positive bacteria. Although no clear-cut function has been proposed for these elements, they seem to represent regulatory DNA domains probably controlling competence-specific and virulence-related genes (van Belkum *et al.,* 1996). These repeats were found to consist of three discriminate regions: *box*A, *box*B, *and box*C, which are 59, 45, and 50 base pairs in length respectively (Martin *et al.,* 1992). Koeuth and co-workers (1995) developed several primers for the box A, B and C subunits, and found the BoxA element to be the most conserved among bacterial species. The BOX A1R primer amplifies specific genomic regions located between BOX elements (154 bp) and its natural inverted repeats (Martin *et al.,* 1992), typically occurring in approximately 25 copies. These primers have been used to classify bacteria in accordance with the band profiles and is a powerful tool for determining and clustering inter and intra-species, as well as being a promising candidate for pathogen profiling and microbial source tracking (MST) (Johnson *et al.,* 2004; Yang and Yen, 2012). The features that make BOX PCR a frequently used tool in biogeography studies in environmental microbiology is that it is quick and cheap, the band patterns consistent, not affected by the culture age of the strain to be analysed (Kang and Dunne, 2003) and the fingerprints can be easily analysed by computer assisted methods (Tuang *et al.,* 1999). However, variation in the brand of thermostable DNA polymerase and the nature of the buffer and gel concentration caused the banding patterns to change, limiting its consistent reproducibility (Yang and Yen, 2012).

BOX PCR, independent from the other rep-PCR techniques, has revealed the possibility of delineating *P. syringae* genomospecies (Marques *et al.,* 2008), for identification of races and biovars of *Ralstonia solanacearum* (Galal *et al.,* 2003) and for typing strains of *Aeromonas* species (Tacao *et al.,* 2005).

Implementation of BOX PCR along with other typing methods has been useful in epidemiological studies of *V. cholerae* revealing clonal origin and relatedness among clinical and environmental strains (Singh *et al.,* 2001; Kumar *et al.,* 2009; Goel *et al.,* 2010). The molecular diversity based on BOX PCR combined with additional fingerprinting methods were analysed to cluster and compare intra and inter species variation in *V. parahaemolyticus* (Maluping *et al.,* 2008), *V. harveyi* (Gomez-Gil *et al.,* 2004) and *V. vulnificus* (Gordan, 2008). The technique has also been used to define new species of *Vibrio* as a part of a polyphasic molecular approach (Rameshkumar and Nair, 2007; Rameshkumar *et al.,* 2011).

2.9. Characteristics and Classification Scheme of Toxigenic *Vibrio cholerae*

V. cholerae is classified on the basis of its somatic antigens (O antigens) into serovars or serogroups and there are at least 200 known serogroups (Baumann *et al.,* 1984; Shimada *et al.,* 1993; 1994). Until 1992, the only serogroup known to cause epidemic cholera was O1. Strains belonging to the O1 serogroup were further classified into two biotypes, namely the Classical and El Tor and these are differentiated by phenotypic traits and by precise genetic markers (Kaper *et al.,* 1995). In addition to this, *V. cholerae* O1 is classified into 3 serotypes, namely Ogawa, Inaba and Hikojima, the last of which is a rare and inadequately described

serotype (Shimada *et al.,* 1994). Seven recorded pandemics of cholera have occurred globally and there is firm evidence that at least the 5th and 6th were caused by the classical biotype O1 strains (Blake, 1994).

The El Tor biotype was responsible for the 7th pandemic (Blake, 1994). In 1992, another serogroup, namely O139 caused outbreaks of cholera in India and Bangladesh (Ramamurthy *et al.,* 1993). Currently, these 2 serogroups are associated with endemic and epidemic cholera, while the other *V. cholerae* serogroups, not associated with epidemics or pandemics, are collectively referred to as non-O1, non-O139 *V. cholerae* or also as non-epidemic serogroups. These non-O1/non-O139 strains have been divided into serogroups O2 through O200 on the basis of variation in lipopolysaccharide (LPS) somatic antigen.

The great majority of these strains does not produce the cholera toxin (CT) and are not associated with epidemic diarrhoea (Morris, 1990). These strains are occasionally isolated from cases of diarrhoea (usually associated with consumption of seafood) and have also been implicated with a variety of extra-intestinal infections (Ko *et al*., 1998; Sharma *et al*., 1998; Restrepo *et al*., 2006; Rozemeijer *et al*., 2009). They are regularly found in estuarine environments, and infections due to these strains are commonly of environmental origin (Kaper *et al*., 1995). While the great majority of these strains do not produce CT, some strains may produce toxins yet unidentified. However, for many strains of *V. cholerae* non-O1/non-O139 isolated from cases of gastroenteritis, the pathogenic mechanisms are unknown. Toxigenic non-O1/non-O139 strains belonging O144, O19, O12, O39, O6, O11 serogroups carrying *ctx*A/B have been reported previously from Calcutta, India (Sharma *et al*., 1998).

V. cholerae serogroup O75 strains possessing the cholera toxin gene were isolated from patients with severe diarrhoea, in Georgia, Alabama, and South Carolina (Tobin-D'Angelo *et al*., 2008). The genes of CTXΦ genome could be transferred from O1 strains to non-O1/non-O139 strains suggesting that this mechanism might be responsible for spread of *ctxA/B* genes in aquatic environment (Choi *et al*., 2010). Although environmental non-O1/non-O139 strains may acquire virulence-associated genes and become human pathogens, most of these strains are unlikely to attain pandemic potential by acquisition of TCP and CT genes alone. It thus appears that the toxigenic non-O1/non-O139 strains and the pandemic O1 and O139 strains may have distinct lineages (Faruque *et al*., 2004).

2.10. Virulence Factors of *V. cholerae*

The pathogenesis of cholera is a complex process and involves a number of factors which aid the pathogen to reach and colonize the epithelium of the small intestine and produce the enterotoxin that disrupts ion transport by intestinal epithelial cells. Although production of cholera toxin encoded by the *ctx*AB genes is directly responsible for the manifestation of diarrhoea, cholera pathogenesis relies on the synergistic action of a number of other genes, including the genes for one or more colonization factors (Kaper *et al.,* 1995). Several bacterial pathogens have acquired clusters of virulence genes that display a typical base composition and these Pathogenicity Islands are not present in related non-pathogenic species (Basu *et al.,* 2000). In *V. cholerae*, the major virulence genes appear to exist in clusters and there are at least 2 regions of the *V. cholerae* chromosome in which genes encoding virulence factors are clustered (Ogierman *et al.,* 1993; Pearson *et al.,* 1993; Trucksis *et al.,* 1993; Everiss *et al.,* 1994; Harkey *et al.,* 1994).

2.10.1. Cholera toxin and CTX phage

Cholera toxin activates the adenylate cyclase enzyme in cells of the intestinal mucosa leading to increased levels of intracellular cAMP, and the secretion of H_20, Na^+, K^+, Cl^-, and HCO_3^- into the lumen of the small intestine. The effect is dependent on a specific receptor, monosialosyl ganglioside (GM1 ganglioside) present on the surface of intestinal mucosal cells. The bacterium produces an invasin, neuraminidase, during the colonization stage which has the interesting property of degrading gangliosides to the monosialosyl form, which is the specific receptor for the toxin (Collier and Mekalanos, 1980). The toxin has been characterized and contains 5 binding (B) subunits of 11,500 daltons, an active (A1) subunit of 23,500 daltons, and a bridging piece (A2) of 5,500 daltons that links A1 to the 5B subunits. Once it has entered the cell, the A1 subunit enzymatically transfers ADP ribose from NAD to a protein (called Gs or Ns), that regulates the adenylate cyclase system which is located on the inside of the plasma membrane of mammalian cells. The effect of the toxin is to cause cAMP to be produced at an abnormally high rate which stimulates mucosal cells to pump large amounts of Cl^- into the intestinal contents. H_2O, Na^+ and other electrolytes follow due to the osmotic and electrical gradients caused by the loss of Cl. The lost H_2O and electrolytes in mucosal cells are replaced from the blood. Thus, the toxin-damaged cells become pumps for water and electrolytes causing the diarrhoea, loss of electrolytes, and dehydration that are characteristic of cholera.

Toxigenic *V. cholerae* carries one or more copies of CT encoding genes, *ctx*A and *ctx*B (Waldor and Mekalanos, 1996). The A and B subunits of CT are encoded by 2 separate but overlapping open reading frames

(ORFs). The genes encoding CT form part of the genome of a lysogenic filamentous bacteriophage designated CTXΦ. CTXΦ DNA is generally found integrated at either one (El Tor) or two (Classical) loci within the *V. cholerae* genome (Mekalanos, 1983; Pearson *et al.*,1993; Waldor and Mekalanos,1996). CTXΦ is unusual among filamentous phages because the phage genome encodes the functions necessary for a site specific integration system and thus can integrate into the *V. cholerae* chromosome at a site-specific attachment site known as *att*RS, forming stable lysogens. The phage genome consists of 2 regions, RS2 and core. The RS2 represents a site-specific recombination system that allows the lysogenic phage to integrate at specific sites on the host chromosome and the core consists of number of genes, including the *ctx*A, *ctx*B, *zot, ace, psh, cep* and *orf*U (Waldor *et al*., 1996; 1997). The RS2 region consists of the *rst*R, *rst*A and *rst*B genes. The *rst*A gene product is responsible for phage DNA replication, *rst*B is involved in site-specific integration of the phage and *rst*R is a repressor of *rst*A function (Waldor *et al*., 1997).

It has been showed that under suitable conditions toxigenic *V. cholerae* strains can be induced to produce extracellular CTXΦ particles (Waldor *et al*., 1996; Faruque *et al*., 1998b).The phage can be propagated in recipient *V. cholerae* strains in which the CTXΦ genome either integrates chromosomally at a specific site forming stable lysogens or maintained extra-chromosomally as a replicative form of phage DNA (Faruque *et al*., 1998a). Studies have established that some naturally occurring non-toxigenic strains of *V. cholerae* are infected by CTXΦ and converted to toxigenic strain with epidemic potential (Faruque *et al*., 1998b). The bacteriophage uses the Toxin Coregulated Pilin (TCP) as a receptor and therefore expression of TCP by the bacterium is a requirement for its

susceptibility to the phage. Consequently, a virulence factor of the bacterium in humans also serves as a receptor for CTXΦ, demonstrating a co-evolution of genetic elements mediating the transfer of virulence genes with the pathogenic bacterial species they infect (Faruque *et al*., 1998a).

2.10.2. Accessory Cholera Toxin (Ace)

The Ace toxin is an integral membrane protein that consists of 96 amino acids (9-11.3 kDa) (Kaper *et al.,* 1995; Chatterjee *et al.,*2011). This toxin increases the potential difference (PD) across the intestinal epithelium, alters transcellular ion transport and increases the short-circuit current in rabbit ileal tissues that have been mounted in Ussing chambers (Trucksis *et al.,* 1997; Somarny *et al.,* 2004). It is an amphipathic molecule, which when inserted into the eukaryotic cell membrane creates an ion-permeable pore. It acts synergistically with a Ca^{2+}-dependent acetylcholine analog (carbachol) and stimulation secretion has been shown to be dependent on extracellular and intracellular Ca^{2+} (Trucksis *et al.,* 2000). The gene encoding this toxin *ace*, identified by Trucksis *et al.,* (1993) is located immediately upstream of the gene encoding Zot (Zonula occludens toxin) and cholera toxin.

2.10.3. Hemolysin/Cytolysin

Hemolysin, often designated as cytolysin, is a widely distributed toxin among pathogenic *Vibrio* sp. and exerts various roles in the infection process (Iida and Hond, 1997; Shinoda, 1999). They act on erythrocyte membranes leading to cell lysis, which in turn, frees the iron-binding proteins like haemoglobin, transferrin and lactoferrin. The pore forming activity of hemolysin is not restricted to erythrocytes, but extends to a wide range of other cell types including mast cells, neutrophils and polymorphonuclear cells

and enhances virulence by causing cell damage (Ludwig and Goebel, 1997; Shinoda, 1999).

There are four representative hemolysin families produced by *Vibrio* spp. which includes the El Tor hemolysin of *V. cholerae* (Hly A) (Yamamoto *et al.,* 1986; Fallarino *et al.,* 2002), the thermostable direct hemolysins (TDH and δVPH) and the thermolabile hemolysin (TLH) of *V. parahemolyticus* (Taniguchi *et al.,* 1990; Zhang *et al.,* 2001). Hly A hemolysin lyses erythrocytes and other mammalian cells and exhibits enterotoxicity in experimental diarrhoea models, thus manifesting a major role in the pathogenesis of gastroenteritis caused by *V. cholerae* strains (Ichinose *et al.,* 1987). The hemolysin is exported by the majority of *V. cholerae* O1 and non-O1 strains to the culture supernatant as the 79 kDa precursor, prohaemolysin, which on proteolytic removal of the N-terminal 15 kDa pro-domain converts to the fully mature 65 kDa toxin (Nagamune *et al.,* 1996). The much-studied mechanism of cell lysis (hemolysis) by HlyA seems to involve intercalation of single monomeric HlyA proteins into cell membranes; their coalescence to form highly ordered multimeric pores that allow selective leakage of certain ions, and eventual cell disruption (Zitzer *et al.,* 1993). HlyA protein exhibits remarkable differences in potency for erythrocytes from different animal species and cell types (Zitzer *et al.,* 1997) and a dual specificity for cholesterol and ceramides (Zitzer *et al.,* 1999).

2.10.4. Outer Membrane Proteins

Six major outer membrane proteins have been reported in *V. cholerae* (Chakrabarti *et al.,* 1996). The 45-kDa protein OmpS is a maltoporin induced upon growth on maltose and similar to the LamB porin of *Escherichia coli*

(Lang and Palwa, 1993). OmpV (25 kDa) is a heat-induced, highly immunogenic protein associated with peptidoglycan (Sahu *et al.,* 1994). The 35-kDa outer membrane protein, OmpA, is heat modifiable and reminiscent of OmpA of *E. coli* (Alm *et al.,* 1986). The OmpX protein (27 kDa), although osmoregulated and trypsin-resistant, does not have pore-forming properties and is not classified as a porin (Chakrabarti *et al.,* 1996). OmpU (38 kDa) and OmpT (40 kDa) have been shown to allow the transport of hydrophilic solutes in liposome swelling assays (Chakrabarti *et al.,* 1996) and are porins that form water-filled channels across the outer membrane. They are thought to function primarily as channels for entry and exit of hydrophilic low-molecular-mass (600-Da) molecules. Similar proteins have also been described in other *Vibrio* species and shown to have expression patterns that are sensitive to medium composition or external conditions (Davey *et al.,*1998; Nitzan *et al.,*1999; Provenzano and Klose, 2000). In the genus *Vibrio*, OMPs function as porins for iron, phosphate, and sugar transport and in attachment to inanimate surfaces, although their role in adhesion to host epithelia remains controversial (Aeckersberg *et al.,* 2001). OmpU mediates resistance to bile and anionic detergents such as sodium dodecyl sulfate, while OmpT increases susceptibility to anionic detergents (Provenzano and Klose, 2000; Provenzano *et al.,* 2000).

The fact that expression of OmpU is coregulated with expression of critical virulence factors such as CT and the TCP suggests that OmpU is also an important virulence factor (Miller and Mekalanos, 1988).

2.10.5. RTX Toxin

The RTX toxins represent a family of important virulence factors that have disseminated widely among gram-negative bacteria (Coote, 1992). The RTX toxin gene cluster in *V. cholerae* encodes the presumptive

cytotoxin (RtxA), an acyltransferase (RtxC), and an associated ATP-binding cassette transporter system (RtxB and RtxD, two proteins for toxin transportation) (Chow *et al.,* 2001) and is physically linked to the cholera toxin prophage (Lin *et al.,* 1999). Because genes of common pathogenic function are often genetically linked, it is proposed that the *V. cholerae* RTX toxin, like cholera toxin, may play a role in the gastrointestinal virulence properties of *V. cholerae* (Chow *et al.,* 2001).

It has been proved that the interaction of RTX toxin with cells leads to depolymerization of stress fibers and cross-linking of actin, causing the cell rounding effect and increased permeability through paracellular tight junctions (Lin *et al*., 1999; Fullner *et al*., 2001). It was demonstrated that the actin cross linking domain of RTX directly catalyzes covalent cross linking of actin monomers and G-actin or short actin oligomers might be the cytoskeletal substrates of the actin cross-linking reaction *in vivo* (Cordero *et al*., 2007). Classical strains of *V. cholerae* carry a deletion of DNA sequences that overlap the *rtx*A, *rtx*C, and *rtx*B genes and hence defective in production of cytotoxic activity associated with the RTX gene cluster (Lin *et al*., 1999). Thus, the intact RTX gene cluster might have provided a selective advantage for the emergence of El Tor O1 and O139 strains. The RTX toxin is considered to be one of the factors contributing to the reactogenicity of some of the cholera vaccine strains in human subjects (Fullner *et al*., 2001).

2.10.6. Zot Enterotoxin

Zonula occludens toxin (Zot) is an enterotoxin elaborated by *V. cholerae* that increases intestinal permeability by interacting with a mammalian cell receptor with subsequent activation of intracellular signalling leading to the

disassembly of the intercellular tight junctions (Fasano *et al.,* 1991). The gene encoding Zot (*zot*) is a 4.5 kb dynamic region of the *V. cholerae* chromosome located immediately upstream of the CT operon and the size of the Zot protein predicted from the DNA sequence is 44.8 kDa (Baudry *et al.,* 1992).The toxin seems to be involved in the CTXΦ morphogenesis because Zot mutagenesis studies demonstrated the inability of CTX elements to be self-transmissible under appropriate conditions (Walder and Mekalanos, 1996). The high concurrence among *V. cholerae* strains of the *zot* gene and the *ctx* genes (Johnson *et al.,* 1993; Karasawa *et al.,*1993) also suggests a possible synergistic role of Zot in the causation of acute dehydrating diarrhoea typical of cholera. Beside its role in phage morphogenesis, Zot also increases the permeability of the small intestine by affecting the structure of the intercellular tight junctions, the effect initially described on rabbit ileal tissues mounted in Ussing chambers by using filtered supernatants from *V. cholerae O1*strains, (Fasano *et al.,* 1991; Baudry *et al*, 1992). Zot also possesses a cell specificity related to the toxin interaction with a specific receptor whose surface expression differs on various cells (Fasano *et al.,* 1997; Uzzau *et al.,* 2001). It also induces modifications of cytoskeletal organization that lead to the opening of tight junctions secondary to the transmembrane phospholipase C and subsequent protein kinase Ca-dependent polymerization of actin filaments strategically localized to regulate the paracellular pathway (Di Pierro *et al.,* 2001).

2.10.7. NAG-ST

The NAG-ST is a 17-amino-acid peptide (Arita *et al.,*1986) of *V. cholerae* non-O1 strains which exhibits remarkable similarity, especially in the carboxyl-terminal toxic domain, to the heat-stable enterotoxins (STs)

produced by enterotoxigenic *E. coli* (Takao *et al.*,1983; Yoshimura *et al.*,1986). Morris *et al.,* 1990) showed that a non-O1 strain of *V. cholerae* which produced this toxin could cause diarrhoea in volunteers. NAG-ST causes IP3-mediated calcium release from intracellular calcium store, which then stimulates nitric oxide production by activating nitric oxide synthase and the nitric oxide through cGMP activates calcium influx (Hoque *et al*., 2004). The genes encoding this enterotoxin (*sto*) in *V. cholerae* O1 and non-O1 are flanked by 123-bp direct repeats (Ogawa and Takeda, 1993), thus suggesting that this toxin has been introduced into *V. cholerae* via a transposon.

2.10.8. Toxin Co-regulated Pilus (TCP)

The type IV pilus encoded by *V. cholerae*, named toxin coregulated pilus (TCP) (Taylor *et al.,* 1987), is composed of 7 nm filaments of the TcpA pilin subunit, which laterally associate into bundles, leading to intestinal colonization in both animal and human volunteer studies (Attridge *et al*., 1996) as a result of autoagglutination of the cells (Kirn *et al.,* 2000). In addition, it acts as a receptor for the CTXΦ (Waldor and Mekalanos, 1996) and also facilitates biofilm formation on chitinaceous surfaces (Reguera and Kolter, 2005). The major subunit of TCP, encoded by the *tcp*A gene is assembled into a cell-surface colonization determinant by the action of at least seven accessory proteins (Peterson and Mekalanos, 1988; Shaw *et al.,* 1990; Kaufman *et al.,* 1991; 1993; Ogierman *et al.,* 1993). The *tcp*A gene is considered to be part of a larger genetic element known as the Vibrio Pathogenicity Island (Karaolis *et al*., 1998) residing on chromosome I (Heidelberg *et al.,* 2000) of *V. cholerae*. Even though the cholera toxin (CT) and the TCP are exclusively associated with clinical

strains of *V. cholerae*, especially those belonging to the serogroups O1 and O139, their presence and expression in environmental strains of *V. cholerae* has also been reported (Chakraborty *et al.,* 2000; Kumar *et al.,* 2008).

2.10.9. Neuraminidase

V. cholerae neuraminidase, a sialidase, is considered a virulence factor (Staerk *et al.,* 1974; Galen *et al.,* 1992) that releases sialic acid from higher gangliosides present on eukaryotic cells surface, exposing ganglioside GM1, which is the cholera toxin receptor. The neuraminidase gene, *nan*H, is present in the Vibrio Pathogenicity Island 2 of toxigenic strains of *V. cholerae* (Jermyn and Boyd, 2002) and has also been detected in *V. mimicus* (Jermyn and Boyd, 2005) and *V. vulnificus* (Jeong *et al.,* 2009). NanH, a large neuraminidase (83 kDa), with a three-domain protein consisting of two lectin wings and a central active neuraminidase domain of six β-sheets (Crennel *et al.,* 1994), is also known to have cellular roles such as sialic acid metabolism (Vimr *et al.,* 2004) and is a part of the mucinase complex that acts on the mucus gel protecting the underlying intestinal cells (Stewart-Tull *et al.,* 1986). The breakdown of sialomucin allows the bacteria to reach and colonize the epithelium.

2.11. Vibrio Pathogenicity Island of *Vibrio cholerae*

Vibrio Pathogenicity Island (VPI) is considered to be one of the initial genetic factors required for the emergence and pathogenesis of epidemic and pandemic *V. cholerae*. VPI has many features characteristic of pathogenicity islands (PAIs); low G+C content compared to the rest of the genome, phage like attachment sites (*att*) at its termini, inserted site specifically in the chromosome of epidemic *V. cholerae* strains downstream of tRNA-like locus, genes with potential roles in DNA mobility at its left

and right ends and a phage like integrase gene (*int*) which belongs to the family of site specific recombinases (Karaolis *et al*., 1998). The chromosomal VPI of *V. cholerae* is 41.2 kb in size and is divided into three functional regions (Karaolis *et al*., 2001); the left region containing a gene encoding a potential transposase and several ORFs (Open Reading Frames) of unknown function, the central region containing many *tcp* genes encoding proteins for the TCP structure as well as *tcp*PH genes that regulate TCP and CT expression and the right region including the remaining transport and assembly *tcp* genes, the *tcp*J gene (gene required for processing), *int* gene (high homology to phage like integrase), and the *acf* gene cluster (which appears to have a role in colonization). The VPI encodes 29 potential proteins (Karaolis *et al*., 2001) playing major roles in virulence of *V. cholerae,* including those involved in the synthesis of the toxin-coregulated pilus (TCP) (Taylor *et al.,*1987), accessory colonization factors (Peterson and Mekalanos, 1988) that also acts as receptor for cholera toxin bacteriophage, CTXΦ (Waldor and Mekalanos, 1996) and virulence gene regulators, ToxT (DiRita *et al.,*1991; Higgins *et al.,*1992), TcpH (Carroll *et al.,* 1997) and several ORFs with no known or demonstrated function (Hase and Mekalanos, 1998).

The sequence variations exhibited by the VPI genes of different strains of *V. cholerae* suggest that the VPI is mosaic in nature resulting from recombination events (Karaolis *et al.,* 2001; Vital-Brazil *et al.,* 2002). The presence of VPI in *V. mimicus* isolates suggest that it can be horizontally transferred between *V. cholerae* and *V. mimicus* (Boyd *et al.,* 2000). The finding that the VPI is flanked by phage-like attachment sites and contain a phage-like integrase which can excise from the chromosome (Kovach *et al.,* 1996; Karolis *et al.,*1998; 1999; 2001) and that it can be transferred

by generalized transduction (O'Shea and Boyd, 2002) substantiates that the VPI can undergo horizontal transfer.

Several additional genomic regions have also been identified, predominantly among epidemic O1 and O139 serogroup isolates; these include RS1Φ, Vibrio Seventh Pandemic island-I (VSP-I), VSP-II and VPI-2 (Dziejman *et al.,* 2002; Jermyn and Boyd, 2002; O'Shea *et al.,* 2004). RS1 Φ is associated with the CTX prophage in *V. cholerae* ElTor isolates and is required for the production of CTX Φ (Davis and Waldor, 2003). VSP-I and VSP-II are genomic islands identified by microarray analysis among *V. cholerae* El Tor isolates (Dziejman *et al.,* 2002). It was suggested by Dziejman and colleagues (2002) that the genes encoded on the VSP-I and VSP-II islands are likely to be responsible for the unique characteristics of the seventh pandemic (ElTor) strains. VPI-2 is a 57.3 kb chromosomal region encoding ORFs with all the characteristics of a pathogenicity island (Jermyn and Boyd, 2002). It was suggested that all toxigenic *V. cholerae* O1 and O139 serogroup isolates possessed VPI-2 region, whereas non-O1/ nonO139/ non-toxigenic isolates lacked the region.

2.12. Regulation of Virulence Genes by ToxR Regulon

The gene *tox*R encodes the transmembrane transcription regulator ToxR, first identified to play a role in the coordinate regulation of virulence gene expression (Miller *et al.,*1987; Miller and Mekalanos, 1988) and was subsequently shown to play a pivotal role in the coordinate regulation of *ctx* and many other genes including the *tcp* gene encoding toxin-coregulated pili and the *omp*U and *omp*T genes encoding major outer membrane proteins (OMPs) in *V. cholerae* (Miller *et al.,*1987; Miller and Mekalanos, 1988; DiRita, 1992; Crawford *et al.,*1998). The *tox*R gene encodes a 294

amino acid polypeptide of 32,527-dalton transmembrane, DNA-binding protein that is sensitive to a variety of environmental signals that include osmolarity, pH, temperature, and the presence of certain amino acids (Miller *et al.,* 1987). The activity of ToxR has been reported to be enhanced by another 19 kDa transmembrane protein ToxS, which serves to assemble ToxR monomers into the dimeric form (DiRita *et al*., 1991). Among the genes of the *tox*R regulon, only *ctx*AB is directly regulated by ToxR protein while the other genes are controlled by another regulatory transcription activator, ToxT, a 32kDa protein (Higgins and DiRita, 1996). ToxR controls the transcription of *tox*T gene which encodes ToxT. Consequently, the enhanced expression of ToxT leads to the activation of about 17 distinct virulence related genes in the ToxR regulon. The existence of a regulatory cascade in the ToxT- ToxR regulatory proteins allows the bacterium to bring diversity in the expression of its genes to survive different environmental conditions i.e. the human intestine and its natural estuarine environment (Bina *et al.,* 2003).

The *tox*R gene was also detected in *V. parahaemolyticus* (Lin *et al.,* 1993), *V. fischeri* (Reich and Schoolnik, 1994), *V. vulnificus* (Lee *et al.,* 2000), *V. alginolyticus*, *V. hollisae*, *V. mimicus* and *V. fluvialis* (Osorio and Klose, 2000) and *V. anguillarum* (Okuda *et al.,* 2001). Conejero and Hedreyda (2003) were able to isolate and sequence a 576-bp *tox*R gene homologue from *V. harveyi*. Sequence analysis and alignment of the full length *tox*R gene of *V. harveyi* revealed that it shares 87% sequence similarity with *tox*R of *V. parahaemolyticus*, 84% similarity with *V. fluvialis*, 83% with *V. vulnificus* and partial sequence of *V. campbellii* (Franco and Hedreyda, 2006). A partial sequence of the *V. campbellii tox*R gene was obtained and *tox*R-targeted PCR primers for *V. campbellii* detection was

designed based on the partial *tox*R sequence (Castroverde *et al.,* 2006). Nucleotide sequence analysis of the full-length 873-bp *toxR of V. campbellii* revealed 100% sequence similarity with the partial *tox*R gene of *V. campbellii* (Franco and Hedreyda, 2006) and exhibited lower sequence similarity with *tox*R from *V. harveyi* (79%), *V. parahaemolyticus* (75%), *V. anguillarum* (64%), *V.vulnificus* (63%), *V. cholerae* (59%), *V. fischeri* (53%), *V.hollisae* (50%), and *Photobacterium profundum* (26%) (Orata and Hedreyda, 2011). The usefulness of *tox*R gene for phylogenetic and evolutionary analysis on *Vibrio* isolates belonging to *V. parahaemolyticus* and *V. alginolyticus* is evidenced from the work of Montieri and colleagues (2010). Sequence analysis of the virulence regulatory gene *tox*R is considered as a useful tool for the distinction of various species (Osorio and Klose, 2000) particularly in relation to the significant gap between its maximun intraspecific and minimum interspecific distances (Pascual *et al.*, 2010).

Comparison of the toxR amino acid sequences from several *Vibrio* species showed the presence of the highly divergent membrane tether regions flanked by relatively conserved transcription activation and transmembrane domains (Conejero and Hedreyda, 2003). For species-specific detection, the variable or divergent sequences may be useful in the development of PCR primers effective in identifying the target organism. It is also suggested that the *tox*R gene proposed as an ancestral gene of Vibrionaceae family, is a useful target for *Vibrio* species identification (Kim *et al.,* 1999; Conejero and Hedreyda, 2003).

2.13. Characteristics and Pathogenicity of *Vibrio vulnificus*

Vibrio vulnificus is a Gram-negative halophilic bacterium that inhabits warm coastal and estuarine waters worldwide, and the numbers are

substantially high in marine filter-feeders like bivalve molluscs (Strom & Paranjpye, 2000; Oliver, 2006). Two primary routes of *V. vulnificus* infections in humans are through the exposure of open flesh wounds and ingestion of the pathogen in raw or undercooked seafood. The consequent effects are septicaemia and wound infections sometimes leading to necrotizing fasciitis (Janda *et al.,* 1988; Bisharat *et al.,* 1999). Septicaemia or blood poisoning occurs when toxin-producing bacteria proliferate in the bloodstream. The highest percentage of individuals who experience septicaemia suffer from chronic liver disease (Haq and Dayal, 2005), followed by immuno-compromised people with other chronic diseases such as diabetes (Strom and Paranjpye, 2000; Ho *et al.,* 2003; Kuhnt-Lenz *et al.,* 2004; Haq and Dayal, 2005). *V. vulnificus* causes gastroenteritis much less frequently, following raw or under cooked oyster consumption (Strom & Paranjpye, 2000). Besides host susceptibility, epidemiological data also suggest that a small percentage of *V. vulnificus* strains in oysters are virulent (Warner and Oliver, 2008; Jones andOliver, 2009).

A distinction has been made between various strains of *V. vulnificus* into different biotypes initially based on differing phenotypes, serological properties, and host range (Tison *et al.,* 1982). Three different biotypes have been identified. Biotype 1 is an opportunistic human pathogen that is commonly isolated from environment and clinical samples (Biosca *et al.,* 1997; Hoi *et al.,* 1998; Watanabe *et al.,* 2004). The eel pathogen primarily cultured from eels or humans handling eels and responsible for eel mortality in aquaculture, belongs to Biotype 2 (Tison *et al.,* 1982; Esteve-Gassant and Amaro, 2004) and has rarely been isolated from the environment (Esteve-Gassant and Amaro, 2004; Marco-Noales *et al.,* 2004). *V. vulnificus* isolated from human wound aspirates was distinguishable from

both the aforementioned biotypes (Bisharat *et al.,* 1999), leading to the recognition of Biotype 3.

2.14. Virulence Factors of *V. vulnificus*

Several potential virulent factors have been examined for *V. vulnificus* which included the capsular polysaccharide (CPS), lipopolysaccharide (LPS) and flagella (Strom and Paranjpye, 2000; Lee *et al.*, 2004). Extracellular toxins like haemolysin, enterotoxins and enzymes like metalloprotease, chitinase, and phospholipase have been attributed as possible virulence factors (Strom and Paranjpye, 2000). Besides the virulence factors present in the pathogen, it was observed that an increased iron in the host resulted in increased susceptibility to infection, establishing the significant contribution of the host to the virulence of *V. vulnificus* (Wright *et al.,* 1981).

2.14.1. Capsular Polysaccharide (CPS)

The capsular polysaccharide (CPS) protects the pathogen from bactericidal mammalian serum as well as phagocytosis by macrophages (Yoshida *et al.,* 1985; Simpson *et al.,* 1987; Strom and Paranjpye, 2000) and is considered a major virulence factor for *V. vulnificus* (Chatzidaki-Livanis *et al.*, 2006). Presence of the capsular polysaccharide is related to the colony morphology, with encapsulated strains being opaque and unencapsulated strains being translucent (Yoshida *et al*., 1985; Wright *et al*., 1999). As with other bacteria that rely on their capsule to resist host defences during systemic disease, antibodies to the *V. vulnificus* capsule are protective in animal models (Devi *et al*., 1995; 1996). It exhibits a wide range of heterogeneity at the biochemical level (Bush *et al*., 1997), however, CPS-based vaccines have been developed that imparts protection

against *V. vulnificus* infection, albeit only against strains of the same carbotype (Devi *et al.,* 1995; 1996) and antibodies against *V. vulnificus* capsule have been found in infected patients, underlining the importance of CPS for *V. vulnificus* infection (Fiore *et al.,* 1996).

The identification of genes involved with capsular production was accomplished primarily through transposon insertion mutagenesis. Initially, a gene encoding a sugar epimerase enzyme, essential for capsule production and virulence was identified (Zuppardo and Siebeling, 1998) followed by the elucidation of the group 1-like capsular biosynthetic system (Wright *et al.,* 2001).Subsequently, Smith and Siebeling (2003) performed extensive transposon insertion mutagenesis and identified four genes (*wcv*A, *wcv*F, *wcv*I, and *orf*) essential for capsule biosynthesis and virulence, in addition to other genes with supporting roles in capsule biosynthesis. Phase variation associated with opaque to translucent colony type was also investigated by Wright *et al*. (1999) who determined that capsule biosynthesis was not constitutive, and that capsule production was decreased in stationary phase. Production of the capsule was also found to be inversely related to the ability of *V. vulnificus* to form biofilms *in vitro* (Joseph and Wright, 2004).

2.14.2. Hemolysin/ Cytolysin

The hemolytic and cytolytic activity of *V. vulnificus* was first described by Kreger and Lockwood (1981) and Johnson and Calia (1981). The hemolysin, a putative virulence factor in *V. vulnificus,* acts by forming pores in host cell membranes (Kim *et al*., 1993) and stimulates guanylate cyclase (Kook *et al.,* 1996) in endothelial cells of blood vessels leading to vasodilation and edema, which are characteristic of infection. However,

Kim and team (2006) suggested that hemolysin may play a greater role in environmental survival than in virulence and was supported by Smith and Oliver (2006) who suggested the role of hemolysin in osmotic and/or cold shock responses by some strains appearing to be temperature-regulated in natural environments.

Three types of hemolysin/cytolysins have been reported to be produced by *V. vulnificus.* The most widely studied hemolysin (VVH) is a water-soluble polypeptide (51 kDa) that binds to cholesterol on the membrane and forms small pores in the erythrocyte membrane (Gray and Kreger, 1987). It can induce apoptosis by elevating cytosolic free Ca^{2+}, release of cytochrome C from mitochondria, activate caspase-3, degrade poly-ADP ribose-polymerase (PARP) and induce fragmentation of DNA. The toxin increases vascular permeability and causes skin damage. *V. vulnificus* has been shown to have two other hemolysins apart from VvhA: VllY and HlyIII (Chang *et al.,* 1997; Chen *et al.,* 2004). The *hlyIII* knockout has a minor increase in LD_{50}, but still displays hemolytic activity on blood agar, possibly due to VllY and VvhA (Chen *et al.,* 2004). All three hemolysins could contribute to cytotoxicity in *V. vulnificus.*

2.14.3. Metalloprotease

The *V. vulnificus* metalloprotease, originally discovered because of its collagenase activity (Smith and Merkel, 1982) was identified with additional activities of elastase, caseinase and metalloprotease (Zn2+) (Kothary and Kreger, 1985; Miyoshi *et al.,* 1987). The metalloprotease was instrumental in increasing vascular permeability by stimulating the generation of inflammatory mediators, resulting in septicaemia accompanied by edematous lesions (Miyoshi and Shinoda, 2000; Miyoshi *et al.,* 2004). Metalloprotease

is a 45-kDa Zinc containing protease: the N-terminal 35-kDa part mediates proteolysis and the C-terminal 10-kDa part binds to protein substrates on erythrocytes and blood cells, thus effecting membrane permeability enhancement and tissue hemorrhage.

2.14.4. RTX Toxin

RTX toxins are made of repeated structural subunits which form pores in cellular membranes and are found in a broad range of Gram-negative bacteria. The amino acid sequence of *V. vulnificus* RtxA1 shows high homology to the RtxA sequence of *V. cholerae*, and organization of the Rtx gene cluster is also similar between the two species (Lee *et al.,* 2007; Kim *et al.,* 2008). The toxin has been associated with multiple cytotoxic and cytopathic activities including actin depolymerization, apoptosis, necrosis, induction of reactive oxygen species, and activation of caspase-1 (Lee *et al.,* 2007; 2008; Kim *et al.,* 2008; Chung *et al.,* 2010; Toma *et al.,* 2010). The toxin is encoded by an operon of four genes: *rtx*C, *rtx*A, *rtx*B and rtxD. Genes, *rtx*B and *rtx*D, encode a transport system for the RtxA toxin. RtxC participates in the acylation of RtxA, which is necessary for its activation (Lally *et al.,* 1999). RtxA toxins share a repeated nine amino acid sequence motif. An *rtxA* targeted mutant in *V. vulnificus* showed decreased cytotoxicity towards INT-147 human intestinal epithelial cells and an increase of 457 in the LD_{50} in the iron treated mouse model, suggesting that RtxA is a virulence factor (Lee *et al.,* 2007).

2.14.5. Iron Acquisition Systems

Studies on *V. vulnificus* pathogenesis have indicated that increased iron in the host resulted in increased susceptibility to infection. The pathogen expresses phenolate, hydroxamate, and vulnibactin siderophores

to scavenge iron from the host transferrin, hemin, and lactoferrin (Wright *et al.,* 1981; Helms *et al*., 1984). Hosts with high levels of iron such as those suffering from chronic hemochromatosis or cirrhosis of liver are highly susceptible to *V. vulnificus* infection. Wright and colleagues (1981) showed that iron treatment of mice reduced the intraperitoneal incidence of the pathogen. Liver damage by carbon tetrachloride increased susceptibility of mice in a manner proportional to the levels of iron in serum (Bullen *et al.,* 1991). A couple of studies indicated that the protease produced by *V. vulnificus* could be involved in acquisition of iron from heme proteins (Nishina *et al*., 1992; Okujo *et al*., 1996). *hupA* and *fur* genes regulate iron acquisition (Litwin and Byrne, 1998) and as might be expected with an organism whose pathogenesis is centred on iron imbalanced hosts and acquisition of iron, this element of *V. vulnificus* pathogenesis is complex.

2.14.6. Flagella

In addition to adhesion, flagellum-based motility is important for a variety of bacterial processes including biofilm formation and pathogenesis. In *V. vulnificus*, several flagellar genes have been mutated in order to investigate the role of flagella in pathogenesis. A loss of two flagellar structural components (encoded by *flg*C and *flg*E) each resulted in significant decreases in motility, cellular adhesion, and cytotoxicity compared to those of the parent strains (Ran-Kim & Rhee, 2003; Lee *et al.,* 2004). Injection of *flg*C and *flg*E mutants into mice resulted in increased LD_{50}, indicating that the flagellum is necessary for virulence. However, the use of a polar mutation and problems with complementation tempered the significance of these studies (Gulig *et al.,* 2005). It has been suggested that decreases in motility, adhesion, and cytotoxicity may play a concerted role in reducing

virulence, in that a loss of motility may lead to decreased adhesion and to an inhibition of cytotoxin delivery (Ran-Kim and Rhee, 2003; Lee *et al.,* 2004). Overall, studies investigating the role of attachment in *V. vulnificus* virulence lend support to the hypothesis that host cell contact is required for *V. vulnificus* toxin secretion and pathogenicity.

2.15. *In vivo* Models for Pathogenicity Studies

The molecular basis of the pathogenicity of infectious agents, and of the corresponding mechanisms of host defence can be well elucidated using model systems. Animal virulence models have been used for decades to determine the pathogenesis of bacteria. There is a continuing need for the development of simple animal models for the study of host-pathogen interactions (Finlay, 1999) as well as facilitating the identification and study of virulence mechanisms associated with pathogenesis (Mahajan-Miklos, 2000). Intuitively, the more closely related a model of an infectious disease is to the natural pathology, the greater is its relevance in virulence and pathogenicity assays. Animal virulence models and mammalian cell culture, as *in vivo* and *in vitro* systems respectively, have been used for decades to determine the pathogenesis of bacteria. Mammalian models have the disadvantages of long reproductive cycles, difficult handling, high cost and the ethical constraints on their use in such studies. Alternative models, such as the amoeba *Dictyostelium discoideum*, the nematode *Caenorhabditis elegans*, the insect *Drosophila melanogaster* and the fish *Danio rerio* are however complementary systems for such studies (Vodovar *et al.,* 2004; Steinert and Heuner, 2005; Sifri *et al.,* 2005). The nematode *Caenorhabditis elegans* has been used as a virulence model to study several human pathogens in a number of experiments and is now recognized as a

suitable host to examine aspects of bacterial pathogenesis (Sifri *et al.*, 2005).

2.15.1. *Caenorhabditis elegans*- The Model Organism

The soil nematode *Caenorhabditis elegans* is an amenable model organism for investigations in pathogenesis, and fundamental biological processes like development, reproduction, senescence and functioning of the immune system in humans. Its use was pioneered by Sydney Brenner (Brenner, 1974) and it remains the only multicellular organism in which the entire cell lineage has been traced, from egg to adult (Sulston *et al.,* 1983). Most of the biochemical pathways identified in *C. elegans* have proved to be evolutionarily conserved, such that they have counterparts across the entire spectrum of relatedness, from plant parasitic nematodes (Bird and Opperman, 1998; Costa *et al*., 2007) to humans (Sonnhammer and Durbin, 1997; Chalfie and Jorgensen, 1998). This conservation of genes and metabolic pathways, and the mechanisms of invasion and host responses paralleled in mammalian cells, has given rise to the use of *C. elegans* in studies of human pathogenesis and drug action (Link *et al.,* 2000; Westlund *et al.,* 2004; Kaletta and Hengarter, 2006).

When grown in the laboratory *C. elegans* are fed on a food source of the *E. coli* strain OP50. This food source is replaced with other bacteria or fungi and the subsequent life span of the worms gives an indication of the pathogenicity of the bacterium, interpreted as a pathogenic process similar to that in humans. It has been suggested that virulence of bacteria has developed as a defence against microbivores such as *C. elegans* (Vaitkevicius *et al.*, 2006). When fed on *E. coli* OP50, *C. elegans* has a life-span of about two weeks; this is significantly shortened when grown on these pathogenic

bacteria. The killing process can be divided into slow killing, where live bacteria (which have passed intact through the pharynx), accumulates in the intestine of the worm and over time kills it, and fast killing where the killing is mediated by toxins secreted by the bacteria. This simple feeding-based pathogenicity model, monitoring the health and survival of the nematodes overtime, has developed *C. elegans* as an attractive infection model for several pathogenic bacteria (Couillault and Ewbank, 2002).

2.15.1.1. Life Cycle of *C. elegans*

C. elegans is a free-living nematode found in soil and in compost heaps and feeds on microorganisms like bacteria. The population is dominated by self-fertilising hermaphrodites (XX) with a rare occurrence of males (X0).When self-fertilizing, the hermaphrodite produces approximately 300 progeny; when cross-fertilized by a male, the male sperm will out compete the hermaphrodites own sperm and ~1000 progeny will be produced (Hope, 1999).

C. elegans has a short life cycle that is temperature-dependent. It goes through a reproductive life cycle (egg to egg-laying parent) in 5.5 days at 15^{o}C, 3.5 days at 20^{o}C, and 2.5 days at 25^{o}C. The eggs hatch and animals proceed through 4 larval stages (Fig 2.1) developing into to an adult over three days, and has a lifespan of approximately 10-15 days.

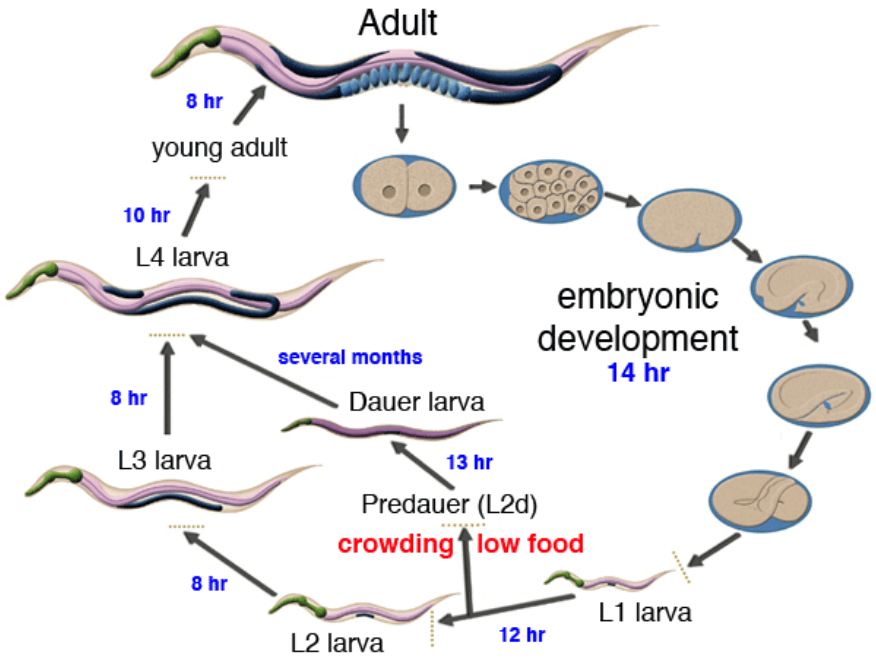


Fig.2.1. Life cycle of *C. elegans* at 22˚C (Artwork by Altun and Hall, Wormatlas)

The body plan of the adult worm (Fig. 2.2) is transparent, unsegmented and cylindrical with an outer and inner tube. The outer tube consists of cuticle, hypodermis, excretory system, neurons and muscles, while the inner tube consists of pharynx, intestine and gonads. In the pharynx there is a grinder which crushes its feed. The nervous system of *C. elegans* consists of ganglia in head and tail and a ventral and a lateral nerve cord (Altun & Hall, 2009).

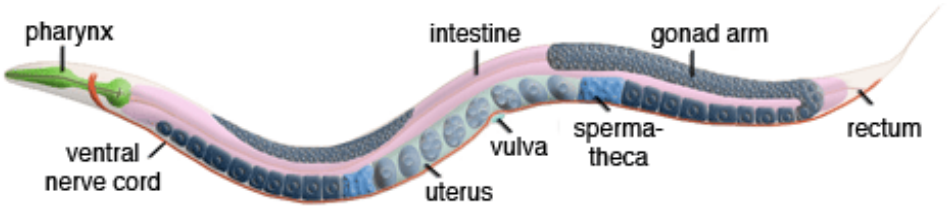


Fig. 2.2. Anatomy of *C. elegans* (Artwork by Altun and Hall, Wormatlas)

Under unfavorable life conditions, the larva will go into Dauer stage where it is isolated from the environment for up to four months. This stage can occur when the larva is about to turn into the second larval stage. During the Dauer stage feeding is arrested and locomotion reduced for as long as the conditions are unfavourable. The lifecycle of the worm is advantageous in the laboratory as the growth can be halted and synchronized between L1 and L2 stages. Another advantage compared to other test organisms is that the worm can be kept as frozen stocks (Hope, 1999; Altun and Hall, 2009).

2.15.1.2. *C. elegans* as *In Vivo* Model for Pathogenicity Studies

Many bacterial and fungal pathogens of clinical importance cause intestinal infections in *C. elegans* that result in death of the nematodes (Sifri *et al.,* 2005). *Pseudomonas aeruginosa*, a broad host-range pathogen, was the first human pathogen shown to kill worms (Mahajan-Miklos, 1999; Tan *et al.,*1999). *C. elegan*s is also affected by ingestion of many other broad host range pathogens, including *Serratia marcescens*, multiple *Burkholderia* species, and an assortment of entomo- and phytopathogens (O'Quinn *et al.,* 2001; Gan *et al.,* 2002; Couillault and Ewbank, 2002; Kurz *et al.,* 2003). Pathogens with narrower ecological niches, such as *Salmonella enterica*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* have been reported to kill worms as well (Aballay *et al.,* 2000; Labrousse *et al.,* 2000; Jansen *et al.,* 2002; Sifri *et al.,* 2003). The *C. elegans* pathogenesis model has also been extended to explore *C. elegans*–yeast relationships. The worms were shown to undergo normal maturation and have normal fecundity while feeding on the non-pathogenic yeasts, *Cryptococcus laurentii* and *Cryptococcus*

kuetzingii (Mylonakis, 2002) while *Cryptococcus neoformans*, the opportunistic yeast pathogen was shown to kill worms (Mylonakis, 2002).

C. elegans has been used as an effecient host model to identify and assess virulence factors of *V. cholerae* (Vaitkevicius *et al.,* 2006). *V. cholerae* causes lethal infection in the nematode via a cholera toxin (Ctx) and toxin co-regulated pili (Tcp) independent process, providing a useful host model system to screen for the virulence factors other than Ctx and Tcp. Worm lethality effect inflicted by *V. cholerae*, is mediated by LuxO-regulated genes in the quorum sensing (QS) pathway, such as *hap*R, *V. cholerae* metalloprotease gene *Prt*V (Vaitkevicius *et al.,* 2006), and VCC encoding gene *hly*A (Cinar *et al*., 2010). The haemolysin, HlyA, also causes developmental delay and intestinal vacuolation in *C. elegans* (Cinar *et al*., 2010). *C. elegans* has also been studied as a model experimental host for *V. vulnificus* (Dhakal *et al.,* 2006), *V. parahaemolyticus* (Durai *et al.,* 2011a) and *V. alginolyticus* (Durai *et al.,* 2011b) infections.

Chapter 3

MATERIALS AND METHODS

3.1. Isolation and Identification of Vibrios

3.1.1. Sources of Samples

Vibrios were isolated from seafood samples like prawn, squid and mussel; and from water and sediment samples from aquafarms and mangroves along the coastal regions of Cochin, South India. List of sampling stations, types of samples/ sampling sites are listed in Table 3. 1.

Table 3. 1. List of sample source, sampling station and strain names assigned

Source/Sample	Type/Station	Strain Names
Seafood	Prawn	BTPR
	Mussel	BTMU
	Squid	BTSQ
Aquafarms	Njarakkal	BTMA
Water & Sediments	Ochanthuruthu	BTOK
	Puthuvype	BTPF, BTAS, BTPS
	Vypeen	BTVM
Mangroves	Njarakkal	BTMM
Water & Sediments	Ochanthuruthu	BTOS
	Puthuvype	BTPM, BTMS
	Vypeen	BTVE

3.1.2. Collection of samples

Fresh seafood samples i.e. prawn, mussel and squid were obtained from a local market at Cochin, Kerala. They were transported immediately in sterile polythene bags in an ice-box to the laboratory within 4 hours of collection. Water and sediment samples from aquafarms and mangrove areas were collected in sterile polythene bags, sealed and transported to the laboratory in an ice-box within 4 hours of collection.

3.1.3. Preparation of serial dilutions of the sample

The tissue samples from prawn, mussel and squid were each homogenized with sterile physiological saline. 10 g of tissue was weighed and transferred aseptically to a sterile mortar, ground well with a pestle and mixed with 90mL of physiological saline.

In the case of water samples, 10mL aliquot of thoroughly mixed sample was added aseptically to 90mL of physiological saline. For sediment samples, 10g of sediment was weighed and transferred aseptically to 90mL of physiological saline. A series of 10 fold dilutions of these samples was prepared using physiological saline and 50µLof the prepared dilution was used as inoculum.

3.1.4. Isolation and purification of *Vibrio* sp.

The serially diluted samples were plated onto Thiosulphate Citrate Bile salt Sucrose (TCBS) agar (HiMedia, India) plates by the spread plate method and incubated at 37^{o}C for 24 hours. Isolated single colonies were picked, purified on nutrient agar (NA) plates supplemented with 1% NaCl, sub-cultured and stocked in nutrient agar slants with 1% NaCl for further studies.

3.1.5. Identification of Vibrios

The isolates were subjected to morphological and biochemical characterization for genus level identification as outlined in Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974).

3.1.5.1. Gram staining, Oxidase and MOF test

The isolates were subjected to Gram staining and observed under the microscope.

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme that catalyzes the oxidation of cytochrome *c* while reducing oxygen to form water. The oxidase test uses a reagent, tetra-methyl-*p*-phenylenediaminedihydrochloride (Kovac'sreagent), as an artificial electron donor for cytochrome *c*. When the reagent is oxidized by cytochrome *c*, it changes from colorless to a dark blue or purple compound, indophenol blue. A piece of filter paper is immersed in 1% Kovács oxidase reagent and allowed to dry. A well-isolated colony from a fresh (18- to 24-hour culture) culture is spotted onto treated filter paper and observed for color changes. Isolates were segregated as oxidase positive when the color changed to dark purple within 5 to 10 seconds.

The oxidative-fermentative test determines if the isolate metabolizes glucose by fermentation or aerobic respiration. During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will turn the bromothymol blue indicator in MOF media from green to yellow in the presence or absence of oxygen. MOF medium (HiMedia, India) was sterilized by autoclaving at 15 lbs for 15 minutes. Added 1% glucose to the sterile basal medium and transferred

4mL aliquots aseptically into sterile tubes and autoclaved at 10 lbs for 8 minutes. The medium was converted to slants with a long butt. The tubes were then stab inoculated and streaked with fresh culture and incubated at 37^{o}C for 24 hours. The results were recorded as follows:

O- Oxidation (yellow coloration in the butt)

F- Fermentation (yellow coloration throughout the tube)

(F)- Fermentation with gas production

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

The isolates segregated as vibrios after a presumptive screening by Gram staining, oxidase, and MOF tests, were subjected to molecular characterization.

3.2. Preservation and Stocking

The presumptive *Vibrio* isolates were purified and maintained as permanent stock cultures employing two methods, viz. paraffin overlay method and glycerol stocking.

3.2.1. Paraffin overlay method

A single colony was inoculated into Nutrient agar supplemented with 1 % sodium chloride in glass vials and incubated at 37^{o}C for 18 hours. Sterile liquid paraffin wax was added under aseptic conditions and the vials were covered with sterile rubber caps. These vials were then stored at room temperature in the dark for further use.

3.2.2. Glycerol stocking

Cultures were grown in nutrient broth, supplemented with 1 % sodium chloride. After 18h growth, sterile glycerol was added to a final concentration of 15% and stored at -80^{o}C.

3.3. Molecular Characterization and 16S rRNA Gene Sequence Analysis for Identification of Species

The presumptive *Vibrio* isolates were subjected to molecular characterization using 16S rRNA partial gene sequencing and the sequences were analysed using bioinformatic tools for species level identification.

3.3.1. Genomic DNA isolation

Genomic DNA isolation was done following the protocol of Esteban *et al.*, (1993).

- A single colony of the isolate was inoculated into Luria Broth (Hi media,, India) and incubated at 37^{o}C overnight with constant shaking
- 1.5 mL culture was taken in a microfuge tube and centrifuged at 8000 rpm (Sigma, Germany) for 5 min.
- The pellet was resuspended in 567µL Tris-EDTA (TE) buffer (pH 8)
- To the suspension, 30µL of 10% sodium dodecyl sulphate (SDS) and 3µL Proteinase K (20mg/mL) were added and mixed well
- The culture was then incubated for 1h at 37^{o}C in a water bath.
- 100µL of 5M NaCl and 80µL Hexadecyltrimethylammonium bromide (CTAB) (10mg/mL) were added and incubated for 10min at 65^{o}C in a water bath.
- The tubes were allowed to cool to room temperature and an equal volume of chloroform-isoamyl alcohol (24:1) was added.
- The contents were mixed gently and centrifuged at 10000 rpm for 10min.
- The aqueous layer at the top, containing the DNA, was carefully transferred to a fresh microfuge tube using a blunt end sterile tip.

- An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the aqueous layer and centrifuged again at 10000 rpm for 10 min.
- The aqueous layer was collected in a fresh tube and 0.6 volume of isopropanol was added to it.
- The contents were mixed gently and centrifuged at 12000 rpm for 10min.
- The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged.
- The supernatant was discarded and the pellet was dried at room temperature.
- The purified DNA was then dissolved in 100µL TE buffer (pH 8)

The concentration of genomic DNA thus obtained was estimated by the spectrophotometric method (Shimadzu UV-VIS Spectrophotometer, Japan). The purity of the DNA was assayed spectrophotometrically using the automatic calculation of the ratio of optical densities at 260 and 280 nm. The quantification of DNA was done using DNA/Protein pack® software of spectrophotometer. DNA was stored at -20^{0}C until further use. An appropriate dilution (~80-100ng) of genomic DNA was used as template for PCR reactions.

3.3.2. 16S rDNA sequence analysis

A PCR based method using a primer pair for 16S rDNA was used for species identification of the *Vibrio* strains (Shivaji *et al*., 2000; Reddy *et al*., 2000, 2002a; b). A portion of the 16S rRNA gene (1.5kb) was amplified from the genomic DNA. Products after PCR amplification was

purified by gene clean kit (Genei, India) and subsequently sequenced, followed by homology analysis.

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

3.3.2.1. Polymerase Chain Reaction (PCR)

PCR was performed using the genomic DNA (~100ng) as template and16S rDNA specific primers as detailed above.

3.3.2.2. PCR Mix composition

PCR buffer (10X)	2.0µL
dNTPs (2.5mM)	2.0µL
Forward primer (10µM)	1.0µL
Reverse primer (10µM)	1.0µL
Taq DNA polymerase (1U)	0.2 µL
Template DNA	0.5 µL
$MgCl_2$ (1.5mM)	1.2µL
Sterile distilled water to make the final volume to	20µL

3.3.2.3. PCR conditions

Annealing	- 56^{o}C	-30 sec.
Extension	- 72^{o}C	-2 min.

PCR assays were performed in MJ Mini Thermal cycler (BioRad, USA).

3.3.2.4. Agarose gel electrophoresis

The agarose gel electrophoresis was carried out for the visualization of PCR products. Agarose gels of appropriate strength (1 - 2%) depending on the size of the PCR product were prepared in Tris-Acetate-EDTA (TAE)

buffer. Ethidium bromide (EtBr) solution was added at a concentration of 10mg/mL. Definite volume of PCR product was mixed with gel loading dye and loaded into the wells. DNA markers were run along with the products for confirmation of amplicon size. Electrophoresis was performed at a constant volt (5V/cm) (Genei, India) and the gel pictures were captured with Gel documentation system (Syngene, UK).

3.3.3. *In-silico* analysis of the 16S rDNA sequences

3.3.3.1. DNA sequencing and analysis

Products after PCR amplification were purified by gene clean kit (Genei, India). The products were sequenced by Sanger's Dideoxy method using ABI 3730 Excel at SciGenom Labs Pvt Ltd, Cochin, Kerala. The sequenced PCR products were analyzed online using BLAST software (http://www.ncbi.nlm.nih.gov/blast) and the identity of the sequences were established (Altschul *et al.,* 1980).

3.3.3.2. Multiple sequence alignment and phylogenetic tree construction based on partial 16S rDNA sequences

The nucleotide sequences obtained after PCR of the 16S rDNA of the vibrios were converted into FASTA format and multiple sequence alignment for the assembled nucleotide sequences was done by using the Clustal X program (Thompson *et al.,* 1997) in BIOEDIT software (Hall 1999). Aligned sequences were imported into a MEGA5: Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura *et al.,* 2007) software for further analysis. The ends of the alignment were trimmed to obtain equal lengths for all sequences and the aligned sequences were converted into MEGA format for phylogenetic analysis. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) using nucleotide based TN84 evolutionary model for estimating genetic

distances based on synonymous and non-synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps. Phylogenetic trees were constructed with all the strains segregated as vibrios and also with *V. cholerae* and *V. vulnificus* strains.

3.4. Phenotypic Characterization

Phenotypic characterization of the *Vibrio* isolates was done based on various biochemical tests and antibiotic susceptibility tests.

3.4.1. Biochemical characterization

3.4.1.1. Hi-Vibrio identification system

Biochemical characterization of *Vibrio* isolates were performed using KB007 Hi-Vibrio^{TM} Identification Kit (Himedia, India) which involved 12 biochemical tests. The tests included Voges-Proskauers's test (VP test), arginine dihydrolase test, 3% salt tolerance test, ONPG test that detects β-galactosidase activity, and tests for utilization of citrate, ornithine, mannitol, arabinose, sucrose, glucose, salicin and cellobiose. The results of these biochemical tests were also used to support the identification of the isolates upto the species level.

3.4.1.1.1. Preparation of inoculum and inoculation of the strip

Each isolate was grown on a nutrient agar plate and a single, well-isolated colony was inoculated into 5mL alkaline peptone water and incubated for 6-8 hours until the inoculum turbidity reached $O.D_{620}$= 0.5 or greater. The kit was opened aseptically and 50µL of bacterial suspension was added to the wells of the strips containing the different substrates using a sterile microtip. The strips were incubated at 37^{o}C for 24 hours.

3.4.1.1.2. Reading of the strips and interpretation of the results

The Voges-Proskauers's tests (VP), which required the addition of reagents after overnight incubation were performed and interpreted after adding 2-3 drops of VP reagent 1 (Himedia, India) and 1 drop of VP reagent 2 (Himedia, India). The development of red color within 10 minutes indicated a positive reaction. In the case of the other tests, reading of reactions were carried out after 24h incubation and the results were interpreted and recorded using the identification table provided in the chart, supplied by the manufacturer.

3.4.2. Antibiotic susceptibility test

Antibiotic susceptibility of the isolates were determined by the disc diffusion method (Bauer *et al.*, 1966). Isolates were screened for susceptibility to a panel of 12 antibiotics belonging to the various antimicrobial classes as given in (Table 3.2). Susceptibility to the selected antibiotics was determined on Mueller-Hinton agar (HiMedia, India) by the disc diffusion method as described below.

- A single, isolated colony of the test strain was picked and transferred to 3mL of sterile physiological saline.
- Turbidity of the cell suspension was adjusted to 0.5 McFarland standards either by adding new inoculum or physiological saline.
- A uniform smear of the culture was made on Mueller- Hinton agar plate using a sterile cotton swab.
- Antibiotic impregnated discs (HiMedia) were placed on to the plates, each plate holding not more than five discs and incubated for 24h at 37^0C.

- Results were interpreted based on the inhibition zone around the discs as provided by the manufacturer (HiMedia) (Table 3.2).

Table 3.2. Antibiotic disc concentration, classification and zone interpretation chart. This chart is adapted from the zone-size interpretative chart published by HiMedia Pvt. Laboratories Ltd., India.

Antimicrobial agent	Class	Disc content (µg/disc)	Resistant (mm)
Gentamicin	Aminoglycoside	10	12
Streptomycin	Aminoglycoside	10	11
Ampicillin	Penicillins	10	13
Nalidixic Acid	Quinolone	30	13
Co-Trimoxazole	Sulfonomide	1.25 mcg	10
Vancomycin	Cyclic peptides	30	14
Trimethoprim	Folate pathway inhibitor	5	10
Cefixime	Cephalasporins	5	15
Azithromycin	Macrolide	15	13
Rifampicin	Rifamycin	5	16
Tetracycline	Tetracyclines	30	14
Chloramphenicol	Phenicol	30	12

3.4.3. Multiple Antibiotic Resistance (MAR) Index

MAR index of cultures gives an indication of the antibiotic stress of the environment from which they were isolated.

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

3.5. Serogrouping of *V. cholerae* Isolates Using Marker Genes

Identification of the serogroup among *V. cholerae* strains was by screening the isolates by PCR amplification for O1*rfb* and O139 *rfb* marker genes (Hoshino *et al*., 1998).

The genes O1*rfb and* O139*rfb* codes for the component protein of bundle forming pili of the O1 and O139 serotypes respectively, of the pandemic causing *V. cholerae.* A positive amplification indicates that the isolate belongs to the respective serotype.

Gene	Primers	Amplicon size (bp)	Reference
O1*rfb*	F- 5'- GTTCACTGAACAGATGGG 3' R- 5'- GGTCATCTGTAAGTACCAAC 3'	450	Hoshino *et al*., 1998
O139*rfb*	F-5'- AGCCTCTTTATTACGGGTGG 3' R-5'- GTCAAACCCGATCGTAAAGG 3'	190	Hoshino *et al*., 1998

Annealing - 62^{o}C for 1 min

Extension - 72^{o}C for 2min

3.6. Molecular Typing

Molecular typing was using PCR, with primers targeting specific conserved sequences in the bacterial genome. The isolates identified as *V. cholerae* and *V. vulnificus* were subjected to molecular typing methods such as ERIC PCR and BOX PCR. The amplified products produced a banding profile, depending upon the number and position of the repeated units, on an agarose gel. The gel pictures were captured and the banding patterns were analysed using POPGENE software (Version 1.32). Clustering was performed using unweighted pair group method with arithmetic averages (UPGMA). Reproducibility of ERIC-PCR and BOX-PCR results were assessed by repetition in 3 independent assays.

3.6.1. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR

ERIC sequences are 126 bp long, highly conserved at the nucleotide level and present in multiple copies in the genome (Stern *et al.,*1984).

Primer	Sequence (5´- 3´)	Reference
ERIC 1	ATGTAAGCTCCTGGGGATTCAC	Versalovic *et al.*, 1991
ERIC 2	AAGTAAGTGACTGGGGTGAGCG	

Annealing - 52^0C for 30 sec.

Extension - 72^0C for 4 min

3.6.2. BOX PCR

Molecular typing using BOX elements, a class of multi-copied and conserved repetitive sequences in the genomes of bacteria, was done using BOX A1R primer amplifying specific genomic regions located between BOX elements (154 bp) and its natural inverted repeats (Martin *et al*., 1992).

Primer	Sequence 5'-3'	Reference
BOX A1R	CTACGGCAAGGCGACGCTGACG	Martin *et al.,* 1992

Annealing - 45^oC for 30 sec

Extension - 72^oC for 4 min

3.7. Screening for Virulence/Virulence Associated Genes

The screening for virulence/virulence associated genes was performed employing PCR in which gene specific primers were used. The *V. cholerae* and *V. vulnificus* strains were screened for 13 genes namely *ctx*A, *ctx*B, *ace*, VPI, *hly*A, *omp*U, *rtx*A, *tox*R, *zot*, *nag*st, *tcp*A, *nin* and *nan*H. The *V. vulnificus* strains, in addition, were subjected to PCR screening of three genes specific to *V. vulnificus* viz., *cps*, *vvh* and *viu*.

3.7.1. Polymerase Chain Reaction (PCR) assays

All PCR reaction mixtures were prepared in the following manner unless otherwise stated. The reactions were performed containing 200µM of each dNTP, 1.5mM $MgCl_2$, 1X *Taq* Buffer (10mM Tris-HCl, (pH 8.3),

50mM KCl), 0.5μM each of the specific primers, 1U *Taq* DNA Polymerase, 1μL (100ng) template DNA and H_2O to a final volume of 20μL. PCR assays were performed in MJ Mini (BioRad) Thermal cycler. The thermal profile for PCR reactions may vary with primers and the size of amplicons.

3.7.1.1. Agarose gel electrophoresis

Electrophoresis of the agarose gel was performed as outlined in section 3.3.2.4.

3.7.1.2. PCR detection of *ctx*A and *ctx*B

Toxigenic *V. cholerae* carries one or more copies of the cholera toxin gene *ctx*A and *ctx*B coding respectively for the subunit A and subunit B of the cholera toxin. The presence of these genes can be detected using primers targeted at these sequences and a positive amplification confirms the toxigenicity of the isolate.

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
*ctx*A	F- CGGGCAGATTCTAGACCTCCTG R- CGATGATCTTGGAGCATTCCCAC	564	Singh *et al.* (2002)
*ctx*B	F- GGTTGCTTCTCATCATCGAACCAC R- GATACACATAATAGAATTAAGGAT	460	Olsvik *et al.* (1993)

Annealing - 60°C 1 min

Extension - 72°C 2 min

3.7.1.3. PCR detection of *ace*

The gene *ace* is responsible for producing an accessory cholera toxin that increases potential difference across membranes. Its presence is confirmed by amplification of the 289 bp fragment of the gene using the specific primers (Trucksis *et al.*, 1993).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
ace	F- TAAGGATGTGCTTATGATGGACACCC R- CGTGATGAATAAAGATACTCATAGG	289	Singh *et al.* (2001)

Annealing	-	62°C	1 min
Extension	-	72°C	2 min

3.7.1.4. PCR detection of Vibrio Pathogenicity Island (VPI)

Vibrio Pathogenicity Island is considered to be one of the initial genetic factors required for the emergence of epidemic and pandemic cholera and encodes proteins playing roles in virulence of *V. cholerae* (Karaolis *et al*., 2001).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
VPI	F –GCAATTTAGGGGCGCGACGT R-CCGCTCTTTCTTGATCTGGTAG	680	Mukhopadhyay *et al.*(2001)

Annealing	-	52°C	1 min
Extension	-	72°C	3 min

3.7.1.5. PCR detection of *hly*A

The *hly*A gene, which encodes a cytotoxic haemolysin, exhibits leukocidal activity contributing to the pathogenesis of gastroenteritis caused by some *V. cholerae* strains lacking the gene coding for cholera toxin (Yamamoto *et al*., 1990).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
hlyA	F- GGCAAACAGCGAAACAAATACC R- CTCAGCGGGCTAATACGGTTTA	481	Hall *et al*. (1990)

Annealing	-	60°C	1 min
Extension	-	72°C	3 min

3.7.1.6. PCR detection of *omp*U

The product of the gene *omp*U produces an outer membrane protein OmpU, which acts as an adherence factor and is involved in colonization of epithelial cells by *V. cholerae* (Chakrabarti *et al.,* 1996).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
ompU	F- ACGCTGACGGAATCAACCAAAG R- GCGGAAGTTTGGCTTGAAGTAG	869	Karunasagar *et al.* (2003)

Annealing	-	64°C	1 min
Extension	-	72°C	3 min

3.7.1.7. PCR detection of *rtx*A

The toxin produced by *rtx*A gene belongs to the RTX (repeat in toxin) family of toxins that displays cytotoxic activity and causes actin crosslinking (Chow *et al*., 2001).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
rtxA	F- CTGAATATGAGTGGGTGACTTACG R- GTGTATTGTTCGATATCCGCTACG	417	Chow *et al.*(2001)

Annealing	-	55°C	1 min
Extension	-	72°C	3 min

3.7.1.8. PCR detection of *tox*R

The ToxR protein acts as the master regulator that regulates at least 17 distinct virulence associated genes in *V. cholerae*. The cholera toxin gene, *ctx*AB is directly regulated by the ToxR protein (Miller *et al*., 1987).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
toxR	F- CCTTCGATCCCCTAAGCAATAC R- AGGGTTAGCAACGAGCGTAAG	779	Rivera *et al.* (2001)

Annealing	-	60°C	1 min
Extension	-	72°C	3 min

3.7.1.9. PCR detection of *zot*

The gene *zot* produces a toxin that increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction, or zonula occludens (Fasano *et al.,* 1991).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
zot	F- TCGCTTAACGATGGCGCGTTTT R- AACCCCGTTTCACTTCTACCCA	947	Shi *et al.(*1997)

Annealing	-	60°C	1 min
Extension	-	72°C	4 min

3.7.1.10. PCR detection of *nag*st

NagST is a heat stable enterotoxin produced by some strains of *V. cholerae* capable of producing diarrhoea in humans (Morris *et al*., 1990).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
nagst	F- TCGCATTTAGCCAAACAGTAGAA R- GCTGGATTGCAACATATTTCGC	172	Rivera *et al.* (2001)

Annealing	-	55°C	1 min
Extension	-	72°C	1 min

3.7.1.11. PCR detection of *tcp*A

The toxin-coregulated pilus, encoded by the *tcp*A gene, is considered to be the most important colonization factor in *V. cholerae* required for intestinal colonization and plays a significant role in pathogenesis (Taylor *et al*., 1987).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
tcpA	F- CACGATAGAAAACCGGTCAAGAG R- CCAAATGCAACGCCGAATGGAGC	618	Keasler and Hall (1993)

Annealing	-	60°C	1 min
Extension	-	72°C	3 min

3.7.1.12. PCR detection of *nin*

The gene *nin*, a bacteriophage like integrase gene, is part of the virulence profile in *V. cholerae* (Figueiredo *et al.*, 2005).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
nin	F- TTACGTAACGCTACGGCAT R- CAAGGTGCCATCGATCAG	1000	Figueiredo *et al.* (2005)

Annealing	-	62°C	1 min
Extension	-	72°C	3 min

3.7.1.13. PCR detection of *nan*H

The *nan*H gene in *V. cholerae* produces neuraminidase, a virulence factor which releases sialic acid from higher gangliosides present on eukaryotic cells surface, exposing ganglioside GM1, the cholera toxin receptor (Holmgren *et al.*, 1975).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
nanH	F- TTTTTACAGCGTCTATGATG R- GGTTTCCTTGTGGGTTAGTA	1100	Figueiredo *et al.* (2005)

Annealing	-	55°C	1.5 min
Extension	-	72°C	3 min

3.7.2. PCR detection of virulence genes specific to *V. vulnificus*

The *V. vulnificus* strains were screened for the presence of three species specific virulence associated genes, *cps* (Capsular Polysaccharide), *vvh* (Haemolysin) and *viu* (Iron acquisition).

3.7.2.1. PCR detection of *cps*

The capsular polysaccharide in *V. vulnificus* is the most important virulence factor that helps to avoid phagocytosis by host defence cells and complement-mediated killing by the host immune system. The capsule is related to the colony morphology, with encapsulated strains being opaque and unencapsulated strains being translucent (Yoshida *et al*., 1985).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
cp	F- TTTGGGATTTGAAAGGCTTG R- GTGCCTTTGCGAATTTTGAT	342	Han *et al.,* (2009)

Annealing	-	55^{o}C	1min
Extension	-	72^{o}C	2 min

3.7.2.2. PCR detection of *vvh*

Hemolyin/cytolysin (Vvh) is one of the representative exotoxins produced by *V. vulnificus* that lyses red blood cells and exhibits cytolytic activity (Gray and Kreger, 1987).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
vvh	F- TTCCAACTTCAAACCGAACTATGAC R- TTCCAACTTCAAACCGAACTATGAC	205	Panicker *et al.,* (2004)

Annealing	-	65^{o}C	1min
Extension	-	72^{o}C	2 min

3.7.2.3. PCR detection of *viu*

The acquisition of iron is directly correlated to virulence of *V. vulnificus* and the *viu* gene is associated with iron acquisition from heme proteins (Nishina *et al*., 1992).

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
viu	F- GGTTGGGCACTAAAGGCAGATATA R- CGGCAGTGGACTAATACGCAGC	504	Panicker *et al.* (2004)

Annealing - 65°C 1min
Extension - 72°C 2 min

3.8. Genetic Analysis and Comparison of Toxin-regulatory Gene *tox*R in Environmental Isolates of *V. cholerae* and *V. vulnificus*

3.8.1. Strains used in the study

The environmental *Vibrio* isolates that produced amplicons on screening for the *toxR* gene were further subjected to genetic analysis of the *tox*R gene. The strains used for the study are given Table3.3

Table 3.3. Environmental isolates of *V. cholerae* and *V. vulnificus* subjected to genetic analysis of the *toxR* gene

Sl.No.	Species	Strain
1	*V. cholerae*	BTPR5
2		BTMA1
3		BTMA5
4		BTMA9
5		BTOK7
6		BTOK9
7		BTOK11
8		BTMS4
9		BTVE2
1	*V. vulnificus*	BTAS3
2		BTPS6
3		BTOS7

3.8.1.1. Amplification of the *toxR* gene

The *tox*R gene from the positive strains of *V. cholerae* and *V. vulnificus* was amplified following the protocol described in section 3.7.1.8.

3.8.1.2. A-Tailing of the PCR product

The final extension step was prolonged to 20 minutes to ensure efficient 3'dA tailing of the PCR product.

3.8.1.3. Gel analysis and confirmation of PCR product

The PCR-amplified DNA fragments were observed after agarose gel electrophoresis using a UV transilluminator and the image was captured by Gel Documentation system (Syngene, USA) as mentioned under section 3.3.2.4.

3.8.1.4. TA Cloning

Cloning of the *toxR* amplicon was performed using the InsTAclone™ PCR Cloning Kit (Fermentas Ltd.). The procedure followed was according to the manufacturer's instructions. The following steps were involved:

3.8.1.4.1. Ligation

The ligation mixture was set up with 2 µL of 5X ligation buffer, 1µL of vector pTZ57R/T,0.5 µL of T4 DNA ligase enzyme, 150ng of PCR product and nuclease free water to a final volume of 10µL. Ligation mixture was incubated over night at 4°C.

3.8.1.4.2. Preparation of competent cells

The TransformAid Bacterial Transformation Kit (Fermentas Ltd.) supplied by the manufacturer was used to provide fast and efficient preparation of chemically competent cells. *E.coli* JM 109 cells were used for transformation. A single colony of the *E.coli* JM109 was inoculated into

2mL of C-medium supplied with the kit and incubated at 37°C in a shaker (Orbitek, Scigenics India) for 24 hours.

3.8.1.4.3. Transformation

The C-medium was pre-warmed and 150 µL of the overnight bacterial culture was inoculated to 1.5mL of C medium. This was incubated for 20 min at 37°C in a shaker. The bacterial cells were pelleted by centrifugation and the cells resuspended in 300µL of T- solution supplied with the kit. It was incubated on ice for 5 min and centrifuged for 1 min. The supernatant was discarded, the cells resuspended in 120 µL of T-solution and incubated on ice for 5 min. To fresh microcentrifuge tubes, 2.5µL of ligation mixture was added and chilled on ice for 2 min. 50µL of the prepared cells were added to the ligation mixture, mixed and incubated on ice for 5 min. This was plated onto pre-warmed LB plates containing appropriate amount of ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D thiogalactoside) and screened for α-complementation after overnight incubation at 37°C.

3.8.1.4.4. Plasmid Isolation

Plasmids were isolated from the positive clones by the alkaline lysis method (Bimboim and Doly, 1979).

- A single bacterial colony was inoculated into 2mL of LB broth with ampicillin and incubated at 37°C overnight
- The culture was centrifuged at 6000 rpm for 5 min and the pellet resuspended in 100µL of ice-cold solution I (50mM glucose, 25mM TrisCl, pH-8.0 and 10mM EDTA, pH 8.0). It was kept on ice for 5 min.

- 200µL of freshly prepared solution II (0.2N NaOH and 1% SDS) was added and kept on ice for 10 min.
- Mixed gently and 150µL of ice cold solution III was added (5M Potassium acetate, glacial acetic acid and water).
- Mixed gently and stored on ice for 15 min.
- Centrifuged at 12,000 rpm at 4^{o}C for 10 min.
- Supernatant was transferred to fresh tube and double volume of absolute alcohol was added. Allowed to stand at -20^{o}C for 30 min.
- Centrifuged at 12,000 rpm at 4^{o}C for 10 min, the supernatant was removed and dried
- Pellet was rinsed with 1mL 70% ethanol and centrifuged at 12,000 rpm for 3 min.
- The pellet was dried and dissolved in 50µL TE (pH-8.0)

3.8.1.4.5. Confirmation of insert by PCR using vector specific primers

The plasmids were subjected to PCR amplification of the insert with the *tox*R gene, using vector specific primers for obtaining the full length sequence of the insert. The vector specific primers were deduced from DNA sequences of the vector provided with the kit.

Primer Sequence (5'-3')	Amplicon size (bp)
F- GTAAAACGACGGCCAGT	1000
R- CAGGAAACAGCTATGAC	

The amplicons were visualized on 1% agarose gel for confirmation of insert.

3.8.1.5. Sequencing of the Insert

Products after PCR amplification were purified by gene clean kit (Genei) and were sequenced by Sanger's Dideoxy method using ABI 3730 Excel at SciGenom Labs Pvt Ltd, Cochin, Kerala.

3.8.1.6. *In-silico* analysis of the *tox*R gene sequence

Products after PCR amplification were purified by gene clean kit (Genei) and were sequenced by Sanger's Dideoxy method using ABI 3730 Excel. The sequenced PCR products were compared with those available from GenBank using online BLAST tools – nucleotide blast (http://www.ncbi.nlm.nih.gov/blast). Nucleic acid sequences of other *toxR* genes were obtained from NCBI database. Multiple sequence alignment for the assembled nucleotide sequences was done by using the Clustal X program (Thompson *et al.,* 1997) in BIOEDIT software (Hall 1999). The unrooted phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) using nucleotide based TN84 evolutionary model in MEGA5: Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura *et al.,* 2007). The evolutionary distances were computed using the Tamura-Nei method (1993) and are in the units of the number of base substitutions per site. Statistical support for branching was estimated using 1000 bootstrap steps.

The nucleic acid sequences was translated *in-silico* into its corresponding protein sequence by Expasy (http://web.expasy.org/translate) and the deduced aminoacid sequences were compared with those available from GenBank using online BLAST tool- blastx (http://www.ncbi.nlm.nih.gov/blast). Aminoacid sequences of ToxR of other related *Vibrio* sp. were obtained from NCBI database and multiple sequence alignment for the assembled

deduced aminoacid sequences was done by using the Clustal W program in BIOEDIT software (Hall 1999). The unrooted phylogenetic tree based on aminoacid sequence of the ToxR was constructed as detailed above with 1000 bootstrap steps. The evolutionary distances were computed (Nei and Kumar, 2000) and are in the units of the number of aminoacid differences per sequence.

The conserved domain of the ToxR protein was predicted using the Conserved Domain Database. Conserved Domain Database is a collection of sequence alignments and profiles representing protein domains conserved in molecular evolution. It also includes alignments of the domains to known 3-dimensional protein structures in the MMDB database. The deduced aminoacid sequence of ToxR gene of *V. cholerae* strain BTMA5 (Accession no. KF420400) was compared with other protein sequences using Conserved Domain Database available in NCBI (Marchler-Bauer *et al.,* 2011).

3.8.1.7. 3D structure prediction/homology modelling

Topology of the predicted protein was computed using Phyre^2 (Protein homology/analogy recognition engine) server (http:// www.sbg.bio.ic.ac.uk/ phyre2/html/page.cgi?id=index). The PHYRE automatic fold recognition server is used for predicting the structure and/or function of the protein sequence.

3.9. Pathogenicity Studies with the Nematode *Caenorhabditis elegans* as Model Organism

The soil nematode *Caenorhabditis elegans* was used as a model organism to test the pathogenicity of representative putative virulent and non- virulent strains of *V. cholerae* and *V. vulnificus.*

3.9.1. *C. elegans* strain and maintenance

The nematode *C. elegans* Bristol N2 wild type used in the present study was kindly supplied by Dr Sandhya P Koushika, National Centre for Biological Sciences, Bangalore. The nematodes were propagated and maintained at 21°C on modified solid Nematode Growth Medium (NGM) and fed with *E. coli* strain OP50 (Brenner, 1974). Medium size plates (6 cm diameter) were used for general strain maintenance and small plates of 3.5 cm diameter were used for the bioassay. *E. coli* OP50 is a uracil auxotroph whose growth is limited on NGM plates. A limited bacterial lawn is desirable as it allows easier observation of the worms. A starter culture of *E. coli* was prepared by aseptically inoculating a single colony into LB broth to grow overnight at 37°C. This overnight culture was used for seeding NGM plates. Worms were observed under a simple dissecting stereomicroscope equipped with a transmitted light source.

3.9.2. Pathogenicity studies using *Vibrio* isolates

3.9.2.1. *Vibrio* strains for pathogenicity studies

The experiment was set up with 3 strains of *V. cholerae* and 2 strains of *V. vulnificus*. Plate with *E. coli* OP50 as the food source was used as the control. The *V. cholerae* strains used for the study are given in Table.3.4.

Table.3.4. Environmental isolates of *V. cholerae* selected for pathogenicity studies with their virulence profile.

Strain	Virulence Profile	Source of strain
V. cholerae Co 366 ElTor	O1 serotype, *ctx*+, VPI$^+$, *hly*A$^+$, *omp*U$^+$, *tox*R$^+$, *rtx*A$^+$, Virulent	Clinical
V. cholerae strain BTPR5	VPI$^+$, *hly*A$^+$, *omp*U$^+$, *tox*R$^+$, *rtx*A$^+$ (Putative virulent)	Environmental (Seafood-Prawn)
V. cholerae strain BTOS6	Negative for all screened genes (Putative non-virulent)	Environmental (Mangrove)

The environmental isolates of *V. vulnificus* subjected to genetic analysis of the *tox*R gene are given in Table 3.5.

Table.3.5. Environmental isolates of *V. vulnificus* selected for pathogenicity studies with their virulence profile.

Strain	Virulence Profile	Source of strain
V. vulnificus strain BTPS6	*cps*$^+$, VPI$^+$, *tox*R$^+$ (Putative virulent)	Environmental (Mangrove)
V. vulnificus strain BTMM7	Negative for all screened genes(Putative non-virulent)	Environmental (Aquafarm)

3.9.2.2. Seeding of *C. elegans*

10µL of *E. coli* OP50 culture grown overnight in LB was spread on Nematode Growth Medium (NGM) in 6 cm diameter plates. Plates were incubated at 37°C for 12-14 hours, allowed to equilibrate to room temperature and 15 to 20 worms (L 4 stage) were placed on each plate. The plates were incubated at 25°C for 24 hours.

3.9.2.3. Pathogenicity studies and bioassays with *C. elegans*

E. coli OP50 and vibrio lawns were grown for *C. elegans* bioassays as follows: The bacterial strains were inoculated into 5mL of LB broth and grown at 28°C for 24 h; 10µL of the grown culture was spread onto 3.5cm petriplates containing NG agar medium. The plates were incubated at 28°C overnight. Evaluation of the life span of *C. elegans* when feeding on the experimental strains was carried out as per Aballay *et al.*,(2000). The plates were equilibrated to room temperature and 10 L4 stage larvae of *C. elegans* were transferred from a lawn of *E. coli* OP50 to the lawn of the test strains. The plates were incubated at 25°C and scored for live and dead worms every 24 hours. A worm was considered dead when it failed to respond to

plate tapping or gentle touch with a platinum wire. Worms that died as a result of getting stuck to the wall of the plate were censored from the analysis. Each experimental condition was tested in triplicate. This was compared with the life span of *E. coli* OP50 feeding worms which served as the control. The worms were transferred to fresh plate seeded with the corresponding bacterial strain every 24 hours for the next 4 days of the assay or until no more progeny were evident. This is done in order to prevent loosing track of the original worms due to crowding by reproduction. The specific mechanisms and the morphological modifications relating to pathogenesis were noted.

3.9.2.4. Calculation of LD_{50}

The time taken for 50% of the nematodes to die (time to death 50, LD_{50}) was calculated using the PRISM (version 5.04) computer program using the equation: Y = Bottom + (Top – Bottom)/(1 + 10^((LogEC50 – X)*Hill Slope)), where X is the logarithm of days and Y is the average of dead worms. The data represents the mean ± the standard error.

3.9.3. Microscopic pictures of *C. elegans*

Agarose pads were prepared on microscope slides using a 2% (w/v) agarose solution. 1-2uL of M9 containing 10-25mM sodium azide (NaN_3), that anesthetizes the worm, was placed at the center of the agar pad. The worms to be viewed were transferred onto the pad with the help of a worm pick, and a cover slip was gently placed on the worm to be observed under the microscope. Photographs of the anaesthetized worms were taken for anatomical analysis.

Chapter 4

RESULTS

4.1. Isolation and Identification of Vibrios

4.1.1. Isolation of vibrios from various samples

Vibrio- like organisms were isolated from marine environments like aquafarms, mangroves and from seafood. A total of 134 strains were isolated from these various marine samples.

4.1.2. Identification of Vibrios

The isolates which were Gram negative, oxidase positive, fermentative, with or without gas production on MOF media, and which showed yellow/green coloured colonies on TCBS (Thiosulfate Citrate Bile salt Sucrose) agar were segregated as *Vibrio* sp. . The distribution of these isolates among the various samples is summarized in Table 4.1.

Table 4.1. Distribution of *Vibrio* sp. in the different marine samples

Sample	Source/ Station	Total No.of Isolates	No. of *Vibrio* sp.
Seafood	Prawn	13	1
	Mussel	11	1
	Squid	13	0
Aquafarms	Njarakkal	11	3
Water & Sediment	Ochanthuruthu	25	9
	Puthuvype	11	8
	Vypeen	5	2
Mangroves	Njarakkal	7	4
Water & Sediment	Ochanthuruthu	22	7
	Puthuvype	9	5
	Vypeen	7	5
	Total	**134**	**45**

4.1.3. 16S rRNA gene sequence analysis for identification of species

The 45 isolates recognised as *Vibrio* sp. were further subjected to species level identification based on partial 16S rDNA sequence analysis. The sequences were submitted to GenBank database and accession numbers were obtained. The species identity of the 45 strains and the source from which they were obtained are detailed in Table 2. The strains were identified as *V. cholerae* (N=21), *V. vulnificus* (N=18), *V. parahaemolyticus* (N=3), *V. alginolyticus* (N=2) and *V. azureus* (N=1).

Table 4.2. Species identification of marine environmental *Vibrio* sp. (N=45) based on 16S rDNA sequence analysis and their distribution

		V.cholerae	***V.vulnificus***	***V. parahaemolytics***	***V. alginolyticus***	***V. azureus***
Seafood	Prawn	1	-	-	-	-
	Mussel	-	-	1	-	-
	Squid	-	-	-	-	-
		-	-	-	-	-
Aquafarms	Njarakkal	3	-	-	-	-
	Ochanthuruthu	4	5	-	-	-
	Puthuvype	3	4	-	1	-
	Vypeen	2	-	-	-	-
Mangroves	Njarakkal	2	2	-	-	-
	Ochanthuruthu	2	3	1	1	
	Puthuvype	3	1	1	-	-
	Vypeen	1	3	-	-	1
	Total	**21**	**18**	**3**	**2**	**1**

The presence of *V. cholerae* was observed not only in the aquafarm and mangrove environmental samples but also in prawn among the seafood samples analysed. *V. cholerae* strains occurred at the highest frequency of 46.67 % followed by *V. vulnificus* at 40%.

Strains of *V. vulnificus* were obtained only from aquafarm and mangrove samples and were not observed in the seafoods analysed. *V. parahaemolyticus* was present only in mussel and mangrove samples, but absent in the aquafarm samples. While *V. alginolyticus* was identified only in aquafarm and mangrove samples, it was not observed in seafoods. The only strain of *V. azureus* was obtained from the mangrove environment.

4.2. Phylogenetic analysis based on 16S rDNA sequences

The phylogenetic relationship based on partial 16S rDNA sequences of the 45 strains of *Vibrio* sp. isolated from marine environments is represented in Fig. 4.1. From the phylogram it was evident that strains of the same species grouped together to form distinct clades. The 18 isolates of *V. vulnificus* cladded to form two groups, one clade with14 isolates and the other with 4, exhibiting a very high level of sequence similarity. Likewise, the sequences of the 21 *V. cholerae* strains also exhibited a high degree of homology with each other and cladded separately from other species. The strains of *V. parahaemolyticus*, *V. alginolyticus* and *V. azureus* exhibited very high similarity scores among themselves, however in this analysis, *V. alginolyticus* strain BTAS2 from aquafarm cladded with the *V. cholerae* group rather than with its close relative, strain BTOS1 isolated and identified from mangrove environments.

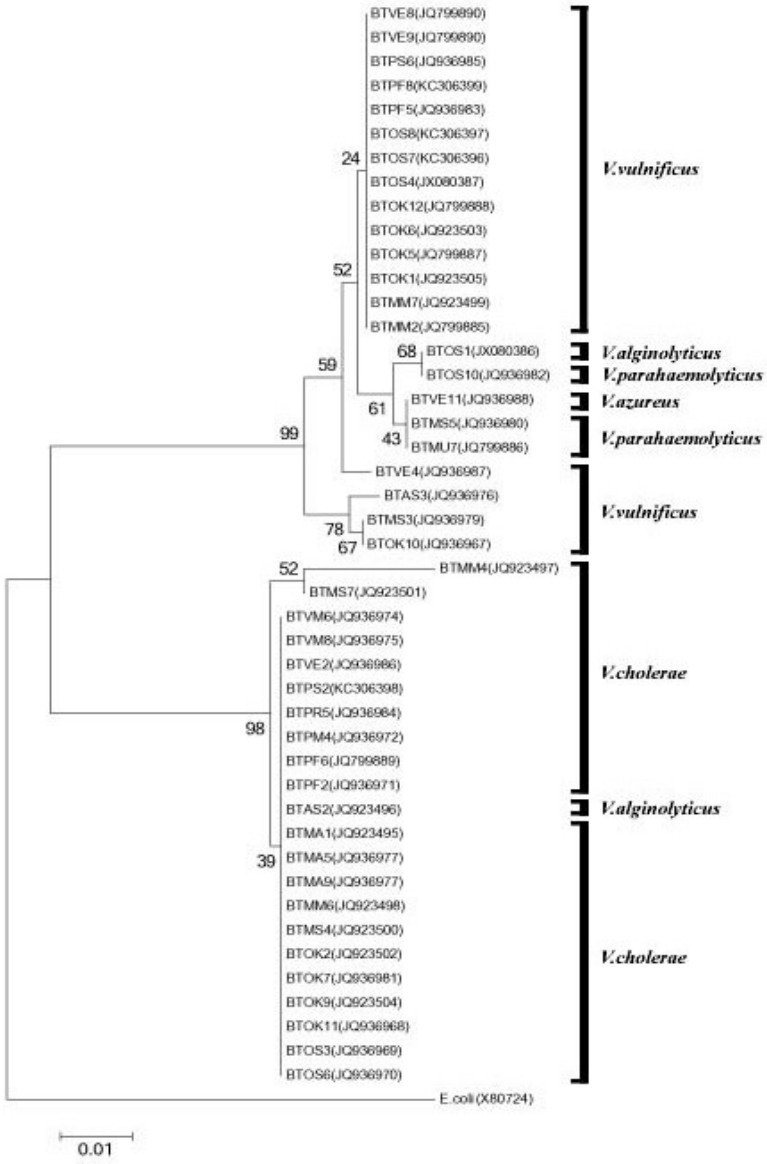


Fig.4.1. Dendrogram showing phylogenetic relationship based on partial 16S rDNA sequences of marine environmental isolates of *Vibrios* (N=45).

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved nucleotide sequences from 45 environmental isolates of *Vibrios*. *E.coli* (Accession No.X80724) was used as outgroup. Accession numbers are given in parentheses.

4.2.1. Phylogenetic analysis of *V. cholerae* strains

Phylogenetic tree was constructed to analyse the intra species variation among the 21 strains of *V. cholerae.* (Fig 4.2) and all the strains grouped together into a major clade, except BTMM4 from mangrove environment, which formed a separate clade, but distinct from the outgroup. Although strains isolated from the same samples exhibited intraspecies variations, their clonal origin was evidenced from the phylogram.

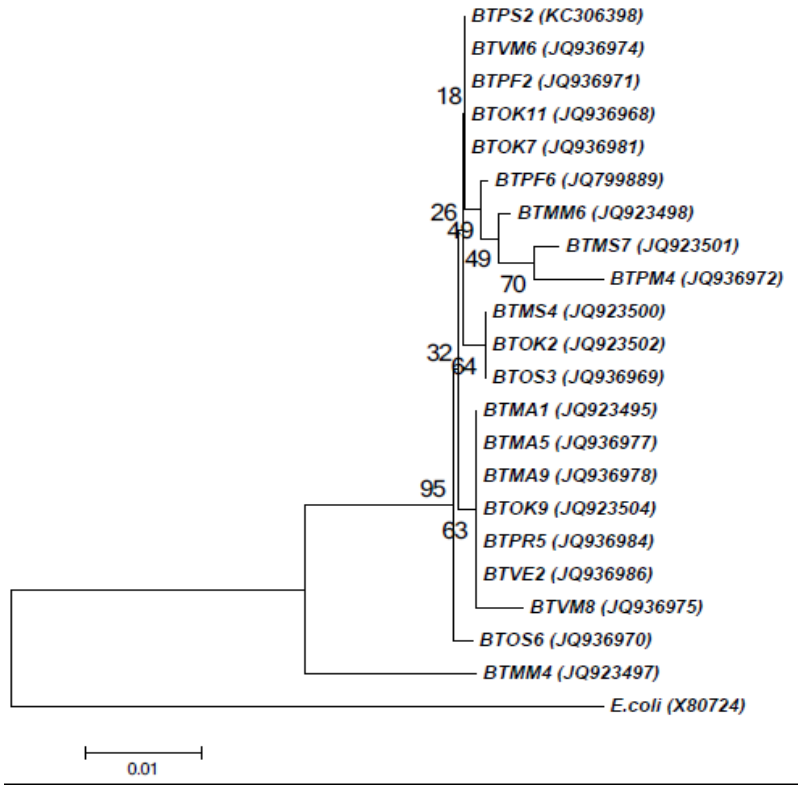


Fig.4.2. Phylogenetic relationship based on partial 16S rDNA sequences of *Vibrio cholerae (N=21)*.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved nucleotide sequences from 21 environmental *V. cholerae* isolates and *E.coli* (Accession numberX80724) was used as outgroup. Accession numbers are given in parentheses.

4.2.2. Phylogenetic analysis of *V. vulnificus* strains

The *V. vulnificus* group (N=18) cladded into 2 separate groups, with 15 strains in one clade and 3 in another, but in general exhibited close relationship to one another as depicted in Fig 4.3. The strains from various sources cladded together indicating their relatedness as a single species; nevertheless, intraspecies variations were exhibited among strains from similar environments.

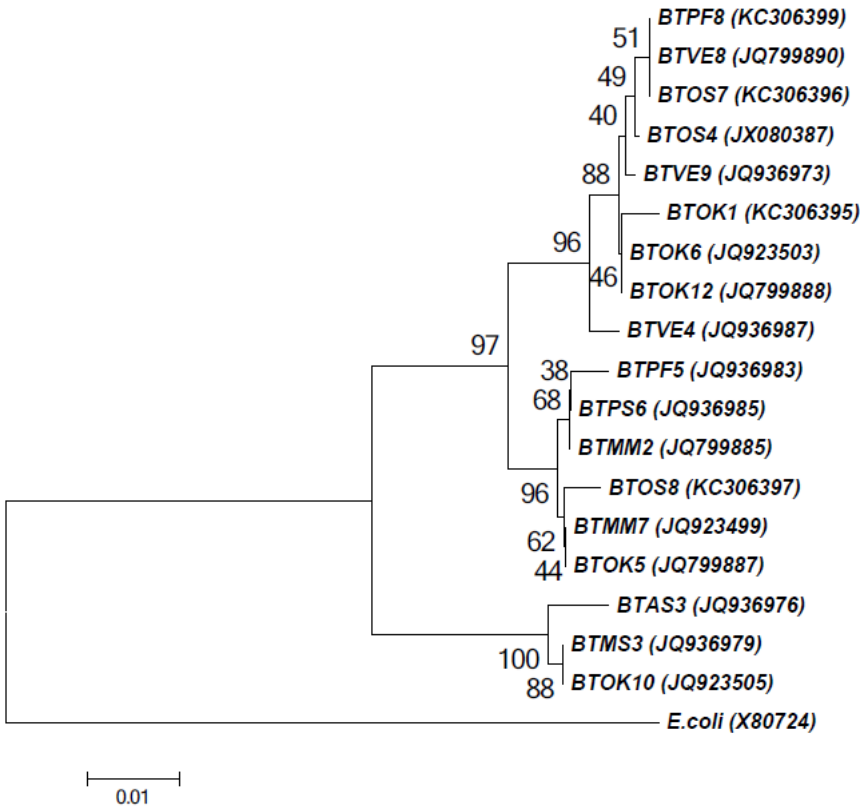


Fig.4.3. Phylogenetic relationship of *Vibrio vulnificus* (N=18) strains based on partial 16S rDNA sequences.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved nucleotide sequences from 18 marine environmental *V. vulnificus* isolates and *E.coli* (Accession numberX80724) was used as outgroup. Accession numbers are given in parentheses.

4.3. Phenotypic Characterization

4.3.1. Biochemical characterization of *V. cholerae* isolates

The species wise biochemical profile of the isolates was constructed and comparison of the biochemical characteristics of the strains was performed.

Table 4.3 details the biochemical profile of *V. cholerae* strains from various samples. All the strains produced yellow coloured colonies on TCBS agar plates, were able to tolerate 3% NaCl, were ONPG positive and able to utilise carbohydrates like sucrose and glucose for growth. The VP test was positive for 15 strains, negative for 5 strains and variable for 1 strain. Citrate was consumed by all strains, except strain BTMA9 from aquafarm. All the isolates were ornithine positive except for strains BTMA9 and BTOK2 from aquafarm. Mannitol utilisation was observed by all the strains except BTMA9, BTOK7 and BTOK9 isolated from aquafarm and BTMS4 from mangroves. Arabinose was used as sole source of carbon only by 6 strains i.e., strains BTPR5 from prawn, BTMA1, BTMA5, BTOK2 and BTOK11from aquafarm and BTVE2 from mangroves. Strains isolated from aquafarms, BTOK2, BTOK7 and BTOK11 showed ability to utilise salicin and these along with BTMA5 were capable of cellobiose degradation.

Table 4.3. Biochemical tests of *V. cholerae* isolates from marine environments (N= 21). (Hi-Vibrio identification kit, HiMedia, India)

		Strain	TCBS	VP	Arginine	NaCl 3%	ONPG	Citrate	Ornithine	Mannitol	Arabinose	Sucrose	Glucose	Salicin	Cellobiose
Seafood	Prawn	BTPR5	Y	+	+	+	+	+	+	+	+	+	+	-	-
Aquafarms	Njarakkal	BTMA1	Y	+	-	+	+	+	+	+	+	+	+	-	-
		BTMA5	Y	-	-	+	+	+	+	+	+	+	+	-	+
		BTMA9	Y	+	+	+	+	-	-	-	-	+	+	-	-
	Ochanthuruthu	BTOK2	Y	-	+	+	+	+	-	+	+	+	+	+	+
		BTOK7	Y	+	-	+	+	+	+	-	-	+	+	+	+
		BTOK9	Y	+	+	+	+	+	+	-	-	+	+	-	-
		BTOK11	Y	-	-	+	+	+	+	+	+	+	+	+	+
	Puthuvype	BTPF2	Y	+	-	+	+	+	+	+	-	+	+	-	-
		BTPF6	Y	+	-	+	+	+	+	+	-	+	+	-	-
		BTPS2	Y	+	-	+	+	+	+	+	-	+	+	-	-
	Vypeen	BTVM6	Y	+	-	+	+	+	+	+	-	+	+	-	-
		BTVM8	Y	+	-	+	+	+	+	+	-	+	+	-	-
Mangroves	Njarakkal	BTMM4	Y	+	-	+	+	+	+	+	-	+	+	-	-
		BTMM6	Y	+	-	+	+	+	+	+	-	+	+	-	-
	Ochanthuruthu	BTOS3	Y	-	-	+	+	+	+	+	-	+	+	-	-
		BTOS6	Y	+/-	-	+	+	+	+	+	-	+	+	-	-
	Puthuvype	BTPM4	Y	+	-	+	+	+	+	+	-	+	+	-	-
		BTMS4	Y	+	-	+	+	+	+	-	-	+	+	-	-
		BTMS7	Y	-	+/-	+	+	+	+	+	-	+	+	-	-
	Vypeen	BTVE2	Y	+	+	+	+	+	+	+	+	+	+	-	-

Y= Yellow; + = positive; - = negative; VP = Voges-Proskauer test

4.3.2. Biochemical characterization of *V. vulnificus* isolates

The biochemical characteristics of *V. vulnificus* isolates are summarised in Table 4. 4. Yellow coloured colonies were produced by all strains on TCBS agar plate except for strain BTPF8 from aquafarm and BTOS7 and BTOS8 from mangroves that showed green colonies. All the

tested strains were VP negative, 3% NaCl tolerant, ONPG positive and capable of utilizing the sugars, sucrose and glucose as their carbon source. Eight strains produced arginine dihydrolases. Fifty percent of the strains utilized citrate, mannitol and arabinose as the sole carbon source. Six of the tested strains, BTOK6 and BTPF5 from aquafarms and BTMM2, BTOS7, BTVE4 and BTVE9 from mangroves tested positive for ornithine decarboxylase. Cellobiose could be utilized by 10 strains whereas salicin utilization was observed only in 3 strains.

Table4.4. Biochemical tests of *V. vulnificus* isolates from marine environments (N= 18). (Hi-Vibrio identification kit, HiMedia, India)

		Strain	TCBS	VP	Arginine	NaCl 3%	ONPG	Citrate	Ornithine	Mannitol	Arabinose	Sucrose	Glucose	Salicin	Cellobiose
Aquafarms	Ochanthuruthu	BTOK1	Y	-	+	+	+	+	-	+	+	+	+	+	+
		BTOK5	Y	-	-	+	+	-	-	-	+	+	+	-	+
		BTOK6	Y	-	+	+	+	-	+	+	+	+	+	-	+
		BTOK10	Y	-	+	+	+	+	-	+	+	+	+	-	-
		BTOK12	Y	-	-	+	+	-	-	-	-	+	+	-	+
	Puthuvype	BTPF5	Y	-	-	+	+	+	+	+	-	+	+	-	-
		BTPF8	G	-	+	+	+	+	-	+	-	+	+	-	+
		BTAS3	Y	-	+	+	+	+	-	+	+	+	+	+	-
		BTPS6	Y	-	-	+	+	-	-	-	-	+	+	-	-
Mangroves	Njarakkal	BTMM2	Y	-	-	+	+	-	+	+	-	+	+	-	+
		BTMM7	Y	-	-	+	+	-	-	-	+	+	+	-	-
	Ochanthuruthu	BTOS4	Y	-	-	+	+	-	-	-	-	+	+	-	+
		BTOS7	G	-	-	+	+	+	+	-	-	+	+	-	+
		BTOS8	G	-	-	+	+	-	-	+	+	+	+	-	-
	Puthuvype	BTMS3	Y	-	+	+	+	+	-	+	+	+	+	+	-
	Vypeen	BTVE4	Y	-	+	+	+	+	+	-	+	+	+	-	+
		BTVE8	Y	-	-	+	+	-	-	-	-	+	+	-	+
		BTVE9	Y	-	+	+	+	+	+	-	-	+	+	-	-

Y= Yellow; G = Green;+ = positive; - = negative; VP = Voges-Proskauer test

4.3.3. Biochemical characterization of *V. parahaemolyticus*, *V. alginolyticus* and *V. azureus* isolates

Table 4.5 summarizes the biochemical characteristics of *V. parahaemolyticus*, *V. alginolyticus* and *V. azureus.* All the three strains of *V. parahaemolyticus* produced green colonies on TCBS agar, were VP negative, able to tolerate 3% NaCl in the medium and could utilize arabinose and glucose as the carbon source. The strains gave negative results for all the other tests. Strain BTMU7 from mussel tested negative for ONPG and mannitol, but was able to consume sucrose and cellobiose from the medium.

The two strains of *V. alginolyticus* though similar in their ability to utilize arginine, mannitol, arabinose, glucose and cellobiose and their tolerance to 3% NaCl, were diverse in other biochemical characteristics like VP and ONPG tests and utilization of citrulline, ornithine and salicin as the carbon source. The colour of the colony that developed on TCBS agar were also markedly different, BTAS2 being yellow and BTOS1 green coloured.

The single strain of *V. azureus*, BTVE11 from mangroves, produced yellow colonies on TCBS agar, was tolerant of 3% NaCl and capable of utilising the citrate, ornithine, arabinose and glucose in the medium. The strain tested negative for VP and ONPG tests and were unable to utilize arginine, mannitol, salicin and cellobiose when provided as the carbon source in the medium.

Table 4.5. Results of biochemical tests of *V. parahaemolyticus, V. alginolyticus and V. azureus* isolates from marine environments (Hi-Vibrio identification kit, HiMedia, India).

		Strain	TCBS	VP	Arginine	NaCl 3%	ONPG	Citrate	Ornithine	Mannitol	Arabinose	Sucrose	Glucose	Salicin	Cellobiose
V. parahaemolyticus															
Seafood	Mussel	BTMU7	G	-	-	+	-	-	-	-	+	+	+	-	+
Mangroves	Puthuvype	BTMS5	G	-	-	+	+	-	-	+	+	-	+	-	-
	Ochanthuruthu	BTOS10	G	-	-	+	+	-	-	+	+	-	+	-	-
V. alginolyticus															
Aquafarm	Puthuvype	BTAS2	Y	+	-	+	+	+	+	+	-	+	+	+	-
Mangrove	Ochanthuruthu	BTOS1	G	-	-	+	-	-	-	+	-	-	+	-	-
V. azureus															
Mangrove	Vypeen	BTVE11	Y	-	-	+	-	+	+	-	+	-	+	-	-

Y= Yellow; G = Green;+ = positive; - = negative; VP = Voges-Proskauer test

4.4. Antibiotic Susceptibility Test

The analysis of the antibiogram obtained suggests that drug resistance is widespread among the marine environmental isolates of vibrios. All the 45 isolates were subjected to antibiotic susceptibility tests against 12 commonly administered drugs and the strains exhibited varying degrees of resistance to gentamicin (2.22%), ampicillin (62.22%), nalidixic acid (4.44%), vancomycin (86.66), cefixime (17.77%), rifampicin (20%), tetracycline (42.22%) and chloramphenicol (2.22%) (Fig. 4.4). All the isolates were susceptible to streptomycin, co-trimoxazole, trimethoprim and azithromycin.

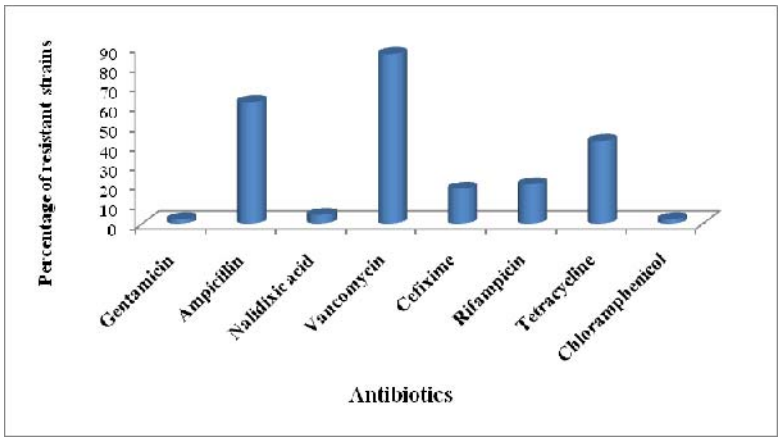


Fig.4.4. The antibiotic resistance profile of *Vibrio* isolates (N=45) from marine environments (expressed in percentage)

4.4.1. Sample-wise antibiotic resistance pattern

The antibiotic resistance exhibited by the strains isolated from the various samples were analysed and is depicted in Fig 4.5. The isolates from seafood exhibited resistance to ampicillin, vancomycin, rifampicin and chloramphenicol. The aquafarm hosted isolates resistant to ampicillin, vancomycin, cefixime, rifampicin and tetracycline. Isolates from the mangrove environment were resistant to gentamicin, ampicillin, nalidixic acid, vancomycin, cefixime, rifampicin and tetracycline.

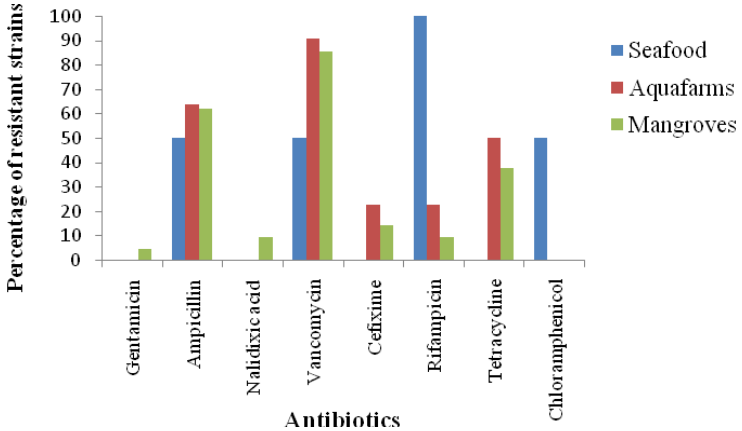


Fig.4.5. Antibiotic resistance pattern exhibited by *Vibrio* spp. from various marine environments (expressed in percentage).

4.4.2. Species–wise antibiotic resistance pattern

The drug resistance profile of the isolates suggests that multidrug resistance is widespread among the various species of *Vibrio* obtained. Among the *V. cholerae* strains (N=21), resistance to antibiotics like vancomycin (19 strains; 90.47%), tetracycline (12 strains; 57%), ampicillin (12 strains; 57%), rifampicin (5 strains; 24%), cefixime (3 strains; 14%), nalidixic acid (1 strain; 5%) and chloramphenicol (1 strain; 5%) were observed. Except for 4 strains, all the other *V. cholerae* isolates were multidrug resistant (Table 4.6). Five strains were resistant to more than three antibiotics.

Table 4.6. Drug resistance profile of *V. cholerae* strains

Sl. No.	Strains	Drug Resistance Profile*
1	BTPR5	Am, Va, Ri, Ch
2	BTMA1	Am, Va, Ri, Te
3	BTMA5	Am, Va, Te
4	BTMA9	Am, Va, Ri, Te
5	BTOK2	Va
6	BTOK7	Am, Ce
7	BTOK9	Va
8	BTOK11	Am, Va, Ce
9	BTPF2	Va, Te
10	BTPF6	Va, Te
11	BTPS2	Va
12	BTVM6	Am, Va, Ri, Te
13	BTVM8	Am, Va, Ri, Te
14	BTMM4	Va, Te
15	BTMM6	Va
16	BTOS3	Am, Va, Te
17	BTOS6	Am, Va, Te
18	BTPM4	Na, Va
19	BTMS4	Am, Va
20	BTMS7	Ce, Te
21	BTVE2	Am, Va, Te

*Am- Ampicillin; Va- Vancomycin; Ri- Rifampicin; Ce- Cefixime; Ch- Chloramphenicol; Te- Tetracycline; Na- Nalidixic acid

The strains of *V. vulnificus* (N=18) exhibited varying degrees of resistance to the tested antibiotics. Seventeen (94%) strains were resistant to vancomycin, 11 strains (61%) were resistant to ampicillin, 7 strains (39%) to tetracycline and 5 strains (28%) to cefixime. One strain each (6%) were resistant to gentamicin, nalidixic acid and rifampicin. All strains except strain BTOS7 were multidrug resistant, with 6 strains exhibiting resistance to 3 or more antibiotics (Table 4.7).

Table 4.7. Drug resistance profile of *V. vulnificus* strains

Sl. No.	Strain	Drug Resistance Profile
1	BTOK1	Am, Va, Ce, Ri
2	BTOK5	Am, Va
3	BTOK6	Am, Ce
4	BTOK10	Am, Va, Ce
5	BTOK12	Am, Va
6	BTPF5	Va, Te
7	BTPF8	Va, Te
8	BTAS3	Am, Va, Te
9	BTPS6	Va, Te
10	BTMM2	Va, Te
11	BTMM7	Va, Te
12	BTOS4	Am, Va
13	BTOS7	Va
14	BTOS8	Am, Va
15	BTMS3	Na, Va
16	BTVE4	Am, Va, Ce
17	BTVE8	Am, Va, Te
18	BTVE9	Ge, Am, Va, Ce

*****Am- Ampicillin; Va- Vancomycin; Ri- Rifampicin; Ce- Cefixime; Ch- Chloramphenicol; Te- Tetracycline; Na- Nalidixic acid; Ge- Gentamicin

Among the *V. parahaemolyticus* strains (N=3), all were resistant to rifampicin (100%), 2 were resistant to ampicillin and one strain to

vancomycin. Both strains of *V. alginolyticus* (N=2) were resistant to ampicillin (100%), with strain BTAS2 from aquafarm also resistant to vancomycin. The *V. azureus* strain, BTVE11, was resistant to ampicillin and vancomycin (Table 4.8).

Table 4.8. Drug resistance profile of *V. parahaemolyticus, V. alginolyticus* and *V. azureus*

Sl. No.	Strain	Drug Resistance Profile[1]
1	BTMU7*	Ri
2	BTOS10*	Am, Va, Ri
3	BTMS5*	Am, Ri
4	BTAS2**	Am, Va
5	BTOS1**	Am
6	BTVE11***	Am, Va

*- *V. parahaemolyticus;* **- *V. alginolyticus;* ***- *V. azureus*

[1]Am- Ampicillin; Va- Vancomycin; Ri- Rifampicin

4.4.3. Multiple Antibiotic Resistance (MAR) Index

The multiple antibiotic resistance index was calculated from the results of the antibiotic susceptibility tests. MAR index value >0.2 is an indication of the antibiotic stress of the environment from which they were isolated. It was seen that 39 strains (87%) were multi- drug resistant, exhibiting resistance to at least two of the antibiotics tested.

4.4.3.1. Sample- wise Multiple Antibiotic Resistance (MAR) Index

Isolates from all the three marine environments exhibited multiple antibiotic resistance, having a high MAR index value, suggestive of the use of antibiotics in these environments (Fig.4.6). Of the two strains isolated from seafood, BTPR5 from prawn had a MAR index value of 0.3 and it was <0.1 for the strain BTMU7 from mussel.

Among the 22 isolates from aquafarms, 3 strains (14%) gave a value of <0.1, 10 strains (46%) had a value between 0.1- 0.2 and 9 strains (40%) had a MAR index value that was greater than 0.2.

Seven strains (33%) out of the 21 isolates from mangroves were resistant to at least three tested antibiotics and showed a value of >0.2, 11 strains (53%) had a value from 0.1-0.2 and the remaining 3 strains (14%) had a value lesser than 0.1.

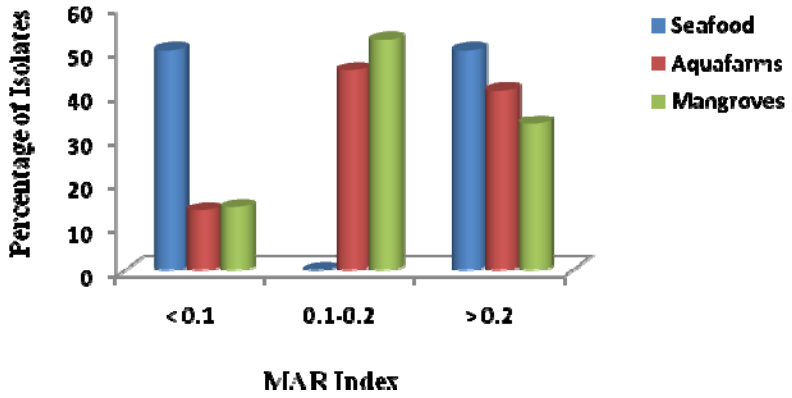


Fig.4.6. The MAR Index pattern exhibited by *Vibrio* spp. isolated from various marine environments (N=45).

4.4.3.2. Species-wise Multiple Antibiotic Resistance (MAR) Index

The MAR index pattern among the different species of *Vibrio* is depicted in Fig.4.7. Ten out of the 21 *V. cholerae* strains (48%) had a high MAR value of >0.2, for 7 strains (33%) it was from 0.1-0.2 and 4 strains (19%) showed a value <0.1. Among the strains of *V. vulnificus*, 33% (6 strains) had a high MAR value of >0.2, in 61% (11 strains) it was from 0.1-0.2 and in one strain the value was <0.1.

One strain among *V. parahaemolyticus* isolates BTOS10 had a MAR value of greater than 0.2 and the strains of *V. alginolyticus* and *V. azureus* gave values lesser than 0.2.

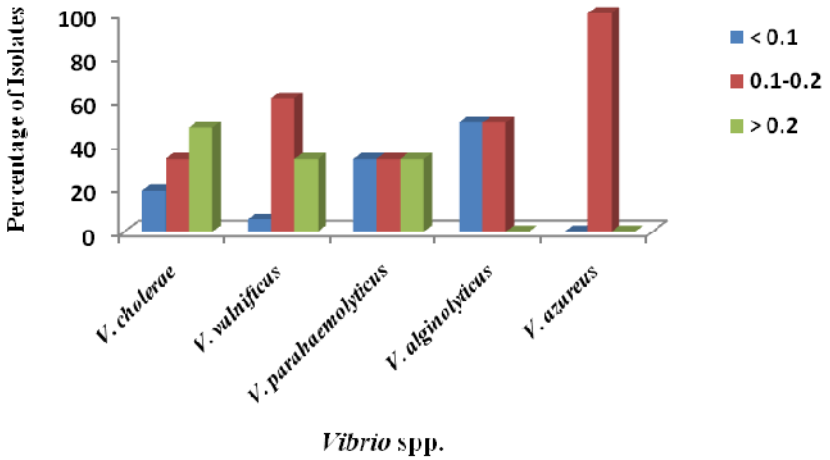


Fig.4.7. MAR Index pattern exhibited by different species of *Vibrio* isolated from marine environments.

4.5. Serogrouping of *V. cholerae* Using Marker Genes

The 21 strains of *V. cholerae* were serogrouped by screening for the presence O1*rfb* and O139 *rfb* marker genes (Hoshino *et al.*, 1998) by PCR amplification that should produce a 450 bp or 190 bp amplicon for O1 and O139 specific *rfb* genes respectively. The specific amplicons were absent in all the tested strains and hence all the *V. cholerae* environmental isolates obtained in this study were categorised as belonging to non O1/ non O139 serogroups.

4.6. Molecular Typing

The 21 isolates identified as *V. cholerae* and 18 isolates of *V. vulnificus* were subjected to molecular typing methods such as ERIC PCR and BOX PCR.

4.6.1. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR

ERIC sequences are 126 bp long sequences, highly conserved at the nucleotide level and present in multiple copies in the genome of vibrios.

The analysis of the amplified products of these repeated sequences produced specific banding profiles revealing the clonal relationship between the strains.

4.6.1.1. ERIC-PCR fingerprints of *V. cholerae*

Genomic fingerprinting analysis by ERIC-PCR of the 21 *V. cholerae* strains resulted in amplification of multiple fragments (6-10) of DNA, with sizes ranging from 100 bp to 1500 bp, yielding specific fingerprint patterns (Fig.4.8). Two fragments, one of ~100 bp and the other of ~300 bp, were common to all the tested strains. A fragment of ~500 bp was present in all strains except strains BTMA1 and BTPS2 from aquafarm and strain BTOS6 from mangrove environment.

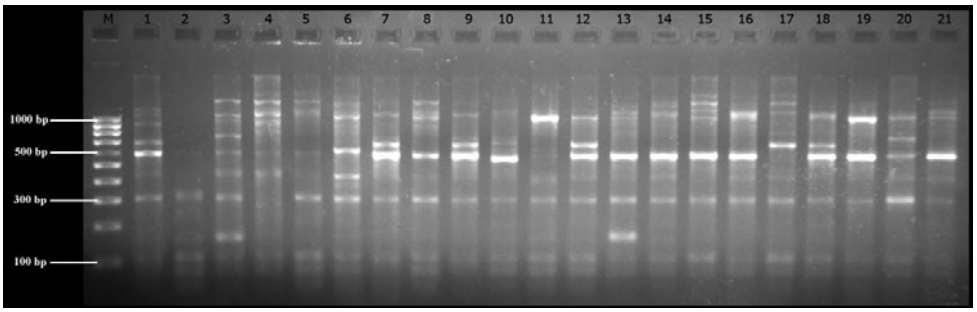


Fig.4.8. ERIC- PCR fingerprints of *V. cholerae* strains with ERIC oligonucleotide primer sets.

Lane M-100 bp ladder; Lane 1-BTPR5; Lane 2-BTMA1; Lane 3-BTMA5; Lane 4-BTMA9; Lane 5-BTOK2; Lane 6-BTOK7; Lane 7-BTOK9; Lane 8-BTOK11; Lane 9-BTPF2; Lane 10-BTPF6; Lane 11-BTPS2; Lane 12-BTVM6; Lane 13-BTVM8; Lane 14-BTMM4; Lane 15-BTMM6; Lane 16-BTOS3; Lane 17-BTOS6; Lane 18-BTPM4; Lane 19-BTMS4; Lane 20-BTMS7; Lane 21-BTVE2

The fingerprint pattern was used to generate a distance matrix and the dendrogram (Fig 4.9) generated showed that the isolates were divided into

2 major clusters. Cluster 1 included 19 strains in 3 groups; Group1 including 11 strains exhibiting close similarity in their fingerprint pattern. A set of five strains, BTPR5 from prawn, BTOK7, BTOK9 and BTVM6 from aquafarms and BTMS7 from mangrove environments exhibited 100% similarity in their fingerprint pattern. Similarly, 6 strains exhibited homology in their banding profile, of which two (BTMA9 and BTOK11) were from aquafarms and the remaining four (BTMM4, BTMM6, BTOS3 and BTVE2) were from mangrove environments. Strains BTMA5 and BTVM8 from aquafarms, with 100% homology in banding patterns, were included in Group 2. The single strain BTPS2 from aquafarm exhibited ~15% dissimilarity from the members of Group 2. Group 3 of Cluster 1 comprised of 5 strains that shared >95% similarity; the banding profile was similar in the strains BTOK2 from aquafarm and BTMS4 from mangrove environment and in the three strains, BTPF2 and BTPF6 from aquafarm and BTPM4 from mangroves. Cluster 2 included the two strains, BTMA1 from aquafarm and BTOS6 from mangroves with distinct fingerprint patterns from the rest of the strains. Generally, the environmental strains of *V. cholerae* exhibited ~70% homology in the ERIC PCR fingerprint pattern, indicative of the importance of this technique as a molecular typing tool in ecological studies.

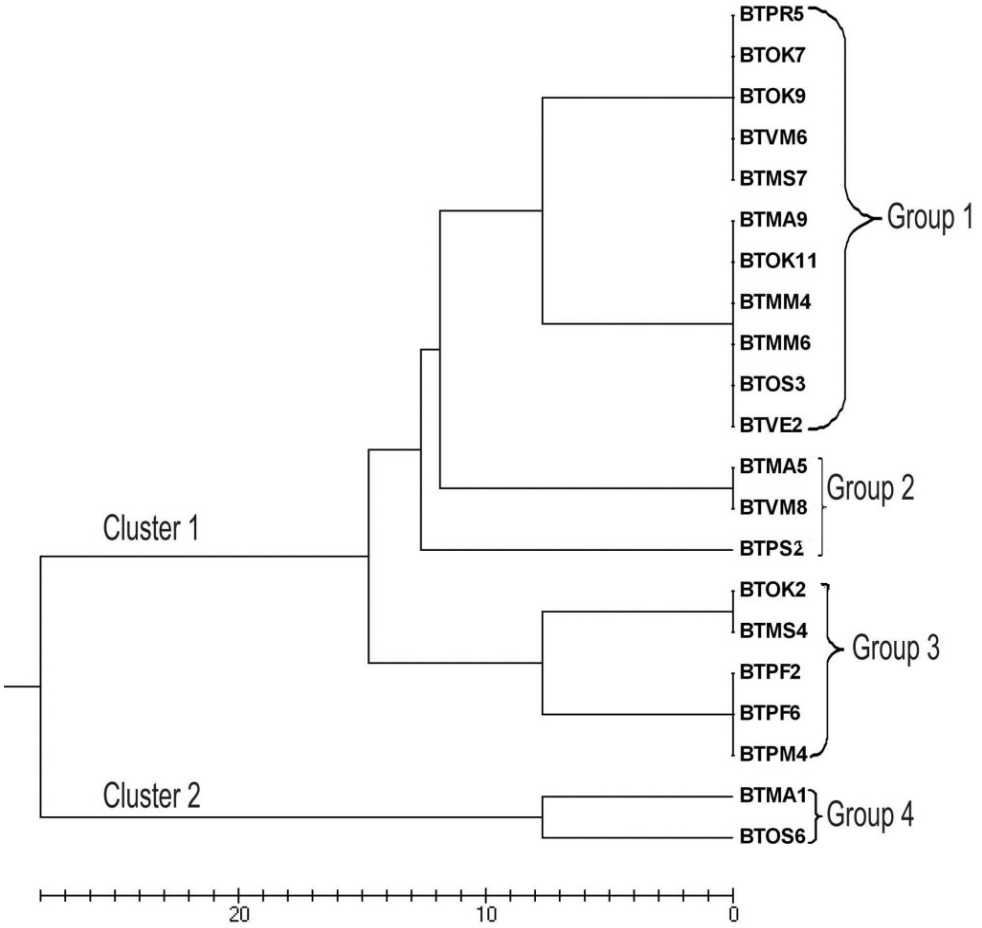


Fig.4.9. Dendrogram constructed on the basis of the ERIC PCR profiles of environmental strains *of V. cholerae* generated by the POPGENE software.

4.6.1.2. ERIC-PCR fingerprints of *V. vulnificus*

The 18 isolates of *V. vulnificus* obtained from marine environments were subjected to ERIC-PCR fingerprinting analysis. The fingerprints consisted of three to eight amplification bands, ranging in size from <100 bp to 1500 bp (Fig. 4.10). Two bands, one below 100 bp and the other at ~300 bp, were found to be common to all the tested strains.

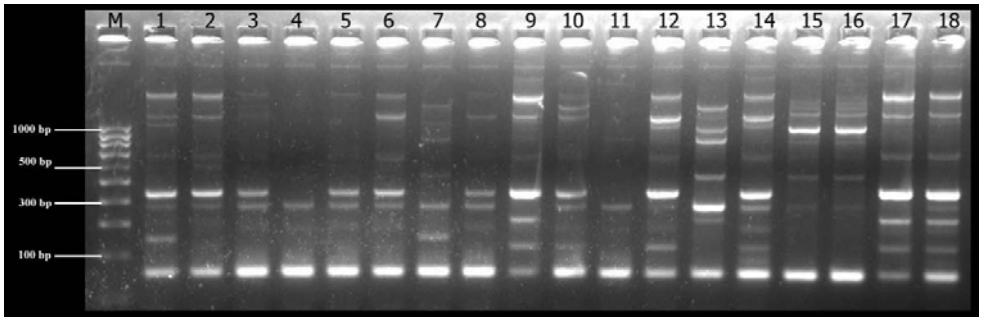


Fig.4.10. ERIC-PCR genomic fingerprinting pattern of *V. vulnificus* with ERIC oligonucleotide primer sets.

Lane M-100 bp ladder; Lane 1-BTOK1; Lane 2-BTOK5; Lane 3-BTOK6; Lane 4-BTOK10; Lane 5-BTOK12; Lane 6-BTPF5; Lane 7-BTPF8; Lane 8-BTAS3; Lane 9-BTPS6; Lane 10-BTMM2; Lane 11-BTMM7; Lane 12-BTOS4; Lane 13-BTOS7; Lane 14-BTOS8; Lane 15-BTMS3; Lane 16-BTVE4; Lane 17-BTVE8; Lane 18-BTVE9

The dendrogram depicting the analysis of the banding pattern obtained by ERIC-PCR fingerprinting in *V. vulnificus* is given in Fig. 4.11. The dendrogram illustrated two Clusters, wherein Cluster 1 comprised 12 strains, in 2 groups; Group 1 with 2 sets of three strains each, BTOK1 from aquafarm and BTMM2 and BTOS8 from mangroves and BTOK5, BTOK6 and BTPF5 from aquafarms, exhibiting 100% similarity among strains of each set. The strains BTOK12 and BTAS3 from aquafarms displayed ~15% divergence from members of Group 1. Group 2 included strains BTPS6 from aquafarm and BTOS4 from mangroves and BTVE8 and BTVE9 from mangroves exhibiting identical fingerprint patterns. Cluster 2 included 6 strains in 2 groups; Strains BTOK10 from aquafarm and BTMM7 from mangrove included in Group 3 exhibited 100% homology in banding profile and were separated from BTPF8 from aquafarm and BTOS7 from mangrove with ~15% dissimilarity. The two strains BTMS3 and BTVE4 from mangroves included

in Group 4 exhibited >90% similarity among themselves but segregated from the rest of the strains in the cluster with ~20% dissimilarity.

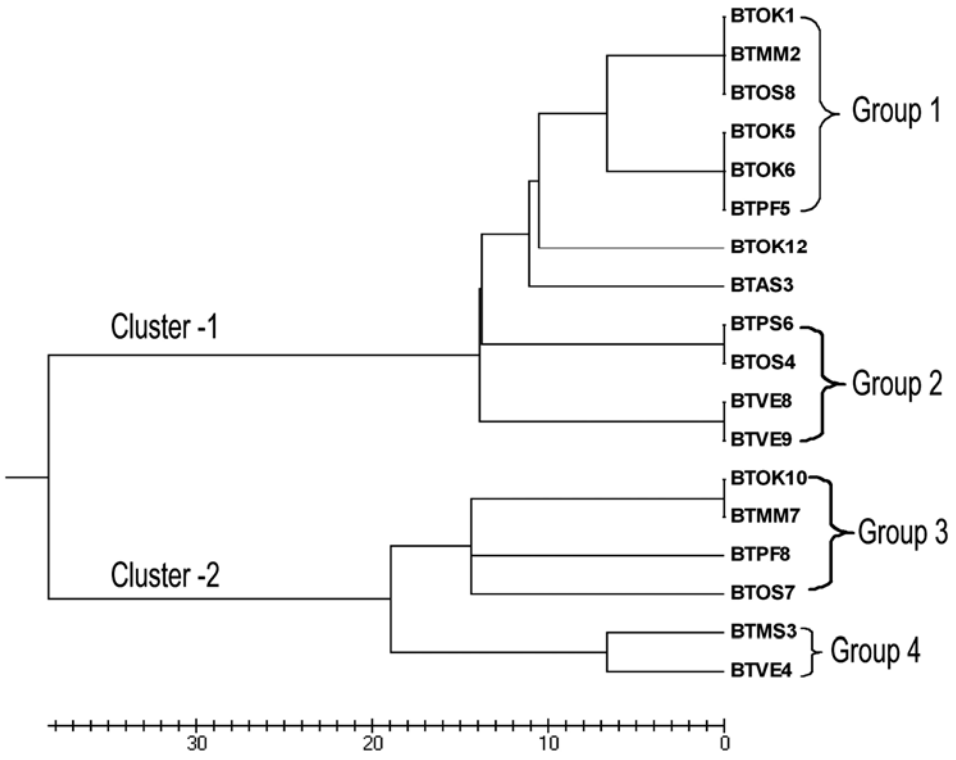


Fig.4.11. Dendrogram constructed on the basis of the ERIC PCR profiles of environmental strains *of V. vulnificus* generated by the POPGENE software.

4.6.2. BOX-PCR fingerprinting

BOX elements, are naturally occurring, multi-copied, conserved, repetitive DNA sequences present in the genomes of most bacteria. The BOX A1R primer amplifies specific genomic regions located between BOX elements (154bp) and its natural inverted repeats.

4.6.2.1. BOX-PCR fingerprints of *V. cholerae*

BOX-PCR fingerprinting method produced fingerprints with 6–13 well spaced bands in the approximate size region of 150–2000 base pairs

(Fig. 4.12). One characteristic band was prominent in all the strains at ~700 bp region.

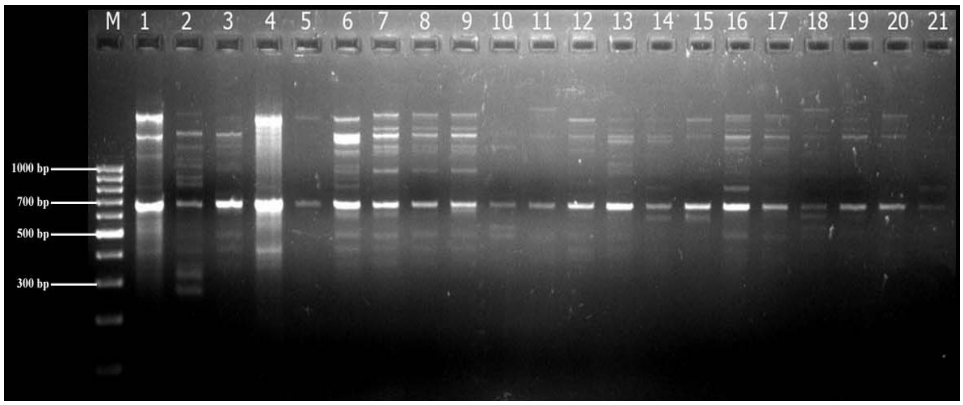


Fig.4.12. BOX PCR fingerprints obtained for 21 environmental *V. cholerae* strains using BOX A1 primer.

Lane M-100 bp ladder; Lane 1-BTPR5; Lane 2-BTMA1; Lane 3-BTMA5; Lane 4-BTMA9; Lane 5-BTOK2; Lane 6-BTOK7; Lane 7-BTOK9; Lane 8-BTOK11; Lane 9-BTPF2; Lane 10-BTPF6; Lane 11-BTPS2; Lane 12-BTVM6; Lane 13-BTVM8; Lane 14-BTMM4; Lane 15-BTMM6; Lane 16-BTOS3; Lane 17-BTOS6; Lane 18-BTPM4; Lane 19-BTMS4; Lane 20-BTMS7; Lane 21-BTVE2

The cluster analysis of the fingerprints of *V. cholerae* strains generated by BOX-PCR revealed 2 main Clusters in the dendrogram (Fig 4.13). Cluster 1 including 12 strains diverged into 3 groups. In Group 1, three strains exhibited identical fingerprint pattern, i.e., BTMA1 and BTMA5 from aquafarms and BTMM4 from mangrove and strain BTPR5 from prawn clustered with group 1 with > 95% homology. Strain BTMS4 from mangrove diverged from Group 1 with 20% dissimilarity. Group 2 included 3 strains with 100% similarity, i.e., BTOK2, BTPF6 and BTPS2 from aquafarms and the closely related BTMA9 from the same environment. Group 3 comprised of BTPM4 and BTVE2 from mangroves that exhibited homology in banding profile and strain BTVM8

from aquafarm that exhibited ~90% similarity to other members of the group. Cluster 2 included 9 strains clustered into Group 4, with 100% similarity exhibited between a set of six strains and a set of 3 strains. Although the isolates were from diverse environmental sources, a high degree of similarity in banding profile was displayed among the strains.

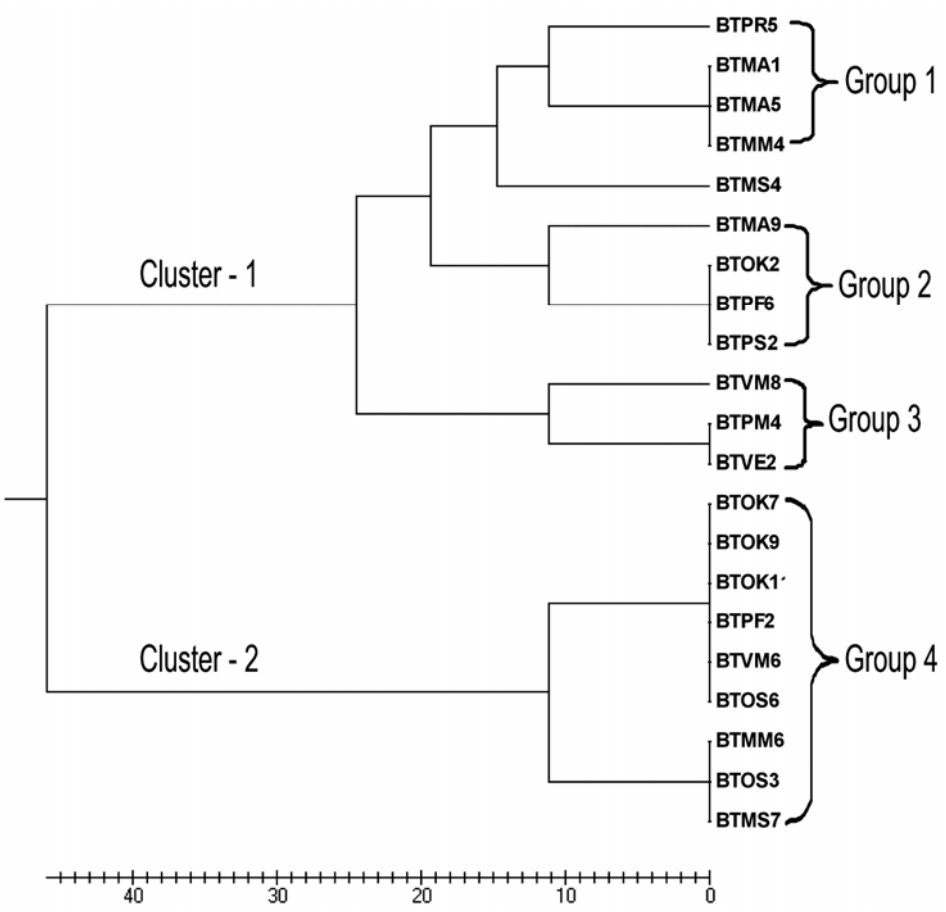


Fig.4.13. Dendrogram obtained by comparison of BOX-PCR fingerprinting patterns from 21 environmental strains belonging to *V. cholerae.*

The dendrogram was established by the POPGENE software using the unweighted pair group method by arithmetic mean (UPGMA) on the basis of the BOX PCR profiles obtained with BOX A1primer.

4.6.2.2. BOX-PCR fingerprints of *V. vulnificus*

The BOX-PCR genomic fingerprints of the 18 environmental strains of *V. vulnificus* yielded 3-11fragments, in the ~300 bp to 2000 bp range. (Fig 4.14). A distinct band at ~700 bp was observed in all the tested strains.

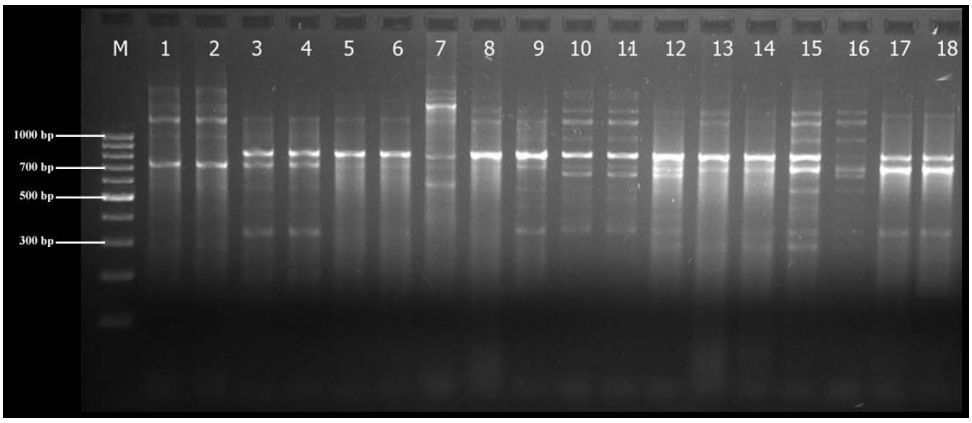


Fig.4.14. BOX PCR fingerprints obtained using BOX A1 primer for 18 *V. vulnificus* strains from marine environments.

Lane M-100 bp ladder; Lane 1-BTOK1; Lane 2-BTOK5; Lane 3-BTOK6; Lane 4-BTOK10; Lane 5-BTOK12; Lane 6-BTPF55; Lane 7-BTPF8; Lane 8-BTAS3; Lane 9-BTPS6; Lane 10-BTMM2; Lane 11-BTMM7; Lane 12-BTOS4; Lane 13-BTOS7; Lane 14-BTOS8; Lane 15-BTMS3; Lane 16-BTVE4; Lane 17-BTVE8; Lane 18-BTVE9

Analysing the similarity among the banding profiles from the dendrogram (Fig 4.15), it was observed that two distinct clusters emerged among the isolates, Cluster 1 which included 2 strains and Cluster 2 which grouped the remaining 16 strains. Cluster 1 comprised Group 1with 2 strains, BTOK1 and BTOK5 from mangrove with 100% homology in banding profile. Cluster 2 included 4 groups of strains with nearly identical fingerprint patterns. Group 2 included 4 strains from aquafarms (BTOK6, BTOK10, BTOK12 and BTPF5) and Group 3, a homologous pair (BTOS4

and BTOS8) from mangroves. Group 4 clustered 8 strains; the first homologous set represented by BTPS6 from aquafarm and BTMM2, BTMM7, BTVE8 and BTVE9 from mangroves and the second including the three closely related strains, BTOS7, BTMS3 and BTVE4 from mangroves. Group 5 with two strains, BTPF8 and BTAS3 from aquafarms, exhibited ~75% similarity with other members of the cluster.

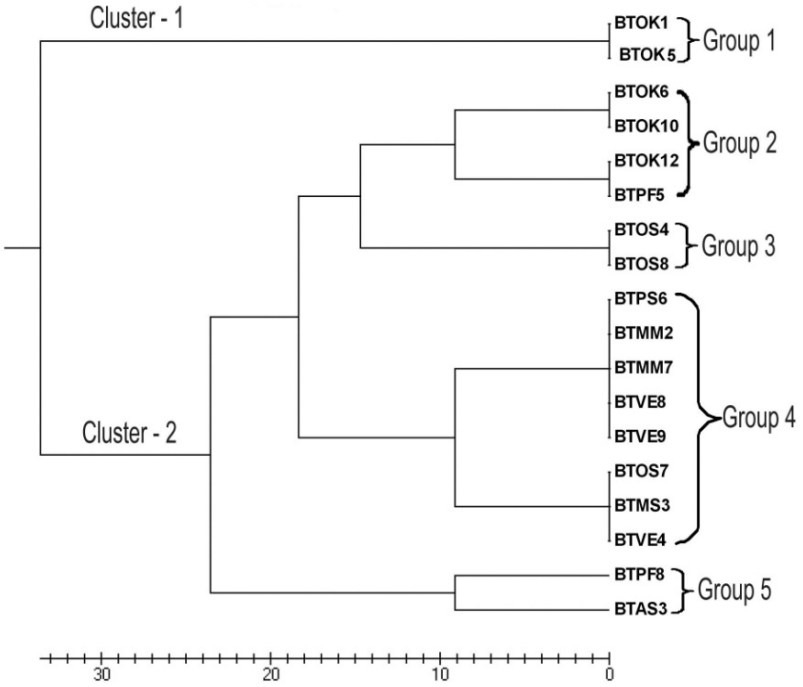


Fig.4.15. Dendrogram obtained by comparison of BOX-PCR fingerprinting patterns from 18 environmental strains belonging to *V. vulnificus.*

The dendrogram was established by the POPGENE software using the unweighted pair group method by arithmetic mean (UPGMA) on the basis of the BOX PCR profiles obtained with BOX A1primer.

4.7. Screening for Virulence/ Virulence Associated Genes in *V. cholerae*

The *V. cholerae* strains were screened for the presence of 13 virulence/ virulence associated genes like *ctx*A, *ctx*B, *ace*, VPI, *hly*A, *omp*U, *rtx*A, *tox*R,

zot, *nag*st, *tcp*A, *nin* and *nan*H. The amplification of the genes was by using gene specific primers.

4.7.1. Detection of Vibrio Pathogenicity Island (VPI)

VPI region of the genomic DNA was targeted using a primer set that specifically produces a ~680 bp amplicon indicating the presence of the pathogenicity island in the environmental strains. Out of the 21 environmental isolates of *V. cholerae*, only 9 strains tested VPI positive (Fig.4.16). Representative strains from all tested samples hosted the VPI i.e., strain BTPR5 from seafood (Prawn), strains BTOK7, BTOK11 and BTPF2 from aquafarms and BTMM4, BTMM6, BTOS3, BTPM4 and BTVE2 from mangrove environments.

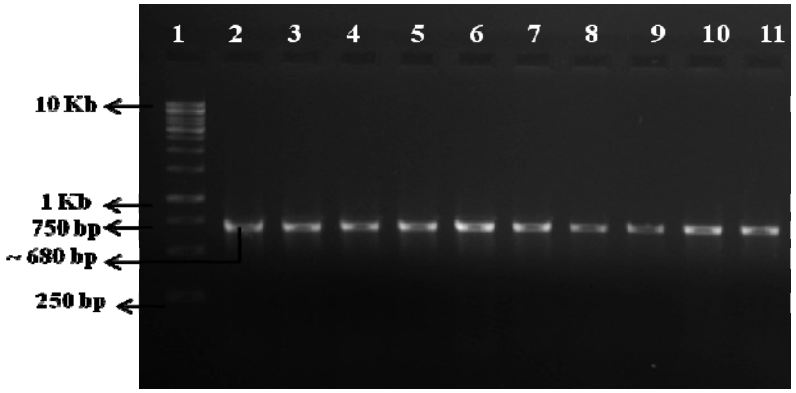


Fig.4.16. Detection of VPI gene by PCR in strains of *V. cholerae* isolated from marine environments.

Lane 1- 1 kb ladder; Lane 2- Positive control (*V.cholerae* CO366 Eltor strain); Lane 3- BTPR5; Lane 4- BTOK7; Lane 5- BTOK11; Lane 6- BTPF2; Lane 7-BTMM4; Lane 8- BTMM6; Lane 9- BTOS3; Lane 10- BTPM4; Lane 11- BTVE2

4.7.2. Detection of *hly*A

The *hly*A gene responsible for the leukocidal activity contributing to the pathogenesis of gastroenteritis caused by some *V. cholerae* strains was

detected by PCR in 14 environmental isolates (Fig 4.17) producing ~481 bp amplicon corresponding to the *hly*A gene. The strains hosting the *hly*A gene were isolated from seafood (strain BTPR5), aquafarms (BTMA1, BTMA5, BTOK2, BTOK7, BTOK11, BTPF6 and BTPS2) and mangrove environments (BTVM6, BTVM8, BTMM6, BTPM4, BTMS4and BTMS7).

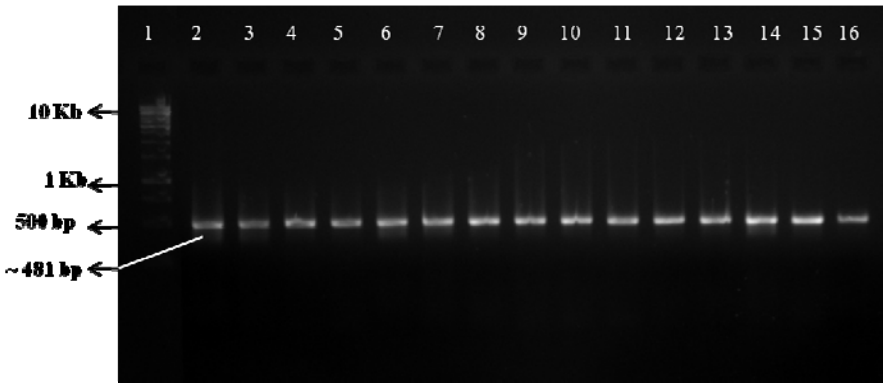


Fig.4.17. Detection of *hly*A in strains of *V. cholerae* isolated from marine environments.

Lane 1- 1 kb ladder; Lane 2- Positive control (*V.cholerae* CO366 Eltor strain); Lane 3- BTPR5; Lane 4- BTMA1; Lane 5- BTMA5; Lane 6-BTOK2; Lane 7- BTOK7; Lane 8- BTOK11; Lane 9- BTPF6; Lane 10- BTPS2; Lane 11- BTVM6; Lane 12 –BTVM8; Lane 13- BTMM6; Lane 14- BTPM4; Lane 15- BTMS4; Lane 16-BTMS7

4.7.3. Detection of *omp*U

Only 5 strains out of the 21 strains of *V. cholerae* screened, tested positive for the *omp*U gene giving amplification products correlating in size to ~869bp. (Fig 4.18). *omp*U gene produces an outer membrane protein which acts as an adherence factor and is involved in the colonization of epithelial cells by *V. cholerae*. Strains, BTPR5 (seafood), BTPF6 and BTVM6 (aquafarms) and BTMM4 and BTMM6 (mangroves) tested positive for this gene.

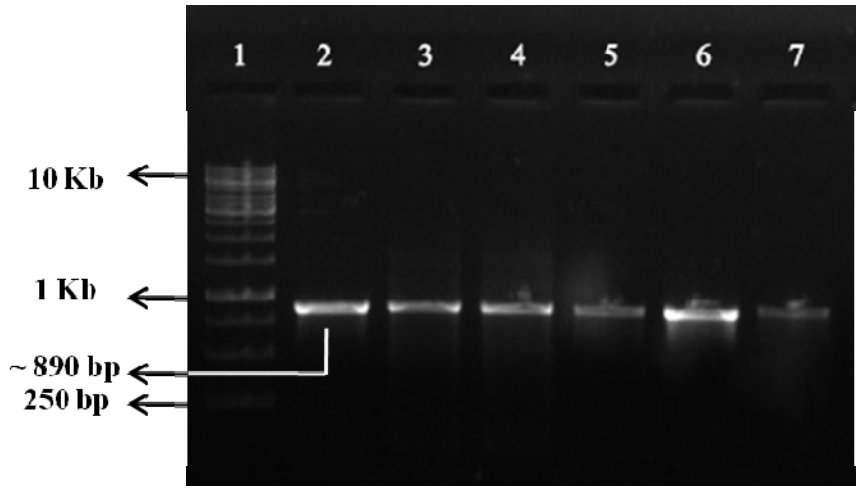


Fig.4.18. Detection of *omp*U gene in strains of *V. cholerae* isolated from marine environments.

Lane 1- 1 kb ladder; Lane 2- Positive control (*V.cholerae* Co 366 Eltor strain) ; Lane 3- BTPR5; Lane 4- BTPF6; Lane 5- BTVM6; Lane 6- BTMM4; Lane 7- BTMM6

4.7.4. Detection of *rtx*A

The *rtx*A gene belongs to the RTX (repeat in toxin) family of toxins, present in *Vibrios*, that display cytotoxic activity and causes actin crosslinking in mammalian epithelial cells leading to symptoms of gastroenteritis in humans. The PCR detection protocol for *rtx*A gene using a set of specific primers revealed its presence in 8 environmental isolates of *V. cholerae* (Fig 4.19). In the present study the strains of *V. cholerae* with the *rtx*A gene were BTPR5 from seafood, BTOK2, BTPF6 and BTVM6 from aquafarms and BTMM4, BTMM6, BTMS7 and BTVE2 from mangrove environments.

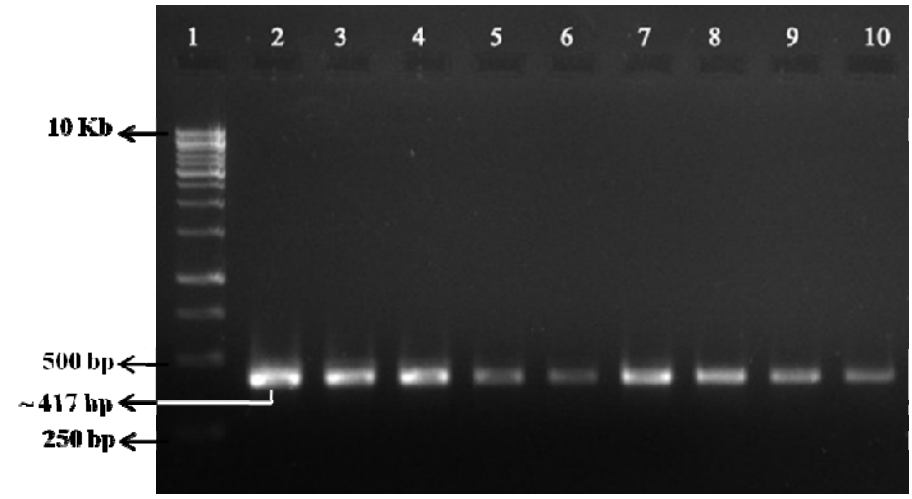


Fig.4.19. Detection of *rtx*A gene in strains of *V. cholerae* isolated from marine environments.

Lane 1- 1 kb ladder; Lane 2- Positive control (*V.cholerae* CO366 Eltor strain); Lane 3-BTPR5; Lane 4- BTOK2; Lane 5- BTPF6; Lane 6- BTVM6; Lane 7-BTMM4; Lane 8- BTMM6; Lane 9- BTMS7; Lane 10- BTVE2

4.7.5. Detection of *tox*R

The product of the *tox*R gene, acts as the master regulator of at least 17 distinct virulence associated genes in *V. cholerae* and its presence in the environmental isolates was detected by PCR by targeting the ~779bp region of the genomic DNA, using the gene specific primers at annealing temperature of 60^{o}C. The *tox*R gene was detected in nine *V. cholerae* strains of environmental origin (Fig. 4.20) viz. strains BTPR5 (Seafood), BTMA1, BTMA5, BTMA9, BTOK7, BTOK9 and BTOK11 (aquafarms) and BTMS4 and BTVE2 (mangroves).

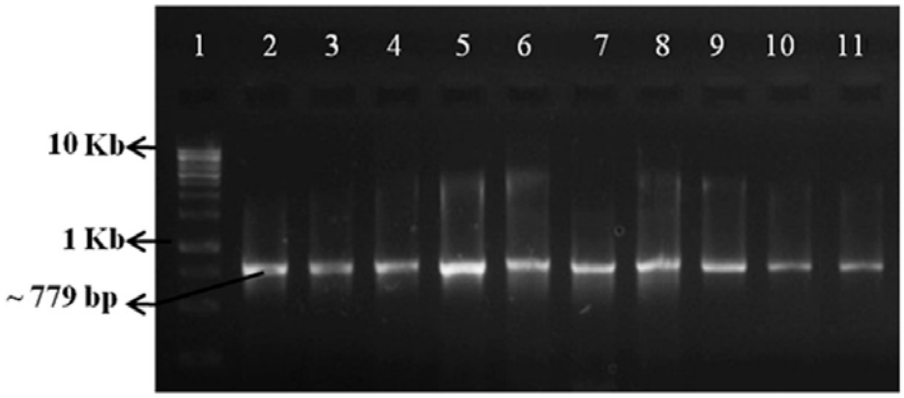


Fig.4.20. Detection of *tox*R gene in strains of *V. cholerae* isolated from marine environments.

Lane 1- 1 kb ladder; Lane 2- Positive control (*V.cholerae* CO366 Eltor strain); Lane 3- BTPR5; Lane 4-BTMA1; Lane 5- BTMA5; Lane 6- BTMA9; Lane 7- BTMS4; Lane 8- BTOK7; Lane 9- BTOK9; Lane 10- BTOK11; Lane 11- BTVE2

4.7.6. Detection of *ctx*A, *ctx*B, *ace*, *zot*, *nag*st, *tcp*A, *nin* and *nan*H

The *V. cholerae* environmental isolates were also screened for the presence of *ctx*A, *ctx*B, *ace*, *zot*, *nag*st, *tcp*A, *nin* and *nan*H by PCR using gene specific primers. However none of the isolates tested positive for these eight virulence genes.

4.7.7. Distribution of virulence genes among *V. cholerae* strains

The 21 environmental isolates of *V. cholerae* were screened for the presence of 13 virulence/virulence associated genes namely, *ctx*A, *ctx*B, *ace*, VPI, *hly*A, *omp*U, *rtx*A, *tox*R, *zot*, *nag*st, *tcp*A, *nin* and *nan*. Only 5 of the tested virulence/virulence associated genes were found among the various isolates in this study. PCR screening for the remaining 8 genes yielded negative results, suggesting their absence in these environmental strains. The distribution of these tested virulence genes among the isolates were analysed and is as given in Fig. 4.21. The gene *hly*A was present in

the highest frequency i.e., 67% in the tested strains, whereas the *tox*R gene and the VPI were present in only 43% of the isolates. The *rtx*A gene was detected in 38% and *omp*U gene in 24% of the environmental strains. Out of the 21 isolates, only 1 (5%) did not produce amplicons for any of the genes tested.

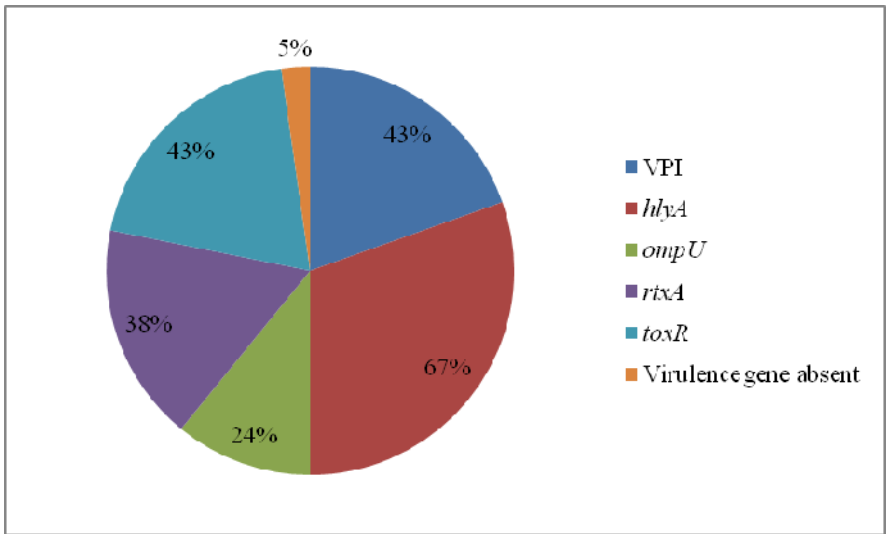


Fig.4.21. Distribution of the virulence/virulence associated genes among *V. cholerae* isolates from marine environments

4.7.8. Virulence profile of *V. cholerae* strains

The virulence profile of the tested *V. cholerae* strains is represented in Table 4.9. Among the isolates, BTPR5 from seafood (Prawn) hosted 5 out of the 13 screened genes, followed by BTMM6, from mangroves, which yielded positive results for 4 genes. Three genes each were detected in 6 strains (BTOK7, BTOK11, BTPF6, BTVM6, BTMM4 and BTVE2). Six strains (BTOK2, BTMA1, BTMA5, BTPM4, BTMS4 and BTMS7) yielded amplicons for 2 genes each and six strains, BTMA9, BTOK9, BTPF2, BTPS2, BTVM8 and BTOS3 were positive for a single gene each. The

strain, BTOS6, from mangroves did not yield amplicons for any of the tested genes.

Table 4.9. Virulence profile of *V. cholerae* strains isolated from marine environments.

Strain	VPI	*hlyA*	*ompU*	*rtxA*	*toxR*
BTPR5	+	+	+	+	+
BTMA1	-	+	-	-	+
BTMA5	-	+	-	-	+
BTMA9	-	-	-	-	+
BTOK2	-	+	-	+	-
BTOK7	+	+	-	-	+
BTOK9	-	-	-	-	+
TOK11	+	+	-	-	+
BTPF2	+	-	-	-	-
BTPF6	-	+	+	+	-
BTPS2	-	+	-	-	-
BTVM6	-	+	+	+	-
BTVM8	-	+	-	-	-
BTMM4	+	-	+	+	-
BTMM6	+	+	+	+	-
BTOS3	+	-	-	-	-
BTOS6	-	-	-	-	-
BTPM4	+	+	-	-	-
BTMS4	-	+	-	-	+
BTMS7	-	+	-	+	-
BTVE2	+	-	-	+	+

'+' indicates presence and '–' indicates absence.

4.8. Screening for Virulence/ Virulence Associated Genes in *V. vulnificus*

The *V. vulnificus* strains were screened for the presence of 13 virulence/virulence associated genes like *ctx*A, *ctx*B, *ace*, VPI, *hly*A,

*omp*U, *rtx*A, *tox*R, *zot*, *nag*st, *tcp*A, *nin* and *nan*H together with three species specific virulence associated genes, *cps* (Capsular Polysaccharide), *vvh* (Haemolysin) and *viu* (Iron Aqusition).

4.8.1. Detection of Vibrio Pathogenicity Island (VPI)

The presence of the Vibrio Pathogenicity Island (VPI) was detected by PCR amplification of the gene in 6 strains of *V. vulnificus*, which yielded an amplicon of size ~680 bp (Fig.4.22). The amplicon was detected in strains BTAS3 and BTPS6 from aquafarms and BTMM2, BTOS7, BTVE4 and BTVE9 from mangrove environments.

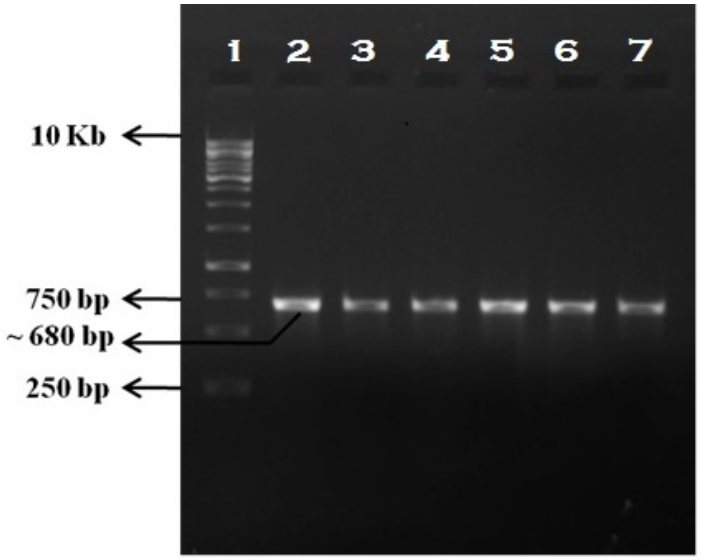


Fig.4.22. Detection of VPI in strains of *V. vulnificus* isolated from marine environments.
Lane 1 – 1 Kb ladder ; Lane 2- BTAS3; Lane 3- BTPS6; Lane 4-BTMM2; Lane 5-BTOS7; Lane 6-BTVE4; Lane 7-BTVE9

4.8.2. Detection of *tox*R

The PCR screening for the presence of the *tox*R gene was by using a set of specific primers yielding ~779 bp amplicon. Three strains tested

positive for *tox*R gene, namely, BTAS3 and BTPS6 from aquafarms and BTOS7 isolated from mangroves (Fig. 4.23).

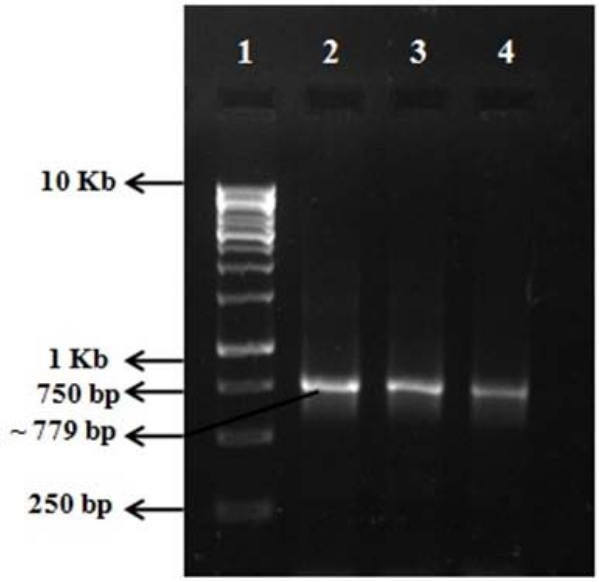


Fig.4.23. Detection of *tox*R gene in strains of *V. vulnificus* isolated from marine environments.
Lane 1- 1 Kb ladder; Lane 2-BTAS3; Lane 3-BTPS6; Lane 4- BTOS7

4.8.3. Detection of *cps*

In *V. vulnificus*, the product of the capsular polysaccharide gene (*cps*) is the most important virulence factor, helping to avoid phagocytosis by host defence cells and complement-mediated killing by the host immune system. The presence of this gene was indicated by the ~342 bp amplicon produced by a set of primers targeting the specific site on the genomic DNA. Three strains tested positive for *cps* gene, namely, BTPS6 from aquafarm and strains BTVE4 and BTVE8 from mangrove environments (Fig.4.24).

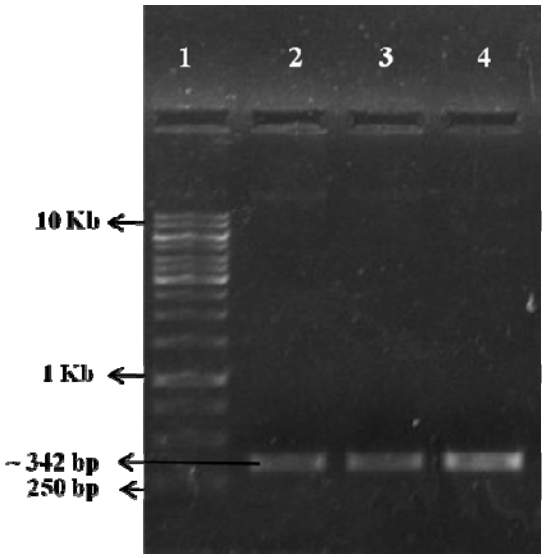


Fig.4.24. Detection of *cps* gene in strains of *V. vulnificus* isolated from marine environments.
Lane 1-1Kb ladder; Lane 2- BTPS6; Lane 3-BTVE4; Lane 4-BTVE8

4.8.4. Detection of *ctx*A, *ctx*B, *ace*, *zot*, *nag*st, *tcp*A, *nin*, *nan*H, *vvu* and *viu*

Gene specific primers were used for the PCR detection of other virulence/virulence associated genes like *ctx*A, *ctx*B, *ace*, *zot*, *nag*st, *tcp*A, *nin* and *nan*H and *V. vulnificus* specific genes like *vvu* and *viu* in the 18 environmental isolates of *V. vulnificus*, but the specific amplicons were not produced, indicating the absence of these genes in the tested isolates.

4.8.5. Distribution of virulence genes among *V. vulnificus* strains

The virulence/virulence associated genes detected in the environmental isolates of *V. vulnificus* were VPI, *tox*R and *cps* genes. The pathogenicity island was detected in 33% and the *tox*R and *cps* genes in 17% each of the tested isolates. The virulence genes screened were not detected in 33% of the isolates (Fig 4.25).

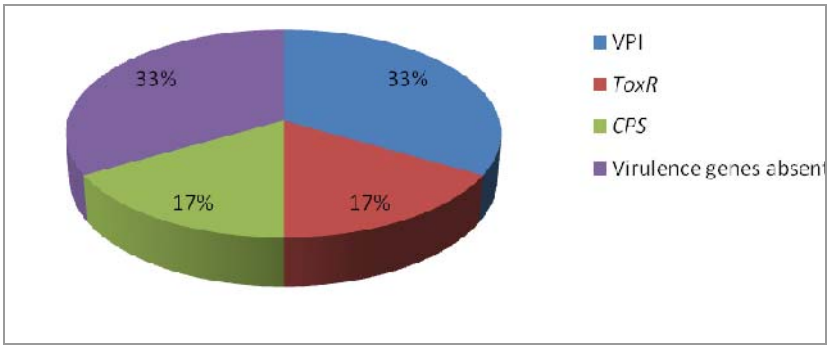


Fig.4.25. Distribution of tested virulence genes among *V. vulnificus* isolates from marine environments

4.8.6. Virulence profile of *V. vulnificus* isolates

Of the 18 environmental strains of *V.vulnificus* screened for the presence of virulence genes, only 7 strains produced positive results (Table 4.10). Strains BTPS6 isolated from aquafarms and BTVE4 from mangroves tested positive for 3 virulence genes. Strain BTAS3 from aquafarm hosted 2 virulence genes. Strains BTMM2, BTOS7, BTVE8 and BTVE9 tested positive for a single gene in the screening process.

Table 4.10. Virulence profile of *V. vulnificus* isolates from marine environments.

Strain	VPI	*toxR*	*cps*
BTAS3	+	+	-
BTPS6	+	+	+
BTMM2	+	-	-
BTOS7	+	+	-
BTVE4	+	-	+
BTVE8	-	-	+
BTVE9	+	-	-

'+' indicates presence and '–'indicates absence.

4.9. Genetic Analysis of Toxin-regulatory Gene *tox*R

4.9.1. Genetic analysis and comparison of Toxin-regulatory gene *tox*R in environmental isolates of *V. cholerae*

PCR amplification of the *tox*R gene of the environmental isolates of *V. cholerae* using the forward primer (*tox*R F) and reverse primer (*tox*R R)

(Section 3.7.1.8) generated the expected amplicon of approximately 779 bp in nine strains (Section 4.7.5). These amplicons were cloned and sequenced, to investigate the genetic variation in the *tox*R gene and to determine the evolutionary genetic relationships among the environmental isolates of *V. cholerae***.** The GenBank accession numbers and the closest identities after nucleotide BLAST of *tox*R genes are as detailed in Table.4.11.

Table 4.11. Nucleotide blast results and GenBank accession numbers obtained for the toxin-regulatory gene *tox*R of environmental isolates of *V. cholerae*

Sl. No.	Strain No.	Gene sequence to which submitted sequence exhibited maximum similarity after nucleotide blast	GenBank accession number
1	*V.cholerae* strain BTMA1	*Vibrio cholerae* strain 310/08 ToxR (*toxR*) gene, partial cds (HM042641.1) 99% identity	KF420399
2	*V.cholerae* strain BTMA5	*Vibrio cholerae* strain 933/08 ToxR gene, partial cds (HQ452872.1)- 99% identity	KF420400
3	*V.cholerae* strain BTMA9	Synthetic construct *Vibrio cholerae* clone FLH198266.01F *toxR* gene, complete sequence (DQ774024.1)- 99% identity	KF420401
4	*V.cholerae* strain BTMS4	*V.cholerae toxR* gene encoding a transcription-activating transmembrane DNA-binding protein, complete cds (M21249.1)- 98% identity	KF420402
5	*V.cholerae* strain BTOK7	*Vibrio cholerae* strain 82/08 ToxR (*toxR*) gene, partial cds(HM042637.1)- 99% identity	KF420404
6	*V.cholerae* strain BTOK9	*Vibrio cholerae* strain 35/04 ToxR (*toxR*) gene, partial cds(HM042640.1)- 99% identity	KF420405
7	*V.cholerae* strain BTOK11	*Vibrio cholerae* strain GB/08 ToxR (*toxR*) gene, partial cds(HQ452873.1)- 99% identity	KF420403
8	*V.cholerae* strain BTPR5	*Vibrio cholerae* strain 35/04 ToxR *(toxR*) gene, partial cds(HM042640.1)- 98% identity	KF420406
9	*V.cholerae* strain BTVE2	*V.cholerae toxR* gene encoding a transcription-activating transmembrane DNA-binding protein, complete cds (M21249.1) - 98% identity	KF420407

Multiple sequence alignment using ClustalW (Fig 4.26) revealed that all the 9 environmental strains included in this study showed 99 % similarity in their nucleotide sequences. Close comparison of the *tox*R gene of the environmental isolates revealed 100% sequence similarity to the *tox*R gene of the near relative, *V. cholerae* (M21249), except that of strain BTVE2 which exhibited 98% similarity. Sequences of strains BTMA9, BTOK7, BTOK9, BTOK11, BTPR5 and BTMA5 displayed 100%, whereas, BTMA1 and BTMS4 exhibited 86% and strain BTVE2 showed 75 % similarity in nucleotide sequences to the *tox*R gene sequence of *V. harveyi* (DQ503438). When compared to the gene sequence of *V. parahaemolyticus* (L11929), strains BTMA9 and BTOK11 shared 87%, BTOK7, BTOK9, BTPR5 and BTMA5 showed 85% and BTMA1 and BTMS4 displayed 83% sequence similarity. Strain BTVE2 recorded the lowest homology (75%) among the strains. All the strains displayed sequence resemblance of 82 % to the gene sequence of *V. vulnificus* (AF170883) except BTPR5 and BTMA5 with 81% identity.

```
BTMA1                TGAATGTAATTCAGCGTTAACTGGTTATTTTGTCCACCTGTGGCAATGACTTCTATCGGC 83
BTMS4                TGAATGTAATTCAGCGTTAACTGGTTATTTTGTCCACCTGTGGCAATGACTTCTATCGGC 86
BTVE2                TGAATGTAATTCAACGTTAACTGGTTATTTTGTCCACCCGTGGCAATCACTTCTATCGGC 101
BTMA9                TGAATGTAATTCAACGTTAACTGGTTATTTTGTCCACCCGTGGCAATCACTTCTATCGGC 110
BTOK7                TGAATGTAATTCAACGTTAACTGGTTATTTTGTCCACCCGTGGCAATGACTTCTATCGGC 122
BTOK11               TGAATGTAATTCAACGTTAACTGGTTATTTTGTCCACCCGTGGCAATCACTTCTATCGGC 97
BTOK9                TGAATGTAATTCAACGTTAACTGGTTATTTTGTCCACCCGTGGCAATGACTTCTATCGGC 105
BTPR5                TGAATGTAATTCAACGTTAACTGGTTATTTTGTCCACCCGTGGCAATGACTTCTATCGGC 98
BTMA5                TGAATGTAATTCAGCGTTAACTGGTTATTTTGTCCACCCGTGGCAATCACTTCTATCGGC 99
V.harveyi            TGATTGACAAAGAAGATAGTGAAGAGATCATTAGATTAGGCAGCAATGAAAGCCGAATTC 128
V.cholera            TGATTGACAAAGAAGATAGTGAAGAGATCATTCGATTAGGCAGCAACGAAAGCCGAATTC 360
V.parahemolyticus    TCGCTGACCAACAAAGCGGCAACGAAGTTGTACGATTAGGAAGCAACGAAAGCCGTATAC 274
V.vulfinus           ------------------------------------------------------------

BTMA1                TTGAGCCCACCAGTATGCTTTTCATTGTATTTTTTAACGCACAGTTCGATTGACGGTAGC 143
BTMS4                TTGAGCCCACCAGTATGCTTTTCATTGTATTTTTTAACGCACAGTTCGATTGACGGTAGC 146
BTVE2                TTGAGCCCACCAGTATGCTTTTCATTGTATTTTTTAACGCACAGTTCGATTGACGGTAGC 161
BTMA9                TTGAGCCCACCAGTATGCTTTTCATTGTATTTTTTAACGCACAGTTCGATTGACGGTAGC 170
BTOK7                TTGAGCCCACCAGTATGCTTTTCATTGTATTTTTTAACGCACAGTTCGATTGACGGTAGC 182
BTOK11               TTGAGCCCACCAGTATGCTTTTCATTGTATTTTTTAACGCACAGTTCGATTGACGGTAGC 157
BTOK9                TTGAGCCCACCAGTATGCTTTTCATTGTATTTTTTAACGCACAGTTCGATTGACGGTAGC 165
BTPR5                TTGAGCCCACCAGTATGCTTTTCATTGTATTTCTTAACGCACAGTTCGATTGACGGTAGC 158
BTMA5                TTGAGCCCACCAGTATGCTTTTCATTGTATTTTTTAACGCACAGTTCGATTGACGGTAGC 159
V.harveyi            TT-TGGCTGCTGGCCCAACGTCCAAACGAGGTGATTTCTCGCAATGATTTGCATG-ACTT 186
V.cholera            TT-TGGCTGCTGGCCCAACGTCCAAACGAGGTAATTTCTCGCAATGATTTGCATG-ACTT 418
V.parahemolyticus    TC-CTGATGTTGGCGGAGAGACCAAACGAAGTTTTAACCCGTAACGAGCTTCACG-AGTT 332
V.vulfinus           ------------------------------------------------------------

BTMA1                CAGTTTGAAAGATCAGGGTGGTTATTCGGCATATTGACGGCT-ACGCCATCGACAACCGT 202
BTMS4                CAGTTTGAAAGATCAGGGTGGTTATTCGGCATATTGACGGCT-ACGCCATCGACAACCGT 205
BTVE2                CAGTTTGAAAGATCAGGGTGGTTATTCGGCATATTGACGGCT-ACGCCATCGACAACCGT 220
BTMA9                CAGTTTGAAAGATCAGGGTGGTTATTCGGCATATTGACGGCT-ACGCCATCGACAACCGT 229
BTOK7                CAATTTGAAAGATCAGGGTGGTTATTCGGCATATTGACGGCT-ACGCCATCGACAACCGT 241
BTOK11               CAATTTGAAAGATCAGGGTGGTTATTCGGCATATTGACGGCT-ACGCCATCGACAACCGT 216
BTOK9                CAATTTGAAAGATCAGGGTGGTTATTCGGCATATTGACGGCT-ACGCCATCGACAACCGT 224
BTPR5                CAATTTGAAAGATCAGGGTGGTTATTCGGCATATTGACGGCT-ACGCCATCGACAACCGT 217
BTMA5                CAATTTGAAAGATCAGGGTGGTTATTCGGCATATTGACGGCT-ACGCCATCGACAACCAT 218
V.harveyi            TGTTTGGCGAGAGCAAGGTTTTGAAGTCGATGATTCCAGCTTAACCCAAGCCATTTCGAC 246
V.cholera            TGTTTGGCGAGAGCAAGGTTTTGAAGTCGATGATTCCAGCTTAACCCAAGCCATTTCGAC 478
V.parahemolyticus    TGTTTGGCGTGAGCAAGGTTTTGAGGTGGATGACTCAAGCCTGACTCAAGCGATTTCTAC 392
V.vulfinus           ----------GAGCAGGGGTTTGAGGTGGATGATTCCAGCCTAACTCAAGCGATTTCTAC 50
                               ** **.**   * *    *.: * * ..*  * ** *.* * * ::* .

BTMA1                TAGGGGTTTAAAGCTGGTTTGGCTCGGGTTAGTGAGCAGTAATACTGCGAGGGGAAGTAA 262
BTMS4                TAGGGGTTTAAAGCTGGTTTGGCTCGGGTTAGTGAGCAGTAATACTGCGAGGGGAAGTAA 265
BTVE2                TAGGGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGCAGTAATACTGCGAGGGGAAGTAA 280
BTMA9                TAGGGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGCAGTAATACTGCGAGGGGAAGTAA 289
BTOK7                TAGGGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGCAGTAATACTGCGAGGGGAAGTAA 301
BTOK11               TAGGGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGCAGTAATACTGCGAGGGGAAGTAA 276
BTOK9                TAGGGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGCAGTAATACTGCGAGGGGAAGTAA 284
BTPR5                TAGGGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGCAGTAATACTGCGAGGGGAAGTAA 277
BTMA5                TAGTGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGCAGTAATACTGCGAGGGGAAGTAA 278
V.harveyi            TCTGCGCAAAATGCT-CAAAGATTCGACAAAGTCCCCACAATACGTCAAAACGGTTCCGA 305
V.cholera            TCTGCGCAAAATGCT-CAAAGATTCGACAAAGTCCCCACAATACGTCAAAACGGTTCCGA 537
V.parahemolyticus    TCTGCGTAAGATGTT-GAAGGATTCAACCAAATCTCCAGAGTTTGTTAAAACCGTTCCAA 451
V.vulfinus           CTTACGCAAAATGCT-CAAAGACTCTACAAAGTCCCCTGAGTTTGTGAAAACGGTTCCAA 109
                          * ::.*:* *  :: *. ** .  :*.*   *: :.::  * ..*.  *::  .*
```

```
BTMA1                GAC--CGCTATCAGAATAAGCAGTCGATTCCCCAAGTTTGGAGCCGATTTATTCGTCACA 320
BTMS4                GAC--CGCTATCAGAATAAGCAGTCGATTCCCCAAGTTTGGAGCCGATTTATTCGTCACA 323
BTVE2                GAC--CGCTATCAGAATAAGCAGTCGATTCCCCAAGTTTGGAGCCGATTTATTCGCCACG 338
BTMA9                GAC--CGCTATCAGAATAAGCAGTCGATTCCCCAAGTTTGGAGCCGATTTATTCGCCACG 347
BTOK7                GAG--CGCTATCAGAATAAGCAGTCGATTCCCCAAGTTTGGAGCCGATTTATTCGCCACG 359
BTOK11               GAG--CGCTATCAGAATAAGTAGTCGATTCCCCAAGTTTGGAGCCGATTTATTCGCCACG 334
BTOK9                GAG--CGCTATCAGAATAAGCAGTCGATTCCCCAAGTTTGGAGCCGATTTATTCGCCACG 342
BTPR5                GAG--CGCTATCAGAATAAGCAGTCGATTCCCCAAGTTTGGAGCCGATTTATTCGCCACG 335
BTMA5                GAC--CGCTATCAGAATAAGCAGTCGATTCCCCAAGTTTGGAGCCGATTTATTCGCCACG 336
V.harveyi            AACGCGGTTACCAATTGATCG---------CCCGAGTGG--AAACGGTTGAAGA--AGAG 352
V.cholera            AGCGCGGTTACCAATTGATCG---------CCCGAGTGG--AAACGGTTGAAGA--AGAG 584
V.parahemolyticus    AACGAGGCTATCAACTCATTTGTACTGTTGAACGCCTAA--GCCCGCTTTCTTC--AGAC 507
V.vulfinus           AACGTGGTTATCAGTTGATCTGTTCGGTTGAGCGCATTA--ACCCGCTCCTGTC--AGAT 165
                     ..    * ** **. : *:           . *.. *    . .** *     .  ...

BTMA1                ACATTGGCTGGCTGCGGTGTGTTCACTACAGTGGCTGATGAAGGCACACTG-CTTGACTC 379
BTMS4                ACATTGGCTGGCTGCGGTGTGTTCACTACAGTGGCTGATGAAGGCACACTG-CTTGACTC 382
BTVE2                ACATTGGCTGGCTGCGGTGTGTTCACTACAGTGGCTGATGAAGGCACTCTG-CTTGATTC 397
BTMA9                ACATTGGCTGGCTGCGGTGTGTTCACTACAGTGGCTGATGAAGGCACTCTG-CTTGATTC 406
BTOK7                ACATTGGCTGGCTGCGGTGTGTTCACTACAGTGGCTGATGAAGGCACACTG-CTTGACTC 418
BTOK11               ACATTGGCTGGCTGCGGTGTGTTCACTACAGTGGCTGATGAAGGCACACTG-CTTGACTC 393
BTOK9                ACATTGGCTGGCTGCGGTGTGTTCACTACAGTGGCTGATGAAGGCACACTG-CTTGACTC 401
BTPR5                ACATTGGCTGGCTGCGGTGTGTTCACTACAGTGGCTGATGAAGGCACACTG-CTTGACTC 394
BTMA5                ACATTGGCTGGCTGCGGTGTGTTCACTACAGTGGCTGATGAAGGCACACTG-CTTGACTC 395
V.harveyi            ATGG------CTCGCG------AAAGCGAAGCTGCTCATGACATCTCTCAG-CCAGAATC 399
V.cholera            ATGG------CTCGCG------AAAACGAAGCTGCTCATGACATCTCTCAG-CCAGAATC 631
V.parahemolyticus    TCAAGCTCAATTGAAG------TTGAAGAACCTGCTTCTGATAACAATGACGCCTCTGCT 561
V.vulfinus           TCAA---CCAACAACG------TGAATGACGCAGCTTCTGAAGCATTAGAT--CAAGAAG 214
                     : .          ..*      : .  ...   *** .*** . .: : :    :
```

```
BTMA1                TGCGTATTCATTGACAGATTCTGGCTG--AGAGATGTCATGAGCAGCTTCGCTTTCGCGA 437
BTMS4                TGCGTATTCATTGACAGATTCTGGCTG--AGAGATGTCATGAGCAGCTTCGCTTTCGCGA 440
BTVE2                TGCGTATTCATTGACAGATTCTGGCTG--AGAGATGTCATGAGCAGCTTCGCTTTCGCGA 455
BTMA9                TGCGTATTCATTGACAGATTCTGGCTG--AGAGATGTCATGAGCAGCTTCGCTTTCGCGA 464
BTOK7                TGCGTATTCATTGACAGATTCCGGTTG--AGAGATGTCATGAGCAGCTTCGCTTTCGCGA 476
BTOK11               TGCGTATTCATTGACAGATTCCGGTTG--AGAGATGTCATGAGCAGCTTCGCTTTCGCGA 451
BTOK9                TGCGTATTCATTGACAGATTCCGGTTG--AGAGATGTCATGAGCAGCTTCGCTTTCGCGA 459
BTPR5                TGCGTATTCATTGACAGATTCCGGTTG--AGAGATGTCATGAGCAGCTTCGCTTTCGCGA 452
BTMA5                TACGTATTCATTGACAGATTCCGGTTG--AGAGATGTCATGAGCAGCTTCGTTTTCGCGA 453
V.harveyi            TGTCAATGAATACGCAGAGTCAAGCAG-----TGTGCCTTCATCAGC--CACTGTAG-TG 451
V.cholera            TGTCAATGAATACGCAGAATCAAGCAG-----TGTGCCTTCATCAGC--CACTGTAG-TG 683
V.parahemolyticus    AATGAGGTAGAAACGATCGTAGAGCCGTCTTTAGCGACGTCTTCTGA--CGCAATCGTTG 619
V.vulfinus           AATTAGAAAACGAAATCAGTACTGACG-CGGTTCAAACATCCTCGTC--AGAAATTGGTA 271
                     :.  :.  ..     : . *.  *  *        . * *   *  .  .. : * *  .

BTMA1                GCCATCTCTTCTTCAACCGTTTCCACTCGGGCGATCAATTGGTAACCGCGTTTCGGAACC 497
BTMS4                GCCATCTCTTCTTCAACCGTTTCCACTCGGGCGATCAATTGGTAACCGCGTTTCGGAACC 500
BTVE2                GCCATCTCTTCTTCAACCGTTTCCACTCGGGCGATCAATTGGTAACCGCGTTTCGGAACC 515
BTMA9                GCCATCTCTTCTTCAACCGTTTCCACTCGGGCGATCAATTGGTAACCGCGTTTCGGAACC 524
BTOK7                GCCATCTCTTCTTCAACCGTTTCCACTCGGGCGATCAATTGGTAACCGCGTTTCGGAACC 536
BTOK11               GCCATCTCTTCTTCAACCGTTTCCACTCGGGCGATCAATTGGTAACCGCGTTTCGGAACC 511
BTOK9                GCCATCTCTTCTTCAACCGTTTCCACTCGGGCGATCAATTGGTAACCGCGTTTCGGAACC 519
BTPR5                GCCATCTCTTCTTCAACCGTTTCCACTCGGGCGATCAATTGGTAACCACGTTTCGGAACC 512
BTMA5                GCCATCTCTTCTTCAACCGTTTCCACTCGGGCGATCAATTGGTAACCGCGTTTCGGAACC 513
V.harveyi            AACACACCGCAGCCAGCCAATGTCGTGGCGAA--TAAATCGGCTCC--------AAACTT 501
V.cholera            AACACACCGCAGCCAGCCAATGTCGTGGCGAA--TAAATCGGCTCC--------AAACTT 733
V.parahemolyticus    AACCAGAAGCG-CCAGTAGTACCTGAAAAAGC--ACATGTGGCTTCTGCTGTGAATCCTT 676
V.vulfinus           GAGATGTCGCG-CATAATGCTGGCACGTCAAC--AAAGATGGCCGCGTCGCAAAAAAATT 328
                     .. .   .     .:.  . :   .    ...  :.*   **   *        . ..
```

```
BTMA1                 G-TTTTGACGTATTGTGGGGACTTTGTCGAATCTTTGAGCATTTTGCGCAGAGTCGAAAT 556
BTMS4                 G-TTTTGACGTATTGTGGGGACTTTGTCGAATCTTTGAGCATTTTGCGCAGAGTCGAAAT 559
BTVE2                 G-TTTTGACGTATTGTGGGGACTTTGTCGAATCTTTGAGCATTTTGCGCAGAGTCGAAAT 574
BTMA9                 G-TTTTGACGTATTGTGGGGACTTTGTCGAATCTTTGAGCATTTTGCGCAGAGTCGAAAT 583
BTOK7                 G-TTTTGACGTATTGTGGGGACTTTGTCGAATCTTTGAGCATTTTGCGCAGAGTCGAAAT 595
BTOK11                G-TTTTGACGTATTGTGGGGACTTTGTCGAATCTTTGAGCATTTTGCGCAGAGTCGAAAT 570
BTOK9                 G-TTTTGACGTATTGTGGGGACTTTGTCGAATCTTTGAGCATTTTGCGCAGAGTCGAAAT 578
BTPR5                 GTTTTTGACGTATTGTGGGGACTTTGTCGAATCTTTGAGCATTTTGCGCAGAGTCGAAAT 572
BTMA5                 G-TTTTGACGTATTGTGGGGACTTTGTCGAATCTTTGAGCATTTTGCGCAGAGTCGAAAT 572
V.harveyi             G-----GGGAATCGACTGCTTATTCTG-ATAGCGGTCTTACTTCCCCTCGCAGTATTACT 555
V.cholera             G-----GGGAATCGACTGTTTATTCTG-ATAGCGGTCTTACTTCCCCTCGCAGTATTACT 787
V.parahemolyticus     G-----GATTCCACGCGTTATTTTATTTTTGGCACTATTACTACCGATTTGCGTACTGCT 731
V.vulfinus            G-----GCTAATTAAAGGGCTATTCTTGTTAGCCGCACTGCTGCCACTCTGCGTTGTCTT 383
                      *     *       .    : **    :. *       .*    .    .**  :  *

BTMA1                 GGCTTGGGTTAAGCTGGAATCATCGACTTCAAAACCTTGCTCTCGCCAAACAAAGTCATG 616
BTMS4                 GGCTTGGGTTAAGCTGGAATCATCGACTTCAAAACCTTGCTCTCGCCAAACAAAGTCATG 619
BTVE2                 GGCTTGGGTTAAGCTGGAATCATCGACTTCAAAACCTTGCTCTCGCCAAACAAAGTCATG 634
BTMA9                 GGCTTGGGTTAAGCTGGAATCATCGACTTCAAAACCTTGCTCTCGCCAAACAAAGTCATG 643
BTOK7                 GGCTTGGGTTAAGCTGGAATCATCGACTTCAAAACCTTGCTCTCGCCAAACAAAGTCATG 655
BTOK11                GGCTTGGGTTAAGCTGGAATCATCGACTTCAAAACCTTGCTCTCGCCAAACAAAGTCATG 630
BTOK9                 GGCTTGGGTTAAGCTGGAATCATCGACTTCAAAACCTTGCTCTCGCCAAACAAAGTCATG 638
BTPR5                 TGCCTGGGTTAAGCTGAAATCATCGACTTCAAAACCTTGCTCTCGCCAAACAAAGTCATG 632
BTMA5                 GGCTTGGGTTAAGC---------------------------------------------- 586
V.harveyi             GCTCACTAACCCGAGCCAATCCAGCTTTAAACC-CCTAACGGTTGTCGAT-GGCGTAGCC 613
V.cholera             GCTCACTAACCCAAGCCAATCCAGCTTTAAACC-CCTAACGGTTGTCGAT-GGCGTAGCC 845
V.parahemolyticus     GTTTACAAACCCTGCGGAATCTCAGTTCCGTCAGATTGGTGAGTATCAG--AACGTACCA 789
V.vulfinus            ACTCACCAATCCGTCGGAGTCTAAGTTCCGTTT-ACTGGAAAATGTCAAT-GGTGTGGAA 441
                          :  .: ..
```

Fig.4.26. Multiple sequence alignment of *toxR* nucleotide sequences from thirteen *Vibrio* species using the ClustalW program.

The partial *toxR* nucleotide sequences of the environmental isolates of *V.cholerae* are represented as BTMA1, BTMS4, BTVE2, BTMA9, BTOK7, BTOK11, BTOK9, BTPR5 and BTMA5. Other *toxR* gene sequences of *Vibrio* species used in the alignment are represented with GenBank accession numbers in parenthesis: *V. harveyi* (DQ503438), *V. cholerae* (M21249),*V. parahaemolyticus* (L11929) and *V. vulnificus* (AF170883). Consensus regions are denoted by an asterisk.

4.9.1.1. Phylogenetic analysis of *V. cholerae* strains based on the *toxR* gene sequences

Unrooted trees were constructed by the neighbor-joining method to study the phylogenetic interrelationships of environmental *V. cholerae* isolates with related *Vibrio* sp. based on *tox*R gene nucleotide sequences. The phylogenetic tree (Fig.4.27) clustered the nine environmental isolates of *Vibrio* into a core group divided into three clusters. The first cluster comprised four isolates BTMA5, BTOK7, BTOK9, BTOK11 from aquafarms,

and one BTPR5 from seafood (Prawn). The second cluster included isolates BTMA1 from aquafarm and BTMS4 from mangroves, while the third cluster grouped BTMA9 from aquafarm and BTVE2 from mangroves. *V. cholerae* (M21249) and *V. harveyi* (DQ503438) grouped into a separate cluster supported by a bootstrap value of 100%. The same goes with *V. vulnificus* (AF170883) *and V. parahaemolyticus* (L11929) forming more distinct, separate clusters represented by relatively lower boostrap value (59%).

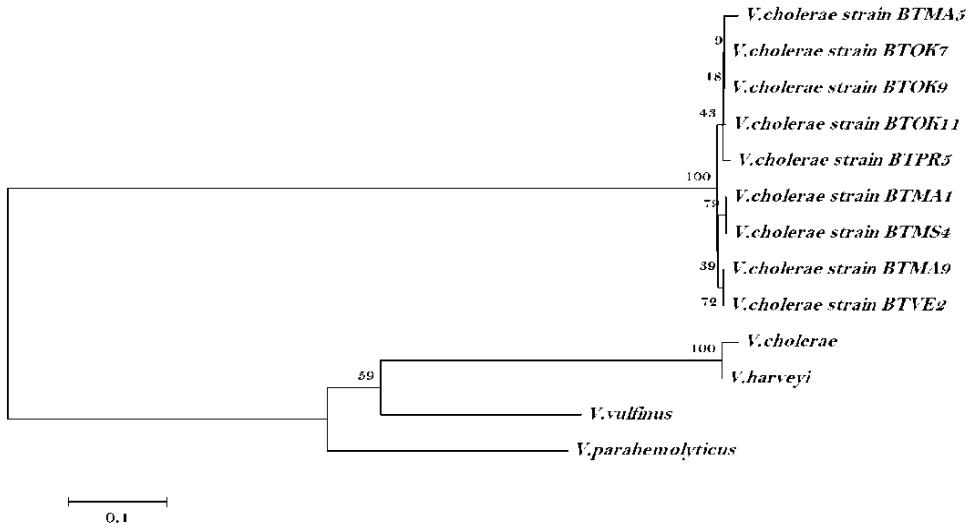


Fig.4.27. Unrooted tree constructed by the neighbor-joining method showing the phylogenetic interrelationships of *V. cholerae* strains with related *Vibrio* sp. based on *toxR* gene nucleotide sequences.

Bootstrap values are given at the branching points and bars show sequence divergence.

4.9.1.2. Multiple sequence alignment of the ToxR amino acid sequences of environmental isolates of *V. cholerae*

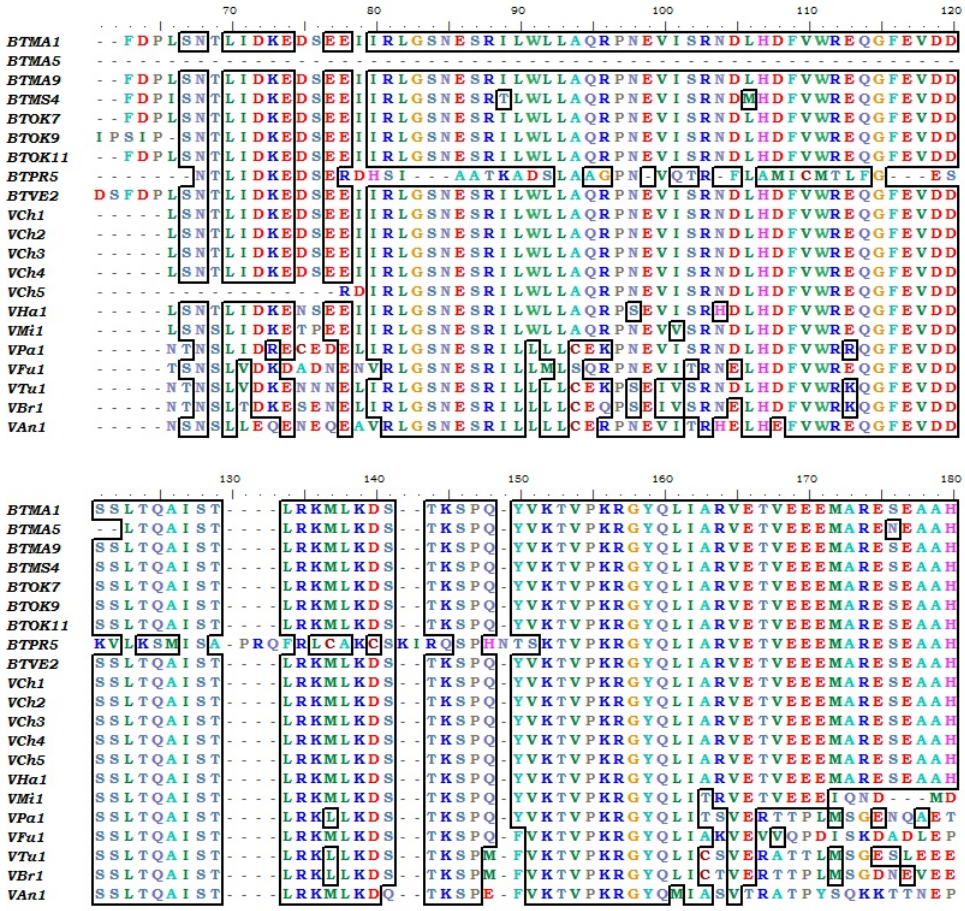
The nucleic acid sequence of the environmental isolates of *V. cholerae* was translated *in-silico* into its corresponding protein sequence by Expasy (http://web.expasy.org/translate) and the deduced amino acid sequences were compared with those available from GenBank using online BLAST tool- blastx (http://www.ncbi.nlm.nih.gov/blast). The GenBank accession

numbers and the closest identities after protein blast of deduced amino acid sequences of *tox*R genes of *V. cholerae* strains are as detailed in Table.4.12.

Table 4.12. Protein blast results and GenBank accession numbers of deduced aminoacid sequences of *toxR* gene of *V. cholerae* strains from marine environments

Sl No	Strain no	Aminoacid sequence to which submitted deduced aminoacid sequence exhibited maximum similarity after protein blast	GenBank accession number of *tox*R gene of *V. cholerae* strains
1	*V.cholerae* strain BTMA1	Transcriptional regulator toxR *V. cholerae* (WP000018134.1) – 99% identity	KF420399
2	*V.cholerae* strain BTMA5	ToxR *Vibrio cholerae* (AED88311.1) – 99% identity	KF420400
3	*V.cholerae* strain BTMA9	Cholera toxin homolog transcriptional activator (*Vibrio cholerae*)(WP000018138.1)- 99% identity	KF420401
4	*V.cholerae* strain BTMS4	Cholera toxin homolog transcriptional activator (*Vibrio cholerae*) (WP000394385.1) - 98% identity	KF420402
5	*V.cholerae* strain BTOK7	Transcriptional regulator (*Vibrio cholerae*)(WP 000018133.1) - 96% identity	KF420404
6	*V.cholerae* strain BTOK9	Transcriptional regulator (*Vibrio cholerae*)(WP 000018133.1) - 96% identity	KF420405
7	*V.cholerae* strain BTOK11	Transcriptional regulator (*Vibrio cholerae*)(WP 000018133.1) - 99% identity	KF420403
8	*V.cholerae* strain BTPR5	ToxR (*Vibrio cholerae*) (ADG44920.1) – 100% identity	KF420406
9	*V.cholerae* strain BTVE2	Transcriptional regulator (*Vibrio cholerae*)(WP001893768.1) – 99% identity	KF420407

Multiple sequence alignment of the deduced ToxR amino acid sequence of the environmental strains of *V. cholerae* with ToxR of other *Vibrio* species obtained from the GenBank database revealed a high degree of sequence similarity within strains of the same species and also the ToxR amino acid sequences of related species like *V. harveyi, V. parahaemolyticus, V. mimicus, V. furnissii, V. tubaishii, V. brasiliensis* and *V. alginolyticus* (Fig. 4.28).The sequences of strain BTPR5 exhibited variation in the amino acid sequences up to the 150th amino acid and thereafter showed 100% sequence homology to other related strains.



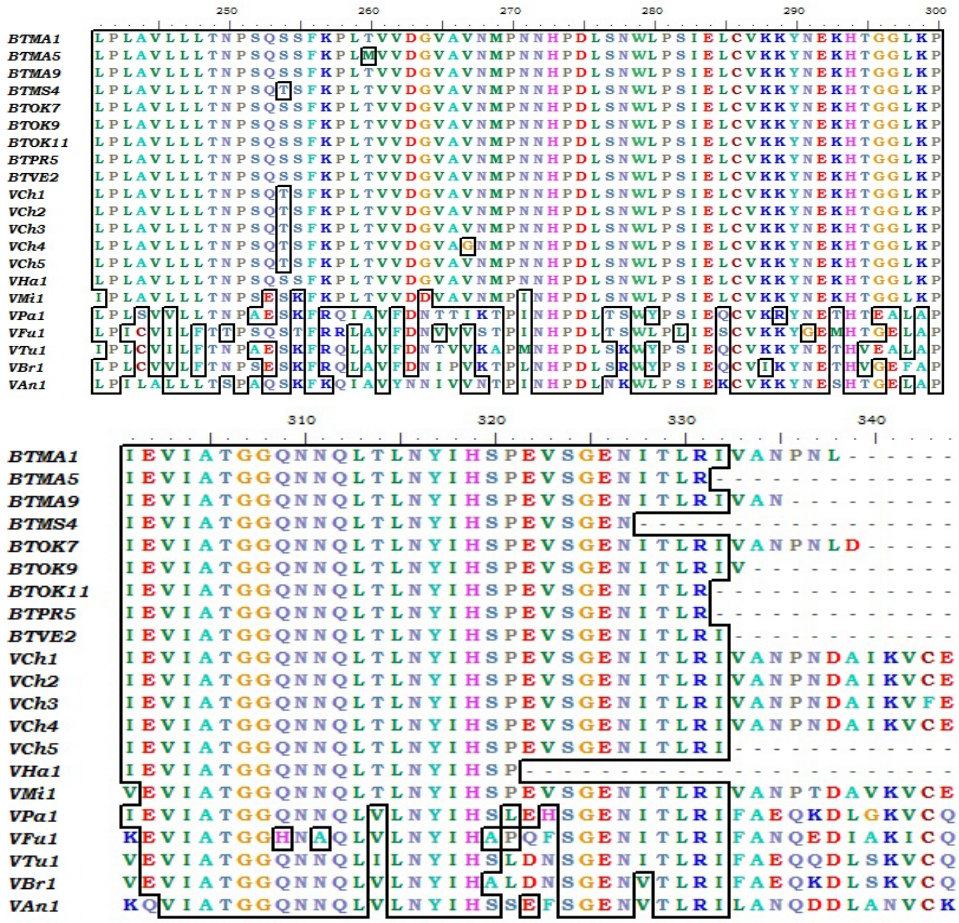


Fig.4.28. Multiple sequence alignment of deduced ToxR amino acid sequences of environmental isolates of *V. cholerae* with ToxR amino acid sequences of related *Vibrio* sp.

The amino acid sequences of the environmental isolates of *V.cholerae* are represented as BTMA1, BTMS4, BTVE2, BTMA9, BTOK7, BTOK11, BTOK9, BTPR5 and BTMA5. Other *toxR* amino acid sequences of *Vibrio* species used in the alignment are represented with GenBank accession numbers in parenthesis:VCh1- *V.cholerae* (WP000018138), VCh2- *V.cholerae* (WP000394385), VCh3- *V.cholerae* (WP002039486), VCh4- *V.cholerae* (WP002028070), VCh5- *V.cholerae* (ADG44921), VHa1- *V.harveyi* (ADA54890), VMi1- *V.mimicus* (ABS42980), VPa1- *V.parahaemolyticus* (WP 005474711), VFu1- *V.furnissii* (WP 004726677), VTu1- *V.tubiashii* (WP 004748979), VBr1- *V.brasiliensis* (WP 006881610) and VAn1- *V. alginolyticus* (WP 005398060).

4.9.1.3. Phylogenetic analysis of environmental isolates of *V. cholerae* based on the ToxR amino acid sequences

The unrooted tree constructed by the neighbor-joining method revealed the similarity of deduced amino acid sequences of ToxR in the environmental strains of *V. cholerae* obtained in the present study (Fig.4. 29). The amino acid sequence of *tox*R genes of all the environmental strains together formed a major clade including other *V.cholerae* strains and *V.harveyi* strain. They showed divergence from the amino acid sequence of ToxR of other *Vibrio* sp. used for tree construction.

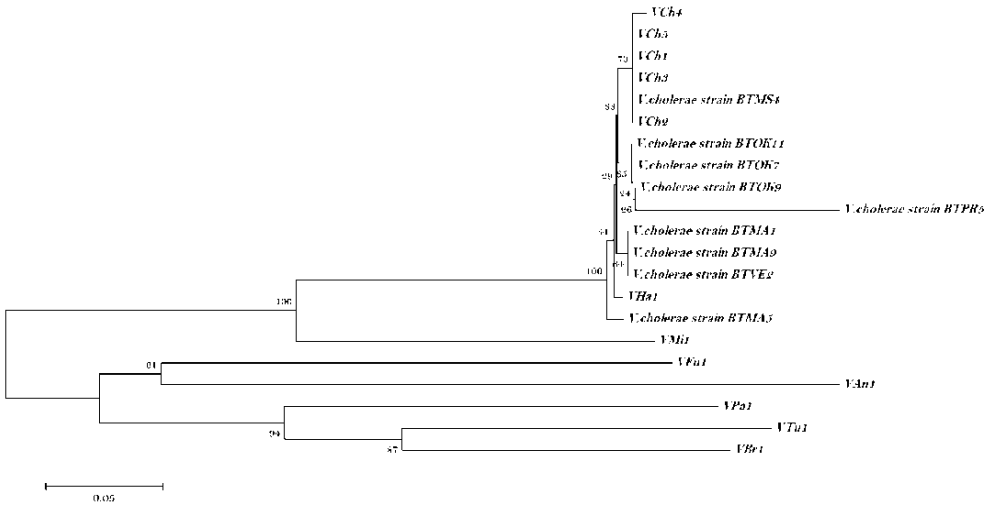


Fig.4.29. Unrooted trees constructed by the neighbor-joining method showing the phylogenetic interrelationships of environmental *V. cholerae* strains with related *Vibrio* sp. based on ToxR amino acid sequences.

Bootstrap values are given at the branching points and bars show sequence divergence. Environmental isolates of *V. cholerae* strains are represented as BTMA1, BTMA5, BTMA9, BTMS4, BTOK7, BTOK9, BTOK11, BTPR5 and BTVE2. Other strains used for tree construction, with their accession numbers in parenthesis, are VCh1- *V.cholerae* (WP000018138), VCh2- *V.cholerae* (WP000394385), VCh3- *V.cholerae* (WP002039486), VCh4- *V.cholerae* (WP002028070), VCh5- *V.cholerae* (ADG44921), VHa1- *V.harveyi* (ADA54890), VMi1- *V.mimicus* (ABS42980), VPa1- *V.parahaemolyticus* (WP 005474711), VFu1- *V.furnissii* (WP 004726677), VTu1- *V.tubiashii* (WP 004748979), VBr1- *V.brasiliensis* (WP 006881610) and VAn1- *V. alginolyticus* (WP 005398060).

4.9.2. Genetic analysis and comparison of Toxin-regulatory gene *tox*R in environmental isolates of *V. vulnificus*

The *tox*R gene sequences obtained after cloning and sequencing the *tox*R gene amplicons from three of the environmental isolates of *V. vulnificus* were analysed to understand the genetic variation at the *tox*R locus. The sequences were compared with the *tox*R sequences of *V. harveyi, V. cholerae, V. parahaemolyticus* and *V. vulnificus* from NCBI GenBank database.The GenBank accession numbers and the closest identities after nucleotide blast of *tox*R genes are as detailed in Table. 4.13.

Table 4.13.Nucleotide blast results and GenBank accession numbers obtained for the toxin-regulatory gene *tox*R of environmental isolates of *V. vulnificus*

Sl. No.	Strain No.	Gene sequence to which submitted sequence exhibited maximum similarity after nucleotide blast (~ 99% identity)	GenBank accession number of Submitted *toxR* gene sequence
1	BTOS7	*V.cholerae toxR* gene encoding a transcription-activating transmembrane DNA-binding protein, complete cds (M21249.1)	KF322109
2	BTAS3	*V.cholerae toxR* gene encoding a transcription-activating transmembrane DNA-binding protein, complete cds (M21249.1)	KF322108
3	BTPS6	*V.cholerae toxR* gene encoding a transcription-activating transmembrane DNA-binding protein, complete cds (M21249.1)	KF322110

Comparison of the *tox*R gene sequences of *V. vulnificus* strains by multiple sequence alignment revealed that all three strains, BTOS7, BTAS3 and BTPS6 exhibited 100% sequence resemblance to the *tox*R nucleotide sequences of *V. cholerae* (M21249) and *V. harveyi* (DQ503438). They displayed sequence homology of 85% with *V. parahaemolyticus* (L11929) and 82% with the close relative *V. vulnificus* (AF170883) (Fig.4.30).

```
BTOS7                ACGGCTACGCCATCGACAACCGTTAGGGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGC 276
BTAS3                ACGGCTACGCCATCGACAACCGTTAGGGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGC 276
BTPS6                ACGGCTACGCCATCGACAACCGTTAGGGGTTTAAAGCTGGATTGGCTCGGGTTAGTGAGC 247
V.harveyi            ATGACTTTGT--TTGGCGAGAGC-AAGGTTTTGAAG-TCGAT-GATTCCAGCT--TAACC 232
V.cholera            ATGACTTTGT--TTGGCGAGAGC-AAGGTTTTGAAG-TCGAT-GATTCCAGCT--TAACC 464
V.vulnificus         -------------------GAGC-AGGGGTTTGAGG-TGGAT-GATTCCAGCC--TAACT 36
V.parahemolyticus    ACGAGTTTGT--TTGGCGTGAGC-AAGGTTTTGAGG-TGGAT-GACTCAAGCC--TGACT 378
                                         *  * ** *** * * * *** *  **  *    * *

BTOS7                AGTAATACTGCGAGGGGAAGTAAGACCGCTATCAGAATAAGCAGTCGATTCCCCAAGTTT 336
BTAS3                AGTAATACTGCGAGGGGAAGTAAGACCGCTATCAGAATAAGCAGTCGATTCCCCAAGTTT 336
BTPS6                AGTAATACTGCGAGGGGAAGTAAGACCGCTATCAGAATAAGCAGTCGATTCCCCAAGTTT 307
V.harveyi            CAAGCCATTTCGACTCTGCGCAAAAT-GCTCAAAGATTCGACAA--AGTCCCCACAATAC 289
V.cholera            CAAGCCATTTCGACTCTGCGCAAAAT-GCTCAAAGATTCGACAA--AGTCCCCACAATAC 521
V.vulnificus         CAAGCGATTTCTACCTTACGCAAAAT-GCTCAAAGACTCTACAA--AGTCCCCTGAGTTT 93
V.parahemolyticus    CAAGCGATTTCTACTCTGCGTAAGAT-GTTGAAGGATTCAACCA--AATCTCCAGAGTTT 435
                           * * * *      * ** *  * *    ** *   *      *  **  * *

BTOS7                G--GAGCCGATTTATTCGCCACGACATTGGCTGGCTGCGGTGTGTTCACTACAGTG---G 391
BTAS3                G--GAGCCGATTTATTCGCCACGACATTGGCTGGCTGCGGTGTGTTCACTACAGTG---G 391
BTPS6                G--GAGCCGATTTATTCGCCACGACATTGGCTGGCTGCGGTGTGTTCACTACAGTG---G 362
V.harveyi            GTCAAAACGGTT------CCGAAACGC-GGTTACCAAT----TGATCGCCCGAGTGGAAA 338
V.cholera            GTCAAAACGGTT------CCGAAGCGC-GGTTACCAAT----TGATCGCCCGAGTGGAAA 570
V.vulnificus         GTGAAAACGGTT------CCAAAACGT-GGTTATCAGT----TGATCT-----GT----T 133
V.parahemolyticus    GTTAAAACCGTT------CCAAAACGA-GGCTATCAAC----TCATTT-----GT----A 475
                     *   *  *  **      **    *   ** *  *       *  *       **
```

```
BTOS7                CTGATGAA-----GGCACTCTGCTTGATTCTGCGTATTCATTGACAGATTCTGGCT---- 442
BTAS3                CTGATGAA-----GGCACTCTGCTTGATTCTGCGTATTCATTGACAGATTCTGGCT---- 442
BTPS6                CTGATGAA-----GGCACTCTGCTTGATTCTGCGTATTCATTGACAGATTCTGGCT---- 413
V.harveyi            CGGTTGAAGAAGAGATGGCTCGCGAAAGCGAAGCTGCTCAT-GACATCTCTCAGCC---- 393
V.cholera            CGGTTGAAGAAGAGATGGCTCGCGAAAACGAAGCTGCTCAT-GACATCTCTCAGCC---- 625
V.vulnificus         CGGTTGAG------------CGCATTAAC----CCGCTCCT-GTCAGAT-TCAACC---- 171
V.parahemolyticus    CTGTTGAA------------CGCCTAAGC----CCGCTTTC-TTCAGAC-TCAAGCTCAA 517
                     * * ***               **   *          *      **

BTOS7                ----------GAGAGATGTC----------ATGAGCAGCTTCGCTTTCG----------- 471
BTAS3                ----------GAGAGATGTC----------ATGAGCAGCTTCGCTTTCG----------- 471
BTPS6                ----------GAGAGATGTC----------ATGAGCAGCTTCGCTTTCG----------- 442
V.harveyi            ----------AGAATCTGT---------CAATGAATA----CGC---------------A 415
V.cholera            ----------AGAATCTGT---------CAATGAATA----CGC---------------A 647
V.vulnificus         ---------AACAACGTG----------AATGA-------CGC---------------- 188
V.parahemolyticus    TTGAAGTTGAAGAACCTGCTTCTGATAACAATGA-------CGCCTCTGCTAATGAGGTA 570
                               *  **           ****       ***

BTOS7                -----------CGAGCCATCT----------CTTCT---TCAACCGTTTCCACTCGGGCG 507
BTAS3                -----------CGAGCCATCT----------CTTCT---TCAACCGTTTCCACTCGGGCG 507
BTPS6                -----------CGAGCCATCT----------CTTCT---TCAACCGTTTCCACTCGGGCG 478
V.harveyi            GA-------GTCAAGCAGTGT--------GCCTTC---ATCAGCCA-CTGTAGTGAACAC 456
V.cholera            GA-------ATCAAGCAGTGT--------GCCTTC---ATCAGCCA-CTGTAGTGAACAC 688
V.vulnificus         -------------AGC----------------TTCTGAAGCA-----TTAGATCAAGA-- 212
V.parahemolyticus    GAAACGATCGTAGAGCCGTCTTTAGCGACGTCTTCTGACGCAATCG-TTGAACCAGAAGC 629
                               ***               ***     **      *  *
```

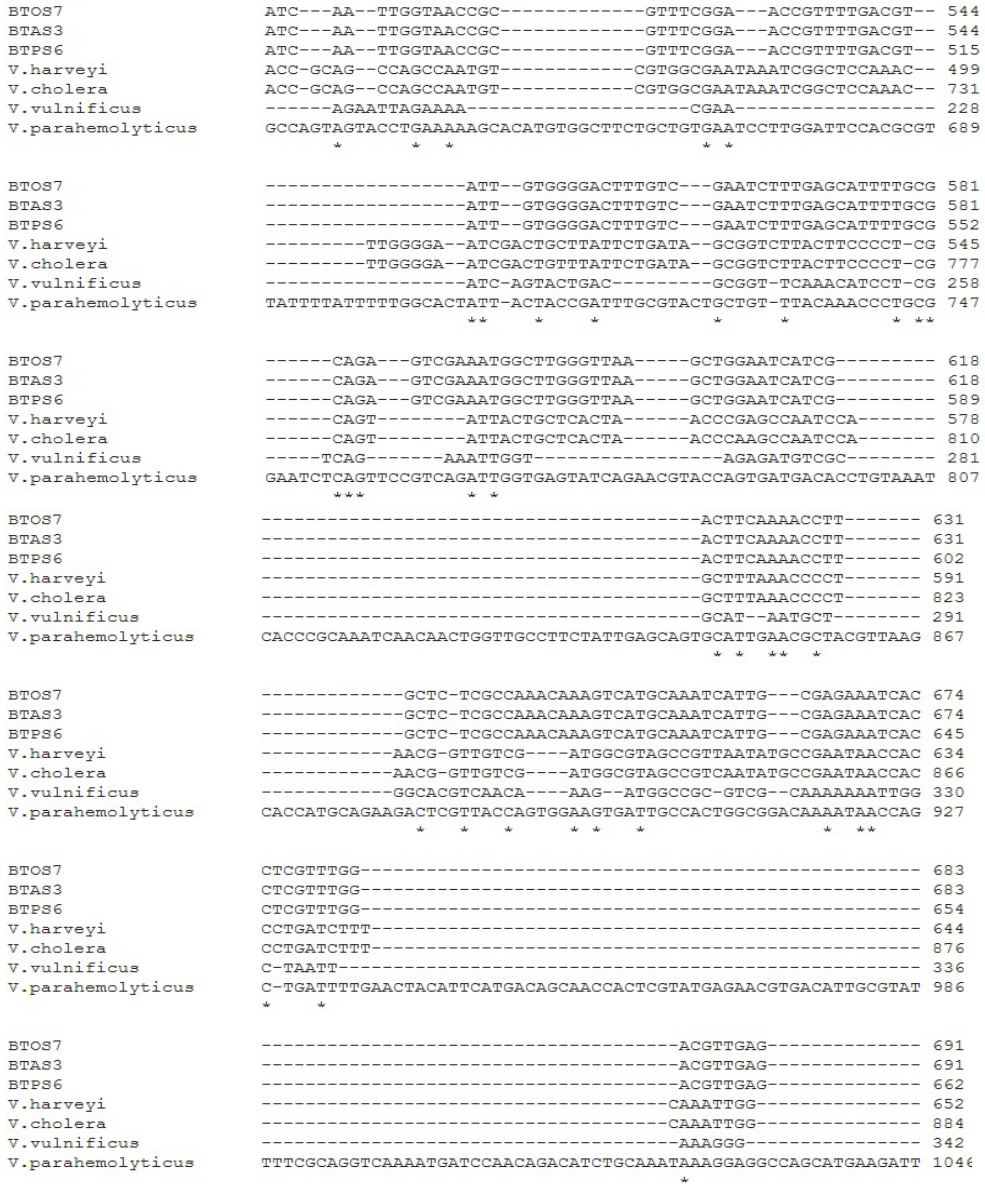


Fig.4.30. Multiple sequence alignment of *toxR* nucleotide sequences from seven *Vibrio* species using the ClustalW program.

The partial *toxR* nucleotide sequence of *V. vulnificus* strains represented byBTOS7, BTAS3 and BTPS6 with accession numbers KF322109, KF322108 and KF322110 respectively were aligned with *toxR* gene sequences from related *Vibrio* species with GenBank accession numbers in parenthesis: *V. cholerae (*M21249), *V. parahaemolyticus* (L11929), *V. vulnificus* (AF170883) and *V. harveyi* (DQ503438). Consensus regions are denoted by an asterisk.

4.9.2.1. Phylogenetic analysis of *V. vulnificus* strains based on the *toxR* gene sequences

An unrooted phylogenetic tree was constructed by the neighbor-joining method to study the interrelationships based on *tox*R gene nucleotide sequences of the environmental isolates of *V. vulnificus* strains with related *V.cholerae, V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* strains obtained from the GenBank database.

Isolates BTAS3, BTOS7 and BTPS6 clustered into a single clade supported by a bootstrap value of 100%. The reference strains formed a separate clade with *V. cholerae* (M21249) and *V. harveyi* (DQ503438) clustering together separated from *V. parahaemolyticus* (L11929) and *V. vulnificus* (AF170883) (Fig 4.31).

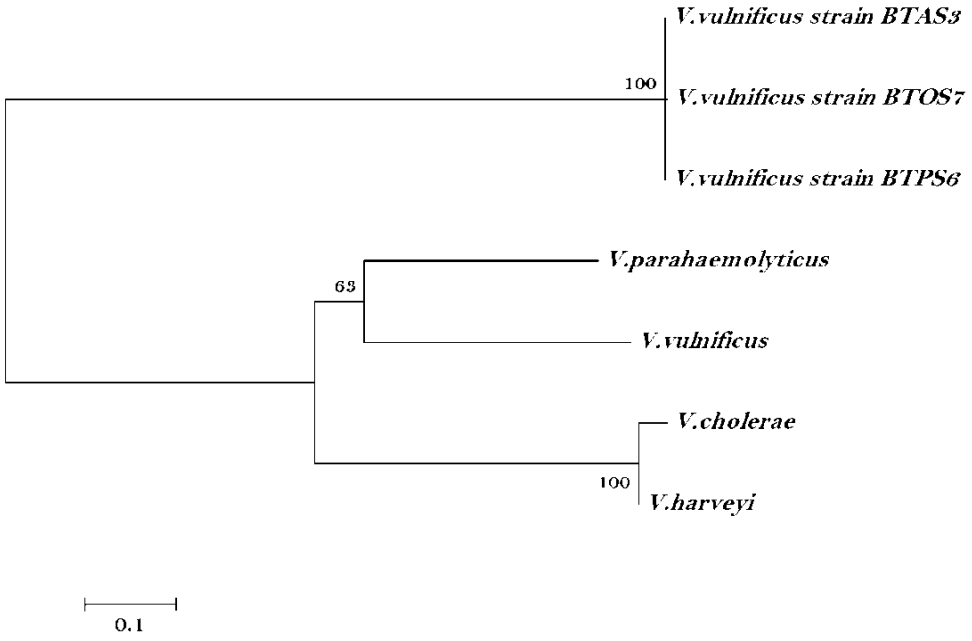


Fig.4.31. Unrooted tree constructed by the neighbor-joining method showing the phylogenetic interrelationships of *V.vulnificus* strains with related *Vibrio* sp. based on *toxR* gene nucleotide sequences.

Bootstrap values are given at the branching points and bars show sequence divergence.

4.9.2.2. Multiple sequence alignment of ToxR amino acid sequences of environmental isolates of *V. vulnificus*

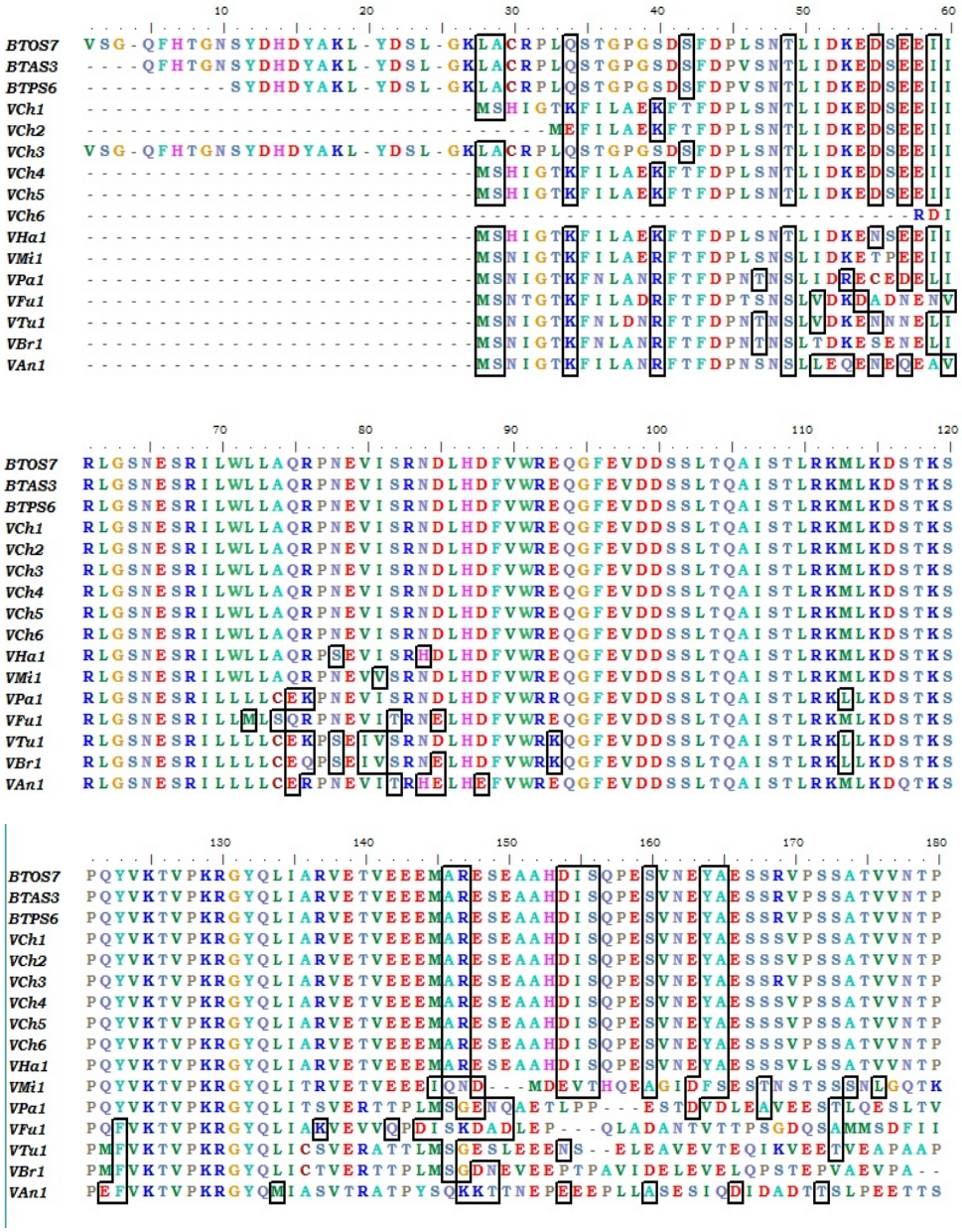
The deduced amino acid sequences obtained by *in-silico* analysis of the nucleic acid sequence of the environmental isolates of *V. vulnificus* was compared with similar sequences available in the GenBank using online BLAST tool- blastx (http://www.ncbi.nlm.nih.gov/blast). The GenBank accession numbers and the closest identities after protein blast of deduced amino acid sequences of *tox*R genes of *V. vulnificus* strains are as detailed in Table.4.14.

Table 4.14. Protein blast results and GenBank accession numbers of deduced amino acid sequences of ToxR protein of *V. vulnificus* strains

Sl. No.	Strain No.	Amino acid sequence to which submitted deduced amino acid sequence exhibited maximum similarity after protein blast	GenBank accession number of *toxR* genes of *V. vulnificus* strains
1	BTOS7	Cholera toxin homolog transcriptional activator (*Vibrio cholerae*) (WP000018138.1) – 99% similarity	KF322109
2	BTAS3	Transcriptional regulator (*Vibrio cholerae*) (WP001884534.1) – 98% similarity	KF322108
3	BTPS6	Transcriptional regulator (*Vibrio cholerae*) (WP001884534.1) – 99% similarity	KF322110

Multiple amino acid sequence alignment of deduced amino acid sequences of ToxR protein from environmental strains of *V. vulnificus* using ClustalW with ToxR of other *Vibrio* species obtained from the GenBank database(Fig. 4.32), revealed highest percentage of sequence

similarity with *V. cholerae* strains. They exhibited minor differences in the amino acid sequences of other compared *Vibrio* species.



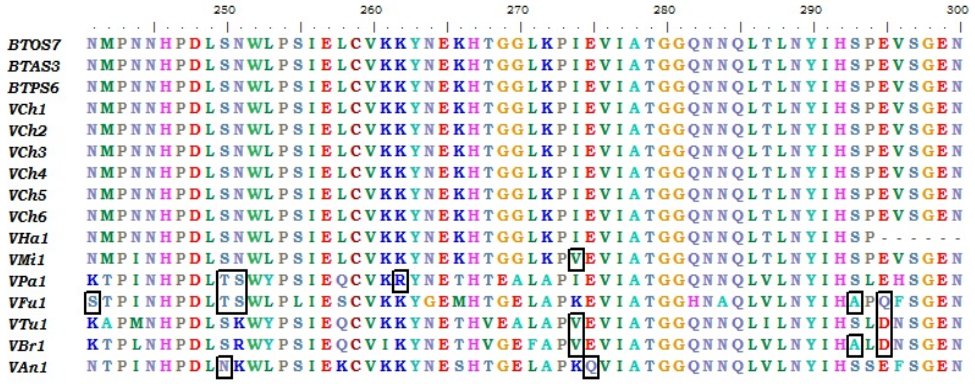


Fig.4.32. Multiple sequence alignment of ToxR amino acid sequences of environmental isolates of *V. vulnificus* strains with related *Vibrio* sp.

The amino acid sequences of the environmental isolates of *V.vulnificus* are represented as BTOS7, BTAS3 and BTPS6. Other *toxR* amino acid sequences of *Vibrio* species used in the alignment are represented with GenBank accession numbers in parenthesis:VCh1- *V.cholerae* (WP000018138), VCh2- *V.cholerae* (WP000394385), VCh3- *V.cholerae* (WP002039486), VCh4- *V.cholerae* (WP002028070), VCh5- *V.cholerae* (ADG44921), VHa1- *V.harveyi* (ADA54890), VMi1- *V.mimicus* (ABS42980), VPa1- *V.parahaemolyticus* (WP 005474711), VFu1- *V.furnissii* (WP 004726677), VTu1- *V.tubiashii* (WP 004748979), VBr1- *V.brasiliensis* (WP 006881610) and VAn1- *V. alginolyticus* (WP 005398060).

4.9.2.3. Phylogenetic analysis of the environmental isolates of *V. vulnificus* based on the ToxR amino acid sequences

The phylogenetic relationship of the environmental isolates of *V. vulnificus* and related *Vibrio* sp. based on ToxR amino acid sequences was deduced from the unrooted tree constructed by the neighbour joining method. It was evident that the ToxR amino acid sequences of the environmental isolates BTOS7, BTAS3 and BTPS6 were closely related to the sequences of *V. cholerae* and *V. harveyi* reference strains, elucidated by their grouping into a single clade (Fig 4.33).

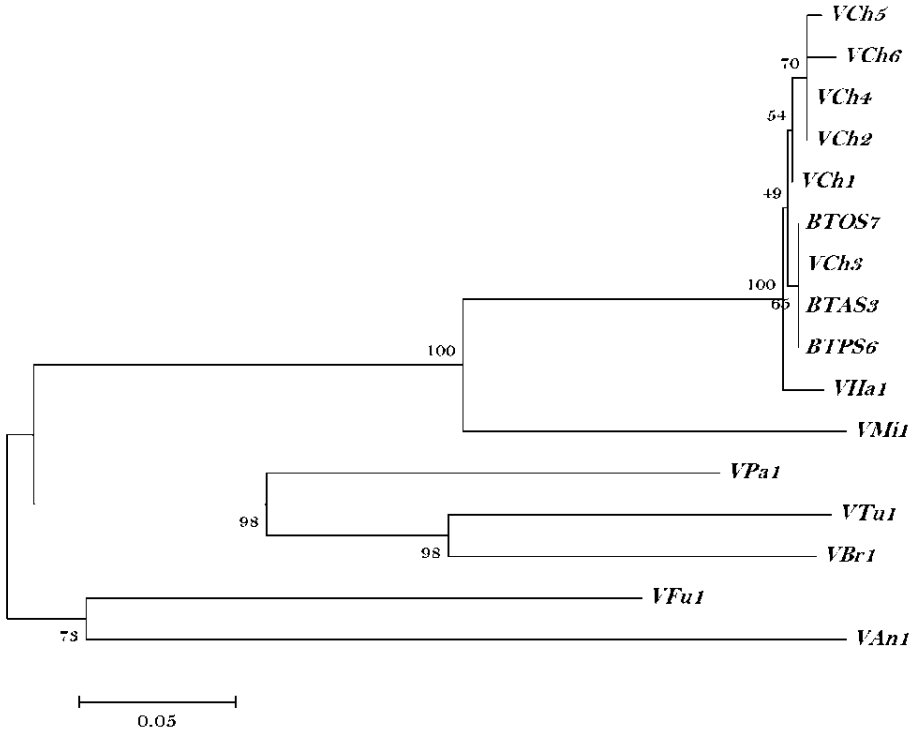


Fig.4.33. Unrooted trees constructed by the neighbor-joining method showing the phylogenetic interrelationships of *V. vulnificus* strains with related *Vibrio* sp. based on ToxR amino acid sequences.

Bootstrap values are given at the branching points and bars show sequence divergence. The environmental isolates of *V. vulnificus* are represented as BTOS7, BTAS3 and BTPS6. Other *Vibrio* species used for tree construction are represented with GenBank accession numbers in parenthesis:VCh1- *V.cholerae* (WP000018138), VCh2- *V.cholerae* (WP000394385), VCh3- *V.cholerae* (WP002039486), VCh4- *V.cholerae* (WP002028070), VCh5- *V.cholerae* (ADG44921), VHa1- *V.harveyi* (ADA54890), VMi1- *V.mimicus* (ABS42980), VPa1- *V.parahaemolyticus* (WP 005474711), VFu1- *V.furnissii* (WP 004726677), VTu1- *V.tubiashii* (WP 004748979), VBr1- *V.brasiliensis* (WP 006881610) and VAn1- *V. alginolyticus* (WP 005398060).

4.9.3. *In-silico* characterization studies of ToxR protein

Multiple sequence alignment of the deduced aminoacid sequence of ToxR protein of all the environmental isolates of *Vibrio* strains (9 strains of *V.cholerae* and 3 strains of *V.vulnificus*) revealed a high degree of homology among all the strains with similar amino acid sequences. The deduced amino acid sequence of ToxR protein of *V.cholerae* strain BTMA5 (KF420400) was selected for further *in-silico* characterization studies.

4.9.3.1. Elucidation of conserved domain database

Conserved Domain Database is a protein annotation resource that consists of a collection of well-annotated multiple sequence alignment models for ancient domains and full-length proteins. CD-Search is NCBI's interface to searching the Conserved Domain Database with protein query sequences. It uses RPS-BLAST to quickly scan a set of pre-calculated position-specific scoring matrices (PSSMs) with a protein query. The deduced aminoacid sequence of the ToxR protein of *V.cholerae* strain BTMA5 was analysed using the putative conserved domains search service (RPS-BLAST) to find conserved domains. Results indicate that the protein sequence showed maximum resemblance with conserved domain model cl17355 (PSSM Id - 247909), which represented the trans_reg_C superfamily, with an E value of 8.57e-14.

The conserved domain model cl17355 represents effector domain of response regulator. They are seen in bacteria and certain eukaryotes like protozoa and in higher plants. Phosphorylation of highly conserved receiver domain of response regulator activates a variable effector domain of the response regulator, which triggers the cellular response. The C-terminal effector domain contains DNA and RNA polymerase binding sites.

4.10. Structure Prediction of the ToxR Protein

The amino acid sequence of ToxR protein of *V.cholerae* strain BTMA5 (Accession no. KF420400) was exported to PHYRE^2 software (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and the resultant PDB file is depicted in Fig.4.34. The deduced aminoacid sequence showed maximum resemblance with the structure of DNA-binding domain of response regulator2 from *Escherichia coli* k-12 (Library id - c3zq7A).

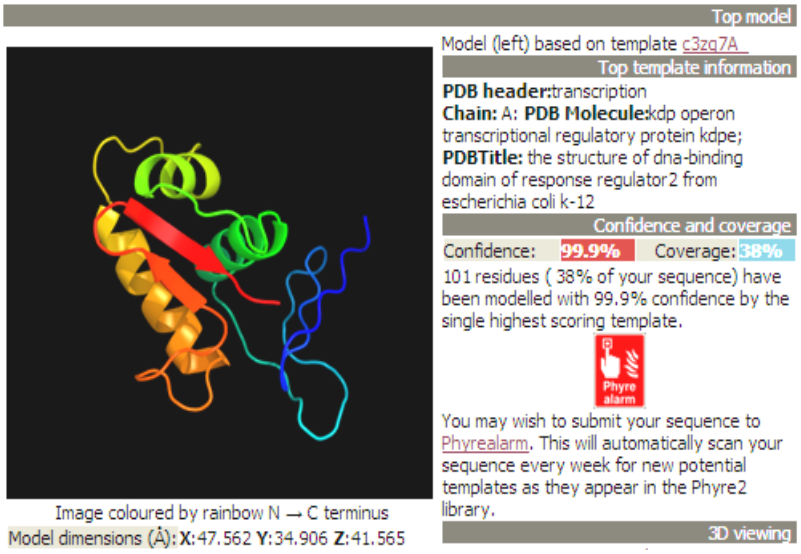


Fig.4.34. The structure predicted by PHYRE^2 software for the deduced amino acid sequence of *V. cholerae* strain BTMA5 (KF420400**).**

Template based homology modelling in Phyre^2 successfully modelled the predicted protein based on protein data bank (PDB) template c3zq7A which consists of chain A representing kdp operon transcriptional regulatory protein kdpe. The secondary structure and disorder prediction of the given template by Phyre^2 is represented in Fig.4.35. The alignment of amino acid sequences of the structure of DNA-binding domain of response regulator2 of the predicted protein (residues from 6-110) and template has

shown highly conserved residues between them with a confidence value of 99.9% within query coverage of 38%.

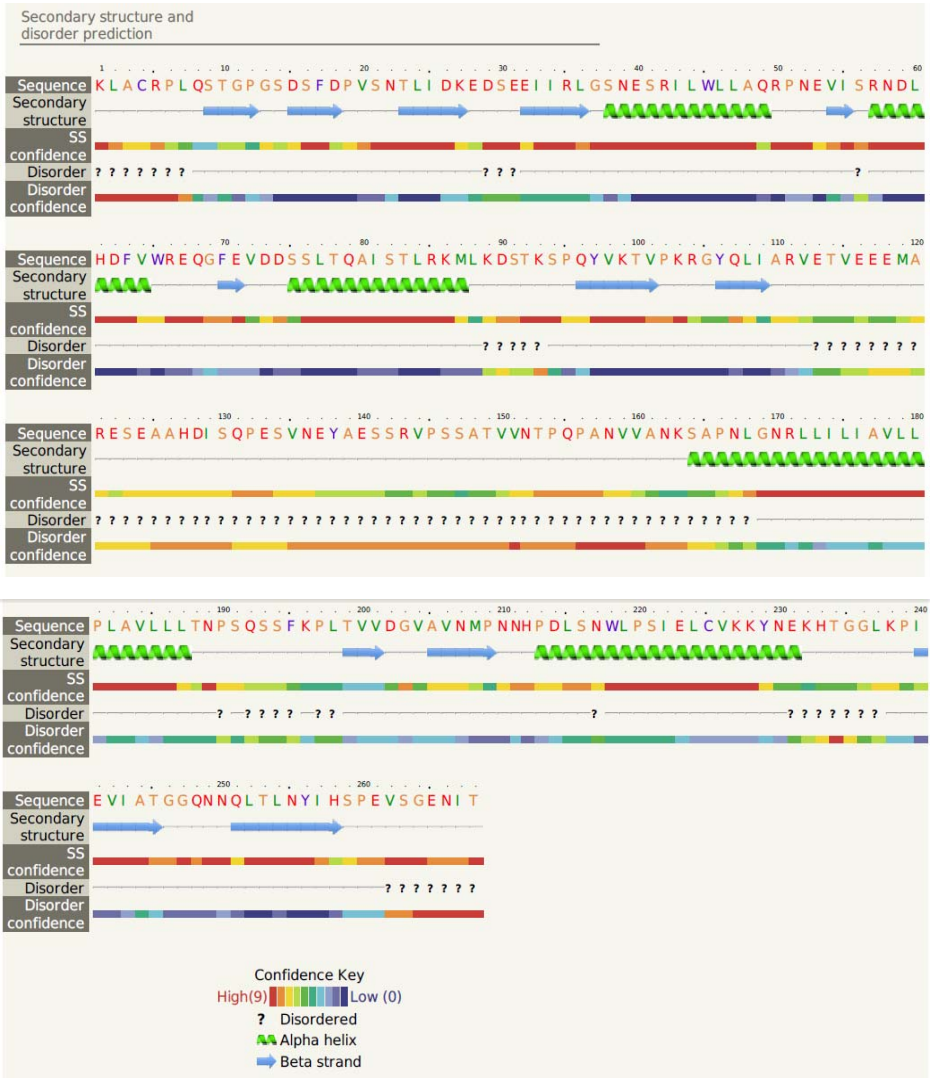


Fig.4.35. The secondary structure and disorder prediction of the deduced protein of *V. cholerae* strain BTMA5 by PHYRE[2].

4.11. Pathogenicity Studies with the Nematode *Caenorhabditis elegans* as model organism

The pathogenicity studies was performed using the nematode *Caenorhabditis elegans* as a model system to determine whether *Vibrio* sp. could serve as a food source for *C. elegans*, and to assess the pathogenicity of the test strains on the nematode.

4.11.1. Assessment of pathogenicity of *V. cholerae* strains

4.11.1.1. Bioassay using *V. cholerae* strains

The bioassay was conducted with the following strains of bacteria: *E. coli* strain OP50 as the food source in control plates, environmental *V. cholerae* strain BTOS6, negative for all tested virulence genes, to check for the suitability of *Vibrio* sp. as a food source for the nematode, *V. cholerae* Co 366 ElTor, a clinical pathogenic strain and *V. cholerae* strain BTPR5, isolated from seafood (Prawn) and positive for 5 of the tested virulence genes. It was observed that the growth and viability of the nematodes when fed with *V. cholerae* strain BTOS6 was almost at par with the results obtained when fed with its food source, *E.coli* OP50, suggesting the ability of *Vibrio* sp. to serve as a food source for the nematode. The results of the bioassay is illustrated in Fig 4.36. The figure represents the percentage mortality of *C. elegans* over a span of 16 days when exposed to the test strains (*V. cholerae* Co 366 El Tor and *V. cholerae* strain BTPR5) in comparison to the control worms fed on *E. coli* OP50 and *V. cholerae* BTOS6.

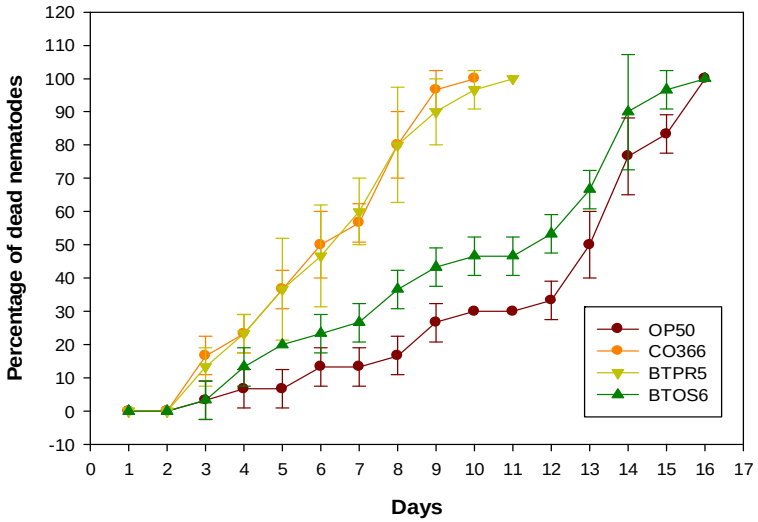


Fig.4.36. Bioassay using *C. elegans* fed with *V. cholerae* strains

The time required for the mortality of 50% of the nematodes (LD_{50}) was calculated from the results obtained using the PRISM (version 5.04) computer program. $LD_{50}$was calculated in three independent experiments and values were represented as mean ± standard error. The time required for 50% of nematodes to die when fed on an *E. coli* OP50 lawn was 12.35±0.2 days and the LD_{50} of the nematodes when fed with the alternate food source, *V. cholerae* strain BTOS6 was 11.86±0.26 days. The lifespan of the nematodes reduced to 5.24±0.19 when fed with *V. cholerae* Co 366 El Tor strain and it was 5.618±0.19 with *V. cholerae* strain BTPR5, indicating the pathogenicity of these strains.

Behavioural abnormalities were noted in the nematodes when fed with *V. cholerae* Co366 El Tor strain and *V. cholerae* strain BTPR5. The motility of the worms and the rate of pharyngeal pumping gradually declined when feeding on these lawns. The worms also exhibited lawn avoidance behaviour by the second day, where they entered into the lawns initially but migrated out to remain at the periphery of the lawn.

4.11.1.2. Microscopy of *C. elegans* fed with test strains of *V. cholerae*

As the nematode, *C. elegans* is a transparent organism, microscopy of the healthy as well as the infected worms was performed to observe and confirm the pathogenesis in the infected worms when compared to the healthy ones. The normal morphological as well as anatomical features of a healthy adult nematode fed on *E.coli* OP50 and *V. cholerae* strain BTOS6 are shown in Fig 4.37 and Fig 4.38 respectively.

Fig.4.37. Micrograph of a healthy *C. elegans* fed on *E. coli* OP 50

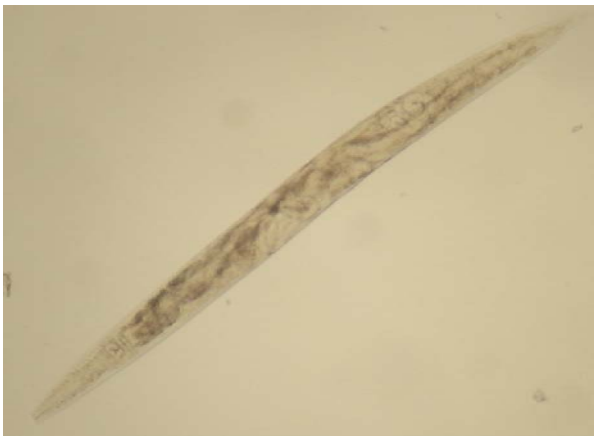


Fig.4.38. Micrograph of *C. elegans* fed on *V. cholerae* strain BTOS6

When the worms were fed with *V. cholerae* Co366 El Tor strain and *V. cholerae* strain BTPR5, distension of the pharyngeal region and the intestinal lumen from the terminal bulb to the anus were observed. These abnormalities were observed from the fourth day of feeding on these lawns. Bagging or internal hatching was another notable feature observed in the adult nematodes when fed with the presumptive pathogenic test strains (Fig. 4.39 & Fig. 4.40). Live larval worms actively moving inside the moribund adult could also be observed.

Fig.4.39. Intestinal colonisation and bagging exhibited by *C. elegans* fed with *V. cholerae* strain BTPR5

Fig.4.40. Bagging in *C. elegans* fed with *V. cholerae* Co366 El Tor clinical strain

4.11.2. Assessment of pathogenicity of *V. vulnificus* strains

4.11.2.1. Bioassay using *V. vulnificus* strains

Environmental *V. vulnificus* strain BTPS6 which tested positive for 3 virulence genes, namely, *cps*, *tox*R and VPI, and *V. vulnificus* strain BTMM7 that did not possess any of the tested virulence gene, were used as test strains for the bioassay on *C. elegans*. The control food source provided was *E.coli* OP50. The results of the killing assay is depicted in Fig 4.41 which represents the percentage mortality of *C. elegans* over a span of 16 days when exposed to the test strains in comparison to the control worms fed on *E. coli* OP50.

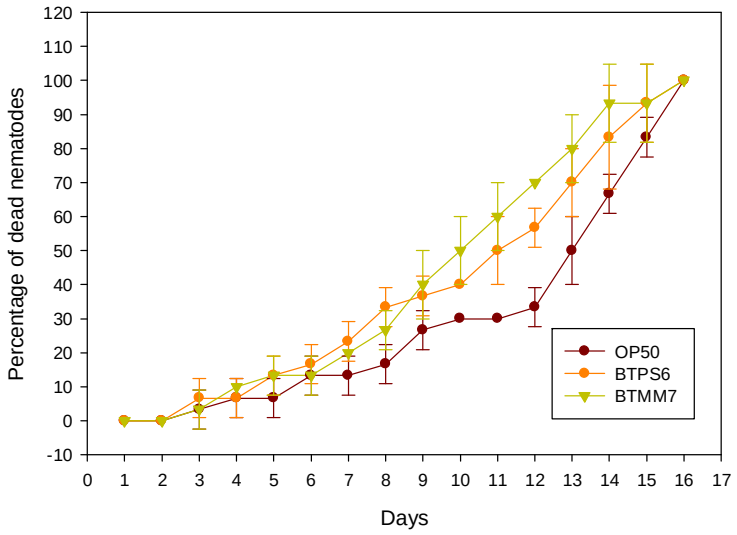


Fig.4.41. Bioassay using *C. elegans* fed with test strains of *V. vulnificus*

The time required for the mortality of 50% of the nematodes when fed with the test strains of *V. vulnificus*, BTPS6 and BTMM7, was compared with that of *E.coli* OP50. The LD_{50} when fed on an *E. coli* OP50 lawn was 12.35±0.2 days and the LD_{50} of the nematodes when fed with the test strains *V. vulnificus* BTMM7 and BTPS6 were 8.90±0.19 and 9.04±0.17 days respectively. There was a reduction in the life span of the worms fed with the test strains of *V. vulnificus* when compared to *E. coli* OP50 strain.

Worms fed with *V. vulnificus* strain BTPS6 which hosted virulence genes and strain BTMM7, negative for tested virulence genes, exhibited a somewhat similar mortality rate, suggesting that the presence of the virulence genes does not greatly affect the lifespan of the nematode. Nevertheless, it is important to note that there was > 26% reduction in the life span of the worms fed with strains of *V. vulnificus* in this study.

However, behavioural abnormalities were observed in the nematode when fed with *V. vulnificus* strain BTPS6. The worms were sluggish in their behaviour and exhibited lawn avoidance.

4.11.2.2. Microscopy of *C. elegans* fed with test strains of *V. vulnificus*

Microscopical observations of the worms revealed that the worms fed with *V. vulnificus* strain BTPS6 and BTMM7 had normal morphological and anatomical features with intact pharynx and intestine. However, intestinal colonisation and bagging were common features observed in the worms fed with *V. vulnificus* strain BTPS6 (Fig 4.42) indicating the pathogen's antagonistic effect on the nematodes.

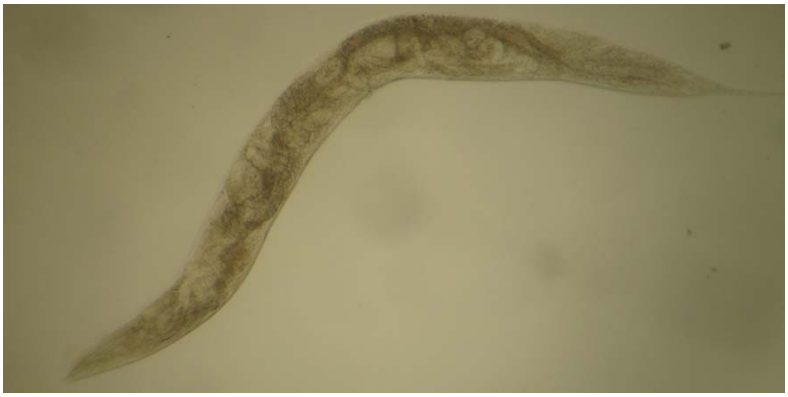


Fig.4.42. Intestinal colonisation and bagging in *C. elegans* fed with *V. vulnificus* strain BTPS6

Chapter 5
DISCUSSION

The marine environment, which includes both native and externally introduced microbial contaminants, plays a pivotal role in ecological and epidemiological studies as it acts as a reservoir not only for the persistence, dissemination and evolution, but also transmission of pathogenic microbes to humans. Vibrios are highly abundant in marine environments, including estuaries, marine coastal waters and sediments, and aquaculture settings (Ortigosa *et al.,* 1989; 1994; Barbieri *et al.,* 1999; Vandenberghe *et al.,* 2003). They also appear at particularly high densities in and/or on marine organisms, e.g., corals (Rosenberg and Ben-Haim, 2002), fishes (Huys *et al.,* 2001; Senderovich *et al.,* 2010; Adeleye *et al.,* 2010; Bakr *et al.,* 2011), molluscs (Roque *et al.,* 2009; Odu *et al.,* 2011), sponges (Taylor *et al.,* 2007; Hoffmann *et al.,* 2010) shrimp (Tran *et al.,* 2002; Gopal *et al.,* 2005; Kannapiran *et al.,* 2009; Walling *et al.,* 2010) and zooplankton (Tamplin *et al.,* 1990; Baffone *et al.,* 2006; Rehnstam-Holm *et al.,* 2010; de Magny *et al.,* 2011). Although vibrios persist as natural components of the marine microbial flora, a small percentage of environmental isolates carry the genetic determinants for human pathogenesis (Boer *et al*., 2013; Gavilan *et al.,* 2013; Khouadja *et al.,* 2013) and are known to be commonly associated with outbreaks of vibrio infections due to consumption of food and water contaminated with human faeces or sewage, raw fish and seafood or with exposure of skin lesion such as cuts, open wounds and abrasions to aquatic environments and marine animals (Igbinosa and Okoh, 2008).

Kerala State with its vast aquatic reservoir, consisting of widespread estuarine system, provides a suitable environment for survival of *Vibrio sp.* and has also experienced several outbreaks of cholera in the past (Radhakutty *et al.,* 1997; Sabeena *et al.,* 2001; http://ibnlive.in.com/news/cholera-outbreak-expert-team-visits-wayanad/163928-60-116.html). Hence studies on the ecology, distribution, virulence potential and antibiotic resistance of vibrios in the marine environment were therefore taken up to understand the relationships of these pathogens to their environments and their potential threat to human health, as it is pertinent and assumes significance in the above context.

5.1.Isolation and identification of vibrios from marine environments

Habitat preferences and selected environmental factors such as sea-surface temperature and salinity have been associated with higher rates of isolation of vibrios from environmental samples (Lipp *et al.,* 2002; Randa *et al.,* 2004; de Magny *et al.,* 2009). In the present study, vibrios were isolated from marine environments like seafood (Prawn and Mussel), aquafarms and mangroves from the coastal areas of Cochin, Kerala. The various species of *Vibrio* isolated and identified by phenotypic and molecular methods, in order of their frequencies were *V. cholerae, V. vulnificus, V. parahaemolyticus, V. alginolyticus* and *V. azureus.*

Among the seafood samples studied, a strain of *V. cholerae* was isolated from prawn and a strain of *V. parahaemolyticus* from mussel. Although squid harboured bacteria, none of them belonged to the genus *Vibrio*. The genus *Vibrio* is reported to be ubiquitous in the marine and estuarine aquatic ecosystems in which shrimp occur naturally and are farmed (Vanderzant *et al.,* 1971; Ruangpan and Kitao, 1991; Otta *et al*.,

1999) and some of them associated with bacterial infections in shrimp (Lightner, 1993; Jiravanichpaisal and Miyazaki, 1995; Lavilla-Pitogo, 1995) are generally considered to be opportunistic pathogens causing disease when shrimp are compromised. There are reports on the occurrence of *V. alginolyticus* (Felix and Devaraj, 1993), *V. anguillarum* (Nammalwer and Thangaraj 1980), *V. cholerae* (Premanand *et al.,*1996), *V. fluvialis* (Ponnuraj *et al.,*1995), *V. parahaemolyticus* (Abraham *et al.*, 1993), *V. mimicus, V. vulnificus* (Karunasagar *et al.,*1990), *V. damsela* (Aravindan and Kalavati, 1997) and *V. harveyi* (Abraham and Manley, 1995) in prawn and shrimp larvae from India. In the present study, only *V. cholerae* was present at a frequency of 7% of the total bacterial community in the prawn sample. Although *V. cholerae* non-O1/ non O139 appeared to constitute the normal microflora of prawns (Nair *et al.,* 1991), there are only a few records of isolation of choleragenic *V. cholerae* from prawn associated with cholera like disease in isolated cases (Eberhart-Phillips *et al.,* 1996; Jiménez *et al.,* 2011) as well as in outbreaks through ingestion of contaminated seafood (Daniels and Shafaie, 2000).

Mussels and oysters are especially prone to bacterial contamination as they are filter feeders that concentrate bacteria in their filtration systems and are ideally suited to trap all bacteria and viruses, pathogenic or otherwise, that live in their environment (Popovic *et al*., 2010). Some *Vibrio* species, including *V. harveyi*, *V. splendidus*, *V. tubiashii*, *V. tapetis* and *V. pectenicida* are pathogenic to bivalve molluscs (Hada *et al*. 1984; Fujiwara *et al.,* 1993; Sutton and Garrick, 1993; Borrego *et al.,*1996; Lambert *et al.,* 1998), while others like *V. parahaemolyticus* , *V. vulnificus* and *V. cholerae* cause disease outbreaks like gastroenteritis, wound infection, and septicaemia following ingestion of poorly cooked or raw

shellfish contaminated with these bacteria (Weber *et al.,* 1994; McLaughlin *et al.,* 2005; Haq and Dayal, 2005). *V. parahaemolyticus* was present in the mussel sample in this study at a frequency of 9% and although Nair and co-workers (2007) are of opinion that pathogenic isolates capable of inducing gastroenteritis in humans are rare in environmental samples (2 to 3%), it has been established that the presence of toxigenic strains can lead to sporadic outbreak of food borne diseases (CDC, 1999; DePaola *et al.,* 2000).

Detection of *V. cholerae* in seafood harvested in Indian coastal waters suggests that this commodity should be considered a source of potential risk to consumers and is supported by other studies (Senderovich *et al.* 2010; Hill *et al.,* 2011), which demonstrate that fish should be considered a major reservoir and a vector for *V. cholerae*. The occurrence of pathogenic *Vibrio* species in seafood is to be viewed with caution as seafood may serve as a vehicle for most pathogenic *Vibrios,* because for public health concern for seafood consumers, and also a reason for export bans, detentions and rejections in international fish trade.

The aquaculture farms selected for the present study were all brackish water aquafarms, where shrimps were the major species cultivated. The water and the sediment samples were screened for the presence of *Vibrio* like organisms. A total of 52 isolates were obtained and the vibrios constituted 42% of total isolates from this environment. *V. cholerae* occurred in the highest frequency of 54% followed by *V. vulnificus (*41%) and *V. alginolyticus (*5%). This was in accordance with similar studies from India (Otta *et al.*, 1999; Vaseeharan and Ramasamy, 2003), where vibrios accounted for 38–81% of the bacterial biota in the brackish water pond

ecosystem. Aquafarms are considered to be stressful environments mainly due to high organic matter and dissolved oxygen fluctuations (Direkbusarakom *et al*, 1998) and *V. cholerae* is found associated with a number of biotic and abiotic substrates where the pathogen can exist in high abundance, survive long periods of stress and, when conditions are right, cause human infection (Colwell, 1996; Nair, 2008). Since vibrios are important bacterial pathogens for animals reared in aquaculture (Hjeltnes and Roberts, 1993; Lightner and Redman, 1998; Austin and Austin, 2007) the risk of sudden outbreak of diseases is considered a significant problem to the development of aquaculture sector and it is crucial to ensure that such ventures are protected from the impact of diseases.

The development of mangrove-friendly aquaculture techniques utilize the positive attributes of natural mangrove forests as nursery and breeding grounds for fish, crabs, molluscs and shrimps. In the present study, mangroves associated with aquafarms along the Cochin coast were selected for screening for vibrios. From the vibrio like organisms obtained, 47% belonged to the genus *Vibrio*, *V. vulnificus* being predominant (43%) followed by *V. cholerae* (38%), *V. parahaemolyticus* (10%), *V. alginolyticus* (5%) and *V. azureus* (5%). Although there are several reports on the vibrios associated with mangrove rhizosphere and their role in the biodegradation and mineralisation process (Rameshkumar and Nair, 2007; 2009; Rameshkumar *et al.*, 2008; 2010; Lara *et al.,* 2011; Manjusha *et al.,* 2013), studies on their prevalence in the mangrove aquaculture integrated systems and their potential pathogenicity are rare (Vandenberge *et al.,* 2003; Gopal *et al.,* 2005). The occurrence of *Vibrio* species in fishes reared in mangrove systems and their potential health risks have been elaborated (Ramamurthy and Raveendran, 2009), consequently justifying the significance of this study.

It is evident on comparing the incidence of vibrios in the various samples studied, that the aquaculture environment hosts the highest frequency of *Vibrio* sps., followed by the mangrove environment. However, the mangrove environment exhibited species diversity represented by *V. cholerae, V. vulnificus, V. alginolyticus* and *V. azureus* strains. Of the 45 strains isolated from the marine environments, the predominant species were *V. cholerae* and *V. vulnificus*. The incidence of *V. parahaemolyticus*, *V. alginolyticus* and *V. azureus* was comparatively lower. The ability of these *Vibrio* species in causing serious and often fatal infections in humans and seafood animals should serve as a warning to a need for constant monitoring of this species in the marine environment. It is a fact that the development of large outbreaks is the result of the interplay of local environmental factors with the mixing of genes via lateral gene transfer between *Vibrio* strains in the environment, which evolves into epidemic strains (Faruque *et al.,* 2004). This logic is largely predicated on the assumption that the causative agent is one of a limited number of clones harbouring particular suites of virulence or virulence-associated factors.

A remarkable observation during the present study was the incidence of non-*Vibrio* isolates on TCBS agar, with characteristic biochemical features of the genus *Vibrio*. The doubt on the reliability and reproducibility of TCBS, as a medium for isolation of environmental *Vibrios* (McLaughlin, 1995) prompted the use of 16S rDNA sequence analysis along with the biochemical tests for proper identification at the species level. Strains which were isolated along with vibrios and showed typical *Vibrio* reactions on TCBS agar were positive for cytochrome oxidase test, fermentative on MOF medium, Gram negative rods/cocci were assigned to the genera, *Aeromonas*, *Pleisiomonas*, *Proteus*, and *Klebsiella* after16S rDNA sequence

analysis (Joseph *et al.*, 2013). This study therefore substantiates the doubt aired previously by other workers on the reliability of TCBS for screening *Vibrio* species.

5.2. 16S rRNA gene based taxonomy and phylogeny

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most commonly used housekeeping genetic marker, since it is present in almost all bacteria, as a multigene family or operon. Furthermore, the 16S rRNA gene of ~1,500 bp is large enough for informatics purposes and its function over time has not changed, suggesting that random sequence changes are a more accurate measure of evolution (Janda and Abbott, 2007). The data generated using the universal 16S rRNA gene segment primers in the present study was accurate, reproducible and less time consuming compared to the conventional phenotypic identification schemes. Since 16S rRNA gene sequence similarity of ≥97% is a reasonable level for grouping bacteria into species (Hagstrom *et al*., 2002) this method was adopted for the identification of the *Vibrio* isolates to the species level.

Analysis of 16S rRNA gene sequences allowed construction of phylograms using the neighbour joining method. Strains of *V. cholerae* from the different environments exhibited both variability and relatedness among themselves. Within the tree, many of the isolates from aquafarms and mangroves claded distinctly into clusters representing their geographical origin, however, some clusters grouped together representative strains from diverse sources also. Strain BTPR5 isolated from prawn exhibited genetic similarity to the strains isolated from aquafarm and mangrove environments. Strain BTOS6 isolated from mangroves appeared to be distinct

phylogenetically from other strains isolated from the same environment. Similarly, BTMM4 isolated from mangroves formed a distinct clade separated from all the other strains, but distinct from the outgroup *E. coli*.

The phylogram constructed using the 16S rRNA gene sequences of the strains of *V. vulnificus* also displayed homogeneity and heterogeneity in their genetic content irrespective of their geographic origin. Strains from different sources claded together with minor variations in their genomic sequences. The relatedness of the strains is suggestive of their clonal origin. However, BTAS3 from aquafarm and BTMS3 and BTOK10 from mangroves formed a distinct clade more remotely related to other isolates from the same samples.

The ecological differentiation of co-occurring bacterial populations with small-scale differences in 16S rRNA gene sequences remains an open question (Rappe and Giovannoni, 2003). Given the variable evolutionary structure of the 16S rRNA gene sequence, however, this subtle difference belies significant genetic diversity between strains. According to Hoffmann and colleagues (2010), along with the notable genetic homogeneity evident among the geographically disparate strains, it is reasonable to postulate a panmictic population structure (that is, members of the population move freely across habitats, likely are able to exchange DNA and share a common gene pool), whereby members freely associate in spite of the physical distances separating these strains.

5.3. Biochemical characterization

The biochemical characterization of *Vibrio* spp. is an important determinant in food and environmental microbiology taking into account that these micro-organisms, considered non-pathogens for a long time have remarkable clinical importance. The development of a practical system for

the identification has involved the use of commercial kits, which work properly, only in the identification of vibrios of clinical importance, but their utility in identifying environmental strains is limited. In the present study, Hi-Vibrio identification kit was used for biochemical characterization of the isolates which involved 12 biochemical tests. The commercial kit could not be utilized for the accurate species level identification of the isolates; nevertheless, the results obtained supplemented the polythetic approach adopted for identification of the isolates. The ability of the isolates to grow on an array of substrates and their multi-enzyme activities proves that these organisms respond cohesively to the ecology of their environment. The biochemical profile of environmental isolates depicts the physicochemical conditions, the specific association of the bacteria with aquatic plants or animals, and/or the existence of specific ecological associations involving several components of the environmental niche of the organism (Faruque *et al.,* 1998a).

5.4. Antibiotic Resistance

The antibiotic profile reveals the high individual and multiple antibiotic resistances among the test strains of *Vibrio.* The isolates exhibited varying degrees of resistance to gentamicin, ampicillin, nalidixic acid, vancomycin, cefixime, rifampicin, tetracycline and chloramphenicol. All the isolates were susceptible to streptomycin, co-trimoxazole, trimethoprim and azithromycin. Although previous studies have shown that *Vibrio* species are susceptible to rifampicin, kanamycin, tetracycline, polymyxin B (Li *et al.,* 2012), chloramphenicol and norfloxacin (Bernard, 2006), it is in variance with this study where resistances to streptomycin, tetracycline, rifampicin and chloramphenicol were observed in the *Vibrio*

isolates. The incidence of resistance in *Vibrio* sp. towards ampicillin, vancomycin and rifampicin have been reported from Kerala, for *Vibrio* sp. isolated from water and fishery samples along south west coast of India (Manjusha *et al.,* 2005) and in PHA accumulating *Vibrio* sp. (Raghul, 2012).

The antibiogram results of *V. cholerae* isolates indicate the higher frequency of antibiotic resistance amongst these strains with 81% of the isolates exhibiting multidrug resistance. The resistance spectrum of *V. cholerae* is reported to vary in different locales, as in the area around Delhi, extensive resistance to furazolidone, cotrimoxazole and nalidixic acid was noted, while tetracycline remained effective (Sharma *et al.,* 2007); while in Bangladesh tetracycline resistance was more frequent (Saha *et al.,* 2006). Tetracycline, chloramphenicol and nalidixic acid have been generally considered as the drugs of choice for the treatment of cholera (Ramamurthy *et al.,* 2000). However, resistance to these drugs was observed in the present study, as also corroborated by Jagadeeshan and co-workers (2009) in *V.cholerae* non O1/non O139 from South Indian waters.

Report of drug-resistant *V. cholerae* strains are appearing with increasing frequency (Ramamurthy, 2008) and the progressive increase in antimicrobial resistance is a serious impediment in the treatment and containment of cholera. Antibiotics are not required to resolve cholera symptoms and cannot be used as a sole treatment for the disease; however, there are advantages in combining oral rehydration therapy with antibiotic treatment (Kitaoaka *et al.,* 2011). Nevertheless, the wide use of antibiotics has increased the sporadic appearance of multidrug resistant strains which is a cause for concern. It is recommended that use of antibiotics to treat cholera should be strictly relegated to patients suffering from severe dehydration.

The *V. vulnificus* strains obtained in this study were resistant to vancomycin (94%), ampicillin (61%) tetracycline (39%) cefixime (28%), gentamicin, nalidixic acid and rifampicin (6%). Since the antibiotic therapy prescribed for *V. vulnificus* infections include tetracyclines, aminoglycosides, cephalosporins and chloramphenicol (Morris and Tenney, 1985), resistance exhibited by the isolates in this study and similar studies (Manjusha *et al.*,2005; Kim *et al.,* 2011; Elhadhi, 2012) is a cause of great concern.

The resistance exhibited by *V. parahaemolyticus* and *V. alginolyticus* to the generally applied antibiotic, ampicillin, suggests that this antibiotic will only have a potentially low efficiency in empirical treatment of *Vibrio* infections.

According to Matyar and fellow workers (2007), MAR index values >0.2 indicates existence of isolates in high risk contaminated source with higher frequency of the use of antibiotics while values <0.2 is indicative of the lesser application of antibiotics. The MAR index of >0.2 observed in the *Vibrio* isolates from the marine environments in the present study, points to the fact that multiple resistances may arise from selective pressure due to indiscriminate use of antibiotics and the need therefore to oversee the judicious use of antibiotics as therapeutics and as prophylactic agents.

Antimicrobial drug resistance in *Vibrio* spp. can develop through mutation or through acquisition of resistance genes on mobile genetic elements, such as plasmids, transposons, integrons, and integrating conjugative elements (ICEs). ICEs integrate and replicate with the host chromosome and can excise themselves and effect horizontal gene transfer of resistance genes. The indiscriminate use of antibiotics as growth promoters,

prophylactics and therapeutic agents in medicine, agriculture and aquaculture has increased the reservoir of resistance genes. The accumulated scientific evidence is that use of antibiotics in food producing plants, animals and fishes can lead to antibiotic resistance in intestinal bacteria, and this resistance can then be transmitted to the general population, causing treatment-resistant illness (Serrano, 2005). The use of antibiotics can also create antibiotic resistance in non-pathogenic bacteria, the resistance genes of which can be transferred to disease-causing bacteria, resulting in antibiotic-resistant infections (Kitaoka *et al*., 2011). Continued surveillance for antimicrobial resistance from clinical and environmental sources may be useful in identifying reservoirs for this pathogen and practices that encourage development of resistance.

The comparatively higher incidence of *V. cholerae* and *V. vulnificus* from marine environments and their potential pathogenic implications on humans and aquatic organisms justifies the selection of isolates of these species for further scrutiny and characterization in this study.

5.5. Molecular typing

A large number of methods have been employed for typing both clinical and environmental *Vibrio* strains. PCR-based methods of fingerprinting take advantage of the presence of repetitive sequences that are interspersed throughout the genome of diverse bacterial species. The fingerprinting methods, ERIC PCR and BOX PCR used in this study are well established and have been applied to both clinical and environmental strains for their identification and molecular typing.

Fingerprinting analysis has been widely applied for *V. cholerae* research to understand the molecular epidemiology of cholera disease

(Faruque *et al*., 1998b). ERIC PCR and BOX PCR along with other fingerprinting methods have been successfully employed in the discrimination of strains of *V. cholerae* (Rivera *et al.,* 1995; Goel and Jiang, 2011; Shuan-Ju *et al.,* 2011), *V. vulnificus* (Radu *et al.,* 2000; Chatzidaki-Livanis, 2006*)*, *V. parahaemolyticus* (Maluping *et al.,* 2005; Alitheen *et al.,* 2009), *V. halioticoli* (Sawabe *et al*., 2002), *V. tapetis* (Rodriguez *et al.,* 2006) and *V. harveyi* and *V. alginolyticus* (Kumar *et al.,* 2007).

In the present study, ERIC PCR fingerprinting analysis of *V. cholerae* strains showed amplification of multiple fragments of DNA ranging between 100 and 1500 bp in size, the results being in accordance with that obtained with *V. cholerae* isolates associated with an outbreak in India (Goel and Jiang, 2011). This fingerprinting technique was reported to distinguish between toxigenic O1/O139 and non-toxigenic non O1*V. cholerae* strains (Sailes *et al.,* 1994; Riviera *et al.,* 1995; Goel and Jiang, 2011; Shuan-Ju *et al.,* 2011), however this study could only resolve genotypic intraspecific variability among *V. cholerae* strains from different regions as observed by Jiang and co-workers (2000). Intraspecific variability has also been observed in *V. vulnificus* isolates in the present study; nevertheless there seemed to be a greater degree of homology between strains from the same source. Similar results have been observed earlier within many species of the genus *Vibrio*, including some pathogenic for marine organisms such as *V. anguillarum*, *V. vulnificus*, and *V. halioticoli* (Aznar *et al*., 1993; Sawabe *et al.,* 2002).

The BOX PCR of genomic DNA from *V. cholerae* isolates resulted in amplification of different fragments of DNA of varying length ranging

from 150 to 2000 bp. One band of ~700 bp was characteristically present in all the *V. cholerae* strains. Moreover, the dendrogram generated from the banding profile grouped the 21 strains into five distinct groups with 100% similarity in the banding pattern of the strains. The BOX PCR fingerprints of *V. vulnificus* also yielded a banding pattern from 300 to 2000 bp with a distinct band at ~700 bp region and the banding pattern was identical or nearly identical in 16 out of the 18 environmental isolates as evidenced from the dendrogram. The characteristic similarity in banding pattern exhibited by environmental *V. cholerae* strains and *V. vulnificus* strains can be used in the development of species specific genetic amplification assay for diagnostic purposes (Maluping *et al.,* 2008).

Both ERIC PCR and BOX PCR fingerprinting methods revealed molecular genetic heterogeneity within the environmental isolates of *V. cholerae* and *V. vulnificus*. It has been pointed out that *Vibrio* strains isolated from even a single source could have genomic variations that could be exhibited by fingerprinting methods (Kumar *et al.,* 2007; Waturangi *et al.,* 2012). The present study also supports the above finding that intraspecies variation in *V. cholerae* and *V. vulnificus* strains were present even among strains isolated from the same environmental source. This probably could be an indicator of the nature of variability happening in the bacterial strains when present in the environment where the bacteria are subjected to various types of stressors and also due to the ability of bacterial strains in mutation and recombination to promote genetic divergence for adaptation to stressors and functional repair system (Vulic *et al.,* 1999). However, some of the strains though isolated from different sources, exhibited identical fingerprint patterns in the present study. The mangrove environments selected were those that are physically linked to

the aquafarms under study and it has been reported that identical fingerprint profiles may occur for strains from different environmental sources and this can result from the geographical proximity of the sources of isolation (Jiang *et al.,* 2000; Waturangi *et al.,* 2012). The emergence of genetic diversity among *V. cholerae* strains has been attributed to various factors, including the roles of mobile genetic elements, bacteriophages, and the competence of *V. cholerae* to take up and assimilate free DNA from the environment (Udden *et al.,* 2008). The results reported herein for the different strains of *V. cholerae* and *V. vulnificus* could also be a combination of natural factors that lead to the emergence of genetic variants among these environmental strains.

The reproducibility of ERIC and BOX fingerprints were determined by repeated examinations. DNA was extracted from each isolate and amplified at least three times and reproducible and stable profiles were obtained for both techniques. This is in agreement with the findings of other groups of researchers who reported that different PCR typing strategies may result in detection of different amounts of genetic diversity (van Belkum, 1994; van Belkum *et al.,* 1996).

Many methods have been developed for molecular typing of *Vibrio* species including the widely used ribotyping and PFGE; however, ribotyping is cumbersome and costly, and PFGE takes several days to reveal the fingerprinting pattern (Currie *et al.,* 2007). PCR-based methods of fingerprinting take advantage of the presence of repetitive sequences that are interspersed throughout the genome of diverse bacterial species. The high degree of evolutionary conservation of the repetitive sequences are a result of natural selection that constrain variation in these sequences as they represent sites of essential protein-DNA interactions and the propagation of

these elements as retroposons (Gordon and Wright, 2000). The PCR methods of fingerprinting employed in this study, ERIC and BOX, are well established and have been applied to both clinical and environmental strains of *Vibrio* for their identification and molecular typing. The distribution of fingerprints based on ERIC and BOX PCR among environmental *V. cholerae* and *V. vulnificus* strains was diverse and independent of sample type but revealed a consistent pattern, demonstrating that the fingerprint is stable and specific to a given bacterial strain. Both ERIC and BOX methods presented a unique profile for each strain and the comparison of similarities among the different patterns confirmed the clonal origin of the species, at the same time, revealing the genetic heterogeneity among the strains of the same species. Although these two methods did not allow the establishment of well-defined genetic clusters to differentiate between toxigenic and non-toxigenic strains, such methods can be used to follow the spreading of bacterial strains responsible for a particular outbreak. It is speculated that the application of ERIC PCR and BOX PCR techniques to environmental samples may aid in understanding the molecular ecology of the cholera agent and related enteric pathogens in the environment.

5.6. Screening for marker and virulence genes by PCR

The bundle forming pili genes, *01rfb* and *0139rfb,* are surface marker genes for pandemic strains of *V. cholerae* O1 and O139 serotypes respectively and screening for their presence can endorse the reliability of the genes as marker candidates as well as indicate the serotype of the strains. This PCR is an alternative for serotyping and hence known as serotyping PCR. Since none of the tested *Vibrio* isolates in this study showed a positive amplification for these genes, it was inferred that the 21

isolates identified as *V. cholerae* belonged to either the non O1 or the non O139 serogroup. Although the mechanism of pathogenesis in *V. cholerae* O1 and O139 is well understood, reports on the localized cholera-like outbreaks due to non-O1, non-O139 strains in tropical countries (Sharma *et al.,* 1998; Cheasty *et al*., 1999) pose a serious doubt regarding the possibilities of different pathogenicity mechanisms prevalent in non-O1 and non-O139 strains. It is also important to emphasize that an infection by potentially pathogenic non-O1/non-O139 strains may not always lead to disease due to factors such as the susceptibility or immune status of the exposed individual and the infectious dose of the microorganism (Ottaviani *et al*., 2009). This might be the reason for rare occurrence of gastroenteritis and bacteraemia in humans as compared to diarrhoea. Hence, non O1/non O139 strains can no longer be ignored since they may contribute to both intestinal and extra intestinal infections.

The *V. cholerae* strains were screened for the presence of 13 virulence/ virulence associated genes using gene specific primers for *ctx*A, *ctx*B, *ace*, VPI, *hly*A, *omp*U, *rtx*A, *tox*R, *zot*, *nag*st, *tcp*A, *nin* and *nan*H. Positive amplicons were obtained for five genes namely; VPI, *hlyA, ompU, rtxA* and *toxR*, indicating their presence in some strains. Of the 21 strains screened for harbouring virulence genes, only BTOS6 did not yield amplicons for any of the tested genes. The genes for *ctxA*, *ctxB*, *ace*, *zot*, *nagst*, *tcpA*, *nin* and *nan*H were not detected in any of the tested strains.

The *V. vulnificus* strains were screened for the presence of 13 virulence/virulence associated genes like *ctx*A, *ctx*B, *ace*, VPI, *hly*A, *omp*U, *rtx*A, *tox*R, *zot*, *nag*st, *tcp*A, *nin* and *nan*H together with three species specific virulence associated genes, *cps* (Capsular Polysaccharide),

vvh (Haemolysin) and *viu* (Iron Acquisition). Only three genes namely VPI (33%), *tox*R (17%) and *cps* (17%) were present in the environmental strains.

The production of cholera toxin, encoded by the *ctx*AB genes, is directly responsible for the manifestation of diarrhoea; however, cholera pathogenesis relies on the synergistic action of a number of other genes, including the genes for one or more colonization factors (Kaper *et al.*, 1995). It has been presumed that the cholera toxin (CT) and toxin co-regulated pilus (TCP) are exclusively associated with clinical strains of *V. cholerae*, notably those belonging to serogroups O1 and O139, whereas reports on the incidence of CT and TCP among environmental strains of *V. cholerae* from the coastal areas of Kerala (Kumar *et al.,* 2008) and in tropical seafood in Cochin (Kumar and Lalitha, 2013) necessitated the screening for these genes in the environmental non O1 and non O139 strains, but yielded negative results.

Although the presence of virulence associated genes like *ace, zot, nagst, nin* and *nan*H were screened by PCR, no positive amplicons were obtained indicating their absence in these strains. Earlier works on *V. cholerae* non O1/ non O139 strains from Kerala have also reported negative results for these genes (Singh *et al.,* 2001; 2002; Kumar *et al.,* 2008) whereas they were reported from toxigenic O1 strains from South India (Balaji *et al.,* 2013).

Of the 21 environmental isolates of *V. cholerae* tested for the presence of the Vibrio Pathogenicity Island (VPI), positive PCR products were obtained with 9 strains (43%). It is hypothesized that all the genes on the VPI are likely to be important in disease, either having a direct role in

cholera pathogenesis or an indirect role in the transfer and mobility of the VPI, thereby creating the potential for the emergence of new epidemic and pandemic strains (Karolis *et al.,* 1998).The VPI is flanked by *att* sites that presumably function as specific attachment sites between this element and the host bacterial chromosome. It appears that possession of the VPI has allowed specific environmental strains of *V. cholerae* to become adapted to the human intestinal environment and successfully colonize it. In addition, the VPI facilitates infection by CTXΦ. The identification of potential integrase and transposase genes at each end of the VPI suggests that these genes may have had a role in the transfer and integration of the VPI into epidemic and pandemic strains (Jermyn and Boyd, 2005).

The presence of VPI was also detected in 6 out of the 18 *V. vulnificus* strains tested. O'Shea and colleagues (2004) found that the VPI of *V. cholerae* is a region with some homology to the *V. vulnificus* island-1 (VVI-1), a region unique to *V. vulnificus* strain YJ016 and is inserted at the same genome location as that of VPI, at the tRNA-met site. Both *V. cholerae* and *V. vulnificus* occupy similar environmental niches and it is possible that horizontal gene transfer between these species appears to be an emerging theme in their evolution.

Hly A hemolysin (encoded by the gene *hly*A) belongs to the El Tor family of hemolysin that lyses erythrocytes and other mammalian cells and exhibits enterotoxicity in experimental diarrhoea models, thus manifesting a major role in the pathogenesis of gastroenteritis caused by *V. cholerae* strains (Ichinose *et al.,* 1987). On screening for the presence of *hly*A gene in the environmental strains of *V. cholerae*, this gene was found to be present in 67% of the isolates under study. None of the *V. vulnificus* strains yielded

amplicons for the *hly*A gene. The relevance of the presence of this gene is due to the fact that the hemolysin produced by *V. cholerae* non-O1 is both structurally and immunologically indistinguishable from the toxigenic El Tor cytolysin (Yamamoto *et al.,* 1986) and has enterotoxic activity contributing to the pathogenesis of gastroenteritis caused by some *V. cholerae* strains lacking the gene coding for cholera toxin (Ichinose *et al.,* 1987).

The hemolysin gene of *V. vulnificus* (*vvh*) encodes the VVH protein capable of lysing erythrocytes by forming small pores in the cytoplasmic membrane of animal species (Kim *et al*., 1993). Though the *V. vulnificus* strains were tested for their characteristic *vvh* gene, no positive strains were detected.

The gene o*mp*U that produces an outer membrane protein involved in the colonization of the epithelial cells by *V. cholerae*, was detected in 5 of the environmental *V. cholerae* isolates. Moreover, OmpU mediates resistance to bile as it is cation-selective. The bile salts being negatively charged, lowers the flux of such salts through OmpU. Bile is essentially deleterious to cells because of the detergent-induced damage caused to the inner membrane. The reduced accessibility of the inner membrane to bile in OmpU-containing strains will provide protection and thus will impart such strains the ability to better survive bile-containing environments (Simonet *et al.,* 2003). Moreover, the presence of the OmpU porin has been reported to offer resistance to antimicrobial peptides in *V. cholerae* (Mathur and Waldor, 2004) suggesting the crucial role of this protein in offering antibiotic resistance to positive strains.

The RTX (repeat in toxin) belongs to a group of related exotoxins produced by a variety of pathogenic Gram-negative bacteria with hemolytic,

leukotoxic, and leukocyte-stimulating activities (Boardman and Satchell, 2004). The gene *rtx*A encoding this toxin was detected in 8 of the environmental *V. cholerae* strains and was absent in strains of *V. vulnificus*. Reports on the presence of this gene from clinical *V. cholerae* O1 biotype El Tor serotype Inaba strains isolated in Trivandrum (Mohapatra *et al.,* 2007) and V. cholerae non O1/non O139 from waters in South India (Jagadeeshan et al., 2009) have been published. It is reported that a V. *cholerae* O1 El Tor strain from which all known toxin genes excluding the *rtx* gene cluster have been deleted was capable of triggering an inflammatory response (Silva *et al.,* 1996). Chow and co-workers (2001) have also reported that strains, which cause only sporadic, milder cases of diarrhoea, do secrete the RTX cytotoxins but do not secrete CT. The role of this toxin in actin depolymerization and cross-linking in HEp-2 cells has also been demonstrated (Fullner and Mekalanos, 2000) emphasizing the importance of this gene in pathogenesis of *V. cholerae*.

The ToxR regulon, the set of genes whose expression is under the control of ToxR, regulates the cholera toxin ctxAB, the *tcp* genes required for the production of the TCP pilus, the genes encoding the outer membrane proteins OmpU and OmpT, the *acf* genes specifying an accessory colonization factor (ACF), and a group of genes, the *tag* genes (ToxR-activated genes), which have not yet been associated with a definite virulence property (Peterson and Mekalanos, 1988). The *tox*R genes are considered to be universally distributed in the family Vibrionaceae, the nucleotide sequences reflecting their phylogenetic relationship (Osorio and Klose, 2000) and the gene is regarded as an effective taxonomic marker for identification of *Vibrio spp*. (Kim *et al.*, 1999). Although the *toxR* genes have been reported in all toxigenic strains of *V. cholerae* tested

(Chakraborty *et al.,* 2000; Kondo and Ajawatanawong, 2009; Bhowmick *et al.,* 2009), their presence was detected only in certain strains of non O1/ non O139 isolates of environmental origin (Bernard, 2006; Kumar and Lalitha, 2013). Here in this study also, out of the 22 strains of *V. cholerae* tested, 9 strains harboured this upstream toxic cascade regulator.

The *tox*R regulon seems to play an important role in the survival and host-microorganism interaction of *V. vulnificus* (Lee *et al.,* 2000) and this gene was detected in three of the environmental strains studied, namely, BTAS3, BTPS6 and BTOS7. The variation regarding the detection of the *tox*R gene, considered to be universal among Vibrionaceae, indicates that the virulence genes in environmental strains may have significantly divergent nucleotide sequences or may exist with truncated or modified forms as observed in *V. alginolyticus* genome (Osorio and Klose, 2000).

The polysaccharide capsule (CPS) encoded by the *cps* gene has been revealed to be a major virulence factor of *V. vulnificus* and is reported to protect the bacteria from phagocytosis and complement-mediated killing by the host immune system (Gulig *et al.,* 2005). Three strains of *V. vulnificus*, BTPS6 from aquafarm and BTVE4 and BTVE8 from mangrove environments produced amplicons for the *cps* gene thus confirming their potential pathogenicity in these environments.

The present study indicated that VPI, *hly*A, *rtx*A, *omp*U and *tox*R are the major virulence associated genes in environmental isolates of *V. cholerae* and VPI, *cps* and *tox*R were present in *V. vulnificus*. The combination of all these toxins or individually may be responsible for causing occasional diarrhoea by non O1/non O139 strains. Non O1/non O139 strains exist as part of the normal bacterial flora of marine

environments, being considered of negligible microbiological significance for a long time (Cheasty *et al*., 1999). However, in the last decades it has been demonstrated that non cholera vibrios (NCVs) in marine environments can cause sporadic cases or occasional outbreaks of diarrhoea (Faruque *et al*., 2004), acute septicemia (Restrepo *et al*., 2006) and skin infections (Blake *et al*., 1980) in humans. Apart from these, several uncharacterized toxins might be present in non O1/non O139 strains. It should also be emphasized that an infection by potentially pathogenic non O1/non O139 strains may not always lead to disease, due to factors such as the susceptibility or immune status of the exposed individual and the infectious dose of the microorganism (Ottaviani *et al*., 2009) and this may be the reason for the rare occurrence of gastroenteritis and bacteraemia in humans as compared to diarrhoea. Molecular characterization of microbial ecosystems provides useful information about the ecology of vibrios, and environmental studies of pathogenic vibrios have been done with the expectation that strains possessing the entire complement of virulence genes would be isolated. Now it is concluded that virulence genes are dispersed among environmental strains of vibrios and may be ferried about, given the fact that most of the virulence genes that were studied are located on mobile elements. Indeed, the potential for "mixing and matching" of genes in the environment or in the human intestine, leading to new pathogenic variants, are to be addressed.

5.7. Genetic analysis of toxin-regulatory gene *tox*R

The *tox*R gene, a global regulator of virulence and membrane porin gene expression, was found to be in the ancestral chromosome of bacteria under the Vibrionaceae family making it a candidate gene for comparison and detection of *Vibrio* species. Partial to complete *tox*R gene sequences from different *Vibrio* species, including *V. cholerae* (Miller and Mekalanos,

1988; DiRita, 1992), *V. parahaemolyticus* (Lin *et al.*, 1993), *V. fischeri* (Reich and Schoolnik, 1994), *V. vulnificus*(Lee *et al.*,2000), *V. alginolyticus*, *V. fluvialis*, *V. mimicus* (Osorio and Klose, 2000), *V. hollisae* (Vuddhakul *et al.,* 2000), *V. anguillarum* (Okuda *et al.*, 2001), and *V. harveyi* (Franco and Hedreyda, 2006) have been reported. Studies on the *tox*R gene sequences have been focused mainly on the type strains of *Vibrio* species and their role in species identification and phylogeny. Gene sequence analysis of the environmental isolates of *Vibrio* species are rare (Pang *et al.,* 2006). In the present study, the amplification products of the *tox*R gene in 9 strains of *V. cholerae* and 3 strains of *V. vulnificus* were cloned and sequenced. Cloning enabled inclusion of maximum number of nucleotide sequences for analysis. The nucleotide sequences and the deduced amino acid sequences were compared with reported *tox*R sequences from other species of *Vibrio* to facilitate comparison of the gene in the environmental isolates with their close relatives.

Multiple sequence alignment of the nucleotide sequences of the environmental strains of *V. cholerae* revealed that the *tox*R gene in the environmental strains are 100% homologous to the *V. cholerae* (M21249) *tox*R gene sequence available in the GenBank database. It is also noteworthy that the environmental strains exhibited only about 75-85% sequence homology with other species of *Vibrio*, revealing higher sequence divergence with phylogenetically related *Vibrio* species. The phylogenetic tree constructed based on the nucleotide sequences also revealed the relatedness among the *V. cholerae* strains with reference to the *tox*R gene, though the strains were from different environmental sources. This result is particularly interesting as it reinforces the role of *tox*R as an ancestral gene and its use as a gene marker for species detection and classification.

Multiple sequence alignment of the amino acid sequences of the ToxR protein of *V. cholerae* also demonstrated a high level of similarity among themselves and with the *V. cholerae* strains from GenBank database. An exception was the amino acid sequence of strain BTPR5 which exhibited a marked difference in amino acid sequence up to the 150th amino acid, after which it exhibited 100% homology with other strains. This can be attributed either to a sequencing error that might have occurred or to a deletion or insertion of a nucleotide that has resulted in a frame shift mutation in the reading frame. Changes in amino acids of *tox*R may lead to changes of the expression of toxin genes, which could subsequently affect the severity of cholera infection. However, further investigations of their effects on the virulence gene expression should be conducted. Phylogenetic analysis based on ToxR amino acid sequences also clustered the environmental strains of *V. cholerae* together revealing their monophyletic nature. This result is in accordance with the study on *V. alginolyticus* by Montieri and co-workers (2010), who attributed the low genetic variability to the cohabitation of the same ecological niche where there is an apparently low evolutionary pressure.

The *tox*R gene of the environmental isolates of *V. vulnificus* were also analysed by Multiple Sequence Alignment of the nucleotide sequences and the amino acid sequences. The 3 strains of *V. vulnificus* displayed high nucleotide and amino acid sequence similarity among themselves and were also similar to the sequences of *V. cholerae* (M21249) and *V. harveyi* (DQ503438) obtained from the GenBank database, but exhibited only 72% homology to the sequences of its close relative *V. vulnificus* (AF170883). This may be due to the fact that the primers used for detection of the *tox*R gene in this study was specific for the *V. cholerae tox*R gene. Specific primers need to be designed for the *V. vulnificus* strains and this is possible

only if a considerable number of environmental strains with the *tox*R gene are available for analysis.

Availability and analysis of complete sequences of the *tox*R gene from vibrios will aid to generate baseline information important in distinguishing and identifying known, unknown, and variant environmental *Vibrio* strains. Sequence analysis of this ancestral marker gene also contributes data that could be used for phylogenetic analysis of the various strains. Moreover the hypervariable region of *tox*R could be targeted in primer design for species detection.

In an effort to elucidate the structure of the ToxR protein, the amino acid sequence of *V. cholerae* strain BTMA5 (Accession No.KF420400) was analysed using PHYRE^2 software. The results indicated maximum resemblance with the structure of DNA-binding domain of response regulator2 from *Escherichia coli* k-12 (Library id - c3zq7A). From earlier studies, it is elucidated that the ToxR is a bitopic membrane protein whose cytoplasmic domain is homologous to the winged helix-turn-helix ('winged helix') DNA-binding/transcription activation domain found in a variety of prokaryotic and eukaryotic regulators (Krukonis *et al*., 2000; Crawford *et al.,* 2003). Its periplasmic domain exists as dimer due to the presence of an interchain disulfide linkage that homo-dimerizes the native ToxR. The protein is anchored in the membrane by a single membrane-spanning segment, its N- and C-terminal domains facing the cytoplasm and the periplasm, respectively. Fengler and colleagues (2012) have revealed the role of cysteine residues in dimerization of ToxR using site-directed mutagenesis.

The transmembrane protein, ToxR, is significant in the pathogenesis of *Vibrio* infections. The master regulator for the expression of *ctx*AB in *V.*

cholerae, it further regulates at least 17 distinct genes including the TCP colonization factor (Taylor *et al.,* 1987), the accessory colonization factor (Peterson and Mekalanos, 1988), the OMPs, OmpT and OmpU (Miller and Mekalanos, 1988), and three other lipoproteins (Parsot *et al.,* 1991). Except for the *ctx*AB genes, other genes in the ToxR regulon are controlled through ToxT, which in turn is controlled by ToxR. The ToxR protein binds to a tandemly repeated 7 bp DNA sequence found upstream of the *ctx*AB structural gene and increases the transcription of *ctx*AB, resulting in higher levels of CT expression (DiRita *et al.,* 1991). The activity of ToxR is enhanced by ToxS that assemble ToxR monomers into the dimeric form (Miller and Mekalanos, 1988). ToxR is at the top of the regulatory cascade that controls the expression of CT and other important virulence factors in *V. cholerae*, while the expression of ToxR itself remains under the control of environmental factors (Skorupski and Taylor, 1997).

The complete sequences of the *tox*R gene from a number of clinical and environmental isolates are necessary to elucidate the exact structure and function of the ToxR protein. Mutation analysis would also help to identify ToxR-regulated genes. Since the ToxR protein has been found to regulate virulence gene expression in *Vibrio* species, future studies on the *tox*R gene and the function of the protein will provide valuable insight into the pathogenic mechanisms of these environmental isolates.

5.8. Pathogenicity studies with the nematode *Caenorhabditis elegans* as model organism

The pathogenicity studies was performed using the nematode *Caenorhabditis elegans* as a model system, to determine whether *Vibrio* sp. could serve as a food source for *C. elegans* and to assess the pathogenicity

of the test strains of *V. cholerae* and *V. vulnificus* on the nematode. Practically and ethically attractive as a model system, *C. elegans* is increasingly recognized as relevant for the study of bacterial pathogenesis as the nematode is susceptible to a surprisingly broad range of bacteria and may constitute a useful model for the study of both pathogens and symbionts (Couillault and Ewbank, 2002). *C. elegans* has been used as an invertebrate host model to identify and assess virulence factors of several *Vibrio* species including *V. cholerae* (Cinar *et al.,* 2010; Sahu *et al.,* 2012.), *V. vulnificus* (Dhakal *et al.,* 2006), *V. parahaemolyticus* (Durai *et al.,* 2011a) and *V. alginolyticus* (Durai *et al.,* 2011b).

The bioassay was conducted with *E. coli* strain OP50 as the food source in control plates, environmental *V. cholerae* strain BTOS6, negative for all tested virulence genes, to check for the suitability of *Vibrio* sp. as a food source for the nematode; *V. cholerae* Co366 El Tor, a clinical pathogenic strain and *V. cholerae* strain BTPR5 from seafood (Prawn) and positive for the tested virulence genes like VPI, *hly*A, *omp*U, *rtx*A and *tox*R. From the results, it was concluded that *V. cholerae* strain BTOS6 could serve as a food source in place of *E. coli* strain OP50, indicated by nearly similar LD_{50} values in both cases. Behavioural aberrations like sluggish movement and lawn avoidance behaviour and morphological abnormalities like pharyngeal and intestinal distensions and bagging were exhibited by the worms fed on *V. cholerae* Co366 El Tor strain and environmental BTPR5 indicating their pathogenicity on the nematode. Earlier works have reported that *V. cholerae* causes lethal infection in the nematode *C. elegans* via a cholera toxin (Ctx) and toxin co-regulated pili (Tcp) independent process, providing a useful host model system to screen for the virulence factors other than Ctx and Tcp ((Vaitkevicius *et al.,* 2006; Sahu *et al.,* 2012). It is reported that worm lethality effect inflicted by *V.*

cholerae, is mediated by LuxO-regulated genes in the quorum sensing (QS) pathway, such as *hap*R, *V. cholerae* metalloprotease gene *Prt*V (Vaitkevicius *et al.,* 2006), and VCC encoding gene *hly*A (Cinar *et al.,* 2010). *hly*A is also testified to cause developmental delay and intestinal vacuolation in *C. elegans* (Cinar *et al.,* 2010). This study also supports the work of Vaitkevicius and colleagues (2006) stating that CTX and TCP are not mandatory for lethal infections in the nematode, however, mutant strains expressing individual genes should necessarily be included in the assay to elucidate the role of each gene in the pathogenesis.

The pathogenicity of *V. vulnificus*, a human opportunistic pathogen which causes fatal septicemia and necrotic wound infection, was also tested on the nematode *C. elegans*. Environmental *V. vulnificus* strain BTPS6 which tested positive for 3 virulence genes, namely, *cps*, *tox*R and VPI, and *V. vulnificus* strain BTMM7 that did not possess any of the tested virulence gene, were used as test strains for the bioassay. The results obtained were in accordance with the study of Dhakal and co-workers (2006), where a marked reduction was observed in the life span of worms fed on environmental strains of *V. vulnificus* rather than on the ordinary laboratory food source, *E. coli* OP50. Behavioural abnormalities like sluggish movement, lawn avoidance and bagging were observed in the worms fed with strain BTPS6, but the pharynx and the intestine were intact indicating that the contribution of the virulence genes to the pathogenesis of gastroenteritis by *V. vulnificus* has to be elucidated by further studies.

The most apparent manifestation of pathogenicity by *V. cholerae* strain BTPR5 was the pharyngeal and intestinal distensions. This is a typical symptom shown by *C. elegans* infected by pathogenic *Salmonella* (Aballay

et al.,2000) and virulent bacterial strains like *V. vulnificus (*Dhakal *et al.*, 2006), *V.cholerae* (Vaitkevicius *et al.*, 2006), *V. parahaemolyticus* (Durai *et al.*, 2011a) and *V. alginolyticus* (Durai *et al.*, 2011b). The distension of the intestine may be due to clumping of pathogenic microorganisms accumulated within and probably the primary cause of early deaths in the worm (Dhakal *et al.*, 2006). Ingestion of the pathogen leads to an intestinal infection characterized by the collapse of the intestinal epithelial cells, the proliferation (or accumulation) of the pathogenic microbe in the alimentary tract and premature death of the infected animals. Normally, the pharyngeal grinder of the worm efficiently disrupts the *E. coli* on which it feeds and essentially no intact bacterial cells are found within the intestinal lumen.

Another behavioural aberration exhibited by *C. elegans* when fed with *V. cholerae* strain BTPR5 and *V. vulnificus* strain BTPS6 was the lawn avoidance behaviour. Recognition and ability to distinguish among pathogenic bacteria represents a potentially valuable behavioural adaptation in *C. elegans.* The worms can discriminate between different species of bacteria and modify its olfactory preferences after exposure to pathogenic bacteria (Zhang *et al.*, 2005). It is established that some pathogenic bacteria elicit lawn avoidance by *C. elegans*, where the worms initially enter the bacterial lawn but later exit and remain near the edge of the lawn (Pujol *et al.*, 2001). Lawn avoidance deprives the nematode of bacterial food, which is otherwise consumed continuously. In the present study, the lawn avoidance behaviour was observed only when the worms were in lawns of *V. cholerae* strain BTPR5 and *V. vulnificus* strain BTPS6, both of which possessed virulence factors. This behaviour may be regulated by the sensory cues that are received by the chemosensory and thermosensory neurons of the worm (Shtonda and Avery, 2006).

Bagging, a phenomenon where the worms became laden with eggs, which in some cases hatched internally, was an abnormality observed when the worms were fed with *V. cholerae* strain BTPR5 and *V. vulnificus* strain BTPS6. This phenomenon has not been observed in earlier works on *Vibrio- C. elegans* interaction studies. Bagging is suggested to be caused by a weakening of the infected worm, rendering it unable to lay eggs normally (Sifri *et al.,* 2003). Although it is not known why bagging is prevalent when nematodes feed on bacterial pathogens, one possibility is that infected worms may become too weak to lay eggs normally.

While much can be learned about bacterial host interactions using *C. elegans* as an invertebrate host model, the model has its strengths and weaknesses. One drawback of using the nematode as a model to study bacterial physiology and pathogenesis is the fact that *C. elegans* is grown at temperatures below 25^{o}C, whereas some virulence genes involved in mammalian pathogenesis are only expressed at 37^{o}C. Furthermore, the immune responses to infection are relatively dissimilar between invertebrates and mammals. Nevertheless, no animal model completely simulates all features of human disease. *C. elegans* is a convenient animal model since the genome is fully sequenced and different phenotypes and mutants are easily available. The strains can be kept as frozen stocks for long periods of time. In addition, the size and short generation time of *C. elegans* enable studies that require a large number of animals, which are unfeasible with mammals due to ethical constraints, cost and space requirements.

This study has revealed that although the environmental strains of *V. cholerae* (BTPR5) and *V. vulnificus* (BTPS6) harboured none of the major virulence genes, they possessed genes that had rendered them

potential pathogenicity as revealed by their antagonistic effect on the life of *C. elegans*. Further studies could be done to identify *V. cholerae* and *V. vulnificus* virulence factors important for mammalian pathogenesis and of the *C. elegans* response to individual gene mutants of these pathogens to provide a unique starting point to identify previously unknown signalling pathways and molecular mechanisms of host immune response to bacterial virulence. Understanding how these pathogens disrupts host defence and causes host damage and death is critical to identifying new therapeutic targets to treat infectious diseases.

Briefly, the present study revealed the presence of *Vibrio* species in marine environmental samples like seafood, aquafarms and mangroves. These *Vibrio* species were adapted to their ecological niche as evidenced by their biochemical profile and their antibiotic susceptibility. The 16S rDNA sequence analysis of the environmental isolates aided in their identification at the species level and enabled a basic study of the phylogenetic relationship among the *Vibrio* isolates. *Vibrio cholerae* and *Vibrio vulnificus* were found to be the predominant species in the marine environment and molecular typing revealed the relatedness and variability among strains of the same species. The occurrence of virulence associated genes among the *V. cholerae* and *V. vulnificus* strains is a cause of concern as these strains constitute a reservoir of diverse virulence genes in the marine environment that play a crucial role in pathogenicity. The genetic analysis of the *tox*R gene in *V. cholerae* and *V. vulnificus* will aid in the detection and identification of environmental strains of these species. The potential pathogenicity of *V. cholerae* strain BTPR5 from prawn is alarming as evidenced by the pathogenicity test on *C. elegans* and this strain has to be subjected to further analysis to monitor its role in ecology and epidemiology.

Chapter 6

SUMMARY AND CONCLUSION

The marine environment plays a significant role in ecological and epidemiological studies of pathogenic forms of bacteria, being a cauldron or reservoir of prospective bacteria, allowing interactions and exchange of genetic material. Human dependence on marine environments for fisheries and aquaculture implicates the importance of monitoring of these environments to prevent possible health risks. This study was an attempt to screen the marine environments like aquafarms and mangroves, seafood samples like prawn, squid and mussel for the presence of *Vibrios.* Of the 134 isolates obtained from the various samples, 45 were segregated as *Vibrio* species as they were Gram negative, oxidase positive, fermentative, with or without gas production on MOF media, and appeared as yellow/green coloured colonies on TCBS (Thiosulfate Citrate Bile salt Sucrose).

Partial 16S rDNA sequence analysis was utilized for species level identification of the isolates segregated as *Vibrios.* The strains were identified as *V. cholerae* (N=21), *V. vulnificus* (N=18), *V. parahaemolyticus* (N=3), *V. alginolyticus* (N=2) and *V. azureus* (N=1).

The genetic relatedness and variations among the 45 *Vibrio* isolates were elucidated based on 16S rDNA sequences. Strains of the same species claded together revealing their monophyletic nature. Dendrograms depicting the phylogenetic and evolutionary relationships among the 21 *V. cholerae*

strains and 18 *V. vulnificus* strains were analyzed. In the study involving *V. cholerae* strains, 20 of them formed a single clade, while strain BTMM4 was separated from the others. The *V. vulnificus* group cladded into 2 separate groups, with 15 strains in one clade and 3 in another, but in general, exhibited close relationship to one another. This study substantiates the use of 16S rDNA sequences both as a phylogenetic marker and as an identification tool.

Phenotypic characterization of the isolates was based on their response to 12 biochemical tests namely Voges-Proskauers's (VP test), arginine dihydrolase , tolerance to 3% NaCl, ONPG test that detects β-galactosidase activity, and tests for utilization of citrate, ornithine, mannitol, arabinose, sucrose, glucose, salicin and cellobiose. The isolates exhibited diverse biochemical patterns, some specific for the species and others indicative of the environmental source from which they were isolated.

Antibiogram for the isolates was determined subsequent to testing their susceptibility to 12 antibiotics i.e. gentamycin, ampicillin, nalidixic acid, vancomycin, cefixime, rifampicin, tetracycline and chloramphenicol, by the disc diffusion method. Varying degrees of resistance to gentamycin (2.22%), ampicillin (62.22%), nalidixic acid (4.44%), vancomycin (86.66), cefixime (17.77%), rifampicin (20%), tetracycline (42.22%) and chloramphenicol (2.22%) was exhibited. All the isolates were susceptible to streptomycin, co-trimoxazole, trimethoprim and azithromycin. Isolates from all the three marine environments exhibited multiple antibiotic resistance, with high MAR index value, suggestive of the use of antibiotics and the stressful nature in these environments.

The 21 strains of *V. cholerae* were serogrouped by screening for the presence O1*rfb* and O139*rfb* marker genes by PCR. As the specific

amplicons were absent in all the tested strains, all the *V. cholerae* environmental isolates obtained in this study were categorised as belonging to non O1/ non O139 serogroups.

Molecular typing was by PCR based method, with primers targeting specific conserved sequences in the bacterial genome. The isolates identified as *V. cholerae* and *V. vulnificus* were subjected to molecular typing methods such as ERIC PCR and BOX PCR. Both the molecular fingerprinting methods revealed genetic heterogeneity within the environmental isolates of *V. cholerae* and *V. vulnificus*. The analysis of the banding pattern obtained in the two methods aided clustering of the strains and highlighted the intraspecies relatedness and variability and their phylogenetic lineages.

Polymerase Chain Reaction was employed as a rapid screening method to verify for the presence of selected virulence genes. The screening for virulence/virulence associated genes was performed in *V. cholerae* and *V. vulnificus* strains for 13 genes namely *ctx*A, *ctx*B, *ace*, VPI, *hly*A, *omp*U, *rtx*A, *tox*R, *zot*, *nag*st, *tcp*A, *nin* and *nan*H. The *V.vulnificus* strains were also screened for three species specific genes viz., *cps*, *vvh* and *viu*. In *V. cholerae* strains, the virulence associated genes like VPI, *hly*A, *rtx*A, *omp*U and *tox*R were confirmed by PCR. All the isolates, except for strain BTOS6, harbored at least one or a combination of the tested genes and *V. cholerae* strain BTPR5 isolated from prawn hosted the highest number of virulence associated genes. Among the *V. vulnificus* strains, only 3 virulence genes, VPI, *tox*R and *cps*, were confirmed out of the 16 tested and only 7 of the isolates had these genes in one or more combinations. Strain BTPS6 from aquafarm and strain BTVE4 from mangrove samples yielded positive amplification for the three genes.

The *toxR* gene from 9 strains of *V. cholerae* and 3 strains of *V. vulnificus* were cloned and sequenced for genetic analysis. Phylogenetic analysis was done based on the nucleotide sequences and the amino acid sequences. Multiple sequence alignment of the nucleotide sequences and amino acid sequences of the environmental strains of *V. cholerae* revealed that the *toxR* gene in the environmental strains are 100% homologous to themselves and to the *V. cholerae tox*R gene sequence available in the Genbank database. The sequences exhibited only 75-85% sequence homology with other related species of *Vibrio*s. The 3 strains of *V. vulnificus* displayed high nucleotide and amino acid sequence similarity among themselves and to the sequences of *V. cholerae* and *V. harveyi* obtained from the GenBank database, but exhibited only 72% homology to the sequences of its close relative *V. vulnificus*.

Structure prediction of the ToxR protein of *Vibrio cholerae* strain BTMA5 was done using PHYRE^2 software. The deduced aminoacid sequence showed maximum resemblance with the structure of DNA-binding domain of response regulator2 from *Escherichia coli* k-12 (Library id - c3zq7A).Template based homology modelling in PHYRE^2 successfully modelled the predicted protein and its secondary structure based on protein data bank (PDB) template c3zq7A which consists of chain A representing kdp operon transcriptional regulatory protein kdpe.

The pathogenicity studies were performed using the nematode *Caenorhabditis elegans* as a model system. The assessment of pathogenicity of environmental strain of *V. cholerae* was conducted with *E. coli* strain OP50 as the food source in control plates, environmental *V. cholerae* strain BTOS6, negative for all tested virulence genes, to check for the suitability

of *Vibrio* sp. as a food source for the nematode; *V. cholerae* Co 366 El Tor, a clinical pathogenic strain and *V. cholerae* strain BTPR5 from seafood (Prawn) and positive for the tested virulence genes like VPI, *hly*A, *omp*U, *rtx*A and *tox*R. It was concluded that *V. cholerae* strain BTOS6 could serve as a food source in place of *E. coli* strain OP50 but behavioural aberrations like sluggish movement and lawn avoidance behaviour and morphological abnormalities like pharyngeal and intestinal distensions and bagging were exhibited by the worms fed on *V. cholerae* Co 366 El Tor strain and environmental BTPR5 indicating their pathogenicity to the nematode.

Assessment of pathogenicity of the environmental strains of *V. vulnificus* was performed with *V. vulnificus* strain BTPS6 which tested positive for 3 virulence genes, namely, *cps*, *tox*R and VPI, and *V. vulnificus* strain BTMM7 that did not possess any of the tested virulence genes. A reduction was observed in the life span of worms fed on environmental strain of *V. vulnificus* BTMM7 rather than on the ordinary laboratory food source, *E. coli* OP50. Behavioural abnormalities like sluggish movement, lawn avoidance and bagging were also observed in the worms fed with strain BTPS6, but the pharynx and the intestine were intact indicating that the contribution of the virulence genes to the pathogenesis of gastroenteritis by *V. vulnificus* has to be elucidated by further studies.

Conclusion

Environmental reservoirs are hot spots in the global environment supporting survival, persistence and dissemination of pathogens. The isolation and abundance of *Vibrio* species in the marine environments recognizes this milieu as a putative environmental reservoir where these pathogens persist and determine their ecology. The presence of multi drug resistant strains in

environmental isolates cannot be ignored and future epidemics may be imminent. The unique biological features of *Vibrio* species and the rapid development of molecular biological techniques present an accessible gateway for investigating the molecular events leading to genetic diversity in the marine environment. Using nucleic acids as targets, the methods of fingerprinting like ERIC PCR and BOX PCR, have revealed that the marine environment is a largely unexplored frontier harbouring a virtual pantheon of phenotypic and genetic diversity for this potential pathogenic group of bacteria.

The distribution of virulence associated genes, which aid in the bacterial invasion, process of cell adhesion, colonization, cytotoxicity, and tissue destruction, in the environmental isolates of *V. cholerae* and *V. vulnificus* provide tangible material for further investigation. These environmental strains are to be dealt with caution as they constitute a major reservoir of diverse virulence genes in the marine environment and play a crucial role in pathogenicity and horizontal gene transfer. The genomic information on the *tox*R gene of these *Vibrios* would be valuable for future studies on phylogeny, virulence, and genes regulated by the *tox*R gene product. The practical applications of protein structure prediction are many and varied, including guiding the development of functional hypotheses about hypothetical proteins, improving phasing signals in crystallography, selecting sites for mutagenesis and the rational design of drugs.

The pathogenicity studies on the nematode, *C. elegans,* underscores the virulence potential of non-pandemic environmental strains of *V. cholerae* and *V. vulnificus.*

The current study emphasizes the need for constant surveillance involving systematic environmental sampling and multigene analyses of

loci encoding virulence determinants to detect possible emergence of virulent and variant strains of these capricious pathogens. Strong regional commitment to surveillance, continuous monitoring of the changing trends in antimicrobial resistance patterns and emergence of new toxigenic clones is highly warranted in the context of the epidemiology of infectious diseases caused by environmental *Vibrios*.

REFERENCES

Aballay, A., Yorgey, P., & Ausubel, F. M. (2000). *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Current Biology*, 10(23), 1539-1542.

Abbott, S. L., & Janda, J. M. (1994). Severe gastroenteritis associated with *Vibrio hollisae* infection: report of two cases and review. *Clinical Infectious Diseases*, 18(3), 310-312.

Abraham T.J., Shanmugam S.A., & Sundararaj V. (1993) Microbial disease diagnosis and control in shrimp culture systems. *Seafood Export Journal* XXV, 13-17.

Abraham T.J., & Manley R. (1995) Luminous and non-luminous *Vibrio harveyi* associated with shell disease in cultured *Penaeus indicus*. *Journal of Aquaculture in Tropics*, 10, 273-276.

Acosta, C. J., Galindo, C. M., Kimario, J., Senkoro, K., Urassa, H., Casals, C. & Alonso, P. L. (2001). Cholera outbreak in southern Tanzania: Risk factors and patterns of transmission. *Emerging infectious diseases*, *7*(3 Suppl), 583.

Actis, L.A., Tolmasky, M.E., & Crosa, J.H. (1999). *Vibriosis*. In: Woo, P.T.K., & Bruno, D.W. (Eds.), *Fish Diseases and Disorders*, Vol. 3, CAB International Publication, United Kingdom, pp. 523– 558.

Adams, M.R. & Moss, M.O. (2008). *Bacterial Agents of Foodborne Illness*. In: *Food Microbiology*, 3rd Edition, RSC, Cambridge United Kingdom, pp. 182-269.

Adebayo-Tayo, B. C., Okonko, I. O., Esen, C. U., Odu, N. N., Onoh, C. C., & Igwiloh, N. J. P. (2011). Incidence of potentially pathogenic *Vibrio* spp. in fresh seafood from Itu Creek in Uyo, Akwa Ibom State, Nigeria. *World Applied Sciences Journal*, *15*(7), 985-991.

Adeleye, I.A., Daniels, F.V., & Enyinnia, V.A. (2010). Characterization and pathogenicity of *Vibrio spp*. contaminating seafoods in Lagos, Nigeria. *Internet Journal of Food Safety*, Vol.12, 1-9

Aeckersberg, F., Lupp, C., Feliciano, B., & Ruby, E. G. (2001). *Vibrio fischeri* outer membrane protein OmpU plays a role in normal symbiotic colonization. *Journal of Bacteriology*, 183(22), 6590-6597.

Agarwal, V., Biswas, M., Pathak, A. A., & Saoji, A. M. (1995). Rapid detection of *Vibrio cholerae* 0139 in faecal specimens by coagglutination. *The Indian Journal of Medical Research*, 101, 55-56.

Alagappan, K. M., Deivasigamani, B., Somasundaram, S. T., & Kumaran, S. (2010). Occurrence of *Vibrio parahaemolyticus* and its specific phages from shrimp ponds in east coast of India. *Current Microbiology*, 61(4), 235-240.

Alam, M., Sultana, M., Nair, G. B., Siddique, A. K., Hasan, N. A., Sack, R. B., Sack, A.D., & Colwell, R. R. (2007). Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proceedings of the National Academy of Sciences*, 104(45), 17801-17806.

Alavandi, S. V., Manoranjita, V., Vijayan, K. K., Kalaimani, N., & Santiago, T. C. (2006). Phenotypic and molecular typing of *Vibrio harveyi* isolates and their pathogenicity to tiger shrimp larvae. *Letters in Applied Microbiology*, *43*(5), 566-570.

Albert, M.J., Hossain, M.A., Alam, K., Kabir, I., Neogi, P.K.B., & Tzipori, S. (1991). A fatal case associated with shigellosis and *Vibrio fluvialis* bacteremia. *Diagnosis of Microbiological Infectious Diseases*, 14, 509-510.

Albert, M.J. (1996). Epidemiology & molecular biology of *Vibrio cholerae* O139 Bengal. *Indian Journal of Medical Research,* 104, 14-27.

Alitheen, M., Banu, N., Radu, S., Abdul Rahim, R., Lihan, S., & Yeap, S. K. (2009). Random amplified polymorphic DNA-PCR and ERIC PCR analysis on *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. *International Food Research Journal*, 16, 141-150.

Allain, G., Arzul, I., Chollet, B., Cobret, L., de Decker, S., Faury, N., & Renault, T. (2009). Summer mortality outbreaks of French Pacific oysters, *Crassostrea gigas,* in 2008: Research and detection of pathogens. In *Proceedings of 14th EAFP International Conference, Diseases of Fish and Shellfish* (Vol. 127).

Allam, B., Paillard, C., Auffret, M., & Ford, S. E. (2006). Effects of the pathogenic *Vibrio tapetis* on defence factors of susceptible and non-susceptible bivalve species: II. Cellular and biochemical changes following in vivo challenge. *Fish & Shellfish Immunology*, 20(3), 384-397.

Alm, R. A., Braun, G., Morona, R., & Manning, P. A. (1986). Detection of an OmpA-like protein in *Vibrio cholerae*. *FEMS Microbiology Letters*, 37(1), 99-104.

Alsina, M., & Blanch, A. R. (1994a). A set of keys for biochemical identification of environmental *Vibrio* species. *Journal of Applied Microbiology*, *76*(1), 79-85.

Alsina, M., & Blanch, A. R. (1994b). Improvement and update of a set of keys for biochemical identification of *Vibrio* species. *Journal of Applied Microbiology*, *77*(6), 719-721.

Alsina, M., Martinez-Picado, J., Jofre, J., & Blanch, A. R. (1994c). A medium for presumptive identification of *Vibrio anguillarum*. *Applied and Environmental Microbiology*, *60*(5), 1681-1683.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403-410.

Altun, Z. F., & Hall, D. H. (2009). *Hermaphrodite Introduction*, <http://wormatlas. org/hermaphrodite/introduction/ Introframeset.html>

Andrade, J. R., da-Silva, J. L., Barbosa, M. F., & Caldas, C. A. (1991). Preliminary report on the application of the coagglutination test for rapid diagnosis of cholera in the upper Solimoes River area in the Brazilian Amazon region. *Brazilian Journal of Medical and Biological Research* 25(4), 375-378.

Aravindan, N., & Kalavati, C. (1997). The impact of the nutritional status on disease resistance in the early stages of Indian white prawn, *Penaeus indicus*. *Proceedings of the Zoological Society of Calcutta, India* 50, 96-101.

Arboleda, M., & Reichardt, W. (2009). Epizoic communities of prokaryotes on healthy and diseased scleractinian corals in Lingayen Gulf, Philippines. *Microbial Ecology*, 57(1), 117-128.

Arias, C. R., Garay, E., & Aznar, R. (1995). Nested PCR method for rapid and sensitive detection of *Vibrio vulnificus* in fish, sediments, and water. *Applied and Environmental Microbiology*, 61(9), 3476-3478.

Arias, C. R., Verdonck, L., Swings, J., Garay, E., & Aznar, R. (1997a). Intraspecific differentiation of *Vibrio vulnificus* biotypes by amplified fragment length polymorphism and ribotyping. *Applied and Environmental Microbiology*, 63(7), 2600-2606.

Arias, C. R., Verdonck, L., Swings, J., Aznar, R., & Garay, E. (1997b). A polyphasic approach to study the intraspecific diversity amongst *Vibrio vulnificus i*solates. *Systematic and Applied Microbiology*, 20(4), 622-633.

Arita, M., Takeda, T., Honda, T., & Miwatani, T. (1986). Purification and characterization of *Vibrio cholerae* non-O1 heat-stable enterotoxin. *Infection and Immunity*, 52(1), 45-49.

Asakura, H., Ishiwa, A., Arakawa, E., Makino, S. I., Okada, Y., Yamamoto, S., & Igimi, S. (2007). Gene expression profile of *Vibrio* cholerae in the cold stress induced viable but non□culturable state. Environmental Microbiology, 9(4), 869-879.

Attridge, S. R., Manning, P. A., Holmgren, J., & Jonson, G. (1996). Relative significance of mannose-sensitive hemagglutinin and toxin-coregulated pili in colonization of infant mice by *Vibrio cholerae* El Tor. *Infection and Immunity*, 64(8), 3369-3373.

Austin, B., & Austin, D.A. (1993). *Bacterial Fish Pathogens: Disease in Farmed Wild Fish.* 2nd Edition, Ellis Harwood Ltd. England, pp. 266-296.

Austin, B., Stuckey, L. F., Robertson, P. A. W., Effendi, I., & Griffith, D. R. W. (1995). A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii*. *Journal of Fish Diseases*, 18(1), 93-96.

Austin B., & Austin, D. (2007). *Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish*. 4th Edition, Springer, Chechester, England, pp. 342-357.

Aznar, R., Ludwig, W., & Schleifer, K. H. (1993). Ribotyping and randomly amplified polymorphic DNA analysis of *Vibrio vulnificus* Biotypes. *Systematic and Applied Microbiology*, 16(2), 303-309.

Bachellier, S., Clement, J. M., & Hofnung, M. (1999). Short palindromic repetitive DNA elements in Enterobacteria: a survey. *Research in Microbiology*, 150(9), 627-639.

Baffone, W., Tarsi, R., Pane, L., Campana, R., Repetto, B., Mariottini, G. L., & Pruzzo, C. (2006). Detection of free□living and plankton□bound *Vibrios* in coastal waters of the Adriatic Sea (Italy) and study of their pathogenicity□associated properties. Environmental Microbiology, 8(7), 1299-1305.

Bakr, W. M.K., Hazzah, W.A., & Abaza, A.F. (2011). Detection of *Salmonella* and *Vibrio* species in some seafood in Alexandria. *Journal of American Science*, 7(9), 663.

Balaji, K., Okonjo, P. A., Thenmozhi, R., & Karutha Pandian, S. (2013). Virulence and multidrug resistance patterns of *Vibrio cholerae* O1 isolates from diarrheal outbreaks of South India during 2006–2009. *Microbial Drug Resistance*, 19(3), 198-203.

Balcazar, J. L., Loureiro, S., Da Silva, Y. J., Pintado, J., & Planas, M. (2010). Identification and characterization of bacteria with antibacterial activities isolated from seahorses (*Hippocampus guttulatus*). *The Journal of Antibiotics*, 63(5), 271-274.

Bally, M., & Garrabou, J. (2007). Thermodependent bacterial pathogens and mass mortalities in temperate benthic communities: a new case of emerging disease linked to climate change. *Global Change Biology*, 13(10), 2078-2088.

Banin, E., Israely, T., Kushmaro, A., Loya, Y., Orr, E., & Rosenberg, E. (2000). Penetration of the coral-bleaching bacterium *Vibrio shiloi* into *Oculina patagonica*. *Applied and Environmental Microbiology*, 66(7), 3031-3036.

Barbieri, E., Falzano, L., Fiorentini, C., Pianetti, A., Baffone, W., Fabbri, A., Matarrese, P., Casiere, A., Katouli, M., Kuhn, I., Mollby, R., Bruscolini, F., & Donelli, G. (1999). Occurrence, diversity, and pathogenicity of halophilic *Vibrio* spp. and non-O1 *Vibrio cholerae* from estuarine waters along the Italian Adriatic coast. *Applied Environmental Microbiology*, 65, 2748–2753.

Basu, A., Garg, P., Datta, S., Chakraborty, S., Bhattacharya, T., Khan, A., Ramamurthy, T., Bhattacharya, S.K., Yamasaki, S., Takeda, Y., & Nair, G.B. (2000). *Vibrio cholerae* O139 in Calcutta, 1992-1998: incidence, antibiograms, and genotypes. *Emerging Infectious Diseases*, 6(2), 139-147.

Baudry, B., Fasano, A., Ketley, J., & Kaper, J. B. (1992). Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. *Infection and Immunity*, 60(2), 428-434.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C. T., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, 45(4), 493.

Baumann, P., Baumann, L., Bang, S. S., & Woolkalis, M. J. (1980). Reevaluation of the taxonomy of *Vibrio*, *Beneckea*, and *Photobacterium*: Abolition of the genus *Beneckea*. *Current Microbiology*, 4(3), 127-132.

Baumann, P., Furniss, A.L., & Lee, J.V. (1984). *Vibrio*. In: Kreig, N.R., Holt, J.G. (Eds.), *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkins Co., Baltimore, pp. 518–538.

Beaz□Hidalgo, R., Balboa, S., Romalde, J. L., & Figueras, M. J. (2010). Diversity and pathogenecity of *Vibrio* species in cultured bivalve molluscs. Environmental Microbiology Reports, 2(1), 34-43.

Beazley, W. A., & Palmer, G. G. (1992). TCI–a new bile free medium for the isolation of *Vibrio* species. *Australian Journal of Medical Science*, 13, 25-27.

Ben-Haim, Y., & Rosenberg, E. (2002). A novel *Vibrio* sp. pathogen of the coral *Pocillopora damicornis*. *Marine Biology*, 141(1), 47-55.

Ben-Haim, Y., Thompson, F. L., Thompson, C. C., Cnockaert, M. C., Hoste, B., Swings, J., & Rosenberg, E. (2003). *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. *International Journal of Systematic and Evolutionary Microbiology*, *53*(1), 309-315.

Bernard, R.S.W. (2006). Studies on ribotyping, integron genes and pathogenecity of marine *Vibrios*. PhD Thesis. Cochin University of Science and Technology, India.

Bhanumathi, R., Sabeena, F., Isac, S. R., Radhakutty, G., & Singh, D. V. (2002). Characterization of a toxigenic *Vibrio cholerae* O139 strain belonging to a new ribotype and isolated from a diarrheal patient. *Journal of Clinical Microbiology*, 40(12), 4779-4781.

Bhowmick, T. S., Das, M., Ruppitsch, W., Stoeger, A., Pietzka, A. T., Allerberger, F., Rodrigues, D.P., & Sarkar, B. L. (2009). Detection of virulence-associated and regulatory protein genes in association with phage typing of human *Vibrio cholerae* from several geographical regions of the world. *Journal of Medical Microbiology*, 58(9), 1160-1167.

Bimboim, H.C., & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 7(6), 1513-1523.

Bina, J., Zhu, J., Dziejman, M., Faruque, S., Calderwood, S., & Mekalanos, J. (2003). ToxR regulon of *Vibrio cholerae* and its expression in *Vibrios* shed by cholera patients. *Proceedings of the National Academy of Sciences*, 100(5), 2801-2806.

Biosca, E. G., Amaro, C., Marco-Noales, E., & Oliver, J. D. (1996). Effect of low temperature on starvation-survival of the eel pathogen *Vibrio vulnificus* Biotype 2. *Applied and Environmental Microbiology*, 62(2), 450-455.

Biosca, E. G., Amaro, C., Larsen, J. L., & Pedersen, K. (1997). Phenotypic and genotypic characterization of *Vibrio vulnificus*: proposal for the substitution of the subspecific taxon biotype for serovar. *Applied and Environmental Microbiology*, 63(4), 1460-1466.

Bird, D. M., & Opperman, C. H. (1998). *Caenorhabditis elegans*: A genetic guide to parasitic nematode biology. *Journal of Nematology*, 30(3), 299.

Bisharat, N., Agmon, V., Finkelstein, R., Raz, R., Ben-Dror, G., & Lerner, L. (1999). Clinical, epidemiological, and microbiological features of *Vibrio vulnificus* biotype 3 causing outbreaks of wound infection and bacteraemia in Israel. *The Lancet* 354, 1421-1424.

Biyela, P., Lin, J., & Bezuidenhout, C. (2004). The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Science & Technology*, 50(1), 45-50.

Blackwell, K. D., & Oliver, J. D. (2008). The ecology of *Vibrio vulnificus*, *Vibrio cholerae* and *Vibrio parahaemolyticus* in North Carolina estuaries. *The Journal of Microbiology*, 46(2), 146-153.

Blake, P. A., Merson, M. H., Weaver, R. E., Hollis, D. G., & Heublein, P. C. (1979). Disease caused by a marine *Vibrio*. Clinical characteristics and epidemiology. *The New England Journal of Medicine*, 300(1), 1.

Blake, P. A., Weaver, R. E., & Hollis, D. G. (1980). Diseases of humans (other than cholera) caused by *Vibrios*. *Annual Reviews in Microbiology*, 34(1), 341-367.

Blake, P. A. (1994). *Historical perspectives on pandemic cholera: Vibrio cholerae and cholera- Molecular to global perspectives.* ASM Press, Washington, DC. pp. 293-295.

Boardman, B. K., & Satchell, K. J. F. (2004). *Vibrio cholerae* strains with mutations in an atypical type I secretion system accumulate RTX toxin intracellularly. *Journal of Bacteriology*, 186(23), 8137-8143.

Bode, R. B., Brayton, P. R., Colwell, R. R., Russo, F. M., & Bullock, W. E. (1986). A new *Vibrio* species, *Vibrio cincinnatiensis*, causing meningitis: successful treatment in an adult. *Annals of Internal Medicine*, 104(1), 55-56.

Boer, S. I., Heinemeyer, E. A., Luden, K., Erler, R., Gerdts, G., Janssen, F., & Brennholt, N. (2013). Temporal and spatial distribution patterns of potentially pathogenic *Vibrio* spp. at recreational beaches of the German North Sea. *Microbial Ecology*, 1-16.

Bonner, J. R., Coker, A. S., Berryman, C. R., & Pollock, H. M. (1983). Spectrum of *Vibrio* infections in a Gulf Coast community. *Annals of Internal Medicine*, 99(4), 464-469.

Borenstein, M., & Kerdel, F. (2003). Infections with *Vibrio vulnificus*. *Dermatologic Clinics*, 21(2), 245-248.

Borrego, J.J., Castro, D., Luque, A., Paillard, C., Maes, P., Garcia, M.T., & Ventosa, A. (1996). *Vibrio tapetis* sp. nov., the causative agent of the brown ring disease affecting cultured clams. *International Journal of Systematic Bacteriology*, 46, 480–484.

Boyd, E. F., Moyer, K. E., Shi, L., & Waldor, M. K. (2000). Infectious CTXΦ and the Vibrio Pathogenicity Island prophage in *Vibrio mimicus*: evidence for recent horizontal transfer between *V. mimicus* and *V. cholerae*. *Infection and Immunity*, 68(3), 1507-1513.

Brayton, P. R., West, P. A., Russek, E., & Colwell, R. R. (1983). New selective plating medium for isolation of *Vibrio vulnificus* biogroup 1. *Journal of Clinical Microbiology*, 17(6), 1039-1044.

Brayton, P. R., Bode, R. B., Colwell, R. R., MacDonell, M. T., Hall, H. L., Grimes, D. J., West, P.A.,& Bryant, T. N. (1986). *Vibrio cincinnatiensis* sp. nov., a new human pathogen. *Journal of Clinical Microbiology*, 23(1), 104-108.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 77(1), 71-94.

Brenner, D. J., Hickman-Brenner, F. W., Lee, J. V., Steigerwalt, A. G., Fanning, G. R., Hollis, D. G., Farmer, J. J., Weaver, R. E., Joseph, S. W., & Seidler, R. J. (1983). *Vibrio furnissii* (formerly aerogenic biogroup of *Vibrio fluvialis*), a new species isolated from human faeces and the environment. *Journal of Clinical Microbiology*, 18(4), 816-824.

Brock, J. A., & Lightner, D. V. (1990). Diseases of crustaceans- Diseases caused by microorganisms. *Diseases of Marine Animals*, 3, 245-349.

Bruant, G., Watt, S., Quentin, R., & Rosenau, A. (2003). Typing of nonencapsulated *Haemophilus strains* by repetitive-element sequence-based PCR using intergenic dyad sequences. *Journal of Clinical Microbiology*, 41(8), 3473-3480.

Bruno, D. W. (1986). Histopathology of bacterial kidney disease in laboratory infected rainbow trout, *Salmo gairdneri* Richardson, and Atlantic salmon, *Salmo salar* L., with reference to naturally infected fish. *Journal of Fish Diseases*, 9(6), 523-537.

Buchanan, R. E., & Gibbons, N. E. (1974). *Bergey's Manual of Determinative Bacteriology*. 8th Edition, The Williams and Wilkins Co. Baltimore, pp. 1246.

Buck, J. D., & McCarthy, S. A. (1994). Occurrence of non□O1 Vibrio cholerae in Texas Gulf Coast dolphins *(Tursiops truncatus). Letters in Applied Microbiology*, 18(1), 45-46.

Buck, J. D., Wells, R. S., Rhinehart, H. L., & Hansen, L. J. (2006). Aerobic microorganisms associated with free-ranging bottlenose dolphins in coastal Gulf of Mexico and Atlantic Ocean waters. *Journal of Wildlife Diseases*, 42(3), 536-544.

Bullen, J. J., Spalding, P. B., Ward, C. G., & Gutteridge, J. (1991). Hemochromatosis, iron, and septicemia caused by *Vibrio vulnificus*. *Archives of Internal Medicine*, 151(8), 1606.

Bush, C. A., Patel, P., Gunawardena, S., Powell, J., Joseph, A., Johnson, J. A., & Morris, J. G. (1997). Classification of *Vibrio vulnificus* strains by the carbohydrate composition of their capsular polysaccharides. *Analytical Biochemistry*, 250(2), 186-195.

Caburlotto, G., Lleo, M. M., Gennari, M., Balboa, S., & Romalde, J. L. (2011). The use of multiple typing methods allows a more accurate molecular characterization of *Vibrio parahaemolyticus* strains isolated from the Italian Adriatic Sea. *FEMS Microbiology Ecology*, 77(3), 611-622.

Cai, J., Han, Y., & Wang, Z. (2006). Isolation of *Vibrio parahaemolyticus* from abalone (*Haliotis diversicolor* supertexta *L.*) postlarvae associated with mass mortalities. *Aquaculture*, 257(1), 161-166.

Cai, S. H., Lu, Y. S., Wu, Z. H., Jian, J. C., & Huang, Y. C. (2009). A novel multiplex PCR method for detecting virulent strains of *Vibrio alginolyticus*. *Aquaculture Research*, 41(1), 27-34.

Cao, S. Y., Wang, M. S., Cheng, A. C., Qi, X. F., Yang, X. Y., Deng, S. X., & Chen, X. Y. (2008). Comparative analysis of intestinal microbial community diversity between healthy and orally infected ducklings with *Salmonella enteritidis* by ERIC-PCR. *World Journal of Gastroenterology*, 14(7), 1120.

Carroll, P. A., Tashima, K. T., Rogers, M. B., DiRita, V. J., & Calderwood, S. B. (1997). Phase variation in tcpH modulates expression of the ToxR regulon in *Vibrio cholerae*. *Molecular Microbiology*, 25(6), 1099-1111.

Case, R. J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W. F., & Kjelleberg, S. (2007). Use of 16S rRNA and *rpoB* genes as molecular markers for microbial ecology studies. *Applied and Environmental Microbiology*, 73(1), 278-288.

Castroverde, C. D. M., San Luis, B. B., Monsalud, R. G., & Hedreyda, C. T. (2006). Differential detection of *Vibrios* pathogenic to shrimp by multiplex PCR. *The Journal of General and Applied Microbiology*, 52(5), 273-280.

Cazorla, C., Guigon, A., Noel, M., Quilici, M. L., & Lacassin, F. (2011). Fatal *Vibrio vulnificus* infection associated with eating raw oysters, New Caledonia. *Emerging Infectious Diseases*, 17(1), 136.

CDC (Centers for Disease Control and Prevention), (1999). Outbreak of *Vibrio parahaemolyticus* infection associated with eating raw oysters and clams harvested from Long Island Sound-Connecticut, New Jersey, and New York, 1998. *Morbidity and Mortality Weekly Report*, 48, 48-51.

Cervino, J. M., Hayes, R. L., Polson, S. W., Polson, S. C., Goreau, T. J., Martinez, R. J., & Smith, G. W. (2004). Relationship of *Vibrio* species infection and elevated temperatures to yellow blotch/band disease in Caribbean corals. *Applied and Environmental Microbiology*, 70(11), 6855-6864.

Chakrabarti, S. R., Chaudhuri, K., Sen, K., & Das, J. (1996). Porins of *Vibrio cholerae*: purification and characterization of OmpU. *Journal of Bacteriology*, 178(2), 524-530.

Chakraborty, S., Mukhopadhyay, A. K., Bhadra, R. K., Ghosh, A. N., Mitra, R., Shimada, T., Yamasaki, S., & Nair, G. B. (2000). Virulence genes in environmental strains of *Vibrio cholerae. Applied and Environmental Microbiology*, 66(9), 4022-4028.

Chakraborty, R., Sinha, S., Mukhopadhyay, A. K., Asakura, M., Yamasaki, S., Bhattacharya, S. K., & Ramamurthy, T. (2006). Species-specific identification of *Vibrio fluvialis* by PCR targeted to the conserved transcriptional activation and variable membrane tether regions of the *toxR* gene. *Journal of Medical Microbiology*, 55(6), 805-808.

Chalfie, M., & Jorgensen, E. M. (1998). *C. elegans* neuroscience: Genetics to genome. *Trends in Genetics*, 14(12), 506-512.

Chander, J., Kaistha, N., Gupta, V., Mehta, M., Singla, N., Deep, A., & Sarkar, B. L. (2009). Epidemiology & antibiograms of *Vibrio cholerae* isolates from a tertiary care hospital in Chandigarh, North India. *Indian Journal of Medical Research*, 129(5): 613-617.

Chandrasekhar, M. R., Krishna, B. V. S., & Patil, A. B. (2008). Changing characteristics of *Vibrio cholerae*: emergence of multidrug resistance and non-O1, non-O139 serogroups. *The Southeast Asian Journal of Tropical Medicine and Public Health*, 39(6): 1092-1097.

Chang, C. I., Lee, C. F., Wu, C. C., Cheng, T. C., Tsai, J. M., & Lin, K. J. (2011). A selective and differential medium for *Vibrio alginolyticus*. *Journal of Fish Diseases*, 34(3), 227-234.

Chang, T. M., Chuang, Y. C., Su, J. H., & Chang, M. C. (1997). Cloning and sequence analysis of a novel hemolysin gene (*vllY*) from *Vibrio vulnificus*. *Applied and Environmental Microbiology*, 63(10), 3851-3857.

Chatterjee, S., & Haldar, S. (2012). *Vibrio* related diseases in aquaculture and development of rapid and accurate identification methods. *Journal of Marine Science and Research Development,* S1:002. doi:10.4172/2155-9910.

Chatterjee, T., Mukherjee, D., Dey, S., Pal, A., Hoque, K. M., & Chakrabarti, P. (2011). Accessory cholera enterotoxin, Ace, from *Vibrio cholerae*: structure, unfolding, and virstatin binding. *Biochemistry*, 50(14), 2962-2972.

Chatzidaki-Livanis, M., Hubbard, M. A., Gordon, K., Harwood, V. J., & Wright, A. C. (2006). Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. *Applied and Environmental Microbiology*, 72(9), 6136-6141.

Chavez□Dozal, A., & Nishiguchi, M. K. (2011). Variation in biofilm formation among symbiotic and free□living strains of *Vibrio fischeri*. Journal of Basic Microbiology, 51(5), 452-458.

Cheasty, T., Saif, B., & Threlfall, E. J. (1999). *V cholerae* non-01: implications for man? *The Lancet*, 354(9173), 89-90.

Chen, F. R., Liu, P. C., & Lee, K. K. (2000). Lethal attribute of serine protease secreted by *Vibrio alginolyticus* strains in kuruma prawn *Penaeus japonicus*. *Zeitschrift Fur Naturforschung C*, 55(1/2), 94-99.

Chen, Y. C., Chang, M. C., Chuang, Y. C., & Jeang, C. L. (2004). Characterization and virulence of hemolysin III from *Vibrio vulnificus*. *Current Microbiology*, 49(3), 175-179.

Choi, S., Dunams, D., & Jiang, S. C. (2010). Transfer of cholera toxin genes from O1 to non□O1/O139 strains by vibriophages from California coastal waters. *Journal of Applied Microbiology*, 108(3), 1015-1022.

Chow, K. H., Ng, T. K., Yuen, K. Y., & Yam, W. C. (2001). Detection of RTX toxin gene in *Vibrio cholerae* by PCR. *Journal of Clinical Microbiology*, 39(7), 2594-2597.

Chowdhury, N., Asakura, M., Neogi, S. B., Hinenoya, A., Haldar, S., Ramamurthy, T., & Yamasaki, S. (2010). Development of simple and rapid PCR□fingerprinting methods for *Vibrio cholerae* on the basis of genetic diversity of the superintegron. *Journal of Applied Microbiology*, 109(1), 304-312.

Chun, J., Grim, C. J., Hasan, N. A., Lee, J. H., Choi, S. Y., Haley, B. J., & Colwell, R. R. (2009). Comparative genomics reveals mechanism for short-term and long-term clonal transitions in pandemic *Vibrio cholerae*. *Proceedings of the National Academy of Sciences*, 106(36), 15442-15447.

Chung, K. J., Cho, E. J., Kim, M. K., Kim, Y. R., Kim, S. H., Yang, H. Y., & Lee, T. H. (2010). RtxA1-induced expression of the small GTPase Rac2 plays a key role in the pathogenicity of *Vibrio vulnificus*. *Journal of Infectious Diseases*, 201(1), 97-105.

Cinar, H. N., Kothary, M., Datta, A. R., Tall, B. D., Sprando, R., Bilecen, K., Yildiz, F., & McCardell, B. (2010). *Vibrio cholerae* hemolysin is required for lethality, developmental delay, and intestinal vacuolation in *Caenorhabditis elegans*. *PloS one*, *5*(7), e11558.

Clarridge, J. E., & Zighelboim-Daum, S. (1985). Isolation and characterization of two hemolytic phenotypes of *Vibrio damsela* associated with a fatal wound infection. *Journal of Clinical Microbiology*, 21(3), 302-306.

Collier, R. J., & Mekalanos, J. J. (1980) *Multifunctional Proteins*. Bisswanger, H. & Schmincke-Ott, E. (Eds.), Wiley, New York, pp. 261-291.

Colwell, R.R. (1996) Global climate and infectious disease: The cholera paradigm. *Science* 274: 2031–2025.

Conejero, M. J. U., & Hedreyda, C. T. (2003). Isolation of partial toxR gene of Vibrio harveyi and design of toxR□targeted PCR primers for species detection. *Journal of Applied Microbiology*, 95(3), 602-611.

Coote, J. G. (1992). Structural and functional relationships among the RTX toxin determinants of gram-negative bacteria. *FEMS Microbiology Letters*, 88(2), 137-161.

Cordero, C. L., Sozhamannan, S., & Satchell, K. J. F. (2007). RTX toxin actin cross-linking activity in clinical and environmental isolates of *Vibrio cholerae*. *Journal of Clinical Microbiology*, 45(7), 2289-2292.

Cortes, J. (Ed.). (2003). *Latin American coral reefs*. Elsevier Science, Amsterdam, Netherlands.

Costa, J. C., Lilley, C. J., & Urwin, P. E. (2007). *Caenorhabditis elegans* as a model for plant-parasitic nematodes. *Nematology*, 9(1), 3-16.

Costinar, L.V., Herman, C., Pascu C., Mareu, A.D., & Faur B. (2010). Isolation and characterisation of *Vibrio alginolyticus* and *Pasteurella* spp. from Siberian sturgeon (*Acinpenser baerii*). *Lucran Stuntifice Medicina Veterinaria*Vol. XLIII(1). 125-127.

Couillault, C., & Ewbank, J. J. (2002). Diverse bacteria are pathogens of *Caenorhabditis elegans*. *Infection and Immunity*, 70(8), 4705-4707.

Crawford, J. A., Kaper, J. B., & DiRita, V. J. (1998). Analysis of ToxR□dependent transcription activation of *omp*U, the gene encoding a major envelope protein in *Vibrio cholerae*. *Molecular Microbiology*, 29(1), 235-246.

Crawford, J. A., Krukonis, E. S., & DiRita, V. J. (2003). Membrane localization of the ToxR winged□helix domain is required for TcpP□mediated virulence gene activation in *Vibrio cholerae*. *Molecular Microbiology*, 47(5), 1459-1473.

Crennell, S., Garman, E., Laver, G., Vimr, E., & Taylor, G. (1994). Crystal structure of *Vibrio cholerae* neuraminidase reveals dual lectin-like domains in addition to the catalytic domain. *Structure*, 2(6), 535-544.

Croci, L., Suffredini, E., Cozzi, L., Toti, L., Ottaviani, D., Pruzzo, C., & Mioni, R. (2007). Comparison of different biochemical and molecular methods for the identification of *Vibrio parahaemolyticus*. *Journal of Applied Microbiology*, 102(1), 229-237.

Currie, B. J., Gal, D., Mayo, M., Ward, L., Godoy, D., Spratt, B. G., & LiPuma, J. J. (2007). Using BOX-PCR to exclude a clonal outbreak of melioidosis. *BMC Infectious Diseases*, 7(1), 68.

Dalmasso, A., Civera, T., & Bottero, M. T. (2009). Multiplex primer-extension assay for identification of six pathogenic *Vibrios*. *International Journal of Food Microbiology*, 129(1), 21-25.

Dalsgaard, A., Glerup, P., Hoybye, L. L., Paarup, A. M., Meza, R., Bernal, M., Shimada, T., & Taylor, D. N. (1997). *Vibrio furnissii* isolated from humans in Peru: a possible human pathogen? *Epidemiology and Infection*, 119(2), 143-149.

Dalsgaard, I., Hoi, L., Siebeling, R. J., & Dalsgaard, A. (1999). Indole-positive *Vibrio vulnificus* isolated from disease outbreaks on a Danish eel farm. *Diseases of Aquatic Organisms*, 35(3), 187.

Daniels, N. A., & Shafaie, A. (2000). A review of pathogenic *Vibrio* infections for clinicians. *Infections in Medicine*, 17(10), 665-685.

Daniels, N. A., MacKinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., Sharon, T., & Slutsker, L. (2000). *Vibrio parahaemolyticus* infections in the United States, 1973–1998. *Journal of Infectious Diseases*, 181(5), 1661-1666.

Davey, M. L., Hancock, R. E., & Mutharia, L. M. (1998). Influence of culture conditions on expression of the 40-kilodalton porin protein of *Vibrio anguillarum* serotype O2. *Applied and Environmental Microbiology*, 64(1), 138-146.

Davis, B. M., & Waldor, M. K. (2003). Filamentous phages linked to virulence of *Vibrio cholerae*. *Current Opinion in Microbiology*, 6(1), 35-42.

de la Pena, L. D., Tamaki, T., Momoyama, K., Nakai, T., & Muroga, K. (1993). Characteristics of the causative bacterium of vibriosis in the kuruma prawn, *Penaeus japonicus*. *Aquaculture*, 115(1), 1-12.

de Magny, G. C., Long, W., Brown, C. W., Hood, R. R., Huq, A., Murtugudde, R., & Colwell, R. R. (2009). Predicting the distribution of *Vibrio* spp. in the Chesapeake Bay: A *Vibrio cholerae* case study. *EcoHealth*, 6(3), 378-389.

de Magny, G. C., Mozumder, P. K., Grim, C. J., Hasan, N. A., Naser, M. N., Alam, M., & Colwell, R. R. (2011). Role of zooplankton diversity in *Vibrio cholerae* population dynamics and in the incidence of cholera in the Bangladesh Sundarbans. *Applied and Environmental Microbiology*, 77(17), 6125-6132.

Demircan, D., & Candan, A. (2006). Identification of *Vibrio anguillarum* by PCR (*rpo*N gene) associated with vibriosis in marine fish in Turkey. *Turkish Journal of Veterinary and Animal Sciences*, 30, 305-310.

DePaola, A., Kaysner, C. A., Bowers, J., & Cook, D. W. (2000). Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas, and New York (1997 and 1998). *Applied and Environmental Microbiology*, 66(11), 4649-4654.

DePaola, A., Nordstrom, J. L., Dalsgaard, A., Forslund, A., Oliver, J., Bates, T., Bourdade, K.L.,& Gulig, P. A. (2003). Analysis of *Vibrio vulnificus* from market oysters and septicemia cases for virulence markers. *Applied and Environmental Microbiology*, 69(7), 4006-4011.

Derber, C., Coudron, P., Tarr, C., Gladney, L., Turnsek, M., Shankaran, S., & Wong, E. (2011). *Vibrio furnissii*: an unusual cause of bacteremia and skin lesions after ingestion of seafood. *Journal of Clinical Microbiology*, 49(6), 2348-2349.

Desenclos, J. C. A., Klontz, K. C., Wolfe, L. E., & Hoecheri, S. (1991). The risk of *Vibrio* illness in the Florida raw oyster eating population, 1981–1988. *American Journal of Epidemiology*, 134(3), 290-297.

Desmond, E. P., Janda, J. M., Adams, F. I., & Bottone, E. J. (1984). Comparative studies and laboratory diagnosis of *Vibrio vulnificus*, an invasive *Vibrio* sp. *Journal of Clinical Microbiology*, 19(2), 122-125.

Devi, S. J. N., Hayat, U., Frasch, C. E., Kreger, A. S., & Morris, J. G. (1995). Capsular polysaccharide-protein conjugate vaccines of carbotype 1 *Vibrio vulnificus*: Construction, immunogenicity, and protective efficacy in a murine model. *Infection and Immunity*, 63(8), 2906-2911.

Devi, S. J. N., Hayat, U., Powell, J. L., & Morris, J. G. (1996). Preclinical immunoprophylactic and immunotherapeutic efficacy of antisera to capsular polysaccharide-tetanus toxoid conjugate vaccines of *Vibrio vulnificus*. *Infection and Immunity*, 64(6), 2220-2224.

Dhakal, B. K., Lee, W., Kim, Y. R., Choy, H. E., Ahnn, J., & Rhee, J. H. (2006). *Caenorhabditis elegans* as a simple model host for *Vibrio vulnificus* infection. *Biochemical and Biophysical Research Communications*, 346(3), 751-757.

Di Pierro, M., Lu, R., Uzzau, S., Wang, W., Margaretten, K., Pazzani, C., Maimone, F., & Fasano, A. (2001). Zonula occludens toxin structure-function analysis: Identification of the fragment biologically active on tight junctions and of the zonulin receptor binding domain, *Journal of Biological Chemistry*, 276(22), 19160-19165.

Di Pinto, A., Ciccarese, G., Tantillo, G., Catalano, D., & Forte, V. T. (2006). A collagenase-targeted multiplex PCR assay for identification of *Vibrio alginolyticus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus*. *Journal of Food Protection*, 68(1), 150-153.

Dieng, M. T., Niang, S. O., Ly, F., Bathily, T., & Ndiaye, B. (2001). Necrotizing dermatitis due to *Vibrio vulnificus*. In *Annales de dermatologie et de vénéréologie* , 128(5), 653.

DiGaetano, M., Ball, S. F., & Straus, J. G. (1989). *Vibrio vulnificus* corneal ulcer. *Archives of Ophthalmology*, 107(3), 323.

Diggles, B. K., Carson, J., Hine, P. M., Hickman, R. W., & Tait, M. J. (2000). *Vibrio* species associated with mortalities in hatchery-reared turbot (*Colistium nudipinnis*) and brill (*C. guntheri*) in New Zealand. *Aquaculture*, 183(1), 1-12.

Direkbusarakom, S., Yoshimizu, M., Ezura, Y., Ruangpan, L., & Danayadol, Y. (1998). *Vibrio* spp., the dominant flora in shrimp hatchery against some fish pathogenic viruses. *Journal of Marine Biotechnology*, *6*, 266-267.

DiRita, V. J., Parsot, C., Jander, G., & Mekalanos, J. J. (1991). Regulatory cascade controls virulence in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences*, 88(12), 5403-5407.

DiRita, V. J. (1992). Co□ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. *Molecular Microbiology*, 6(4), 451-458.

Doyle, M. P., & Beuchat, L. R. (2007). *Food microbiology: Fundamentals and frontiers*. 3rd Edition, ASM Press.

Drake, S. L., DePaola, A., & Jaykus, L. A. (2007). An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. *Comprehensive Reviews in Food Science and Food Safety*, 6(4), 120-144.

Durai, S., Pandian, S. K., & Balamurugan, K. (2011a). Changes in *Caenorhabditis elegans* exposed to *Vibrio parahaemolyticus*. *Journal of Microbiology and Biotechnology*, 21(10), 1026-1035.

Durai, S., Pandian, S. K., & Balamurugan, K. (2011b). Establishment of a *Caenorhabditis elegans* infection model for *Vibrio alginolyticus*. *Journal of Basic Microbiology*, 51(3), 243-252.

Dziejman, M., Balon, E., Boyd, D., Fraser, C. M., Heidelberg, J. F., & Mekalanos, J. J. (2002). Comparative genomic analysis of *Vibrio cholerae*: genes that correlate with cholera endemic and pandemic disease. *Proceedings of the National Academy of Sciences*, *99*(3), 1556-1561.

Dziejman, M., Serruto, D., Tam, V. C., Sturtevant, D., Diraphat, P., Faruque, S. M., & Mekalanos, J. J. (2005). Genomic characterization of non-O1, non-O139 *Vibrio cholerae* reveals genes for a type III secretion system. *Proceedings of the National Academy of Sciences of the United States of America*, 102(9), 3465-3470.

Eberhart-Phillips, J., Bessser, R. E., Tormey, M. P., Feikin, D., Araneta, M. R., Wells, J., & Mascola, L. (1996). An outbreak of cholera from food served on an international aircraft. *Epidemiology and Infection*, 116(01), 9-13.

Egidius, E., Wiik, R., Andersen, K., Hoff, K. A., & Hjeltnes, B. (1986). *Vibrio salmonicida* sp. nov., a new fish pathogen. *International Journal of Systematic Bacteriology*, 36(4), 518-520.

Eilers, H., Pernthaler, J., Glockner, F. O., & Amann, R. (2000). Culturability and in situ abundance of pelagic bacteria from the North Sea. *Applied and Environmental Microbiology*, 66(7), 3044-3051.

El-Galil, M., Abd, A. A., & Mohamed, M. H. (2012). First isolation of *Vibrio alginolyticus* from ornamental bird Wrasse Fish (*Gomphosus caeruleus*) of the Red Sea in Egypt. *Journal of Fisheries and Aquatic Science*, 7(6), 461-467.

Elhadi, N. (2012). Antibiotic resistance and plasmid profiling of clinically significant *Vibrio vulnificus* isolated from coastal water in Eastern Province of Saudi Arabia. *British Journal of Pharmacology and Toxicology*, 3(2), 93-97.

English, V. L., & Lindberg, R. B. (1977). Isolation of *Vibrio alginolyticus* from wounds and blood of a burn patient. *The American Journal of Medical Technology*, *43*(10), 989.

Enos□Berlage, J. L., Guvener, Z. T., Keenan, C. E., & McCarter, L. L. (2005). Genetic determinants of biofilm development of opaque and translucent *Vibrio parahaemolyticus*. *Molecular Microbiology*, 55(4), 1160-1182.

Esteban, E., Snipes, K., Hird, D., Kasten, R., & Kinde, H. (1993). Use of ribotyping for characterization of *Salmonella* serotypes. *Journal of Clinical Microbiology*, 31(2), 233-237.

Esteve-Gassent, M. D., & Amaro, C. (2004). Immunogenic antigens of the eel pathogen *Vibrio vulnificus* serovar E. *Fish & Shellfish Immunology*, 17(3), 277-291.

Everiss, K. D., Hughes, K. J., & Peterson, K. M. (1994). The accessory colonization factor and toxin-coregulated pilus gene clusters are physically linked on the *Vibrio cholerae* 0395 chromosome. *Mitochondrial DNA*, 5(1), 51-55.

Fallarino, A., Attridge, S. R., Manning, P. A., & Focareta, T. (2002). Cloning and characterization of a novel haemolysin in *Vibrio cholerae* O1 that does not directly contribute to the virulence of the organism. *Microbiology*, 148(7), 2181-2189.

Farmer, J. J., Hickman-Brenner, F. W., Fanning, G. R., Gordon, C. M., & Brenner, D. J. (1988). Characterization of *Vibrio metschnikovii* and *Vibrio gazogenes* by DNA-DNA hybridization and phenotype. *Journal of Clinical Microbiology*, 26(10), 1993-2000.

Faruque, S. M., Albert, M. J., & Mekalanos, J. J. (1998a). Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae. Microbiology and Molecular Biology Reviews*, 62(4), 1301-1314.

Faruque, S. M., Saha, M. N., Alim, A. A., Albert, M. J., Islam, K. N., & Mekalanos, J. J. (1998b). Analysis of clinical and environmental strains of nontoxigenic *Vibrio cholerae* for susceptibility to CTXΦ: molecular basis for origination of new strains with epidemic potential. *Infection and Immunity*, 66(12), 5819-5825.

Faruque, S. M., Chowdhury, N., Kamruzzaman, M., Dziejman, M., Rahman, M. H., Sack, D. A., Nair, G.B., & Mekalanos, J. J. (2004). Genetic diversity and virulence potential of environmental *Vibrio cholerae* population in a cholera-endemic area. *Proceedings of the National Academy of Sciences of the United States of America*, 101(7), 2123-2128.

Fasano, A., Baudry, B., Pumplin, D. W., Wasserman, S. S., Tall, B. D., Ketley, J. M., & Kaper, J. B. (1991). *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proceedings of the National Academy of Sciences*, 88 (12), 5242-5246.

Fasano, A., Uzzau, S., Fiore, C., & Margaretten, K. (1997). The enterotoxic effect of zonula occludens toxin on rabbit small intestine involves the paracellular pathway. *Gastroenterology*, 112(3), 839-846.

Feachem, R. G. (1981). Environmental aspects of cholera epidemiology. I. A review of selected reports of endemic and epidemic situations during 1961-1980. *Tropical Diseases Bulletin*, 78(8), 675.

Feghali, R., & Adib, S. M. (2011). Two cases of *Vibrio cholerae* non-O1/non-O139 septicaemia with favourable outcome in Lebanon. *Eastern Meditteranean Health Journal*, 17(8), 722-724.

Felix, S., & Devaraj, M. (1993) Incidence of destructive MBV and IHHNV in a commercial shrimp hatchery. *Sea Food Export Journal* 25, 13-18.

Fengler, V. H., Boritsch, E. C., Tutz, S., Seper, A., Ebner, H., Roier, S., & Reidl, J. (2012). Disulfide bond formation and ToxR activity in *Vibrio cholerae*. *PloS one*, 7(10), e47756.

Fidopiastis, P. M., von Boletzky, S., & Ruby, E. G. (1998). A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. *Journal of Bacteriology*, 180(1), 59-64.

Figueiredo, S. C. D. A., Neves-Borges, A. C., & Coelho, A. (2005). The neuraminidase gene is present in the non-toxigenic *Vibrio cholerae* Amazonia strain: a different allele in comparison to the pandemic strains. *Memórias do Instituto Oswaldo Cruz*, 100(6), 563-569.

Finlay, B. B. (1999). Bacterial disease in diverse hosts. *Cell*, 96(3), 315-318.

Fiore, A., Hayat, U., Wasserman, S. S., Wright, A., Bush, C. A., & Morris Jr, J. G. (1996). Antibodies that react with the capsular polysaccharide of *Vibrio vulnificus* are detectable in infected patients, and in persons without known exposure to the organism. *Diagnostic Microbiology and Infectious Disease*, 24(3), 165-167.

Fong, J. C., Syed, K. A., Klose, K. E., & Yildiz, F. H. (2010). Role of *Vibrio* polysaccharide (*vps)* genes in VPS production, biofilm formation and *Vibrio cholerae* pathogenesis. *Microbiology*, 156(9), 2757-2769.

Ford, L. A., Alexander, S. K., Cooper, K. M., & Hanlon, R. T. (1986). Bacterial populations of normal and ulcerated mantle tissue of the squid, *Lolliguncula brevis*. *Journal of Invertebrate Pathology*, 48(1), 13-26.

Forssman, B., Mannes, T., Musto, J., Gottlieb, T., Robertson, G., Natoli, J. D., Shadbolt, Biffin,B., & Gupta, L. (2007). *Vibrio cholerae* O1 El Tor cluster in Sydney linked to imported whitebait. *Medical Journal of Australia*, 187(6), 345.

Fouz, B., Larsen, J. L., Nielsen, B., Barja, J. L., & Toranzo, A. E. (1992). Characterization of *Vibrio damsela* strains isolated from turbot *Scophthalmus maximus* in Spain. *Diseases of Aquatic Organisms*, 12(3), 155-166.

Franco, P. F., & Hedreyda, C. T. (2006). Amplification and sequence analysis of the full length *toxR* gene in *Vibrio harveyi*. *The Journal of General and Applied Microbiology*, 52(5), 281-287.

Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B., & Rediers, H. (2011). *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. *Journal of Fish Diseases*, 34(9), 643-661.

Fujioka, R. S., Greco, S. B., Cates, M. B., & Schroeder, J. P. (1988). *Vibrio damsela* from wounds in bottlenose dolphins *Tursiops truncatus*. *Diseases of Aquatic Organisms*, 4(1), 1-8.

Fujiwara, M., Uyeno, Y. and Iwao, A. (1993) A *Vibrio* sp. associated with mortalities in cockle larvae *Fulvia mutica* (Mollusca: Cardiidae). *Gyobyo Kenkyu* 28, 83–89.

Fullner, K. J., & Mekalanos, J. J. (2000). *In vivo* covalent cross-linking of cellular actin by the *Vibrio cholerae* RTX toxin. *The EMBO Journal*, 19(20), 5315-5323.

Fullner, K. J., Lencer, W. I., & Mekalanos, J. J. (2001). *Vibrio cholerae*-induced cellular responses of polarized T84 intestinal epithelial cells are dependent on production of cholera toxin and the RTX toxin. *Infection and Immunity*, 69(10), 6310-6317.

Gaafar, H. A. (1970). *Vibrio*, In: Blair, J. E., Lennette, E. H., & Truant, J. P (Eds.), *Manual of Clinical Microbiology*. 1st Edition, American Society for Microbiology, Washington, pp. 232-235.

Galal, A. A., Kehil, Y., El-Daoudi, Y. H., Shihata, Z. A., & Ouf, M. F. (2003). A comparative study on the identification of races and biovars of some Egyptian isolates of *Ralstonia solanacearum*. *Egyptian Journal of Phytopathology*, 31(1-2), 103-117.

Galen, J. E., Ketley, J. M., Fasano, A., Richardson, S. H., Wasserman, S. S., & Kaper, J. B. (1992). Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. *Infection and Immunity*, 60(2), 406-415.

Gan, Y. H., Chua, K. L., Chua, H. H., Liu, B., Hii, C. S., Chong, H. L., & Tan, P. (2002). Characterization of *Burkholderia pseudomallei* infection and identification of novel virulence factors using a *Caenorhabditis elegans* host system. *Molecular Microbiology*, 44(5), 1185-1197.

Garnier, M., Labreuche, Y., Garcia, C., Robert, M., & Nicolas, J. L. (2007). Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. *Microbial Ecology*, 53(2), 187-196.

Gavilan, R. G., Zamudio, M. L., & Martinez-Urtaza, J. (2013). Molecular epidemiology and genetic variation of pathogenic *Vibrio parahaemolyticus* in Peru. *PLOS Neglected Tropical Diseases*, 7(5), e2210.

Gay, M., Renault, T., Pons, A. M., & Le Roux, F. (2004). Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: Taxonomy and host alterations. *Diseases of Aquatic Organisms*, 62(1-2), 65-74.

Geeta, M. G., & Krishnakumar, P. (2005). Cholera in Kerala. *Indian Pediatrics Journal*, 42, 89.

George, M. R., John, K. R., Iyappan, T., & Jeyaseelan, M. J. P. (2005). Genetic heterogeneity among *Vibrio alginolyticus* isolated from shrimp farms by PCR fingerprinting. *Letters in Applied Microbiology*, 40(5), 369-372.

Goarant, C., Ansquer, D., Herlin, J., Domalain, D., Imbert, F., & De Decker, S. (2006a). "Summer Syndrome" in *Litopenaeus stylirostris* in New Caledonia: Pathology and epidemiology of the etiological agent, *Vibrio nigripulchritudo*. *Aquaculture*, 253(1), 105-113.

Goarant, C., Reynaud, Y., Ansquer, D., Decker, S. D., Saulnier, D., & Roux, F. L. (2006b). Molecular epidemiology of *Vibrio nigripulchritudo*, a pathogen of cultured penaeid shrimp (*Litopenaeus stylirostris*) in New Caledonia. *Systematic and Applied Microbiology*, 29(7), 570-580.

Goel, A. K., Jain, M., Kumar, P., & Jiang, S. C. (2010). Molecular characterization of *Vibrio cholerae* outbreak strains with altered El Tor biotype from southern India. *World Journal of Microbiology and Biotechnology*, 26(2), 281-287.

Goel, A. K., & Jiang, S. C. (2011). Association of heavy rainfall on genotypic diversity in *V. cholerae* isolates from an outbreak in India. *International Journal of Microbiology*, Article ID 230597.

Gomez-Gil, B., Tron-Mayen, L., Roque, A., Turnbull, J. F., Inglis, V., & Guerra-Flores, A. L. (1998). Species of *Vibrio* isolated from hepatopancreas, haemolymph and digestive tract of a population of healthy juvenile *Penaeus vannamei*. *Aquaculture*, 163(1), 1-9.

Gomez-Gil, B., Soto-Rodriguez, S., García-Gasca, A., Roque, A., Vazquez-Juarez, R., Thompson, F. L., & Swings, J. (2004). Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. *Microbiology*, 150(6), 1769-1777.

Gonzalez-Escalona, N., Martinez-Urtaza, J., Romero, J., Espejo, R. T., Jaykus, L. A., & DePaola, A. (2008). Determination of molecular phylogenetics of *Vibrio parahaemolyticus* strains by multilocus sequence typing. *Journal of Bacteriology*, 190(8), 2831-2840.

Gopal, S., Otta, S. K., Kumar, S., Karunasagar, I., Nishibuchi, M., & Karunasagar, I. (2005). The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety. *International Journal of Food Microbiology*, 102(2), 151-159.

Gordon, G. S., & Wright, A. (2000). DNA segregation in bacteria. *Annual Reviews in Microbiology*, 54(1), 681-708.

Gordon, K. V. (2008). Multilocus virulence typing of clinical and environmental *Vibrio vulnificus* isolates. PhD Thesis, University of South Florida.

Gray, L. D., & Kreger, A. S. (1985). Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus*. *Infection and Immunity*, 48(1), 62-72.

Gray, L. D., & Kreger, A. S. (1987). Mouse skin damage caused by cytolysin from *Vibrio vulniflcus* and by *V. vulnificus* Infection. *Journal of Infectious Diseases*, 155(2), 236-241.

Grimes, D. J., Stemmler, J., Hada, H., May, E. B., Maneval, D., Hetrick, F. M., Jones R. T., Stoskopf, M., & Colwell, R. R. (1984). *Vibrio* species associated with mortality of sharks held in captivity. *Microbial Ecology*, 10(3), 271-282.

Grisez, L., Sorgeloos, P., & Ollevier, F. (1996). Mode of infection and spread of *Vibrio anguillarum* in turbot *Scophthalmus maximus* larvae after oral challenge through live feed. *Diseases of Aquatic Organisms*, 26(3), 181-187.

Grisez, L., Reyniers, J., Verdonck, L., Swings, J., & Ollevier, F. (1997). Dominant intestinal microflora of sea bream and sea bass larvae, from two hatcheries, during larval development. *Aquaculture*, 155(1), 387-399.

Grossart, H. P., Levold, F., Allgaier, M., Simon, M., & Brinkhoff, T. (2005). Marine diatom species harbour distinct bacterial communities. *Environmental Microbiology*, 7(6), 860-873.

Gubala, A. J. (2006). Multiplex real-time PCR detection of *Vibrio cholerae*. *Journal of Microbiological Methods*, 65(2), 278-293.

Gubala, A. J., & Proll, D. F. (2006). Molecular-beacon multiplex real-time PCR assay for detection of *Vibrio cholerae*. *Applied and Environmental Microbiology*, 72(9), 6424-6428.

Guerrero□Ferreira, R. C., & Nishiguchi, M. K. (2007). Biodiversity among luminescent symbionts from squid of the genera *Uroteuthis, Loliolus* and *Euprymna* (Mollusca: Cephalopoda). *Cladistics*, 23(5), 497-506.

Gulig, P. A., Bourdage, K. L., & Starks, A. M. (2005). Molecular pathogenesis of *Vibrio vulnificus*. *Journal of Microbioogy*, 43, 118-131.

Hada, H.S., West, P.A., Lee, J.V., Stemmler, J., & Colwell, R.R. (1984) *Vibrio tubiashii* sp. nov., a pathogen of bivalve mollusks. *International Journal of Systematic Bacteriology*, 34, 1–4.

Hagstrom, A., Pommier, T., Rohwer, F., Simu, K., Stolte, W., Svensson, D., & Zweifel, U. L. (2002). Use of 16S ribosomal DNA for delineation of marine bacterioplankton species. *Applied and Environmental Microbiology*, 68(7), 3628-3633.

Haldar, S., Mody, K. H., & Jha, B. (2011). Abundance, diversity and antibiotics resistance pattern of *Vibrio* spp. in coral ecosystem of Kurusadai Island. *Journal of Basic Microbiology*, 51(2), 153-162.

Hall, R. H., & Drasar, B. S. (1990). *Vibrio cholerae* HlyA hemolysin is processed by proteolysis. *Infection and Immunity*, 58(10), 3375-3379.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.

Halpern, M., Gancz, H., Broza, M., & Kashi, Y. (2003). *Vibrio cholerae* hemagglutinin/protease degrades chironomid egg masses. *Applied and Environmental Microbiology*, 69(7), 4200-4204.

Halpern, M., Senderovich, Y., & Izhaki, I. (2008). Waterfowl-The missing link in epidemic and pandemic cholera dissemination? *PLoS Pathogens*, 4(10), e1000173.

Han, F., Walker, R. D., Janes, M. E., Prinyawiwatkul, W., & Ge, B. (2007). Antimicrobial susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana Gulf and retail raw oysters. *Applied and Environmental Microbiology*, 73(21), 7096-7098.

Han, F., Pu, S., Hou, A., & Ge, B. (2009). Characterization of clinical and environmental types of *Vibrio vulnificus* isolates from Louisiana oysters. *Foodborne Pathogens and Disease*, 6(10), 1251-1258.

Hanlon, R. T., Claes, M. F., Ashcraft, S. E., & Dunlap, P. V. (1997). Laboratory culture of the sepiolid squid *Euprymna scolopes*: a model system for bacteria-animal symbiosis. *The Biological Bulletin*, 192(3), 364-374.

Hanlon, R. T., Forsythe, J. W., & Lee, P. G. (1988). External pathologies of cephalopods in captivity. In *Proceedings of the International Colloquium on Pathology in Marine Aquaculture*, 3, 17-18.

Hansen, G. H., & Olafsen, J. A. (1999). Bacterial interactions in early life stages of marine cold water fish. *Microbial Ecology*, 38(1), 1-26.

Haq, S. M., & Dayal, H. H. (2005). Chronic liver disease and consumption of raw oysters: A potentially lethal combination—a review of *Vibrio vulnificus* septicemia. *The American Journal of Gastroenterology*, 100(5), 1195-1199.

Harkey, C. W., Everiss, K. D., & Peterson, K. M. (1994). The *Vibrio cholerae* toxin-coregulated-pilus gene *tcp*I encodes a homolog of methyl-accepting chemotaxis proteins. *Infection and Immunity*, 62(7), 2669-2678.

Harris, K. A., & Hartley, J. C. (2003). Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. *Journal of Medical Microbiology*, 52(8), 685-691.

Harris, L., Owens, L., & Smith, S. (1996). A selective and differential medium for *Vibrio harveyi*. *Applied and Environmental Microbiology*, 62(9), 3548-3550.

Harth, E., Romero, J., Torres, R., & Espejo, R. T. (2007). Intragenomic heterogeneity and intergenomic recombination among *Vibrio parahaemolyticus* 16S rRNA genes. *Microbiology*, 153(8), 2640-2647.

Harwood, V. J., Gandhi, J. P., & Wright, A. C. (2004). Methods for isolation and confirmation of *Vibrio vulnificus* from oysters and environmental sources: A review. *Journal of Microbiological Methods*, 59(3), 301-316.

Hasan, J. A., Huq, A., Tamplin, M. L., Siebeling, R. J., & Colwell, R. R. (1994). A novel kit for rapid detection of *Vibrio cholerae* O1. *Journal of Clinical Microbiology*, 32(1), 249-252.

Hasan, J. A., Huq, A., Nair, G. B., Garg, S., Mukhopadhyay, A. K., Loomis, L., & Colwell, R. R. (1995). Development and testing of monoclonal antibody-based rapid immunodiagnostic test kits for direct detection of *Vibrio cholerae* O139 synonym Bengal. *Journal of Clinical Microbiology*, 33(11), 2935-2939.

Hase, C. C., & Mekalanos, J. J. (1998). TcpP protein is a positive regulator of virulence gene expression in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences*, 95(2), 730-734.

Hazen, T. H., Pan, L., Gu, J. D., & Sobecky, P. A. (2010). The contribution of mobile genetic elements to the evolution and ecology of *Vibrios*. *FEMS Microbiology Ecology*, 74(3), 485-499.

Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft,D.H., Hickey, E.K., Peterson, J.D., Umayam, L., Gill, S.R., Nelson, K.E., Read, T.D., Tettelin, H., Richardson, D., Ermolaeva, M.D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R.D., Nierman, W.C., White, O., Salzberg, S.L., Smith, H.O., Colwell, R.R., Mekalanos, J.J., Venter, J.C., & Fraser, C. M. (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature*, 406(6795), 477-483.

Heidelberg, J. F., Heidelberg, K. B., & Colwell, R. R. (2002). Bacteria of the γ-subclass Proteobacteria associated with zooplankton in Chesapeake Bay. *Applied and Environmental Microbiology*, 68(11), 5498-5507.

Helms, S. D., Oliver, J. D., & Travis, J. C. (1984). Role of heme compounds and haptoglobin in *Vibrio vulnificus* pathogenicity. *Infection and Immunity*, 45(2), 345-349.

Hendriksen, R. S., Price, L. B., Schupp, J. M., Gillece, J. D., Kaas, R. S., Engelthaler, D. M., Bortolaia, V., Pearson, T., Wters, A.E., Updhyay, B.P., Shrestha, S.D., Adhikari, S., Shakya, D., Keim, P.S., & Aarestrup, F. M. (2011). Population genetics of *Vibrio cholerae* from Nepal in 2010: Evidence on the origin of the Haitian outbreak. *MBio*, 2(4): 1-6.

Hernandez, G., & Olmos, J. (2004). Molecular identification of pathogenic and nonpathogenic strains of *Vibrio harveyi* using PCR and RAPD. *Applied Microbiology and Biotechnology*, 63(6), 722-727.

Hervio□Heath, D., Colwell, R. R., Derrien, A., Robert□Pillot, A., Fournier, J. M., & Pommepuy, M. (2002). Occurrence of pathogenic Vibrios in coastal areas of France. *Journal of Applied Microbiology*, 92(6), 1123-1135.

Hickman, F. W., Farmer, J. J., Hollis, D. G., Fanning, G. R., Steigerwalt, A. G., Weaver, R. E., & Brenner, D. J. (1982). Identification of *Vibrio hollisae* sp. nov. from patients with diarrhea. *Journal of Clinical Microbiology*, 15(3), 395-401.

Higgins, C. F., Ames, G. F. L., Barnes, W. M., Clement, J. M., & Hofnung, M. (1982). A novel intercistronic regulatory element of prokaryotic operons. *Nature* 298:760-762.

Higgins, D. E., Nazareno, E., & DiRita, V. J. (1992). The virulence gene activator ToxT from *Vibrio cholerae* is a member of the AraC family of transcriptional activators. *Journal of Bacteriology*, 174(21), 6974-6980.

Higgins, D. E., & DiRita, V. J. (1996). Genetic analysis of the interaction between *Vibrio cholerae* transcription activator ToxR and ToxT promoter DNA. *Journal of Bacteriology*, 178(4), 1080-1087.

Hill, V. R., Cohen, N., Kahler, A. M., Jones, J. L., Bopp, C. A., Marano, N., & Tauxe, R. V. (2011). Toxigenic *Vibrio cholerae* O1 in water and seafood, Haiti. *Emerging Infectious Diseases*, 17(11), 2147.

Hjelm, M., Bergh, O., Riaza, A., Nielsen, J., Melchiorsen, J., Jensen, S., & Gram, L. (2004). Selection and identification of autochthonous potential probiotic bacteria from Turbot larvae (*Scophthalmus maximus*) rearing units. *Systematic and Applied Microbiology*, 27(3), 360-371.

Hjeltnes, B., & Roberts, R. J. (1993). *Vibriosis*, In: Roberts, R. J., Bromage, N. R., & Inglis, V. (Eds.), *Bacterial Diseases of Fish*. Blackwell Scientific, Oxford, United Kingdom. pp. 109–121.

Hlady, W. G., & Klontz, K. C. (1996). The epidemiology of *Vibrio* infections in Florida, 1981–1993. *Journal of Infectious Diseases*, 173(5), 1176-1183.

Ho, H., Do, T.H., Ho, T.T., Wu, W., & Lee, D. (2003) *Vibrio* infections. In *E Journal*: *eMedicine.com*.

Hoffmann, M., Fischer, M., Ottesen, A., McCarthy, P. J., Lopez, J. V., Brown, E. W., & Monday, S. R. (2010). Population dynamics of *Vibrio* spp. associated with marine sponge microcosms. *The ISME Journal*, 4(12), 1608-1612.

Hoi, L., Larsen, J. L., Dalsgaard, I., & Dalsgaard, A. (1998). Occurrence of *Vibrio vulnificus* biotypes in Danish marine environments. *Applied and Environmental Microbiology*, 64(1), 7-13.

Holcombe, D. J. (1991). *Vibrio vulnificus* peritonitis. A unique case. *The Journal of the Louisiana State Medical Society,* 143(12), 27.

Holmgren, J., Lonnroth, I., Mansson, J., & Svennerholm, L. (1975). Interaction of cholera toxin and membrane GM1 ganglioside of small intestine. *Proceedings of the National Academy of Sciences*, 72(7), 2520-2524.

Holt, J. G., Krieg, N. R., Sneath, P. H., Staley, J. T., & Williams, S. T. (1994). *Bergey's manual of determinative bacteriology*. The Williams and Wilkins Co. Baltimore, pp. 787.

Honda, T., Sornchai, C., Takeda, Y., & Miwatani, T. (1982). Immunological detection of the Kanagawa phenomenon of *Vibrio parahaemolyticus* on modified selective media. *Journal of Clinical Microbiology*, 16(4), 734-736.

Hope, I. A. (1999). *C. elegans: A practical approach* (Ed.), Oxford University Press.

Hoque, K. M., Saha, S., Gupta, D. D., & Chakrabarti, M. K. (2004). Role of nitric oxide in NAG-ST induced store-operated calcium entry in rat intestinal epithelial cells. *Toxicology*, 201(1), 95-103.

Hoshino, K., Yamasaki, S., Mukhopadhyay, A. K., Chakraborty, S., Basu, A., Bhattacharya, S. K., Nair, G.B., Shimada, T., & Takeda, Y. (1998). Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. *FEMS Immunology and Medical Microbiology*, 20(3), 201-207.

Hsueh, P. R., Lin, C. Y., Tang, H. J., Lee, H. C., Liu, J. W., Liu, Y. C., & Chuang, Y. C. (2004). *Vibrio vulnificus* in Taiwan. *Emerging Infectious Diseases*, 10(8), 1363.

http://ibnlive.in.com/news/cholera-outbreak-expert-team-visits-wayanad/163928-60-116.html retrieved on 24-05- 2013.

Huang, C. Y., Liu, P. C., & Lee, K. K. (2001). Withering syndrome of the small abalone, *Haliotis diversicolor supertexta*, is caused by *Vibrio parahaemolyticus* and associated with thermal induction. *Zeitschrift fur Naturforschung*, 56(9/10), 898-901.

Hughes, J. M., Hollis, D. G., Gangarosa, E. J., & Weaver, R. E. (1978). Non-cholera *Vibrio* infections in the United States - clinical, epidemiologic, and laboratory features. *Annals of Internal Medicine*, 88(5), 602-606.

Hulet, W. H., Villoch, M. R., Hixon, R. F., & Hanlon, R. T. (1979). Fin damage in captured and reared squids. *Laboratory Animal Science*, 29(4), 528.

Hulton, C. S. J., Higgins, C. F., & Sharp, P. M. (1991). ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other Enterobacteria. *Molecular Microbiology*, 5(4), 825-834.

Huq, A., Small, E. B., West, P. A., Huq, M. I., Rahman, R., & Colwell, R. R. (1983). Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Applied and Environmental Microbiology*, 45(1), 275-283.

Huq, A., & Colwell, R. R. (1996). Environmental factors associated with emergence of disease with special reference to cholera. *Eastern Mediterranean Health Journal*, 2, 37-45.

Huq, A., Sack, R. B., Nizam, A., Longini, I. M., Nair, G. B., Ali, A.J., Morris, G., & Colwell, R. R. (2005). Critical factors influencing the occurrence of *Vibrio cholerae* in the environment of Bangladesh. *Applied and Environmental Microbiology*, 71(8), 4645-4654.

Huq, A., Whitehouse, C. A., Grim, C. J., Alam, M., & Colwell, R. R. (2008). Biofilms in water, its role and impact in human disease transmission. *Current Opinion in Biotechnology*, 19(3), 244-247.

Huq, M. I., Huber, D., & Kibryia, G. (1979). Isolation of urease producing *Vibrio parahaemolyticus* strains from cases of gastroenteritis. *Indian Journal of Medical Research,* 70, 549-553.

Huq, M. I., Aziz, K. M., & Colwell, R. R. (1985). Enterotoxigenic properties of *Vibrio fluvialis* (Group F *Vibrio*) isolated from clinical and environmental sources. *Journal of Diarrhoeal Disease Research*, 3(2), 96-99.

Huys, L., Dhert, P., Robles, R., Ollevier, F., Sorgeloos, P., & Swings, J. (2001). Search for beneficial bacterial strains for turbot (*Scophthalmus maximus* L.) larviculture. *Aquaculture*, 193(1), 25-37.

Ichinose, Y., Yamamoto, K., Nakasone, N., Tanabe, M. J., Takeda, T., Miwatani, T., & Iwanaga, M. (1987). Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae*. *Infection and Immunity*, 55(5), 1090-1093.

Igbinosa, E. O., & Okoh, A. I. (2008). Emerging *Vibrio* species: an unending threat to public health in developing countries. *Research in Microbiology*, 159(7), 495-506.

Iida, T., & Honda, T. (1997). Hemolysins produced by *Vibrios*. *Toxin Reviews*, 16(4), 215-227.

Ito, I., Osawa, M., Arita, M., Hashimoto, T., Ishida, T., Hongo, T., & Fujii, H. (1999). *Vibrio vulnificus* infection: Clinical and bacteriological analysis of four cases. *The Journal of the Japanese Association for Infectious Diseases*, 73(2), 149.

Iwamoto, Y., Suzuki, Y., Kurita, A., Watanabe, Y., Shimizu, T., Ohgami, H., & Yanagihara, Y. (1995). *Vibrio trachuri* sp. nov., a new species isolated from diseased Japanese horse mackerel. *Microbiology and Immunology*, 39(11), 831.

Jagadeeshan, S., Kumar, P., Abraham, W. P., & Thomas, S. (2009). Multiresistant Vibrio cholerae non□O1/non□O139 from waters in South India: resistance patterns and virulence□associated gene profiles. *Journal of Basic Microbiology*, 49(6), 538-544.

Jain, M., Kumar, P., Goel, A. K., Kamboj, D. V., & Singh, L. (2008). Class 1 integrons and SXT elements conferring multidrug resistance in *Vibrio cholerae* O1 strains associated with a recent large cholera outbreak in Orissa, Eastern India. *International Journal of Antimicrobial Agents*, 32(5), 459-460.

Janda, J. M., Brenden, R., DeBenedetti, J. A., Constantino, M. O., & Robin, T. (1986). *Vibrio alginolyticus* bacteremia in an immunocompromised patient. *Diagnostic Microbiology and Infectious Disease*, 5(4), 337-340.

Janda, J. M., Powers, C., Bryant, R. G., & Abbott, S. L. (1988). Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clinical Microbiology Reviews*, 1(3), 245-267.

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, 45(9), 2761-2764.

Jansen, W. T. M., Bolm, M., Balling, R., Chhatwal, G. S., & Schnabel, R. (2002). Hydrogen peroxide-mediated killing of *Caenorhabditis elegans* by *Streptococcus pyogenes*. *Infection and Immunity*, 70(9), 5202-5207.

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). Foodborne gastroenteritis caused by *Vibrio, Yersinia*, and *Campylobacter* species. *Modern Food Microbiology*, 657-678.

Jayaprakash, N. S., RejishKumar, V. J., Philip, R., & Bright Singh, I. S. (2006). *Vibrios* associated with *Macrobrachium rosenbergii* (De Man, 1879) larvae from three hatcheries on the Indian southwest coast. *Aquaculture Research*, 37(4), 351-358.

Jayasree, L., Janakiram, P., & Madhavi, R. (2006). Characterization of *Vibrio* spp. associated with diseased shrimp from culture ponds of Andhra Pradesh (India). *Journal of the World Aquaculture Society*, 37(4), 523-532.

Jean-Jacques, W., Rajashekaraiah, K. R., Farmer, J. J., Hickman, F. W., Morris, J. G., & Kallick, C. A. (1981). *Vibrio metschnikovii* bacteremia in a patient with cholecystitis. *Journal of Clinical Microbiology*, 14(6), 711-712.

Jeong, H. G., Oh, M. H., Kim, B. S., Lee, M. Y., Han, H. J., & Choi, S. H. (2009). The capability of catabolic utilization of N-acetylneuraminic acid, a sialic acid, is essential for *Vibrio vulnificus* pathogenesis. *Infection and Immunity*, 77(8), 3209-3217.

Jermyn, W. S., & Boyd, E. F. (2002). Characterization of a novel *Vibrio* pathogenicity island (VPI-2) encoding neuraminidase (nanH) among toxigenic *Vibrio cholerae* isolates. *Microbiology*, 148(11), 3681-3693.

Jermyn, W. S., & Boyd, E. F. (2005). Molecular evolution of Vibrio pathogenicity island-2 (VPI-2): mosaic structure among *Vibrio cholerae* and *Vibrio mimicus* natural isolates. *Microbiology*, 151(1), 311-322.

Jesudason, M. V., Thangavelu, C. P., & Lalitha, M. K. (1984). Rapid screening of faecal samples for *Vibrio cholerae* by a coagglutination technique. *Journal of Clinical Microbiology*, 19(5), 712-713.

Ji, H., Chen, Y., Guo, Y., Liu, X., Wen, J., & Liu, H. (2011). Occurrence and characteristics of *Vibrio vulnificus* in retail marine shrimp in China. *Food Control*, 22(12), 1935-1940.

Jiang, P., Li, J., Han, F., Duan, G., Lu, X., Gu, Y., & Yu, W. (2011). Antibiofilm activity of an exopolysaccharide from marine bacterium *Vibrio* sp. QY101. *PloS one*, *6*(4), e18514.

Jiang, S. C., Louis, V., Choopun, N., Sharma, A., Huq, A., & Colwell, R. R. (2000). Genetic diversity of *Vibrio cholerae* in Chesapeake Bay determined by amplified fragment length polymorphism fingerprinting. *Applied and Environmental Microbiology*, 66(1), 140-147.

Jimenez, M. L., Apostolou, A., Suarez, A. J. P., Meyer, L., Hiciano, S., Newton, A., & Pimentel, R. (2011). Multinational cholera outbreak after wedding in the Dominican Republic. *Emerging Infectious Diseases*, 17(11), 2172.

Jiravanichpaisal, P., & Miyazaki, T. (1995). *Comparative histopathology of vibriosis in black tiger shrimp, Penaeus monodon*. In: Shariff, M., Subasinghe, R.P., Arthur, J.R. (Eds.), *Diseases in Asian Aquaculture II*. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 123– 130.

John, T. J., Rajappan, K., & Arjunan, K. K. (2004). Communicable diseases monitored by disease surveillance in Kottayam district, Kerala state, India. *Indian Journal of Medical Research*, 120, 86-93.

Johnson, D. E., & Calia, F. M. (1981). Hemolytic reaction of clinical and environmental strains of *Vibrio vulnificus*. *Journal of Clinical Microbiology*, 14(4), 457-459.

Johnson, J. A., Morris, J. G., & Kaper, J. B. (1993). Gene encoding zonula occludens toxin (*zot*) does not occur independently from cholera enterotoxin genes (*ctx*) in *Vibrio cholerae*. *Journal of Clinical Microbiology*, 31(3), 732-733.

Johnson, L. K., Brown, M. B., Carruthers, E. A., Ferguson, J. A., Dombek, P. E., & Sadowsky, M. J. (2004). Sample size, library composition, and genotypic diversity among natural populations of *Escherichia coli* from different animals influence accuracy of determining sources of faecal pollution. *Applied and Environmental Microbiology*, 70(8), 4478-4485.

Johnston, J. M., Andes, W. A., & Glasser, G. (1983). *Vibrio vulnificus*: A gastronomic hazard. *The Journal of the American Medical Association*, 249(13), 1756-1757.

Jones, B. W., & Nishiguchi, M. K. (2004). Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Marine Biology*, 144(6), 1151-1155.

Jones, M. K., & Oliver, J. D. (2009). *Vibrio vulnificus*: Disease and pathogenesis. *Infection and Immunity*, 77(5), 1723-1733.

Joseph, A. V., Sasidharan, R. S., Nair, H. P., Bhat, S. G., (2013). Occurrence of potential pathogenic *Aeromonas* species in tropical seafood, aquafarms and mangroves off Cochin coast in South India. *Veterinary World*, *6*(6), 300-306.

Joseph, L. A., & Wright, A. C. (2004). Expression of *Vibrio vulnificus* capsular polysaccharide inhibits biofilm formation. *Journal of Bacteriology*, 186(3), 889-893.

Kaletta, T., & Hengartner, M. O. (2006). Finding function in novel targets: *C. elegans* as a model organism. *Nature Reviews Drug Discovery*, 5(5), 387-399.

Kamal, A. M. (1974). *The seventh pandemic of cholera*. In *Cholera.* The WB Saunders Co., Philadelphia. pp. 1-14.

Kang, H. P., & Dunne, W. M. (2003). Stability of repetitive-sequence PCR patterns with respect to culture age and subculture frequency. *Journal of Clinical Microbiology*, 41(6), 2694-2696.

Kannapiran, E., Ravindran, J., Chandrasekar, R.,& Kalaiarasi, A. (2009). Studies on luminous, *Vibrio harveyi* associated with shrimp culture system rearing *Penaeus monodon*. *Journal of Environmental Biology*, 30(5), 791-795.

Kanungo, S., Sah, B. K., Lopez, A. L., Sung, J. S., Paisley, A. M., Sur, D., Clemens , J.D., & Nair, G. B. (2010). Cholera in India: an analysis of reports, 1997-2006. *Bulletin of the World Health Organization*, 88(3), 185-191.

Kaper, J. B., Morris, J. G., & Levine, M. M. (1995). Cholera. *Clinical Microbiology Reviews*, 8(1), 48-86.

Karaolis, D. K., Johnson, J. A., Bailey, C. C., Boedeker, E. C., Kaper, J. B., & Reeves, P. R. (1998). A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. *Proceedings of the National Academy of Sciences*, 95(6), 3134-3139.

Karaolis, D. K., Somara, S., Maneval, D. R., Johnson, J. A., & Kaper, J. B. (1999). A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. *Nature*, 399(6734), 375-379.

Karaolis, D. K., Lan, R., Kaper, J. B., & Reeves, P. R. (2001). Comparison of *Vibrio cholerae* pathogenicity islands in sixth and seventh pandemic strains. *Infection and Immunity*, 69(3), 1947-1952.

Karasawa, T., Mihara, T., Kurazono, H., Nair, G. B., Garg, S., Ramamurthy, T., & Takeda, Y. (1993). Distribution of the *zot (*zonula occludens toxin) gene among strains of *Vibrio cholerae* O1 and non-O1. *FEMS Microbiology Letters*, 106(2), 143-145.

Karunasagar I., Susheela M., Malathi G.R., & Karunasagar, I.(1990) Incidence of human pathogenic *Vibrio* in seafoods harvested along the coast of Karnataka (India). *Export Inspection Journal,* VI: 53-56.

Karunasagar, I., Rivera, I., Joseph, B., Kennedy, B., Shetty, V. R., Huq, A., & Colwell, R. R. (2003). OmpU genes in non□toxigenic *Vibrio cholerae* associated with aquaculture. *Journal of Applied Microbiology*, 95(2), 338-343.

Katz, B. Z. (1988). *Vibrio vulnificus* meningitis in a boy with thalassemia after eating raw oysters. *Pediatrics*, 82(5), 784-786.

Kaufman, M. R., Seyer, J. M., & Taylor, R. K. (1991). Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by gram-negative bacteria. *Genes & Development*, 5(10), 1834-1846.

Kaufman, M. R., Shaw, C. E., Jones, I. D., & Taylor, R. K. (1993). Biogenesis and regulation of the *Vibrio cholerae* toxin-coregulated pilus: analogies to other virulence factor secretory systems. *Gene*, 126(1), 43-49.

Keasler, S., & Hall, R. (1993). Detecting and biotyping *Vibrio cholerae* 01 with multiplex polymerase chain reaction. *The Lancet*, 341(8861), 1661.

Kelly, M. T., & Avery, D. M. (1980). Lactose-positive *Vibrio* in seawater: a cause of pneumonia and septicemia in a drowning victim. *Journal of Clinical Microbiology*, 11(3), 278-280.

Kenzaka, T., Yamaguchi, N., Tani, K., & Nasu, M. (1998). rRNA targeted fluorescent in situ hybridization analysis of bacterial community structure in river water. *Microbiology*, 144(8), 2085-2093.

Khouadja, S., Suffredini, E., Spagnoletti, M., Croci, L., Colombo, M. M., & Amina, B. (2013). Presence of pathogenic *Vibrio parahaemolyticu*s in waters and seafood from the Tunisian Sea. *World Journal of Microbiology and Biotechnology*, 1-8.

Kierek, K., & Watnick, P. I. (2003). The *Vibrio cholerae* O139 O-antigen polysaccharide is essential for Ca2+-dependent biofilm development in sea water. *Proceedings of the National Academy of Sciences*, 100(24), 14357-14362.

Kim, C. M., Park, R. Y., Park, J. H., Sun, H. Y., Bai, Y. H., Ryu, P. Y., & Shin, S. H. (2006). *Vibrio vulnificus* vulnibactin, but not metalloprotease VvpE, is essentially required for iron-uptake from human holotransferrin. *Biological and Pharmaceutical Bulletin*, 29(5), 911-918.

Kim, H. B., Wang, M., Ahmed, S., Park, C. H., LaRocque, R. C., Faruque, A. S., & Hooper, D. C. (2010). Transferable quinolone resistance in *Vibrio cholerae*. *Antimicrobial Agents and Chemotherapy*, 54(2), 799-803.

Kim, H. R., Rho, H. W., Jeong, M. H., Park, J. W., Kim, J. S., Park, B. H., Kim, U.H., & Park, S. D. (1993). Hemolytic mechanism of cytolysin produced from *V. vulnificus*. *Life Sciences*, 53(7), 571-577.

Kim, J. H., Shin, S. P., Han, J. E., Jun, J. W., & Park, S. C. (2011). Occurrence and antibiotic resistance of *Vibrio vulnificus* in seafood and environmental waters in Korea. *Journal of Food Safety*, 31(4), 518-524.

Kim, Y. B., Okuda, J., Matsumoto, C., Takahashi, N., Hashimoto, S., & Nishibuchi, M. (1999). Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. *Journal of Clinical Microbiology*, 37(4), 1173-1177.

Kim, Y. R., Lee, S. E., Kook, H., Yeom, J. A., Na, H. S., Kim, S. Y., Chung, S.S., Choy, H.E., & Rhee, J. H. (2008). *Vibrio vulnificus* RTX toxin kills host cells only after contact of the bacteria with host cells. *Cellular Microbiology*, 10(4), 848-862.

King, A. A., Ionides, E. L., Pascual, M., & Bouma, M. J. (2008). Inapparent infections and cholera dynamics. *Nature*, 454(7206), 877-880.

Kirn, T. J., Lafferty, M. J., Sandoe, C. M., & Taylor, R. K. (2000). Delineation of pilin domains required for bacterial association into microcolonies and intestinal colonization by *Vibrio cholerae*. *Molecular Microbiology*, 35(4), 896-910.

Kitaoka, M., Miyata, S. T., Unterweger, D., & Pukatzki, S. (2011). Antibiotic resistance mechanisms of *Vibrio cholerae*. *Journal of Medical Microbiology*, 60(4), 397-407.

Ko, W. C., Chuang, Y. C., Huang, G. C., & Hsu, S. Y. (1998). Infections due to non-O1 *Vibrio cholerae* in southern Taiwan: Predominance in cirrhotic patients. *Clinical Infectious Diseases*, 27(4), 774-780.

Koenig, J. E., Bourne, D. G., Curtis, B., Dlutek, M., Stokes, H. W., Doolittle, W. F., & Boucher, Y. (2011). Coral-mucus-associated *Vibrio* integrons in the Great Barrier Reef: genomic hotspots for environmental adaptation. *The ISME Journal*, 5(6), 962-972.

Koeuth, T., Versalovic, J., & Lupski, J. R. (1995). Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Research*, 5(4), 408-418.

Kondo, S., & Ajawatanawong, P. (2009). Distribution and sequence analysis of virulence associated genes in *Vibrio cholerae* O1, O139 and non-O1/non-O139 isolates from Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, 40(5), 1015.

Kook, H., Lee, S. E., Baik, Y. H., Chung, S. S., & Rhee, J. H. (1996). *Vibrio vulnificus* hemolysin dilates rat thoracic aorta by activating guanylate cyclase. *Life Sciences*, 59(3), 41-47.

Kothary, M. H., & Kreger, A. S. (1985). Production and partial characterization of an elastolytic protease of *Vibrio vulnificus*. *Infection and Immunity*, 50(2), 534-540.

Kovach, M.E., Shaffer, M.D., Peterson, K.M. (1996) A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in *Vibrio cholerae*. *Microbiology,* 142, 2165-2174.

Kreger, A., & Lockwood, D. (1981). Detection of extracellular toxin (s) produced by *Vibrio vulnificus*. *Infection and Immunity*, 33(2), 583-590.

Kreger, A. S. (1984). Cytolytic activity and virulence of *Vibrio damsela*. *Infection and Immunity*, 44(2), 326-331.

Krieg, N. R., & Holt, J. G. (1984). *Bergy's manual of determinative bacteriology*. Baltimore, The Williams and Wilkins Co. Baltimore.

Krishna, B. V. S., Patil, A. B., & Chandrasekhar, M. R. (2006). Fluoroquinolone-resistant *Vibrio cholerae* isolated during a cholera outbreak in India. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 100(3), 224-226.

Krukonis, E. S., Yu, R. R., & DiRita, V. J. (2000). The *Vibrio cholerae* ToxR/TcpP/ToxT virulence cascade: Distinct roles for two membrane□localized transcriptional activators on a single promoter. *Molecular Microbiology*, 38(1), 67-84.

Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. *Applied and Environmental Microbiology*, 46(1), 165-170.

Kuhnt-Lenz, K., Krengel, S., Fetscher, S., Heer-Sonderhoff, A., & Solbach, W. (2004). Sepsis with bullous necrotizing skin lesions due to *Vibrio vulnificus* acquired through recreational activities in the Baltic Sea. *European Journal of Clinical Microbiology and Infectious Diseases*, 23(1), 49-52.

Kumamoto, M. D., Kenneth, S., Vukich, M. D., & David, J. (1998). Clinical Infections of *Vibrio vulnificus*: A case report and review of the literature. *The Journal of Emergency Medicine*, 16(1), 61-66.

Kumar, H. S., Parvathi, A., Karunasagar, I., & Karunasagar, I. (2006). A *gyrB* based PCR for the detection of *Vibrio vulnificus* and its application for direct detection of this pathogen in oyster enrichment broths. *International Journal of Food Microbiology*, 111(3), 216-220.

Kumar, P. A., Patterson, J., & Karpagam, P. (2009). Multiple antibiotic resistance profiles of *Vibrio cholerae* non-O1 and non-O139. *Japanese Journal of Infectious Diseases*, 62(3), 230-232.

Kumar, P., Peter, W. A., & Thomas, S. (2008). Detection of virulence genes in *Vibrio cholerae* isolated from aquatic environment in Kerala, Southern India. *Applied Biochemistry and Biotechnology*, 151(2-3), 256-262.

Kumar, R., & Lalitha, K. V. (2013). Prevalence and molecular characterization of *Vibrio cholerae* O1, non-O1 and non-O139 in tropical seafood in Cochin, India. *Foodborne Pathogens and Disease*, 10(3), 278-283.

Kumar, S., George, M. R., John, K. R., & Jeyaseelan, M. P. (2007). Molecular typing of bacteria *Vibrio harveyi* and *V. alginolyticus* from shrimp farming systems. *Indian Journal of Marine Sciences*, 36(1), 43-50.

Kumaran, S., Deivasigamani, B., Alagappan, K. M., & Sakthivel, M. (2010). Infection and immunization trials of Asian seabass (*Lates calcarifer*) against fish pathogen *Vibrio anguillarum*. *Journal of Environmental Biology*, 31(4), 539-541.

Kurz, C. L., Chauvet, S., Andrès, E., Aurouze, M., Vallet, I., Michel, G. P., Uh, M., & Ewbank, J. J. (2003). Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. *The EMBO Journal*, 22(7), 1451.

Kvennefors, E. C. E., Sampayo, E., Ridgway, T., Barnes, A. C., & Hoegh-Guldberg, O. (2010). Bacterial communities of two ubiquitous Great Barrier Reef corals reveals both site-and species-specificity of common bacterial associates. *PloS one*, 5(4), e10401.

Labrousse, A., Chauvet, S., Couillault, C., Leopold Kurz, C., & Ewbank, J. J. (2000). *Caenorhabditis elegans* is a model host for *Salmonella typhimurium*. *Current Biology*, 10(23), 1543-1545.

Lally, E. T., Hill, R. B., Kieba, I. R., & Korostoff, J. (1999). The interaction between RTX toxins and target cells. *Trends in Microbiology*, 7(9), 356-361.

Lambert, C., Nicolas, J.L., Cilia, V., & Corre, S. (1998) *Vibrio pectenicida* sp. nov., a pathogen of scallop (*Pecten maximus*) larvae. *International Journal of Systematic Bacteriology*, 48, 2481–2487.

Lan, R., & Reeves, P. R. (2002). Pandemic spread of cholera: Genetic diversity and relationships within the seventh pandemic clone of *Vibrio cholerae* determined by amplified fragment length polymorphism. *Journal of Clinical Microbiology*, 40(1), 172-181.

Lancelot, C., & Billen, G. (1984). Activity of heterotrophic bacteria and its coupling to primary production during the spring phytoplankton bloom in the Southern Bight of the North Sea. *Limnology and Oceanography*, 29(4), 721-730.

Lang, H., & Palva, E.T. *(*1993*)* The *ompS* gene of *Vibrio cholerae* encodes a growth-phase-dependent maltoporin. *Molecular Microbiology,*10, 891–901.

Lara, R. J., Neogi, S. B., Islam, M. S., Mahmud, Z. H., Islam, S., Paul, D., & Kattner, G. (2011). *Vibrio cholerae* in waters of the Sunderban mangrove: Relationship with biogeochemical parameters and chitin in seston size fractions. *Wetlands Ecology and Management*, 19(1), 109-119.

Larsen, M. H., & Boesen, H. T. (2001). Role of flagellum and chemotactic motility of *Vibrio anguillarum* for phagocytosis by and intracellular survival in fish macrophages. *FEMS Microbiology Letters*, 203(2), 149-152.

Larsen, M. H., Larsen, J. L., & Olsen, J. E. (2001). Chemotaxis of *Vibrio anguillarum* to fish mucus: Role of the origin of the fish mucus, the fish species and the serogroup of the pathogen. *FEMS Microbiology Ecology*, 38(1), 77-80.

Lavilla-Pitogo, C. R., Baticados, M. C. L., Cruz-Lacierda, E. R., & de la Pena, L. D. (1990). Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Aquaculture*, 91(1), 1-13.

Lavilla-Pitogo, C.R. (1995). *Bacterial diseases of penaeid shrimps: An Asian view*. In: Shariff, M., Arthur, J.R., Subasinghe, R.P. (Eds.), *Diseases in Asian Aquaculture II*. Fish Health Section, Asian Fisheries Society, Manila. pp. 107– 121.

Lavilla-Pitogo, C. R., Leano, E. M., & Paner, M. G. (1998). Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent *Vibrios* in the rearing environment. *Aquaculture*, 164(1), 337-349.

Le Roux, W., Masoabi, D., de Wet, C., & Venter, S. (2004). Evaluation of a rapid polymerase chain reaction based identification technique for *Vibrio cholerae* isolates. *Water Science & Technology*, 50(1), 229-232.

Lee, C. Y., Pan, S. F., & Chen, C. H. (1995). Sequence of a cloned pR72H fragment and its use for detection of *Vibrio parahaemolyticus* in shellfish with the PCR. *Applied and Environmental Microbiology*, 61(4), 1311-1317.

Lee, J. H., Rho, J. B., Park, K. J., Kim, C. B., Han, Y. S., Choi, S. H., & Park, S. J. (2004). Role of flagellum and motility in pathogenesis of *Vibrio vulnificus*. *Infection and Immunity*, 72(8), 4905-4910.

Lee, J. H., Kim, M. W., Kim, B. S., Kim, S. M., Lee, B. C., Kim, T. S., & Choi, S. H. (2007). Identification and characterization of the *Vibrio vulnificus rtx*A essential for cytotoxicity in vitro and virulence in mice. *Journal of Microbiology-Seoul-*, 45(2), 146.

Lee, J. V., Shread, P., Furniss, A. L., & Bryant, T. N. (1981). Taxonomy and description of *Vibrio fluvialis* sp. nov. (synonym group F *Vibrios*, group EF6). *Journal of Applied Microbiology*, 50(1), 73-94.

Lee, K. K., Liu, P. C., & Chuang, W. H. (2002). Pathogenesis of gastroenteritis caused by *Vibrio carchariae* in cultured marine fish. *Marine Biotechnology*, 4(3), 267-277.

Lee, S. E., Kim, S. Y., Kim, S. J., Kim, H. S., Shin, J. H., Choi, S. H., & Rhee, J. H. (1998). Direct identification of *Vibrio vulnificus* in clinical specimens by nested PCR. *Journal of Clinical Microbiology*, 36(10), 2887-2892.

Lee, S. E., Shin, S. H., Kim, S. Y., Kim, Y. R., Shin, D. H., Chung, S. S., Lee, Z.H., & Rhee, J. H. (2000). *Vibrio vulnificus* has the transmembrane transcription activator ToxRS stimulating the expression of the hemolysin gene *vvhA*. *Journal of Bacteriology*, 182(12), 3405-3415.

Lemonnier, H., Herbland, A., Salery, L., & Soulard, B. (2006). "Summer syndrome" in *Litopenaeus stylirostris* grow out ponds in New Caledonia: Zootechnical and environmental factors. *Aquaculture*, 261(3), 1039-1047.

Levine, W. C., & Griffin, P. M. (1993). *Vibrio* infections on the Gulf Coast: results of first year of regional surveillance. *The Journal of Infectious Diseases*, 479-483.

Li, Y., Ma, X. L., & Zhang, L. Y. (2012). Analysis on drug resistance of pathogenic *Vibrio* in food. *Modern Preventive Medicine*, 3, 026.

Lightner, D.V. (1993). Diseases of cultured penaeid shrimps. In: Mc Vey, J.P. (Ed.), CRC Handbook of Mariculture, 2nd ed.,CRC Press, Boca Raton, pp. 393– 486.

Lightner, D. V., & Redman, R. M. (1998). Shrimp diseases and current diagnostic methods. *Aquaculture,*164, 201–220.

Lin, W., Fullner, K. J., Clayton, R., Sexton, J. A., Rogers, M. B., Calia, K. E., Calderwood, S. B., Fraser, C., & Mekalanos, J. J. (1999). Identification of a *Vibrio cholerae* RTX toxin gene cluster that is tightly linked to the cholera toxin prophage. *Proceedings of the National Academy of Sciences*, 96(3), 1071-1076.

Lin, Z., Kumagai, K., Baba, K., Mekalanos, J. J., & Nishibuchi, M. (1993). *Vibrio parahaemolyticus* has a homolog of the *Vibrio cholerae toxRS* operon that mediates environmentally induced regulation of the thermostable direct hemolysin gene. *Journal of Bacteriology*, 175(12), 3844-3855.

Linde, H. J., Kobuch, R., Jayasinghe, S., Reischl, U., Lehn, N., Kaulfuss, S., & Beutin, L. (2004). *Vibrio metschnikovii*, a rare cause of wound infection. *Journal of Clinical Microbiology*, 42(10), 4909-4911.

Link, E. M., Hardiman, G., Sluder, A. E., Johnson, C. D., & Liu, L. X. (2000). Therapeutic target discovery using *Caenorhabditis elegans*. *Pharmacogenomics*, 1(2), 203-217.

Lipp, E.K., Huq, A., & Colwell, R.R. (2002). Effects of global climate on infectious disease: The cholera model. *Clinical Microbiology Reviews,* 15, 757–770.

Lipp, E. K., Rivera, I. N., Gil, A. I., Espeland, E. M., Choopun, N., Louis, V. R., & Colwell, R. R. (2003). Direct detection of *Vibrio cholerae* and *ctxA* in Peruvian coastal water and plankton by PCR. *Applied and Environmental Microbiology*, 69(6), 3676-3680.

Litwin, C. M., & Byrne, B. L. (1998). Cloning and characterization of an outer membrane protein of *Vibrio vulnificus* required for heme utilization: Regulation of expression and determination of the gene sequence. *Infection and Immunity*, 66(7), 3134-3141.

Liu, C. H., Cheng, W., Hsu, J. P., & Chen, J. C. (2004a). *Vibrio alginolyticus* infection in the white shrimp *Litopenaeus vannamei* confirmed by polymerase chain reaction and 16S rDNA sequencing. *Diseases of Aquatic Organisms*, 61(1), 169-174.

Liu, P. C., Lee, K. K., Yii, K. C., Kou, G. H., & Chen, S. N. (1996). News & Notes: Isolation of *Vibrio harveyi* from diseased Kuruma prawns, *Penaeus japonicus*. *Current Microbiology*, 33(2), 129-132.

Liu, P. C., Chen, Y. C., & Lee, K. K. (2001). Pathogenicity of *Vibrio alginolyticus* isolated from diseased small abalone *Haliotis diversicolor supertexta*. *Microbios*, 104(408), 71.

Liu, P. C., Chuang, W. H., & Lee, K. K. (2003). Infectious gastroenteritis caused by *Vibrio harveyi* (*V. carchariae*) in cultured red drum, *Sciaenops ocellatus*. *Journal of Applied Ichthyology*, 19(1), 59-61.

Liu, P. C., Lin, J. Y., Chuang, W. H., & Lee, K. K. (2004b). Isolation and characterization of pathogenic *Vibrio harveyi* (*V. carchariae*) from the farmed marine cobia fish *Rachycentron canadum* L. with gastroenteritis syndrome. *World Journal of Microbiology and Biotechnology*, 20(5), 495-499.

Long, R. A., & Azam, F. (2001). Microscale patchiness of bacterioplankton assemblage richness in seawater. *Aquatic Microbial Ecology*, 26, 103.

Long, R. A., Rowley, D. C., Zamora, E., Liu, J., Bartlett, D. H., & Azam, F. (2005). Antagonistic interactions among marine bacteria impede the proliferation of *Vibrio cholerae*. *Applied and Environmental Microbiology*, 71(12), 8531-8536.

Love, M., Teebken-Fisher, D., Hose, J. E., Farmer III, J. J., Hickman, F. W., & Fanning, G. R. (1981). *Vibrio damsela*, a marine bacterium, causes skin ulcers on the damselfish *Chromis punctipinnis*. *Science*, 214(4525), 1139-1140.

Lowry, P. W., McFarland, L. M., & Threefoot, H. K. (1986). *Vibrio hollisae* septicemia after consumption of catfish. *Journal of Infectious Diseases*, 154(4), 730-731.

Lozano□Leon, A., Torres, J., Osorio, C. R., & Martínez□Urtaza, J. (2003). Identification of tdh□positive *Vibrio parahaemolyticus* from an outbreak associated with raw oyster consumption in Spain. *FEMS Microbiology Letters*, 226(2), 281-284.

Luan, X. Y., Chen, J. X., Zhang, X. H., Jia, J. T., Sun, F. R., & Li, Y. (2007). Comparison of different primers for rapid detection of *Vibrio parahaemolyticus* using the polymerase chain reaction. *Letters in Applied Microbiology*, 44(3), 242-247.

Ludwig, A., & Goebel, M. (1997). Hemolysins of *Escherichia coli.* In: Sussman, M. (Ed.) *Escherichia coli*: *Mechanisms of virulence.* Cambridge University Press, pp. 281-329.

MacRae, S. H., Clements, T., & Horan, J. H. (1983). Non-O1 *Vibrio cholerae* gastroenteritis in New Hampshire: a case report. *American Journal of Medical Technology*, *49*, 327-329.

Magalhaes, V., Castello Filho, A., Magalhaes, M., & Gomes, T. T. (1993). Laboratory evaluation on pathogenic potentialities of *Vibrio furnissii*. *Memórias do Instituto Oswaldo Cruz*, 88(4), 593-597.

Mahajan-Miklos, S., Tan, M. W., Rahme, L. G., & Ausubel, F. M. (1999). Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa–Caenorhabditis elegans* pathogenesis model. *Cell*, 96(1), 47-56.

Mahajan□Miklos, S., Rahme, L. G., & Ausubel, F. M. (2000). Elucidating the molecular mechanisms of bacterial virulence using non□mammalian hosts. *Molecular Microbiology*, 37(5), 981-988.

Maiti, B., Shekar, M., Khushiramani, R., Karunasagar, I., & Karunasagar, I. (2009). Evaluation of RAPD-PCR and protein profile analysis to differentiate *Vibrio harveyi* strains prevalent along the southwest coast of India. *Journal of Genetics*, 88(3), 273-279.

Maluping, R. P., Ravelo, C., Lavilla□Pitogo, C. R., Krovacek, K., & Romalde, J. L. (2005). Molecular typing of *Vibrio parahaemolyticus* strains isolated from the Philippines by PCR□based methods. Journal of Applied Microbiology, 99(2), 383-391.

Maluping, R. P., Lavilla-Pitogo, C. R., Romalde, J. L., & Krovacek, K. (2008). Molecular typing and antimicrobial susceptibility of *Vibrio parahaemolyticus* strains. *Diseases in Asian Aquaculture*, 6, 451-468.

Manjusha, S., Sarita, G. B., Elyas, K. K., & Chandrasekaran, M. (2005). Multiple antibiotic resistances of *Vibrio* isolates from coastal and brackish water areas. *American Journal of Biochemistry and Biotechnology*, 1(4), 201-206.

Manjusha, K., Jayesh, P., Jose, D., Sreelakshmi, B., Priyaja, P., Gopinath, P., & Singh, I. B. (2013). Alkaline protease from a non-toxigenic mangrove isolate of *Vibrio* sp. V26 with potential application in animal cell culture. *Cytotechnology*, 65(2), 199-212.

Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., & Bryant, S. H. (2011). CDD: A Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Research*, 39 (suppl 1), D225-D229.

Marco□Noales, E., Biosca, E. G., Rojo, C., & Amaro, C. (2004). Influence of aquatic microbiota on the survival in water of the human and eel pathogen *Vibrio vulnificus* serovar E. *Environmental Microbiology*, 6(4), 364-376.

Marhual, N. P., Das, B. K., Pradhan, J., Swain, P., Mishra, B. K., & Eknath, A. E. (2012). RAPD-PCR and outer membrane protein characterization of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* isolated from diseased shrimp. *The Israeli Journal of Aquaculture*, 64, 8p.

Marques, A. S., Marchaison, A., Gardan, L., & Samson, R. (2008). BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae*: *P. viridiflava* group. *Genetics and Molecular Biology*, 31(1), 106-115.

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., & Claverys, J. P. (1992). A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Research*, 20(13), 3479-3483.

Maruyama, A., Honda, D., Yamamoto, H., Kitamura, K., & Higashihara, T. (2000). Phylogenetic analysis of psychrophilic bacteria isolated from the Japan Trench, including a description of the deep-sea species *Psychrobacter pacificensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, 50(2), 835-846.

Massad, G., & Oliver, J. D. (1987). New selective and differential medium for *Vibrio cholerae* and *Vibrio vulnificus*. *Applied and Environmental Microbiology*, 53(9), 2262-2264.

Mathur, J., & Waldor, M. K. (2004). The *Vibrio cholerae* ToxR-regulated porin OmpU confers resistance to antimicrobial peptides. *Infection and Immunity*, 72(6), 3577-3583.

Matyar, F., Kaya, A., & Dincer, S. (2007). Distribution and antibacterial drug resistance of *Aeromonas* spp. from fresh and brackish waters in Southern Turkey. *Annals of Microbiology*,57, 443–447.

Matz, C., McDougald, D., Moreno, A. M., Yung, P. Y., Yildiz, F. H., & Kjelleberg, S. (2005). Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *Proceedings of the National Academy of Sciences of the United States of America*, 102(46), 16819-16824.

Mccoubrey, D. J. (1996). Risk of *Vibrio vulnificus* infection following consumption of raw commercially harvested North Island oysters. PhD Thesis. University of Auckland.

McFall-Ngai, M. J. (1994). Animal-bacterial interactions in the early life history of marine invertebrates: The *Euprymna scolopes/Vibrio fischeri* symbiosis. *American Zoologist*, 34(4), 554-561.

McFall-Ngai, M. J., & Ruby, E. G. (1998). Sepiolids and *Vibrios*: When first they meet. *BioScience*, 48(4), 257-265.

McLaughlin, J. B., DePaola, A., Bopp, C. A., Martinek, K. A., Napolilli, N. P., Allison, C. G., Murray, S.L., Thompson, E.C.,Bird, M.M., & Middaugh, J. P. (2005). Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. *New England Journal of Medicine*, 353(14), 1463-1470.

McLaughlin, J. C. (1995). *"Vibrio."* In: Baron, P, M., Tenover, E, J., Yolken, F. C. (Eds.) 6th Edition, *Manual of clinical microbiology*. ASM Press, Washington.

Mekalanos, J.J. (1983). Duplication and amplification of toxin genes in *Vibrio cholerae*. *Cell*, 35(1), 253-263.

Middelboe, M., Sondergaard, M., Letarte, Y., & Borch, N. H. (1995). Attached and free-living bacteria: Production and polymer hydrolysis during a diatom bloom. *Microbial Ecology*, 29(3), 231-248.

Mienda, B. S. (2012). Proteolytic activity of *Vibrio parahaemolyticus* isolated from *Epinephilus* spp. A preliminary report. *Research in Biotechnology*, 3(1), 36-40.

Mihajlovic, L., Bockemuhl, J., Heesemann, J., & Laufs, R. (1982). Imported mussels as a cause of *Vibrio parahaemolyticus* gastroenteritis. *Infection*, 10(5), 285-289.

Miller, M. B., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Reviews in Microbiology*, 55(1), 165-199.

Miller, V. L., Taylor, R. K., & Mekalanos, J. J. (1987). Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. *Cell*, 48(2), 271-279.

Miller, V. L., & Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: Osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. *Journal of Bacteriology*, 170(6), 2575-2583.

Mishra, A., Taneja, N., Sharma, R., Kumar, R., Sharma, N., & Sharma, M. (2011). Amplified fragment length polymorphism of clinical and environmental *Vibrio cholerae* from a freshwater environment in a cholera-endemic area, India. *BMC Infectious Diseases*, 11(1), 249.

Miyake, M., Honda, T., & Miwatani, T. (1988). Purification and characterization of *Vibrio metschnikovii* cytolysin. *Infection and Immunity*, 56(4), 954-960.

Miyoshi, N., Shimizu, C., Miyoshi, S., & Shinoda, S. (1987). Purification and characterization of *Vibrio vulnificus* protease. *Microbiology and Immunology*, 31(1), 13.

Miyoshi, S. I., & Shinoda, S. (2000). Microbial metalloproteases and pathogenesis. *Microbes and Infection*, 2(1), 91-98.

Miyoshi, S. I., Watanabe, H., Kawase, T., Yamada, H., & Shinoda, S. (2004). Generation of active fragments from human zymogens in the brady kinin-generating cascade by extracellular proteases from *Vibrio vulnificus* and *V. parahaemolyticus, Toxicon*, 44(8), 887-893.

Mohapatra, S. S., Ramachandran, D., Mantri, C. K., & Singh, D. V. (2007). Characterization of the genetic background of *Vibrio cholerae* O1 biotype El Tor serotype Inaba strains isolated in Trivandrum, Southern India. *Journal of Medical Microbiology*, 56(2), 260-265.

Monsur, A. K. (1961). A highly selective gelatin-taurocholate-tellurite medium for the isolation of *Vibrio cholerae*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 55(5), 440-442.

Montes, M., Pérez, M. J., & Nieto, T. P. (1999). Numerical taxonomy of Gram-Negative, facultative anaerobic bacteria isolated from skin of Turbot (*Scophthalmus maximus*) and surrounding water. *Systematic and Applied Microbiology*, 22(4), 604-618.

Montieri, S., Suffredini, E., Ciccozzi, M., & Croci, L. (2010). Phylogenetic and evolutionary analysis of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* isolates based on *toxR* gene sequence. *The New Microbiologica*, 33(4), 359.

Moreno, C., Romero, J., & Espejo, R. T. (2002). Polymorphism in repeated 16S rRNA genes is a common property of type strains and environmental isolates of the genus *Vibrio*. *Microbiology*, 148(4), 1233-1239.

Morris, J. G. Jr., Wilson, R., Hollis, D., Weaver, R., Miller, H., Tacket, C., & Blake, P. (1983). Illness caused by *Vibrio damsela* and *Vibrio hollisae*. *The Lancet*, 319(8284), 1294-1297.

Morris, J. G. Jr, & Tenney, J., (1985). Antibiotic therapy for *Vibrio vulnificus* infection. *JAMA*, 253, 1121–1122.

Morris, J. G. Jr., (1990). Non-O group 1 *Vibrio cholerae*: A look at the epidemiology of an occasional pathogen. *Epidemiologic Reviews*, 12, 179.

Morris, J. G. Jr., Takeda, T., Tall, B. D., Losonsky, G. A., Bhattacharya, S. K., Forrest, B. D., Kay, B. A., & Nishibuchi, M.(1990). Experimental non-O group 1 *Vibrio cholerae* gastroenteritis in humans. *Journal of Clinical Investigations,* 85, 697-705.

Morris, J. G. Jr., & Acheson, D. (2003). Cholera and other types of vibriosis: a story of human pandemics and oysters on the half shell. *Clinical Infectious Diseases*, 37(2), 272-280.

Mukhopadhyay, A. K., Saha, P. K., Garg, S., Bhattacharya, S. K., Shimada, T., Takeda, T., & Nair, G. B. (1995). Distribution and virulence of *Vibrio cholerae* belonging to serogroups other than O1 and O139: A nationwide survey. *Epidemiology and Infection*, 114(1), 65-70.

Mukhopadhyay, A. K., Basu, I., Bhattacharya, S. K., Bhattacharya, M. K., & Nair, G. B. (1998). Emergence of fluoroquinolone resistance in strains of *Vibrio cholerae* isolated from hospitalized patients with acute diarrhea in Calcutta, India. *Antimicrobial Agents and Chemotherapy*, 42(1), 206-207.

Mukhopadhyay, A. K., Chakraborty, S., Takeda, Y., Nair, G. B., & Berg, D. E. (2001). Characterization of VPI Pathogenicity Island and CTXφ prophage in environmental strains of *Vibrio cholerae*. *Journal of Bacteriology*, 183(16), 4737-4746.

Murphree, R. L., & Tamplin, M. L. (1995). Uptake and retention of *Vibrio cholerae* O1 in the Eastern oyster, *Crassostrea virginica*. *Applied and Environmental Microbiology*, 61(10), 3656-3660.

Mylonakis, E., Ausubel, F. M., Perfect, J. R., Heitman, J., & Calderwood, S. B. (2002). Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis. *Proceedings of the National Academy of Sciences*, 99(24), 15675-15680.

Nagamune, K., Yamamoto, K., Naka, A., Matsuyama, J., Miwatani, T., & Honda, T. (1996). *In vitro* proteolytic processing and activation of the recombinant precursor of ElTor cytolysin/hemolysin (pro-HlyA) of *Vibrio cholerae* by soluble hemagglutinin/protease of *V. cholerae*, trypsin, and other proteases. *Infection and Immunity*, 64(11), 4655-4658.

Nair, G.B., Bhadra, R. K., Ramamurthy, T., Ramesh, A., & Pal, S. C. (1991). *Vibrio cholerae* and other *Vibrios* associated with paddy field cultured prawns. *Food Microbiology*, 8(3), 203-208.

Nair, G. B., Ramamurthy, T., Bhattacharya, S. K., Dutta, B., Takeda, Y., & Sack, D. A. (2007). Global dissemination of *Vibrio parahaemolyticus* serotype O3: K6 and its serovariants. *Clinical Microbiology Reviews*, 20(1), 39-48.

Nair, G. B. (2008). The new emerging strain of cholera: One step ahead of genomics. *International Journal of Infectious Diseases*, 12, e5-e6.

Nakashima, Y., Oho, M., Kusaba, K., Nagasawa, Z., Komatsu, O., Manome, I., & Nakashima, M. (2007). A chromogenic substrate culture plate for early identification of *Vibrio vulnificus* and isolation of other marine *Vibrios*. *Annals of Clinical & Laboratory Science*, 37(4), 330-334.

Nammalwar, P., & Thangaraj, T. (1980). Problems of diseases on marine fish and prawn farming in India. *Seafood Export Journal*, 12(12), 9-12.

Nandi, B., Nandy, R. K., Mukhopadhyay, S., Nair, G. B., Shimada, T., & Ghose, A. C. (2000). Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. *Journal of Clinical Microbiology*, 38(11), 4145-4151.

Nato, F., Boutonnier, A., Rajerison, M., Grosjean, P., Dartevelle, S., Guenole, A., & Chanteau, S. (2003). One-step immunochromatographic dipstick tests for rapid detection of *Vibrio cholerae* O1 and O139 in stool samples. *Clinical and Diagnostic Laboratory Immunology*, 10(3), 476-478.

NeiNei, M., & Kumar, S. (2000). *Molecular evolution and phylogenetics*. Oxford University Press.

Ngandjio, A., Tejiokem, M., Wouafo, M., Ndome, I., Yonga, M., Guenole, A., Lemee, L., Quilici, M., & Fonkoua, M. C. (2009). Antimicrobial resistance and molecular characterization of *Vibrio cholerae* O1 during the 2004 and 2005 outbreak of cholera in Cameroon. *Foodborne Pathogens and Disease*, 6(1), 49-56.

Nicolas, J. L., Corre, S., Gauthier, G., Robert, R., & Ansquer, D. (1996). Bacterial problems associated with scallop *Pecten maximus* larval culture. *Diseases of Aquatic Organisms*, 27(1), 67-76.

Nicolas, J. L., Basuyaux, O., Mazurie, J., & Thebault, A. (2002). *Vibrio carchariae*, a pathogen of the abalone *Haliotis tuberculata*. *Diseases of Aquatic Organisms*, 50(1), 35-43.

Nishiguchi, M. K., Lopez, J. E., & Boletzky, S. V. (2004). Enlightenment of old ideas from new investigations: More questions regarding the evolution of bacteriogenic light organs in squids. *Evolution & Development*, 6(1), 41-49.

Nishimori, E., Hasgawa, O., Numata, T., & Wakabayashi, H. (1998).*Vibrio carchariae* (*harveyi*) causes mass mortalities in Japanese abalone, *Suculus diversicolor suprasexta*. *Fish Pathology*, 33, 495-502.

Nishina, Y., Miyoshi, S. I., Nagase, A, & Shinoda, S. (1992). Significant role of an exocellular protease in utilization of heme by *Vibrio vulnificus*. *Infection and Immunity*, 60(5), 2128-2132.

Nitzan, Y., Gribun, A., & Pechatnikov, I. (1999). Characterization of porins isolated from the outer membrane of *Vibrio damsela*. *Current Microbiology*, 38(1), 1-8.

Noorlis, A., Ghazali, F. M., Cheah, Y. K., Tuan Zainazor, T. C., Ponniah, J., Tunung, R., Tang,J.Y.H., Nishibuchi, M., Nakaguchi, Y., & Son, R. (2011). Prevalence and quantification of *Vibrio* species and *Vibrio parahaemolyticus* in freshwater fish at hypermarket level. *International Food Research Journal*, 18, 673-679.

Novotny, L., Dvorska, L., Lorencova, A., Beran, V., & Pavlik, I. (2004). Fish: a potential source of bacterial pathogens for human beings. *Veterinarni Medicina*, 49(9), 343-358.

Nyholm, S. V., & McFall-Ngai, M. (2004). The winnowing: Establishing the squid–*Vibrio* symbiosis. *Nature Reviews Microbiology*, 2(8), 632-642.

Odu, N.N., Igwiloh, N.J.P., Okonko, I.O., & Njoku, H.O. (2011). Occurrence of potentially pathogenic *Vibrio species* in some edible molluscs from Kalarugbani creek in Okirika LGA, River State, Nigeria. *Archives of Applied Science Research*, 3 (4), 248-255.

Ogawa, A., & Takeda, T. (1993). The gene encoding the heat-stable enterotoxin of *Vibrio cholerae* is flanked by 123-base pair direct repeats. *Microbiology and Immunology*, 37(8), 607.

Ogierman, M. A., Zabihi, S., Mourtzios, L., & Manning, P. A. (1993). Genetic organization and sequence of the promoter-distal region of the *tcp* gene cluster of *Vibrio cholerae*. *Gene*, 126(1), 51-60.

O'Hara, C. M., Sowers, E. G., Bopp, C. A., Duda, S. B., & Strockbine, N. A. (2003). Accuracy of six commercially available systems for identification of members of the family Vibrionaceae. *Journal of Clinical Microbiology*, 41(12), 5654-5659.

Okada, K., Iida, T., Kita-Tsukamoto, K., & Honda, T. (2005). *Vibrios* commonly possess two chromosomes. *Journal of Bacteriology*, 187(2), 752-757.

Okuda, J., Nakai, T., Chang, P. S., Oh, T., Nishino, T., Koitabashi, T., & Nishibuchi, M. (2001). The *toxR* gene of *Vibrio (Listonella) anguillarum* controls expression of the major outer membrane proteins but not virulence in a natural host model. *Infection and Immunity*, 69(10), 6091-6101.

Okujo, N., Akiyama, T., Miyoshi, S. I., Shinoda, S., & Yamamoto, S. (1996). Involvement of vulnibactin and exocellular protease in utilization of transferrin-and lactoferrin-bound iron by *Vibrio vulnificus*. *Microbiology and Immunology*, 40(8), 595.

Olafsen, J. A. (2001). Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture*, 200(1), 223-247.

Oliver, J.D., Wear, J.E., Thomas, M.B., Warner, M., & Linder, K. (1986). Production of extracellular enzymes and cytotoxicity by *Vibrio vulnificus*. *Diagnostic Microbiology and Infectious Diseases,* 5, 99-111.

Oliver, J.D., & Kaper, J.B. *(*1997*).Vibrio* species. In: Doyle, M.P., Beuchat, L.R. & Montville T.J. (Eds.), *Food Microbiology, Fundamentals and Frontiers.* Washington DC, USA: ASM Press, pp. 228–264.

Oliver, J. D. (2005). The viable but nonculturable state in bacteria. *Journal of Microbiology*, 43(1), 93-100.

Oliver, J. D. (2006). *Vibrio vulnificus.* In*:* Thompson, F. L., Austin, B., & Swings, J. (Eds.), *The Biology of Vibrios.* Washitonton, DC: ASM press. pp. 349-366.

Olsvik, O., Wahlberg, J., Petterson, B., Uhlen, M., Popovic, T., Wachsmuth, I. K., & Fields, P. I. (1993). Use of automated sequencing of polymerase chain reaction-generated amplicons to identify three types of cholera toxin subunit B in *Vibrio cholerae* O1 strains. *Journal of Clinical Microbiology*, 31(1), 22-25.

O'Quinn, A. L., Wiegand, E. M., & Jeddeloh, J. A. (2001). Burkholderia pseudomallei kills the nematode Caenorhabditis elegans using an endotoxin□mediated paralysis. *Cellular Microbiology*, 3(6), 381-393.

Orata, F. D., & Hedreyda, C. T. (2011). Isolation and sequence analysis of the full-length *toxR* gene of type strain *Vibrio campbellii* and use of the *toxR* gene sequence to evaluate variation and relatedness with other *Vibrio* species. *Philippine Science Letters*, 4(1), 70-80.

Ortigosa, M., Esteve, C., & Pujalte, M. J. (1989). *Vibrio* species in seawater and mussels: Abundance and numerical taxonomy. *Systematic and Applied Microbiology*, 12, 316–325.

Ortigosa, M., Garay, E., & Pujalte, M. J. (1994). Numerical taxonomy of Vibrionaceae isolated from oysters and seawater along an annual cycle. *Systematic and Applied Microbiology,*17, 216–225.

O'Shea, Y. A., & Boyd, E. F. (2002). Mobilization of the Vibrio pathogenicity island between *Vibrio cholerae* isolates mediated by CP□T1 generalized transduction. FEMS Microbiology *Letters*, 214(2), 153-157.

O'shea, Y. A., Reen, F. J., Quirke, A. M., & Boyd, E. F. (2004). Evolutionary genetic analysis of the emergence of epidemic *Vibrio cholerae* isolates on the basis of comparative nucleotide sequence analysis and multilocus virulence gene profiles. *Journal of Clinical Microbiology*, 42(10), 4657-4671.

Osorio, C. R., & Klose, K. E. (2000). A region of the transmembrane regulatory protein ToxR that tethers the transcriptional activation domain to the cytoplasmic membrane displays wide divergence among *Vibrio* species. *Journal of Bacteriology*, 182(2), 526-528.

Otta, S. K., Karunasagar, I., & Karunasagar, I. (1999). Bacterial flora associated with shrimp culture ponds growing *Penaeus monodon* in India. *Journal of Aquaculture in the Tropics*, 14(4), 309-318.

Ottaviani, D., Bacchiocchi, I., Masini, L., Leoni, F., Carraturo, A., Giammarioli, M., & Sbaraglia, G. (2001). Antimicrobial susceptibility of potentially pathogenic halophilic *Vibrios* isolated from seafood. *International Journal of Antimicrobial Agents*, 18(2), 135-140.

Ottaviani, D., Leoni, F., Rocchegiani, E., Santarelli, S., Masini, L., Di Trani, V., Canonico C., Pianetti A., Tega L.,& Carraturo, A. (2009). Prevalence and virulence properties of non-O1 non-O139 *Vibrio cholerae* strains from seafood and clinical samples collected in Italy. *International Journal of Food Microbiology*, 132(1), 47-53.

Oxley, A. P. A., Shipton, W., Owens, L., & McKay, D. (2002). Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis*. *Journal of Applied Microbiology*, 93(2), 214-223.

Pal, D., & Das, N. (2010). Isolation, identification and molecular characterization of *Vibrio parahaemolyticus* from fish samples in Kolkata. *European Review for Medical and Pharmacological Sciences*, 14(6), 545.

Pan, T. M., Wang, T. K., Lee, C. L., Chien, S. W., & Horng, C. B. (1997). Food-borne disease outbreaks due to bacteria in Taiwan, 1986 to 1995. *Journal of Clinical Microbiology*, 35(5), 1260-1262.

Pan, J. C., Ye, R., Wang, H. Q., Xiang, H. Q., Zhang, W., Yu, X. F., & He, Z. S. (2008). *Vibrio cholerae* O139 multiple-drug resistance mediated by *Yersinia pestis* pIP1202-like conjugative plasmids. *Antimicrobial Agents and Chemotherapy*, 52(11), 3829-3836.

Pang, L., Zhang, X. H., Zhong, Y., Chen, J., Li, Y., & Austin, B. (2006). Identification of *Vibrio harveyi* using PCR amplification of the *toxR* gene. *Letters in Applied Microbiology*, 43(3), 249-255.

Panicker, G., Myers, M. L., & Bej, A. K. (2004). Rapid detection of *Vibrio vulnificus* in shellfish and Gulf of Mexico water by real-time PCR. *Applied and Environmental Microbiology*, 70(1), 498-507.

Parker, R. W., & Lewis, D. H. (1995). Sandwich enzyme-linked immunosorbent assay for *Vibrio vulnificus* hemolysin to detect *V. vulnificus* in environmental specimens. *Applied and Environmental Microbiology*, 61(2), 476-480.

Parsot, C., Taxman, E., & Mekalanos, J. J. (1991). ToxR regulates the production of lipoproteins and the expression of serum resistance in *Vibrio cholerae*. *Proceedings of the National Academy of Sciences*, 88(5), 1641-1645.

Pascual, J., Macian, M. C., Arahal, D. R., Garay, E., & Pujalte, M. J. (2010). Multilocus sequence analysis of the central clade of the genus *Vibrio* by using the 16S rRNA, *recA*, *pyrH*, *rpoD*, *gyrB*, *rctB* and *toxR* genes. *International Journal of Systematic and Evolutionary Microbiology*, 60(1), 154-165.

Pavia, A. T., Bryan, J. A., Maher, K. L., Hester, T. R., & Farmer, J. J. (1989). *Vibrio carchariae* infection after a shark bite. *Annals of Internal Medicine*, 111(1), 85-86.

Pearson, G. D., Woods, A., Chiang, S. L., & Mekalanos, J. J. (1993). CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. *Proceedings of the National Academy of Sciences*, 90(8), 3750-3754.

Peterson, K. M., & Mekalanos, J. J. (1988). Characterization of the *Vibrio cholerae* ToxR regulon: Identification of novel genes involved in intestinal colonization. *Infection and Immunity*, 56(11), 2822-2829.

Petti, C. A., Polage, C. R., & Schreckenberger, P. (2005). The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods. *Journal of Clinical Microbiology*, 43(12), 6123-6125.

Pfeffer, C., & Oliver, J. D. (2003). A comparison of thiosulphate□citrate□bile salts□sucrose (TCBS) agar and thiosulphate□chloride□iodide (TCI) agar for the isolation of Vibrio species from estuarine environments. *Letters in Applied Microbiology*, 36(3), 150-151.

Piergentili, P., Castellani-Pastoris, M., D'fellini, R., Farisano, G., Bonello, C., Rigoli, E., & Zampieri, A. (1984). Transmission of non O group 1 *Vibrio cholerae* by raw oyster consumption. *International Journal of Epidemiology*, 13(3), 340-343.

Pomeroy, L. R., & Wiebe, W. J. (2001). Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. *Aquatic Microbial Ecology*, 23(2), 187-204.

Ponnuraj M., Murugesan A.G., Sukumaran N. & Palaniappan R. (1995). A preliminary report on shrimp culture problems in Vedaranyam (Tamil Nadu). *Seafood Export Journal*, 21-25.

Pontes, D. S., Lima-Bittencourt, C. I., Chartone-Souza, E., & Nascimento, A. M. A. (2007). Molecular approaches: Advantages and artifacts in assessing bacterial diversity. *Journal of Industrial Microbiology & Biotechnology*, 34(7), 463-473.

Popovic, N. T., Skukan, B. A., Dzidara, P., Coz-Rakovac, R., Strunjak-Perovic, I., Kozacinski, L., Jadan, M., & Brlek-Gorski, D. (2010). Microbiological quality of marketed fresh and frozen seafood caught off the Adriatic coast of Croatia. *Veterinarni Medicina,* 55(5), 233-241.

Prado, S., Romalde, J. L., Montes, J., & Barja, J. L. (2005). Pathogenic bacteria isolated from disease outbreaks in shellfish hatcheries. First description of *Vibrio neptunius* as an oyster pathogen. *Diseases of Aquatic Organisms*, 67(3), 209.

PremAnand T., Patterson E.J.K., & Ayyakannu, K. (1996) Monitoring of a shrimp culture system with special reference to*Vibrio* and fungi. *Indian Journal of Marine Sciences* 25, 253-258.

Prociv, P. (1978). *Vibrio alginolyticus* in Western Australia. *Medical Journal of Australia*, 2, 296.

Provenzano, D., & Klose, K. E. (2000). Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes *Vibrio cholerae* bile resistance, virulence factor expression, and intestinal colonization. *Proceedings of the National Academy of Sciences*, 97(18), 10220-10224.

Provenzano, D., Schuhmacher, D. A., Barker, J. L., & Klose, K. E. (2000). The virulence regulatory protein ToxR mediates enhanced bile resistance in *Vibrio cholerae* and other pathogenic *Vibrio* species. *Infection and Immunity*, 68(3), 1491-1497.

Pruzzo, C., Gallo, G., & Canesi, L. (2005). Persistence of *Vibrios* in marine bivalves: The role of interactions with haemolymph components. *Environmental Microbiology*, 7(6), 761-772.

Pujalte, M. J., Ortigosa, M., Macián, M. C., & Garay, E. (2010). Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. *International Microbiology*, 2(4), 259-266.

Pujol, N., Link, E. M., Liu, L. X., Kurz, C. L., Alloing, G., Tan, M. W., Ray, K.P., Solari, R., Johnson, C.D., & Ewbank, J. J. (2001). A reverse genetic analysis of components of the Toll signaling pathway in *Caenorhabditis elegans.Current Biology*, 11(11), 809-821.

Qadri, F., Hasan, J. A., Hossain, J., Chowdhury, A., Begum, Y. A., Azim, T., & Albert, M. J. (1995). Evaluation of the monoclonal antibody-based kit Bengal SMART for rapid detection of *Vibrio cholerae* O139 synonym Bengal in stool samples. *Journal of Clinical Microbiology*, 33(3), 732-734.

Qian, R., Xiao, Z., Zhang, C., Chu, W., Mao, Z., & Yu, L. (2008). Expression and purification of two major outer membrane proteins from *Vibrio alginolyticus*. *World Journal of Microbiology and Biotechnology*, 24(2), 245-251.

Quintol, N. M., Beeshmi, P., & Porteen, K. (2008). Occurrence of *Vibrio parahaemolyticus* in fishes sold in markets. *Indian Veterinary Journal*, 85(7), 105-106.

Rad, M., & Shahsavani, D. (2010). Isolation and characterization of *Vibrio (Listonella) anguillarum* from catfish. *Turkish Journal of Veterinary and Animal Sciences*, 34(4), 413-415.

Radhakutty, G., Sircar, B. K., Mondal, S. K., Mukhopadhyay, A. K., Mitra, R. K., Basu, A., & Bhattacharya, S. K. (1997). Investigation of the outbreak of cholera in Alleppey & Palghat districts, South India. *Indian Journal of Medical Research*, 106, 455-457.

Radu, S., Rusul, G., Yeang, L. K., & Nishibuchi, M. (2000). Detection and molecular characterization of *Vibrio vulnificus* from coastal waters of Malaysia. *The Southeast Asian Journal of Tropical Medicine and Public Health,* 31(4): 668-673.

Raghul, S. S., & Bhat, S. G. (2011). Seasonal variation in the hydrolytic exo-enzyme profile of *Vibrio* sp. associated with the marine benthic environment of South India. *Indian Journal of Marine Sciences*, 40, 826-833.

Raghul, S.S. (2012). Characterization of polyhydroxyalkanoates accumulating *Vibrios* from marine benthic environments and production studies of polyhydroxyalkanoates by *Vibrio* sp. BTKB33. PhD Thesis. Cochin University of Science and Technology, India.

Raguenes, G., Christen, R., Guezennec, J., Pignet, P., & Barbier, G. (1997). *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. *International Journal of Systematic Bacteriology*, 47(4), 989-995.

Raissy, M., Momtaz, H., Moumeni, M., Ansari, M., & Rahimi, E. (2011). Molecular detection of *Vibrio* spp. in lobster hemolymph. *African Journal of Microbiological Research*, 5(13), 1697-1700.

Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya, S.K., Nair, G.B., Shimada, T., Takeda, T., Karasawa, T., Kurazano, H., Pal, A., & Takeda, Y. (1993). Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in Southern and Eastern India. *The Lancet,* 341, 303–704.

Ramamurthy, T., Rajendran, K., Garg, P., Shimada, T., Basu, A., Chowdhury, N.R., Nandy, R.K., Yamasaki, S., Bhattacharya, S.K., Takeda, Y., &Nair, G.B. (2000). Cluster-analysis and patterns of dissemination of multidrug resistance among clinical strains of *Vibrio cholerae* in Calcutta, India. *Indian Journal of Medical Research*, 112, 78–85.

Ramamurthy, T. (2008). Antibiotics resistance in *Vibrio cholerae*. In: Faruque, S.M., Nair, G.B. (Eds.) *Vibrio cholerae: Genomic and Molecular Biology*, Horizon Scientific Press, pp. 195-208.

Ramamurthy, V., & Raveendran, S. (2009). Isolation and identification of *Vibrio* sp. from mangrove fish. *Journal of Ecobiology*, 24(1), 57-64.

Rameshkumar, N., & Nair, S. (2007) *Vibrio rhizosphaerae* sp. nov., a novel red-pigmented species that antagonizes phytopathogenic bacteria. *International Journal of Systematic and Evolutionary Microbiology*, 57, 2241–2246.

Rameshkumar, N., Fukui, Y., Sawabe, T., & Nair, S. (2008) *Vibrio porteresiae* sp. nov., a novel diazotrophic bacterium isolated from a mangrove associated wild rice (*Porteresia coarctata*Tateoka). *International Journal of Systematic and Evolutionary Microbiology,* 58, 1608–1615.

Rameshkumar, N., & Nair, S. (2009) Isolation and molecular characterization of genetically diverse antagonistic, diazotrophic red-pigmented *Vibrios* from different mangrove rhizospheres. *FEMS Microbiology Ecology*, 67, 455–467.

Rameshkumar, N., Lang, E., & Nair, S. (2010). *Mangrovibacter plantisponsor* gen. nov., sp. nov., a novel diazotrophic bacterium isolated from a mangrove associated wild rice (*Porteresia coarctata* Tateoka). *International Journal of Systematic and Evolutionary Microbiology*, 60, 179–186.

Rameshkumar, N., Gomez-Gil, B., Sproer, C., Lang, E., Dinesh Kumar, N., Krishnamurthi, S., & Roque, A. (2011). *Vibrio plantisponsor* sp. nov., a diazotrophic bacterium isolated from a mangrove associated wild rice (*Porteresia coarctata* Tateoka). *Systematic and Applied Microbiology*, 34(7), 487-493.

Randa, M. A., Polz, M. F., & Lim, E. (2004). Effects of temperature and salinity on *Vibrio vulnificus* population dynamics as assessed by quantitative PCR. *Applied and Environmental Microbiology*, 70(9), 5469-5476.

Rank, E. L., Smith, I. B., & Langer, M. (1988). Bacteremia caused by *Vibrio hollisae*. *Journal of Clinical Microbiology*, 26(2), 375-376.

Ran-Kim, Y., & Rhee, H.J. (2003). Flagellar basal body *flg* operon as a virulence determinant of *Vibrio vulnificus*. *Biochemical and Biophysical Research Communications*, 304(2), 405-410.

Ranoa, D. R. E., & Hedreyda, C. T. (2005). Sequence analysis of partial *toxR* gene from Philippine *Vibrio* isolates and design of *toxR*-targeted primers for detection. *The Journal of General and Applied Microbiology*, 51(6), 343-351.

Rappe, M. S., & Giovannoni, S. J. (2003). The uncultured microbial majority. *Annual Reviews in Microbiology*, 57(1), 369-394.

Reddy, G. S.N., Aggarwal, R. K., Matsumoto, G. I., & Shivaji, S. (2000). *Arthrobacter flavus* sp. nov., a psychrophilic bacterium isolated from a pond in McMurdo Dry Valley, Antarctica. *International Journal of Systematic and Evolutionary Microbiology*, 50(4), 1553-1561.

Reddy, G.S.N., Prakash, J.S.S., Vairamani, M., Prabhakar, S., Matsumoto, G.I., & Shivaji, S. (2002a). *Planococcus antarcticus* and *Planococcus psychrophilus* spp. nov. isolated from cyanobacterial mat samples collected from ponds in Antarctica. *Extremophiles*, 6(3), 253-261.

Reddy, G.S.N., Prakash, J.S.S., Matsumoto, G.I., Stackebrandt, E., Shivaji, S. (2002b). *Arthrobacter roseus* sp. nov., a psychrotolerant bacterium isolated from an Antarctic cyanobacterial mat sample. *International Journal of Systematic and Evolutionary Microbiology*, 52, 1017-1021.

Reguera, G., & Kolter, R. (2005). Virulence and the environment: a novel role for *Vibrio cholerae* toxin-coregulated pili in biofilm formation on chitin. *Journal of Bacteriology*, 187(10), 3551-3555.

Rehnstam-Holm, A. S., Godhe, A., Harnstrom, K., Raghunath, P., Saravanan, V., Collin, B., & Karunasagar, I. (2010). Association between phytoplankton and *Vibrio* spp. along the southwest coast of India: A mesocosm experiment. *Aquatic Microbial Ecology*, 58(2), 127-139.

Reich, K. A., & Schoolnik, G. K. (1994). The light organ symbiont *Vibrio fischeri* possesses a homolog of the *Vibrio cholerae* transmembrane transcriptional activator ToxR. *Journal of Bacteriology*, 176(10), 3085-3088.

Rennels, M. B., Levine, M. M., Daya, V., Angle, P., & Young, C. (1980). Selective vs. nonselective media and direct plating vs. enrichment technique in isolation of *Vibrio cholerae*: Recommendations for clinical laboratories. *Journal of Infectious Diseases*, 142(3), 328-331.

Restrepo, D., Huprikar, S. S., VanHorn, K., & Bottone, E. J. (2006). O1 and non-O1 *Vibrio cholerae* bacteremia produced by hemolytic strains. *Diagnostic Microbiology and Infectious Disease*, 54(2), 145-148.

Riemann, L., Steward, G. F., & Azam, F. (2000). Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Applied and Environmental Microbiology*, 66(2), 578-587.

Ripabelli, G., Sammarco, M. L., McLauchlin, J., & Fanelli, I. (2003). Molecular characterisation and antimicrobial resistance of *Vibrio vulnificus* and *Vibrio alginolyticus* isolated from mussels (*Mytilus galloprovincialis*). *Systematic and Applied Microbiology*, 26(1), 119-126.

Rivera, I. G., Chowdhury, M. A., Huq, A., Jacobs, D., Martins, M. T., & Colwell, R. R. (1995). Enterobacterial repetitive intergenic consensus sequences and the PCR to generate fingerprints of genomic DNAs from *Vibrio cholerae* O1, O139, and non-O1 strains. *Applied and Environmental Microbiology*, 61(8), 2898-2904.

Rivera, I. N., Chun, J., Huq, A., Sack, R. B., & Colwell, R. R. (2001). Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. *Applied and Environmental Microbiology*, 67(6), 2421-2429.

Robert□Pillot, A., Guenole, A., & Fournier, J. M. (2002). Usefulness of R72H PCR assay for differentiation between *Vibrio parahaemolyticus* and *Vibrio alginolyticus* species: Validation by DNA–DNA hybridization. *FEMS Microbiology Letters*, 215(1), 1-6.

Rodríguez, J. M., López-Romalde, S., Beaz, R., Alonso, M. C., Castro, D., & Romalde, J. L. (2006). Molecular fingerprinting of *Vibrio tapetis* strains using three PCR-based methods: ERIC-PCR, REP-PCR and RAPD. *Diseases of Aquatic Organisms*, 69(2/3), 175.

Rogers, R. C., Cuffe, R. G. C. J., Cossins, Y. M., Murphy, D. M., & Bourke, A. T. C. (1980). The Queensland cholera incident of 1977: 2. The epidemiological investigation. *Bulletin of the World Health Organization*, 58(4), 665.

Rohwer, F., Breitbart, M., Jara, J., Azam, F., & Knowlton, N. (2001). Diversity of bacteria associated with the Caribbean coral *Montastraea franksi*. *Coral Reefs*, 20(1), 85-91.

Roque, A., Lopez-Joven, C., Lacuesta, B., Elandaloussi, L., Wagley, S., Furones, M.D., Ruiz-Zarzuela, I., de Blas, I., Rangdale, R., & Gomez-Gil, B. (2009). Detection and identification of *tdh*- and *trh*-positive *Vibrio parahaemolyticus* strains from four species of cultured bivalve molluscs on the Spanish Mediterranean coast. *Applied and Environmental Microbiology*, 75 (23),7574–7577.

Rosenberg, E., & Ben□Haim, Y. (2002). Microbial diseases of corals and global warming. *Environmental Microbiology,* 4(6), 318-326.

Rosenberg, E., & Loya, Y. (Eds.). (2004). *Coral health and disease*. Springer, Verlag KG, Berlin, Germany.

Roszak, D. B., & Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiological Reviews*, 51(3), 365.

Roychowdhury, A., Pan, A., Dutta, D., Mukhopadhyay, A. K., Ramamurthy, T., Nandy, R. K., Bhattacharya, S.K., & Bhattacharya, M. K. (2008). Emergence of tetracycline resistant *Vibrio cholerae* O1 serotype Inaba, in Kolkata, India. *Japanese Journal of Infectious Diseases*, 61(2), 128.

Rozemeijer, W., Korswagen, L. A., Voskuyl, A. E., & Budding, A. E. (2009). *Vibrio cholerae* non-O1 non-O139 infection in an immunocompromised patient returning from Spain, July 2009. *Euro Surveillance*, 14(32), 492.

Ruangpan, L., & Kitao, T. (1991). *Vibrio* bacteria isolated from black tiger shrimp, *Penaeus monodon Fabricius*. *Journal of Fish diseases*, 14(3), 383-388.

Ruby, E. G., & McFall-Ngai, M. J. (1992). A squid that glows in the night: Development of an animal-bacterial mutualism. *Journal of Bacteriology*, 174(15), 4865.

Ruby, E. G., & Lee, K. H. (1998). The *Vibrio fischeri-Euprymna scolopes* light organ association: Current ecological paradigms. *Applied and Environmental Microbiology*, 64(3), 805-812.

Ruiz, G. M., Rawlings, T. K., Dobbs, F. C., Drake, L. A., Mullady, T., Huq, A., & Colwell, R. R. (2000). Global spread of microorganisms by ships. *Nature*, 408(6808), 49-50.

Ruiz-Bolivar, Z., Carrascal-Camacho, A. K., Neuque-Rico, M. C., Gutierrez-Trivino, C., Rodríguez-Bocanegra, M. X., Poutou-Pinales, R. A., & Mattar, S. (2011). Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) fingerprinting reveals intra-serotype variations among circulating *Listeria monocytogenes* strains. *African Journal of Microbiology Research*, 5(13), 1586-1598.

Sabeena, F., Thirivikramji, G., Radhakutty, G., Indu, P., & Singh, D. V. (2001). *In vitro* susceptibility of *Vibrio cholerae* O1 biotype ElTor strains associated with an outbreak of cholera in Kerala, Southern India. *Journal of Antimicrobial Chemotherapy*, 47(3), 361-362.

Sadok, K., Mejdi, S., Nourhen, S., & Amina, B. (2013). Phenotypic characterization and RAPD fingerprinting of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* isolated during Tunisian fish farm outbreaks. *Folia Microbiologica*, 58(1), 17-26.

Safrin, S., Morris, J. G., Adams, M., Pons, V., Jacobs, R., & Conte, J. E. (1988). Non-O l *Vibrio cholerae* bacteremia: Case report and review. *Review of Infectious Diseases*, 10(5), 1012-1017.

Saha, D., Karim, M.M., Khan, W.A., Ahmed, S., Salam, M.A., & Bennish, M.L. (2006). Single-dose azithromycin for the treatment of cholera in adults. *New England Journal of Medicine,*354 , 2452–2462

Sahu, G. K., Chowdhury, R., & Das, J. (1994). Heat shock response and heat shock protein antigens of *Vibrio cholerae*. *Infection and Immunity*, 62(12), 5624-5631.

Sahu, S. N., Lewis, J., Patel, I., Bozdag, S., Lee, J. H., LeClerc, J. E., & Cinar, H. N. (2012). Genomic analysis of immune response against *Vibrio cholerae* hemolysin in *Caenorhabditis elegans*. *PloS one*, 7(5), e38200.

Sailes, C. A., Momen, H., Coelho, A. M., de Oliveira, E. F., Vicente, A. C. P., & Nair, G. B. (1994). Bengal: El Tor cholera *Vibrio* in a new robe. *Mem Inst Oswaldo Cruz, Rio de Janeiro*, 89(1), 115-116.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4(4), 406-425.

Sakai, T., Hirae, T., Yuasa, K., Kamaishi, T., Matsuyama, T., Miwa, S., & Iida, T. (2007). Mass mortality of cultured kuruma prawn *Penaeus japonicus* caused by *Vibrio nigripulchritudo*. *Fish Pathology*,42(3), 141-147.

Sakata, T., Matsuura, M., & Shimokawa, Y. (1989). Characteristics of *Vibrio damsela* isolated from diseased yellowtail *Seriola quinqueradiata*. *Bulletin of the Japanese Society of Scientific Fisheries*, 55.

Salvesen, I., Reitan, K. I., Skjermo, J., & Oie, G. (2000). Microbial environments in marine larviculture: Impacts of algal growth rates on the bacterial load in six microalgae. *Aquaculture International*, 8(4), 275-287.

Sangster, C. R., & Smolowitz, R. M. (2003). Description of *Vibrio alginolyticus* infection in cultured *Sepia officinalis, Sepia apama*, and *Sepia pharaonis*. *The Biological Bulletin*, 205(2), 233-234.

Sanjeev, S., & Stephen, J. (2012). Occurrence of enteropathogenic, Kanagawa positive strains of *Vibrio parahaemolyticus* in fresh finfish and shellfish. *Fishery Technology*, 32(1), 64-66.

Satchell, K. J. F. (2007). MARTX, multifunctional autoprocessing repeats-in-toxin toxins. *Infection and Immunity*, 75(11), 5079-5084.

Sawabe, T., Thompson, F. L., Heyrman, J., Cnockaert, M., Hayashi, K., Tanaka, R., Yoshimizu, M., Hoste, B., Swings, J., & Ezura, Y. (2002). Fluorescent amplified fragment length polymorphism and repetitive extragenic palindrome-PCR fingerprinting reveal host-specific genetic diversity of *Vibrio halioticoli*-like strains isolated from the gut of Japanese abalone. *Applied and Environmental Microbiology*, 68(8), 4140-4144.

Sawabe, T., Setoguchi, N., Inoue, S., Tanaka, R., Ootsubo, M., Yoshimizu, M., & Ezura, Y. (2003). Acetic acid production of *Vibrio halioticoli* from alginate: a possible role for establishment of abalone-*V. halioticoli* association. *Aquaculture*, 219(1), 671-679.

Sawabe, T., Kita-Tsukamoto, K., & Thompson, F. L. (2007). Inferring the evolutionary history of *Vibrios* by means of multilocus sequence analysis. *Journal of Bacteriology*, 189(21), 7932-7936.

Schmidt, U., Chmel, H., & Cobbs, C. (1979). *Vibrio alginolyticus* infections in humans. *Journal of Clinical Microbiology*, 10(5), 666-668.

Scimeca, J. M., & Oestmann, D. J. (1995). Selected diseases of captive and laboratory reared cephalopods. *Proceedings of the International Association of Aquatic Animal Medicine* 26, 83.

Scimeca, J. M., & Oestmann, D.J. (1996). Trauma associated lesions in the cuttlefish, *Sepia officinalis*. *Proceedings of the International Association of Aquatic Animal Medicine*, 27, 88.

Senderovich, Y., Izhaki. I., Halpern, M. (2010). Fish as reservoirs and vectors of *Vibrio cholerae*. *PLoS One* 5(1): e8607.

Serrano, P. H. (2005). Responsible use of antibiotics in aquaculture. *Fisheries Technological Paper*, FAO, 469.

Shandera, W. X., Johnston, J. M., Davis, B. R., & Blake, P. A. (1983). Disease from infection with *Vibrio mimicus*, a newly recognized *Vibrio* species-Clinical characteristics and epidemiology. *Annals of Internal Medicine*, 99(2), 169-171.

Shangkuan, Y. H., Lin, H. C., & Wang, T. M. (1997). Diversity of DNA sequences among *Vibrio cholerae* O1 and non□O1 isolates detected by whole□cell repetitive element sequence□based polymerase chain reaction. Journal of Applied Microbiology, 82(3), 335-344.

Shangkuan, Y. H., Yang, J. F., Lin, H. C., & Shaio, M. F. (2000). Comparison of PCR–RFLP, ribotyping and ERIC–PCR for typing *Bacillus anthracis* and *Bacillus cereus* strains. *Journal of Applied Microbiology*, 89(3), 452-462.

Sharma, C., Thungapathra, M., Ghosh, A., Mukhopadhyay, A.K., Basu, A., Mitra, R., Basu, I., Bhattacharya, S.K., Shimada, T., Ramamurthy, T., Takeda, T., Yamasaki, S., Takeda, Y., Nair, G.B. (1998). Molecular analysis of non-O1, non-O139 *Vibrio cholerae* associated with an unusual upsurge in the incidence of cholera-like disease in Calcutta, India. *Journal of Clinical Microbiology*, 36, 756-763.

Sharma, N.C., Mandal, P.K., Dhillon, R., & Jain, M. (2007). Changing profile of *Vibrio cholerae* 01, 0139 in Delhi and periphery (2003–2005). *Indian Journal of Medical Research,* 125, 633–640.

Shaw, C. E., Peterson, K. M., Mekalanos, J. J., & Taylor, R. K. (1990). Genetic studies of *Vibrio cholerae* TCP pilus biogenesis. *Advanced Research in Cholera Related Diarrheas*, *7*, 51-58.

Shi, L., Miyoshi, S. I., Hiura, M., Tomochika, K. I., Shimada, T., & Shinoda, S. (1997). Detection of genes encoding cholera toxin (CT), zonula occludens toxin (ZOT), accessory cholera enterotoxin (ACE) and heat-stable enterotoxin (ST) in *Vibrio mimicus* clinical strains. *Microbiology and Immunology*, 42(12), 823-828.

Shikongo-Nambabi, M. N. N. N. (2010). Occurrence and control of Vibrio spp. as contaminants of processed marine fish. PhD Thesis, University of Pretoria.

Shimada, T., Nair, G. B., Deb, B.C., Albert, M. J., Sack, R.B., & Takeda, Y. (1993). Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh. *The Lancet,* 341, 1346-1347.

Shimada, T., Arakawa, E., Itoh, K., Okitsu, T., Matsushima, A., Asai, Y., Yamai, S., Nakajato, T.,& Takeda, Y. (1994). Extended serotyping scheme for *Vibrio cholerae*. *Current Microbiology*, 28(3), 175-178.

Shinoda, S. (1999). Protein toxins produced by pathogenic *Vibrios*. *Journal of Natural Toxins*, 8(2), 259.

Shivaji, S., Vijaya Bhanu, N., & Aggarwal, R. K. (2000). Identification of *Yersinia pestis* as the causative organism of plague in India as determined by 16S rDNA sequencing and RAPD□based genomic fingerprinting. FEMS Microbiology Letters, 189(2), 247-252.

Shtonda, B. B., & Avery, L. (2006). Dietary choice behavior in *Caenorhabditis elegans*. *Journal of Experimental Biology*, 209(1), 89-102.

Shuan- Ju T. C., Thong, K. L., Osawa, R., & Heng Chua, K. (2011). Comparative PCR-based fingerprinting of *Vibrio cholerae* isolated in Malaysia. *The Journal of General and Applied Microbiology*, 57(1), 19-26.

Sifri, C. D., Begun, J., & Ausubel, F. M. (2005). The worm has turned–microbial virulence modeled in *Caenorhabditis elegans*. *Trends in Microbiology*, 13(3), 119-127.

Sifri, C. D., Begun, J., Ausubel, F. M., & Calderwood, S. B. (2003). *Caenorhabditis elegans* as a model host for *Staphylococcus aureus* pathogenesis. *Infection and Immunity*, 71(4), 2208-2217.

Silva, T. M., Schleupner, M. A., Tacket, C. O., Steiner, T. S., Kaper, J. B., Edelman, R., & Guerrant, R. (1996). New evidence for an inflammatory component in diarrhea caused by selected new, live attenuated cholera vaccines and by ElTor and O139 *Vibrio cholerae*. *Infection and Immunity*, 64(6), 2362-2364.

Simidu, U., Kaneko, E., & Taga, N. (1977). Microbiological studies of Tokyo Bay. *Microbial Ecology*, 3(3), 173-191.

Simidu, U., Tsukamoto, K., & Akagi, Y. (1982). Heterotrophic bacterial population in Bengal Bay and the South China Sea. *Bulletin of the Japanese Society of Scientific Fisheries*, 48(3), 425-431.

Simonet, V. C., Basle, A., Klose, K. E., & Delcour, A. H. (2003). The *Vibrio cholerae* porins OmpU and OmpT have distinct channel properties. *Journal of Biological Chemistry*, 278(19), 17539-17545.

Simpson, L. M., White, V. K., Zane, S. F., & Oliver, J. D. (1987). Correlation between virulence and colony morphology in *Vibrio vulnificus*. *Infection and Immunity*, 55(1), 269-272.

Sindermann, C. J. (1990). *Principal diseases of marine fish and shellfish: diseases of marine fish.* (Vol. 1). Academic Press.

Singh, D. V., Matte, M. H., Matte, G. R., Jiang, S., Sabeena, F., Shukla, B. N., Sanyal, S.C., Huq, A., & Colwell, R. R. (2001). Molecular analysis of *Vibrio cholerae* O1, O139, non-O1, and non-O139 strains: Clonal relationships between clinical and environmental isolates. *Applied and Environmental Microbiology*, 67(2), 910-921.

Singh, D. V., Isac, S. R., & Colwell, R. R. (2002). Development of a hexaplex PCR assay for rapid detection of virulence and regulatory genes in *Vibrio cholerae* and *Vibrio mimicus*. *Journal of Clinical Microbiology*, 40(11), 4321-4324.

Skorupski, K., & Taylor, R. K. (1997). Control of the ToxR virulence regulon in *Vibrio cholerae* by environmental stimuli. *Molecular Microbiology*, 25(6), 1003-1009.

Smith, A. B., & Siebeling, R. J. (2003). Identification of genetic loci required for capsular expression in *Vibrio vulnificus*. *Infection and Immunity*, 71(3), 1091-1097.

Smith, B., & Oliver, J. D. (2006). *In situ* and *in vitro* gene expression by *Vibrio vulnificus* during entry into, persistence within, and resuscitation from the viable but nonculturable state. *Applied and Environmental Microbiology*, 72(2), 1445-1451.

Smith, G. C., & Merkel, J. R. (1982). Collagenolytic activity of *Vibrio vulnificus*: potential contribution to its invasiveness. *Infection and Immunity*, 35(3), 1155-1156.

Snoussi, M., Noumi, E., Cheriaa, J., Usai, D., Sechi, L. A., Zanetti, S., & Bakhrouf, A. (2008). Adhesive properties of environmental *Vibrio alginolyticus* strains to biotic and abiotic surfaces. *New Microbiology*, 31(4), 489-500.

Sochard, M. R., Wilson, D. F., Austin, B., & Colwell, R. R. (1979). Bacteria associated with the surface and gut of marine copepods. *Applied and Environmental Microbiology*, 37(4), 750-759.

Somarny, W. M. Z., Mariana, N. S., Rozita, R., & Raha, A. R. (2004). Cloning and expression of *Vibrio cholerae* virulence gene, accessory cholera enterotoxin (*ace*). *Southeast Asian Journal of Tropical Medicine and Public Health*, 35(4), *856-862.*

Sonnhammer, E. L., & Durbin, R. (1997). Analysis of protein domain families in *Caenorhabditis elegans*. *Genomics*, 46(2), 200-216.

Soto-Rodriguez, S. A., Gomez-Gil, B., & Lozano, R. (2010). 'Bright-red' syndrome in Pacific white shrimp *Litopenaeus vannamei* is caused by *Vibrio harveyi*. *Diseases of Aquatic Organisms*, 92(1), 11-19.

Spira, W. M., & Fedorka-Cray, P. J. (1983). Production of cholera toxin-like toxin by *Vibrio mimicus* and non-O1 *Vibrio cholerae*: batch culture conditions for optimum yields and isolation of hypertoxigenic lincomycin-resistant mutants. *Infection and Immunity*, 42(2), 501-509.

Stabb, E. V. (2006). The *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. *The biology of Vibrios.* ASM Press, Washington, DC, pp. 204-218.

Staerk, J., Ronneberger, H. J., & Wiegandt, H. (1974). Neuraminidase, a virulence factor in *Vibrio cholerae* infection. *Behring Inst Mitt*, *55*, 145-146.

Steinert, M., & Heuner, K. (2005). *Dictyostelium* as host model for pathogenesis. *Cellular microbiology*, 7(3), 307-314.

Stephenson, D. P., Moore, R. J., & Allison, G. E. (2009). Comparison and utilization of repetitive-element PCR techniques for typing *Lactobacillus* isolates from the chicken gastrointestinal tract. *Applied and Environmental Microbiology*, 75(21), 6764-6776.

Stern, M. J., Ames, G. F. L., Smith, N. H., Clare Robinson, E., & Higgins, C. F. (1984). Repetitive extragenic palindromic sequences: A major component of the bacterial genome. *Cell*, 37(3), 1015-1026.

Stewart-Tull, D. E. S., Ollar, R. A., & Scobie, T. S. (1986). Studies on the *Vibrio cholerae* mucinase complex. I. Enzymic activities associated with the complex 325. *Journal of Medical Microbiology*, 22(4), 325-333.

Strom, M. S., & Paranjpye, R. N. (2000). Epidemiology and pathogenesis of *Vibrio vulnificus*. *Microbes and Infection*, 2(2), 177-188.

Strunjak-Perovic, I., Kozacinski, L., Jadan, M., & Brlek-Gorski, D. (2010). Microbiological quality of marketed fresh and frozen seafood caught off the Adriatic coast of Croatia. *Veterinarni Medicina*, 55(5), 233-241.

Sugumar, G., Nakai, T., Hirata, Y., Matsubara, D., & Muroga, K. (1998). *Vibrio splendidus* biovar II as the causative agent of bacillary necrosis of Japanese oyster *Crassostrea gigas* larvae. *Diseases of Aquatic Organisms*, 33(2), 111-118.

Sulston, J. E., Schierenberg, E., White, J. G., & Thomson, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Developmental Biology*, 100(1), 64-119.

Sung, H. H., Hsu, S. F., Chen, C. K., Ting, Y. Y., & Chao, W. L. (2001). Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. *Aquaculture*, 192(2), 101-110.

Sussman, M., Loya, Y., Fine, M., & Rosenberg, E. (2003). The marine fireworm *Hermodice carunculata* is a winter reservoir and spring□summer vector for the coral□bleaching pathogen Vibrio shiloi. Environmental Microbiology, 5(4), 250-255.

Sussman, M., Willis, B. L., Victor, S., & Bourne, D. G. (2008). Coral pathogens identified for white syndrome (WS) epizootics in the Indo-Pacific. *PLoS One*, 3(6), e2393.

Sussman, M., Mieog, J. C., Doyle, J., Victor, S., Willis, B. L., & Bourne, D. G. (2009). *Vibrio* zinc-metalloprotease causes photoinactivation of coral endosymbionts and coral tissue lesions. *PLoS One*, 4(2), e4511.

Sutherland, K. P., Porter, J. W., & Torres, C. (2004). Disease and immunity in Caribbean and Indo-Pacific zooxanthellate corals. *Marine Ecology Progress Series*, 266, 265-272.

Sutton, D.C., & Garrick, R. (1993) Bacterial disease of cultured giant clam *Tridacna gigas* larvae. *Diseases of Aquatic Organi*sms 16, 47–53.

Tacao, M., Alves, A., Saavedra, M. J., & Correia, A. (2005). BOX-PCR is an adequate tool for typing *Aeromonas* spp. *Antonie van Leeuwenhoek*, 88(2), 173-179.

Takahashi, H., Hara-Kudo, Y., Miyasaka, J., Kumagai, S., & Konuma, H. (2005). Development of a quantitative real-time polymerase chain reaction targeted to the *toxR* for detection of *Vibrio vulnificus*. *Journal of Microbiological Methods*, 61(1), 77-85.

Takao, T., Hitouji, T., Aimoto, S., Shimonishi, Y., Hara, S., Takeda, T., & Miwatani, T. (1983). Amino acid sequence of a heat-stable enterotoxin isolated from enterotoxigenic *Escherichia coli* strain 18D. *FEBS Letters*, 152(1), 1-5.

Talkington, D., Bopp, C., Tarr, C., Parsons, M. B., Dahourou, G., Freeman, M., Joyce, K.& Gerner-Smidt, P. (2011). Characterization of toxigenic *Vibrio cholerae* from Haiti, 2010–2011. *Emerging Infectious Diseases*, 17(11), 2122.

Tamplin, M. L., Gauzens, A. L., Huq, A., Sack, D. A., & Colwell, R. R. (1990). Attachment of *Vibrio cholerae* serogroup O1 to zooplankton and phytoplankton of Bangladesh waters. *Applied and Environmental Microbiology*, 56(6), 1977-1980.

Tamplin, M. L., Martin, A. L., Ruple, A. D., Cook, D. W., & Kaspar, C. W. (1991). Enzyme immunoassay for identification of *Vibrio vulnificus* in seawater, sediment, and oysters. *Applied and Environmental Microbiology*, 57(4), 1235-1240.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, 10(3), 512-526.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24(8), 1596-1599.

Tan, M. W., Rahme, L. G., Sternberg, J. A., Tompkins, R. G., & Ausubel, F. M. (1999). *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. *Proceedings of the National Academy of Sciences*, 96(5), 2408-2413.

Taniguchi, H., Kubomura, S., Hirano, H., Mizue, K., Ogawa, M., & Mizuguchi, Y. (1990). Cloning and characterization of a gene encoding a new thermostable hemolysin from *Vibrio parahaemolyticus*. *FEMS Microbiology Letters*, 67(3), 339-345.

Tao, Z., Bullard, S., & Arias, C. (2011). High numbers of *Vibrio vulnificus* in tar balls collected from oiled areas of the north-central Gulf of Mexico following the 2010 BP Deepwater Horizon Oil Spill. *Ecohealth*, 8(4), 507-511.

Taylor, M.W., Radax, R., Steger, D., & Wagner, M. (2007). Sponge-associated microorganisms: Evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews,* 71(2), 295–347.

Taylor, R. K., Miller, V. L., Furlong, D. B., & Mekalanos, J. J. (1987). Use of *pho*A gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proceedings of the National Academy of Sciences*, 84(9), 2833-2837.

Theron, J., Cilliers, J., Du Preez, M., Bröz el, V. S., & Venter, S. N. (2000). Detection of toxigenic *Vibrio cholerae* from environmental water samples by an enrichment broth cultivation–pit□stop semi□nested PCR procedure. Journal of Applied Microbiology, 89(3), 539-546.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25(24), 4876-4882.

Thompson, F. L., Hoste, B., Vandemeulebroecke, K., & Swings, J. (2001). Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism. *Systematic and Applied Microbiology*, 24(4), 520-538.

Thompson, F. L., Iida, T., & Swings, J. (2004). Biodiversity of *Vibrios*. *Microbiology and Molecular Biology Reviews*, 68(3), 403-431

Thompson, F. L., Gevers, D., Thompson, C. C., Dawyndt, P., Naser, S., Hoste, B., & Swings, J. (2005). Phylogeny and molecular identification of *Vibrios* on the basis of multilocus sequence analysis. *Applied and Environmental Microbiology*, 71(9), 5107-5115.

Thompson F.L., & Swings, J. (2006). *Taxonomy of the Vibrios*. In: Thompson F. L., Austin, B., & Swings, J. (Eds,), *The Biology of Vibrios*. ASM Press, Washington D.C., pp. 29-43.

Thompson, F. L., Cleenwerck, I., Swings, J., Matsuyama, J., & Iida, T. (2007a). Genomic diversity and homologous recombination in *Vibrio parahaemolyticus* as revealed by amplified fragment length polymorphism (AFLP) and multilocus sequence analysis (MLSA). *Microbes and Environments*, 22(4), 373-379.

Thompson, F. L., Gomez-Gil, B., Vasconcelos, A. T. R., & Sawabe, T. (2007b). Multilocus sequence analysis reveals that *Vibrio harveyi* and *V. campbellii* are distinct species. *Applied and Environmental Microbiology*, 73(13), 4279-4285.

Thungapathra, M., Sinha, K. K., Chaudhuri, S. R., Garg, P., Ramamurthy, T., Nair, G. B., & Ghosh, A. (2002). Occurrence of antibiotic resistance gene cassettes aac (6′)-Ib, dfrA5, dfrA12, and ereA2 in class I integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrobial Agents and Chemotherapy*, 46(9), 2948-2955.

Tindall, B. J., Rossello-Mora, R., Busse, H. J., Ludwig, W., & Kampfer, P. (2010). Notes on the characterization of prokaryote strains for taxonomic purposes. *International Journal of Systematic and Evolutionary Microbiology*, 60(1), 249-266.

Tison, D. L., Nishibuchi, M., Greenwood, J. D., & Seidler, R. J. (1982). *Vibrio vulnificus* biogroup 2: New biogroup pathogenic for eels. *Applied and Environmental Microbiology*, 44(3), 640-646.

Tobin-D'Angelo, M., Smith, A. R., Bulens, S. N., Thomas, S., Hodel, M., Izumiya, H., Arakawa, E., Morita, M., Watanabe, H., Marin, C., Parsons, M.B., Greene, K., Cooper, K., Haydel, D., Bopp, C., Yu, P., & Mintz, E. (2008). Severe diarrhea caused by cholera toxin–producing *Vibrio cholerae* serogroup O75 infections acquired in the southeastern United States. *Clinical Infectious Diseases*, 47(8), 1035-1040.

Toma, C., Higa, N., Koizumi, Y., Nakasone, N., Ogura, Y., McCoy, A. J., & Suzuki, T. (2010). Pathogenic *Vibrio* activate NLRP3 inflammasome via cytotoxins and TLR/nucleotide-binding oligomerization domain-mediated NF-κB signaling. *The Journal of Immunology*, 184(9), 5287-5297.

Toranzo, A. E., & Barja, J. L. (1993). Virulence factors of bacteria pathogenic for coldwater fish. *Annual Review of Fish Diseases*, 3, 5-36.

Toranzo, A. E., Magarinos, B., & Romalde, J. L. (2005). A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, 246(1), 37-61.

Tracz, D. M., Backhouse, P. G., Olson, A. B., McCrea, J. K., Walsh, J. A., Ng, L. K., & Gilmour, M. W. (2007). Rapid detection of *Vibrio* species using liquid microsphere arrays and real-time PCR targeting the *ftsZ* locus. *Journal of Medical Microbiology*, *56*(1), 56-65.

Tran, T.T.H., Dang, T.H.O., Nguyen, T.P. (2002). Study on diseases in giant freshwater prawns (*Macrobrachium rosenbergii*). A Review for the year 2002. Department of Fisheries Biology, College of Aquaculture and Fisheries, Can Tho University, Cantho City, Vietnam.

Trucksis, M., Galen, J. E., Michalski, J., Fasano, A., & Kaper, J. B. (1993). Accessory cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette. *Proceedings of the National Academy of Sciences*, 90(11), 5267-5271.

Trucksis, M., Conn, T. L., Fasano, A., & Kaper, J. B. (1997). Production of *Vibrio cholerae* accessory cholera enterotoxin (Ace) in the yeast *Pichia pastoris*. *Infection and Immunity*, 65(12), 4984-4988.

Trucksis, M., Conn, T. L., Wasserman, S. S., & Sears, C. L. (2000). *Vibrio cholerae* ACE stimulates Ca2+-dependent Cl−/HCO3− secretion in T84 cells *in vitro*. *American Journal of Physiology-Cell Physiology*, 279(3), C567-C577.

Tuang, F. N., Rademaker, J. L., Alocilja, E. C., Louws, F. J., & Bruijn, F. J. (1999). Identification of bacterial rep□PCR genomic fingerprints using a backpropagation neural network. *FEMS Microbiology Letters*, 177(2), 249-256.

Tubiash, H. S., Colwell, R. R., & Sakazaki, R. (1970). Marine *Vibrios* associated with bacillary necrosis, a disease of larval and juvenile bivalve mollusks. *Journal of Bacteriology*, 103(1), 271.

Tubiash, H. S., & Otto, S. V. (1986). Bacterial problems in oysters. A review. *Pathology in marine aquaculture. European Aquaculture Society, Special Publication*, 8, 233-242.

Udden, S. N., Zahid, M. S. H., Biswas, K., Ahmad, Q. S., Cravioto, A., Nair, G. B., Mekalanos, J.J., & Faruque, S. M. (2008). Acquisition of classical CTX prophage from *Vibrio cholerae* O141 by ElTor strains aided by lytic phages and chitin-induced competence. *Proceedings of the National Academy of Sciences*, 105(33), 11951-11956.

Urakawa, H., Kita□Tsukamoto, K., & Ohwada, K. (1997). 16S rDNA genotyping using PCR/RFLP (restriction fragment length polymorphism) analysis among the family Vibrionaceae. *FEMS Microbiology Letters*, 152(1), 125-132.

Urakawa, H., Kita-Tsukamoto, K., & Ohwada, K. (1999). 16S rDNA restriction fragment length polymorphism analysis of psychrotrophic *Vibrios* from Japanese coastal water. *Canadian Journal of Microbiology*, 45(12), 1001-1007.

Urakawa, H., Yoshida, T., Nishimura, M., & Ohwada, K. (2000). Characterization of depth□related population variation in microbial communities of a coastal marine sediment using 16S rDNA□based approaches and quinone profiling. *Environmental Microbiology*, 2(5), 542-554.

Urdaci, M. C., Marchand, M., Ageron, E., Arcos, J. M., Sesma, B., & Grimont, P. A. (1991). *Vibrio navarrensis* sp. nov., a species from sewage. *International Journal of Systematic Bacteriology*, 41(2), 290-294.

Uzzau, S., Lu, R., Wang, W., Fiore, C., & Fasano, A. (2001). Purification and preliminary characterization of the zonula occludens toxin receptor from human (CaCo2) and murine (IEC6) intestinal cell lines. *FEMS Microbiology Letters*, *194*(1), 1-5.

Vaitkevicius, K., Lindmark, B., Ou, G., Song, T., Toma, C., Iwanaga, M., Zhu, J. & Wai, S. N. (2006). A *Vibrio cholerae* protease needed for killing of *Caenorhabditis elegans* has a role in protection from natural predator grazing. *Proceedings of the National Academy of Sciences*, 103(24), 9280-9285.

van Belkum, A. (1994). DNA fingerprinting of medically important microorganisms by use of PCR. *Clinical Microbiology Reviews*, 7(2), 174-184.

van Belkum, A., Sluijuter, M., de Groot, R., Verbrugh, H., & Hermans, P. W. (1996). Novel BOX repeat PCR assay for high-resolution typing of *Streptococcus pneumoniae* strains. *Journal of Clinical Microbiology*, 34(5), 1176-1179.

Vandenberghe, J., Thompson, F. L., Gomez-Gil, B., & Swings, J. (2003). Phenotypic diversity amongst *Vibrio* isolates from marine aquaculture systems. *Aquaculture*, 219(1), 9-20.

Vanderzant, C., Nickelson, R., & Judkins, P. W. (1971). Microbial flora of pond-reared brown shrimp (*Penaeus aztecus*). *Applied Microbiology*, 21(5), 916-921.

Vartian, C. V., & Septimus, E. J. (1990). Osteomyelitis caused by *Vibrio vulnificus*. *Journal of Infectious Diseases*, 161(2), 363-363.

Vaseeharan, B., & Ramasamy, P. (2003). Abundance of potentially pathogenic micro-organisms in *Penaeus monodon* larvae rearing systems in India. *Microbiological Research*, 158(4), 299-308.

Vengadesh, L., Son, R., & Yoke-Kqueen, C. (2012). Molecular quantitation and characterization of *Vibrio cholerae* from different seafood obtained from wetmarket and supermarket. *International Food Research Journal*, 19(1), 45-50.

Venkateswaran, K., Dohmoto, N., & Harayama, S. (1998). Cloning and nucleotide sequence of the *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimp. *Applied and Environmental Microbiology*, 64(2), 681-687.

Veron, M. (1965). La position taxonomique des *Vibrio* et de certaines bactéries comparables. *CR Acad Sci Hebd Seances Acad Sci*, 261, 5243-5246.

Versalovic, J., Koeuth, T., & Lupski, R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research*, 19(24), 6823-6831.

Verschuere, L., Heang, H., Criwl, G., & Sorgeloos, P. V. (2000) Selected bacterial strains protect *Artemia* spp. from the pathogenic effects of *Vibrio proteolyticus* CW8T2. *Applied Environmental Microbiology*, 66, 1139-1146.

Vezzulli, L., Pruzzo, C., Huq, A., & Colwell, R. R. (2010). Environmental reservoirs of *Vibrio cholerae* and their role in cholera. *Environmental Microbiology Reports*, 2(1), 27-33.

Vimr, E. R., Kalivoda, K. A., Deszo, E. L., & Steenbergen, S. M. (2004). Diversity of microbial sialic acid metabolism. *Microbiology and Molecular Biology Reviews*, 68(1), 132-153.

Visick, K. L. (2009). An intricate network of regulators controls biofilm formation and colonization by *Vibrio fischeri*. *Molecular Microbiology*, 74(4), 782-789.

Vital-Brazil, J. M., Alves, R. M., Rivera, I. N., Rodrigues, D. P., Karaolis, D. K., & Campos, L. C. (2002). Prevalence of virulence□associated genes in clinical and environmental *Vibrio cholerae* strains isolated in Brazil between 1991 and 1999. *FEMS Microbiology Letters*, 215(1), 15-21.

Vodovar, N., Acosta, C., Lemaitre, B., & Boccard, F. (2004). *Drosophila*: A polyvalent model to decipher host–pathogen interactions. *Trends in Microbiology*, 12(5), 235-242.

Vuddhakul, V., Nakai, T., Matsumoto, C., Oh, T., Nishino, T., Chen, C. H., & Okuda, J. (2000). Analysis of *gyrB* and *toxR*gene sequences of *Vibrio hollisae* and development of *gyrB* and *toxR* targeted PCR methods for isolation of *V. hollisae* from the environment and its identification. *Applied and Environmental Microbiology*, 66(8), 3506-3514.

Vulic, M., Lenski, R. E., & Radman, M. (1999). Mutation, recombination, and incipient speciation of bacteria in the laboratory. *Proceedings of the National Academy of Sciences*, 96(13), 7348-7351.

Wagner, K. R., & Crichton, E. P. (1981). Marine *Vibrio* infections acquired in Canada. *Canadian Medical Association Journal*, 124(4), 435.

Wai, S. N., Mizunoe, Y., & Yoshida, S. I. (1999). How *Vibrio cholerae* survive during starvation. *FEMS Microbiology Letters*, 180(2), 123-131.

Waldor, M. K., & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science*, 272(5270), 1910-1914.

Waldor, M. K., Tschape, H., & Mekalanos, J. J. (1996). A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. *Journal of Bacteriology*, 178(14), 4157-4165.

Waldor, M. K., Rubin, E. J., Pearson, G. D., Kimsey, H., & Mekalanos, J. J. (1997). Regulation, replication, and integration functions of the *Vibrio cholerae* CTXΦ are encoded by region RS2. *Molecular Microbiology*, 24(5), 917-926.

Walling, E., Vourey, E., Ansquer, D., Beliaeff, B., & Goarant, C. (2010). *Vibrio nigripulchritudo* monitoring and strain dynamics in shrimp pond sediments. *Journal of Applied Microbiology*, 108(6), 2003-2011.

Wang, L., Zhao, X., Chu, J., Li, Y., Li, C., & Zhong, Q. (2011). Application of an improved loop-mediated isothermal amplification detection of *Vibrio parahaemolyticus* from various seafood samples. *African Journal of Microbiology*, 5(31), 5765-5771.

Warner, E., & Oliver, J. D. (2008). Population structures of two genotypes of *Vibrio vulnificus* in oysters (*Crassostrea virginica*) and seawater. *Applied and Environmental Microbiology*, 74(1), 80-85.

Watanabe, H., Miyoshi, S. I., Kawase, T., Tomochika, K. I., & Shinoda, S. (2004). High growing ability of *Vibrio vulnificus* biotype 1 is essential for production of a toxic metalloprotease causing systemic diseases in humans. *Microbial Pathogenesis*, 36(3), 117-123.

Waturangi, D. E., Joanito, I., Yogi, Y., & Thomas, S. (2012). Use of REP- and ERIC-PCR to reveal genetic heterogeneity of *Vibrio cholerae* from edible ice in Jakarta, Indonesia. *Gut Pathogens*, 4, 1-2.

Weber, J. T., Mintz, E. D., Canizares, R., Semiglia, A., Gomez, I., Sempertegui, R., & Blake, P. A. (1994). Epidemic cholera in Ecuador: Multidrug-resistance and transmission by water and seafood. *Epidemiology and Infection*, 112(1), 1-12.

Webster, N. S., & Blackall, L. L. (2008). What do we really know about sponge-microbial symbioses & quest. *The ISME Journal*, 3(1), 1-3.

Welsh, J., & McClelland, M. (1993). *The characterization of pathogenic microorganisms by genomic fingerprinting using arbitrarily primed polymerase chain reaction (AP-PCR)*. In: Persing D. H. (Ed.), *Diagnostic Molecular Microbiology*. Washington, ASM press. pp. 595- 602.

West, P. A., Lee, J. V., & Bryant, T. N. (1983). A numerical taxonomic study of species of *Vibrio* isolated from the aquatic environment and birds in Kent, England. *Journal of Applied Microbiology*, 55(2), 263-282.

Westlund, B., Stilwell, G., & Sluder, A. (2004). Invertebrate disease models in neurotherapeutic discovery. *Current Opinion in Drug Discovery & Development*, 7(2), 169.

Whitehouse, C. A., Baldwin, C., Sampath, R., Blyn, L. B., Melton, R., Li, F., & Huq, A. (2010). Identification of pathogenic *Vibrio* species by multilocus PCR-electrospray ionization mass spectrometry and its application to aquatic environments of the former Soviet Republic of Georgia. *Applied and Environmental Microbiology*, *76*(6), 1996-2001.

Wiik, R., Stackebrandt, E., Valle, O., Daae, F. L., Rodseth, O. M., & Andersen, K. (1995). Classification of fish-pathogenic *Vibrios* based on comparative 16S rRNA analysis. *International Journal of Systematic Bacteriology*, 45(3), 421-428.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18(22), 6531-6535.

Wolfe, A. J., Millikan, D. S., Campbell, J. M., & Visick, K. L. (2004). *Vibrio fischeri* σ54 controls motility, biofilm formation, luminescence, and colonization. *Applied and Environmental Microbiology*, 70(4), 2520-2524.

Won, K. M., & Park, S. I. (2008). Pathogenicity of *Vibrio harveyi* to cultured marine fishes in Korea. *Aquaculture*, 285(1), 8-13.

Wong, H. C., & Wang, P. (2004). Induction of viable but nonculturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *Journal of Applied Microbiology*, 96(2), 359-366.

Wong, S. Y., & Leong, T. S. (1990). A comparative study of *Vibrio* infections in healthy and diseased marine finfishes cultured in floating cages near Penang, Malaysia. *Asian Fisheries Science*, 3, 353-359.

Wright, A. C., Simpson, L. M., & Oliver, J. D. (1981). Role of iron in the pathogenesis of *Vibrio vulnificus* infections. *Infection and Immunity*, 34(2), 503-507.

Wright, A. C., Hill, R. T., Johnson, J. A., Roghman, M. C., Colwell, R. R., & Morris, J. G. (1996). Distribution of *Vibrio vulnificus* in the Chesapeake Bay. *Applied and Environmental Microbiology*, 62(2), 717-724.

Wright, A. C., Powell, J. L., Tanner, M. K., Ensor, L. A., Karpas, A. B., Morris, J. G., & Sztein, M. B. (1999). Differential expression of *Vibrio vulnificus* capsular polysaccharide. *Infection and Immunity*, 67(5), 2250-2257.

Wright, A. C., Powell, J. L., Kaper, J. B., & Morris, J. G. (2001). Identification of a group 1-like capsular polysaccharide operon for *Vibrio vulnificus*. *Infection and Immunity*, 69(11), 6893-6901.

Yamamoto, K., Ichinose, Y., Nakasone, N., Tanabe, M., Nagahama, M., Sakurai, J., & Iwanaga, M. (1986). Identity of hemolysins produced by *Vibrio cholerae* non-O1 and V. cholerae O1, biotype ElTor. *Infection and Immunity*, 51(3), 927-931.

Yamamoto, K., Wright, A. C., Kaper, J. B., & Morris, J. G. (1990). The cytolysin gene of *Vibrio vulnificus*: Sequence and relationship to the *Vibrio cholerae* ElTor hemolysin gene. *Infection and Immunity*, 58(8), 2706-2709.

Yang, A., & Yen, C. (2012). PCR optimization of BOX-A1R PCR for microbial source tracking of *Escherichia coli* in Waterways. *Journal of Experimental Microbiology and Immunology*, 16, 85-89.

Ye, Y., Wu, Q., Yao, L., Dong, X., Wu, K., & Zhang, J. (2009). Analysis of a consensus fragment in ERIC-PCR fingerprinting of *Enterobacter sakazakii*. *International Journal of Food Microbiology*, 132(2), 172-175.

Yildiz, F. H., & Visick, K. L. (2009). *Vibrio* biofilms: so much the same yet so different. *Trends in Microbiology*, 17(3), 109-118.

Yoshida, S., Ogawa, M., & Mizuguchi, Y. (1985). Relation of capsular materials and colony opacity to virulence of *Vibrio vulnificus*. *Infection and Immunity*, 47(2), 446-451.

Yoshii, Y., Nishino, H., Satake, K., & Umeyama, K. (1987). Isolation of *Vibrio fluvialis*, and unusual pathogen in acute suppurative cholangitis. *The American Journal of Gastroenterology*, 82(9), 903.

Yoshimura, S., Takao, T., Shimonishi, Y., Arita, M., Takeda, T., Imashi, H., Honda, T., and Miwatani. T. (1986). A heat-stable enterotoxin of *Vibrio cholerae* non-O1: Chemical synthesis, and biological and physicochemical properties. *Biopolymers,* 25, S69-S83.

Young, H. K. (1993). Antimicrobial resistance spread in aquatic environments. *Journal of Antimicrobial Chemotherapy*, 31(5), 627-635.

Yu, S. L., & Uy-Yu, O. (1984). *Vibrio parahaemolyticus* pneumonia. *Annals of Internal Medicine*, 100(2), 320-320.

Zamborsky, D. J., & Nishiguchi, M. K. (2011). Phylogeographical patterns among Mediterranean sepiolid squids and their *Vibrio* symbionts: Environment drives specificity among sympatric species. *Applied and Environmental Microbiology*, 77(2), 642-649.

Zanetti, S., Spanu, T., Deriu, A., Romano, L., Sechi, L. A., & Fadda, G. (2001). *In vitro* susceptibility of *Vibrio* spp. isolated from the environment. *International Journal of Antimicrobial Agents*, 17(5), 407-409.

Zhang, X. H., Meaden, P. G., & Austin, B. (2001). Duplication of hemolysin genes in a virulent isolate of *Vibrio harveyi*. *Applied and Environmental Microbiology*, 67(7), 3161-3167.

Zhang, Y., Lu, H., & Bargmann, C. I. (2005). Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature*, 438(7065), 179-184.

Zitzer, A. O., Nakisbekov, N. O., Li, A. V., Semiotrochev, V. L., Kiseliov, Y. L., Muratkhodjaev, J. N.,Krasilnikov, O. V., & Ezepchuk, Y. V. (1993). Entero-cytolysin (EC) from *Vibrio cholerae* Non-O1 (Some properties and pore-forming activity). *Zentralblatt für Bakteriologie*, 279(4), 494-504.

Zitzer, A., Wassenaar, T. M., Walev, I., & Bhakdi, S. (1997). Potent membrane-permeabilizing and cytocidal action of *Vibrio cholerae* cytolysin on human intestinal cells. *Infection and Immunity*, *65*(4), 1293-1298.

Zitzer, A., Zitzer, O., Bhakdi, S., & Palmer, M. (1999). Oligomerization of *Vibrio cholerae* cytolysin yields a pentameric pore and has a dual specificity for cholesterol and sphingolipids in the target membrane. *Journal of Biological Chemistry*, 274(3), 1375-1380.

Zulkifli, Y., Alitheen, N. B., Raha, A. R., Yeap, S. K., Marlina, S. R., & Nishibuchi, M. (2009). Antibiotic resistance and plasmid profiling of *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. *International Food Research Journal*, 16(1), 53-58.

Zuppardo, A. B., & Siebeling, R. J. (1998). An epimerase gene essential for capsule synthesis in *Vibrio vulnificus*. *Infection and Immunity*, 66(6), 2601-2606.

APPENDIX

MEDIA

Alkaline Peptone Water (APW)

Peptone	1 g
NaCl	0.17 M
Distilled water	100 mL

Dissolve the peptone and NaCl in distilled water and adjust the pH 8.5.

Nutrient Medium

Peptone	5 g
Sodium chloride	5 g
Beef extract	1 g
Yeast extract	2 g

Suspend 1.3 g of Nutrient Broth Medium (Himedia, India) in 100mL of distilled water. Mix well and autoclave at 15 lbs pressure for 15 minutes and cool to 50-55°C. When used as solid agar medium, 2.0% agar (w/v) is added to the medium for agar plate preparation. Final pH- 7.4±0.2.

Luria Bertani Broth

Casein enzymic hydrolysate	10 g
Yeast extract	5 g
Sodium chloride	10 g

Suspend 25 grams of LB Broth medium (Himedia, India) in 1000 mL distilled water. The medium is heated to dissolve completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Final pH- 7.5±0.2.

Mueller -Hinton agar (Himedia, India)

Beef, dehydrated infusion	30.0 % (w/v)
Casein hydrolysate	1.75 % (w/v)
Starch	0.15 % (w/v)
Agar	17 g
pH (25^{o}C) 7.4±0.2	

Dissolve 3.8 g of media (Himedia, India) in 100ml of distilled water. Heat to boiling to dissolve the medium completely. Mix well, autoclave at 15 lbs pressure for 15 minutes and cool to 50-55^{o}C. The sterile medium is poured into sterile plates and the plates can be stored at 4^{o}C.

Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar

Proteose peptone	10 g
Yeast extract	5 g
Sodium thiosulphate	10 g
Sodium citrate	10 g
Oxgall	8 g
Sucrose	20 g
Sodium chloride	10 g
Ferric citrate	1 g
Bromothymol blue	4 mg
Thymol blue	4 mg
Agar	15 g
Final pH (at 25°C) 8.6±0.2	

Suspend 89.08 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Do not autoclave. Cool to 50°C and pour into sterile petri plates.

MOF Medium (Marine Oxidation Fermentation Medium)

Casein enzymichydrolysate	1 g
Yeast extract	0.1 g
Trishydroxymethylaminomethane	0.5 g
Boric acid	1.1 mg
Ammonium sulphate	0.5 g
Disodium phosphate	4 mg
Ammonium nitrate	0.08 mg
Sodium chloride	9.7 g
Magnesium chloride	4.4 g
Sodium sulphate	1.6 g
Calcium chloride	0.9 g
Potassium chloride	2.75 mg
Sodium bicarbonate	0.08 g
Potassium bromide	0.04 g
Strontium chloride	1.7 mg
Sodium silicate	2 mg
Sodium fluoride	1.2 mg
Phenol red	0.01 g
Agar	3 g

Final pH (at 25°C) 8.0±0.2

Suspend 22.14 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 55-60°C and aseptically add sterile dextrose solution (or other carbohydrate of choice) to a final concentration of 1%. Mix well and dispense into sterile test tubes.

Nematode Growth Medium (NGM)

NaCl	3 g
Agar	17 g
Peptone	2.5 g
MgSO4 (1M)	1 mL
CaCl (1M)	1 mL
Cholesterol	5 mg
Ethanol	1 mL
KPO_4 buffer (1M)	25 mL
Distilled water	975 mL

Mix 3 g NaCl, 17 g agar, and 2.5 g peptone in a 2 litre Erlenmeyer flask. Add 975 mL distilled water. The mouth of the flask is covered with aluminium foil and autoclaved for 50 min. The flask is cooled at 55°C in a water bath for 15 min. Add 1 ml 1M $CaCl_2$, 1 mL 5 mg/mL cholesterol in ethanol, 1 mL 1M $MgSO_4$ and 25 mL 1M KPO_4 buffer and swirl to mix well. Pour into sterile petriplates.

REAGENTS

Oxidase reagent

Tetramethyl p-phenyldiamine	1 g
Isoamyl alcohol	100 mL

Disodium ethylenediaminetetraacetete (EDTA) - 0.5 M

EDTA	186.1 g
Distilled water	1000 mL
NaOH	~20 g

EDTA is dissolved in 800 mL of distilled water and stirred vigorously on a magnetic stirrer. Adjust pH to 8.0 using NaOH pellets to a final volume to 1000 mL. Autoclave before use.

Tris EDTA (TE) buffer

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

Tris-Acetate EDTA (50 X)

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

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

Agarose gel

Agarose (SRL)	1.0 g
IX TAE	100 mL

The agarose is melted in the microwave oven and cooled to 50°C before pouring the gel.

Bromophenol blue loading dye

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

The loading dye is dispensed in 1 mL aliquots in fresh Eppendorf tubes and stored at 4^{o}C

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

CTAB	10 g
NaCl	4.1 g
Distilled water	80 mL

Dissolve 4.1 g NaCl in 80 mL distilled water and slowly 10 g CTAB is added, while heating and stirring. If necessary, heat to 65^{o}C to dissolve. Adjust final volume to 100mL.

Glycerol Loading Dye (6X)

Bromophenol blue	0.26 g
Glycerol	30 mL

Ethidium Bromide (EtBr) (10 mg/mL)

Ethidium Bromide	0.1 g
Distilled water	10 mL

The 10 mg/mL of solution is kept in a dark bottle and stored at 4^{o}C. For staining agarose gels, a working solution of 0.5µg/mL is prepared.

Sodium dodecyl sulphate (SDS) – 10%

SDS	10 g
Distilled water	100 mL

1M KPO4 buffer (pH 6)

KH_2PO_4	108.3 g
K_2HPO_4	35.6 g
Distilled water	1000 ml

M9 Buffer

Na2HPO4	6 g
KH2PO4 (dibasic)	3 g
NaCl	5g
1 M MgSO4	1mL

Dissolve the above in 800 ml distilled water. Adjust the volume to 1000ml with distilled water and sterilize by autoclaving at 15lbs for 20 minutes.

LIST OF PUBLICATIONS

Peer Reviewed Paper Publication

Joseph, A. V., Sasidharan, R. S., Nair, H. P., Bhat, S. G., (2013). Occurrence of potential pathogenic *Aeromonas* species in tropical seafood, aquafarms and mangroves off Cochin coast in South India.*Veterinary World, 6*(6), 300-306.

Patent Filed

The research work on "**Bio-compatible ZnS:Nanocrystals conjugated with L-citrulline, as fluorescent probes for DNA visualization and for finger print analysis in forensic studies"**, has been filed for patent with the patent application number **4900/CHE/2012.**

Authors- M. Sajimol Augustine, **AlphonsaVijaya Joseph**, Sarita.G.Bhat, S.Jayalekshmi

Genbank submissions

1. GenBank Accession No. JQ799885.1*Vibrio* sp. strain BTMM2 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G.
2. GenBank Accession No. JQ799886.1 *Vibrio* sp. strain BTMU7 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G
3. GenBank Accession No. JQ799887.1 *Vibrio*sp. strain BTOK5 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G.
4. GenBank Accession No. JQ799888.1 *Vibrio*sp. strain BTOK12 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G.
5. GenBank Accession No. JQ799889.1*Vibrio* sp. strain BTPF6 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G.
6. GenBank Accession No. JQ799890.1*Vibrio* sp. strain BTVE8 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G.
7. GenBank Accession No. JQ799891.1*Vibrio* sp. strain BTVE10 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G.
8. GenBank Accession No. JQ923495.1*Vibrio* sp. strain BTMA1 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Siju,V.M. and Sarita,B.G.

9. GenBank Accession No. JQ923496.1*Vibrio* sp. strain BTAS2 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Siju,V.M. and Sarita,B.G.

10. GenBank Accession No. JQ923497.1 *Vibrio* sp. strain BTMM4 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Siju,V.M. and Sarita,B.G.

11. GenBank Accession No. JQ923498.1*Vibrio* sp. strain BTMM6 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Siju,V.M. and Sarita,B.G.

12. GenBank Accession No. JQ923499.1.1*Vibrio* sp. strain BTMM7 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Siju,V.M. and Sarita,B.G.

13. GenBank Accession No. JQ923500.1*Vibrio* sp. strain BTMS4 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Siju,V.M. and Sarita,B.G.

14. GenBank Accession No. JQ923501.1 *Vibrio* sp. strain BTMS7 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

15. GenBank Accession No. JQ923502.1 *Vibrio*sp. strain BTOK2 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

16. GenBank Accession No. JQ923503.1*Vibrio* sp. strain BTOK6 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

17. GenBank Accession No. JQ923504.1 *Vibrio*sp. strain BTOK9 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

18. GenBank Accession No. JQ923505.1 *Vibrio*sp. strain BTOK10 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

19. GenBank Accession No. JQ936967.1Vibrio sp. strain BTOK10 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Raghul,S.S. and Sarita,B.G.

20. GenBank Accession No. JQ936968.1*Vibrio* sp. strain BTOK11 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Raghul,S.S. and Sarita,B.G.

21. GenBank Accession No. JQ936969.1*Vibrio* sp. strain BTOS3 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Raghul,S.S. and Sarita,B.G.

22. GenBank Accession No. JQ936970.1*Vibrio* sp. strain BTOS6 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Raghul,S.S. and Sarita,B.G.

23. GenBank Accession No. JQ936971.1*Vibrio* sp. strain BTPF2 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Raghul,S.S. and Sarita,B.G.

24. GenBank Accession No. JQ936972.1*Vibrio* sp. strain BTPM4 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Smitha,S. and Sarita,B.G.

25. GenBank Accession No. JQ936973.1*Vibrio* sp. strain BTVE9 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G..

26. GenBank Accession No. JQ936974.1*Vibrio* sp. strain BTVM6 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G.

27. GenBank Accession No. JQ936975.1*Vibrio* sp. strain BTVM8 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G.

28. GenBank Accession No. JQ936976.1*Vibrio* sp. strain BTAS3 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph** and Sarita,B.G.

29. GenBank Accession No. JQ936977.1*Vibrio* sp. strain BTMA5 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Smitha,S. and Sarita,B.G.

30. GenBank Accession No. JQ936978.1*Vibrio* sp. strain BTMA9 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Jeena,A. and Sarita,B.G.

31. GenBank Accession No. JQ936979.1*Vibrio* sp. strain BTMS3 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Jeena,A. and Sarita,B.G.

32. GenBank Accession No. JQ936980.1 *Vibrio*sp. strain BTMS5 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Jeena,A. and Sarita,B.G.

33. GenBank Accession No. JQ936981.1*Vibrio* sp. strain BTOK7 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Jeena,A. and Sarita,B.G.

34. GenBank Accession No. JQ936982.1 *Vibrio*sp. strain BTOS10 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Linda,L. and Sarita,B.G.

35. GenBank Accession No. JQ936983.1*Vibrio* sp. strain BTPF5 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Linda,L. and Sarita,B.G.

36. GenBank Accession No. JQ936984.1*Vibrio* sp. strain BTPR5 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Linda,L. and Sarita,B.G.

37. GenBank Accession No. JQ936985.1*Vibrio* sp. strain BTPS6 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Linda,L. and Sarita,B.G.

38. GenBank Accession No. JQ936986.1*Vibrio* sp. strain BTVE2 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Helvin,V. and Sarita,B.G.

39. GenBank Accession No. JQ936987.1*Vibrio* sp. strain BTVE4 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Helvin,V. and Sarita,B.G.

40. GenBank Accession No. JQ936987.1*Vibrio* sp. strain BTVE4 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Helvin,V. and Sarita,B.G.

41. GenBank Accession No. JQ936987.1*Vibrio* sp. strain BTVE4 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Helvin,V. and Sarita,B.G.

42. GenBank Accession No. JQ936988.1*Vibrio* sp. strain BTVE11 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Helvin,V. and Sarita,B.G.

43. GenBank Accession No. JQ964244.1*Aeromonassp*. strain BTSQ7 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

44. GenBank Accession No. JQ964245.1*Aeromonassp*. strainBTSQ9 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

45. GenBank Accession No. JQ964246.1*Aeromonassp*. strainBTSQ10 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

46. GenBank Accession No. JQ964247.1 *Aeromonassp*. strainBTSQ11 16S ribosomal RNA gene, partial sequence AlphonsaVijaya Joseph, Harisree,N.P. and Sarita,B.G.

47. GenBank Accession No. JQ964248.1*Aeromonassp*. strainBTPR1 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

48. GenBank Accession No. JQ964249.1*Aeromonassp*. strainBTPR3 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

49. GenBank Accession No. JQ964250.1 *Aeromonassp*. strainBTPR4 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

50. GenBank Accession No. JQ964251.1*Aeromonassp*. strainBTPR6 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

51. GenBank Accession No. JQ964252.1*Aeromonassp*. strainBTPR7 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

52. GenBank Accession No. JQ964253.1*Aeromonassp*. strainBTMU3 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

53. GenBank Accession No. JQ964254.1*Aeromonassp*. strainBTVE3 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

54. GenBank Accession No. JQ964255.1*Aeromonassp*. strainBTMA11 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

55. GenBank Accession No. JQ964256.1*Aeromonassp*. strainBTPF7 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

56. GenBank Accession No. JQ964257.1*Aeromonassp*. strainBTAS5 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

57. GenBank Accession No. JX080382.1 *Proteus* sp. strainBTVE7 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Mridula,V.G. and Sarita,B.G.

58. GenBank Accession No. JX080383.1*Aeromonassp*. strainBTMA8 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Mridula,V.G. and Sarita,B.G.

59. GenBank Accession No. JX080384.1*Klebsiella sp.*strainBTMA10 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Mridula,V.G. and Sarita,B.G.

60. GenBank Accession No. JX080385.1*Plesiomonassp*. strainBTOK416S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Mridula,V.G. and Sarita,B.G.

61. GenBank Accession No. JX080386.1 *Vibrio* sp. strainBTOS1 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Mridula,V.G. and Sarita,B.G.

62. GenBank Accession No. JX080387.1 *Vibrio* sp. strainBTOS4 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Mridula,V.G. and Sarita,B.G.

63. GenBank Accession No. JX852421.1Uncultured bacterium clone MS106 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., **Alphonsa,V.J.** and Mrithula,V.G.

64. GenBank Accession No. JX852422.1Uncultured bacterium clone MS112 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., **Alphonsa,V.J.** and Mrithula,V.G.

65. GenBank Accession No. JX852423.1Uncultured bacterium clone MS119 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., **Alphonsa,V.J.** and Mrithula,V.G.

66. GenBank Accession No. JX852424.1Uncultured bacterium clone MS135 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., **Alphonsa,V.J.** and Mrithula,V.G.

67. GenBank Accession No. JX852425.1Uncultured bacterium clone MS143 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., **Alphonsa,V.J.** and Mrithula,V.G.

68. GenBank Accession No. JX852426.1Uncultured bacterium clone MS154 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., **Alphonsa,V.J.** and Mrithula,V.G.

69. GenBank Accession No. JX852427.1Uncultured alpha *Proteobacterium* clone MS177 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., **Alphonsa,V.J.** and Mrithula,V.G.

70. GenBank Accession No. JX852428.1Uncultured gamma *Proteobacterium* clone MS118 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., **Alphonsa,V.J.** and Mrithula,V.G.

71. GenBank Accession No. JX852429.1Uncultured bacterium clone MS68 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., Alphonsa,V.J. and Mrithula,V.G.

72. GenBank Accession No. JX852428.1Uncultured gamma *Proteobacterium* clone MS118 16S ribosomal RNA gene, partial sequence. Harisree,P.N., Sarita,G.B., Helvin,V., **Alphonsa,V.J.** and Mrithula,V.G.

73. GenBank Accession No. HM030821.1 Bacillus *licheniformis* strain BTEK16 16S ribosomal RNA gene, partial sequence **AlphonsaVijaya Joseph**, Harisree,N.P. and Sarita,B.G.

74. GenBank Accession No. JQ964256.1*Aeromonassp*. strainBTPF7 16S ribosomal RNA gene, partial sequence Smitha,S., Siju,V.M., **Alphonsa,V.J.** and Sarita,B.G.

75. GenBank Accession No. KF420399*Vibrio cholerae* isolate BTMA1 ToxR (toxR) gene, partial cds **Alphonsa,V.Joseph,** Raghul,S.S., Harisree,P.Nair., Helvin,V. and Sarita,G.Bhat.

76. GenBank Accession No. KF420400*Vibrio cholerae* isolate BTMA5 ToxR (toxR) gene, partial cds **Alphonsa,V.Joseph,** Raghul,S.S., Harisree,P.Nair., Helvin,V. and Sarita,G.Bhat.

77. GenBank Accession No KF420401Vibrio cholerae isolate BTMA9 ToxR (toxR) gene, partial cds **Alphonsa,V.Joseph,** Raghul,S.S., Harisree,P.Nair., Helvin,V. and Sarita,G.Bhat.

78. GenBank Accession No. KF420402*Vibrio cholerae* isolate BTMS4 ToxR (toxR) gene, partial cds **Alphonsa,V.Joseph,** Raghul,S.S., Harisree,P.Nair., Helvin,V. and Sarita,G.Bhat.

79. GenBank Accession No. KF420403*Vibrio cholerae* isolate BTMOK11 ToxR (toxR) gene, partial cds **Alphonsa,V.Joseph,** Raghul,S.S., Harisree,P.Nair., Helvin,V. and Sarita,G.Bhat.

80. GenBank Accession No. KF420404*Vibrio cholerae* isolate BTOK7 ToxR (toxR) gene, partial cds **Alphonsa,V.Joseph,** Raghul,S.S., Harisree,P.Nair., Helvin,V. and Sarita,G.Bhat.

81. GenBank Accession No. KF420405*Vibrio cholerae* isolate BTOK9 ToxR (toxR) gene, partial cds **Alphonsa,V.Joseph,** Raghul,S.S., Harisree,P.Nair., Helvin,V. and Sarita,G.Bhat.

82. GenBank Accession No. KF420406*Vibrio cholerae* isolate BTPR5 ToxR (toxR) gene, partial cds **Alphonsa,V.Joseph,** Raghul,S.S., Harisree,P.Nair., Helvin,V. and Sarita,G.Bhat.

83. GenBank Accession No. KF420407 *Vibrio cholerae* isolate BTMVE2 ToxR (toxR) gene, partial cds **Alphonsa,V.Joseph,** Raghul,S.S., Harisree,P.Nair., Helvin,V. and Sarita,G.Bhat.

84. GenBank Accession No. KF322108 *Vibrio vulnificus* isolate BTAS3 ToxR (toxR) gene, partial cds **Alphonsa,J.Vijaya,** Sarita,B.G., Harisree,N.P. and Raghul,S.Subin.

85. GenBank Accession No. KF322109 *Vibrio vulnificus* isolate BTOS7 ToxR (toxR) gene, partial cds **Alphonsa,J.Vijaya,** Sarita,B.G., Harisree,N.P. and Raghul,S.Subin.

86. GenBank Accession No. KF322110 *Vibrio vulnificus* isolate BTPS6 ToxR (toxR) gene, partial cds **Alphonsa,J.Vijaya,** Sarita,B.G., Harisree,N.P. and Raghul,S.Subin.

doi:10.5455/vetworld.2013.300-306 ORIGINAL RESEARCH

Occurrence of potential pathogenic *Aeromonas* species in tropical seafood, aquafarms and mangroves off Cochin coast in South India

Alphonsa Vijaya Joseph, Raghul Subin Sasidharan, Harisree P. Nair and Sarita G. Bhat

Department of Biotechnology,
Cochin University of Science and Technology, Cochin, India
Corresponding author: Sarita G. Bhat, email:saritagbhat@gmail.com
Received: 02-11-2012, **Accepted:** 01-12-2012, **Published online:** 05-03-2013

How to cite this article: Joseph AV, Sasidharan RS, Nair HP and Bhat SG (2013) Occurrence of potential pathogenic *Aeromonas* species in tropical seafood, aquafarms and mangroves off Cochin coast in South India, *Vet. World* 6(6):300-306, doi:10.5455/vetworld.2013.300-306

Abstract

Background: The genus *Aeromonas* include gram-negative, motile, facultative anaerobic, rod shaped and oxidase positive bacteria comprising several species, associated with the aquatic environment. *Aeromonas* species have been implicated in human pathogenesis and are linked with gastroenteritis, muscle infections, septicemia, and skin diseases. In fish they are renowned as enteric pathogens causing haemorrhagic septicemia, fin rot, soft tissue rot and furunculosis resulting in major die-offs and fish kills.

Aim: This study reports the occurrence of potential pathogenic *Aeromonas* sp. in tropical seafood (Squids, Prawns and Mussels), aquafarms and mangroves of Cochin, Kerala, South India.

Materials and Methods :Tropical seafood (Squid, Prawn and Mussel), sediment and water samples from aquafarms and associated mangroves were screened for *Aeromonas* contamination. The isolates were identified by 16S rDNA sequence analysis and subjected to morphological and biochemical characterization. Haemolytic assay was used for determining pathogenicity of the organisms. Antibiotic susceptibility against 12 antibiotics were performed and the MAR index was calculated.

Results: A total of 134 isolates were recovered from the samples of which 15 were identified as *Aeromonas* species by 16S rDNA sequence analysis and were assigned to 5 species namely, *A. hydrophila*, *A. enteropelogenes*, *A. caviae*, *A. punctata* and *A. aquarorium*. Morphological, biochemical and phylogenetic analyses revealed relatedness and variability among the strains. All the isolates were haemolytic on blood agar indicating their pathogenicity. The isolates exhibited varying degrees of resistance to vancomycin (86.66%), ampicillin (46.66%), nalidixic acid (20%), tetracycline (6.66%), co-trimaxozole (6.66%) and rifampicin (6.66%) and were susceptible to antibiotics like gentamycin, streptomycin, trimethoprim, azithromycin, cefixime and chloramphenicol. 20% of *Aeromonas* sp. showed MAR index $\geq$0.2 indicative of the high risk environment.

Conclusion: The presence of *Aeromonas* sp. has been recognised as a potential health risk and surveillance of this pathogen is crucial for successful disease management and control.

Keywords: 16S rDNA analysis, *Aeromonas* sp., antibiotic susceptibility tests, aquafarm, mangrove, pathogenic, phylogenetic tree, seafood

Introduction

Human demand for seafood has nudged the aquaculture industry to establish as a major source of excellent quality proteins and healthy oils by intensive and semi-intensive culture methods, with production reaching almost 50% of the total fish production including marine and freshwater species for human consumption [1]. Aquaculture farms, associated mangroves and seafood are prospective reservoirs of pathogenic bacteria and these realms play significant roles in ecological and epidemiological studies. Seafood harbours pathogenic microorganisms due to the texture of their flesh and also their microbe laden habitat. Contaminated seafood and their products therefore pose health risks to consumers.

Genus *Aeromonas* comprise non-motile psychrophilic and motile mesophilic Gram negative bacteria and include 15 species, being distributed ubiquitously in aquatic environments and are of increasing importance as seafood and waterborne pathogens [2]. Seven species causing gastroenteritis in adults [3] and in children [4], cellulitis [5] and septicaemia [6] are cause for human concern. They have also been implicated in peritonitis, meningitis and eye infections on rare occassions [7]. In addition, *Aeromonas* species are also etiological agents of fish diseases like furunculosis [8], septicaemia [9] and skin ulcers [10].

A. hydrophila, and *A. caviae* are considered major pathogens most commonly implicated in human intestinal infections [11] and also account for more than 95% of all blood-borne infections [12]. Pathogenicity by *A. enteropelogenes* is attributed to the production of enterotoxins in humans and in fishes [13]. *A. aquariorum* was the first species to be isolated

doi:10.5455/vetworld.2013.300-306

from ornamental fishes and is associated with diarrhoea, bacteremia, wound infections and other extra-intestinal infections [10].

Mesophilic species, namely *A. hydrophila* and *A. veronii,* cause an assortment of diseases in fish, including motile *Aeromonas* septicemia (MAS), fish rot, soft tissue rot and furunculosis resulting in major die-offs and fish kills around the globe [5]. Epizootic ulcerative syndrome (EUS) in fishes due to *A. sobria* caused great damage to fish farms in Bangladesh and India [14]. Most *Aeromonas* species are psychrotrophic and can grow at refrigerator temperatures, replicating at high salt concentrations [2] and preservation techniques seem ineffective in inhibiting the replication of these strains, which can multiply in packed food products and those under refrigeration. *Aeromonas* infections are probably the most common bacterial diseases to infect tropical fishes and prawns, with disease outbreaks being increasingly recognized as a significant constraint on aquaculture production, export and trade, consequently affecting the economic development of the sector in many countries.

Recognition and monitoring of the potential reservoirs of pathogenic bacteria and their drug resistance profile are essential in epidemiological and environmental studies to prevent possible health risks. The present study elaborates the increasing concern on the occurrence of prospective pathogenic species of *Aeromonas* in seafood, aquaculture farms and associated mangroves and the antibiotic resistance in these isolates.

Materials and Methods

Sampling and processing: Squid, prawn and mussel samples from the local fish market, water and sediment samples from aquaculture farms and their associated mangroves along the coastal area of Cochin were collected. All samples were collected in sterile polythene bags and processed within 2 hours of collection.10 gm each of seafood and sediment sample were aseptically inoculated into 90 ml of alkaline peptone water for enrichment, after which 1 ml of enriched culture was plated on Nutrient Broth agar (HiMedia Laboratories, India) plates by the pour plate method at regular intervals of 4 hours. The plates were incubated at 37°C for 18-24 hours and isolated colonies were picked, purified and stored.

Identification of *Aeromonas* sp.: Genomic DNA was isolated [15]. Briefly cells from a 24 hour LB culture were harvested by centrifugation (6000 r.p.m. for 10 min), resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), treated with 10% (w/v) SDS and proteinase K (20mg/ml)(Sigma) and incubated at 37 °C for 1 hour. After incubation, 10% cetyl trimethyl ammonium bromide (CTAB) in 0.5 M NaCl was added and the mixture incubated at 65°C for 10 min. The aqueous phase was treated with double volume of phenol: chloroform: isoamyl alcohol (25:24:1) and the DNA pellet was washed with absolute ethanol and suspended in TE. The concentration was estimated spectrophotometrically and appropriate dilutions (50-100ng) were used as template for PCR reactions.

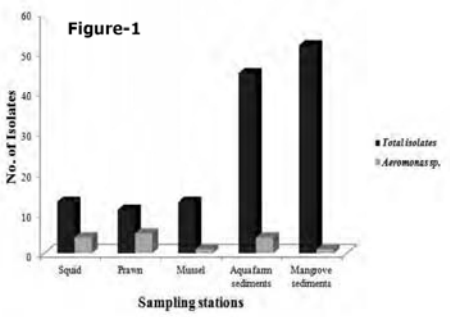
16S rDNA sequence analysis: Identification of the *Aeromonas* strains was done by analysis of partial 16S rDNA sequences. A portion of the 16S rRNA gene (1.5kb) was amplified from the genomic DNA [16-19] using a set of primers (F 5' AGTTTGATCCTGGCTCA 3' and R 5' ACGGCTACCTTGTTACGACTT3') targeting the 16S rRNA gene. Products after PCR amplification were purified by gene clean kit (Bangalore Genei). The products were sequenced by Sanger's Dideoxy method using ABI 3730 Excel and the identity of the sequences was determined using BLAST [20].

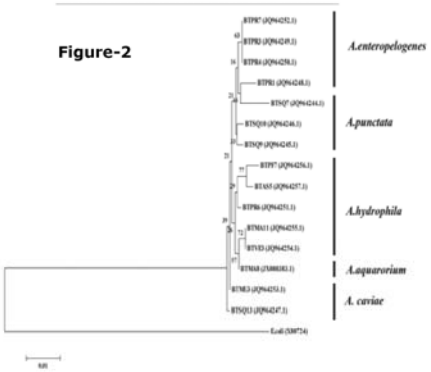
The phylogenetic tree was constructed using the Neighbor-Joining method [21]. The evolutionary distances were computed using the Tajima-Nei method [22] and evolutionary analyses were conducted in MEGA5: Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 [23]. Statistical support for branching was estimated using 1000 bootstrap steps.

Morphological and biochemical characterization : All strains were tested for phenotypic traits like Gram staining, oxidase test, catalase test, Voges Proskauer's test, arginine utilisation, 3% salt tolerance test, ONPG, citrate utilisation, ornithine utilisation, and utilisation of carbohydrates like mannitol, arabinose, sucrose, glucose, salicin and cellobiose [24]. Hemolytic screening on sheep blood agar plates was used to test the pathogenicity of the isolates [25]. Isolates were also checked for growth on thiosulphate-citrate-bile-salt sucrose (TCBS) (HiMedia Laboratories, India) agar plates.

Antibiotic susceptibility tests: Pure cultures of the isolates were grown in Luria Bertani broth and resistance profiles were determined by the Kirby-Bauer disc diffusion method [26]. A single colony of the test strain was transferred into 3 ml normal saline and turbidity adjusted to 0.5 McFarland's standard. The bacterial suspension was swab inoculated onto Mueller- Hinton agar (HiMedia Laboratories, India) plates. Antibiotic discs (HiMedia Laboratories, India) used were Gentamycin (10µg), Streptomycin (10 µg), Ampicillin (10 µg), Nalidixic acid (30 µg), Vancomycin (30 µg) Co-trimoxazole (23.75 µg), Trimethoprim (5 µg), Cefixime (5 µg), Azithromycin (15 µg), Rifampicin (5 µg), Tetracycline (30 µg) and Chloramphenicol (30 µg). The plates were incubated at 37°C for 24 hours and the result was interpreted as resistant, intermediate or sensitive based on the size of the inhibition zone as provided by the manufacturer. The Multiple Antibiotic Resistance (MAR) Index of an isolate was calculated using the formula a/b, where 'a' is the number of antibiotics to which the isolate was resistant and 'b', the number of antibiotics to which the isolate was exposed [27].

doi:10.5455/vetworld.2013.300-306





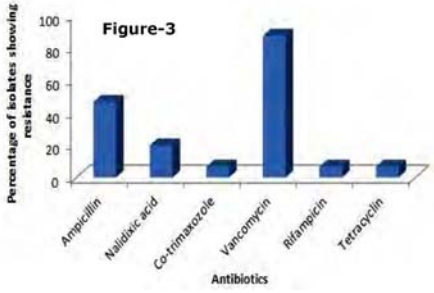


Figure-1. The distribution of the *Aeromonas* species from different sampling stations

Figure-2. Phylogenetic relationship based on partial 16S rDNA sequences of *Aeromonas* strains isolated from tropical seafoods, aquafarms and associated mangroves. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The analysis involved 15 nucleotide sequences and *E.coli* (accession number X80724) as an outgroup. Accession numbers are given in parentheses.

Figure-3. Percentage of isolates showing resistance to tested antibiotics

Results

Identification of *Aeromonas* sp.: The total number of isolates obtained from the various samples was 134. 16S rDNA sequencing identified 15 (11.2%) of the 134 isolates, as *Aeromonas* sp. Their identity was confirmed by comparing the sequences with GenBank entries by BLAST [20] and the sequences were deposited in the GenBank and accession numbers obtained. The 15 isolates of *Aeromonas* sp. were assigned to five species, viz., *A.punctata, A.caviae, A.enteropelogenes, A.hydrophila* and *A.aquarorium.*

The distribution of the *Aeromonas* species in the studied samples is given in Figure 1. The highest incidence of *Aeromonas* strains was in prawns followed by squid and aquafarm sediments. Only one strain each from mussel and mangrove sediment were confirmed as *Aeromonas* sp. *A. punctata* and *A. caviae* were identified from squid, *A. enteropelogenes* and *A. hydrophila* from prawn, *A. caviae* from mussel, *A. hydrophila* from mangroves and *A. hydrophila* and *A. aquarorium* from aquafarms. Among the isolates, *A. hydrophila* was the predominant species followed by *A. enteropelogenes.*

16S rDNA sequence analysis: Phylogenetic relationship based on partial 16S rDNA sequences of the 15 isolates of *Aeromonas* is represented in Fig. 2. All the strains claded together separated from its outgroup in the phylogram. Within the major clades, alignment was such that the sequences of the same species aligned together, exhibiting the relatedness between these strains. The strains BTPR7, BTPR3 and BTPR4 representing *A.enteropelogenes* grouped to form a clade. Two strains of *A. punctata* from squid, BTSQ9 and BTSQ10 grouped together whereas BTSQ7 aligned separately with *A. enteropelogenes* strain BTPR1 from prawn. The *A. hydrophila* strains formed a distinct clade despite the observed sequence diversity at the strain level. *A.aquarorium* strain BTMA8 clustered separately from the rest of the isolates and is placed nearer to *A. hydrophila* strains BTVE3 and BTMA11. The two strains of *A. caviae*, BTMU3 and BTSQ13, aligned together to form a distinct group.

Morphological and Biochemical Characterization: The biochemical characteristics of the 15 aeromonads are given in Table-1. All the isolates were Gram negative, catalase and oxidase positive, Voges-Proskauer negative, able to tolerate 3% NaCl in the medium, ONPG positive, ornithine negative and fermented sucrose and glucose irrespective of the species. They exhibited variations with regard to the other properties tested like utilisation of arginine and citrate and carbohydrates like mannitol, salicin and cellobiose. With regard to the utilisation of arginine by the isolates, variations were observed between strains of the same species. All strains of *A. enteropelogenes* were able to utilise arginine except strain BTPR7 from prawn. Both strains of *A. caviae*, three strains of *A. hydrophila* and two strains of *A. punctata* tested negative for arginine utilisation whereas *A. aquarorium* was positive. Five

doi:10.5455/vetworld.2013.300-306

Table-1. Phenotypic characteristics of *Aeromonas sp.* from seafoods, aquafarms and associated mangroves.

Strains	TCBS	GS	C	O	VP	Arginine	NaCl 3%	ONPG	Citrate	Ornithine	Mannitol	Arabinose	Sucrose	Glucose	Salicin	Cellobiose	Hemolysis
A.enteropelogenes																	
BTPR1	G	-	+	+	-	+	+	+	+	-	+	-	+	+	+	+	β
BTPR3	G	-	+	+	-	+	+	+	+	-	+	-	+	+	+	+	β
BTPR4	Y	-	+	+	-	+	+	+	+	-	+	-	+	+	-	+	β
BTPR7	Y	-	+	+	-	-	+	+	+	-	+	-	+	+	-	+	β
A. caviae																	
BTSQ13	Y	-	+	+	-	-	+	+	+	-	+	+	+	+	+	+	α
BTMU3	Y	-	+	+	-	-	+	+	+	-	+	+	+	+	+	+	α
A. hydrophila																	
BTPR6	G	-	+	+	-	+	+	+	+	-	-	-	+	+	-	+	β
BTAS5	Y	-	+	+	-	-	+	+	+	-	+	-	+	+	-	+	β
BTVE3	Y	-	+	+	-	-	+	+	+	-	+	-	+	+	-	+	β
BTMA11	Y	-	+	+	-	-	+	+	+	-	+	-	+	+	-	+	β
BTPF7	Y	-	+	+	-	+	+	+	-	-	+	-	+	+	+	-	β
A. aquarorium																	
BTMA8	Y	-	+	+	-	+	+	+	-	-	-	-	+	+	-	-	β
BTSQ7	Y	-	+	+	-	-	+	+	+	-	+	-	+	+	-	+	α
BTSQ9	G	-	+	+	-	+	+	+	+	-	+	-	+	+	-	+	α
BTSQ10	Y	-	+	+	-	-	+	+	+	-	+	-	+	+	-	+	α

TCBS-Thiosulphate Citrate Bile Salt Agar;G- Green; Y- Yellow; GS- Gram staining; C-Catalase; O-Oxidase; + = Positive; - = negative

Table-2. Antibiotic profile of *Aeromonas sp.* from seafoods, aquafarms and associated mangroves.

Isolate code	Species identified	G	S	A	N	V	Co	T	Cef	Azi	R	Tetra	Chlora
BTSQ 7	*A.punctata*	S	S	S	S	S	S	S	S	S	S	S	S
BTSQ9	*A.punctata*	S	S	S	R	R	S	S	S	S	S	S	S
BTSQ 10	*A.punctata*	S	S	S	R	R	S	S	S	S	S	S	S
BTSQ 13	*A.caviae*	S	S	R	S	R	S	S	S	S	S	S	S
BTPR 1	*A.enteropelogenes*	S	S	S	S	R	S	S	S	S	S	S	S
BTPR 3	*A.enteropelogenes*	S	S	S	S	R	S	S	S	S	S	S	S
BTPR 4	*A.enteropelogenes*	S	S	S	S	R	S	S	S	S	S	S	S
BTPR 6	*A. hydrophila*	S	S	S	S	R	S	S	S	S	S	S	S
BTPR 7	*A.enteropelogenes*	S	S	S	S	R	S	S	S	S	S	S	S
BTMU 3	*A. caviae*	S	S	R	R	R	S	S	S	S	R	S	S
BTVE 3	*A. hydrophila*	S	S	R	S	R	S	S	S	S	S	S	S
BTMA 8	*A. aquarorium*	S	S	R	S	R	R	S	S	S	S	S	S
BTMA 11	*A. hydrophila*	S	S	R	S	R	S	S	S	S	S	S	S
BTPF 7	*A. hydrophila*	S	S	R	S	R	S	S	S	S	S	R	S
BTAS 5	*A. hydrophila*	S	S	R	S	S	S	S	S	S	S	S	S

G-Gentamycin, S-Streptomycin, A-Ampicillin, N-Nalidixic Acid, V-Vancomycin, Co-Co-Trimoxazole, T-Trimethoprim, Cef-Cefixime, Azi-Azithromycin, R-Rifampicin, Tetra-Tetracycline, Chlora-Chloramphenicol, S = Sensitive; R = Resistant

strains were able to utilise salicin for their growth. All the isolates were haemolytic on blood agar indicating their pathogenic nature. *A. enteropelogenes*, *A. hydrophila* and *A. aquarorium* exhibited β-hemolysis in the form of a clearance zone in the blood agar plate whereas *A. caviae* and *A. punctata* developed green colouration showing α haemolytic activity. All the isolates were able to grow on TCBS agar plates producing yellow or green coloured colonies.

Antibiotic susceptibility tests: The antibiogram of the *Aeromonas* isolates revealed the diverse pattern of resistance to vancomycin (86.66%), ampicillin (46.66%), nalidixic acid (20%), tetracycline (6.66%), co-trimaxozole (6.66%) and rifampicin (6.66%). All the isolates were susceptible to gentamycin, streptomycin, trimethoprim, azithromycin, cefixime and chloramphenicol. The percentage of isolates exhibiting resistance against the tested antibiotics is presented in Fig 3.

The *A. hydrophila* strains from prawn, aquafarms and mangroves exhibited variations in their antibiotic profile with BTPR6 from prawn being resistant to vancomycin and BTVE3 from mangroves being resistant to ampicillin and vancomycin. Among the three isolates from aquafarms, BTMA11 and BTAS5 were resistant to ampicillin and BTPF7 exhibited multiple antibiotic resistance to ampicillin, vancomycin and tetracycline. The *A. enteropelogenes* strains (BTPR1, BTPR3, BTPR4 and BTPR7) from prawn were sensitive to all the classes of antibiotics tested except vancomycin. *A.punctata* strains BTSQ9 and BTSQ10 from squid, were resistant to nalidixic acid and vancomycin, while BTSQ7 was sensitive to all tested antibiotics. Among the *A.caviae* strains, BTSQ13 from squid was resistant to ampicillin and vancomycin whereas BTMU3, the only isolate, from mussel exhibited multiple drug resistance to ampicillin, nalidixic acid, vancomycin and rifampicin. *A. aquarorium* (BTMA8) from aquafarm was resistant to ampicillin,vancomycin and co-trimaxozole.

The antibiogram of the 15 isolates of *Aeromonas* is represented in Table-2. Based on the results of antibiotic profiling, the multiple antibiotic resistance (MAR) index was calculated. 20% of the *Aeromonas* sp. showed MAR Index $\geq$0.2 (data not shown) and this higher MAR index is indicative of the high risk environment.

Discussion

Aeromonas is a genus of growing interest due to its pathogenicity to aquatic organisms, its potential pathogenic effects in humans [2,6] in fishes [28] and its spoilage action in food [29].

Reports are available on the occurrence of

doi:10.5455/vetworld.2013.300-306

Aeromonas species in squid [30], prawn [31] and mussel [32] and their potential as health hazards when associated with aquaculture settings and seafood [12,33] have been well documented. In South India, the prevalence of *Aeromonas* in drinking water [34], finfish and prawn [35] and ornamental fishes [36,37] emphasise the need for the surveillance of this pathogen in the environmental reservoirs.

The present study analysed three tropical seafoods (squid, prawn and mussel), aquaculture and mangrove environments to reveal varying degrees of *Aeromonas* contamination and incidence.

Molecular identification by 16S rDNA sequence analysis is a valuable tool in identification of *Aeromonas* species [38] and has been adopted in this study. The isolates were assigned to five species, namely, *A. hydrophila* (33.3%), *A. enteropelogenes* (26.6%), *A. punctata* (20%), *A. caviae* (13.3%) and *A. aquarorium* (6.6%). The dominance of *A. hydrophila* in seafood samples over other species is in accordance with earlier studies [39,40], where it was the predominant species in the fish samples analysed. Though considered a common inhabitant of healthy fish [41] and aquatic system [42], *A. hydrophila* is also an established opportunistic pathogen infecting fish and man under physiological and environmental stress [40]. *A. enteropelogenes* was isolated from prawn in this study and has been reported earlier from prawn cultured in concrete tanks in Saudi Arabia [43]; also an opportunistic pathogen with the same pathogenicity factors and enterotoxin production as featured by *A. hydrophila* [4]. Squid isolates were *A. punctata*, a species associated with human gastroenteritis and fish septicaemia [44].

A. caviae, associated with gastroenteritis in humans [11] and ulcers in fish [45], predominant in faecal polluted water [46], fresh water fishes [40] and ornamental fishes [47], was obtained from squid and mussel in this study. *A. aquarorium*, of epidemiological significance as reported in clinical specimens from Malaysia [48], was isolated from aquafarm sediment in this study. *A. aquarorium* was reported to be pathogenic to man and fishes causing bacteremia and wound infections [10]. The production of hemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads and the property of hemolysis on blood agar is directly related to enteropathogenicity [2]. β hemolysin has been characterised in *A. hydrophila*, *A. caviae and A. sobria* [31] and α hemolysin in *A. hydrophila* [11] indicating their pathogencity. The high frequency of hemolytic activity among *Aeromonas* strains isolated from the various samples is indicative of the pathogenic potential of these strains and further characterisation of virulence factors are to be done to establish their degree of pathogenicity.

The phylogenetic and evolutionary relationship among the 15 species of *Aeromonas* elucidated using the partial 16S rDNA sequences and species level identification was possible without ambiguity. However, microheterogeneity of the 16S rRNA gene can interfere with the proper identification of species [38] and additional molecular methods can be employed for unambiguous identification and description of new species [49]. The intraspecies diversity based on partial 16S rDNA sequence analysis was observed in the variations in the biochemical reactions and antibiotic susceptibility of these strains.

Aeromonas species demonstrate marked differences in their susceptibilities to antibiotics and are generally considered resistant to ampicillin [50], tetracycline and vancomycin [51]; sensitive to cephalosporins [52] and nalidixic acid [2]. *A. enteropelogenes* has sensitivity to β lactams [13] and our results substantiate this. *A. punctata* strains in this study were also ampicillin sensitive, exhibiting 100% sensitivity to gentamycin, streptomycin, co-trimoxazole, trimethoprim, cefixime, azithromycin and chloramphenicol, the results being nearly in accordance with prior studies [51,53]. Resistance to rifampicin and tetracycline exhibited by two strains should receive ample attention because these are antibiotics commonly used in aqua culture settings in developing countries and has a higher risk potential due to lateral gene transfer [50]. Analysis of the antibiotic profile of a species is significant as it aids in the design of culture media for selective isolation of species.

According to Matyar et. al., [54], MAR index values >0.2 indicates existence of isolates in high risk contaminated source with higher frequency of the use of antibiotics while values <0.2 is indicative of the lesser application of antibiotics. The higher MAR index exhibited by 20% of the isolates indicates the need for the judicious use of antibiotics as therapeutics and as prophylactic agents.

There has been a substantial increase in seafood consumption in the developed world on account of the better taste, nutritional quality and medicinal properties associated with seafood and its products. Kerala, being one of the premier fish producing states in India, holds a major position in Indian seafood exports. As the major quality criteria for seafood export is the biological status of the resource material, presence of pathogens and concentration of aquaculture drugs like antibiotic residues, hormone and pesticide residues, can pose health risks to consumers of fish and fishery products. Food safety regulations have been imposed in the industrialized markets and non-adherence to these as well as the stringency of food safety regulations cause huge losses. Furthermore, outbreaks of bacterial infections in aqua farms also lead to substantial economic loss to farmers.

The prevalence of *Aeromonas* species in the aquatic environment has been recognized as a potential health risk and understanding the natural reservoirs of *Aeromonas* will help in developing methods to monitor and control these bacteria in seafood, aquaculture settings and associated mangroves.

doi:10.5455/vetworld.2013.300-306

Conclusion

The study revealed that seafood, aquafarms and associated mangroves of Cochin, Kerala, harbour potential pathogenic *Aeromonas* species and serve as environmental reservoirs to support the survival, persistence and dissemination of these pathogens. Since Kerala holds a significant position in the fisheries sector, aquaculture industries offer immense scope not only on augmenting the aquatic production but also contributing to the export earnings. There is also a growing demand for ornamental fishes both in the domestic and international markets. Due to the pathogenic nature of *Aeromonas spp.*, monitoring the levels and species distribution of *Aeromonas* in seafoods and aqua culture environments is requisite to prevent risk to human and aquatic animal health. Ensuring appropriate quality control practices and coordination of the surveillance of antibiotic use and multidrug resistance in aquaculture industries are crucial to reducing overall disease incidence and resistance by these pathogens.

Authors' contribution

All authors contributed equally. All authors read and approved the final manuscript.

Acknowledgements

The first author gratefully acknowledges the support from the University Grants Commission, India for sanction of teacher fellowship under the Faculty Development programme.

Competing interests

Authors declare that they have no competing interests.

References

1. Khan, M. A., Khan, S., and Miyan, K. (2011) Aquaculture as Food Production System: A Review. *Biol Med* 3: 291-302.
2. Janda, J. M., and Abbott, S. L. (2010) The genus *Aeromonas*: Taxonomy, Pathogenicity and Infection. *Clin Microbiol Rev.* 23: 35-73.
3. Sinha, S., Shimada, T., Ramamurthy, T., Bhattacharya, S. K., Yamasaki, S., Takeda, Y., and Balakrish Nair, G. (2004) Prevalence, Serotype Distribution, Antibiotic Susceptibility and Genetic Profiles of Mesophilic *Aeromonas* species Isolated from Hospitalized Diarrhoeal Cases in Kolkata, India. *J. Med. Microbiol.* 53: 527–534.
4. Albert, M. J., Ansaruzzaman, M., Talukder, K. A., Chopra, A. K., Kuhn, I., Rahman, M., Faruque, A. S., Islam, M. S., Sack, R. B., and Mollby, R. (2000) Prevalence of Enterotoxin Genes in *Aeromonas* spp. Isolated from Children with Diarrhoea, Healthy Controls, and the Environment. *J. Clin. Microbiol.* 38: 3785-3790.
5. Joseph, S. W., and Carnahan, A. M. (2000) Update on the Genus *Aeromonas*. *ASM News* 66: 218–223.
6. Dwivedi, M., Mishra, A., Prasad, A., Azim, A., Singh, R. K., Baronia, A. K., Prasad, K. N., and Dwivedi, U. N. (2008) *Aeromonas caviae* Septicemia in Immunocompetent Gastrointestinal Carriers. *Braz. J. Infect. Dis.* 12: 547-548.
7. Kelly, K. A., Koehler, J. M., and Ashdown, L. R. (1993) Spectrum of Extraintestinal Disease due to *Aeromonas* Species in Tropical Queensland, Australia. *Clin. Infect. Dis.* 16:574-579.
8. Reith, M. E., Singh, R. K., Curtis, B., Boyd, J. M., Bouevitch, A., Kimball, J., Munholland, J., Murphy, C., Sarty, D., Williams, J., Nash, J. H. E., Johnson, S. C., and Brown, L. L. (2008) The Genome of *Aeromonas salmonicida* subsp. *salmonicida* A449: Insights in to the Evolution of a Fish Pathogen. *BMC Genomics* 9: 427.
9. Singh, D. V., and Sanyal, S. C. (1999) Virulence Patterns of *Aeromonas eucrenophila* Isolated from Water and Infected Fish. *J. Diarrhoeal Dis. Res* 17: 37-42.
10. Figueras, M. J., Alperi, A., Saavedra, M. J., Ko, W., Gonzalo, N., Navarro, M., and Martínez-Murcia, A. J. (2009) Clinical Relevance of the Recently Described Species *Aeromonas aquariorum*. *J. Clin. Microbiol.* 47: 3742–3746.
11. Von Gravenitz, A. (2007) The Role of *Aeromonas* in Diarrhea: a Review. *Infection* 35: 59-64.
12. Ghenghesh, K. S., Ahmed, S. F., El-Khalek, R. A., Al-Gendy, A., and Klena, J. (2008) *Aeromonas*-Associated Infections in Developing Countries. *J. Infect. Dev. Countr.* 2: 81-98.
13. Luca, F. D., Giraud-Morin, C., Rossolini, G. M., Docquier, J.-D., and Fosse, T. (2010) Genetic and Biochemical Characterization of TRU-1, the Endogenous Class C β-Lactamase from *Aeromonas enteropelogenes*. *Antimicrob. Agents Ch.* 54: 1547–1554.
14. Rahman, M., Huys, G., Rahman, M., Albert, M. J., Kühn, I., and Möllby, R. (2007) Persistence, Transmission, And Virulence Characteristics of *Aeromonas* strains in a Duckweed Aquaculture-Based Hospital Sewage Water Recycling Plant in Bangladesh. *Appl. Envir. Microbiol.* 73: 1444–1451.
15. Murray, M. G., and Thompson, W. F. (1980) Rapid Isolation of High Molecular Weight Plant DNA. *Nucl. Acids Res.* 19:4321-4326.
16. Shivaji, S., Vijaya Bhanu, N., and Aggarwal, R. K. (2000) Identification of *Yersinia pestis* as the Causative Organism of Plague in India as Determined by 16S rDNA Sequencing and RAPD Based Genomic Fingerprinting. *FEMS Microbiol. Lett.* 189: 247-252.
17. Reddy, G. S. N., Aggarwal, R. K., Matsumoto, G. T., and Shivaji, S. (2000) *Arthrobacter flavus* sp. nov., a Psychrophilic Bacterium Isolated From a Pond in McMurdo Dry Valley. Antarctica. *Int. J. Syst. Evol. Microbiol.* 50:1553-1561.
18. Reddy, G. S. N., Prakash, J. S. S., Vairamani, M., Prabhakar, S., Matsumoto, G. I., and Shivaji, S. (2002a) *Planococcus antarcticus* and *Planococcus psychrophilus* sp. nov., Isolated From Cyanobacterial Mat Samples Collected from Ponds in Antarctica. *Extremophiles* 6:253-261.
19. Reddy, G. S. N., Prakash, l. S., Matsumoto, G., Stackebrandt, L., and Shivaji, S. (2002b) *Arthrobacter roseus* sp. nov., a Psychrotolerant Bacterium Isolated from an Antarctic Cyanobacterial Mat Sample. *Int. J. Syst. Evol. Microbiol.* 52: 1017-1021.
20. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1980) Basic Local Alignment Search Tool. *J. Mol. Biol.* 215: 403-410.
21. Saitou N, and Nei, M. (1987) The Neighbor-Joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evol.* 4: 406-425.
22. Tajima, F., and Nei, M. (1984) Estimation of Evolutionary Distance Between Nucleotide Sequences. *Mol. Biol. Evol.* 1:269-285.
23. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.* 28:2731–2739.
24. Abbott, S. L., Cheung, W. K. W., and Janda, J. M. (2003) The Genus Aeromonas: Biochemical Characteristics, Atypical Reactions, and Phenotypic Identification Schemes. *J. Clin. Microbiol.* 41: 2348–2357.
25. Russell, F.M., Biribo, S.S.N., Selvaraj, G., Oppedisano, F., Warren, S., Seduadua, A., Mulholland, E.K. and Carapetis, J.R. (2006) As a Bacterial Culture Medium, Citrated Sheep Blood Agar Is a Practical Alternative to Citrated Human Blood Agar in Laboratories of Developing Countries. *J. Clin.*

doi:10.5455/vetworld.2013.300-306

Microbiol. 44(9) 3346–3351.
26. Bauer, A. W., Kirby, W. M., Sherris, J. C., and Turck, M. (1966): Antibiotic Susceptibility Testing by a Standardized Single Disk Method. *Am. J. Clin. Path.* 45: 493-496.
27. Krumperman, P. H. (1985) Multiple Antibiotic Indexing of *E. coli* to Identify High-Risk Sources of Fecal Contamination of Foods. *Appl. Envir. Microbiol.* 46: 165–170.
28. Thampuran, N., Surendran, P. K., Mukundan, M. K., and Gopakumar, K. (1995) Bacteriological Studies on Fish Affected by Epizootic Ulcerative Syndrome (EVS) in Kerala, India. *Asian Fish. Sci.* 8: 103-111.
29. Bezirtzoglou, E., Maipa, V., Voidarou, C., Tsiotsias, A., and Papapetropoulou, M. (2000) Food Borne Intestinal Bacterial Pathogens. *Microb. Ecol. Health Dis.* 2: 96-104.
30. Baldrias, L. R., and Alvero, M. A. C. (1999) *Aeromonas* Species from Raw Squid Balls: A Preliminary Study of a Potential Food-Borne Pathogen and Health Hazard *J. Environ. Sci. Manag.* 2: 48-52.
31. Thayumanavan, T., Vivekhanandhan, G., Savithakumari, K., Subashkumar, R., and Lakshmanaperumalsamy, P. (2003) Incidence of Haemolysin Positive and Drug Resistant *Aeromonas hydrophila* in Freshly Caught Finfish and Prawn Collected from Major Commercial Fishes of Coastal South India. *FEMS Imm. Med. Microbiol.* 36:41-45.
32. Ottaviani, D., Santarelli, S., Bacchiocchi, S., Masini, L., Ghittino, C., and Bacchiocchi, I. (2006) Occurrence and Characterization of *Aeromonas* spp. in Mussels from the Adriatic Sea. *Food Microbiol.* 23: 418-422.
33. Austin, B., and Austin, D. A. (1993) *Bacterial Fish Pathogens.* Ellis Horwood, Chichester, United Kingdom.
34. Mascher, F., Reinthaler, F., Schuhmann, G., Enayat, U., Sixl, W., and Klambauer, B. (1989) Microbiological and Chemical Analysis of Drinking Water in Southern India (with special consideration of *Aeromonas* species). *Geogr Med. Suppl.* 3: 135-140.
35. Vivekanandhan, G., Hatha, A. A. M., and Lakshmanaperumalsamy, C. P. (2005) Prevalence of *Aeromonas hydrophila* in Fish and Prawns from the Seafood Market of Coimbatore, South India. *Food Microbiol.* 22: 133-137.
36. Sreedharan, K., Philip, R., and Singh, I. S. B. (2012) Virulence Potential and Antibiotic Susceptibility Pattern of Motile Aeromonads Associated with Freshwater Ornamental Fish Culture Systems: A Possible Threat To Public Health. *Braz. J. Microbiol.* 43: 754-765.
37. Mohan, L., and Unni , A. C. B. (2012) Pathogenicity of *Aeromonas hydrophila* in Goldfish (*Carassius auratus auratus*) and Development of Vaccine for Control of Infections. *Adv. Bio Tech* 12: 29-31.
38. Janda, J. M., and Abbott, S. L. (2007) 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *J. Clin. Microbiol.* 45: 2761-2764.
39. Hatha, A. A. M., Vivekanandhan, A. A., Joice, G. J., and Christol (2005) Antibiotic Resistance Pattern of Motile Aeromonads From Farm Raised Freshwater Fish. *Int. J. Food Microbiol.* 98: 131-134.
40. Yoganath, N., Bhakyaraj, R., Chanthuru, A., Anbalagan, T., and Mullai Nila, K. (2009) Detection of Virulence Gene in *Aeromonas hydrophila* Isolated from Fish Samples Using PCR Technique. *Gl. J. Biotech. Biochem.* 4: 51-53.
41. Wong, S. Y., and Leong, T. S. (1987) *Current fish disease problems in Malaysia In. Fish quarantine and fish diseases in South and Southeast Asia.* Asian Fisheries Society, Manila.
42. Kaper, J. B., Lockman, H., Colwell, R. R., and Joseph, S. W. (1981) *Aeromonas hydrophila*: Ecology and Toxigenicity of Isolates from an Estuary. *J. Appl. Bacteriol.* 50:359–377.
43. Al-Harbi, A. H. (2003) Bacterial Flora of Freshwater Prawn, *Macrobrachium rosenbergii* (de Man), Cultured in Concrete Tanks in Saudi Arabia. *J. Appl. Aquaculture* 14: 113-124.
44. Gilardi, G. L. (1967) Morphological and Biochemical Characteristics of *Aeromonas punctata (hydrophila, liquefaciens)* Isolated From Human Sources. *Appl. Microbiol.* 15:417-421.
45. Singh, D. V., and Sanyal, S. C. (1992) Haemolysin and Enterotoxin Production by *Aeromonas caviae* Isolated from Diarrhoeal Patients, Fish and Environment. *J. Diarrhoeal Dis. Res.* 10:16–20.
46. Stecchini, M. L., and Domenis, C. (1994) Incidence of *Aeromonas* species in Influent and Effluent of Urban Waste Water Purification Plants. *Lett. Appl. Microbiol.* 19: 237–239.
47. John, N., and Hatha, A. A. M. (2012) Prevalence, Distribution and Drug Resistance of Motile Aeromonads in Freshwater Ornamental Fishes. *Indian J. Fish.* 59: 161-164.
48. Puthucheary, S. D., Puah, S. M., and Chuaz, K. H. (2012) Molecular Characterization of Clinical Isolates of *Aeromonas* Species from Malaysia. *PLoS one.* 7 e30205.
49. Alperi, A., Figueras, M. J., Inza, I., and Martínez-Murcia, A. J. (2008) Analysis of 16S rRNA Gene Mutations in a Subset of *Aeromonas* Strains and their Impact in Species Delineation. *Int. Microbiol.* 11: 185-194.
50. Igbinosa, I. H., and Okoh, A. I. (2012) Antibiotic Susceptibility Profile of *Aeromonas* Species Isolated From Wastewater Treatment Plant. *Scientific World Journal* Article ID 764563, 6 pages.
51. Awan, M. B., Ahmed, M., Abdul, B., and Karel, K. (2009) Antibiotic Susceptibility Profile of *Aeromonas* spp. Isolates from Food in Abu Dhabi, United Arab Emirates. *New Microbiologica* 32: 17-23.
52. Emekdas, G., Aslan, G., Tezcan, S., Serin, M. S., Yildiz, C., Ozturhan, H., and Durmaz, R. (2006) Detection of the Frequency, Antimicrobial Susceptibility and Genotypic Discrimination of *Aeromonas* Strains Isolated From Municipally Treated Tap Water Samples by Cultivation and AP-PCR in Turkey. *Int. J. Food. Microbiol.* 107:310-314.
53. Kaskhedikar, M., and Chhabra, D. (2010) Multiple Drug Resistance in *Aeromonas hydrophila* Isolates of Fish. *Vet. World* 3: 76-77
54. Matyar, F., Kaya, A., and Dincer, S. (2007) Distribution and Antibacterial Drug Resistance of *Aeromonas* spp. from Fresh and Brackish Waters in Southern Turkey. *Ann. Microbiol.* 57: 443–447.

****