Differential induction, isolation, physicochemical and molecular characterization of temperate phages of environmental *Vibrio cholerae* as evidence of phage mediated Horizontal Gene Transfer

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Certificate

This is to certify that the thesis entitled **"Differential induction, isolation, physicochemical and molecular characterization of temperate phages of environmental *Vibrio cholerae* as evidence of phage mediated Horizontal Gene Transfer"** is a record of the bonafide research work done by Mrs. Linda Louis under my supervision and guidance, in partial fulfilment of the requirement for the degree of Doctor of Philosophy, under the Faculty of Sciences of Cochin University of Science and Technology.

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I hereby declare that the thesis entitled **"Differential induction, isolation, physicochemical and molecular characterization of temperate phages of environmental *Vibrio cholerae* as evidence of phage mediated Horizontal Gene Transfer"** 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, 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.

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22/12/ 2014

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'My lord my god'

Your grace and spirit helped me to complete this for your glory

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Abbreviations

%	Percentage
°C	Degree Celsius
Ace	Accessory cholera enterotoxin
Acf	Accessory colonization factor
AFLP	Amplified Fragment Length Polymorphism
APS	Ammonium persulfate
APW	Alkaline Peptone Water
BLAST	Basic Local Alignment Search Tool
bp	base pair
Ca	Calcium
cAMP	cyclic Adenosine Monophosphate
CDC	Centre for Disease Control
CFU	Colony Forming Units
cm	Centimetre
CPS	Capsular Polysaccharide
CT	Cholera Toxin
CTAB	Cetyltrimethylammonium Bromide
Da	Dalton
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic Acid
ERIC	Enterobacterial Repetitive Intergenic Consensus Sequences
EtBr	Ethidium Bromide
Fe	Iron
Fig.	Figure
g	Gram
G+C	guanine + cytosine
HlyA	Haemolysin A
CDC	Centre for Disease Control
kb	Kilobase
kDa	Kilo Dalton
LB	Luria Bertani
Log	Logarithm
LPS	Lipopolysaccharide

M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
mg	milligram
min.	minute
mL	millilitre
mM	Millimolar
MLSA	Multilocus Sequence Analysis
MOF	Marine Oxidation Fermentation
MOI	Multiplicity of Infection
Mg	Magnesium
NICED	National Institute for Cholera and Enteric Diseases
NA	Nutrient agar
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	nanogram
O. D.	Optical density
OD_{600}	Absorbance at 600nm
Omp	Outer membrane proteins
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PFGE	Pulsed Field Gel Electrophoresis
PFU	plaque forming units
pH	Power of Hydrogen
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
SDS	Sodium dodecyl sulphate
sp.	species
TAE	Tris-Acetate-EDTA
TCBS	Thiosulphate-Citrate-Bile Salts-Sucrose

TCP	Toxin co-regulated Pilus
TE	Tris-EDTA
TEM	Transmission Electron Microscopy
TEMED	N-N-N’-N’-Tetramethyl ethylene diamine
TTC	2,3,5-triphenyltetrazolium chloride
UPGMA	Unweighted pair group method with arithmetic average
UV	Ultraviolet
V	Volts
VBNC	viable but non-culturable
VPI	Vibrio Pathogenecity Island
v/v	Volume/volume
viz.	Namely
w/v	Weight/volume
WHO	World Health Organization
Zot	Zonula occcludens toxin
µL	microliter
µM	micromolar
Φ	Phage

With the grace of God Almighty.....

Chapter 1
INTRODUCTION

Viruses contribute significantly to the marine microbial loop and nutrient cycling in the oceans, besides serving as agents of gene transfer in the marine environment (Fuhrman 1999). Viruses (most of them probably phages) outnumber bacteria in the open ocean (Brüssow and Hendrix, 2002). In view of the large volume of the world's oceans and the high titre of phage particles of 10^7/mL of seawater, phage particles are the most abundant biological entities on earth (Jiang and Paul, 1998).

Phages are mainly investigated as the simplest model systems in molecular biology (Bushman, 2001). Three recent trends have renewed the interest in phage research: phages influence the cycling of organic matter in the oceans, they are potential tools for the treatment of antibiotic-resistant bacterial pathogens and they have a major impact on bacterial genome evolution (Carlos *et al*., 2003).

Bacteriophages, whose hosts are bacterial cells, act as agents of 'mobile DNA'. A temperate phage infection on bacteria either results in multiplication of the phage with concomitant lysis of the bacterial host or lysogenization, (*i.e.* integration of the phage DNA into the bacterial chromosome as a prophage). Lysogeny is more the rule than the exception and many bacteria contain multiple prophages. Some temperate phages change the phenotype of the bacterial host ('lysogenic conversion genes', LCG). Lysogeny is a motor of short term bacterial evolution (de la Cruz and Davies, 2000). The production of temperate phages is dependent on the number of lysogenic bacteria and the presence of an inducing agent, whereas virulent phage production depends on the encounter rate between phages and host cells (Weinbauer and Suttle, 1996).

Phage ecology and phage-mediated DNA transfer became a focus in marine microbiology (Wommack and Colwell, 2000). Phage mediated gene transfer takes place at the incredible rate of about 20 million billion times per second in the oceans, if the transduction frequency is 10^{-8} per plaque forming unit (Bushman, 2001). Pathogenic strains of *Vibrio cholerae* also owe much of their pathogenicity to phage conversion, with cholera toxin encoded by the temperate and filamentous phage CTXΦ (Waldor and Mekalanos, 1996).

Cholera, a waterborne gastroenteric infection that can spread rapidly as explosive epidemics, remains a significant threat to public health in the developing world. The disease in its severest form causes copious diarrhoea with flakes and mucus, dehydration, and sometimes death from dehydration is typical in the absence of treatment (Kaper *et al*., 1995; Jay *et al*., 2005; Talkington *et al*., 2011). The curved bacteria in the intestinal contents of cholera victims was first described and named by Pacini (Pollitzer, 1959), in 1854 in Italy as *V. cholerae.* Since the early epidemiological work in 1853 in London by John Snow and later laboratory investigation by Koch (1884), it has been known that water is important in the transmission of cholera. Before the development of intravenous and oral fluid replacement therapies, cholera is estimated to have killed over 100,000 people each year in India alone between 1900-1950. As the disease went pandemic in the early 1800's, it killed millions worldwide. Even though there are effective vaccines, cholera still extracts a death toll that measures in thousands (Mekalanos *et al*., 1997).

Vibrio cholerae belonging to family Vibrionaceae, is a facultatively anaerobic, Gram-negative, non-spore-forming curved rod, about 1.4–2.6μm long (Baumann *et al.*, 1984). *V. cholerae* naturally colonize lakes, rivers, and estuaries where salinity is between 4 to 17% (Colwell *et al*., 1977; Kaper *et al*., 1979); local outbreaks are usually by contamination of local water supplies in areas of poor sanitation. Outbreaks of cholera occur cyclically, usually twice per year in

endemic areas, and the intensity of these outbreaks vary (Faruque *et al*., 2002). Extrinsic factors, such as large-scale weather cycles (Colwell, 1996), and intrinsic factors like the induction of bacteriophages infecting *Vibrios* (also called vibriophages) were shown to correlate in time with components of the epidemic cycle (Faruque *et al*., 2000).

V. cholerae infections can lead to epidemics, pandemics or may be endemic in specific areas. The fatality rate exceeded the survivors' ability to bury the dead, when Asiatic cholera affected India (Graham, 1862). Seven cholera pandemics were reported since its discovery in 1817 (Karaolis *et al*., 1995). The first six pandemics originated in the Indian Subcontinent with the Ganges Delta region as the main reservoir (Colwell, 1996). The seventh pandemic started in 1961 in Indonesia and spread to other parts of the world and the most recent outbreak occurred in Haiti (Hendriksen *et al*., 2011).

The species *V. cholerae* have variety of strains and biotypes, receiving and transferring genes for virulence-associated factors as well as genes for other biochemical functions, including antibiotic resistance, capsular polysaccharides, and new surface antigens (Mekalanos *et al*., 1997; Faruque *et al*., 1998a). Serogroup O1 is responsible for all seven recorded cholera pandemics (Colwell, 1996) and can be further subdivided into two biotypes, El Tor and classical. The classical *V. cholerae* biotype marked the first six cholera pandemics (Blake, 1994), but was replaced by the El Tor biotype during the onset of the seventh pandemic (Mutreja *et al*., 2011).

Many researchers have employed various DNA fingerprinting or genotyping methods to reveal the epidemiological link among *V. cholerae* isolates. Novel PCR based DNA fingerprinting methods like Amplified fragment length polymorphism(AFLP), Random 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) methods permit both phylogenetic inference and clonal differentiation of individual *V. cholerae* strains (Caburlotto *et al*., 2011; Cazorla *et al*., 2011; Hendriksen *et al*., 2011)

O1 serogroups of *V. cholerae* are biochemically indistinguishable from their non-O1 counterparts except for their property to be agglutinated by O1 antiserum (Sakazaki, 1992). Non-O1 *V. cholerae* strains representing a heterogenous group comprising more than 140 serogroups are the natural inhabitants of aquatic environment (Kaper *et al*., 1979; Morris, 1990). Some of these strains are also reported to be responsible for sporadic cases of gastroenteritis and extra intestinal infections (Blake *et al*., 1980). However, in 1992–93, an epidemic of cholera was reported from India and Bangladesh which was caused by a non-O1 strain of *V. cholerae*, (Albert *et al*., 1993; Ramamurthy *et al.,* 1993a) later to be designated as O139 Bengal (Shimada *et al.,* 1993). In recent years several non-O1, non-O139 serogroups were observed to be involved in multiple outbreaks of cholera-like diseases (Morris, 1990; Yamamoto *et al.,* 1990).

The long-term persistence of pathogens in the non-human environment (King *et al.*, 1994) 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). In the aquatic environment, *V. cholerae* is associated with a number of biotic and abiotic substrates (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.

Marine aquaculture settings and mangrove environments of Kerala serve as reservoirs for *V. cholerae.* 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 serogroup O139 has evolved as a result of horizontal transfer of genes from a non-O1 strain to the seventh pandemic clone of *V. cholerae* O1 (Bik *et al.,* 1995; Waldor and Mekalanos, 1994). In this context, the temperate bacteriophages of *V. cholerae* are the most promising candidates for the conversion of avirulent strains to virulent ones, while the transducing phages of *V. cholerae* were probably involved in the emergence of pandemic strains through biotype transition (Ogg *et al.*,1981).

Cholera toxin (CT) with A and B subunits, toxin coregulated pilus (TCP) and the regulatory element ToxR are believed to be the important virulence factors associated with the epidemic causing strains of *V.cholerae* O1 and O139 (Waldor *et al.,* 1997). Revolutionary changes took place in 1996 with the recognition that, genes coding cholera toxin are borne on, and can be infectiously transmitted by CTXΦ, a filamentous lysogenic phage, (Waldor and Mekalanos, 1996). The 7–9.7 kilobase, CTXΦ genome can become incorporated into the *V. cholerae* chromosome, often as an array of tandemly repeated copies, but can also replicate and be vertically transmitted as a plasmid.

Waldor and Mekalanos (1996) demonstrated that the *ctxAB* loci that encode the A and B subunits of cholera toxin can be carried and infectiously transmitted in mice, as well as *in vitro*, by CTXΦ. This single stranded DNA phage infects host bacteria by adsorbing to a ‘toxin co-regulated pilus’ (Boyd and

Waldor, 1999). The gene for the pilus protein is expressed under control of the ToxR regulatory system which also regulates the transcription of the cholera toxin genes (Herrington *et al.*, 1988).

TCP (Toxin Co-regulated Pili) was also reported to be encoded by VPIΦ- another lysogenic, filamentous phage (Karaolis *et al.,* 1999). In addition, recently the horizontal transmission of CTXΦ among different *Vibrio* species has been documented (Faruque *et al.,* 1998a; Boyd *et al.,* 2000). Thus with the simultaneous discovery of a number of temperate vibriophages (Kar *et al.,* 1996) and prophage-like sequences in the *V. cholerae* genome **(**Farque *et al.,* 2002), vibriophages have established themselves as an emerging force in the appearance of the novel pathogenic clones of *V. cholerae.*

Lateral or horizontal gene transfer (HGT) is defined as the exchange of genes between different strains or species. HGT is known as an important mechanism to shape the genomes of bacteria (Ochman *et al*. 2000; Boucher *et al*. 2003). There are evidences of apparent transfer of virulence genes between phages belonging to different phage groups (Mirold *et al*., 2001) or phages infecting different bacterial species (Desiere *et al.*, 2001) which increases the lateral spread of these genes in bacteria. Tailed phages are very efficient gene-transfer element. Phage tail and its associated fibres act as an effective DNA transfer device. It assures both the specific host cell recognition and injection of the phage DNA into the bacterial cell (Zhang *et al*., 2000).

These observations raise a number of questions about the mechanisms and evolution of virulence in *V. cholerae* and of bacterial pathogenesis in general. It is important to understand the various ecological conditions and genetic processes responsible for the evolution and persistence of phage encoded virulence. This in turn can enable comprehension of the reasons for cyclical outbreaks of cholera.

OBJECTIVES

The reassortment of genes plays a major role in the emergence of *V.cholerae* and consequently new epidemics. Gene transfer depends on prophage induction and the initiation of the phage lytic cycle. Environmental interaction may confer enhanced pathogenicity on a subset of an environmental population. These natural phenomena may be further enhanced by anthropogenic activities which results in environmental pollution.

Understanding the lateral or horizontal transfer of genes by phage, pathogenicity islands, and other accessory genetic elements can provide insights into how this bacterial pathogen emerges and evolves to become new strains. The present work was undertaken with the following specific objectives:

1. Screening and identification of environmental isolates of *Vibrio cholerae* with temperate phages.
2. Molecular characterization of environmental isolates of *Vibrio cholerae* with temperate phages.
3. Differential induction and purification of prophages.
4. Physicochemical characterization of vibriophages
5. Bacteriophage genome analysis
6. Transduction studies as evidence for horizontal gene transfer

Chapter 2

REVIEW OF LITERATURE

2.1. Cholera

The term cholera is derived from the Greek, meaning 'bilious', whereas another possible derivation is from the Hebrew, meaning 'bad disease' (Pollitzer, 1959). Cholera as a disease is not as old as tuberculosis or smallpox, it is known for at least a thousand years (Mekalanos *et al.,* 1997). The two distinctive epidemiologic features of cholera are its tendency to appear in explosive outbreaks, often starting in several distinct foci simultaneously, and its propensity to cause true pandemics that progressively affect many countries in multiple continents over the course of many years. Cholera is a major infectious disease in the recent past, with a global increase in its incidence. In 1994 cholera cases were notified from 94 countries, the highest ever number of countries in one year (WHO, 1995).

The intensity of cholera and the explosiveness of cholera epidemics have impressed ancient observers. As noted by Pollitzer (1954), in a translation of a Sanskrit work whose origin is believed to be Tibet around 802 AD, describes cholera: "When the strength of virtues and merits decreases on earth, there appears amongst the people, first among those living on the shores of big rivers, various ailments which gives no time for treatment, but prove fatal immediately after they appear. The various vessels secrete water so that the body becomes empty. Its first signs are dizziness, a numb feeling in the head, and then the most violent purging and vomiting".

Cholera is caused by the Gammaproteobacterium *Vibrio cholerae* that exists as a free-living organism in coastal waters throughout the world (Colwell

and Spira, 1992). *Vibrio cholera* was first described and named by Pacini (Pollitzer, 1959), who found a large number of curved bacteria in the intestinal contents of cholera victims in 1854 in Italy. Robert Koch identified that cholera was caused by comma-shaped organism, which he named *comma bazillen* and the subsequent name *Vibrio comma* was used for several decades before the pioneering work of Pacini was recognized and the name was changed to *Vibrio cholerae* (Koch, 1884). *V. cholerae* O1 is the primary causative agent of cholera (Morris and Black, 1985). In 1992, toxigenic *V. cholerae* O139 (the Bengal strain) was recognized as another cause of cholera (Ramamurthy *et al.*, 1993a). *V. cholerae* O139, was first discovered on the Indian subcontinent (Albert, 1994; Nair *et al.*, 1994).

2.1.1. Cholera pandemics

The first cholera pandemic started in India in 1817 (Graham, 1862; Blake, 1994) and swept through the Middle East and Asia. Since that time there have been six more pandemics, the last starting in 1961 and caused by the El Tor biotype (Gangarosa and Mosley, 1974; Dodin, 1978).

During the second pandemic it was demonstrated that the characteristic rice water stools of patients contained salts and alkali, *i.e*., were high in electrolyte content (Gangarosa and Mosley, 1974). The second pandemic was the first to reach the New World via ships from Ireland carrying immigrants (Chambers, 1938). Spreading from Montreal, cholera moved south over the next 10 weeks to reach, successively, New York, Philadelphia, Baltimore and Washington D.C. (Pyle, 1969; Barua, 1992).

The epidemiologic observations of Snow (1855) and Black (1982) on the waterborne transmission of cholera were made during the third pandemic. On the basis of his epidemiologic observations, Snow deduced that cholera must represent a contagion.

During the fourth pandemic in 1870's, New Orleans and cities and towns along the Mississippi, Missouri, and Ohio rivers experienced considerable cholera (Barnes *et al.,* 1875). In the course of field investigations in Egypt in 1883 during the fifth pandemic, Koch (1884) isolated cholera from the rice water stools of patients with cholera which he referred to as ``comma bacilli''. Moving on to Calcutta in 1884, Koch isolated the same bacteria (Howard-Jones, 1984). The fifth pandemic extensively affected South America, causing large epidemics accompanied by high mortality in Argentina, Chile, and Peru, among other countries (Laval, 2003).

The sixth pandemic, and probably the fifth as well, were caused by *V. cholerae* O1 of the classical biotype (Baine *et al.,* 1974; Glass and Black, 1992; Faruque *et al.,* 1998a). With the exception of a large epidemic in Egypt in 1947 (Shousha, 1948), cholera remained virtually confined to South and Southeast Asia for more than 100 years.

In 1961, the seventh cholera pandemic originated from the island of Sulawesi, in the Indonesian archipelago and spread throughout the world (Griffith *et al.,* 2006), reaching Africa in 1970 and South America in 1991. The pathogen, *V. cholerae* O1 El Tor, accumulated variation, with both random genetic drift and recombination (Karaolis *et al.,* 1995). This pandemic is the most extensive in geographic spread and in time.

Epidemiologic reports of cholera caused by new serogroup, O139 in 1992 in India reveals the severity of the disease (Albert, 1994). Its rapid spread to neighboring countries, and its ability to cause large explosive outbreaks (CDC, 1993; Ramamurthy *et al.,* 1993a; Nair *et al.,* 1994; Popovic *et al.,* 1995) give testimony that it represent the etiologic agent of a new, eighth pandemic of cholera. Between these pandemics, cholera has been present in the Indian subcontinent and Africa largely in endemic foci.

The most recent outbreak occurred in Haiti (Hendriksen *et al*., 2011). The movement of both humans and water has recently been suggested to influence the spatial spread of cholera in Haiti (Dowell and Braden, 2011)

2.1.2. Epidemiology

The occurrence of cholera and its epidemiologic behavior prior to the 19th century are subjects of contentious debate among medical historians, as reviewed by Pollitzer (1959). The World Health Organisation maintains a public database of cholera outbreaks. Cholera is most prevalent in the developing world, particularly in warm climates, and it is seasonal (Rodo *et al.*, 2002).

Upon ingestion of inoculum, the majority of bacteria are killed by the acidic pH in the stomach. Those that survive enter the lumen of the small intestine and begin colonization. Human inoculum size is likely large, since there is approximately a 4–6 log reduction of *V. cholerae* due to the low pH in the stomach (Cash *et al.*, 1974).

The incubation period of cholera is usually 2 to 3 days (range, 6 hours to 5 days) (Azman *et al.*, 2013). Only minorities of people infected with CT producing *V. cholerae* develop the most severe manifestations of the disease, termed cholera gravis (Gangarosa and Mosley, 1974). Cholera infection cause profuse watery diarrhea, vomiting, and muscle cramps. It is a dehydrating diarrheal illness that results in substantial loss of fluid and electrolytes. On occasion, stool volumes may approach 1 L/h (Pierce *et al.*, 1969). The spectrum of illness includes asymptomatic infection (75%), mild illness (18%), moderate illness (5%), and severe illness (2%). Severe diarrhea may result in hypovolemic shock and possibly death within a few hours without treatment (Gangarosa and Mosley, 1974).

Severe illness has been associated with high-dose exposure, low gastric acidity, and blood group O (Blake, 1993). Severe cholera may be characterized by

"rice water" stools, loss of 10% or more of body weight, loss of normal skin turgor, dry mucous membranes, sunken eyes, lethargy, anuria, weak pulse, altered consciousness, and circulatory collapse. Diarrheal fluid loss may result in profound hypokalemia, metabolic acidosis (from bicarbonate loss), and renal failure (Das *et al.*, 2005).

V. cholerae makes a potent enterotoxin, cholera toxin, which modifies a key regulatory protein in intestinal cells. Cholera alters intestinal water and electrolyte transport without structural damage to the gut mucosa (Elliott *et al.,* 1970). Cholera enterotoxin produces its effect on intestinal water and electrolyte transport by direct action upon the luminal surface of gut mucosal cells (Pierce *et al.,* 1971). The ultimate effect of cholera toxin is constitutive cyclic AMP production in intoxicated cells that results in the opening of normally gated channels in the membrane (Kimberg *et al.,* 1971). This leads to loss of chloride and other ions from the cells, followed by water. The result for the infected individual is massive fluid and ion loss in the form of a watery diarrhea that is the hallmark of cholera infection. The diarrhea in cholera can reach volumes of 20L per day and leads to shock and death if not treated by oral rehydration therapy (Wheby, 2014).

Other than *V. cholerae* O139, non-O1 *V. cholerae* usually causes a less severe diarrhea than *V. cholerae* O1, although certain strains, especially those that produce cholera toxin, can cause severe cholera-like disease (Datta *et al.,* 1986). *V. cholerae* non O1/O139 serotypes are occasionally isolated from cases of diarrhoea and 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).

Septicemia with non-O1 *V. cholerae* is seen in immunocompromised hosts, particularly patients with cirrhosis. In one series, all 15 patients with non-O1 *V. cholerae* bacteremia had hepatic cirrhosis and 7 (47%) died (Ko *et al*., 1998). Soft tissue infections, including cellulitis and necrotizing fasciitis, are a less common presentation of non-O1 *V. cholerae* infection. There were 130 cases of non-O1 *V. cholerae* infection reported in Florida from 1981 to 1993 (Colwell *et al*., 1996).

Vibrio cholerae O1 can enter a state in which they remain viable but are non-culturable. Presumably, such bacteria can be pathogenic if they retain the capacity to proliferate in rabbit ligated loops and human intestine following ingestion (Colwell *et al*., 1996).

2.1.3. Ecologic factors

Until the late 1970s and early 1980s, *Vibrio cholerae* was believed to be highly host-adapted and incapable of surviving longer than a few hours or days outside the human intestine (Felsenfeld, 1974). The likelihood of the pathogen survival outside the human body and especially in rivers was explained (Koch, 1884). The autochthonous nature of toxigenic *Vibrio cholerae* is an important factor in the epidemiology of cholera. The environmental and clinical isolates of *Vibrio cholerae* O1 serogroups have been shown to be identical by 5SrRNA sequencing (MacDonell and Colwell, 1984a)

For about 100 years the common wisdom was that human intestinal track was the only reservoir of toxigenic *V. cholerae* O1. “Non-agglutinable vibrios”

non- O1 *V. cholerae*, found in aquatic, predominantly estuarine environments was considered taxonomically separate from "cholera vibrios".

The environment seems to play an important role in epidemics and the establishment of endemic cholera. Environmental characteristics, such as water temperature and salinity, are also known to influence the diversity of *Vibrio* sp. in the environment (Beaz-Hidalgo *et al.,* 2010). *V. cholerae* are found in association with aquatic organisms like algae and mollucs (Snoussi *et al.,* 2008). It survives unfavourable environmental conditions, by the formation of biofilms (Huq *et al.,* 1983; Visick, 2009; Yildiz and Visick, 2009). Viable but non-culturable (VBNC) state is also described (Roszak and Colwell, 1987; Asakura, *et al.,* 2007) where they do not form colonies on any media, but are metabolically active and are resistant to environmental stress (Oliver, 2005).

They are opportunistic pathogens that are widely distributed in marine aquaculture environment (Sung *et al.,* 2001; 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.,* 1993; Pal and Das, 2010; Frans *et al.,* 2011). *Vibrio cholerae* is also known to inhabit freshwater environments (Baumann *et al.,* 1980).

The persistence of *V. cholerae* within the environment may be facilitated by its ability to assume survival forms, including a viable but nonculturable state and a 'rugose' survival form (Nalin *et al.,* 1979; Colwell and Spira, 1992). Other aquatic biota, such as water hyacinths, has also been colonized by *V. cholera,* to promote its growth (Spira *et al.,* 1981).

2.1.4. Therapy

The key to therapy is provision of adequate rehydration until the disease has run its course (usually 1 to 5 days in the absence of antimicrobial therapy).

Rehydration can be accomplished by intravenous infusion of fluid (in severe cases) or by oral rehydration with an oral rehydration solution (ORS) (Black, 1982; Morris and Black, 1985; Swerdlow and Ries, 1992). Antimicrobial agents play a secondary but valuable role in therapy by decreasing the severity of illness and the duration of excretion of the organism.

For adults, about 2 liters of intravenous re-placement solution need to be given and children should receive 30 mL of intravenous fluid per kg of body weight (Bhattacharya, 2003). The WHO recommends Ringer's lactate as the best commercial solution. Isotonic saline corrects hypovolemia, but potassium, base, and glucose must be supplemented (Seas *et al.,* 1996). Patients with mild or moderate dehydration can receive initial fluid replacement to repair water and electrolyte deficits exclusively by the oral route (Black, 1982).

Antimicrobial agents can shorten the duration of cholera diarrhea and the period of excretion of vibrios. Tetracycline is the drug of choice (Greenough *et al.,* 1964; Swerdlow and Ries, 1992; Bennish, 1994). Quinolones (norfloxacin and ciprofloxacin) have excellent *in vitro* activity against *V. cholerae* and may prove to be useful therapeutic agents (Morris *et al.,* 1985; Gotuzzo *et al.,* 1995; Khan *et al.,* 1996).

2.1.5. Surveillance

The WHO maintains a public database of cholera outbreaks and provides outbreak updates and an annual cholera summary of national aggregate data in the Weekly Epidemiologic Record. Official notification of cholera outbreaks by WHO member states is mandatory under the International Health Regulations (IHR). The Program for Monitoring Emerging Diseases (ProMED), initiated in 1994, operates as an on-line forum for infectious disease specialists, microbiologists, and public health officials and has been administered through the International Society for Infectious Diseases since 1999. ProMED disseminates information about

outbreaks of infectious diseases. Its sources include official government and multinational agency reports, print and online media reports and information from local observers.

2.2. Taxonomy and serological classification of *Vibrio cholerae*

Vibrio cholerae, belonging to family Vibrionaceae, is a facultatively anaerobic, Gram-negative, non spore forming curved rod, about 1.4-2.6 µm long, capable of respiratory and fermentative metabolism; it is well defined on the basis of biochemical tests and DNA homology studies (Baumann *et al.,* 1984). The bacterium is oxidase-positive, reduces nitrate, and is motile by means of a single, sheathed, polar flagellum. Growth of *V. cholerae* is stimulated by addition of 1% sodium chloride (NaCl). However, an important distinction from other *Vibrio* sp is the ability of *V. cholerae* to grow in nutrient broth without added NaCl (Feeley, 1965).

Differences in the sugar composition of the heat-stable surface somatic "O" antigen are the basis of the serological classification of *V. cholerae* which was first described by Gardner & Venkatraman (1935). Currently the organism is classified into 206 "O" serogroups (Shimada *et al.,* 1994; Yamai *et al.,* 1997). All strains that were identified as *V. cholerae* on the basis of biochemical tests but that did not agglutinate with "O" antiserum were collectively referred to as non-O1 or NAGs (non agglutinating group) *V. cholerae*.

The O1 serogroup exists as two biotypes, classical and El Tor; antigenic factors allow further differentiation into two major serotypes- Ogawa and Inaba. Strains of the Ogawa serotype are said to express the A and B antigens and a small amount of C antigen, whereas Inaba strains express only the A and C antigens. A third serotype (Hikojima) expresses all three antigens but is rare and unstable. The El Tor biotype differs from the classical in several properties that include its ability to produce hemolysin, presence of mannose-sensitive hemagglutinin, and

resistance to the antibiotic polymyxin (Feeley, 1965; Davis *et al*, 1981; Shimada *et al.*, 1994). The most distinguishing feature of El Tor is its resistance to group-IV phages (Maiti and Chatterjee, 1971).

The key confirmation for identification of *V. cholerae* O1 is agglutination in polyvalent antisera raised against the O1 antigen. Polyvalent antiserum for *V. cholerae* O1 and O139 can be used in slide agglutination or coagglutination tests (Rahman *et al*., 1989).

The simple distinction between *V. cholerae* O1 and *V. cholerae* non-O1 (nonagglutinable vibrios) became obsolete in early 1993 with the first reports of a new epidemic of severe, cholera-like disease in Bangladesh (Albert *et al.,* 1993) and India (Ramamurthy *et al.,* 1993b). At first, the responsible organism was referred to as non-O1 *V. cholerae* because it did not agglutinate with O1 antiserum. The new serogroup was given the designation O139 Bengal after the area where the strains were first isolated (Shimada *et al.,* 1993). Since recognition of the O139 serogroup, the designation non-O1 non-O139 *V. cholerae* has been used to include all the other recognized serogroups of *V. cholerae* except O1 and O139 (Nair *et al.,* 1994).

These non-O1/non-O139 strains have been divided into sero-groups O2 through O138 on the basis of the lipopolysaccharide (LPS) somatic antigen. The great majority of these strains does not produce CT and are not associated with epidemic diarrhea (Morris, 1990).

Citarella and Colwell (1970) proved that both O1 and non-O1 *V. cholerae* comprise a single species, using DNA/DNA hybridization. The evidences including numerical taxonomy (West, 1984), DNA/DNA hybridization (Citarella and Colwell, 1970) and nucleic acid sequence data (MacDonell and Colwell, 1984a; MacDonell and Colwell, 1984b) supports the conclusion. Non-toxigenic *V. cholerae* O1 comprise those isolates which agglutinate in O1 antiserum but do not

possess the cholera toxin gene. These strains may produce diarrheal disease and are designated as *V. cholerae* O1 (CT^-). Some non-O1 *V. cholerae* isolates produces an enterotoxin indistinguishable from cholera toxin.

2.3. Phenotypic and molecular typing of *Vibrio cholerae*

In an outbreak of cholera, it is very important to determine whether the strains have a common origin or different origins. Phage typing and serotyping continue to be useful tools for epidemiological surveillance of *V. cholerae* (Bhowmick *et al*., 2007).

More recently, attention has turned to sequence based approaches which include PCR-ribotyping, RFLP, AFLP, ERIC-PCR, BOX-PCR, Multilocus sequence typing (MLST), PFGE, to name a few. Molecular typing plays a key role in understanding disease transmission and tracking and has been used in identification of clinical strains isolated from different sources (Cooper *et al*., 2006). The molecular typing techniques had the potential for studying the origin and interrelatedness of strains of epidemiological settings.

2.3.1. Phage typing

Phage typing of *V. cholerae* is useful both for differentiating classical and El Tor strains and for epidemiological purposes (Mukerjee, 1963). A number of phage typing schemes has been proposed. The international phage typing scheme of Basu and Mukerjee (Basu and Mukerjee, 1968) includes five phages (I, II, III, IV, and V) by which *V. cholerae* O1 biotype El Tor strains can be differentiated into six different phage types. It has been used routinely for identification of the strains at the Vibriophage Reference Laboratory since 1968. Phage typing methods can test large number of strains rapidly. It is a cost effective and a simple laboratory method that does not require any sophisticated equipment. Because of host specificity, it offers the basic information on identification, biotyping and

discrimination of strains (Ghosh *et al*., 1989). Correlation was observed between the phage types and antibiogram (Chattopadhyay *et al*., 1993).

2.3.2. Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a high resolution genomic fingerprinting method. It has been demonstrated to have both a greater capacity for genome coverage and better reproducibility than other genotyping technologies (Jiang *et al*., 2000a). 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. Evolutionary relationships and molecular diversity of *V. cholerae* serogroup O1, O139, and non-O1, non-O139 isolates were studied using AFLP (Jiang *et al*., 2000b). Environmental isolates of O1 and non-O1 strains could be separated by AFLP, whereas O1 and O139 clinical strains were undistinguishable.

2.3.3. 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* , while Chowdhury and his team (2010) developed an RFLP method targeting sections of the super integron region of the *V. cholerae* genome.

2.3.4. Pulse field gel electrophoresis (PFGE)

DNA fingerprinting by pulsed-field gel electrophoresis (PFGE) is another useful method in epidemiologic studies involving a variety of pathogenic bacteria (Mahalingam *et al*., 1994) including *V. cholerae.* Cameron *et al.*, (1994) were able to detect differences in PFGE patterns between toxigenic and nontoxigenic strains, as well as between epidemiologically unrelated nontoxigenic strains. PFGE showed better agreement with epidemiological data than phage typing, as it divided a single phage type into multiple pulsotypes. Wachsmuth *et al.,* (1994) had concluded that the method appears to be more discriminating than ribotyping for *V. cholerae* O1 and is probably the method of choice within a defined epidemic setting. PFGE had better typeability and concordance with different geographical regions than phage typing. PFGE was reported (Mahalingam *et al*., 1994) to have the most discriminatory power among these methods.

2.3.5. Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST), first described in 1998 (Maiden *et al*., 1998), provides a balance between sequence-based resolution, informativeness and technical feasibility, and has been used to characterize several pathogenic bacteria including *V. cholerae* (Urwin and Maiden, 2003). The technique, as originally described, involves determining the nucleotide sequences of a series of housekeeping genes. MLST detects all genetic variations within the amplified gene fragment (Lee *et al*., 2006). As variation occurs most commonly at the nucleotide level, MLST has better discriminatory ability than PFGE for typing *V. cholerae* (Kotetishvili *et al*., 2003).

2.3.6. 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 (Rodrigue *et al.*, 1994). 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).

This technique along with Rep-PCR has been successfully employed in the discrimination of strains of *V. cholerae* (Rivera *et al.,* 1995). 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).

2.3.7. BOX PCR

BOX elements, a class of repetitive sequences which were discovered in *Streptococcus pneumoniae* (Martin *et al.,* 1992) are naturally occurring, multicopied, conserved, repetitive DNA sequences present in the genomes of most

Gram-negative and Gram-positive bacteria. These repeats 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). 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 intraspecies, 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). 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).

2.4. Virulence factors of *V. cholerae*

In order to colonize the small intestine and cause diarrhea, *V. cholerae* must upregulate and coordinate the expression of a multitude of virulence factors. The two most studied of these factors are cholera toxin (CT) and toxin-coregulated pilus (TCP) (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 (Pearson *et al.,* 1993; Trucksis *et al.,* 1993; Everiss *et al.,* 1994; Harkey *et al.,* 1994).

2.4.1. Cholera toxin

CT is an ADP-ribosylating toxin that binds ganglioside GM1 on absorptive intestinal epithelial cells. 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_2O, Na^+, K^+, Cl^-, and HCO_3^- into the lumen of the small intestine. The bacterium produces an invasin, neuraminidase, during the colonization stage (Collier and Mekalanos, 1980). Once internalized, CT leads to increased intestinal secretion of electrolytes and water into the lumen, and is largely responsible for the profuse watery diarrhea that is the hallmark 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.

2.4.2. 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 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). The CTXΦ encoded Ace protein, reported to account in part for *V. cholerae* enterotoxicity, is thought to be a minor coat protein of the CTXΦ virion. 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.

2.4.3. Toxin Co-regulated Pilus (TCP)

TCP is a type IV pilus that is essential for intestinal colonization in humans and in animal models of cholera (Taylor *et al*., 1987; Herrington *et al*., 1988). TCP serves as the receptor for a lysogenic bacteriophage, CTXΦ, which carries within its genome the structural genes for cholera toxin, *ctxAB* (Waldor and Mekalanos, 1996).

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 (Jiang *et al.*, 2000a). In addition, it 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). 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 of *V. cholerae*. Even though the cholera toxin (CT) and the TCP are exclusively associated with clinical strains, especially those belonging to the serogroups O1 and O139, their presence and expression has also been reported in environmental strains of *V. cholerae* (Chakraborty *et al.*, 2000; Kumar *et al.*, 2008).

TCP is required for CTXΦ infection of *V. cholerae.* It was proposed by Shaw *et al.*, (1990) that there were two crucial sequential steps in the evolution of pathogenic *V. cholerae*. First, *V. cholerae* strains acquired the *tcp* operon and second, these TCP^+ strains were infected with and lysogenized by CTXΦ.

2.4.4. Zonula occludens toxin (Zot)

Zonula occludens toxin increases the permeability of small intestinal mucosa by inducing a modification of the structure of the intercellular tight junction, or zonula occludens (Fasano *et al.*, 1991). The *zot* gene, together with the adjacent CTX operon encoding the two subunits of the classical cholera toxin, belongs to a site specific transposable element (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. 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 (Di Pierro *et al.*, 2001).

2.4.5. Hemolysin (Hly A)

Hemolysin act on erythrocyte membranes leading to cell lysis (Iida and Honda, 1997; Shinoda, 1999). 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). El Tor hemolysin of *V. cholerae* (Hly A) (Yamamoto *et al.*, 1986; Fallarino *et al.*, 2002) lyses erythrocytes and other mammalian cells and exhibits enterotoxicity in experimental diarrhoea models. It has a major role in the pathogenesis of gastroenteritis caused by *V. cholerae* strains. The hemolysin is exported by the

majority of *V. cholerae* O1 and non-O1 strains to the culture supernatant as the 80 kDa precursor, prohaemolysin, which on proteolytic removal of the N-terminal 15 kDa prodomain converts to the fully mature 65 kDa toxin (Nagamune *et al.*, 1996).

2.4.6. Outer Membrane Proteins (Omp)

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 (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 (Alm *et al.,* 1986). The OmpX protein (27 kDa) is osmoregulated and trypsin resistant (Chakrabarti *et al.,* 1996). OmpU (38 kDa) and OmpT (40 kDa) allow the transport of hydrophilic solutes in liposome swelling assays (Chakrabarti *et al.,* 1996). 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). The ompW gene is located in the small chromosome (Chr II) of *V. cholerae*, which contains genes involved in metabolic and regulatory pathways. The modulation of OmpW expression may be linked to such adaptive response under stress conditions (Nandi *et al*., 2005).

2.4.7. RTX Toxin (Repeats in toxin)

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). 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.

2.4.8. 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.

2.5. Vibrio pathogenicity island (VPI)

Pathogenicity islands are large segments of DNA carrying virulence genes that were acquired, at some time in the distant past, by a variety of pathogens. 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* (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*H 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), TcpH (Higgins *et al.*,1992) 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).

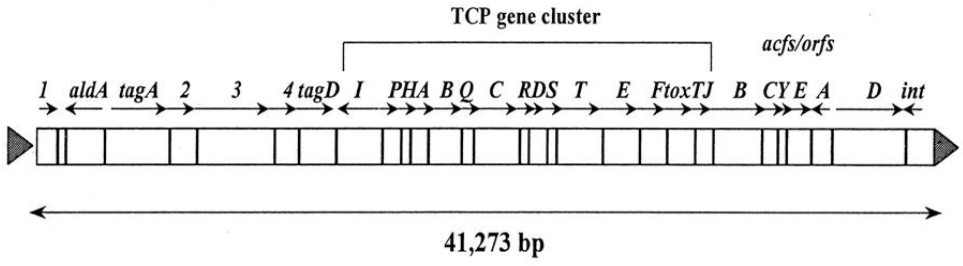


Fig 2.1. Schematic representation of Vibrio Pathogenicity Island (VPI). Triangles flanking VP1 represent phage-like attachment (att) sites. (Figure adapted from Zhang *et al*., 2000).

Several additional genomic regions have also been identified, predominantly among epidemic O1 and O139 serogroup isolates; these include RS1, 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 is associated with the CTXΦ prophage in *V. cholerae* El Tor isolates and is required for the production of pCTXΦ (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 that all toxigenic *V. cholerae* O1 and O139 serogroup isolates possessed VPI-2 region, whereas non-O1/ nonO139/ non-toxigenic isolates lacked the region (Jermyn and Boyd, 2002).

2.6. ToxR Regulon

The gene *tox*R encodes the transmembrane transcription regulator ToxR coordinate regulation of virulence gene expression (Miller *et al.,* 1987; Miller and Mekalanos, 1988). ToxR is a critical component that act early in the regulatory cascade and this virulence gene regulon is referred to as the "ToxR regulon." It 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). Among the genes of the *tox*R regulon, only *ctx*AB is directly regulated by ToxR protein (Higgins and DiRita, 1994). ToxR is a transcription factor located in the inner membrane that directly regulates the expression of CT, ToxT, and the porins OmpU and OmpT (Miller and Mekalanos, 1984; Higgins and DiRita, 1994). Null mutations in these regulatory genes abolish expression of CT and TCP, and result in profound attenuation of virulence in animal models of cholera (Skorupski and Taylor, 1997).

2.7. Bacteriophages

Bacteriophages are natural viral pathogens of bacteria. They are considered as the most abundant and ubiquitous life-like entity in both marine environment (Bergh *et al.,* 1989) and terrestrial soil environments (Ashelford *et al.,* 2003). Hankin (1896), a British bacteriologist working in India reported the existence of unidentified substance responsible for marked activity against the bacterial pathogen *Vibrio cholerae* in the waters of the Ganges and Yamuna. After almost 20 year Frederick Twort, reported a similar phenomenon and put forth the hypothesis that it may have been due to a virus (Twort, 1915). The term "bacteriophage" was proposed by Felix d'Herelle which comes from the words "bacteria" and "phagein" which in Greek means to eat or devour (d'Herelle, 1917).

Bacteriophages offer a special perspective on the diversity, origins, and evolution of viruses, not only in their tremendous abundance but also in their distant origins, probably more than three billion years ago. Moreover, viral ecologists calculate that there are about 10^{23} phage infections per second on a global scale, indicating that the population is not only large and old but also highly dynamic (Hendrix, 2003; Hatfull, 2008). Phage research in more recent years has revealed their dramatic impact on the ecology of our planet (Suttle, 1994; Wommack and Colwell, 2000) and their influence on the evolution of microbial populations (Chibani-Chennoufi *et al.*, 2004). Phage derived proteins are currently being used as therapeutic agents (Nelson *et al*., 2009) and for drug discovery (Liu *et al*., 2004).

2.7.1. Classification of phages

In earlier days, bacteriophages were classified merely based on host specificity (Ackermann, 2007). The advent of electron microscopy enabled scientists to classify phages based on their morphology. Present day classification

by International Committee on Taxonomy of Viruses (ICTV) is derived from the scheme proposed by Bradley (1967) using gross morphology and nature of their nucleic acid. It includes six basic morphological types, exemplified by phages T4, λ, T7, φX174, MS2 and fd.

Table.2.1. Overview of phage families

Shape	Nucleic acid	Family	Genera	Example	Members
Tailed	dsDNA (L)	*Myoviridae*	6	T4	1,320
		Siphoviridae	7	λ	3,229
		Podoviridae	4	T7	771
Polyhedral	ssDNA (C)	*Microviridae*	4	*φX174*	40
	dsDNA (C, S)	*Corticoviridae*	1	PM2	3
	dsDNA (L)	*Tectiviridae*	1	PRD1	19
	dsDNA (L)	SH1∗		SH1	1
	dsDNA (C)	STIV∗		STIV	1
	ssRNA (L)	*Leviviridae*	2	MS2	39
	dsRNA (L, M)	*Cystoviridae*	1	*φ6*	3
Filamentous	ssDNA (C)	*Inoviridae*	2	M13	67
	dsDNA (L)	*Lipothrixviridae*	4	TTV1	7
	dsDNA (L)	*Rudiviridae*	1	SIRV-1	3
	dsDNA (C, S)	*Plasmaviridae*	1	L2	5
	dsDNA (C, S)	*Fuselloviridae*	1	SSV1	11
	dsDNA (L, S)	-	1∗∗	His1	1
	dsDNA (C, S)	*Guttaviridae*	1	SNDV	1
	dsDNA (L)	Ampullaviridae∗		ABV	1
	dsDNA (C)	Bicaudaviridae∗		ATV	1
	dsDNA (L)	Globuloviridae∗		PSV	1

C Circular; L linear; M multipartite; NC nucleocapsid; S supercoiled; _ no name; ∗Awaiting classification (adapted from Ackermann, 2009)

Over the years many new families were added and currently it includes one order, 17 families and three "floating" groups (Ackermann, 2007, 2009). Bacteriophages encompass many types of virion morphologies and nucleic acid compositions, but the large majority are double stranded DNA (dsDNA) tailed phages (Caudovirales) (Ackermann, 2007).

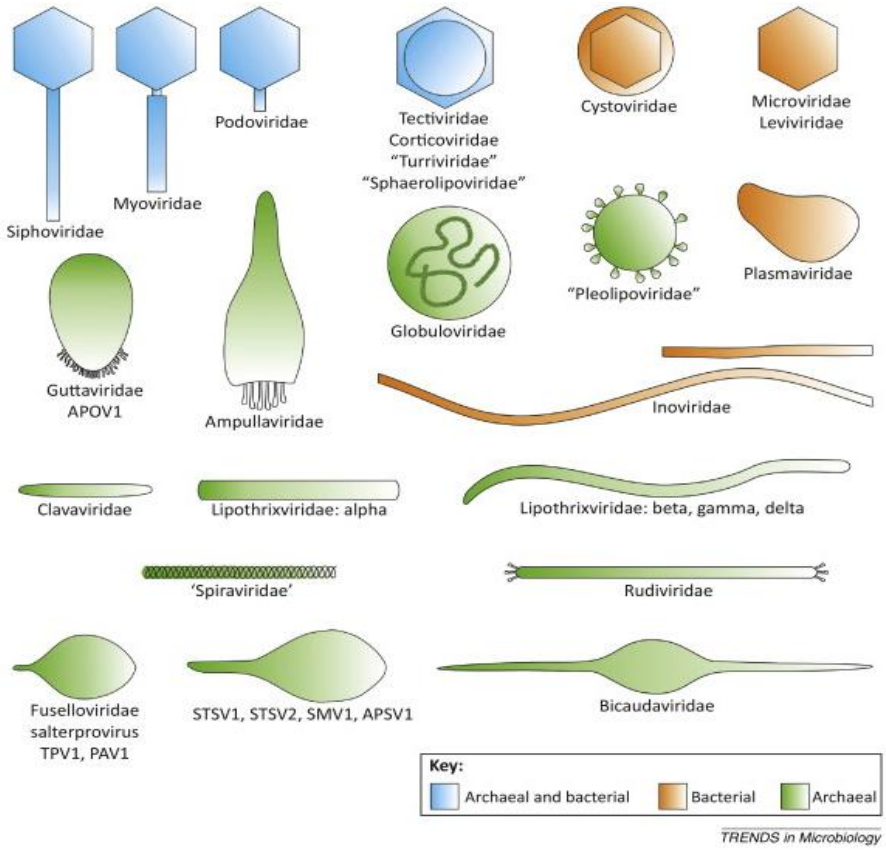


Fig 2.2. Phage morphotypes (Figure adapted from Pietilä *et al*., 2014)

2.7.2. Phage replication

There are two predominant mechanisms of bacteriophage replication, lysis and lysogeny. The environmental factors which determine whether lysogeny is established or whether lysogens re-enter the lytic cycle is currently unknown.

Furthermore, lysis and lysogeny are important factors with the likelihood for the genetic exchange (Chiura and Takagi, 1994). Adsorption of phage on to the host is the first and the most important step in phage multiplication and it is via their tail tips that they recognize the receptor on the host surface. The receptor material on host cell can either be carbohydrate, protein, lipopolysaccharides (LPS) or flagella. The phage genome is introduced into the bacterial cytoplasm either by the contraction of the sheath or by weakening the components of the bacterial envelope by digestive enzymes (Fischetti *et al.*, 2006). Nucleic acid is the only phage component that actually enters the cell.

Most phages produce a lytic infection, which typically involves phage attachment to the cell surface, injection of phage nucleic acid into the cell, intracellular expression of phage genes to produce nascent phage nucleic acid and proteins, assembly of progeny phages, and cell lysis to release progeny phages. In some situations, instead of a lytic infection, some phages remain latent in infected cells – a process called lysogeny (Echols, 1972)

2.7.3. Bacteriophage propelled control of bacterial virulence

Bacteriophages are intimately associated with bacterial virulence genes (Wagner and Waldor, 2002). This relationship likely reflects the mobility that virulence genes derive from being phage encoded, allowing for their widespread dissemination among bacterial populations. Furthermore, virulence genes encoded by phages may withstand environmental exposure better than those encoded by bacteria (Boyd and Brüssow, 2002).

The contribution of bacteriophages to the pathogenicity of their bacterial hosts began to be uncovered as early as 1927, when Frobisher and Brown discovered that nontoxigenic streptococci exposed to filtered supernatants of toxigenic streptococcal cultures acquired the ability to produce scarlatinal toxin (Frobisher and Brown, 1927). Their hypothesis that bacteria acquire virulence

properties had since gained widespread acceptance, as many virulence genes have been transfered by phages among bacteria. Prophage DNA is the major source of DNA sequence diversity between the recently emerged food pathogen *E. coli* O157 and the laboratory *E. coli* strain K-12. The prophage encodes the shiga-like toxin that is responsible for the clinical symptomatology of this enterohemorrhagic *E. coli* strain.

Over time, a number of toxin genes were observed to be phage encoded. This consolidatedthe role of phages in bacterial pathogenesis, emphasizing dissemination of toxin genes among bacterial strains (Bishai and Murphy, 1988). Toxin genes are only a subset of the diverse virulence factors encoded by bacteriophages. Since d'Herelle's time, his notion of the phage as a third variable in bacterial pathogenesis has been proved (d'Hérelle and Smith, 1926). Phages serve as a driving force in bacterial pathogenesis, acting not only in the evolution of bacterial pathogens through gene transfer, but also contributing directly to bacterial pathogenesis during infection.

Historically, exotoxin production has been the most widely recognized bacterial characteristic linked to bacteriophage infection. However, phage infection of bacteria is increasingly associated with additional effects on bacterial virulence. Phages can alter host bacterial properties relevant to all stages of the infectious process including bacterial adhesion, colonization, invasion, and spread through human tissues; resistance to immune defenses; exotoxin production; sensitivity to antibiotics; and transmissibility among humans (Table 2.1).

The most widely recognized examples of phage-encoded virulence factors are exotoxins. Exotoxin production is the major pathogenic mechanism of several bacterial pathogens, including *V. cholerae*, *C. diphtheriae*, and *Clostridium botulinum*

Table 2.2. Bacterial virulence properties altered by bacteriophages

Property	Altered Mechanism	Reference
Adhesion		
E. coli	The λ encoded *lom* gene promotes adhesion to buccal epithelial cells.	Reeve and Shaw, 1979
P. aeruginosa	Phage FIZ15 promotes adhesion to buccal epithelial cells	Vaca *et al.,* 1997
V. cholerae	The toxin-coregulated pilus may be VPIΦ phage encoded.	Karaolis *et al.*, 1999
Invasion		
S. enterica	Phage Gifsy-1 encodes *gipA*, a gene that enhances survival in the Peyer's patch.	Stanley *et al*., 2000
S. pyogenes	Hyaluronidase is phage encoded.	Hynes *et al*., 1995
S. aureus	Fibrinolysin is phage encoded	Carroll *et al.,* 1995
phagocytes		
S. aureus	Phages encode CHIPS, a phagocytotoxin	Rooijakkers *et al.,* 2006
S. pyogenes	Lysogeny up-regulates the antiphagocytic M protein.	Wagner and Waldor, 2002
Exotoxin production		
V. cholerae	Cholera toxin is CTXΦ phage encoded	Davis *et al.,* 2000
C. botulinum	Botulinum toxin is phage encoded.	Hill *et al*., 2007
C. diphtheriae	Diphtheria toxin is phage encoded.	Collier, 2001
E. coli	The Shiga toxins are phage encoded	Wagner *et al*., 2001
Transmission		
V. cholerae	Phage-encoded cholera toxin likely promotes transmission by stimulating copious amounts of watery diarrhea.	Davis and Waldor, 2003

Virulence factors are often produced during lysogeny. Phage-encoded virulence genes are located near the attachment (att) sites of their respective phages, the portion of the integrated phage adjacent to the host chromosome. This finding suggests that they were acquired from the ancestral bacterial chromosome via aberrant phage excision events, perhaps as independently regulated operons. Phage-encoded virulence genes are regulated by chromosome-encoded transcription factors.

2.8. Vibriophages

The first vibrio phages were also identified by d'Herelle in 1926 (d'Hérelle and Smith, 1926), and by the 1950s several distinct types of bacteriophage acting on *V. cholerae* had been described. Most early studies were directed towards the use of cholera phages for treatment or prophylaxis rather than strain discrimination (Pollitzer, 1954). Interest in cholera phages revived with the spread of the seventh pandemic and the appearance in the 1960s of the ElTor biotype, and has proceeded along two distinct lines; the development of phage-typing schemes using lytic phages and classification using lysogeny (Rowe and Frost, 1992). CTXΦ is a *Vibrio cholerae*-specific filamentous bacteriophage that was first identified in 1996 (Waldor and Mekalanos, 1996). In the recent times vibriophages are found to occur in amazingly in large numbers in the environment around the globe.

2.8.1. CTX phage

CTXΦ is a filamentous phage whose single-stranded DNA genome includes *ctx*AB, the genes that encode cholera toxin (CT) (Waldor and Mekalanos, 1996), the primary virulence factor of *Vibrio cholerae* (Kaper *et al*., 1995). CTXΦ produces lysogens, which contain the phage genome integrated into the bacterial chromosome (Mekalanos 1983, Waldor *et al.*, 1997). CTXΦ is unusual among filamentous phages because the phage genome encodes the functions necessary for

a site-specific integration system that can integrate into the *V. cholerae* chromosome at a specific attachment site known as *att*RS (Faruque, 1998a, b). Integration of CTXΦ DNA into the *V. cholerae* chromosome is a site-specific process that does not require RecA (Pearson *et al.*, 1993). Both O1 El Tor and O139 strains of *V. cholerae*, have just one chromosomal locus within which the phage genome is integrated (Waldor and Mekalanos, 1996). The core of this integration site (*attB*) is a 17-bp sequence that is almost identical to an 18-bp sequence within the phage genome. CTXΦ genome has two regions, the core and the RS2. The 4.6-kb core region encodes CT and genes for virion morphogenesis, whereas the 2.5-kb RS2 region encodes genes for the regulation, replication, and integration of the CTXΦ genome. RS2 region consists of three open reading frames (ORFs), *rstR*, *rstA*, and *rstB*, and two intergenic regions, *ig1* and *ig2* (Waldor *et al.*, 1997; Pearson *et al.*, 1993; Rubin *et al.*, 1998). The gene *rstR* encodes the phage repressor, and *rstA* and *rstB* are the phage genes needed for autonomous replication of phage DNA and for phage DNA integration. In toxigenic *V. cholerae*, particularly in El Tor and O139 strains, the CTXΦ genome is integrated in the chromosome flanked by a 2.7-kb repetitive sequence originally referred to as the RS1 element, which is closely related to the RS2 region of CTXΦ, but has an additional ORF termed *rstC* (Campos, 1998, Waldor *et al.*, 1997) (Fig 2.2).

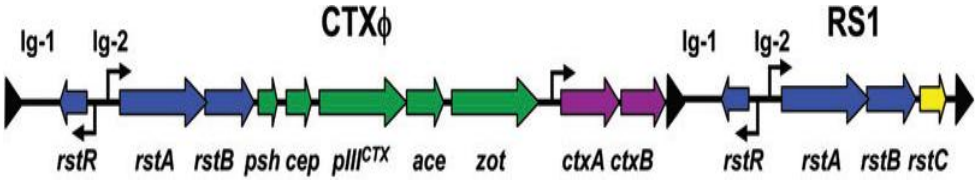


Fig. 2.3. The CTXΦ prophage genome in an array with RS1: The block arrows depict open reading frames and indicate the direction of transcription. The genes coloured green are involved in packaging and secretion of phage DNA. The genes that are almost identical in CTX Φ and RS1 are coloured blue. The genes in purple encode for the A and B subunits of cholera toxin. The one gene in RS1 that does not exist in CTX Φ, *rstC*, is coloured yellow

Phage DNA on integration into the genome of an *attB*$^{+}$, CTXΦ^{-} El Tor strain of *V. cholerae* yields single or tandem prophages flanked by these 17/18-bp sequences, which are known as end repeats (ERs) (Pearson *et al.*, 1993). O1El Tor and O139 strains yield infectious virions at a low but measurable rate, even without induction using mitomycin C (Davis *et al.*, 1999, Kimsey and Waldor 1998). These virions are secreted, rather than released through bacterial lysis, without limiting the growth of the host bacterium. It is likely that virion production by lysogens is preceded by formation of the replicative form (RF) of the phage genome (also known as pCTX). Analysis of the few tranductants derived from these strains suggests that virions were produced after imprecise excision of the single CTX prophage from the chromosome (Davis *et al.*, 1999).

CTXΦ virions are produced for horizontal transmission of phage DNA (transduction) without disrupting the vertical transmission of the prophage (via chromosomal replication). Virion production is not solely an escape mechanism used to release prophage DNA from a damaged or replication-deficient host. CTXΦ is secreted, rather than released by host lysis, is a complementary feature of CTXΦ dissemination. In summary, the CTX genome appears to replicate by a strategy that maximizes stable propagation of the lysogen while still enabling spread of the phage to new hosts (Davis *et al.*, 2000a, Davis and Waldor, 2000)

Production of the filamentous CTXΦ does not destroy its host bacterial cell. In fact, CTXΦ and cholera toxin share a mechanism of nonlethal secretion from the cell.

2.8.2. VPIΦ phage

Karaolis and colleagues (1999) have proposed that the Vibrio pathogenecity island (VPI) is in fact the genome of a filamentous bacteriophage named VPIΦ. VPIΦ is a 39.5kb filamentous bacteriophage that encodes the VPI in *V. cholerae* isolates. It is speculated that VPIΦ encoding TCP is first acquired before CTXΦ

can lysogenize *V. cholerae* (Boyd *et al.,* 2001). As TCP is required for CTXΦ infection of *V. cholerae*, it was proposed that there were two crucial sequential steps in the evolution of pathogenic *V. cholerae*. First, *V. cholerae* strains acquired the *tcp* operon from VPIΦ and second, these TCP^+ strains were infected by CTXΦ and lysogenized.

2.9. Bacteriophage control of bacterial virulence

The contribution of bacteriophages to the pathogenicity of their bacterial hosts were only uncovered when Frobisher and Brown (1927) discovered that nontoxigenic *Streptococci* exposed to filtered supernatants of toxigenic streptococcal cultures acquired the ability to produce scarlatinal toxin as the supernatants contained a bacteriophage encoding the toxin that was transduced. Their hypothesis that bacteria acquire virulence properties has since gained widespread acceptance, as many virulence genes have been transfered by phages among bacteria.

2.9.1 Effect of *in situ* prophage induction in bacterial virulence

Many prophages are induced by environmental conditions that lead to bacterial DNA damage (Freifelder, 1987). The occurrence of lysogeny appears to be under the direct or indirect influence of a variety of environmental and biological parameters such as water temperature, nutrient concentration, primary productivity, and bacterial productivity (Williamson *et al*., 2002).

Pathogenicity of a number of bacteria depends on the presence of extrachromosomal genetic elements in their cells (Wagner and Waldor, 2002). Many pathogenicity factors are encoded in genomes of bacteriophages. Shiga toxin-producing *Escherichia coli* are a group of strains which are pathogenic for humans as they carry prophages bearing genes capable for producing specific

toxins (Schmidt, 2001). Effective production of Shiga toxins occurs only upon prophage induction and its further lytic development (Herold *et al*., 2004).

Phage-inducing agents derived from human cells have been implicated in the pathogenesis of some lysogens. H_2O_2 (a known inducer of λ like phages) can induce Stx-encoding prophages (Livny and Friedman, 2004), which enhances toxin production. *Salmonella* phage Gifsy-2 is also induced by H_2O_2 (Figueroa and Bossi, 1999). Many antibiotics commonly used to treat diarrhea, are known to induce Stx-encoding phages and therefore to promote toxin production (Zhang *et al*., 2000).

2.9.2. Prophage induction in environment

Environmental factors such as inorganic nutrient concentration, temperature, presence of pollutants, nutritional status of the host cell population etc may have a direct influence on viral replication and prophage induction (Wagner and Waldor, 2002). Many environmentally important pollutants are inducing agents for natural lysogenic viral production in the marine environment (Cochran *et al*., 1998).

Induction of the lytic cycle commonly occurs following stimulation of the lysogenic cell by environmental factors such as ultraviolet light or other DNA damaging agents (Weinbauer and Suttle, 1999). It has been demonstrated that polyaromatic hydrocarbons may induce lysogenic marine microbial communities (Jiang and Paul 1996). Changes in temperature acting as an inducing factor have only been observed with specifically isolated temperature-sensitive mutants from enteric host-phage systems (Edgar and Lielausis, 1964). Hydrogen peroxide, produced *in vivo* by attacking phagocytes results in lytic induction of *S. pyogenes* prophages (Brüssow *et al*., 2004).

2.10. Genetic exchange between bacteria in the environment

The acquisition of foreign DNA conferring novel phenotypes to bacteria is a major step in the evolution of pathogenic bacteria from their non-pathogenic ancestors (Hacker and Kaper, 2000; Fitzgerald and Musser, 2001). In bacteria, gene transfer takes place by transformation, transduction, or conjugation (Mordacq and Ellington, 1998). The genomes of bacteria are remarkably fluid. A significant part of the bacterial DNA is not acquired by the descendents from the parental cell ('vertical' transfer), but is acquired horizontally by transformation, conjugation or transduction ('lateral' transfer) (Bushman, 2001).

2.10.1. Transformation

Natural genetic transformation of bacteria encompasses the active uptake by a cell of free (extracellular) DNA (plasmid and chromosomal) and the heritable incorporation of its genetic information (Dubnau, 1999). It is a mechanism of horizontal gene transfer and depends on the function of several genes located on the bacterial chromosome. Bacteria are the only organisms capable of natural transformation. Gene transfer by transformation does not require even a living donor cell, because release of DNA during death and cell lysis suffices to provide free DNA (Chen and Dubnau, 2004). A close genetic relationship between donor and recipient cells is not necessary for transformation with plasmid DNA. Many species of bacteria are naturally transformable. Transformation plays a profound role in the genetic adaptation of bacterial populations to contribute to evolution and speciation (Smith *et al*., 1999).

2.10.2. Conjugation

Conjugation, occurs when one bacteria transfers DNA to another bacteria. Lederberg and Tatum (1946) showed that bacteria could transfer genetic material from one cell to another. Transfer of DNA between bacterial cells was directional

with one cell acting as the donor and the other cell acting as the recipient. It was found that the donor cells contain a fertility factor (F^+) whereas the recipient lacks the fertility factor (F^-). F is a small circular piece of DNA called an episome which can replicate autonomously within the bacterial cell or can integrate into the host chromosome. Genes found on F encode fibrous proteins that make up minute tubules called pili. The F pili enable the F^+ cells to attach to other cells and the F^+ cell transfers a copy of its DNA to the F^- cell. It is also possible for the F factor to integrate into the host chromosome and thus transfer parts of the host chromosome.

There is evidence that bacteria can directly transfer DNA into the cells of plants, fungi, and mammals via the conjugation mechanism (Mizuta, 2012).

2.10.3. Transduction

Transduction is the process by which a bacterial phage (virus) picks up a piece of DNA from one bacterial cell and injects it into another where it is incorporated into the chromosome. In the process of transduction, bacterial genes are incorporated by bacteriophage particles and transferred to another bacterium. Bacteriophages act as agents of 'mobile DNA'. A temperate phage infection on bacteria either results in multiplication of the phage with concomitant lysis of the bacterial host or lysogenization, (i.e. integration of the phage DNA into the bacterial chromosome as a prophage). Lysogeny is more the rule than the exception and many bacteria contain multiple prophages. Some temperate phages change the phenotype of the bacterial host ('lysogenic conversion genes', LCG). Lysogeny is a motor of short term bacterial evolution (Carols, 2003)

Tailed phages are very efficient gene-transfer element and represent densely compacted phage DNA encased in its head. Phage tail and its associated fibres act as an effective DNA transfer device. It assures both the specific host cell recognition and injection of the phage DNA into the bacterial cell. (Zhang *et al*.,

2000; Molineux, 2001; Kanamaru *et al*., 2002) The global population of tailed phages in the environment turns over every few days, from which it is calculated that the number of phages initiating an infection somewhere on Earth every second is on the order of Avagadro's number (Wilhelm *et al.,* 2002).

Transduction may be either "generalized" (e.g., by coliphage P1), whereby any bacterial gene may be transferred, or "specialized" (e.g., by coliphage lambda), where only genes located near the site of prophage integration are transferred.

2.10.3.1. Specialised transduction

Specialized transduction occurs when a small segment of flanking bacterial DNA is copackaged with the phage DNA on prophage excision from the heterologous host. Resolvase-type integrases from phages of Gram-positive bacteria have no requirements for cofactors facilitating their integration into heterologous hosts (Groth *et al.,* 2000*).* Prophages from low GC content Gram-positive bacteria frequently contain extra genes in the vicinity of attR gene. These genes differ in GC-content from the surrounding DNA and suggest a phage-mediated gene transfer from a host differing in GC content (Ferretti *et al.,* 2001). In the case of pathogenic bacteria, these extra genes frequently encoded important virulence factors like bacterial toxins (Smoot *et al*., 2001; Baba *et al.,* 2002; Beres *et al.,* 2002).

LCG were also identified in prophages from Gram-negative bacteria. Some of them were located at the prophage genome ends but the majority of the extra genes or 'morons' (for more DNA) were detected in the centre of the prophage genomes. LCG (lysogenic conversion genes), were shown to respond to environmental cues (Wagner *et al*., 2001).

2.10.3.2. Generalised transduction

Generalised transduction in phages which commit the error to package large amounts of bacterial DNA instead of phage DNA (eg: Salmonella phage P22, coliphage Mu). Upon infection of the next host, this bacterial DNA can be incorporated into the bacterial chromosome.

Transduction of the agarase gene between *V. alginolyticus* and *V. parahaemolyticus* (Baross *et al*., 1978) is a typical example of generalized transduction. Supernatants of the O1El Tor strains were used in transduction assays to determine whether insertion of a prophage fragment could rescue a solitary prophage (i.e., enhance its capacity to yield virions). It was found that insertion of a 1,280-bp sequence, downstream of a single prophage was as effective as insertion of a complete prophage in rescuing virion production (Davis *et al.,* 2000b). Studies with CTXΦ have shown that the efficiency of transduction was considerably higher in vivo, since the phage receptor TCP is known to express more efficiently under in vivo conditions (Faruque *et al.,* 2002).

2.11. Phage genomics

Phage genome size varies enormously, ranging from the ~3300 nucleotide ssRNA viruses of Escherichia coli (Friedman *et al*., 2009) to the almost 500 kbp genome of *Bacillus megaterium* phage. The smallest of the dsDNA tailed phages genomes are ~11.5 kbp *Mycoplasma* phage P1 (Hatfull and Hendrix, 2011), ~21 kbp *Lactococcus* phage c2 (Lubbers *et al*., 1995), and ~30 kbp *Pasteurella* phage F108 (Campoy *et al*., 2006) in *Podoviridae, Siphoviridae* and *Myoviridae* respectively. In general, these genomes are packaged at similar densities into their capsids and the size of the capsid varies as a function of genome size. There are evolutionary pressures to either gain or lose DNA to accommodate packaging and virion stability (Hatfull *et al*., 2010).

Nucleotide sequence comparison of bacteriophage genomes reveals them to be enormously diverse (Hatfull, 2008). There are fewer than 50 each of completely sequenced RNA phages and ssDNA phages, and of the more than 500 sequenced dsDNA tailed phages, ~55% are morphologically members of the *Siphoviridae* with long flexible non-contractile tails; the remainder are *Myoviridae* with contractile tails and *Podoviridae* with short stubby tails (~25% and ~20% respectively). Overall, phage genomes represent only about 15% of all viruses with known unique sequences and are thus vastly under-represented in the genome databases (Hatfull and Hendrix, 2011). The GC% content of the large collection of mycobacteriophages varies between 55% and 70% (Pope *et al*., 2011).

The core genes that are shared by all members of the group fail to engage in horizontal exchange relative to the other genes in the group (Sullivan, 2005). There is little or no evidence of horizontal swapping of the head gene regions of most phages, even though other parts of the genome may be flamboyantly mosaic. Tail genes and lysis genes are similar groups of genes that ‘‘travel together through evolution’’.

Non-core genes are not found in all members of a group of related phages. Non-core genes are found in all tailed phage genomes; they are often in small clusters of genes, with the clusters interspersed among the clusters of core genes. Functions of the non-core genes are unknown, and in phages like coliphage λ, they have been deleted without adverse effect on phage growth under laboratory conditions. The non-core genes are moving in and out of the phage genomes on a much faster time scale than the core genes (Hatfull *et al.*, 2010).

Different segments of phage genome have distinct evolutionary histories, they are pervasively mosaic. Horizontal genetic exchange plays a dominant role in shaping these genome architectures. All genes in a given genome do not participate in mosaicism to the same degree (Juhala *et al*., 2000). Genome

mosaicism can be observed by comparing genes at the amino acid sequence level (Hendrix *et al.,* 1999).

There are many different host-mediated protection systems such as restriction-modification (King and Murray, 1994), CRISPR's (Deveau, 2010), tRNA cleavage (Amitsur *et al.,* 1987), and toxin–antitoxin systems (Fineran *et al.,* 2009), as well as phage-encoded mechanisms for generating genome diversity at high frequency (Medhekar and Miller, 2007).

2.12. Horizontal gene transfer

Lateral or horizontal gene transfer (HGT) is defined as the exchange of genes between different strains or species (Ochman *et al*., 2000). HGT introduces new genes into a recipient genome that are either homologous to existing genes, or belong to entirely new sequence families. During the process of Horizontal Gene Transfer, a piece of DNA (e.g., a gene) is transferred from one organism to another, which is not its offspring (De la Cruz and Davies, 2000). The genetic material is stably incorporated in the acceptor genome, in contrast to the vertical inheritance of genes by descent from one's parents. Whether the genes are transferred by transposons, viruses, bacteria, or other vectors, or perhaps through direct contact or initial hybridization-like events, the horizontal flow of genes is a part of the story of life (Syvanen, 2012).

HGT is known as an important mechanism to shape the genomes of bacteria (Ochman *et al*., 2000; Boucher *et al*., 2003), but recently there is also an accumulation of data indicating that this process also occurs in the evolution of eukaryotes (De la Cruz and Davies, 2000; Andersson, 2005) and archaea (Nelson *et al*., 1999; Diruggiero *et al*., 2000). The amount of adopted genes varies between virtually none in organisms with small genome size, for example, *Rickettsia prowazekii, Borrelia burgdorferi,* and *Mycoplasma genitalium*, to nearly 17% in *Synechocystis* PCC6803 (Ochman *et al*., 2000).

Large-scale genomic sequencing of prokaryotes has revealed that gene transfer is an important evolutionary mechanism for these organisms (Doolittle, 1999). HGT has been linked to the acquisition of drug resistance by benign bacteria (Dutta and Pan, 2002). A key issue for this emergence will be the integration of antibiotic resistance genes in gene-transfer elements (e.g. plasmids), a feature that is favoured by the release of antibiotics in natural ecosystems (Cattoir *et al*., 2008).

2.12.1. Lateral gene transfer between phages

There are evidences of apparent transfer of virulence genes between phages belonging to different phage groups (Mirold *et al.*, 2001) or phages infecting different bacterial species (Desiere *et al.*, 2001) which increases the lateral spread of these genes in bacteria. Analyzis of the sequenced data from coliphages and dairy phages has demonstrated that large phage gene clusters were transferred between distinct groups of phages (Ravin *et al.*, 2000). The strikingly different GC-content of the left and right arm of phage lambda suggests its heterologous origin. The mosaic character of phages was greater in Gram-negative than in Gram-positive bacteria (Bru¨ ssow and Desiere, 2001). This suggested homologous recombination as the driving force for lateral gene transfer between phages (Clark *et al.*, 2001). The comparison of other lambdoid coliphage genomes suggested that non-homologous recombination occurs everywhere and the observed order in phage genome organisation is the consequence of selection forces eliminating all non-viable recombinants (Juhala *et al.*, 2000; Lawrence *et al.*, 2002).

2.12.2. Interkingdom Horizontal Gene Transfer

Interkingdom gene transfer between yeast CTG clade (*C. parapsilosis)* and bacterial species was studied by Fitzpatrick *et al*., (2008). Two strongly supported incidences of HGT, both within the *C. parapsilosis* genome were identified (Struhl *et al*., 1976). Independent transfers into the Pezizomycotina, Basidiomycotina and Protozoan lineages were also identified.

The loss of eukaryote genes and subsequent reacquisition of a prokaryotic copy has previously been described in yeast, and can confer specific metabolic capabilities (Hall and Dietrich, 2007). The acquisition of the *URA1* gene (encoding dihydroorotate dehydrogenase) from *Lactobacillus* and replacement of the endogenous gene in *S. cerevisiae*, have allowed growth under anaerobic conditions. Similarly, acquisition of *BDS1* (alkyl-aryl-sulfatase) from proteobacteria have enabled the survival of *S. cerevisiae* in a harsh soil environment (Anderson *et al*., 2003).

The well-known transfer, by conjugation, of part of the Ti plasmid DNA from *Agrobacterium tumifaciens* to plants and to yeast (Zupan and Zambryski, 1995) demonstrates the horizontal transfer of genes between different phylogenetic kingdoms. Syvanen (1985) announced the creation of the first mouse carrying transgenes from bacteria. There are viruses with a broad host range that grow in different species and hence have the potential to move DNA or RNA from one host species to another. Evidence suggests that host-parasite interactions promote HGT (Gilbert *et al.*, 2010).

2.12.3. Lateral gene transfer in Eukaryotes

HGT events attributed to introgression have been observed between duck species (Kraus, 2012), between butterfly species involved in mimicry (*Heliconius* Genome Consortium), and between human and Neanderthal (Currat and Excoffier, 2011). P element movement into *Drosophila melanogaster* during historic times was already widely accepted two decades ago, as discussed by Kidwel (1993).

HGT among flowering plants is discussed by Syvanen (1994). The chaotic phylogenetic relationships among plants remain unresolved, and a consideration of horizontal gene flow could help solve the puzzle. In plants, one of the important mechanisms of HGT involves pollination between different species (Kane, 2009). There are recent studies of whole chloroplasts moving from one plant species to another when the mature plants are in physical contact with each other (Stegemann, 2012)

2.12.4. Detection of Horizontal gene transfer

The ability to detect gene transfer is dependent on the fate of the transferred DNA once it enters the recipient cell. Many bacteria possess DNA restriction systems which destroy foreign DNA (Brunel and Davison, 1979).Several approaches have been published that discover single HGT events (Lerat *et al*. 2003). Phylogenetic tree analysis will continue to provide the best set of tools for comparative genomics. Finding trees with reticulations or nets (as representations of HGT events) is even more difficult (Collins *et al*, 2011). The substitution of a tree-like by a web-like representation of the phylogenetic relationship between bacteria is an evidence of the impact of HGT on microbial evolution (Huson and Scornavaccao, 2011). Another way of detecting horizontally transferred genes uses bacterial genome sequences to examine the nucleotide composition (GC content) and usage of different codons (Lawrence and Ochman 1997).

Analysis of phylogenetic tree topologies helps to decipher evolutionary scenarios, including horizontal gene transfer events (Syvanen, 1994). The presence of three or more genes in the same order in distant genomes is extremely unlikely unless these genes form an operon (Syvanen, 2012). Genes whose nucleotide or codon compositions are significantly different from the mean for a given genome are considered as probable horizontal acquisitions (Lawrence, 1997).

An analysis, based on the differences in base composition and codon utilization patterns, has permitted an estimation of the extent of this horizontal gene transfer (Lawrence, 1999).

Chapter 3

MATERIALS AND METHODS

3.1. Screening and identification of temperate phage containing environmental isolates of *Vibrio cholerae*

3.1.1. Sources of Samples

Vibrio cholerae were isolated from water and sediment samples from aquafarms and mangroves along the coastal regions of Ernakulam and Alappuzha, Kerala, South India. All environmental isolates and the *Vibrio cholerae* strains from the culture collection in the Microbial Genetics Laboratory of the Department of Biotechnology, CUSAT, were screened for the presence of temperate phages.

3.1.2. Collection of samples

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 h of collection.

3.1.3. Preparation of serial dilutions of the sample

Water samples in 10 mL aliquot were added aseptically to 90 mL of physiological saline and thoroughly mixed. For sediment samples, 10 g was weighed and transferred aseptically to 90 mL of physiological saline. A series of 10 fold dilutions of these samples was prepared in physiological saline (0.95% NaCl) and 50 µL of 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, Mumbai, India) plates by spread plate method and incubated at 37^{o}C for 24 h. Isolated single colonies were picked, purified on nutrient agar (NA) (HiMedia) 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 Marine Oxidation Fermentation (MOF) test

The isolates were Gram stained 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 water. This test uses tetra-methyl-*p*-phenylenediaminedihydrochloride (Kovac's reagent), as an artificial electron donor for cytochrome c. When the reagent is oxidized by cytochrome c, it changes from a colorless to dark blue or purple compound, indophenol blue.

A piece of filter paper was immersed in 1% Kovács oxidase reagent and allowed to dry. A well-isolated colony from a fresh (18 to 24 h culture) culture was spotted on treated filter paper and observed for color change. Isolates were segregated as oxidase positive when the color changed to dark purple within 5 to 10 seconds.

The oxidative-fermentative (O/F) test determines the ability of the isolate to metabolize glucose either 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. 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) was sterilized by autoclaving at 15 lbs for 15 minutes. Added 1% glucose to the sterile basal medium and transferred 4 mL aliquots aseptically into sterile tubes and autoclaved at 10 lbs for 8 minutes. 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 h. 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.

3.1.6. 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.

3.1.6.1. 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 h. 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. 1.6.2. Glycerol stocking

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

3.1.7. Molecular characterization and 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 to identify *Vibrio cholerae*.

3.1.7.1. Genomic DNA isolation

Chromosomal DNA was isolated as described by Ausubel *et al.*, (1987). Log phase culture (2 mL) was taken in sterile microfuge tube and centrifuged at 5000 x g (Sigma 2-16K, Germany) for 10 min at 4 ºC. The supernatant was discarded and the cell pellet was blotted dry. The pellet was resuspended in 875 µL of Tris EDTA (TE) buffer (pH 8; Appendix-2). 5 µL proteinase K (10 mg/mL stock; Sigma- Aldrich, USA) and 100 µL of 10 % SDS was added, mixed gently and incubated at 37 ºC for 1 h. Equal volume of phenol-chloroform mixture (1:1) was added to this, centrifuged at 12000 x g for 10 min at 4 ºC. The supernatant was transferred to another sterile tube using sterile cut tip. The extraction with phenol-chloroform was repeated three times. To this supernatant, 0.1 volume of 5 M sodium acetate (pH 5.2) and double volume of ice-cold isopropanol were added. This was kept at -20 ºC for about 1 h and then centrifuged at 12000 x g for 10 min at 4 ºC. The DNA pellet obtained was washed with 70% ethanol, air dried and dissolved in 50 µL TE buffer (pH 8).

The concentration of genomic DNA thus obtained was estimated by the spectrophotometric method (Shimadzu UV-VIS Spectrophotometer, Japan). Purity of the DNA was also assayed spectrophotometrically using 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^{o}C until further use. An appropriate dilution (~80-100 ng) of genomic DNA was used as template for PCR reactions.

3.1.7.2. Identification of *V. cholerae* isolates using *omp*W marker gene

Among the six outer membrane proteins, OmpW is of marker value as 97% of all *omp*W positive isolates can be identified as *Vibrio cholerae*. The strains positive for *omp*W was recognized by the PCR amplicon of 588 bp size.

Primer	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
***omp*W**	F-ACCAAGAAGGTGACTTTATTGTG R- GAACTTATAACCACCCGCG	588	Nandi *et al*., 2000

Annealing – 65^{o}C for 1 min.

Extension – 72^{o}C for 1.5 min.

3.1.7.3. Polymerase Chain Reaction (PCR)

PCR was performed using the genomic DNA (~100 ng) as template gene specific primers as detailed above. The reactions contained 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1X *Taq* Buffer (10 mM Tris-HCl, (pH 8.3), 50 mM KCl), 0.5 µM each of the specific primers, 1U *Taq* DNA Polymerase, 1 µL (100 ng) template DNA and H_2O to a final volume of 20 µL. Amplifications were carried out in a Thermal Cycler (BioRad MJ Mini Gradient, USA) using the following thermal program: an initial denaturation of 94 ºC for 5 min, followed by 30 cycles of 94 ºC

for 1 minute, annealing at 65 ºC for 1 min and extension at 72 ºC for 90 sec, ending with a final extension step of 72 ºC for 10 minutes.

3.1.7.4. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to visualize the 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 10 mg/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, Bangalore, India) and the gel pictures were captured with gel documentation system (Syngene, UK).

3. 1.7.5. 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). A portion of the 16S rRNA gene (1.5 kb) was amplified from the genomic DNA as described in section 3.1.7.3 at a different temperature for primer annealing. Products after PCR amplification were purified by gene clean kit (Genei, India) and subsequently sequenced, followed by homology analysis.

Primer	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
16S	F- AGTTTGATCCTGGCTCA	1500	Shivaji *et. al.*, 2000
	R- ACGGCTACCTTGTTACGACTT		

Annealing – 56 ºC for 1 min.

Extension – 72 ºC for 1.5 min.

3. 1.7.6. DNA sequencing and *in silico* analysis of the 16S rDNA sequences

Nucleotide sequences of the 16S rRNA gene PCR amplicon were determined by the ABI XL DNA analyzer, using the big dye Terminator kit (Applied Biosystems, USA) at SciGenom Cochin, India Ltd. Identity was determined by comparing the sequences obtained with those available in the GenBank database using Basic Local Alignment Search Tool (BLAST) software (Altschul *et al*., 1990) at NCBI site. (http://blast.ncbi. nlm.nih.gov). The sequence was deposited in the GenBank database and accession number was obtained for the submission

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

Identification of the serogroups of *V. cholerae* strains was done by screening the isolates for O1 *rfb* and O139 *rfb* marker genes by PCR amplification (Hoshino *et al*., 1998). The genes O1 *rfb* and O139 *rfb* code for the component protein of bundle form pili of the O1 and O139 serotypes respectively, the pandemic causing strains of *V. cholerae*. A positive amplification indicates that the isolate belongs to the respective serotype. The gene was amplified as described in section 3.1.7.3 at a different temperature for primer annealing and was visualised using agarose gel electrophoresis as stated in section 3.1.7.4.

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

Annealing - 62 ^{o}C for 1 min

Extension - 72 ^{o}C for 2 min

3.1.8. Screening of *V. cholerae* strains for prophages

The environmental isolates (n=6) and the *V. cholerae* isolates (n=44) from the culture collection in Microbial Genetics Lab were all screened for the presence of lysogenic prophages. The presence of lysogenic phages in *V. cholerae* indicates their inherent potential for horizontal gene transfer. Phage presence may provide the host with additional virulence determinants.

3.1.8.1. Induction of lysogenic phages

Mitomycin C was used for induction of lysogenic phages as per protocol described by Yee *et al.* (1993) with modifications. *V. cholerae* strains were grown in Luria broth (LB) (HiMedia) at 37 °C until an absorbance of 0.5 at 600 nm. Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added at 1 µg/mL and incubated overnight at 37 °C. The culture supernatants were passed through 0.22 µm membrane (Millipore, USA). The filtrate was used as putative phage lysate to screen for plaque forming ability by double agar overlay method. The filtrates were assayed for phage after appropriate dilution (Faruque *et al*., 1998b).

3.1.8.2. Double agar overlay method

The filtrate was then assayed by the double-agar overlay method of Adams (1959) with modification. The filtrate was titrated for infectious phage particles by incubating aliquots of the fitrate with recipient strain, MAK 757 (ATCC® 51352™). The logarithmic phase cells (1 mL) of the recipient bacterial strains in Luria broth (HiMedia) were mixed with 1 mL of the serially diluted lysate and were incubated at 37 ºC in a water bath (Scigenics, Chennai, India) for 30 minutes. After incubation, 2 mL of sterile soft agar (nutrient broth containing 0.8% agarose) was added to this, mixed well and was immediately overlaid on nutrient agar plates (base plates) with ampicillin. After overnight incubation at 37 °C, plates were observed for plaque formation, and expressed as plaque forming

units / mL (PFU/mL) of supernatant. Phage free cultures (containing only bacterial host) and host-free cultures (containing only phage) were used as controls. A sample was scored positive for phages when plaques were observed on the bacterial lawn in the plates.

3. 1.8.3. Tetrazolium staining

Tetrazolium staining helps to improve phage plaque visibility against the backdrop of the bacterial growth, whereby each plaque can be observed as a sharp, clear area against the intense red background produced by the reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC) to the insoluble formazan by the bacterial cells (Pattee, 1966). The petri plates with plaques were flooded with 10 mL of trypticase soy broth (HiMedia, India) TTC. After incubation at 37^{o}C for 20 minutes, the broth was poured off and the plaques were observed.

3.2. Molecular characterization of phage containing environmental *Vibrio cholerae*.

3.2.1. Molecular typing of lysogenic *V. cholerae*

The *V. cholerae* strains with lysogenic phages were further characterised by molecular typing methods such as ERIC PCR and BOX PCR, with specific primers and using genomic DNA (~100 ng) as template. All PCR reactions and the thermal profile for the PCR were performed as described in section 3.1.7.3 unless otherwise stated. The amplified products produced a banding profile, depending upon the number and position of the repeated units, on a 2% agarose gel prepared as described in section 3.1.7.4. The gel pictures were captured and the banding patterns were analysed using Genetool software (Version 1.4). Reproducibility of ERIC-PCR and BOX-PCR results were assessed by repetition in 3 independent assays.

3.2.1.1. Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR

ERIC-PCR is a Rep-PCR that employs the enterobacterial repetitive intergenic consensus (ERIC) sequence as the target for PCR. It has been used to identify *V. cholerae* clonal lineages. 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 ^{o}C for 30 sec
Extension - 72 ^{o}C for 4 min

3.2.1.2. 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 ^{o}C for 30 sec
Extension - 72 ^{o}C for 4 min

3.2.2. PCR based screening for virulence/ virulence associated genes in *V. cholerae* with inducible prophages

Screening for virulence/ virulence associated genes was performed employing PCR in which gene specific primers were used. The genes, *ctx*A and *ctx*B (the A and B subunit of CT gene), *ace* (accessory cholera toxin), *zot* (zonula occludens toxin gene), *tox*R (an essential toxin regulating gene), *tcp*A (the major subunit of TCP gene) and *hly*A (an alternate diarrhoegenic factor) were determined by PCR. Presence of *nan*H (encoding neuraminidase) and *int* (encoding bacteriophage-like integrase), were determined by multiplex PCR. All PCR reactions and the thermal profile for the PCR were performed as described in section 3.1.7.3 unless otherwise stated. Electrophoresis on agarose gel was performed as outlined in section 3.1.7.4.

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

Toxigenic *V. cholerae* carry one or more copies of 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 ^{o}C 1.5 min

Extension - 72 ^{o}C 1.5 min

3.2.2. 2. 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 a 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	316	Trucksis *et al*., 1993

Annealing	-	55 ^{o}C	1 min
Extension	-	72 ^{o}C	0.5 min

3.2.2.3. 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
*hly*A	F- GGCAAACAGCGAAACAAATACC R- CTCAGCGGGCTAATACGGTTTA	481	Hall and Drasar, 1990

Annealing	-	60 ^{o}C	1.5 min
Extension	-	72 ^{o}C	2 min

3.2.2.4. PCR detection of *tox*R

The ToxR protein acts as the master regulator for 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
*tox*R	F- CCTTCGATCCCCTAAGCAATAC R- AGGGTTAGCAACGAGCGTAAG	779	Rivera *et al*., 2001

Annealing	-	60 ^{o}C	1 min
Extension	-	72 ^{o}C	2 min

3.2.2.5. Multiplex PCR detection of *tcp*A and Zot

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). The *zot* gene 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
*tcp*A	F- CACGATAAGAAAACCGGTCAAGAG R- CGAAAGCACCTTCTTTCACACGTTG	451	Keasler and Hall, 1993
zot	F- TCGCTTAACGATGGCGCGTTTT R- AACCCCGTTTCACTTCTACCCA	947	Shi *et al*., 1998

Annealing	-	60^{o}C	1.5 min
Extension	-	72^{o}C	1.5 min

3.2.2.6. Multiplex PCR detection of *nan*H and *int*

The gene *int*, a bacteriophage like integrase gene, is part of the virulence profile in *V. cholerae* (Figueiredo *et al*., 2005). The *nan*H gene in *V. cholerae* produces neuraminidase, a virulence factor that releases sialic acid from higher gangliosides present on eukaryotic cells surface, exposing ganglioside GM1, the cholera toxin receptor (Jermyn and Boyd, 2002). The presence of these two virulence genes in the environmental isolates was identified by multiplex PCR.

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reference
int	F- TTACGTAACGCTACGGCAT R- CAAGGTGCCATCGATCAG	1000	Tapchaisri *et al*., 2007
*nan*H	F- ATCTGATGGCGGCAATC R- GCGGCTTCAATGACATC	1900	

Annealing	-	62 ^{o}C	1 min
Extension	-	72 ^{o}C	2 min

3.2.3. *In silico* analysis of partial gene sequence of TcpA protein of ALPVC3

The *tcp*A gene was sequenced by Sanger's Dideoxy method using ABI 3730 Excel (Applied Biosystems) at Scigenom Labs and analysed using various bioinformatic tools.

The identity of the sequence was determined by comparing with sequences in the NCBI database using the online tool BLAST (Altschul *et al*., 1990). The partial gene sequences were deposited at NCBI and accession numbers were obtained.

The Open Reading Frame (ORF) in the nucleotide sequences was determined using NCBI ORF finder (Wheeler *et al*., 2003). The deduced

aminoacid sequence was obtained by translation of the nucleotide sequence using online tool ExPasy (Gasteiger *et al*., 2003) and the identity of the sequence was confirmed by pBLAST. Amino acid sequences were aligned using ClustalW2 (Larkin *et al*., 2007). Based on the conserved domains present, the TcpA was assigned to families in SCOP database (Rawlings and Barrett, 1993). Protein modeling was done using Phyre^2 (Kelley and Sternberg, 2009) with the deduced amino acid sequences. The secondary structures encoded by the amino acid sequences were also predicted using the software.

3.3. Differential induction and purification of prophages.

3.3.1. Host range studies and identification of indicator strain

Further studies on the lysogenic phages are possible only by identifying a suitable indicator strain for large scale production of phages. The host range of the phage was assessed on the basis of its ability to form plaques (Goodridge *et al*., 2003) on *V. cholerae* strains present in the Microbial Genetics Lab culture collection. A total of 44 cultures were tested including the standard strains of *V. cholerae* O139, CO336 and phage host for El Tor serovar O1 typing scheme, MAK 757 (Appendix-5). All the strains used in the study were grown at 37°C in Luria broth (HiMedia, Mumbai, India). The phage lysate was added to the cultures which were in their exponential phase, incubated for 1 h and plated using the double agar overlay method. All plating was performed in triplicates. The plates were incubated at 37 ºC and were observed for plaques.

The strains producing clear plaques were collected as indicator strain/recipient strain (Mahony *et al*., 1985). The indicator strain yielding the largest number of plaques for a given phage suspension was defined as the propagating strain for that phage suspension. Each phage was then propagated in its propagating strain.

3.3.2. Effect of various biotic and abiotic factors on phage induction and bacterial growth.

The knowledge on specific conditions for induction of the prophages in bacteria occurring in environment is very limited. Different prophages may be induced with different efficiency by various agents, and that their development may be modulated by biotic factors, like temperature, nutritional status, quality of light and growth rate of the host. Differential induction was studied using the four strains of *V. cholerae viz* ALPVC3, ALPVC11, ALPVC12 and EKM14.

V. cholerae strains with lysogenic phages were grown in Luria-Bertani (LB) medium at 37^{o}C for six h with constant shaking (Scigenics, Chennai, India) at 120 rpm. The cells were harvested by centrifuging at 5000 x g for 10 min (Sigma, Germany) and were resuspended in fresh broth.

All the four strains (ALPVC3, ALPVC11, ALPVC12 and EKM14) were equally aliquoted to study the prophage induction under six different treatments. mitomycin C, nalidixic acid, NaCl and H_2O_2 were added in to the cultures at specified concentrations outlined in table 3.1.

The influence of temperature on induction of propages was studied by exposing the mid log phase bacterial culture to 60 °C in water bath (Łoś *et al*., 2009).

To study the effect of UV light, the bacterial cultures in petriplates were irradiated for 1 minute with a 15W Philips TUV P/40 bactericidal lamp of wavelength 254 nm which delivers approximately 1 phage T4 hit per 3 sec (Georghiou *et al*., 1981). After irradiation incubation treated cultures were continued grow in Luria-Bertani (LB) broth at 37 ^{o}C in the dark in order to minimize photoreactivation.

Samples of bacterial cultures were withdrawn at 30 min intervals (up to 5 h) and equal volume of chloroform was added to the sample. Samples were vigorously shaken and immediately centrifuged at 9000 x g for 10 minutes. The culture supernatants were filtered through 0.22 µm membrane and was analyzed for phage by double agar overlay method as described in section 3.1.8.2 using MAK757 as reciepient strain.

Table 3.1. Induction agents used in this study

Treatment	**Mode of action**	**Mode of application**
Mitomycin C (MMC) (Sigma Aldrich, USA)	Inhibits DNA replication by cross-linking DNA strands	1.0 µg/mL
UV (15W philips TUV P/40 bactericidal lamp)	Mutation agent and potent induction agent.	254 nm
Nalidixic acid (HiMedia, Mumbai, India)	Quinolone antibiotic which inhibits DNA replication.	3.0 µg/mL
Temperature	Natural factor associated with prophage induction	60 ° C
NaCl	High salt concentration was demonstrated to induce prophage	2 M
H_2O_2	Agents that induce oxidative stress	3 mM

Strains grown in a similar way but without induction agents were used as controls. Assays were carried out in triplicates. The graph was plotted with log of relative phage titer against time. Relative phage titer is adopted as there are chances of spontaneous induction in case of temperate phages even in absence of

an induction agent. So the presented values were normalized to results of control experiments (without induction), *i.e.* they represent ratios of phage titers in induced and non- induced cultures.

Separate culture tubes with side arm were used to study the effect of induction agents on bacterial growth. This facilitated OD observation at regular intervals (30 min) in colorimeter without opening the tubes. Host cells were inoculated in 5 mL LB broth. All the treatments were given at mid log phase *i.e*, when the OD at 600 nm is ~1. The changes in the growth curve after induction was analysed from the graph. All results are an average of triplicate experiments and standard deviation was determined and graphs were plotted using Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA).

3.3.3. Optimization of different parameters for phage induction using mitomycin C

Various physico-chemical parameters affecting phage were optimized towards maximum phage production. The parameters optimized included mitomycinC C concentration, incubation temperature, incubation time, concentration of ampicillin in base plate and growth phase of bacterial culture. *Vibrio cholerae* strains were grown in Luria broth (LB) at 37 °C. The cells were collected by centrifugation, washed, and resuspended in fresh LB before the inducing agent was added in to the aliquot. After each treatment aliquots of the culture supernatants were filtered through 0.22 μm membrane (Millipore, USA). The filtrate was used as putative phage lysate for assay using double agar overlay to analyse the plaque formation after phage induction (Faruque *et al*., 1998a).

All the results are an average of triplicate experiments and standard deviation was determined and graphs were plotted using Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA).

3.3.3.1 Effect of mitomycin C

The bacterial suspension was divided into aliquots, to which mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added in concentrations ranging from 0.1-2.5 µg/mL and incubated overnight at 37 °C (Faruque *et al*., 1998a).

3.3.3.2 Effect of Incubation temperature

For the optimization of incubation temperature for maximum phage induction, the strains of *V. cholerae* were incubated at different temperature (20 ^{o}C, 30 ^{o}C, 37 ^{o}C, 40 ^{o}C and 50 ^{o}C) after induction with mitomycin C (Capra *et al*., 2010).

3.3.3.3 Effect of Incubation time

Optimum incubation time required for maximum plaque production was determined by incubating the host culture after phage induction with mitomycin C for a total of 10 h and analyzing the samples at a regular interval of 2 h for plaque production.

3.3.3.4 Effect of varying concentration of ampicillin in the base plate

The first step in getting an uncontaminated lysate of a particular bacteriophage strain is obtaining single plaques on the host lawn. This obligatory stage of analysis is in fact, a limiting step. Plates supplemented with ampicillin in the bottom agar were tested at the concentrations ranging from 0.5- 3.0 µg/mL for improving the size and number of plaques (Łoś *et al*., 2008).

3.3.3.5 Effect of Growth phase of bacterial culture

The growth phase of the host culture has an impact on the induction of phages. For optimization the growth phase for maximum phage induction,

mitomycin C was added to the cultures at early log phase, mid log phase and late log phase by withdrawing the growing host cultures when OD at 560 nm was 0.2, 0.5 and 1.0 respectively.

3.3.4. Phage isolation by Double agar overlay method

The filtrate was titrated for infectious phage particles by incubating aliquots of the fitrate with recipient strain according to the double-agar overlay method of Adams (1959) as outlined in section 3.1.8.2.

3.3.5. Phage purification and propogation

A single plaque was picked from the plate with a sterile tooth-pick, introduced into 3 mL of a log phase culture of the *V. cholerae* propagating strain in nutrient broth, and was incubated at 37 ºC in an environmental shaker (Orbitek, Scigenics, India) at 120 rpm for 12 h. This was then centrifuged at 10000 x g (Sigma, 3K30, Germany) followed by filtration through 0.22 μm membrane (Millipore, USA). The lysate obtained was used for double agar overlay.

3.3.6. Large scale production of phage lysate

3.3.6.1. Plate method

The plates prepared as described in the section 3.1.8.2, showing infective centers at the rate of 10^{10} PFU/mL, were washed with 10 mL SM buffer (Appendix -2), and were incubated overnight at 4 ºC, with gentle rocking so that phages could easily diffuse into the buffer. The phage suspension was recovered after incubation from all the plates and pooled. Chloroform was added to this pooled mixture at a final concentration of 5% (v/v), mixed well using a vortex mixer and incubated at room temperature for 15 minutes. The cell debris was then removed by centrifugation at 5000 x g for 10 min (Sigma, 3K30, Germany) and the supernatant was transferred to sterile polypropylene tube. Chloroform was

added to a final concentration of 0.3% (v/v) and this was stored at 4 ºC until use. The titer of this lysate was noted after serial dilution (Sambrook *et al.*, 2000).

3.3.6.2. Broth method

Overnight cultures of *V. cholerae* were diluted 100-fold in fresh LB medium and grown for 6h at 30 °C with shaking. Phage was induced using mitomycin C and incubated for 8h. The cell debris was then removed by centrifugation at 5000 x g for 10 min (Sigma 3K30, Germany) and the supernatant filtered through 0.22 μm membrane (Millipore, USA). Chloroform was added to a final concentration of 0.3% (v/v) and this was stored at 4 ºC until use. To confirm that the filtrates did not contain any bacterial cells, aliquots of the filtrates were streaked on Luria agar plates and incubated overnight at 37 °C. The titer of this lysate was noted after serial dilution (Faruque *et al*., 2005).

3.3.7. Phage concentration

Phage was concentrated using Polyethylene glycol (PEG) 6000 as described by Sambrook *et al*., (2000). Briefly 1% (v/v) of a overnight culture of the host bacteria was transferred to 200 mL nutrient broth (Himedia, Mumbai, India), and incubated at 37 ºC for 3.5 h in an environmental shaker at 100 rpm (Orbitek, Scigenics, India). Phage was added at a multiplicity of infection (MOI) of 0.2 and the incubation at 37 ºC was continued at 100 rpm for 12-15 h. This broth was then centrifuged at 10000 x g for 20 min (Sigma, 3K30, Germany), the supernatant was collected and filtered through 0.22 μm membrane filter (Millipore, USA). DNase I (Bangalore Genei) and RNase (Bangalore Genei) was added, to a final concentration of 1 μg/mL each, and incubated at room temperature for 30 minutes. Solid NaCl was added to a final concentration of 1 M and dissolved by stirring with a sterile glass rod. This mixture was kept in ice for 1 hour, followed by centrifugation at 11000 x g for 10 min at 4 ºC. Solid PEG 6000 (SRL, India) was added to the supernatant at a final concentration of 10% (w/v),

dissolved by slow stirring on a magnetic stirrer at room temperature. This was then kept in ice overnight, followed by centrifugation at 11000 x g for 10 min at 4 ºC. The supernatant was discarded completely, while the pellet was resuspended in 5 mL of Phosphate buffered saline (PBS) (Appendix-2). PEG and cell debris were removed from the phage suspension by the addition of an equal volume of chloroform, vortexing for 30 sec, followed by centrifugation at 3000 x g for 15 min at 4 ºC. The aqueous phase containing the phage particles were recovered and stored at -20 ºC.

3.3.8. Maintenance and storage of phage

Phage lysate for long term storage was maintained as stock cultures employing 2 methods, *viz*. storage at 4 ºC as such and as glycerol stock. Phage lysate obtained after large scale production (Section 3.3.6) was stored in sterile 40 mL polypropylene screw-cap tubes at 4 ºC until use.

Nutrient broth containing 50% glycerol was mixed with filtered phage lysate in a sterile microfuge tube (1.5 mL capacity) and the mixture was frozen at -80 ºC, until use.

3.4. Physicochemical characterization of vibriophages

3.4.1. Characterization of phage

The temperate phages isolated from *V. cholerae* that exhibited excellent and consistent bacterial cell lysis capacity were chosen for further characterization.

3.4.1.1. Morphological analysis by Transmission Electron Microscopy (TEM)

One drop of high titer phage sample was spotted onto a carbon-coated TEM grid, allowed to settle for 2-3 min and excess of sample removed by blotting. A drop of 2% uranyl acetate (pH 7.0) was placed on the spot (Luria *et al.*, 1943),

allowed to react for 2-3 min and the excess stain drained off by touching a blotting paper strip to the edge of the grid. The grids were dried for 3 h, examined and photographed using a Transmission Electron Microscope (Model JOEL JEM-100 X) operated at 80 KV at Indian Institute of Horticulture Research (IIHR) Hesaragatta, Bangalore. Phage morphology was observed from the micrographs.

3.4.1.2. Determination of optimal multiplicity of infection

Multiplicity of infection (MOI) is the ratio of phage particles to host bacteria. It is calculated by dividing the number of phage added (volume in mL x PFU/mL) by the number of bacteria added (volume in mL x colony forming units/mL). Optimal MOI was determined according to Lu *et al*. (2003). Briefly the propagating strains were infected at different MOI (0.01, 0.1, 0.5, 1, 5 and 10 PFU/mL) and were incubated at 37 ºC for one hour. At the end of the incubation period, the mixture was centrifuged (Sigma, 3K30, Germany) at 8000 x g for 10 min and supernatant was passed through 0.22 µm membrane filter (Millipore, USA). The lysate was then assayed to determine the phage titre employing the double agar overlay method described previously. Phage free cultures (containing only bacterial host) and host free cultures (containing only phage) were used as controls. All assays were performed in triplicates. The MOI giving maximum yield was considered as optimal MOI.

3.4.1.3. One step growth curve

The construction of the one-step growth curve of a phage is important as it helps in the calculation of the growth kinetics parameters like latent period, rise period and the burst size. Latent period is the time elapsed between the moment the host culture is infected with phage to the moment the first bacterial cells are lysed. The rise period is the time span starting from the end of latent period until all phages are extra cellular. Burst size is the average number of progeny phage

particles produced per infected bacterium. It is calculated as follows: (final PFU-initial PFU)/ number of infected bacterial cells (Adams, 1959).

One step growth curve experiment was performed according to the protocol of Capra *et al*. (2006). Mid log phase culture of the propagating host *V. cholerae* (200 mL) was harvested by centrifugation at 9000 x g for 10 min and resuspended in 1/5 of the initial volume (40 mL) of pre-warmed Luria broth. The phage was added at the optimal MOI, allowed to adsorb for 15 min at 37 ºC, followed by harvesting of phages by centrifugation at 10000 x g (Sigma, 3K30, Germany) for 5 min and resuspension in 200 mL nutrient broth. This was incubated at 37 ºC.

Samples were taken at 10 min intervals (up to 2 h) and immediately titered by the double agar overlay method. Assays were carried out in triplicate and appropriate controls were maintained. The graph was plotted with log of relative phage titer against time. The latent period, the rise period and the burst size of the phage were calculated from the one step growth curve.

3.4.1.4. Phage adsorption

The first step in the growth of bacteriophage is its attachment to susceptible bacteria. This process is called adsorption.

The adsorption studies were carried out as per Lu *et al*. (2003). Late-log phase culture of host was infected using the optimal MOI of the phage and incubated at 37 ºC. Aliquots of 5 mL were sampled at 0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 min time intervals after infection. All samples drawn were immediately filtered through 0.22 μm membrane filter (Millipore, USA). The phage titer was determined using double agar overlay method after appropriate dilutions. All platings were done in triplicates and appropriate controls were maintained. The percentage of phage adsorption was calculated as follows: [(control titer - residual

titer)/control titer] X 100% (Durmaz *et al.*, 1992). The phage titer observed at time zero was considered as the control titer.

3.4.2. Influence of physical and chemical parameters on phage viability

Physical and chemical parameters have a critical role in maintaining phage viability. Hence the effect of different physical and chemical parameters like temperature, pH, NaCl and different sugars on phage viability was studied.

3.4.2.1. Effect of temperature on phage viability

The influence of temperature on phage viability/propagation was studied following the protocol of Lu *et al*., (2003). 900 µL of sterile distilled water was pre-heated to temperatures ranging from 50 ºC to 100 ºC, followed by the addition of 100 µL of phage sample (10^{10} PFU/mL) to these pre-heated tubes. The tubes were maintained at these temperatures for varying intervals, i.e., 15 sec, 30 sec, 1 minute, 2 min and 3 min. After incubation these phage containing tubes were immediately placed in ice. Samples were assayed using double agar overlay method to determine the number of surviving plaque PFU. All plate assays were done in triplicates and appropriate controls were maintained. The counts of surviving phage were expressed as PFU/mL and plotted against temperature values.

3.4.2.2. Effect of NaCl on phage viability

In order to study the influence of NaCl on phage viability, NaCl solutions of varying molar concentrations such as 0.1 M, 0.25 M, 0.5 M, 0.75 M, 1 M, 2 M and 3 M were prepared in deionised water. Phage sample was added (10^{10} PFU/mL), incubated for 30 min at 37 ºC (Capra *et al*., 2006), then diluted and assayed with the mid-log phase host for surviving phage particles by the double agar overlay method. The plaques obtained were counted. All plate assays were

performed in triplicates and appropriate controls were maintained. The result was expressed as PFU/mL and plotted against concentration of NaCl.

3.4.2.3. Effect of pH on phage viability

Influence of pH on phage viability was evaluated by incubating the phages in suitable buffers of different pH, ranging from 2-13 (Capra *et al*., 2006). Hydrochloric acid-potassium chloride buffer was used for the studies at pH 2, while the citrate – phosphate buffer system was used for pH 3 to 6; Phosphate buffer for pH 7, Tris (hydroxymethylamino methane buffer system for pH 8 and 9; carbonate – bicarbonate buffer for pH 10 and 11; Sodium hydroxide - Potassium chloride buffer (pH 12 and 13) (Appendix-2). The phage was added (10^{10} PFU /mL) to 10 mL of sterilized buffer solutions, incubated at 37 ºC for 30 min and assayed with the mid log phase host using double-layer agar plate method to determine the surviving PFU. The plate assay was done in triplicates and appropriate controls were maintained. The viable phage particles were counted. The results were expressed as PFU/mL and plotted against the values of pH.

3.4.3. Influence of physical and chemical parameters on phage adsorption

Phage adsorption is critical for causing phage infection in bacteria. All factors having an influence on phage adsorption, also affect phage infection. Hence the effect of various physical and chemical parameters on phage adsorption was studied.

3.4.3.1. Effect of temperature on phage adsorption

The adsorption of phages on the host *V. cholerae* was determined at temperatures of 0, 10, 20, 30, 37, 40, 45 and 50 °C. The methodology was adopted from Capra *et al*. (2006). Exponentially growing host culture (O.D 600 = 1) was centrifuged and resuspended (approximately 10^8 CFU/mL) in nutrient broth (pH

8). Phage was added at optimal MOI and was incubated, each at the different temperatures mentioned above for 30 min. After centrifugation at 12000 x g for 5 min (Sigma, 3K30, Germany) at 4 °C, the supernatant after appropriate dilutions were assayed for unabsorbed phages employing double agar overlay method. All plating was done in triplicates and appropriate controls were maintained. The phage titer was compared with the control titer. The results were expressed as percentages of adsorption and plotted against temperature.

3.4.3.2. Effect of NaCl on phage adsorption

Influence of different concentrations of NaCl on adsorption was investigated following Capra *et al.* (2006). Exponentially growing host culture was added to nutrient broth with concentrations of NaCl ranging from 0.1, 0. 25, 0.5, 0.75 and 1 M NaCl, and infected with optimal MOI of phages and incubated at 37 °C for 30 min for adsorption. The mixture was then centrifuged at 10000 x g for 5 min at 4 °C (Sigma, 3K30, Germany) to sediment the phage adsorbed bacteria. The supernatant was then assayed for unadsorbed free phages employing double agar overlay method and the counts were compared with the titre of the control which contained no NaCl in nutrient broth. All plating was done in triplicates and appropriate controls were maintained. The results were expressed as a percentage of adsorption and plotted against NaCl concentration.

3.4.3.3. Effect of pH on phage adsorption

Exponentially growing *Vibrio cholerae* host culture was centrifuged at 10000 x g for 10 min (Sigma, 3K30, Germany) at 4 °C, and the cells resuspended in nutrient broth adjusted to the desired pH. Adsorption was determined at the pH values ranging from 2 to 13. Phage was added at an optimal MOI, incubated at 37°C for 30 min for adsorption, centrifuged at 10000 x g for 5 min, the supernatants assayed to determine surviving PFU employing double agar overlay method and was compared to control (Capra *et al* ., 2006). All experiments were

performed in triplicates and appropriate controls were maintained. The results were expressed as a percentage of adsorption and plotted against pH values.

3.4.3.4. Effect of calcium ions on phage adsorption and propagation

The influence of calcium ions on phage adsorption and propagation was determined following (Lu *et al*., 2003). The protocol involved adding 10 mL of exponentially growing *V. cholerae* host culture to 100 mL nutrient broth, and incubation for 3.5 h at 120 rpm. 10 mL each of this mid log phase host culture was added to five, 15 mL McCartney bottle. Appropriate volumes of filter sterilized 1M $CaCl_2$ (Millipore, USA) solution were added to the host aliquots to make 0, 1, 10, 20, and 30 mM concentrations. After the final volume was adjusted to 15 mL with sterile distilled water, each tube was infected with the phage at optimal MOI. All tubes were incubated at 37 ºC for 2 h. One mL aliquots were drawn and centrifuged at 10000 x g for 10 min (Sigma, 3K30, Germany) at 4 °C. The supernatants were serially diluted and assayed using double agar overlay method to determine plaque formation. The phage titer was determined employing double agar overlay method for the medium with and without $CaCl_2$. All plating was done in triplicates and appropriate controls were maintained. The results were expressed as PFU/mL and plotted against $CaCl_2$ concentrations.

3.4.4. Phage structural protein analysis

3.4.4.1. Non-reductive Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) of purified phage proteins was performed under both non reducing and reducing conditions for evaluating the nature of the phage capsid protein using vertical slab electrophoresis (Genei, Bangalore, India) by the method of Laemmli (1970). Low molecular weight protein marker (BioRad, USA) was used as

standard and molecular weight was determined using Quantity One Software (BioRad, USA).

3.4.4.1.1. Procedure

The gel plates were cleaned and assembled. Resolving gel (10%) was prepared by mixing 10 mL of acrylamide: bis-acrylamide (30:0.8), 3.75 mL of resolving gel buffer stock, 300 µL of 10% SDS and 15.95 mL of water followed by 100 µL of ammonium persulfate solution (10%) and TEMED (15 µL) (Appendix - 4). The mixture was immediately poured into the cast and a layer of water was added over the gel and allowed to polymerize for at least one hour. Water layer was poured out after polymerization. The stacking gel (2.5%) was prepared by combining 2.5 mL of 30: 0.8 acrylamide: bis-acrylamide solution, 5 mL of stacking gel buffer stock, 200 µL 10% SDS and 12.3 mL of distilled water, followed by 100 µL of ammonium persulfate and 15 µL of TEMED (Appendix-4). The stacking gel was then poured into the gel assembly, above the resolving gel and the comb was immediately inserted. Gel was allowed to polymerize for 30 min, placed in the electrophoresis apparatus and upper and lower reservoirs filled with reservoir buffer (Appendix-4) and was pre run for 1 h at 80 V.

3.4.4.1.2. Phage sample preparation

Sample was prepared by mixing 100 µL of 1X sample buffer for non-reductive SDS-PAGE (Appendix-4) with concentrated phage lysate. 25 µL of this sample and 5 µL low molecular weight marker mix was loaded on to the gel and run at 80 V. The current was increased to 100 V, when the dye front entered the resolving gel. The run was stopped when the dye front reached 1 cm from the lower end of the plate, the gel was removed and stained.

3.4.4.2. Silver staining

The gel was fixed for 30 min in fixing solution 1, followed by incubation in fixing solution 2 for 15 minutes. This gel was washed 5 times in water for duration of 5 min each**.** Sensitized the gel in freshly prepared sensitizer for 1 min and washed in water twice for 2 min each. The gel was then incubated in staining solution for 25 min at 4 °C, washed twice for duration of 1 minute each and then incubated in developing solution until the bands appear. To prevent over staining, the gel was treated for 10 min in sodium EDTA (Appendix-4), washed in water twice for duration of 2 min each. The image of gel was captured using gel documentation system (Syngene, UK).

3.5. Bacteriophage genome analysis

3.5.1. Phage DNA isolation

Phage DNA extraction was carried out as previously described (Sambrook *et al.*, 2000). Briefly, 1 mL of the PEG 6000 concentrated phage suspension was incubated at 56 °C for one hour with proteinase K at a final concentration of 50µg/mL and SDS at a final concentration of 0.5%. After incubation, the digestion mix was cooled to room temperature and extracted first by adding equal volume of phenol equilibrated with 50 mM Tris (pH 8.0). The digestion mix in the tube was inverted gently many times until a complete emulsion was formed. The phases were separated by centrifugation at 3000 x g for 5 min at room temperature (Sigma, 3K30, Germany). The aqueous phase was transferred to a clean tube using wide-bore pipette, and then extracted with 50:50 mixtures of equilibrated phenol: chloroform, followed by a final extraction with equal volume of chloroform. Double volume of ethanol and sodium acetate (pH 7) (Appendix - 3) is added to a final concentration of 0.3 M was added to the extract followed by incubation at room temperature for 30 minutes. After incubation, the precipitated DNA was

collected by centrifugation at 10000 x g for 5 min at 4°C. The supernatant was discarded and DNA was dissolved in Tris- EDTA (TE) buffer (pH 7.6) (Appendix-3). DNA was run on 1% agarose gel. Gel was stained with ethidium bromide and visualized in UV light. The image of the gel was captured using gel documentation system (Syngene, UK).

3.5.2. Restriction analysis of phage ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

The restriction pattern of the phage DNA was studied using the enzymes *Eco*RI, *Bam*HI, *Sau*3AI and *Hind*III (Fermentas, USA). Enzyme digestions were performed as recommended by the manufacturer. For digestion, each 20μL digestion solution containing approximately 50 μg of bacteriophage DNA and 5U of the restriction enzyme in reaction buffer was incubated for 1 h at reaction temperature as the protocol prescribed for the enzyme. Before loading on the gel, the digests were heated at 65 °C for 10 min and then quenched on ice. Restricted fragments were separated by agarose (2%) gel electrophoresis. Gel was stained with ethidium bromide and visualized in UV light. The image of gel was captured using gel documentation system (Syngene, UK).

3.5.3. PCR Screening for Virulence/ Virulence associated genes in phage ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

At least a few of the genes in the Pathogenecity Island are believed to be acquired by the *V. cholerae* genome by Horizontal Gene Transfer (HGT) mediated by phages or plasmids or transposones. Screening for virulence/virulence associated genes in phage genome would be able to provide new insights in phage mediated HGT.

ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 genomes were analysed for the presence of virulence/virulence associated genes employing PCR

with the help of same forward and reverse primers used in host genome screening (section 3.2.2.). The presence of genes, *ctx*A and *ctx*B (the A and B subunit of CT gene), *zot* (zonula occludens toxin gene), *tox*R (an essential toxin regulating gene), *tcp*A (the major subunit of TCP gene) and *hly*A (an alternate diarrhoegenic factor) were determined by PCR. Presence of *nan*H (encoding neuraminidase) and *int* (encoding bacteriophage-like integrase), were also determined by multiplex PCR using the same primers and procedures as detailed in secion 3.2.2. All PCR reactions and the thermal profile for the PCR were performed as described in section 3.2.2 unless otherwise stated. Electrophoresis on agarose gel was performed as outlined in section 3.1.7.3.

3.5.4. *In silico* analysis of partial gene sequence of *zot* and *ace* gene of ΦALPVC3 and strainALPVC3

The *zot* and *ace* genes products of ΦALPVC3 and ALPVC3 after PCR amplification was purified by gene clean kit (Genei, India) and subsequently sequenced, followed by homology analysis.

The PCR amplicon was sequenced by Sanger's Dideoxy method using ABI 3730 Excel at Scigenom Labs, Kochi, Kerala. The identity of the sequences was determined by comparing with sequences in the NCBI database using the online tool, BLAST (Altschul *et al*., 1990). For this, the sequences in FASTA format was pasted on the NCBI BLAST page (http://blast.ncbi.nlm.nih.gov) and nBLAST was carried out.The ΦALPVC3 phage sequence of *zot* and *ace* genes was aligned with the *zot* and *ace* genes of *V. cholerae* non O1 strain by using the multiple sequence alignment tool, Clustal W2 (Larkin *et al*., 2007). The partial gene sequences of *zot* and *ace* genes of ΦALPVC3 and ALPVC3 were deposited at NCBI and accession numbers were obtained.

3.6. Transduction studies as evidence for horizontal gene transfer

Tailed phages are the most efficient gene-transfer vehicle developed in evolution. Phage mediated transduction is an efficient DNA transfer device with the phage tail and its associated fibres. The unique structure of phage assures both the specific recognition of the appropriate host cell and the guided injection of the phage DNA into the bacterial cell.

The chances of phage mediated lateral gene transfer in the environmental niche were studied with the help of a transduction experiment (Avitabile *et al*., 1972) using the non-O1 environmental strain ALPVC3 as the recipient cell. The phage with *ctx*A and *ctx*B genes encoding Cholera Toxin (CT) was used as the transducing phage.

3.6.1. Prophage curing

The primary requirement for transduction studies was to obtain prophage-cured derivatives from *V. cholerae* lysogenic strains ALPVC3. Strain ALPVC3 was found positive for the toxin coregulated pilus (TCP), which is essential for CTX phage adsorption. Prophage-cured strains were selected after induction using mitomycin C and ultravictolet light in combination as described by Wiederholt and Steele (1993). These cured strains should not produce the plaques when infected with the indicator strain. Also the prophage cured mutant will act as indicator for the lysogenic phage.

V. cholerae strain ALPVC3 with lysogenic phages were grown in Luria-Bertani (LB) medium at 37 ^{o}C with constant shaking at 120 rpm. Bacterial cells were harvested when optical density at 600 nm was approximately 1 corresponding to about 6 x10^{8} CFU/mL, in 2 - 2.5 h. The cells were harvested by centrifuging at 5000 x g (Sigma) for 10 min and were resuspended in in phosphate buffer at pH 7.

The cell suspension was treated with mitomycin C at 1 µg/mL and incubated for 3 h. One mL of treated culture was spread plated on LB plate and incubated overnight at 37 ^{o}C. The ALPVC3 colonies which survived mitomycin C induction were considered to be free of lysogen and so it was believed to escape cell lysis by the induced phage. The nutrient plate with putative phage cured ALPVC3 colonies was irradiated with UV for 1 min and incubated for 3 h at 37 ^{o}C in dark to avoid photoreactivation. Each isolate was spotted on a lawn of sensitive MAK 757 indicator bacteria to test for the presence of induced bacteriophage by the criterion of production of a zone of lysis.

Those irradiated isolates not producing zones of lysis after 16 h incubation were streaked from the master plate for single colonies, reisolated twice, and then tested for U.V. inducibility and the ability to support the growth of ΦALPVC3. The phage cured *ALPVC3 were further analysed by PCR as a confirmatory step of curing. Phage curing of the other three non-O1 environmental isolates (ALPVC11, ALPVC12 and EKM4) was also done simultaneously to study the role of *tcp*A gene in transduction.

3.6.2. Isolation of *ctx*A^{+} and *ctx*B^{+} (CT^{+}) phage from O139

The induction and isolation of *ctx*A^{+} and *ctx*B^{+} (CT^{+}) phage from O139 was done according to the method described by Faruque *et al*., (1998). Toxigenic *V. cholerae* strain O139 were grown in Luria broth at 30 °C to an absorbance of 0.5 at 600 nm. The cells were collected by centrifugation, washed, and resuspended in fresh LB. The suspension was divided into aliquots, to which mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added at 1 µg/mL and incubated overnight at 30 °C. The culture supernatants were analyzed for extracellular phage carrying the CT toxin by double agar overlay method using MAK 757 as recipient strain. The isolated plaques were further multiplied and purified to make high titer lysates as described in section 3.3.6.

3.6.3. PCR analysis of phage-cured *ALPVC3 and CT positive phage.

The phage-cured strain *ALPVC3 was screened for the presence of various virulence/virulence associated genes which were found in non-O1 environmental strain ALPVC3. The genes, *zot* (zonula occludens toxin gene), *ace* (accessory cholera toxin), *tox*R (an essential toxin regulating gene), *tcp*A (the major subunit of TCP protein) and *hly*A (an alternate diarrhoegenic factor) were determined by PCR. Presence of *nan*H (encoding neuraminidase) and *int* (encoding bacteriophage-like integrase), were also determined by multiplex PCR. CT^+ phage was also screened for the presence of all virulence and virulence associated genes used in this study. PCR reactions and the thermal profile for the PCR were performed as described in section 3.2.2. Electrophoresis of the agarose gel was performed as outlined in section 3.1.7.3.

3.6.4. Transduction protocol/ Phage transduction assays

Transduction experiments were performed with a phage cured *ALPVC3 strain of *V. cholerae* using the CT positive phage isolated from O139 strain to determine whether *ctx*AB was transmissible by transduction to nontoxigenic *V. cholerae* O1 recipient strains adopting the protocol of Boyd and Waldor, (1999).

To increase the frequencies of transduction, CT positive bacteriophage suspension containing 10^8–10^9 PFU were routinely UV irradiated for 2 min prior to infection. PFU were titered before and after UV irradiation to determine the percent inactivation. A level of PFU inactivation of 90 - 99 % was found to be optimal for increased transduction. The logarithmic phase cells (1 mL) of the recipient bacterial strain, *ALPVC3 in Luria broth were mixed with 1 mL of the serially diluted irradiated lysate and were incubated at 37 ^oC in a water bath (Scigenics, Chennai, India) for 30 minutes. Simultaneously the phage and bacteria are simply spread together on the surface of the plating medium.

The putative lysogens after transduction was assayed for phages by double agar overlay method as outlined in section 3.1.8.2. The plates were screened for plaques with bull's eye morphology. The opaque region at the center of the plaques denotes the cells with established lysogeny (Herman and Juni, 1974) in which temperate phage had integrated their genome as a prophage into the bacterial chromosome. Transduction assays were performed simultaneously with phage cured non-O1 environmental isolates (ALPVC11, ALPVC12 and EKM4) to study the role of *tcp*A gene in transduction.

3.6.5. Analysis of transduction

The putative lysogens in the eye of the plaque were picked using sterile tooth pick and were grown in nutrient broth. The DNA was isolated from individual colonies and were analysed with PCR using gene specific primers. PCR reactions and the thermal profile for the PCR were performed as described in section 3.2.2. Electrophoresis of the agarose gel was performed as outlined in section 3.1.7.3. The genes for *ctx*A, *ctx*B and *zot* were screened in the putative lysogens. The clones positive for these genes are considered to be positive transductants which can be a proof for lysogenic conversion by phages.

The transductants in the opaque region were pooled and the transduction frequency was analysed based on the MOI and the number of recipient cells used in transduction (Herman and Juni, 1974). The transductants were also analysed for their ability to produce high frequency transducing lysates upon induction with mitomycin C.

Chapter 4
RESULTS

4.1. Screening and identification of environmental isolates of *Vibrio cholerae* with temperate phages

4.1.1. Isolation and Identification of *Vibrio cholerae*

Vibrio cholerae isolates required as hosts for the isolation of the prophages, were isolated from marine environments like aquafarms and mangroves of Alappuzha and Ernakulam districts of Kerala, South India. Seventeen isolates which were Gram negative, oxidase positive, fermentative, with or without gas production on MOF media and which showed yellow coloured colonies on TCBS (Thiosulfate Citrate Bile salt Sucrose) agar were segregated as *Vibrio* sp.

4.1.2. PCR for *omp*W to identify isolates as *V. cholerae*

The unique presence of *omp*W gene with conserved sequences in *V. cholerae* make it a target gene for species level identification of the *V. cholerae* strains.

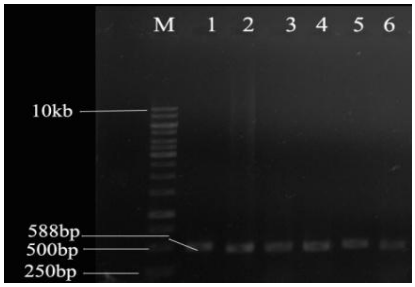


Fig.4.1. Agarose gel showing 588 bp amplification of *omp*W in environmental isolates of *Vibrio spp.* Lane M-1Kb molecular weight ladder; Lanes 1- 6 are environmental isolates of *Vibrio* sp *viz*, AR9, KNM4, KNM12, KNM20, MVN7 and MVN15.

The 588 bp amplicon for *omp*W was observed in strains AR9, KNM4, KNM12, KNM20, MVN7 and MVN15 and also in the strains in the lab *viz*. ALPVC1,

ALPVC2, ALPVC3, ALPVC4, ALPVC5, ALPVC6, ALPVC7, ALPVC8, ALPVC9, ALPVC10, ALPVC11, ALPVC12, ALPVC13, ALPVC14, ALPVC15, EKM1, EKM2, EKM4, EKM6, EKM7, EKM8, EKM9, EKM10, EKM14 and EKM16. These strains were further characterised to confirm the identity at species level.

4.1.3. 16S rRNA gene sequence analysis for identification of *Vibrio cholerae*

The *omp*W positive isolates were further subjected to species level identification based on partial 16S rDNA sequence analysis. Partial 16S rRNA gene was amplified and sequenced from 6 strains and their identity was confirmed by comparing the sequences in Genbank, by BLAST programme. The strains AR9, KNM4, KNM12, KNM20, MVN7 and MVN15 which showed 100% similarity with the classical virulent strains of *V. cholerae* were selected for further studies. The 16S rDNA partial gene sequences were deposited in the Genbank database. The Genbank accession number for AR9, KNM4, KNM12, KNM20, MVN7 and MVN15 are KJ734981, KJ734982, KJ734983, KJ734984, KJ734985 and KJ734986 respectively. These isolates, along with the *V. cholerae* strains in the cultures available in lab collection were used as host for isolating specific lysogenic vibriophages.

4.1.4. Serogrouping of *V. cholerae* using marker genes

The strains of *V. cholerae* were serogrouped by screening for the presence O1 *rfb* and O139 *rfb* marker genes by PCR amplification that should typically produce either 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.

4.1.5. Screening of *Vibrio cholerae* strains for prophages

Twenty two vibriophages were obtained from the *Vibrio cholerae* strains by induction with mitomycin C and were named appropriately as shown in table 4.1

Table 4.1. Vibriophages obtained after induction of *V. cholerae strains* by Mitomycin C

Sl. No	strain	Source	Vibriophage	Strains producing plaques	
				Induction with mitomycin C	without mitomycin C
1	**CO336 (Eltor strain)**	standard strain	Φ CO336	✓	x
2	**O139**	standard strain	Φ O139	✓	✓
3	**KNM4****	surface water	ΦKNM4	✓	✓
4	**KNM12****	surface water	ΦKNM12	✓	x
5	**MVN7****	mangroves	ΦMVN7	✓	x
6	**ALPVC3***	lab isolate	ΦALPVC3	✓	✓
7	**ALPVC4***	lab isolate	ΦALPVC4	✓	✓
8	**ALPVC5***	lab isolate	ΦALPVC5	✓	x
9	**ALPVC6***	lab isolate	ΦALPVC6	✓	x
10	**ALPVC7***	lab isolate	ΦALPVC7	✓	✓
11	**ALPVC8***	lab isolate	ΦALPVC8	✓	x
12	**ALPVC9***	lab isolate	ΦALPVC9	✓	✓
13	**ALPVC10***	lab isolate	ΦALPVC10	✓	✓
14	**ALPVC11***	lab isolate	ΦALPVC11	✓	x
15	**ALPVC12***	lab isolate	ΦALPVC12	✓	x
16	**ALPVC14***	lab isolate	ΦALPVC14	✓	✓
17	**EKM1***	lab isolate	ΦEKM1	✓	x
18	**EKM2***	lab isolate	ΦEKM2	✓	x
19	**EKM4***	lab isolate	ΦEKM4	✓	✓
20	**EKM6***	lab isolate	ΦEKM6	✓	x
21	**EKM7***	lab isolate	ΦEKM7	✓	x
22	**EKM8***	lab isolate	ΦEKM8	✓	x
23	**EKM10***	lab isolate	ΦEKM10	✓	x
24	**EKM14***	lab isolate	ΦEKM14	✓	✓

*Laboratory culture collection **Environmental isolates

Three out of the 6 environmental isolates and 19 of the *V.cholerae* strains from laboratory culture collection, showed the presence of phages on induction by mitomycin C. Inducible prophages were also detected in the two standard virulent strains *V. cholerae* CO 336 and *V. cholerae* O139. The strains that produced translucent plaques with bull's eye morphology on plating (Fig 4.2) were identified as lysogen positive strains. A few strains produced plaques even in control plates indicating spontaneous induction. Ten strains did not yield any plaques after induction, indicating that they did not harbor any prophages.

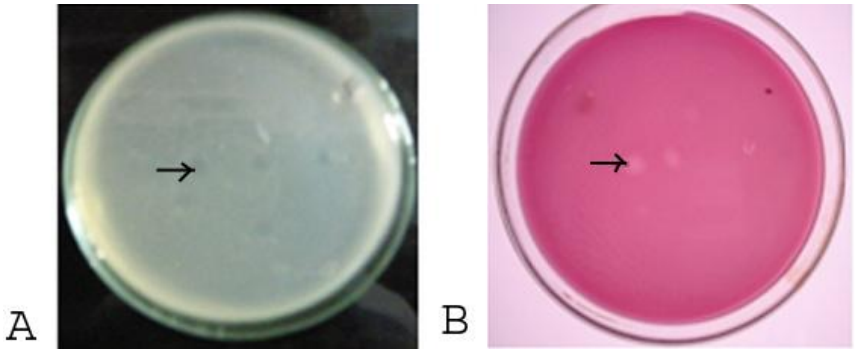


Fig 4.2 Assay for screening temperate phages. A. Translucent plaques on double agar overlay assay B. Tetrazolium stained plates showing plaques formed by phage

4.2. Molecular characterization of environmental isolates of *Vibrio cholerae* with temperate phages.

4.2.1. Molecular Typing

The 22 isolates identified as *V. cholerae* and the 2 standard strains were subjected to molecular typing methods such as ERIC PCR and BOX PCR. ERIC sequences are 126 bp long sequences, highly conserved at the nucleotide level and present in multiple copies in the genome of vibrios. BOX-PCR fingerprinting method produced specific banding patterns depending on the multi-copied,

conserved, repetitive DNA sequences called BOX elements present in the bacterial genome.

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

The analysis of the amplified products of these repeated sequences produced specific banding profiles revealing the clonal relationship between the strains (Fig.4.3).

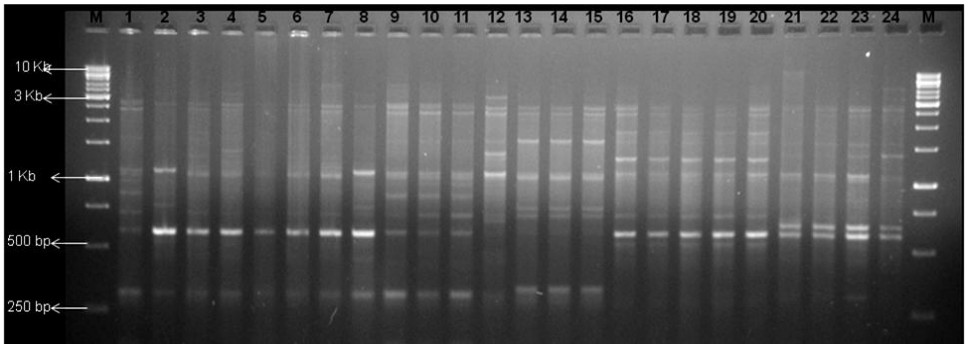


Fig.4.3. ERIC- PCR fingerprints of *V. cholerae* strains with ERIC oligonucleotide primer sets. Lane M-1 kb molecular weight ladder; Lanes 1- 11 are strains ALPVC 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 14 respectively; Lane 12-O139 standard strain; Lane 13 and 14 are strains, KNM4 and KNM12 respectively; Lane 15-MVN7; Lane 15-23 are strains, EKM1, 2, 4, 6, 7, 8, 10 and 14 respectively; Lane 24; El Tor standard stain.

Genomic fingerprinting analysis by ERIC-PCR of the 24 *V. cholerae* strains resulted in amplification of multiple fragments (6-10) of DNA, with sizes ranging from 300 bp to 10 kb, yielding specific fingerprint patterns. Three fragments, one at ~600 bp, other at ~1 kb and another one at ~3 kb were common to all the tested strains.

The fingerprint pattern was used to generate a distance matrix and the dendrogram (Fig 4.4) generated showed that the isolates were divided into 2 major clusters. Cluster 1 included 16 strains in 3 groups including the standard strain *V.*

cholerae CO336; Group1 were cultures isolated from the inland surface waters of Ernakulam. All the 5 strains exhibited close similarity in their fingerprint pattern.

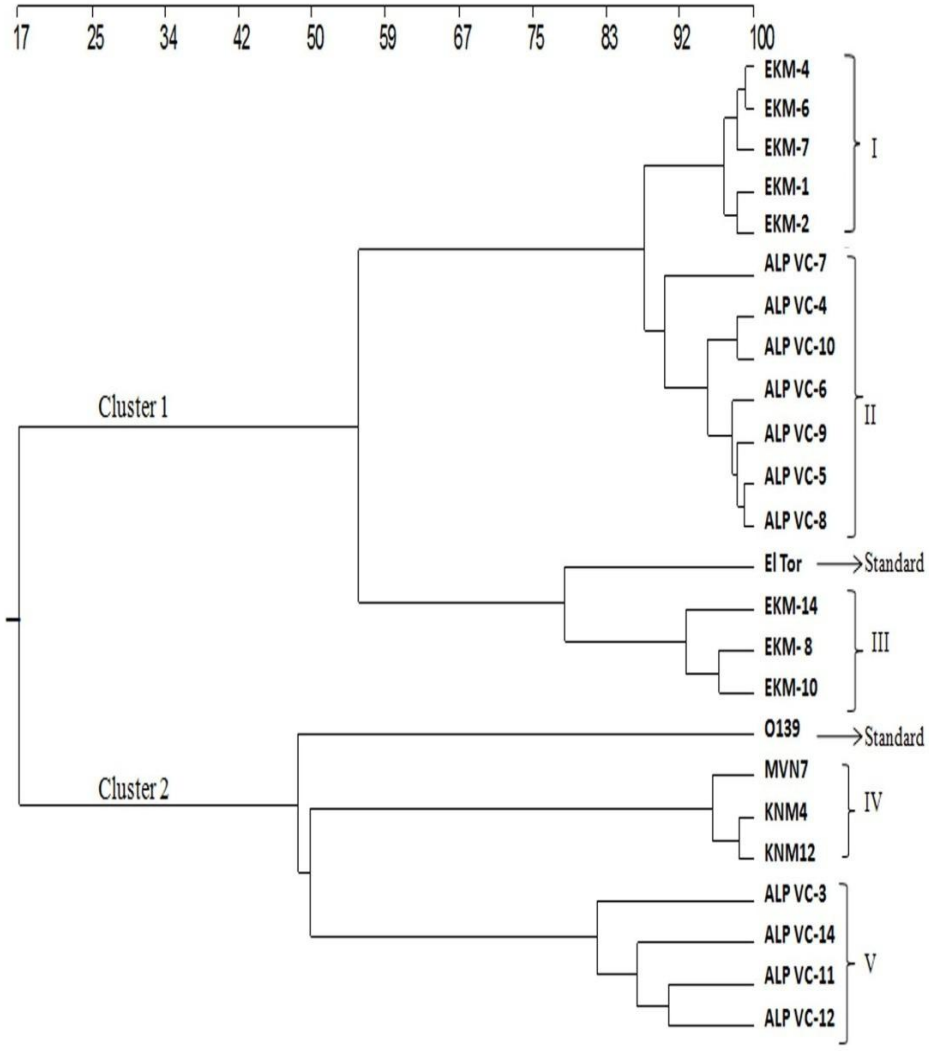


Fig 4.4. Dendrogram constructed on the basis of the ERIC PCR profiles of environmental strains *of V. cholerae* generated by the Genetool software.

The *V. cholerae* strains of group II which showed high percentage similarity were exclusively the non-O1, non-O139 strains from inland lagoons of Alappuzha

district. A set of 3 strains with inducible lysogens isolated from Ernakulam formed the group III. The genomic fingerprint of standard El Tor strain exhibited ~80% similarity to strains EKM8 and EKM10 and ~80% similarity to strain EKM14, all of group III.

Cluster 2 included 8 strains of *V. cholerae* in two groups. The standard strain O139 claded at ~50% similarity with the members of group IV. The *V. cholerae* strains KNM4, KNM12 and MVN7 in group IV, isolated from mangrove environments also exhibited similarity in their fingerprint pattern..

Similarly, 4 strains in group V exhibited homology in their banding profile and all the strains were isolated from Alappuzha. The strain ALPVC3 showed high similarity to ALPVC11 and ALPVC12 of the same group in the dendrogram. The *V. cholerae* strains which with inducible temperate vibriophages were mainly from two locations viz. Alappuzha and Ernakulam. The isolates from both these locations showed two major banding patterns in ERIC PCR fingerprinting. All the isolates from Ernakulam claded together in cluster 1 (group I and III), while those from Alappuzha were found in two groups (II and V); isolates from two regions clading separately in two different clusters. 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.

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

BOX-PCR fingerprinting of lysogenic *V. cholera* used in present study is shown in Fig. 4.5. Two major type of patterns were noticed. The strains isolated from Alappuzha (Lane 2-12) showed homology in profile as did those isolated from Ernakulam, but with more number of bands.

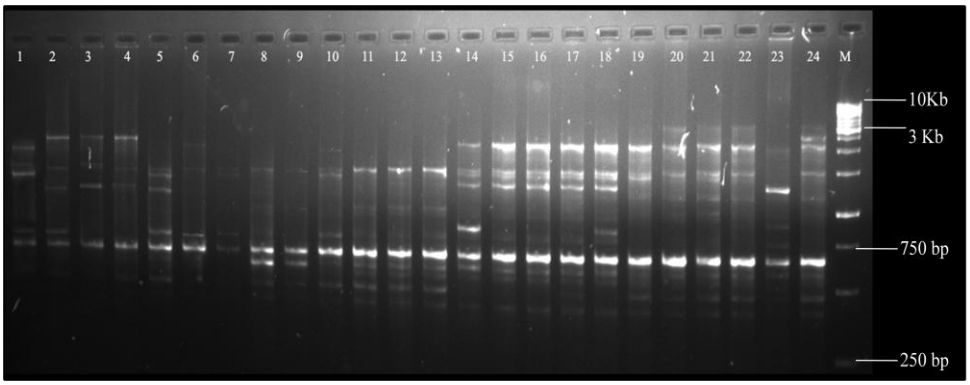


Fig.4.5. BOX PCR fingerprint for *V. cholerae* (N=24) strains using BOX A1 primer.Lane M-1 kb molecular weight ladder; Lane 1-O139 standard strain; Lanes 2- 12 are strains, ALPVC3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and ALPVC14 respectively; Lane 13 and 14 are strains, KNM4 and KNM12 respectively; Lane 15-22 are strains, EKM1, 2, 4, 6, 7, EKM8, 10 and EKM14 respectively; Lane 23; El Tor standard stain; Lane 24-MVN7.

The dendrogram generated after cluster analysis of the fingerprints of *V. cholerae* strains produced by BOX-PCR revealed 1 major cluster with 19 environmental strains and a minor cluster with 3 environmental strains (Fig 4.6). Strains exhibiting >80% similarity were grouped together for analysis. Cluster 1 diverged into 4 types. In type 1, three strains displaying identical fingerprint patternwere ALPVC12 and ALPVC14 from surface water and KNM4 from mangrove. Strain KNM12 from mangrove diverged from group 1 with 20% dissimilarity. Strain ALPVC8 showed very few BOX elements and formed the type II. All the five strains in type III were isolates from Alappuzha. Group IV comprising isolates from Ernakulam, exhibited homology in banding profile and strain MVN7 from Mangalavanam mangroves of Ernakulam exhibited ~90% similarity to other members of the group.

Cluster 2 included four strains together with the standard strain O139. Three strains clustered into Group V, showed ~80% similarity with the standard *V. cholerae* O139 strain used in the study. Although the isolates were from diverse environmental sources, a high degree of similarity in banding profile was displayed among the strains.

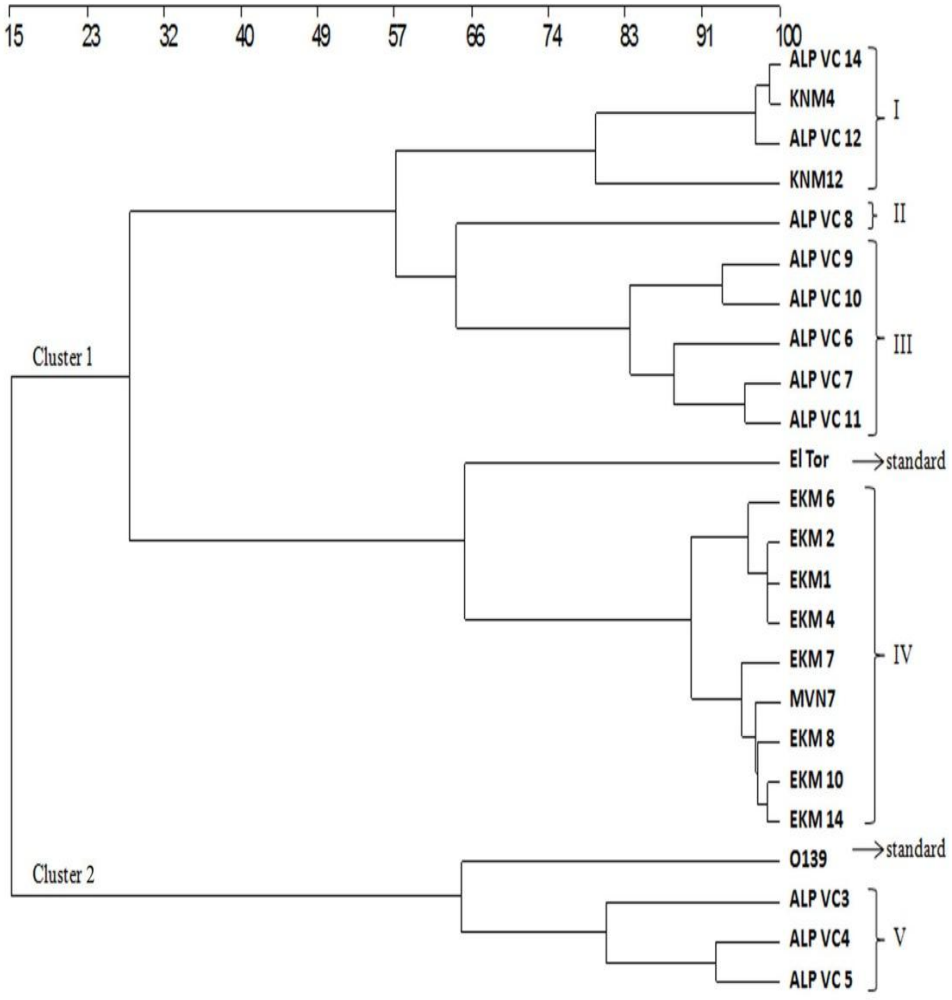


Fig.4.6. Dendrogram constructed on the basis of the BOX- PCR profiles of environmental strains *of V. cholerae* generated by the Genetool software

4.2.2. Screening for Virulence/ Virulence associated genes using PCR

The *V. cholerae* strains with lysogenic phages were further characterized to provide information about the presence of several genes which either encode virulence factors or are genetically linked to virulence genes (*i.e.*, virulence associated genes) and included *ctx*A, *ctx*B, *ace*, *hly*A, *tox*R, *zot*, *tcp*A, *nin*T and *nan*H genes. The amplification of the genes was by using gene specific primers.

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

The ability of pathogenic *V. cholerae* to cause disease depends on the expression of the potent enterotoxin Cholera Toxin (CT) encoded by a transferable filamentous phage, CTXΦ. In this context, environmental isolates of *V. cholerae* were screened for the presence of *ctx*A and *ctx*B gene encoding the A and B subunits of CT, by PCR using gene specific primers. However the *ctx*A and *ctx*B gene were absent in all isolates tested, except in the standard strains included in the study.

4.2.2.2. PCR detection of *ace*

The virulence cassette of toxigenic *V. cholerae* is known to carry at least six genes and *ace* gene encoding accessory cholera enterotoxin is one among them. The Ace toxin is an integral membrane protein consisting of 96 amino acids and increases the potential difference across the intestinal epithelium. It is located immediately upstream of the gene encoding Zot (Zonula occludens toxin) and cholera toxin. Three strains tested positive for *ace* gene (Fig 4.7).

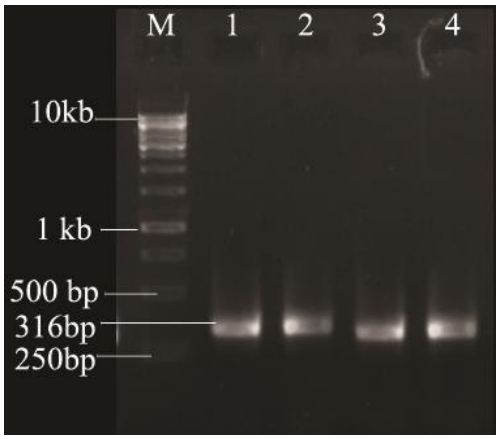


Fig.4.7. Detection of *ace* in environmental isolates of *V. cholerae*

Lane M- 1 kb ladder; Lane 2- Positive control (*V. cholerae* CO366 El Tor strain); Lanes 2-4: PCR amplicons from strains ALPVC 3, 11 and 12 respectively

The strains ALPVC3, ALPVC11 and ALPVC 12 isolated from surface waters of Alappuzha gave amplicons of ~316 bp, typical for *ace* gene.

4.2.2.3. PCR detection of *hly*A

V. cholerae El Tor O1 and non-O1 strains are capable of producing a water soluble cytolytic toxin that has been designated HlyA or El Tor haemolysin. HlyA haemolysin lyses erythrocytes and other mammalian cells. The *hly*A gene which encodes this cytolysin was detected by PCR in 8 environmental isolates (Fig 4.8), and obtained the ~481 bp amplicon corresponding to the *hly*A gene. The strains hosting the *hly*A gene were seven isolates from Alappuzha (ALPVC3, ALPVC4, ALPVC7, ALPVC8, ALPVC11, ALPVC12 and ALPVC14), and two each from Ernakulam (EKM4 and EKM14) and Kannamaly mangroves (KNM4 and KNM12).

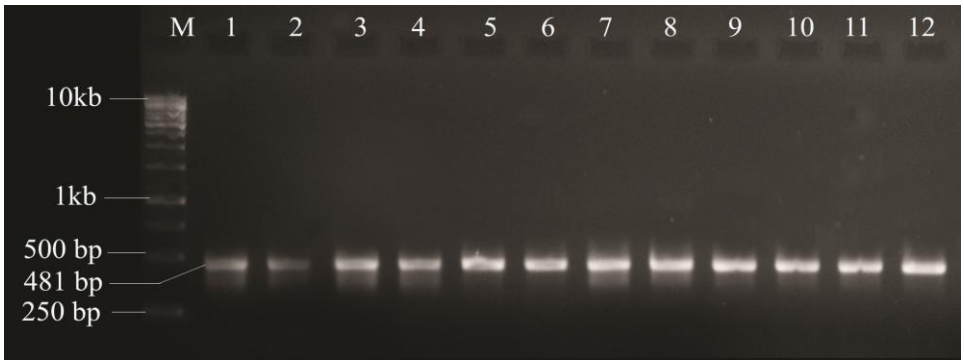


Fig.4.8.Detection of *hly*A in environmental isolates of *V. cholerae*

Lane M-1kb molecular weight ladder; Lane 1; Positive control (*V. cholerae* CO366 El Tor strain); Lanes 2- 8 are strains, ALPVC3, 4, 7, 8, 11, 12 and 14 respectively; Lane 9 and 10 are strains, KNM4 and KNM12 respectively; Lane 11 and 12 are strains, EKM4 and 14 respectively.

4.2.2.4. PCR detection of *tox*R

The *tox*R gene, a global regulator of virulence and membrane porin gene expression, is present in the ancestral chromosome of *V. cholerae.* Its presence in the environmental isolates was detected using PCR by targeting the ~779 bp region of the genomic DNA, using the gene specific primers. The *tox*R gene was detected in nine *V. cholerae* strains of environmental origin (Fig. 4.9), including ALPVC3, ALPVC4, ALPVC7, ALPVC8, ALPVC11, ALPVC12, and ALPVC15 from surface waters of Alappuzha. EKM4 and EKM14 from Ernakulam also gave amplicons of *tox*R gene.

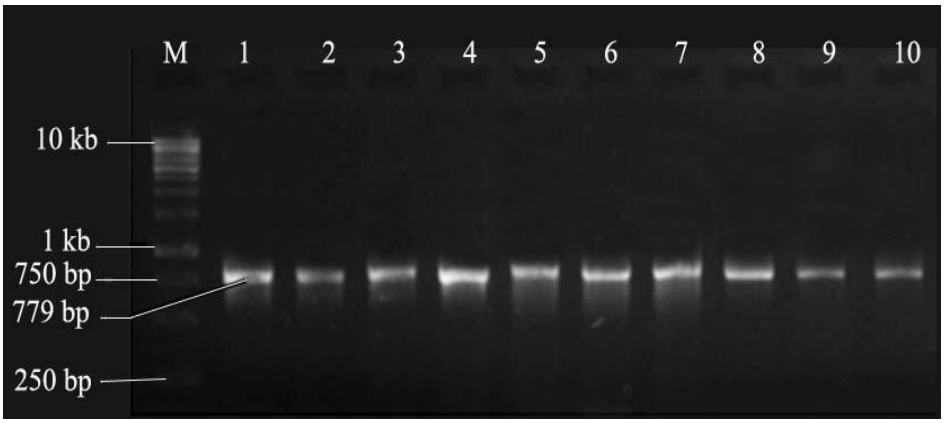


Fig.4.9 Detection of *tox*R gene in environmental isolates of *V. cholerae* Lane M- 1 kb ladder; Lane 1- Positive control (*V. cholerae* CO366 El Tor strain); Lanes 2-10: PCR amplicons from strains ALPVC3, 4, 7, 8, 11, 12, 14, EKM 4 and EKM 14 respectively.

4.2.2.5. Multiplex PCR detection of *tcp*A and *zot*

V. cholerae strains proficient of causing cholera perpetually carry genes for TCP which is an adhesin that is co-ordinately regulated with CT production. Zot (zonula occludens toxin) increases the permeability of mucosa by affecting the structure of the tight junction, or zonula occludens. The multiplex PCR assay for *tcp*A (451 bp) and *zot* (947 bp) gene revealed its presence in the environmental isolates of *V. cholerae* (Fig 4.10).

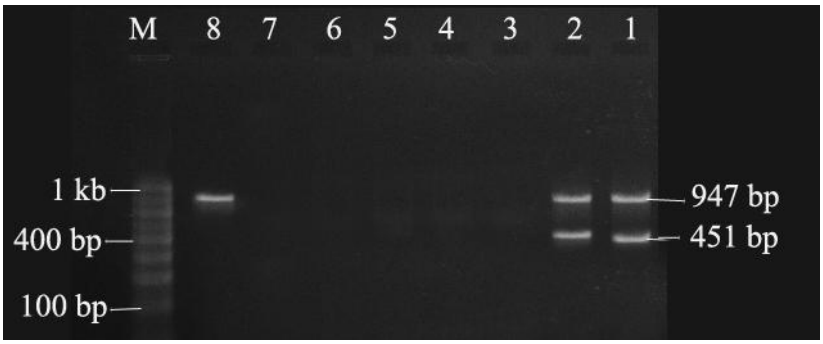


Fig.4.10. Agarose gel of multiplex PCR for *tcp*A and *Zot*

Lane M-100 bp DNA ladder; Lane 1-Positive control (*V. cholerae* CO366 El Tor strain). Lane 2-8; PCR amplicons from strains ALPVC3, ALPVC4, ALPVC5, ALPVC7, ALPVC8, ALPVC11, ALPVC12 respectively.

In this study only strain ALPVC3 was positive for *tcpA* gene as indicated by the 451 bp amplicon in lane 2 (Fig 4.10). Nontoxigenic- nonpathogenic *V. cholerae* with *tcp*A gene is a rare occurrence. The strains hosting the *zot* gene were ALPVC3 and ALPVC12 from Alappuzha.

4.2.2.6. Multiplex PCR for detection of *nanH* and *int*

V. cholerae neuraminidase (NANase) is hypothesized to act synergistically with cholera toxin (CT) to increase the severity of a secretory response by increasing the binding and penetration of CT to enterocytes. Bacteriophage-like integrase gene (*int*), which is located upstream of *nanH* (encoding neuraminidase) was also amplified using specific primers. Multiplex PCR for *nan*H (1.9 kb) and *int* genes demonstrated that all *nan*H-positive *V. cholerae* isolates gave an expected 1.0 kb amplicon, indicating the presence of *int* in these strains (Fig 4.11).

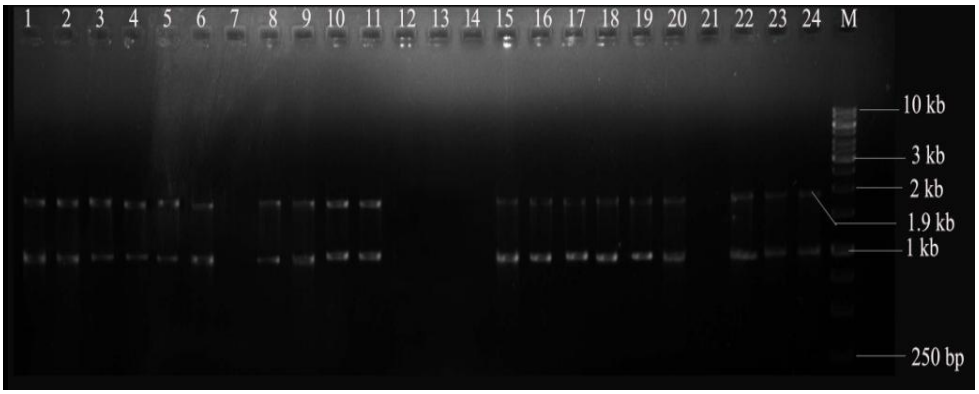


Fig.4.11 Agarose gel of multiplex PCR for *nanH* and *int* genes

Lane M-1 kb molecular weight ladder; Lanes 1- 11 are strains, ALPVC 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 respectively; Lane 12 and 13 are strains, KNM4 and KNM12 respectively; Lane 14-MVN7; Lane 15-22 are strains, EKM1, 2, 4, 6, 7, 8, 10 and 14 respectively; Lane 23-O139 standard strain; Lane 24; Positive control (*V. cholerae* CO366 El Tor strain)

All *V. cholerae* strains from Alappuzha,*i.e.* ALPVC3, ALPVC4, ALPVC5, ALPVC6, ALPVC7, ALPVC8, ALPVC10, ALPVC11, ALPVC12 and ALPVC14, were shown to harbor both *nanH* and *int* genes. None of the isolates from Kannamaly and Mangalavanam mangrooves were host to *nan*H and *int* gene. However, the strains EKM1, EKM2, EKM4, EKM6, EKM7, EKM8 and EKM14 from Ernakulam also gave the typical 1.9 kb and 1 kb amplicons after multiplex PCR.

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

The distribution of these tested virulence genes among the isolates were analysed and is as given in Fig.4.12. The *omp*W gene, a marker gene in *V. cholerae*, was amplified in all strains. The genes encoding cholera toxin (CT), *ctx*A and *ctx*B were not amplified in any of the strains. The genes *nan*H and *int* were present in 72% of the tested strains whereas the *hly*A gene was present in

50% of the isolates. The *tox*R gene was detected in 40% and *ace* in 14% of the environmental strains. The genes *zot* and *tcp*A were present in the least frequency i.e., 9% and 5% respectively in the tested strains. Out of the 22 isolates, MVN7 and EKM10 did not show the presence of any of the virulence genes tested, other than the marker gene *omp*W. 95% of the environmental isolates were found to harbour various virulence genes. The accumulation of virulence genes and the chances of acquiring new virulence genes is evident from the fig 4.12.

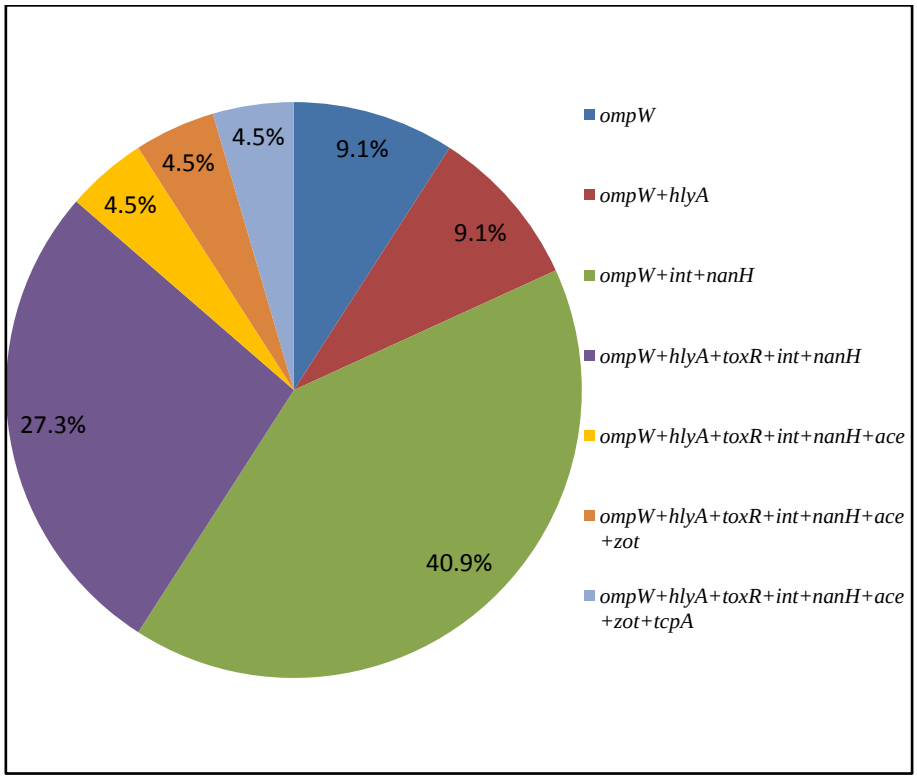


Fig.4.12. Distribution of virulence genes among *V. cholerae* strains

4.2.2.8. Virulence profile of *V. cholerae* strains

The virulence profile of the tested *V. cholerae* strains is represented in Table 4.2.

Table 4.2 Virulence profile of *V. cholerae* strains isolated from marine environments.

Strain	*omp*W	*ctx*A	*ctx*A	*ace*	*hly*A	*zot*	*tox*R	*int*	*nan*H	*tcp*A
KNM4	+	-	-	-	+	-	-	-	-	-
KNM12	+	-	-	-	+	-	-	-	-	-
MVN7	+	-	-	-	-	-	-	-	-	-
ALPVC3	+	-	-	+	+	+	+	+	+	+
ALPVC4	+	-	-	-	+	-	+	+	+	-
ALPVC5	+	-	-	-	-	-	-	+	+	-
ALPVC6	+	-	-	-	-	-	-	+	+	-
ALPVC7	+	-	-	-	+	-	+	+	+	-
ALPVC8	+	-	-	-	+	-	+	+	+	-
ALPVC9	+	-	-	-	-	-	-	+	+	-
ALPVC10	+	-	-	-	-	-	-	+	+	-
ALPVC11	+	-	-	+	+	-	+	+	+	-
ALPVC12	+	-	-	+	+	+	+	+	+	-
ALPVC14	+	-	-	-	+	-	+	+	+	-
EKM1	+	-	-	-	-	-	-	+	+	-
EKM2	+	-	-	-	-	-	-	+	+	-
EKM4	+	-	-	-	+	-	+	+	+	-
EKM6	+	-	-	-	-	-	-	+	+	-
EKM7	+	-	-	-	-	-	-	+	+	-
EKM8	+	-	-	-	-	-	-	+	+	-
EKM10	+	-	-	-	-	-	-	-	-	-
EKM14	+	-	-	-	+	-	+	+	+	-

'+' indicates presence of gene and '-' indicates absence of gene

4.2.3. Structure prediction of TcpA protein in *V. cholerae* strain ALPVC3

Among the environmental isolates of *V. cholerae*, only ALPVC3, a non O1/ non-O139 strain showed the presence of *tcp*A as indicated by the ~480 bp amplicon obtained after multiplex PCR. The 453 bp *tcp*A sequence obtained was shown to be 100% similar to that of other *tcp*A genes of *V. cholerae* after BLAST analysis. The partial gene sequences were deposited at NCBI and the accession number obtained is KJ734987. The amino acid sequence and the open reading frame were identified for further studies and is shown in Fig 4.13.

```
  4 atgacccagacctatcgcgcgctgggcaactatccggcgaccgcg
    M  T  Q  T  Y  R  A  L  G  N  Y  P  A  T  A
 49 aacgcgaacgcggcgacccagctgaccagcggcctggtgagcctg
    N  A  N  A  A  T  Q  L  T  S  G  L  V  S  L
 94 ggcaaagtgagcgcggatgaagcgaaaaacccgtttaccggcacc
    G  K  V  S  A  D  E  A  K  N  P  F  T  G  T
139 gcgatgaacatttttgcgtttccgcgcaacggcgcgccgaacaaa
    A  M  N  I  F  A  F  P  R  N  G  A  P  N  K
184 gcgtttgcgattaccgtggatggcctgacccaggcgcagtgcaaa
    A  F  A  I  T  V  D  G  L  T  Q  A  Q  C  K
229 accctgattaccagcgtgggcgatatgtttccgtatgtgctggtg
    T  L  I  T  S  V  G  D  M  F  P  Y  V  L  V
274 aaagaaggcgcgtttgcggcgtttgcggatctgaccgattttgaa
    K  E  G  A  F  A  A  F  A  D  L  T  D  F  E
319 accacccaggcgaaagcggcggatggcgtgggcgtgattaaaagc
    T  T  Q  A  K  A  A  D  G  V  G  V  I  K  S
364 attgcgccgggcggcaccaacctgaaactgaccgaaattgcgcat
    I  A  P  G  G  T  N  L  K  L  T  E  I  A  H
409 gtggaagcgctgtgcaccggcaccgcgccgtttggcgtggcgttt
    V  E  A  L  C  T  G  T  A  P  F  G  V  A  F
454 ggc 456
    G
```

Fig 4.13 Sequence analysis of *tcp*A gene using ORF Finder

An ORF of 453 bp was detected by the NCBI ORF finder tool. The start codon ATG at position 4 (indicated in blue). This ORF was in the reading frame 1 in 5'→ 3' direction. The 152 aminoacid protein sequence identified as TcpA protein in ExPasy, was aligned with sequences which showed maximum identity

after pBLAST of TcpA protein sequence of ALPVC3. The TcpA protein with accession numbers 3HRV_A of *V. cholerae* (El Tor), AAB86483 of Non-O1 *V. cholerae*, AAM66351 of *V. cholerae* O139, ABC75878 of *V. mimicus* was compared using ClustalW2. The results (Fig. 4.14) showed that the partial protein sequence of TcpA from ALPVC3 was 100% similar that of the protein sequences of Tcp isolated from *V. cholerae* O139 and El Tor. Some sequence variations are noticed when compared with the TcpA of *V. mimicus.*

```
ALPVC3          ------------------------------------------------------SMTQTY
AAB86483        ---KTGQEGMTLLEVIIVLGIMGVVSAGVVTLAQRAIDSQNMTKAAQNLNTVQVSMTQTY
3HRV_A          ---------------MGSSHHHHHHSSGL-VPRGSHMDSQNMTKAAQNLNSVQIAMTQTY
AAM66351        ------------------LGIMGVVSAGVVTLAQRAIDSQNMTKAAQNLNSVQIAMTQTY
ABC75878        HDKKTGQEGMTLLEVIIVLGIMGVVSAGVVTLAQRAIDSQNMTKAAQNLNSVQIAMTQTY
                                                                     :*****

ALPVC3          RALGNYPATANANAATQLTSGLVSLGKVSADEAKNPFTGTAMNIFAFPRNGAPNKAFAIT
AAB86483        RALGNYPATANIAAATKLTSGLVSLGKISSDEAKNPFTGTNMNIFAFPRNGAPNKAFAIA
3HRV_A          RSLGNYPATANANAATQLANGLVSLGKVSADEAKNPFTGTAMGIFSFPRNSAANKAFAIT
AAM66351        RSLGNYPATANANAATQLANGLVSLGKVSADEAKNPFTGTAMGIFSFPRNSAANKAFAIT
ABC75878        RSLGNYPATADANAATQLANGLVSLGKVSADEAKNPFTGTAMGIFSFPRNSAANKAFAIT
                *:********:  ***:*:.:*******:*:********** * **:****.* ******:

ALPVC3          VDGLTQAQCKTLITSVGDMFPYVLVKEGAFAAFADLTDFETTQAKAADGVGVIKSIAPGG
AAB86483        VDGLTQAQCKTLITSVGDMFPYVLIKSAGTIDFADLTDFETTQAKAADGVGVIKSIAPGG
3HRV_A          VGGLTQAQCKTLVTSVGDMFPFINVKEGAFAAVADLGDFETSVADAATGAGVIKSIAPGS
AAM66351        VGGLTQAQCKTLVTSVGDMFPFINVKEGAFAAVADLGDFETSVADAATGAGVIKSIAPGS
ABC75878        VGGLTQAQCKTLVTSVGDMFPFINVKEGAFAAVADLGDFETSVADAATGAGVIKSIAPGS
                * **********:********:: :*...   .*** ****: *.** *.*********.

ALPVC3          TNLKLTEIAHVEALCTGTAPFGVAFG--
AAB86483        TNLKLTEIAHVEALCTGTAPFGVAFG--
3HRV_A          ANLNLTNITHVEKLCTGTAPFTVAFGNS
AAM66351        ANLNLTNITHVEKLCTGTAPFTVAFGNS
ABC75878        ANLNLTNITHVEKLCTGTAPFGVAF---
                :**:**:*:*** ******** ***
```

Fig 4.14. Multiple sequence alignment of TcpA protein using ClustalW

SCOP database provides structural and evolutionary relationships between all proteins whose structure is known. TcpA, a type IV pilin was assigned to TcpA

like pilin family in SCOP database with the following lineage. It is assigned in class of alpha and beta proteins mainly antiparallel beta sheets. The toxin coregulated pilus subunit TcpA of *V. cholerae* contains very long N-terminal helix, whose end is packed against beta-sheets, which is unique for pili subunits. It belongs to the superfamily of bacterial filament proteins. The family of TcpA like pilin family has the common folds decorated with additional structures.

The tertiary structure of the TcpA protein in *V. cholerae* ALPVC3 was predicted using Phyre[2] software, from the deduced amino acid sequence (Fig. 4.15). The residues were modelled by the single highest scoring template and the model was based on the template d1oqva (Fold library id) of PDB database.

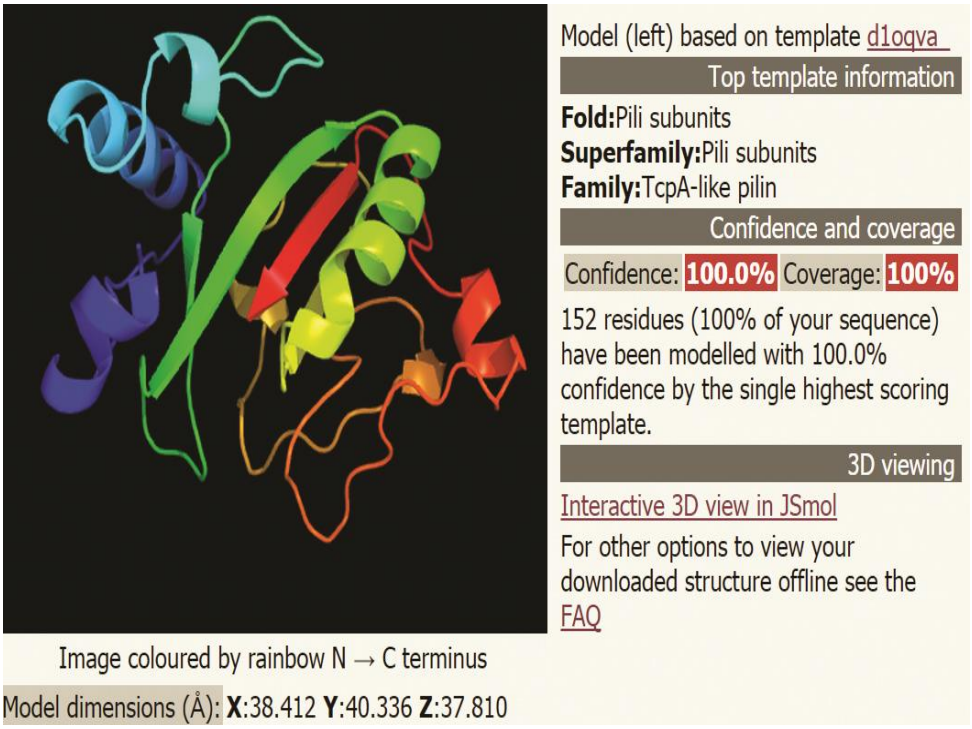


Fig. 4.15 Template based homology modeling of TcpA of ALPVC3
(The dimensions of the model are given in Angstroms)

The model showed very high similarity with the TcpA pilin found in O1 *V. cholera* with pili subunit like fold of pili subunnits super family. The 152 amino acid residues which were identified in the ORF finder were successfully modelled with 100% confidence on the template d1oqva. The secondary structure was also predicted based on the template d1oqva (Fig. 4.16). The amino acid sequence was aligned with the predicted secondary structures. The predicted structure consisted of 42% α-helix and 15% β-strand and the model was predicted with 34% disorder.

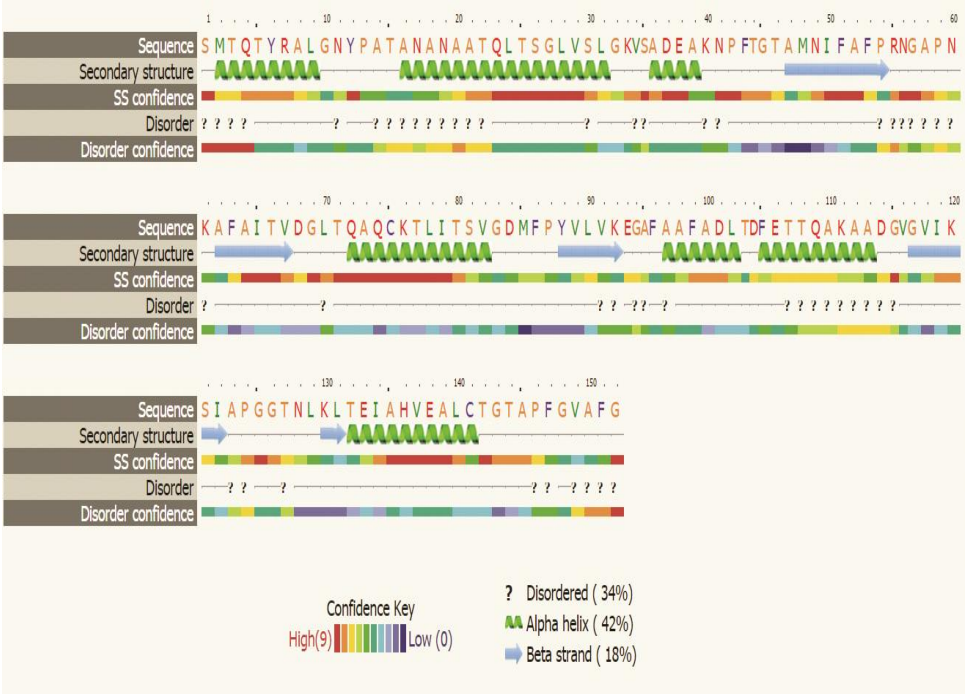


Fig. 4.16. Secondary structures in TcpA aligned with its amino acid sequence

4.3. Differential induction and purification of prophages

Induction studies were conducted using the non-O1 environmental isolates of *V. cholerae* that were found to have inducible lysogens. The indicator strains/ propagating strain are essential for further propagation and characterisation of the phages. These phages were enumerated, purified and characterised.

4.3.1. Identification of indicator /propagating strain

The host range of twenty two vibriophages which were assayed based on the morphology of the plaques produced by the phages on bacterial lawn is as outlined in table 4.3.

Table 4.3. Host range studies of the phages

Phage	Host strains producing translucent plaques	Host strains producing clear plaques
ΦKNM4	KNM4 , MUS 6	MAK757
ΦKNM12	KNM12	-
ΦMVN7	MVN7	-
ΦALPVC3	ALPVC3, MUS 6	MAK757
ΦALPVC4	ALPVC4	-
ΦALPVC5	ALPVC5	-
ΦALPVC6	ALPVC6, MUS 6	-
ΦALPVC7	ALPVC7, MUS 6	MUS 6
ΦALPVC8	ALPVC8	MUS 12
ΦALPVC9	ALPVC9	-
ΦALPVC10	ALPVC10	-
ΦALPVC11	ALPVC11, MUS 6	MAK757
ΦALPVC12	ALPVC12	MAK757
ΦALPVC14	ALPVC14	-
ΦEKM1	EKM1, MUS 12	-
ΦEKM2	EKM2	-
ΦEKM4	EKM4, MUS 12	MUS 12
ΦEKM6	EKM6	-
ΦEKM7	EKM7	-
ΦEKM8	EKM8, MUS 12	-
ΦEKM10	EKM10	-
ΦEKM14	EKM14, MUS 12	MAK757

All the vibriophages produced translucent plaques with bull's eye morphology on its own host. The strain MUS6 (*V. cholerae*) could be infected by ΦKNM4, ΦALPVC3, ΦALPVC6, ΦALPVC7 and ΦALPVC11. The phages ΦEKM1, ΦEKM4, ΦEKM8 and ΦEKM14 produced translucent plaques with MUS12 (*V. cholerae*). The host strains which are completely lysed by the phage therefore forming clear plaques were collected as indicator strain/recipient strain. The indicator strain with largest number of plaques for a given phage suspension was defined as the propagating strain for that phage suspension. The strain MAK757 (*V. cholerae*, ATCC 51352) was identified as a good propagating strain for ΦKNM4, ΦALPVC3, ΦALPVC11, ΦALPVC12 and EKM14. ΦALPVC7 produced a few clear plaques on the lawn of MUS6 whereas ΦALPVC8 produced a similar result with MUS12.

Of the 22 phages, ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 were consistently induced with mitomycin C. These phages produced high phage titre on the indicator strain MAK757 (identified as propagating strain) and were therefore selected for further study.

4.3.2. Effect of various biotic and abiotic factors on phage induction and bacterial growth.

The study on environmental isolates of *V. cholerae* has shown that prophage induction is possible in natural ecosystem due to abiotic factors like pollutants, temperature and UV.

ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 were able to produce plaques by spontaneous induction *i.e.* even in the absence of induction agents. Relative phage titer indicates the actual effect of induction agents after deducting the effect of spontaneous induction. The changes in bacterial growth curve in the presence of induction agents revealed the direct relation between the lysis of bacteria and the increase in phage number. *V. cholerae* under normal

aerobic conditions showed a growth curve with a log phase lasting for ~ 6 h (~10^8-10^9 CFU/mL) after which the bacterial count declined significantly

4.3.2.1. Effect of mitomycin C (MMC) in prophage induction and bacterial growth

Mitomycin C (MMC) produced maximum induction of the lysogens under study (Fig.4.17). MMC is an antibiotic that inhibits DNA synthesis in bacteria by cross-linking DNA strands, eliciting the SOS response and causing prophage induction. ΦALPVC3, ΦALPVC11 and ΦEKM14 produced very high phage titers (~10^6 PFU/mL) on treatment with mitomycin C. ΦALPVC12 produced comparably low phage (~10^4 PFU/mL) and this can be correlated to the minimum bacterial death of strain ALPVC12 in the presence of mitomycin C.

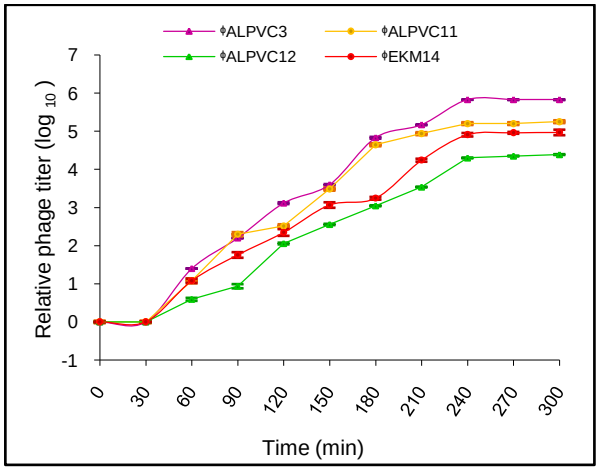


Fig 4.17. Efficiency of Mitomycin C in prophage induction

A rapid drop of absorbance of cell suspensions due to cell lysis was observed midway in cell growth (Fig 4.18). When bacterial cells of strain ALPVC3 and ALPVC11 were incubated in MMC, after little inhibition in cell growth for about two hours, an apparent decrease in absorbance was observed, and followed by a suppression of cell growth thereafter. An analogous change in

absorbance was observed for *V. cholerae* ALPVC12 and EKM14 cells after a period of three hours. The rapid decrease of absorbance might strongly suggest the occurrence of cell lysis by the phage induction from the cells, because MMC has the ability to induce phages from bacteria.

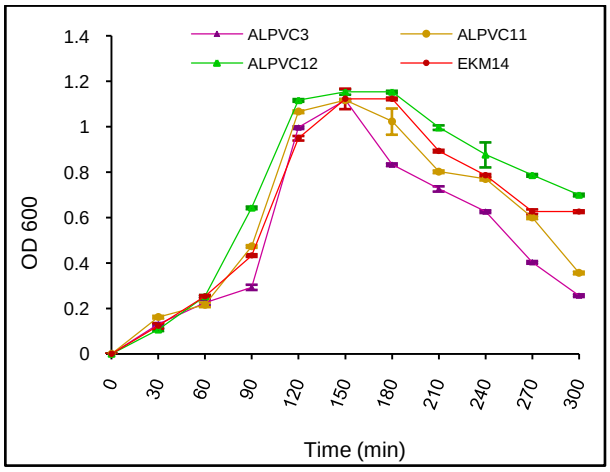


Fig 4.18. Effect of Mitomycin C on the growth of host bacteria

4.3.2.2. Effect of UV in prophage induction and bacterial growth

The traditional and most common approach to study prophages or temperate phages is to induce lysogenic bacteria with mitomycin C treatment or UV exposure. Induction study using 15W bactericidal UV lamp of wavelength 254 nm also produced a high phage titer (~10^3-10^5 PFU/mL) (Fig 4.19) which is clear evidence of prophage induction in nature due to variations in quality of light.

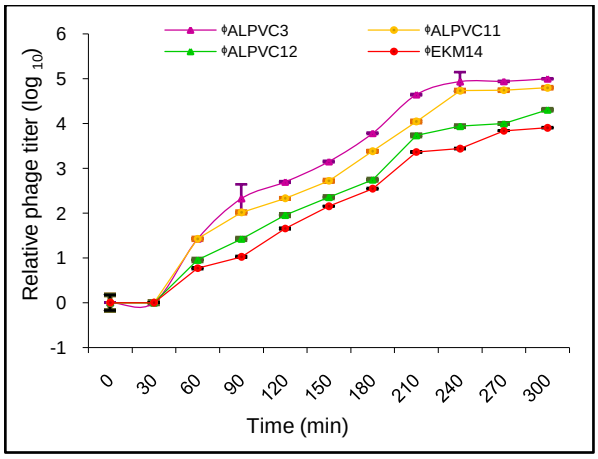


Fig 4.19. Efficiency of UV in prophage induction

In fig 4.19, ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 showed a latent period of 30 minutes. A burst size of ~30 phages per bacterium was obtained for ΦALPVC3 in the presence of UV light. A lower burst size of ~15 phages per bacterium was observed for ΦEM14 under UV induction. The release of prophage ΦEKM14 was also significantly lower (~10^3 PFU/mL) compared to others.

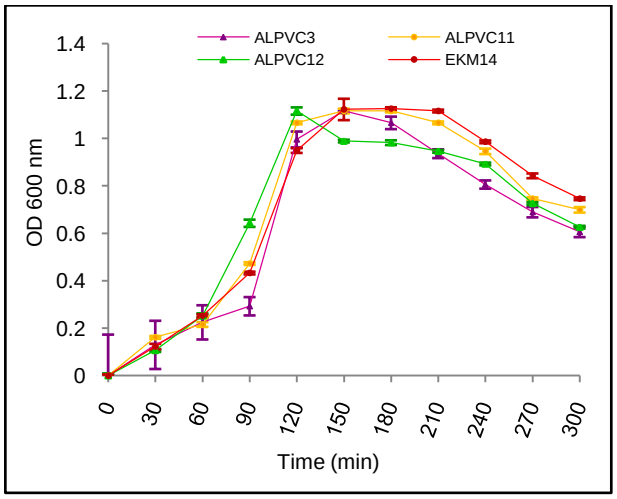


Fig 4.20. Effect of UV on the growth of host bacteria

The growth curve of the host bacteria showed variation when exposed to UV light for different time intervals (Fig 4.20). The presence UV radiation slowed down the multiplication of bacteria. Instead of a steady stationary period of growth, a steady decrease in OD value was noticed after the cultures were exposed to UV. The overall growth of the bacteria was impaired in presence of UV light.

4.3.2.3. Effect of Nalidixic acid in prophage induction and bacterial growth

Nalidixic acid specifically inhibits the synthesis of deoxyribonucleic acid (DNA) in prokaryotes. Nalidixic acid which was not reported earlier as an induction agent was also proved to be a potent inducing agent in this study (Fig 4.21). ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 showed a similar growth curve and relative phage titer was at par with the results of induction by mitomycin C (~10^5 PFU/mL). None of the phages showed a lag in latent period. ΦALPVC12 gave the least burst size of ~15 phages per bacterium compared to the other three phages (~40-45 phages per bacterium)

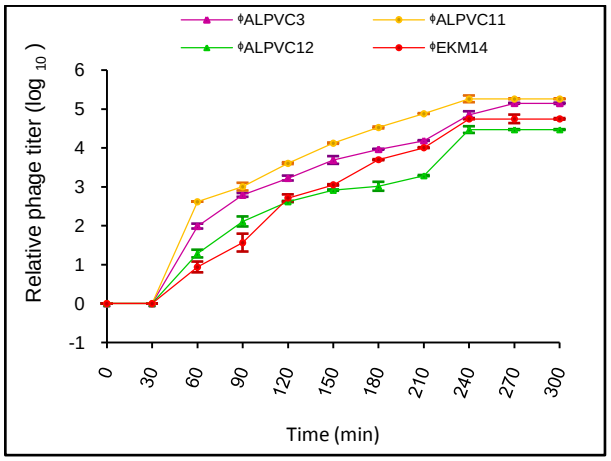


Fig 4.21. Efficiency of Nalidixic acid in prophage induction

The effect of nalidixic acid on the growth of bacterial cultures is as demonstrated in Fig 4.22.

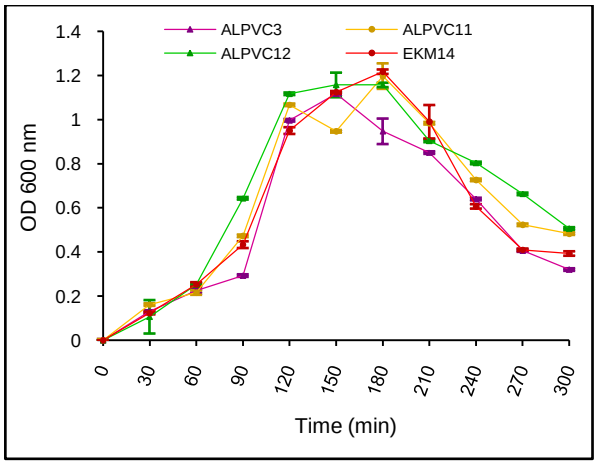


Fig 4.22. Effect of Nalidixic acid on the growth of host bacteria

The inhibition of bacterial growth by nalidixic acid was evident from the rapid fall in the OD values. The bacterial cells almost settled down at the bottom of the tubes after four hours. The action of nalidixic acid on the growth of host can be correlated to the high rates of induction and proliferation of corresponding phages under study.

4.3.2.4. Effect of temperature in prophage induction and bacterial growth

ΦALPVC3, ΦALPVC11 and ΦALPVC12 were induced from their corresponding host after the temperature treatment (Fig 4.23). It was proved that elevated temperature can cause bacteriophage induction and lytic development in the environment. The phage titers obtained were low (~10-10^2 PFU/mL) when compared with the effect of MMC, nalidixic acid and UV. ΦEKM14 was not significantly induced by increase in temperature.

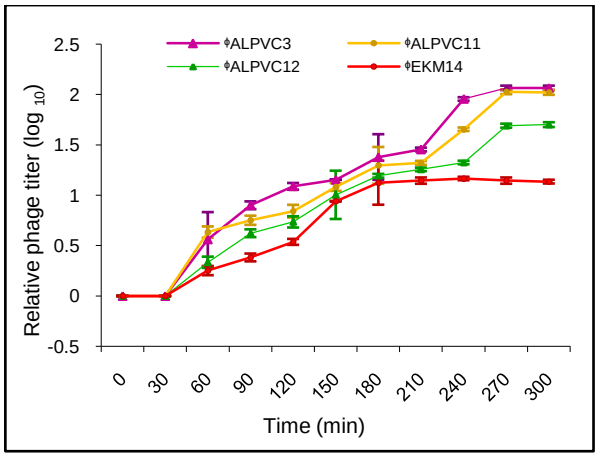


Fig 4.23. Efficiency of temperature (60°C) in prophage induction

The effect of temperature on the growth of bacteria is shown in Fig 4.24 Elevated temperature caused the negative effects on bacterial growth.

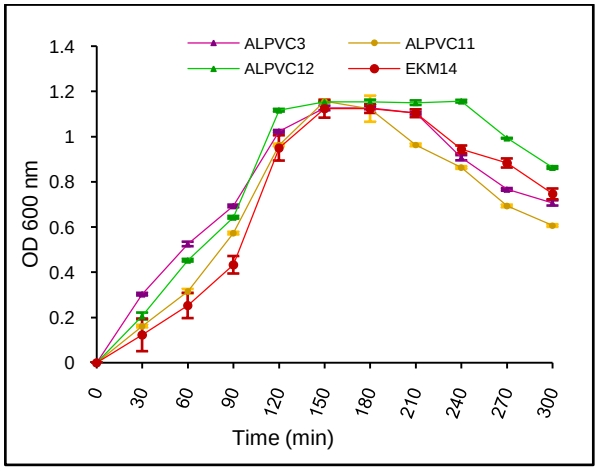


Fig 4.24. Effect of temperature on the growth of host bacteria

The host strains ALPVC3, ALPVC11, ALPVC12 and EKM14 survived the effect of increased temperature for one hour after the given treatment when OD_{600} is 1. ALPVC11 showed an early drop down (after 1 h) in OD compared to

ALPVC3 and EKM14 which showed a decrease in OD after 1½ hours. EKM14 was more tolerant to high temperature compared to others. OD_{600} reached ~0.5 after 5 hours of growth which was significantly lower when compared to control. Present study proves that elevated temperature can cause phage induction.

4.3.2.5. Effect of H_2O_2 in prophage induction and bacterial growth

Hydrogen peroxide produced very low induction of lysogens in this study (Fig.4.25). H_2O_2 appeared to be an effective inducer of at least some prophages, particularly ΦEKM14. On the other hand, significantly lower average efficiency of prophages' induction by hydrogen peroxide was observed relative to mitomycin C and nalidixic acid.

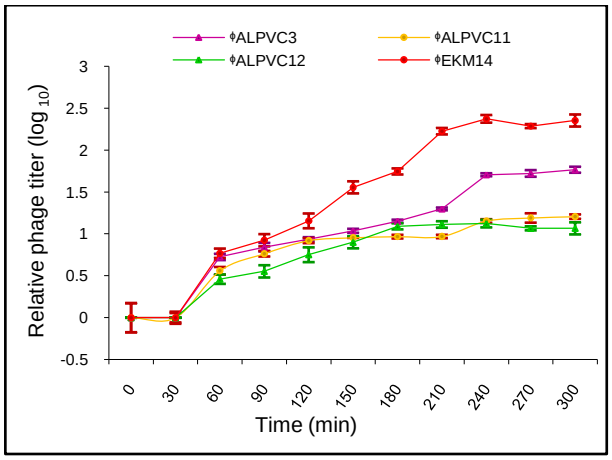


Fig 4.25 Efficiency of H_2O_2 in prophage induction

ΦALPVC3, ΦALPVC11 and ΦEKM14 produced very low phage titers (~10-10^2 PFU/mL) on treatment with H_2O_2.

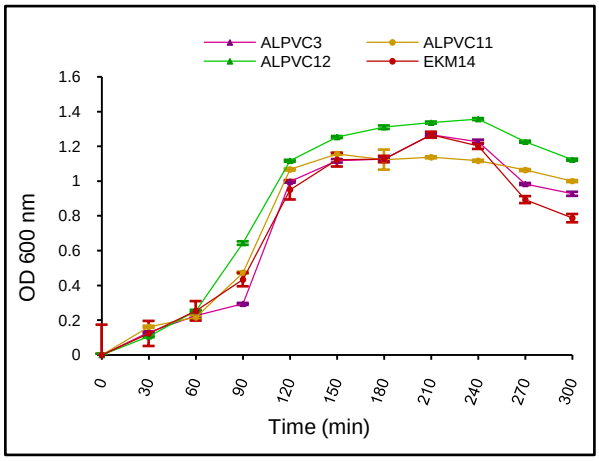


Fig 4.26 Effect of H_2O_2 on the growth of host bacteria

The effect of H_2O_2 on the growth of host strains ALPVC3, ALPVC11 and ALPVC12 is shown in Fig 4.26. The growth of bacteria was slightly impaired, compared to control. The growth of strain EKM14 reduced after one hour of treatment and a steady drop in OD was noticed thereafter. The decrease in cell density was due to lysis of a small percent of host cells by H_2O_2 mediated induction.

4.3.2.6. Effect of NaCl in prophage induction and bacterial growth

The effect of salinity on prophage induction was studied (Fig 4.27). One hour latent period was obtained for all the phages, with very low phage titer.

The vibriophages, ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 showed similar induction curves. All the bacteriophages showed a similar rise period of 3 h followed by a plateau.

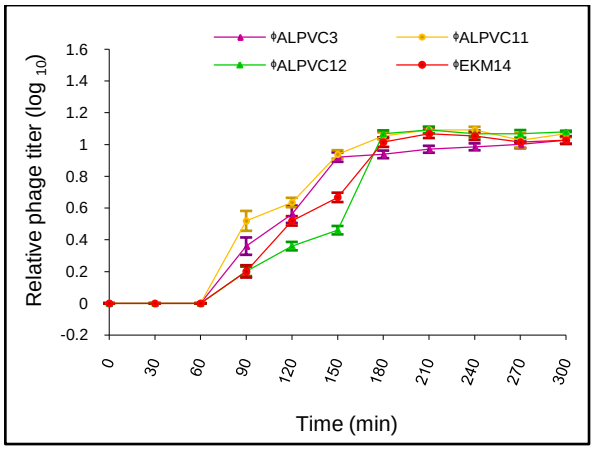


Fig 4.27. Efficiency of NaCl in prophage induction

This study revealed that increase in NaCl concentration did not affect growth of the host *V. cholerae strains* (Fig 4.28).

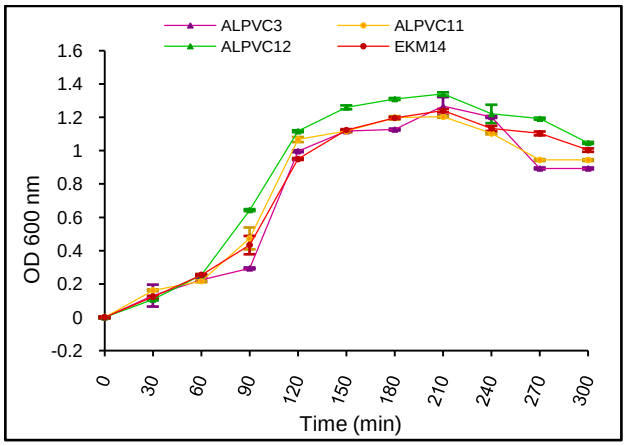


Fig 4.28. Effect of NaCl on the growth of host bacteria

Cell lysis and thereby decrease in turbidity of the culture was evident after 4 hours of growth especially in case of strains ALPVC3 and ALPVC11. In the presence of 2 M concentration of NaCl the bacterial growth curve was similar to control, except that the cell decline was initiated at the fourth hour of growth. The results of differential induction of prophages in terms of growth kinetic parameters

viz latent period rise period and burst size under different treatments are summarised in table 4.4.

Table 4.4. Differential Induction of prophages under different treatments

Treatment	Phage	Latent period (min)	Rise period (min)	Burst size (Phages/ bacterium)
Mitomycin C (MMC) 1.0 µg/mL	ΦALPVC3	30	90	70
	ΦALPVC11	30	90	60
	ΦALPVC12	30	60	50
	ΦEKM14	30	60	45
UV	ΦALPVC3	30	90	60
	ΦALPVC11	30	90	50
	ΦALPVC12	30	90	45
	ΦEKM14	30	90	45
Nalidixic acid 3.0 µg/mL	ΦALPVC3	30	90	60
	ΦALPVC11	30	90	50
	ΦALPVC12	30	90	45
	ΦEKM14	30	90	45
Temperature 60° C	ΦALPVC3	60	60	45
	ΦALPVC11	60	60	30
	ΦALPVC12	60	60	25
	ΦEKM14	60	60	25
H_2O_2 3 mM	ΦALPVC3	90	30	45
	ΦALPVC11	90	30	40
	ΦALPVC12	90	30	20
	ΦEKM14	90	30	30
NaCl 2 M	ΦALPVC3	90	60	25
	ΦALPVC11	90	30	25
	ΦALPVC12	90	30	15
	ΦEKM14	90	30	15

Efficiency of induction of prophages and their further development varied considerably in response to the different induction agents. Mitomycin C produced maximum induction in the host bacteria under study. Induction by UV produced

results similar to that of mitomycin C. Nalidixic acid which was not reported previously as an induction agent in vibriophages, was also shown to be a potent inducing agent. Some differences in the length of the lag phase were evident under different inducing agents.

The latent period of the phages were low in the presence of strong induction agents like mitomycin C, UV and nalidixic acid compared to mild inducing agents like temperature, H_2O_2 and NaCl. Rise period was significantly low in the presence of mild inducing agents compared to others. ΦALPVC3 and ΦALPVC11 were strongly induced after DNA damage whereas ΦALPVC12 and ΦEKM14 showed spontaneous induction only during optimal bacterial growth. It was evident from the results that ΦALPVC3 and ΦALPVC11 are effectively induced in various inducing condition compared to other phages. So these phages may be considered as potent candidates for gene transfer in natural conditions.

4.3.3. Optimisation of different parameters for Phage induction using MMC

Mitomycin C was clearly the most efficient inducer of prophages compared to other induction agents studied. Hence various parameters were optimised to maximize phage inductions by mitomycin C. This was studied in ΦALPVC3 which was observed to produce higher phage titers in the differential induction study. Concentration of mitomycin C, incubation temperature, incubation time, concentration of ampicillin in base plate and growth phase of bacterial culture were the major physico-chemical parameters affecting maximum phage production. The results of optimisation of various factors for maximum induction of phage (ΦALPVC3) in *V. cholerae* strain ALPVC3 using mitomycin C are detailed in Fig 4.29.

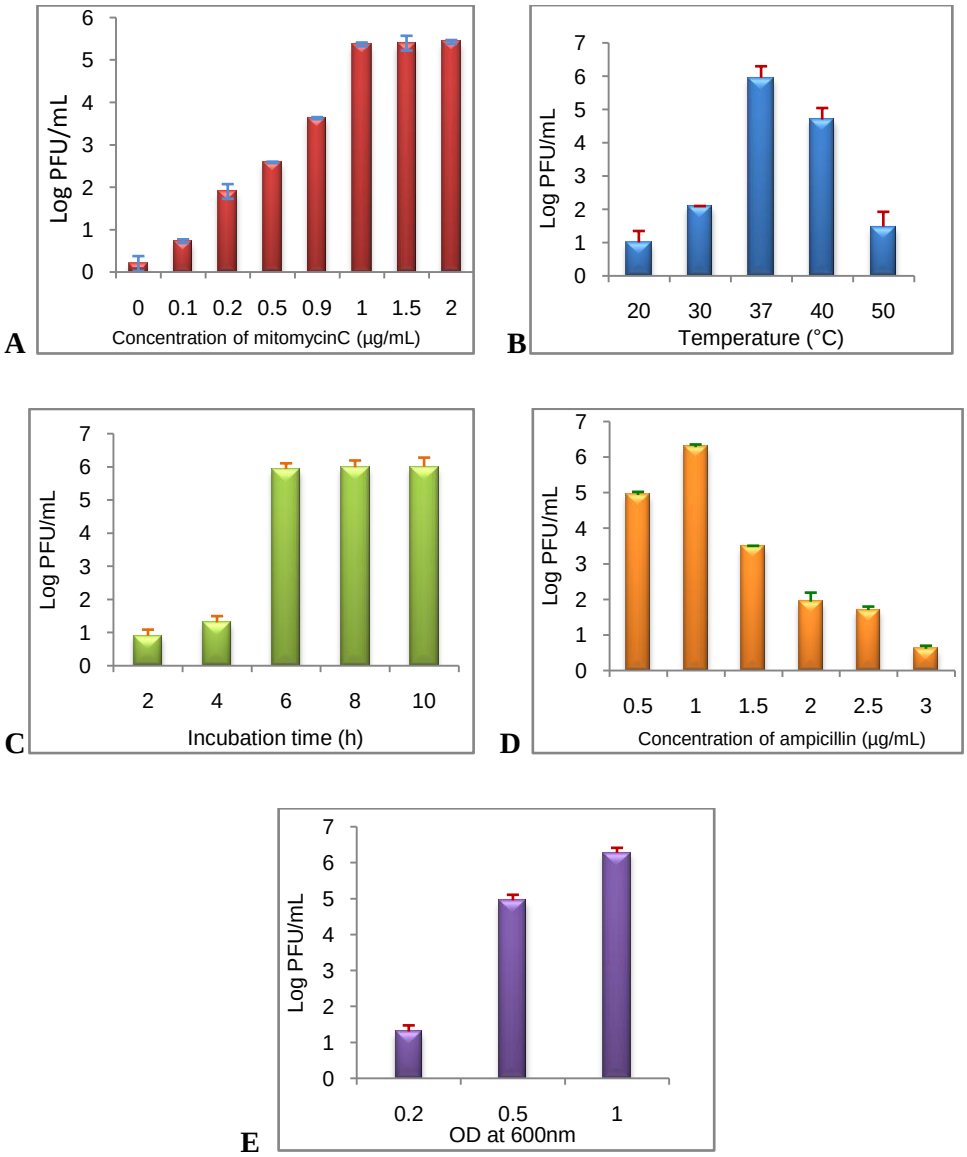


Fig. 4.29 Optimization of culture conditions for induction of phage (ΦALPVC3) in *V. cholerae* strain ALPVC3 using mitomycin C; A Concentration of mitomycin C **B.** Incubation Temperature **C.** Incubation time **D.** Concentration of ampicillin in the base plate **E.** Growth phase of bacterial culture (ALPVC3)

The optimum conditions for maximum phage induction using MMC was carried out taking one-factor at a time. Maximum phage titer was obtained when cultures at OD_{600} is 1.0 was induced using 1 µg/mL mitomycin C and incubated at 37 °C for 6 h and when plated on base plate containing 1 µg/mL amplicillin. The optimised conditions were used for large scale production and concentration of phages.

4.3.4. Concentration of phages

ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 were concentrated up to 1 X 10^{10} PFU/mL using PEG precipitation and these phage concentrates prepared in large quantities were used for all further studies.

4.4. Physicochemical characterization of vibriophages

4.4.1. Characterisation of phage

4.4.1.1. Morphological analysis by Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy based morphological analysis of phages is highly significant as it is the basis for classification of bacteriophages. The TEM elucidated morphology has shown that the four phages studied belong to the three different double stranded DNA phage families, *i.e Myoviridae*, *Siphoviridae* and *Podoviridae*). ΦALPVC3 which was T4-like was a myovirus; ΦALPVC11 and ΦALPVC12 were siphophages and λ-likephages), and ΦEKM14 was a podophage.

TEM image of ΦALPVC 3 (Fig 4.30A) exhibited isometric or elongated head with a diameter of 65 ± 0.50nm and long tail of 75 ± 0.25nm. A few tail fibers or spikes were also noticed.

The TEM pictures of ΦALPVC11 and ΦALPVC12 (Fig 4.30B and 4.30C) show bacteriophages with thin long and flexible tails with icosahedral or prolate virion, which is typical for siphovirus.

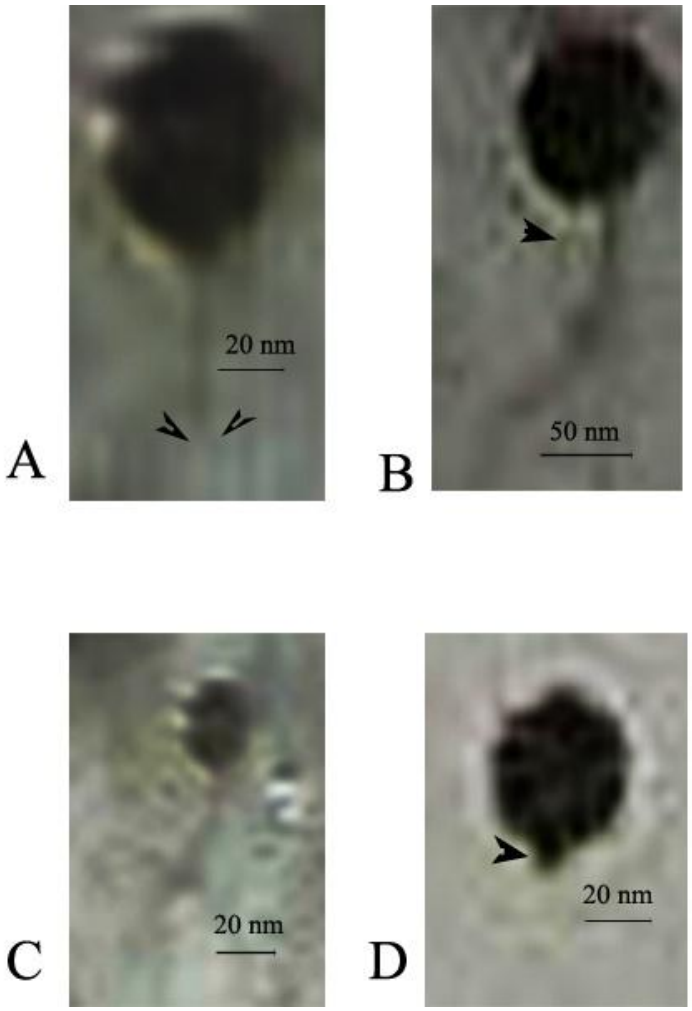


Fig.4.30. Transmission Electron micrograph image of phage stained with 1% uranyl acetate A) ΦALPVC3 with tail fibres (arrows) B) ΦALPVC11 with apical protrusions on head (arrow) C) ΦALPVC12 D) ΦEKM14 with short tail fibres (arrow)

Phage dimensions as observed from the micrographs were as follows- ΦALPVC11 showed 62 ± 0.20 nm head and 159 ± 0.25 nm long tail and that of ΦALPVC12 are 30 ± 0.50 nm (head) and 84 ± 0.40 nm (tail). The vibriophage ΦALPVC11 was also found to have apical protrusions on the head. The two

phages in *Siphoviridae* family thus belonged to two different morphological types.

ΦEKM14 (Fig 4.30D), the podophage was distinguished by short non-contractile tail (12± 0.32 nm) and icosahedral head (44 ± 0.38 nm) which was typical of T-7 like- phages. The phage sizes were determined from the average of 3 independent measurements (mean ± standard deviation).

4.4.1.2. Optimal multiplicity of infection

Multiplicity of infection, defined as the ratio of virus particles to that of the host cells, is an important criterion for the large scale production of bacteriophages, due to its significant impact on phage titre. The MOI of vibriophages ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14, when propagated with the recipient strain MAK757 was three phages per bacterium for ΦALPVC3, ΦALPVC11 and ΦEKM14 and four phages per bacterium for ΦALPVC12. These optimal MOI resulting in the highest phage titre under standard conditions were used in all subsequent large scale phage production of vibriophages, unless otherwise specified.

4.4.1.3. One step growth curve

The parameters required for phage multiplication were calculated from the one-step growth curve. These experiments were performed at 37°C, with optimal MOI. The one step growth curve of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 is as given in Fig 4.31.

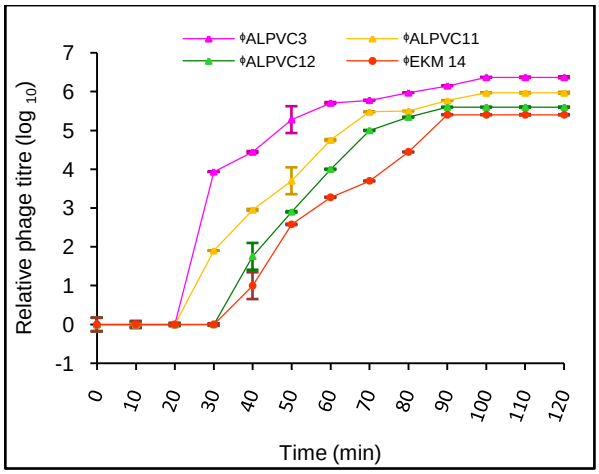


Fig 4.31. One step growth curve of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

The latent period of ΦALPVC3 and ΦALPVC11 was ~20 minutes, while that of ΦALPVC12 and ΦEKM14 was ~30 minutes. Except ΦEKM14, all other vibriophages had a rise period of 70 minutes. The rise period of ΦEKM14 was ~90 minutes. The burst size of all the phages except ΦALPVC3 was roughly similar, and was estimated ~45 phages per bacterium. The calculated burst size was ~60 phages per bacterial cell for ΦALPVC3.

The multiplication period of all the phages under study reached a plateau at about 90 minutes after infection with the indicator strain MAK757. It was noticed from the results that ΦALPVC3 had a larger burst size and a shorter generation period than any other phage studied. A significantly longer lag phase was evident for ΦALPVC12 and ΦEKM14.

4.4.1.4. Phage adsorption

The adsorption curve of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 are shown in Fig 4.32.

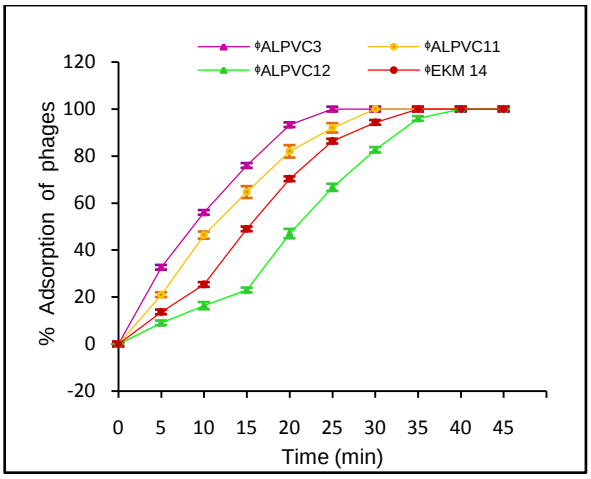


Fig 4.32 Adsorption curves of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

ΦALPVC3 achieved adsorption nearing 100% in 25 min of exposure to host bacteria, where as ΦALPVC11 took 30 min for the same. ΦEKM14 was adsorbed completely in 35 minutes. ΦALPVC12 took the most time for maximum adsorption, *i.e.* about 40 minutes.

4.4.2. Influence of physical and chemical parameters on phage viability

4.4.2.1. Effect of temperature on phage viability

The effect of temperature on the viability of ΦALPVC3, investigated by heat treatment at different temperatures, over varying time intervals is as represented in Fig 4.33. It was evident that phage viability was drastically reduced on exposure to high temperatures. Viable PFU of ΦALPVC3 were highest after exposure to 50 °C. At 60 °C viable PFU were reduced by more than 50% at the end of 3 minutes when compared to the count at 50 °C, whereas complete viability was lost at the end of 2 minutes at 70°C. ΦALPVC3 formed a few plaques at 80°C for 30 sec but failed to survive exposure to 90 °C and 100 °C, even for a few seconds.

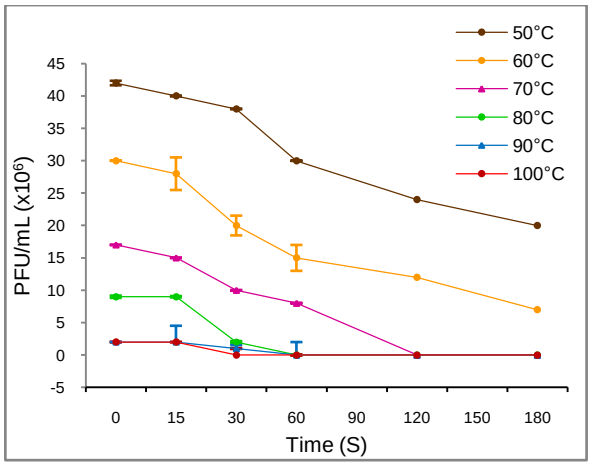


Fig 4.33 Effect of temperature on viability of ΦALPVC3

The influence of temperature variation on viability of phage ΦALPVC11 is as depicted in Fig 4.34. The number of viable PFUs were highest when exposed to 50 ºC, but that was only for a relatively shorter period (up to 60 sec) when compared to that ΦALPVC3. Viability was drastically reduced to a few PFU/mL at 70 ºC, while exposure to temperatures above 70 ºC was fatal for phage ΦALPVC11 as there were no survivors. However, considerable survival was noted at 60 ºC.

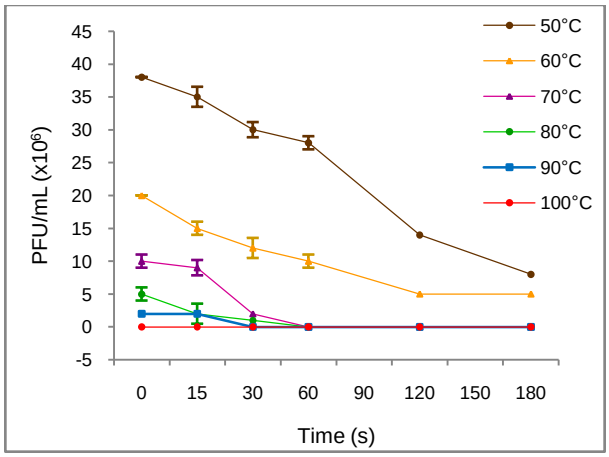


Fig 4.34 Effect of temperature on viability of ΦALPVC11

Fig 4.35 details the effect of temperature on viability of ΦALPVC12. Even though the phage count steadily decreased with increase in exposure time at different temperatures, ΦALPVC12 produced a a small number of viable PFUs even on exposure to 70 °C for 1 hour. A good number of phages survived exposure to 60 °C. However, it wase noted that any temperature above 50 °C was detrimental for phage growth.

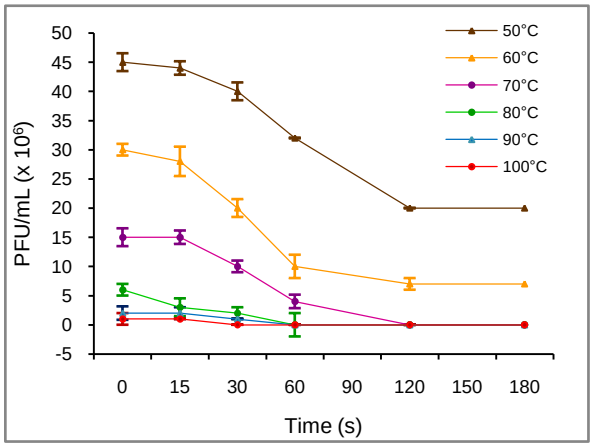


Fig 4.35 Effect of temperature on viability of ΦALPVC12

The temperature tolerance level of ΦEKM14 is represented in Fig 4.36 ΦEKM14 proved to be the most temperature tolerant phage among the four different phages studied. The viable PFUs at 50 °C, 60 °C and 70 °C was maximum for ΦEKM14 after 2 h of incubation at the specified temperatures. Although exposure to high temperatures of 80°C even for 3 minutes was fatal,, there were nevertheless a few survivors at the end of 30 seconds. 100% phage mortality was observed at 90 °C and 100 °C as in case of all other phages.

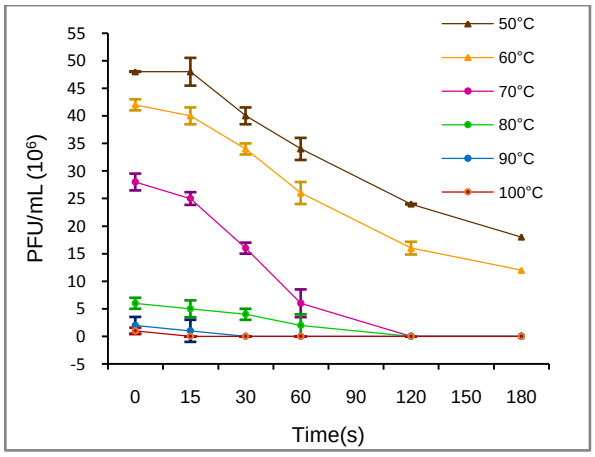


Fig 4.36 Effect of temperature on viability of ΦEKM14

4.4.2.2. Effect of NaCl on phage viability

The effect of varying concentration of NaCl on the viability of the phages ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 are as shown in the Fig 4.37

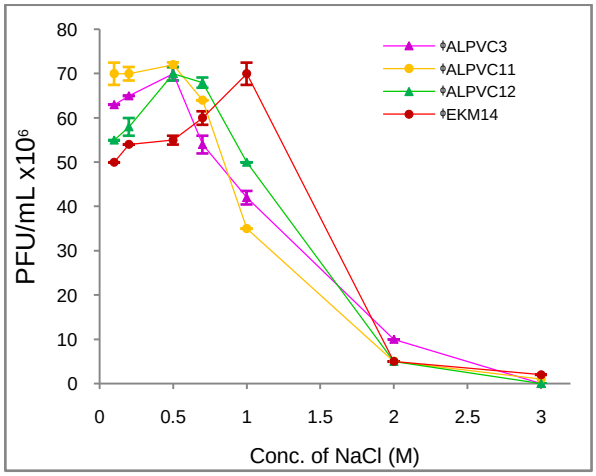


Fig 4.37 Effect of NaCl on viability of phage ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

The study on viability of ΦALPVC3, ΦALPVC11 and ΦALPVC12 in the presence of varying concentration of NaCl revealed 0.5 M NaCl as optimal for

phage survival. At NaCl concentrations below 0.5 M, viable phages were produced in the range of 50 x 10^6 to70 x 10^6 PFU/mL. However, in presence of higher concentrations of NaCl, i.e., above 0.5 M there was a decline in the viability of these phages as observed from Fig 4.33. NaCl concentration above 1M was observed to be lethal.

It was evident that for ΦEKM14**,** the optimum concentration of NaCl required for phage survival was 1 M (Fig 4.38). NaCl concentration below 1 M produced ~50 x 10^6 PFU/mL to ~60 x 10^6 PFU/mL whereas the optimum concentration of NaCl (1 M) produced ~70 x 10^6 PFU/mL. There was considerable reduction in viability of ΦEKM14 at concentrations higher than 1 M NaCl and the phages did not survived concentrations beyond 2 M NaCl.

4.4.2.3. Effect of pH on phage viability

The viability of the phages was studied over a pH range of 2 – 13. Fig 4.38 elucidates the effect of pH on viability of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14**.**

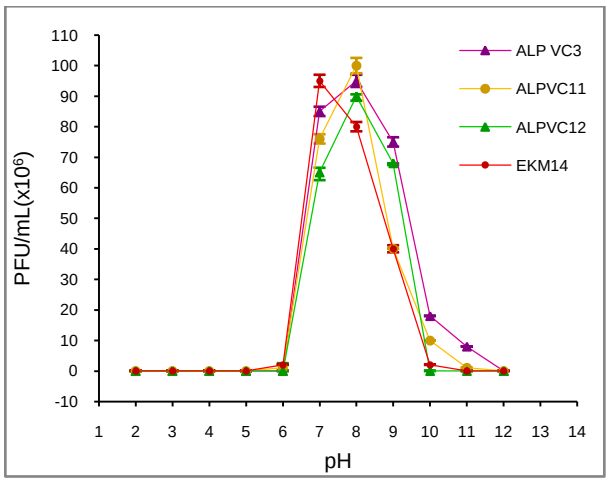


Fig 4.38 Effect of pH on viability of phage ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

The optimum pH for maximum viability of ΦALPVC3, ΦALPVC11 and ΦALPVC12 was pH 8. Even though these three phages shared similar pH affinities, ΦALPVC3 survived better under these conditions which was evident from the higher PFUs at all higher pH studied, starting from pH 7. Viability of ΦALPVC3 was observed even at pH as high as 10, although in smaller numbers.

ΦEKM14 produced maximum viable PFUs at pH 7 even though it gave fairly high PFU at pH 8. ΦEKM14 failed to survive in pH beyond 9. None of the phages survived at the acidic pH of 6 and below, or at pH above 11. ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 had poor tolerance to acidic pH conditions, but comparatively showed greater survival at alkaline pH.

4.4.3. Influence of physical and chemical parameters on phage adsorption

4.4.3.1.Effect of temperature on phage adsorption

The effect of different temperatures ranging from 0 ºC to 50 ºC on the adsorption of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 are as represented in Fig 4.39.

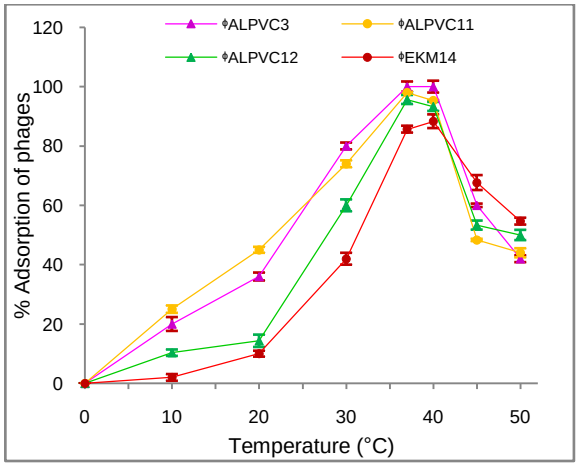


Fig 4.39 Effect of temperature on adsorption of phage ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

The maximum adsorption of ΦALPVC3 nearing 100% was observed at 37ºC and at 40 ºC, while adsorption was 80% at 30 ºC. Adsorption of ΦALPVC3 had drastically reduced to 42% at 50 ºC.

ΦALPVC11and ΦALPVC12 successfully adsorbed on to the bacterial surface to its maximum at 37 ºC – 40 ºC. At higher temperatures both ΦALPVC11and ΦALPVC12 showed adsorption to bacteria, albeit at lower percentiles.

In case of ΦEKM14, maximum adsorption was observed at 40 ºC. There was a steady increase in adsorption as the temperature was raised to 20 ºC. It was interesting to note that moderate adsorption occurred at higher temperatures i.e., 67% adsorption at 45 ºC and 54% adsorption at 50 ºC.

4.4.3.2. Effect of pH on phage adsorption

The influence of pH on the adsorption of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 to MAK757 (recipient strain) is as shown in Fig 4.40.

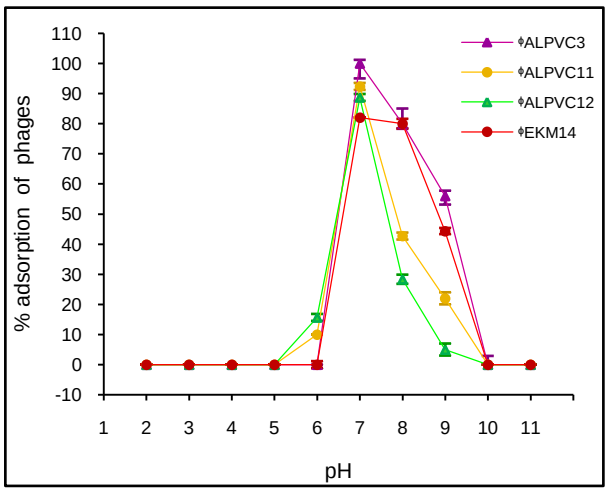


Fig 4.40 Effect of pH on adsorption of phage ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

The pH optimum for maximal adsorption for all the vibriophages was assessed to be 7. ΦALPVC11and ΦALPVC12 showed similarities in adsorption pattern at specified pH. Both the phages showed very low percentage of adsorption at pH above and below pH 7.

ΦALPVC3 gave maximum adsorption nearing 100% at pH 7. In case of ΦEKM14, optimum adsorption was achieved in a pH range of 7- 8. In addition to ΦEKM14, ΦALPVC3 also produced high percentages of adsorption at pH 8 and 9. pH ≤ 7 was detrimental for ΦALPVC3 and ΦEKM14, with no observed adsorption.

4.4.3.3. Effect of NaCl on adsorption

The influence of sodium chloride on adsorption by ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 is as represented in Fig 4.41.

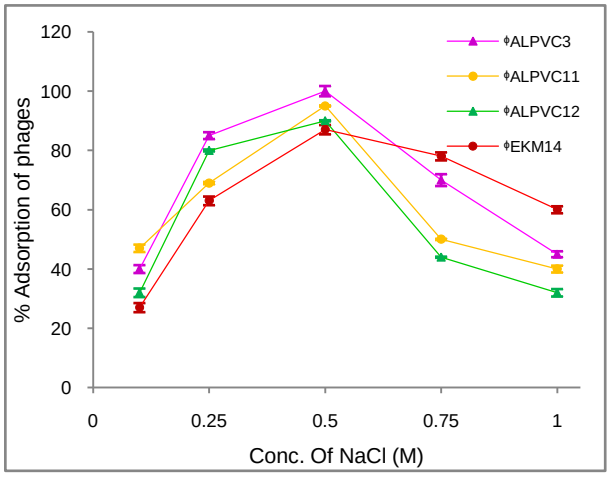


Fig 4.41 Effect of NaCl on adsorption of phage ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

Vibriophages ΦALPVC3, ΦALPVC11 and ΦALPVC12 gave >50% adsorption on the recipient strain in 0.25 M – 0.50 M concentration of NaCl. The most favourable NaCl concentration for maximum adsorption of all the phages

was 0.5 M. Maximum adsorption of 100% was given by ΦALPVC3. Adsorption continued to occur at higher concentration of NaCl *i.e.* 0.75 M and 1 M irrespective of the phage type.

Optimal NaCl concentration for maximal adsorption was 0.5 M – 0.75 M for ΦEKM14, beyond which the efficacy dropped. Adsorption was observed to take place even at concentration of 1 M NaCl, although at comparatively lower levels. The percentage of adsorption dropped to 60% at 1 M NaCl concentration. The ability of ΦEKM14 to successfully adsorb on exposure to a wide range of NaCl concentration was apparent.

4.4.3.4. Effect of calcium ions on phage adsorption and propagation

The propagation of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 in the presence of varying concentration of $CaCl_2$ is as depicted in Fig 4.42.

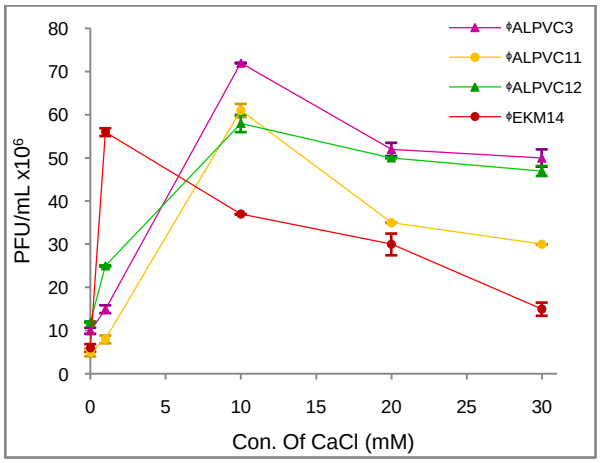


Fig 4.42 Effect of $CaCl_2$ on propagation of phage ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

Optimum $CaCl_2$ concentration for propagation of the temperate vibriophages isolated from surface water of Alappuzha *viz*. ΦALPVC3, ΦALPVC11 and ΦALPVC12 was10 mM. It could be postulated that these three

phages requires a minimum $CaCl_2$ concentration of 10 mM for their propagation. Even at higher concentrations of 20 mM and 30 mM $CaCl_2$, it was noted that >30 x 10^6 PFU/mL phages were formed, whereas very few plaques were obtained at lower concentration of 1mM $CaCl_2$.

On the other hand, 1 mM of $CaCl_2$ was optimum for ΦEKM14. For ΦEKM14, the decline in the number of viable viral particles was gradual beyond 1mM $CaCl_2$.

4.4.4. Analysis of bacteriophage structural protein

The structural protein of the phages, ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 was compared using the protein profile obtained by SDS-PAGE under non reducing conditions (Fig 4.43). Molecular weights of the proteins were determined using Quantity One® software (BioRad, USA).

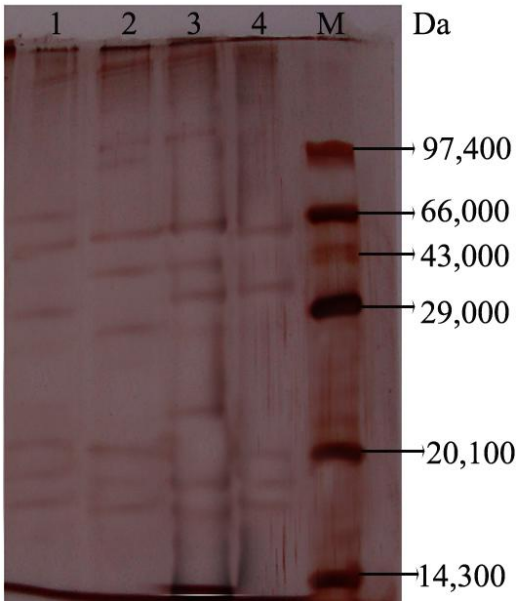


Fig.4.43. SDS PAGE of phage proteins of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 . Lane M: GeNei Protein marker (Medium range). Lane 1-4: Phages ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

Protein profiles of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 showed a total of 6, 8, 7 and 5 bands respectively in the silver stained SDS-PAGE gel. The protein profile of phages produced three protein bands of close similarity at 62150 Da, 18285 Da and 16960 Da. The protein band of ΦEKM14 at 20310 Da is present in the protein profile of ΦALPVC3 and ΦALPVC11while the band with molecular weight 315195 Da is present in ΦALPVC3 and ΦALPVC12

For ΦALPVC3, a unique protein band was observed; with molecular weights 74870 Da. ΦALPVC11 was found to have the maximum bands of which three are common to all phages and two bands with molecular weight 115440 Da and 43220 Da was also noticed in ΦALPVC12. It gave two unique bands; 102200 Da and 27560 Da of molecular weight. The banding profile of ΦALPVC11 showed high similarity with ΦALPVC12.

On the other hand a distinct band was visualized in the protein profile of ΦALPVC12 whose molecular weight was calculated as 22876 Da. The comparison of the structural proteins revealed uniqueness of the phages as well as the chances of similarity of structural protein.

4.5. Bacteriophage genome analysis

4.5.1. Phage DNA isolation

The genomic DNA of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 was successfully isolated. Electrophoresis of DNA isolated from the purified temperate bacteriophages (Fig 4.44) after mitomycin C induction revealed a single band.

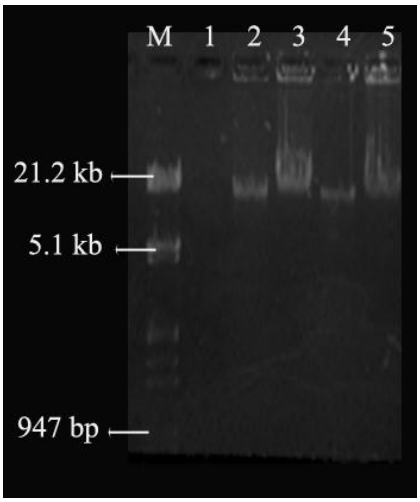


Fig.4.44. Agarose gel (1%) electrophoresis of phage DNA. Lane M – Lambda DNA / *Hind* III Digest; Lane 2-4: Genomic DNA of phages ALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

4.5.2. Restriction analysis of phage DNA

The restriction pattern of the phage DNA revealed the susceptibility of phage genome to the restriction endonuclease *Bam* HI. The restriction pattern of the phage DNA was obtained after 1 hour of incubation in reaction buffer using 5U of enzyme for digestion of 50µg phage DNA. The *Bam* HI restriction analysis of DNA isolated from ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 yielded distinguishable restriction patterns (Fig. 4.45**).**

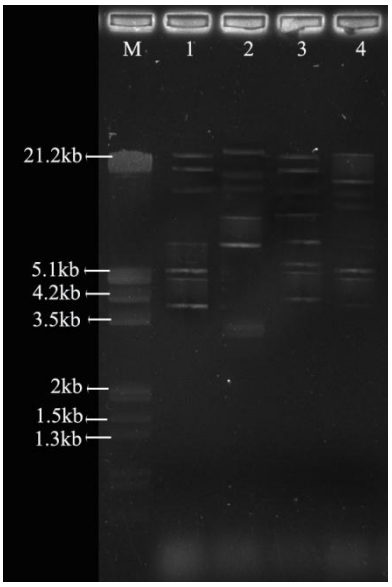


Fig.4.45. Agarose gel showing the restriction digestion of phage DNA

Lane M – Lambda DNA / *Hind* III Digest; Lane 1-4 – Restriction Digestion pattern of phages ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

The nature of the genome of the phages was identified as double stranded DNA on the basis of its sensitivity to digestion by restriction endonuclease. The double stranded nature of the phage DNA places the vibriophages under the order *Caudovirales*. The difference in the fingerprint produced after digestion also revealed that the four vibriophages under study varied a lot in their genetic makeup.

4.5.3. PCR screening for Virulence/ Virulence associated genes in phage ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14

Temperate phages were further characterized using gene specific primers to detect presence of several genes which either code virulence factors or are genetically linked to virulence genes (i.e., virulence associated genes) *viz. ctx*A, *ctx*B, *ace*, *hly*A,

*tox*R, *zot*, *tcp*A, *ninT* and *nan*H. The gel picture of virulence gene amplification in ΦALPVC3 is presented in fig 4.46.

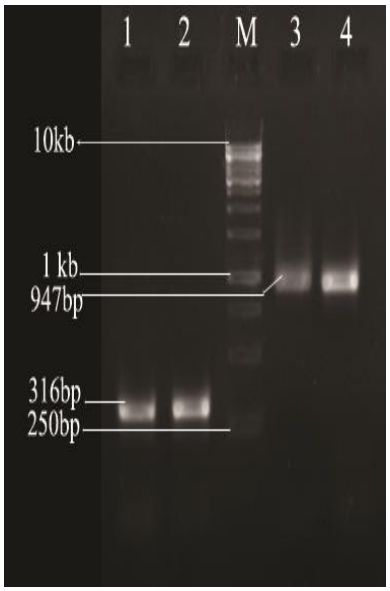


Fig.4.46. PCR amplicons of *zot* and *ace* gene Lane M – 1kb DNA ladder; Lane 1&2 – *ace* gene amplicon in ΦALPVC3 and host *V. cholerae* ALPVC3 respectively; Lane 3 & 4 – *zot* gene amplicon in ΦALPVC3 and host *V. cholerae* ALPVC3 respectively.

ΦALPVC3 harboured the gene encoding accessory cholera enterotoxin (*ace*) and zonula occludens toxin (*zot*). ΦALPVC11, ΦALPVC12 and ΦEKM14 were devoid of any of the virulence genes or virulence associated genes screened in this study. Zot and Ace proteins are required for phage packaging and secretion.

4.5.4. *In silico* analysis of partial gene sequence of *zot* and *ace* gene of ΦALPVC3 and strain ALPVC3

The *ace* and *zot* gene amplicon from ΦALPVC3 and host *V. cholerae* ALPVC3 was sequenced. Both the genes were analysed for sequence similarity

with sequences in NCBI database; *ace* and *zot* gene sequences from ΦALPVC3 was also compared with that amplified from the host *V. cholerae* ALPVC3.

The sequencing of *ace* gene produced a full gene sequence as it is a small protein with 73 amino acids. The identity of the 291 bp long *ace* gene sequence isolated from ΦALPVC3 was determined by comparing with sequences in the NCBI database. The analysis of the sequence using nBLAST of the NCBI database have revealed 99% similarity of the sequence with *ace* gene in the *V. cholerae* genome as well as the *ace* gene of CTXΦ.

Fig.4.47. Multiple sequence alignment of *ace* nucleotide sequences from ΦALPVC3 and strain ALPVC3 using the ClustalW program.

The genetic similarity of the *ace* gene amplicon from ΦALPVC3 and host *V. cholerae* ALPVC3 was determined by its multiple sequence alignment using ClustalW. The consensus regions in the alignment of the gene sequences are denoted by an asterisk (Fig. 4.47). The gene sequences were found to be very similar except at two nucleotides. So it is interesting to speculate that this element

could have evolved from a filamentous bacteriophage or is a part of the temperate phage ΦALPVC3 integrated in the genome of *V. cholerae* ALPVC3.

The partial gene sequence produced after sequencing of *zot* gene was analysed. The identity of the *zot* gene sequence isolated from ΦALPVC3 was determined by comparing with sequences in the NCBI database. The analysis of the sequence using nBLAST of the NCBI database have revealed 99% similarity of the sequence with *ace* gene in the *V. cholerae* genome as well as the *zot* gene of CTXΦ.

The alignment of *zot* gene amplicon from ΦALPVC3 with the host *V. cholerae* ALPVC3 revealed a high level sequence resemblance. The consensus regions in the alignment of the gene sequences are denoted by an asterisk (Fig. 4.48). It is hypothesized that genes for Zot is likely to have evolved from the temperate phage ΦALPVC3 integrated in the genome of *V. cholerae* ALPVC3 and may have a similar transmembrane topology.

Fig.4.48. Multiple sequence alignment of *zot* nucleotide sequences from ΦALPVC3 and strain ALPVC3 using the ClustalW program.

4.6. Transduction studies as evidence for horizontal gene transfer

4.6.1. Prophage curing

Cells cured of ΦALPVC3 were selected after irradiation with UV and treatment with mitomycin C from populations of *V. cholerae* strain ALPVC3. Phage cured cells were unable to produce zones of lysis on the lawn of MAK757 indicator bacteria. Approximately 10% of colonies were cured by using this procedure. The phage cured colonies were further referred as *ALPVC3. Their ability to support the growth of ΦALPVC3 was also confirmed.

4.6.2. Isolation of *ctx*A$^+$ and *ctx*B$^+$ (CT$^+$) phage from O139

*ctx*A$^+$ *ctx*B$^+$ (CT$^+$) phage was induced and isolated from *V. cholerae* strain O139. Extracellular phage carrying the CT toxin was purified using MAK 757 as recipient strain. The purified phages were concentrated up to 1 X 10^{10} PFU/mL using PEG precipitation and these phage concentrates were used for further studies.

4.6.3. PCR analysis of phage-cured *ALPVC3 and CT positive phage.

The strain ALPVC3 was *ctx*A$^-$ *ctx*B$^-$ *zot*$^+$ *tcp*A$^+$ and was confirmed as such previously by PCR. The phage cured colonies *ALPVC3 was further characterised using PCR and the results are shown in fig 4.49. The *zot* gene was absent in those clones without temperate phages indictaing that the *zot* gene is a part of the temperate phage. Curing was confirmed molecularly by PCR with the *zot* specific primer pair for the absence of a phage. Strain *ALPVC3 was found to be negative for *zot* genes and was positive for *tcp*A gene which codes for toxin corregulated pili making it a strong recipient of CTX phage.

CT$^+$ phage was also screened for the presence of *ctx*A and *ctx*B genes as well as *zot* gene which is normally found next to *ctx* genes in the CTX phage. The

CT^+ phage isolated from O139 harboured the genes for *ctx*A and *ctx*B along with zot protein.

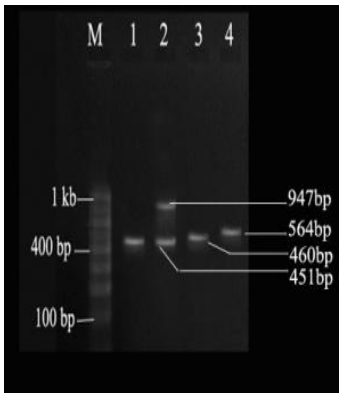


Fig 4.49. PCR analysis of *V. cholerae* strain *ALPVC 3 *(zot*$^-$ *tcp*$\mathbf{A^+}$) and O139 *(zot*$^-$ *tcp*$\mathbf{A^+}$ *ctx*$\mathbf{A^+}$ *ctx*$\mathbf{B^+}$)** Lane M - 100 bp DNA ladder; Lane 1- ALPVC3 after curing (*ALPVC3) with *tcp* gene amplicon; Lane 2 - O139 with *zot* and *tcp*A gene amplicon; Lane 3 - *ctx*B amplicon in O139; Lane 4 - *ctx*A amplicon in O139

4.6.4. Transduction studies

After transduction the opaque region at the center of the plaques with bull's eye morphology were identified as colonies of putative lysogens in which the temperate phage had integrated their genome as a prophage into the bacterial chromosome. The analysis of these transductants proved the likelihood of Horizontal Gene Transfer and the evolution of virulent clones in the environmental pool.

The phage lysate of ctx^+ bacteria lysogenic for CTXΦ was able to transduce the *ctx* gene in to non-O1 environmental strain *ALPVC3 *(zot*$^-$ *tcp*A^+ *ctx*A^- *ctx*B^-). The PCR analysis of *ALPVC3 after transduction is showed in Fig.4.50. The genes *ctx*A, *ctx*B and *zot* were successfully transduced to the

environmental non-O1 strain*ALPVC3 which was only *tcp*$^{+}$. The non-O1 strain, ALPVC3 was therefore converted to an O1 pathogenic strain of *V.cholerae*. The *tcp*$^{-}$ non-O1 strains do not produced any transductants. The lysogenic conversion of the bacteria by CT^{+} transducing phage from O139 was proved to occur similarly in natural conditions.

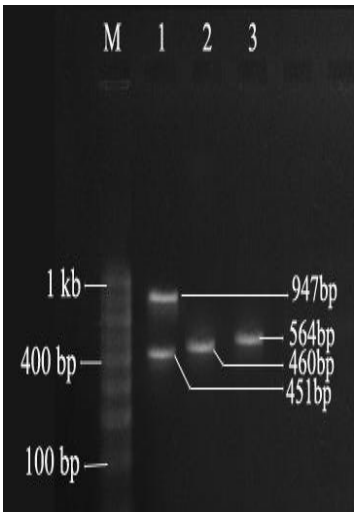


Fig 4.50. PCR analysis of *ALPVC 3 after transduction with CT^{+}phage

Lane M – 100 bp DNA ladder; Lane 3- *zot* (947 bp) and *tcp*A (451 bp) gene amplicon in *ALPVC3 after transduction; Lane 2 – *ctx*B (460 bp) amplicon in *ALPVC3; Lane 3 - *ctx*A (564 bp) amplicon in *ALPVC3

Recovery of transductional clones was enhanced at higher phage multiplicities when the classic pre-adsorption of phage to bacteria was avoided and the phage and bacteria were simply spread together on the surface of the plating medium. Both the methods yielded comparable numbers of transductants at MOI of 2. The frequency of transduction was approximately 20% under the conditions used. At higher MOI the transduction frequency per phage decreased drastically. The choice between lysis and non-lysis of host bacterium after phage infection, affect the recovery of transductional clones. Fifty transductant colonies

were picked, purified and their properties examined. Eighty percent of them produced high frequency transducing lysates upon induction with mitomycin C (1 µg/mL). It was confirmed that environmental non-O1 strain ALPVC3 is a potent strain which can undergo lysogenic conversion and can accept the virulence genes through Horizontal Gene Transfer mediated by phages.

Characterization of the vibriophages had given new insights about the emergence of newer pathogenic clones of *V. cholerae*. Studies on efficiency of prophage induction under treatment with various agents clearly indicate the chances of phage induction and LGT in natural ecosystem. Molecular characteristics of the vibriophages as well as host *V. cholerae* serve as baseline information to trace the emergence of pathogenic variants by horizontal gene transfer.

Bacteriophages offer a special perspective on the diversity and evolution of bacteria. Transduction studies have shown that the efficiency of transduction was considerably higher in that host where the phage receptor, TCP is expressed. Vibriophages are proved to be vector for lateral gene transfer between bacterial genomes of *V. cholerae* and plays a dominant role in shaping the genome architecture of these pathogenic bacteria.

Chapter 5
DISCUSSION

Cholera is the most severe and deadly of diarrheal diseases that has affected millions of people around the world over the centuries. Seven distinct pandemics of cholera have been recorded since the first pandemic in 1817 (Barua, 1992). Toxic factors released by *V. cholerae* are the causes for the manifestations of cholera. Distribution of genes in two different chromosomes in *V. cholerae* is believed to facilitate the survival of the organism in human intestine as well as in the environment (Colwell and Spira, 1992). Pathogenic clones have evolved from aquatic forms that attained the ability to colonize the human intestine by progressive acquisition of virulence-associated genes or gene clusters that are mobile in nature (Davis and Waldor, 2003; O'shea *et al*., 2004). This assumption is supported by the fact that the vast majority of *V. cholerae* strains are still part of the natural flora of the aquatic environment, with only O1 and O139 strains showing pathogenic traits.

The transformation of O1 El Tor strains to O139 occurred by one or more horizontal gene transfer events that resulted in the deletion and replacement of the gene cluster (Faruque *et al*., 2005a; Pal *et al*., 2006; Safa *et al*., 2010). This serogroup transformation of an epidemic clone provides one of the best examples of how pathogenic *V. cholerae* might attain greater evolutionary fitness through continued gene acquisition. Filamentous phages can play critical roles in horizontal gene transfer among *V. cholerae* (Jiang and Paul, 1998; Davis and Waldor, 2003). Bacteriophages being natural viral pathogens of bacteria co-exist with their hosts, sharing the same ecological niches (Goyal *et al.*, 1987). CTXΦ, a lysogenic filamentous bacteriophage encodes *ctx*AB genes producing cholera toxin (CT) (Waldor and Mekalanos, 1996). CTXΦ uses TCP pili as its receptor for

infecting recipient cells; and it appears that acquisition of the TCP island is possibly the initial genetic event required for the evolution of the epidemic strains (Karaolis and Kaper, 1999).

The southern Indian state of Kerala is endemic to cholera. There are reports that in Kerala, most cholera outbreaks are caused by *V. cholerae* O1 El Tor belonging to Ogawa serotype (Thomas *et al*., 2008). It is also not clear what determines the seasonal appearance of epidemic strains and outbreaks of cholera eventhough there are similar reports of cyclic occurrence of cholera in endemic areas, and the intensity of these outbreaks varies (Blake, 1994). Faruque *et al*., (2005b) suggested that during inter-epidemic periods, epidemic *V. cholerae* exist in an unexplained ecological association. The presence of bacterial viruses acting on *V. cholerae* O1 or O139 (cholera phages or vibriophages) inversely correlates with the occurrence of viable *V. cholerae* in the aquatic environment.

Present studies have focused on the assessment of pathogenic potential of environmental *V. cholerae* strains isolated from the endemic areas of Kerala and the role of lysogenic conversion and phage induction in augmenting their pathogenic potential.

5.1. Screening and identification of environmental isolates of *Vibrio cholerae* harboring temperate phages.

The rationale of the present study was to search for temperate phages in the environmental isolates of *V. cholerae.* In the present study, vibrios were isolated from marine environments like aquafarms and mangroves from the coastal areas of Alappuzha and Cochin, Kerala. Seventeen isolates belonging to various species of *Vibrio* were isolated and identified by phenotypic and molecular methods. It is speculated that 90% of viruses are temperate (Freifelder, 1987). *Vibrio cholerae* persists 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). Aquafarms are considered to be stressful environments mainly due to high organic matter and dissolved oxygen fluctuations (Faruque *et al*, 1998a). *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). Prevalence of *V. cholerae* in the mangrove aquaculture integrated systems and their potential pathogenicity are rare (Vandenberghe *et al.*, 2003; Gopal *et al.*, 2005). The non-O1 strains are ubiquitous in estuarine environments, and infections due to these strains are commonly of environmental origin (Morris, 1990).

The sucrose fermenting *V. cholerae* isolates are readily detected on thiosulfate-citrate-bile salts-sucrose (TCBS) agar medium as large, yellow, smooth colonies after enrichment in alkaline peptone water. Alkaline peptone water is the most commonly used enrichment broth to recover low levels of vibrios (Spira and Ahmed, 1981) and thiosulfate-citrate-bile salts-sucrose (TCBS) agar is commonly used plating medium (Massad and Oliver, 1987). The unreliability and non-reproducibility of isolation of environmental *Vibrios* on TCBS medium (McLaughlin, 1995) prompted the use of genetic markers like *omp*W and 16S rDNA sequence analysis for proper identification at the species level.

Genetic marker (*omp*W), was be employed to conclusively state the systematic position of isolated vibrios. By using this PCR marker, it was found that strains AR9, KNM4, KNM12, KNM20, MVN7, MVN15, ALPVC1, ALPVC2, ALPVC3, ALPVC4, ALPVC5, ALPVC6, ALPVC7, ALPVC8, ALPVC9, ALPVC10, ALPVC11, ALPVC12, ALPVC13, ALPVC14, ALPVC15, EKM1, EKM2, EKM4, EKM6, EKM7, EKM8, EKM9, EKM10, EKM14 and EKM16 are *V. cholerae*, as evidenced by a positive PCR reaction with *omp*W primer pairs (Nandi *et al.*, 2000b). PCR based detection using *omp*W is rapid, sensitive and specific for environmental surveillance of *V. cholerae* (Goel *et al.*,

2007). Restriction fragment length polymorphism analysis and nucleotide sequence data revealed that the *omp*W sequence is highly conserved among *V. cholerae* strains belonging to different biotypes and/or serogroups (Nandi *et al.*, 2000a). All the *V. cholerae* strains in the lab collection also tested positive for *omp*W gene. The results support the suggestion that the *omp*W gene can be targeted for the species-specific identification of *V. cholerae* strains.

The 31 *omp*W positive isolates were identified as *V. cholerae* based on partial 16S rDNA sequence analysis using the universal 16S rRNA gene segment primers and their identity was confirmed by comparing the sequences with Genbank entries. The use of 16S rRNA gene sequencing is one of the easiest methods for identifying bacteria (Janda and Abbott, 2007). 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 which was accurate, reproducible and less time consuming compared to the conventional phenotypic identification schemes.

Serogrouping of *V. cholerae* isolates by screening for the presence O1 *rfb* and O139 *rfb* marker genes (Hoshino *et al.*, 1998) is an alternative for serotyping and hence known as serotyping PCR. The bundle forming pili genes, O1 *rfb* and O139 *rfb*, are surface marker genes for pandemic strains of *V. cholerae* O1 and O139 serotypes respectively. Since none of the tested *Vibrio* isolates in this study showed amplification for these genes, it was inferred that all the isolates identified as *V. cholerae* belonged to either the non-O1 or the non-O139 serogroup. 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.

In the present study, vibriophages were induced by the DNA-damaging agent mytomycin C, which is a known inducer of many temperate bacteriophages (Faruque *et al*., 1998b). However, nine environmental strains and O139 strain produced a detectable level of extracellular phage particles, even without treatment with mitomycin C indicating that unidentified environmental factors or possible mutations in the phage or the host bacteria might have caused induction of the phages in these strains. There are similar reports on such spontaneous induction of CTXΦ from toxigenic and environmental isolates of *V. cholerae* (Faruque *et al*., 1999). *Vibrio cholerae* O1El Tor (MAK 757) is widely used as the propagating strain by various workers (Chattopadhyay, 1993; Faruque *et al*., 1999). In the soft agar overlay assay with *V. cholerae* El Tor MAK 757 supernatents of 22 environmental isolates treated with mitomycin C produced turbid plaques characteristic of lysogenic phage as described by Mitra *et al*., (1995). Phages were isolated employing double agar overlay method of Adams (1959).

Further studies were carried out to define possible factors which may play a role in the induction of lysogenic phages in toxigenic *V. cholerae* in the natural habitat.

5.2. Molecular characterization of environmental isolates of *vibrio cholerae* with temperate phages.

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 (Gordon and Wright, 2000). Sequence based approaches for fingerprinting has been widely applied for *V. cholerae* to understand the molecular epidemiology of cholera (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). The standard strain O139 and El Tor strain CO336 show a unique pattern in accordance with the reports that ERIC PCR could be used 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).

In the present study the fingerprint patterns of lysogenic *V. cholerae* strains (KNM4, KNM12, MVN7, ALPVC3, ALPVC4, ALPVC5, ALPVC6, ALPVC7, ALPVC8, ALPVC9, ALPVC10, ALPVC11, ALPVC12, ALPVC14, EKM1, EKM2, EKM4, EKM6, EKM7, EKM8, EKM10 and EKM14) obtained by ERIC-PCR from particular locality showed close similarity. The isolates from inland surface waters of Ernakulam were clustered in group I and III whereas the strains from inland lagoons of Alappuzha were clustered in group II and V. The isolates from mangroves and aquafarms of Mangalavanam and Kannamaly formed a different cluster (group IV). This study could resolve genotypic intraspecific variability among *V. cholerae* strains from different regions as observed by Jiang and co-workers (2000b). The similarity in fingerprint patterns are in accordance with that obtained with *V. cholerae* isolates associated with a seasonal outbreak in India (Goel and Jiang, 2011). Genomic variations of *Vibrio* strains isolated from a single source can be exhibited by fingerprinting methods (Kumar *et al.,* 2007; Waturangi *et al.,* 2012). Intraspecies variation is present even among strains isolated from the same environmental source. The ability of bacteria for mutation and recombination to promote genetic divergence (Vulic *et al*., 1999) can be a reason for this genetic variation. The emergence of genetic diversity among *V. cholerae* strains has been attributed to various factors, including the mobile genetic elements bacteriophages, and the high frequencies of transformations among *V. cholerae* which assimilate free DNA from the environment (Udden *et al.,* 2008).

BOX-PCR fingerprinting method produced specific banding patterns depending on the multicopied, conserved, repetitive DNA sequences called BOX elements. 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). Toxigenic strains gave unique bands in BOX PCR also, but formed different groups when compared to ERIC PCR. The intraspecies genomic variation and differentiation between the isolates from different locales was also evident, in agreement with the reports that different PCR typing strategies may result in detection of different amounts of genetic diversity (van Belkum, 1994; van Belkum *et al.,* 1996). PCR-based methods of fingerprinting take advantage of the presence of repetitive sequences that are interspersed throughout the genome of diverse bacterial species.

Ribotyping using PFGE is cumbersome and costly, and PFGE takes several days to reveal the fingerprinting pattern, (Currie *et al.,* 2007) even though it is more relevant as it gives an understanding of the whole genome. The ribotying of the isolates is an add on tool along with the biochemical identification of strains. Clonal characterisation of *V. cholerae* is also an effective tool in the quarantine measures on imported foods. The results of the present study parallels other studies using a high resolution DNA fingerprinting method to show that clinical toxigenic *V. cholerae* isolates are closely related to non-toxigenic environmental strains (Jiang *et al*., 2000a)

Molecular characterisation of the *V. cholerae* is an effective tool for monitoring the emergence of newer strains. Even though cholera toxin, encoded by the *ctx*AB genes, is directly responsible for 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). The *V. cholerae* strains with lysogenic phages were screened for the presence of several genes which either encode virulence

factors or are genetically linked to virulence genes (i.e., virulence associated genes) *viz*. *ctx*A, *ctx*B, *ace*, *hly*A, *tox*R, *zot*, *tcp*A, *int* and *nan*H genes.

Incidence of CT and TCP among environmental strains of *V. cholerae* was reported from the coastal areas of Kerala (Kumar *et al.,* 2008) and in tropical seafood in Cochin (Kumar and Lalitha, 2013). However the *ctx*A and *ctx*B gene were absent in all isolates tested, except in the standard strains included in the study. The amplification of *tcp*A gene in *V. cholerae* strain ALPVC3 is alarming, as nontoxigenic- nonpathogenic *V. cholerae* with *tcp*A gene is a rare occurrence. Said *et al.,* (1995) showed the presence of the *tcp*A gene in some non-O1 toxigenic strains including O139 strains. Earlier works on *V. cholerae* non O1/ non O139 strains from Kerala have also reported negative results for *tcp*A genes (Singh *et al.,* 2001; 2002; Kumar *et al.,* 2008) whereas it was reported from toxigenic O1 strains from South India (Balaji *et al.,* 2013). The prevalence of the gene for toxin co-regulated pilus among non-O1 *V. cholerae* strains was identified even in fresh water environment (Sharma and Chaturvedi, 2006). Isolation of *V. cholerae* strains with *tcpA* but not *ctxA* was reported earlier as well as in this study; this may denote that the acquisition of these two virulence gene clusters can take place independent of each other. Alternatively, selective loss of CTX genetic element or *tcp*A gene cluster may give rise to evolution of such strains (Said *et al*., 1995; Rodrigue, 1994)

The *tcp*A gene, a determinant of virulence factor for *V. cholerae* O1 and O139 epidemic strains is rarely characterized in non-toxigenic *V. cholerae* environmental isolates. The amplified product of *tcp*A gene in *V. cholerae* strain ALPVC3 was characterized *in silico*. The 460 bp partial *tcp*A gene sequence obtained was shown to be >90% similar to that of other *tcp*A genes of *V. cholerae* O1 (El Tor) and O139. The Bengal O139 isolate carries *tcp*A with a sequence identical to El Tor isolates (Comstock *et al*., 1996). An ORF of 453 bp was detected in the partial sequence. The *tcp*A gene, which encodes major structural

subunit of TCP, is a part of a gene cluster comprising at least 15 open reading frames (Kovach *et al*., 1996). The 152 aminoacid protein sequence identified as TcpA protein was established to have an amino acid sequence with a mosaic pattern of the *tcp*A allele in *V. cholerae* El Tor and that reported in non-O1 environmental isolates. The different *tcp*A alleles may have evolved in response to selective pressures that vary between the environment and the host.

The existence of at least three types of the *tcp*A gene among *V. cholerae* was described by Novais *et al.*, (1999) and the primers specific for the classical *tcp*A gene, amplified all biotypes. They identified TCP cluster in two non O1/ non O139 *V. cholerae* strains. Genetic mosaics in both the CTXΦ and the *tcp*A gene cluster have been detected in non-epidemic environmental *V. cholerae* isolates (Boyd and Waldor, 2002; Manning, 1997). Some of the non-O1 and non-O139 environmental strains possessing novel *tcp*A alleles were able to colonize mice (Faruque and Mekalanos, 1996a). Kumar *et al*., (2011) characterised the nucleotide sequence of four novel alleles of *tcp*A gene from toxigenic and non-toxigenic *V. cholerae* isolated from environmental sources in endemic areas of Kerala. The phylogenetic analysis of *tcp*A revealed that it is related to *tcp*A of newly emerged O139 strain and unrelated to *tcp*A of wild type (classical and El Tor strains).

The template based homology model of TcpA protein of environmental strain ALPVC3 showed a high similarity to the TcpA-like pilin in pilin superfamily. This is in accordance to the findings that, despite the nucleotide and amino acid diversity among the three tcpA, the amino acid sequences are similar to those of the classical phenotypes and pilins (Chiang *et al*., 1995). It is also known that TCP genes, including *tcp*A are similar to genes of the type 4 pilins, produced by a variety of pathogenic Gram-negative bacteria (Strom and Lory, 1993; Kaufman and Taylor, 1994). A domain within the C-terminal region of the TcpA pilin, delineated by the single disulfide loop, is directly responsible for the colonization

function mediated by the toxin coregulated pilus (TCP) of *Vibrio cholerae* (Sun *et al*., 1997).

Karaolis *et al*.suggested that TcpA, which is the major subunit of TCP pili, serves as the VPIΦ coat protein and also the CTXΦ receptor. Studies indicate that the TCP gene cluster is located in a region of the *V. cholerae* chromosome that is analogous to the *E. coli* prophage integration site (Karaolis *et al*., 2001). It can emerge as new epidemic strains with a different genetic background. Ogierman and Manning (1992) concluded that the TCP gene cluster was either recently acquired or was non- *V. cholerae* in origin. Non-toxigenic *V. cholerae* with *tcp*A can be a reservoir of horizontally transferred genes and are potential receptors of the CTXΦ phage.

The two environmental non-O1/non-O139 strains from Alappuzha *viz.* ALPVC3 and ALPVC12 showed the presence of *zot* gene, which adds on to the reports of the presence of *zot* gene in non-toxigenic *V. cholerae* (Colombo, 1994). The *zot* gene encodes the zonula occludens toxin (Zot), which increases intestinal permeability by the disassembly of the intercellular tight junctions (Fasano *et al*., 1991). As noted by Davis and Waldor (2003), the *zot* gene product is homologous to a family nucleoside triphosphate-binding motif of proteins in plasmid and filamentous phage. Waldor and Mekalanos, (1996) identified its role in CTXΦ phage morphogenesis. The *zot* and *ace* genes are among the six genes found in the core of CTX element in *V. cholerae*.

The gene encoding Ace (accessory cholera enterotoxin) is located immediately upstream of the genes encoding Zot and cholera toxin (Trucksis *et al.*, 1993). The *ace* gene product is a minor coat protein of prophage CTXΦ and at the same time also a potent enterotoxin. The strains ALPVC3, ALPVC11 and ALPVC 12 isolated from surface waters of Alappuzha harboured *ace* gene. In a recent study, Kurazono *et al*. (1995) showed that some of the *V. cholerae* O1 El

Tor strains of environmental origin contained *ace* and *zot* genes but not *ctx*A. The *ctx*, *zot*, and *ace* genes (*V. cholerae* "virulence cassette"), which are flanked by RS1 elements is located on a dynamic sector of the chromosome with site specific transposase activity (Goldberg and Mekalanos, 1986). This arrangement can lead to amplification or deletion of all three toxin genes as a unit. ALPVC3 and ALPVC12 are found to have both *ace and zot* gene which suggest translational coupling of the *ace* and *zot* genes as reported by Trucksis *et al*., (1993). The pathogenic potential of strains with incomplete copies of the 'virulence cassette' remains to be determined.

Multiplex PCR for *nan*H and *int* genes demonstrated that all *nan*H positive *V. cholerae* isolates gave amplification for *int* gene also by PCR. All the 10 *V. cholerae* lysogenic strains from Alappuzha and 7 isolates from Ernakulam were positive for both *nan*H and *int* genes. These results again prove that these two genes move together and are part of a single gene cassette. Southern hybridization analysis of chromosomal DNA from *nan*H positive and *nan*H negative isolates with an *int* probe verified the absence of *int* in *nan*H negative *V. cholerae* isolates (Jermyn and Boyd, 2002). In *Vibrio cholerae* neuraminidase, encoded by *nan*H, is thought to increase the sensitivity of host cells to CT by increasing the binding and penetration of the toxin to host enterocytes (Galen *et al*., 1992) and *int* gene encodes a protein related to the integrase family of site-specific recombinases. The *V. cholerae int* is the distal marker of *Tox*R activated colonization determinants TCP and ACF (Kovach *et al.*, 1996).

The *nanH* gene is encoded within a 57±3 kb region that showed all the characteristics of a pathogenicity island which is named VPI-2 (Jermyn and Boyd, 2002). Several pathogenicity islands are flanked by direct repeats of *int* genes and the presence of phage integrase and integration into tRNA genes transfer are of selective value to the bacterial host (Hacker and Kaper 2000). It can be speculated that some pathogenicity islands have integrated from decaying prophages to

achieve mobility.The 35±9 kb bacteriophage-like integrase gene (*int*), is located adjacent to a serine tRNA gene, upstream of *nan*H. The presence of *nan*H and *int* genes on a pathogenicity island suggests that it was acquired by horizontal transfer from a number of bacterial pathogens (Davis and Waldor, 2003).

Seven isolates from Alappuzha and two each from Ernakulam and Kannamaly mangroves showed the presence of *hly*A gene. This is in accordance with earlier findings, that most environmental isolates were non-toxigenic, but possessed the *hly*A gene, therefore having the potential to cause mild gastroenteritis (Rivera *et al*., 2001). *hly*A is the structural gene for 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). 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). Mukhopadhyay and Ghosh (2000) reported that the genome of temperate phage PS166 is at the *hly*A locus which can be a reason for the presence of the *hly*A gene environmental non-O1/ non-O139 lysogenic strains of *V. cholerae.* The *hly*A gene is the most conserved genetic element in *V. cholerae* independent of biotypes and serogroups (Kurazono *et al.,* 1995).

The transmembrane protein, ToxR, is 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). Although the *toxR* genes have been reported in all toxigenic strains of *V. cholerae* tested (Chakraborty *et al.,* 2000; 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, only 9 strains harboured this upstream toxic cascade regulator. 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).

V. cholerae is assumed to have existed long before their human host. The pathogenic clones might have evolved from aquatic forms which later colonized the human intestine by progressive acquisition of genes. This is supported by the fact that the vast majority of *V. cholerae* strains are still part of the natural aquatic environment (Colwell and Spira, 1992). Non O1/non O139 strains of marine environments were considered of negligible microbiological significance for a long time (Cheasty *et al*., 1999). The unusual transformation of *V. cholerae* strains associated with epidemics and the emergence of *V. cholerae* O139 demonstrates the evolutionary success of the organism in attaining greater fitness. Genetic changes in pathogenic *V. cholerae* constitute a natural process for developing immunity within an endemically infected population.

The purpose of this study was to examine the prevalence of *Vibrio cholerae* in environmental samples using molecular approach. The results were unanticipated since it was considered that environmental isolates lack the virulence genes (Tamrakar *et al*., 2006). In recent years cholera has become endemic in large number of geographical areas including the southern Indian state of Kerala (Thomas *et al*., 2008). It is possible that these factors are essential, but not sufficient, prerequisites for a strain to cause cholera epidemics. The analyses of virulence profile of environmental strains will help in timely preventive steps to contain the disease.

5.3. Differential induction and purification of prophages.

Phages have capacity to switch hosts by a variety of mechanisms, even though it is unclear at what rate this occurs in natural populations. The indicator strains/ propagating strain are essential for propagation and characterisation of the phages. The host range of 22 non-O1environmental isolates of *V. cholerae* with inducible lysogens was studied. The indicator strain that produced largest number of clear plaques formed by complete lysis of host for a given phage suspension was defined as the propagating strain for that phage suspension. The strain MAK 757 (*V. cholerae*, ATCC 51352) was identified as a good propagating strain for ΦKNM4, ΦALPVC3, ΦALPVC11, ΦALPVC12 and EKM14. ΦALPVC7 produced a few clear plaques on the lawn of MUS6 whereas ΦALPVC8 produced a similar result with MUS12. Phage infecting a common host can also exhibit substantial diversity. 'Natural' or 'preferred' host of the virus associated in its most recent ecological and evolutionary past may not be the same as the host used for its isolation (Pope *et al*., 2011).

Of the 22 phages, only ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 were consistently induced with mitomycin C and therefore characterized in great detail. The physiologic conditions that control phage production have been studied in these temperate phages, which consistently produced, clear and round plaques with well-defined edges in lawns of indicator strains. As concentration and purification of virus particles are prerequisites for structural and functional characterization of phages (Boulanger, 2009), these four phages were purified and concentrated before further characterization. Concentration was done employing PEG-NaCl precipitation method as described by Sambrook *et al.*, (2000). The efficiency of this method is almost independent of phage concentration and is therefore useful in order to concentrate even phage lysates with very low titer (Yamamoto *et al.*, 1990). This mild, but fast procedure

allows a 100-fold phage concentration, even after low speed centrifugation with negligible loss of infectivity (Boulanger, 2009).

Present study indicates that prophage induction is possible in natural ecosystem due to abiotic factors like pollutants, temperature and UV. *Vibrio cholerae* under normal aerobic conditions showed a growth curve with a log phase lasting for ~ 6 h (~10^8-10^9 CFU/mL) after which the bacterial count declined significantly (Henrici, 1925; Krishnan *et al*., 2004). The growth curve of lysogenic *Vibrio cholerae* used in the study drastically varied in the presence of strong prophage inducers like antibiotics and UV. A directly proportional relation between the bacterial cell lysis of and increase in phage number was noticed in accordance with the earlier reports that, on average, approximately 20% of the standing stock of bacteria is lysed by phages on a daily basis (Suttle, 1994; Weinbauer and Suttle, 1996). A transition of the phage genome from the prophage state to an extra-chromosomal genetic element, and its further propagation, is crucial for transduction and horizontal gene transfer events (Łoś *et al*., 2009).

Efficiency of induction of prophages and their further development varied considerably in response to the different induction agents. Mitomycin C produced maximum induction in the host bacteria under study. It is one of the most effective and widely used inducing agents (Ackermann and DuBow, 1987). It was used by many researchers to examine lysogeny in marine viral communities (Jiang and Paul, 1994; Tapper and Hicks, 1994), and increased production of CTXΦ virions and cholera toxin by *V. cholerae* (Davis and Waldor, 2000). The optimum conditions for maximum phage induction using MMC was obtained when cultures at OD_{600} is 1.0 was induced using 1 µg/mL mitomycin C and incubated at 37 °C for 6 hours and when plated on base plate containing 1 µg/mL amplicillin.

Although induction by UV produced results similar to that of mitomycin C, it occasionally resulted in phage production less than that caused by the

addition of mitomycin C as reported by Weinbauer and Suttle, (1996). Barksdale *et al.* (1960) discovered that UV light is a potent prophage-inducing agent. They suggested that prophage induction and replication could amplify diphtheria toxin production. UV irradiation is used for phage induction in various bacteria like *E. coli* (Łoś *et al*., 2009), *Salmonella typhimurium* (Walker, 1978), *Burkholderia cepacia* (Hens *et al*., 2005) etc. UV light enhances phage and *Streptococcal* scarlatinal toxin production.

CTXΦ production is controlled by a cellular repressor whose activity is regulated by the cell's response to DNA damage. Quinones *et al.*, (2005) found that the *V. cholerae* SOS response regulates CTXΦ production. UV stimulated CTXΦ production in lysogens of *V. cholerae* required RecA dependent autocleavage of LexA, a repressor that controls expression of numerous host DNA repair genes (Miller and Kokjohn, 1990). Environmental conditions can lead to activation of RecA, which in turn catalyzes cleavage of phage repressors including temperature, reactive oxygen species and exogenous agents such as antibiotics (Kelley, 2006).

Nalidixic acid (NAL), which was not reported previously as an induction agent for vibriophages was also shown to be a potent inducing agent. The kinetics of induction of λ prophage has been studied with nalidixic acid (Cowlishaw and Ginoza, 1970), an apparently specific and reversible inhibitor of DNA synthesis. NAL was found to cause inducing conditions in an exponentially growing culture of *Escherichia coli.* It was suggested that abnormal termination of chromosome replication by NAL converted the cell irreversibly to the induced state (Bourguignon *et al*., 1973). The efficiency of antibiotics in prophage induction was also well established in the present study. The *stx* encoding coliphages are found to be induced by antibiotics used to treat diarrhea (Zhang *et al*., 2000). It has been shown for bacterial communities that environmental pollutants can be more efficient inducing agents than universal inducer such as mitomycin C

(Cochran *et al*., 1998). The capacity of polynuclear aromatic hydrocarbons to cause induction of' natural prophage populations also indicates the effect of pollutants in the environment (Jiang and Paul, 1996).

The kinetics of induction indicates that ΦALPVC3, ΦALPVC11 and ΦALPVC12 were induced from their corresponding host after the temperature treatment. Researchers have studied the effect of environmental factors on phage induction in natural bacterial populations (McDaniel and Paul, 2005). Environmental conditions affect the switch between the lytic and lysogenic life styles of the well-studied temperate coliphage (Echols, 1972). Even though, there is evidence that high temperature treatment of lysogenic bacteria leads to prophage induction (Schuster *et al*., 1972), it was not as evident in temperate vibriophages as with antibiotics and UV.

Even though the efficiency of prophage induction by H_2O_2 was very low, it appeared to be an effective inducer for ΦEKM14 which was comparably insensitive to temperature variations; a characteristic also supported by other studies (Goerlich *et al.,* 1989), demonstrating that strains may be induced differently under specific conditions in their natural habitats. The activity of hydrogen peroxide in inducing the development of prophages was first reported by Lwoff and Jacob (1952). DNA damage by hydrogen peroxide is mediated through the Fenton reaction, in which ferrous iron reduces hydrogen peroxide to a reactive radical that generates DNA strand breaks (Imlay *et al.,* 1988). H_2O_2 is produced in the aquatic ecosystem as a result of pollution and it induces oxidative stress on the bacterial cells causing prophage induction (Stickler *et al*., 1965)**.** The transition from viral lysogeny to lytic growth involves complex interactions among host and viral factors. H_2O_2 can induce Stx-encoding prophages (Livny and Friedman, 2004), which enhances toxin production. *Salmonella* phage Gifsy-2 is also induced by H_2O_2 (Figueroa and Bossi, 1999).

The latent period of the phages were low in the presence of strong induction agents like mitomycin C, UV and nalidixic acid. Some differences in the length of the lag phase were evident under different inducing agents. ΦALPVC3 and ΦALPVC11 were strongly induced after DNA damage whereas ΦALPVC12 and ΦEKM14 showed spontaneous induction only during optimal bacterial growth which is in accordance to the reports that efficiency of progeny phage production might be modulated by bacterial growth rate (Łoś *et al*., 2009). An efficient induction of prophages in bacterial medium containing high concentration NaCl was not observed in the present study. Łoś *et al*., (2009) suggested that binding of repressor is effective even at high levels of NaCl concentrations. An increase in NaCl concentration, however did not affect growth of hosts carrying temperate phage.

Differential induction of the phages ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 by UV, mitomycin C, nalidixic acid, temperature (60 °C), 200 mM NaCl and 3 mM H_2O_2 is a clear indication of the impact of environmental pollution and global changes on phage induction. It was evident from the results that ΦALPVC3 and ΦALPVC11 are effectively induced in various inducing condition compared to other phages. So these phages may be considered as potent candidates for gene transfer in natural conditions. Under certain condition these bacteria (lysogens) can spontaneously produce virions of the temperate virus.

5.4. Physicochemical characterization of vibriophages

The morphological features of bacteriophages greatly aid in their classification (Ackermann, 2009). Therefore, transmission electron microscopy was employed to elucidate the morphotype of phages. All the four phages have hexagonal heads. Vibriophage ΦALPVC3 exhibited isometric and contractile tails and falls in the family of *Myoviridae,* while phages ΦALPVC11 and ΦALPVC12

demonstrated a hexagonal head with non-contractile tail and falls within the family *Siphoviridae.* Vibriophages with similar morphology were previously reported (Sen and Ghosh, 2005a). Ackermann and DuBow, (1987) reported two non-cultivated rumen bacteriophages with such long tails as seen in ΦALPVC11. It is also reported that the average head diameter for phages of family *Siphoviridae* is 62.5 nm with 120 nm long tail (De Lappe *et al.,* 2009). Bacteriophage ΦEKM14 is a podophage similar to the N5 vibriophage (Sen and Ghosh, 2005b) showing an isomeric head with an extremely short non-contractile tail. It is reported that the average head diameter is 62.5 nm and tail length 13 nm for phages belonging to family *Podoviridae* (De Lappe *et al.,* 2009). *Podoviridae* bacteriophages have also been previously reported (Kropinski *et al.*, 2007).

MOI is the ratio of the phage particles to the infected bacteria (Adams, 1959) and is one of the first and important factors to be deduced in the study of bacteriophages. The MOI resulting in the highest phage titre under standard conditions was three phages per bacterium for vibriophages ΦALPVC3, ΦALPVC11 and ΦEKM14, and four phages per bacterium for ΦALPVC12. Two principal factors determine establishment of lysogeny by a temperate phage, namely nutritional conditions and multiplicity of infection (MOI). High MO1 favours lysogeny and low MO1 promotes lysis (Herskowitz and Hagen, 1980). If only a few bacteriophages are used for infection, it may be difficult to detect or measure the response being tested (Abedon, 2011).

The one step growth experiment developed by Ellis and Delbruck (1939) depends on the production of synchronous phage infection. A typical curve from the experiment is triphasic, with an initial latent phase when the number of infectious centres is constant, followed by a rise period, and then a plateau, indicating little variation. The average number of phage particles released per infected cell (burst size) can also be calculated from the curve. The one step growth curve experiments used log phase cells of MAK 757 at 37 ºC. Vibriophage

ΦALPVC3 had a larger burst size and a shorter generation period than any other phage studied. The calculated burst size was ~60 phages per bacterial cell for ΦALPVC3. A significantly longer lag phase was evident for ΦALPVC12 and ΦEKM14. The latent period of ΦALPVC3 and ΦALPVC11 was low (~20 minutes) compared to the latent period of ΦALPVC12 and ΦEKM14 (~30 minutes). Phages will have a shorter latent period when either host density is high or host quality is good (Abedon, 1989; Wang *et al*., 2011). It has been argued that the average burst size from all infected cells in a sample represents a minimum estimate, since the number of viruses within a cell may still increase (Weinbauer, 2004).

The intra cellular kinetics of virus growth has been modelled for several phages (Srinivasan and Rangan, 1970; Gáspár *et al*., 1979; Buchholtz and Schneider, 1987; Rabinovitch *et al*., 1999). However, the results obtained can only be used with caution as the phage multiplication kinetics may vary, depending on the physiological state of the host cell (You *et al.*,2002), as well as on the environmental conditions which will vary dramatically in natural milieu, compared to the highly controlled lab conditions (Hadas, 1997). In addition, it is suggested that the timing of phage induced host cell lysis may be subject to a host quantity and host quality dependent selection.

Phage adsorption on the susceptible host is another significant factor affecting successful phage host interaction. Adsorption is described as the first step of phage infection and may be defined as the attachment of phage particles to bacterial surfaces so that phage and bacteria can sediment together (Adams, 1959). The phage extracellular search is a time of free diffusion that delays the onset of virus attachment (Shao and Wang, 2008). ΦALPVC3 achieved adsorption nearing 100% in 25 min of exposure to host bacteria, where as ΦALPVC11, ΦEKM14 and ΦALPVC12 were adsorbed completely in 30, 35 and 40 min respectively. Various environmental properties such as temperature and the chemical makeup of the

phage-host ecology have a substantial influence (Abedon, 1990; Stent, 1963) not only on the phage viability, but also most importantly on phage adsorption, a very crucial step in phage infection (Capra *et al.,* 2006).

The influence of both physical and chemical parameters on phage viability/propagation and phage adsorption was studied, including varying temperature, pH, salinity and calcium ions. The viability of all the four vibriophages was drastically reducing at a temperature above 50ºC. ΦEKM14 was able to survive better at temperatures above 50 ºC. But the viability of vibriophages was significantly reduced to a few PFU/ml at 70 ºC and above this temperature there were no survivors. Phage response to varying temperature exposure is considered as a key model for understanding the ability of the organism under question to adapt to novel environments (Capra *et al.,* 2006).

The marked difference in NaCl optima is in accordance to the previous findings (West and Kelly, 1962) that had shown difference in the NaCl tolerance of free phages. . It was observed in this study that the four vibriophages were viable at 0.5 M-1.0 M NaCl irrespective of their place of occurrence. There was a decline in the viability above 0.5 M NaCl and the concentration above 1 M was observed to be lethal. Optimal concentration of NaCl in phage preparation is known to confer protection to phages especially against high temperature (Krueger and Fong, 1937) and is therefore very crucial factor affecting phage viability. Temperature dependant phage resistance was also reported earlier (Kim and Kathariou, 2009).

Survival of phage particles was maximum at pH 8, with phages also showing viability at pH from 7-10, although in fewer numbers. The affinity of vibriophages for the alkaline environment is easily explained, as they were isolated from inland waters of Kerala, where pH is slightly alkaline (Nair and Sreedharan, 1986; Colwell and Spira, 1992). The optimum pH range for

bacteriophage viability and adsorption is between 5 and 8 (Adams, 1959). Present studies not only helped in optimization of the large scale phage propagation process (Augustine *et al.,* 2013) but also gave insights about the factors that influence phage population in the environmental niches.

The knowledge on environmental factors that influence the binding of phage to sensitive cells is very important to understand the frequency of gene transfer in the natural habitat. The first step in the transduction is a highly specific interaction between phage proteins located at the tip of the tail (Ogg *et al*., 1981) and specific binding sites (receptors) on the cell wall. A successful attachment is followed by the penetration of the phage nucleic acid into the cell and its intracellular replication (Lindberg, 1973). The specificity of phage adsorption to cell surface receptors has been well studied in *Escherichia coli* and other Gram negative bacteria (Taylor *et al.,* 1987). In Gram negative bacteria, the receptors have been identified as protein and lipopolysaccharide components of the outer membrane layer surrounding the peptidoglycan. A particular phage or group of phages adsorb to a specific site, and different phages adsorb at different sites.

Capra *et al.,* (2006) reported that thermal inactivation of cells decreased adsorption rates. In agreement with this, the adsorption rates increased between 0ºC and 40 ºC with maximum adsorption of all the four vibriophages *viz.* ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 at 37 ºC – 40 ºC. At higher temperatures adsorption to bacteria was at lower percentiles. Loss of adsorption occurring under mild elevations in temperature tends to suggest that it is dependent on active processes of the whole cell (Fischetti and Zabriskie, 1968). Adsorption takes place only on live intact cell and not on disrupted cell wall. Temperature dependant phage resistance has been reported earlier (Kim and Kathariou, 2009). The formation of irreversibly bound phage-cell complexes was reported to be inhibited at lower temperatures (Zhang *et al.,* 2000).

Regarding the effect of pH on the adsorption process, the vibriophages under study showed very low percentage of adsorption at pH above and below 7, and is in accordance to the studies of Campos (1998) that pH 6 to 8 is optimum for vibriophages. Most proteins are stable at neutral pH. However, at extreme pH values certain proteins begin to swell and unfold (Leverentz *et al.,* 2004). The ionic environments in which the phage and host interact have a profound influence on the irreversible phage adsorption on host surface (Adams, 1959). Penetration of phages in to the bacterial cell wall is aided by lysozyme, the enzyme located in the phage tail. Finding the optimum pH for this enzyme activity is important as pH can interfere with lysozyme or protein coat, thereby preventing phage attachment to the receptor sites of the host cell (Leverentz *et al.*, 2001; Leverentz *et al*., 2004).

The most favourable NaCl concentration for maximum adsorption of all the phages isolated from Alappuzha (ΦALPVC3, ΦALPVC11and ΦALPVC12) was 0.5 M. Even though the efficacy of adsorption dropped beyond 0.5M – 0.75M for ΦEKM14, it was observed to successfully adsorb in a wide range of NaCl concentration, mainly attributed to the ionic environments in which the phage and host interact in the marine milieu that has a profound influence on the irreversible phage adsorption on host surface (Adams, 1959). It was reported that bacteria grown in salt-free Witte's peptone resisted lysis by phage, but that the addition of sodium chloride or calcium chloride resulted in phage infection followed by bacterial cell lysis (Faruque *et al.*, 1998b; Lindberg, 1973). The electrolytes in the phage- bacteria growth medium have an intense effect on the adsorption efficiency of the phages.

The results obtained for all the phages in the present study, showed that Ca^{2+} was not indispensable for the completion of the lytic cycle, although lysis was faster in the presence of the cation, and very small plaques were yielded in its absence. The three phages, ΦALPVC3, ΦALPVC11 and ΦALPVC12 required minimum $CaCl_2$ concentration of 10 mM for their propagation, while 1 mM of $CaCl_2$ was optimum for ΦEKM14. Propagation and adsorption depend on the

presence of certain cations in the media (Watanabe and Takesue, 1972). The calcium requirement for successful phage-host interaction varies from phage to phage (Brodetsky and Romig, 1965). Numerous studies have proved the positive influence of calcium on phage- host interaction (Potter and Nelson, 1952; Shafia and Thompson, 1964; Watanabe and Takesue, 1972). Ca_2^+ ions are reported to be required for lysing the phage-infected cultures (Augustine *et al.*, 2013). According to Taylor *et al.*, (1987), the Ca_2^+ requirement not only stabilizes the coiled DNA inside the phage capsid and greatly improves the adsorption rate, but also controls the penetration efficiency of phage DNA into the bacterial cells. The optimized parameters not only led to an increase in the titres of these phages for its further studies, but also retained information about the favourable conditions that is naturally required for a phage 'bloom'.

Five to eight structural proteins were detected in all the phages in SDS-PAGE analysis. The banding pattern of the phages revealed differences among them. Three protein bands of close similarity at 62150 Da, 18285 Da and 16960 Da in all the vibriophages can be credited to the relatedness of these vibriophages owing to its lysogenic behavior. It has been observed previously that the major structural proteins of phages classified in the same DNA homology group are conserved (Prevots *et al*., 1990). Difference in protein profile found in this study may be responsible for the diversity of host range, exhibited by these phages, as the same case was reported for T-even phages of *E. coli* (Hantke, 1978). Protein profiling can be used as a molecular signature tag of a phage, helping in identifying as well as differentiating it from other phages, as phages even within the same family tend to have different structural proteins owing to differences in phage specificity (Shivu *et al.*, 2007).

5.5. Bacteriophage genome analysis

The restriction fragment length polymorphism analysis distinguished the four vibriophages isolated with double stranded DNA genome on the basis of its

sensitivity to digestion by restriction endonuclease, *Bam*HI. Restriction digestion pattern of genomic DNA indicated a high degree of similarity between the temperate phages though they appeared to be distinct from one another. Similar results have been reported in *Erwinia amylovora* (Davis *et al*., 2000). Another important observation made was the insensitivity of phages to some of commonly used restriction endonucleases like *Eco*RI, *Sau*3AI and *Hind*III. Similar results have also been reported for the *Campylobacter* phages (Atterbury *et al*., 2003).

Bacteriophages refractory to restriction enzyme are not unusual and have been reported previously (Prevots *et al*., 1990; Johansson *et al*., 1995). A number of explanations have been proposed to explicate phage DNA resistance to restriction enzyme, referred to as 'anti- restriction mechanism' (Moineau *et al*., 1993). Foremost among these explanations is that, phage genomes lose restriction sites naturally during evolution (Moineau *et al*., 1993) and another proposal for the refractory nature of some phages is the integration of unusual bases in the viral DNA (Jensen *et al*., 1998). Instead, the phages may encode methyltransferase that modify specific nucleotides within the recognition site (Sails *et al*., 1998).

Temperate bacteriophages play a significant role in horizontal gene transfer among bacteria. This is seen mostly in temperate phages that encode virulence determinants. The existence of vibriophages that infect *V. cholerae* has been known for over 70 years (Pollitzer, 1959). There are confirmed reports of vibriophages like CTXΦ (Waldor and Mekalanos, 1996) and VPIΦ (Karaolis *et* al., 1999) carrying genes enhancing virulence.

This study was also focused on the role of vibriophages in the evolution of new *V. cholerae* strains by phage mediated lateral gene transfer. To this end, the vibriophages were screened for the presence of several genes *viz. ctx*A, *ctx*B, *ace*, *hly*A, *tox*R, *zot*, *tcp*A, *int* and *nan*H to identify the likelihood of these lysogenic phages in cholera pathogenesis. Temperate phage ΦALPVC3 harboured the gene encoding accessory cholera enterotoxin (*ace*) and zonula occludens toxin (*zot*).

Both these genes were already identified as a part of core region CTX element in *V. cholerae* virulence cassette.

Accessory cholera enterotoxin (ace) increases the potential difference (PD) across the intestinal epithelium (Trucksis *et al.,* 1997), and is a minor coat protein of the CTXΦ virion. Zonula occludens toxin (zot) is involved in the CTXΦ morphogenesis (Walder and Mekalanos, 1996) and this toxin is recognized for increasing the permeability of small intestinal mucosa (Fasano *et al.,* 1991). Trucksis *et al.,* (1993) reported that the gene *ace* is located immediately upstream of the gene *zot* and cholera toxin genes (*ctx*A and *ctx*B). These observations lead to some interesting evolutionary implications of temperate phages in non-O1 environmental isolates of *V. cholerae.*

ALPVC3 has already tested positive for all virulence gene tested in this study (*ace, hly*A, *tox*R, *zot*, *tcp*A, *int* and *nan*H) except *ctx*A and *ctx*B. Translational coupling of the *ace* and *zot* genes is reported by Trucksis *et al*., (1993). The pathogenic potential of strains with incomplete copies of the 'virulence cassette' is highly alarming as ALPVC3 also posses *tcp*A (toxin coregulated pilus) gene, a determinant of virulence casette for *V. cholerae* O1 and O139 epidemic strains, along with *tox*R regulon. The phage integrase genes (*int*) in ALPVC3 genome suggests that these phage recombination genes involved in lateral gene transfer (Carlos *et al.,* 2003) are of selective value to the bacterial host for acquisition of remaining genes from the environmental pool by transformation, conjugation or transduction, to complete its virulence cassette.

The analysis of the *ace* and *zot* gene sequence using bioinformatic tools have revealed 99% similarity of the sequence with the corresponding gene in the ALPVC3, *V. cholerae* genome as well as the CTXΦ genome. This element could have evolved from the temperate phage ΦALPVC3 by generalised transduction, as *zot* gene together with the adjacent *ace* gene belongs to a site-specific transposable

element (Di Rita, 1992). It is speculated phage DNA can contribute to as much as 10-20% of bacterium's genome (Carlos *et al*., 2003).

5.6. Transduction studies as evidence for horizontal gene transfer

A tailed bacteriophage can be thought of as little more than nucleic acid encapsulated in a protein coat. Phage tail and its associated fibres are an efficient DNA transfer device. It assures both the specific host cell identification and injection of the phage DNA into the bacterial cell. (Zhang *et al*., 2000; Molineux, 2001; Kanamaru *et al*., 2002) If a phage infecting a new host carries genetic material from the previous host other than its own DNA, the additional genetic information may be transmitted to the new host, ensuing from transduction (Jiang and Paul, 1998). Bacteriophage mediated horizontal transduction has been known for nearly half a century and observed to occur in many phage-host systems Jiang and Paul, 1998 (Zinder and Lederberg, 1952).

The non-O1/non-O139 environmental *V. cholerae* strain ALPVC3 from Alappuzha track was chosen for phage curing and further transduction studies for the following reasons.

(1) ALPVC3 was tested positive for virulence genes; *ace*, *hly*A, *tox*R, *zot*, *tcp*A, *int* and *nan*H.

(2) The tertiary structure of toxin coregulated pilus subunit TcpA of ALPVC3 was highly similar to TcpA like pilin of O1 and O139 *V. cholerae*, which can act as receptor pilin for CTXΦ.

(3) Temperate phage ΦALPVC3 isolated from ALPVC3 was also positive for accessory cholera enterotoxin (*ace*) and zonula occludens toxin (*zot*).

(4) ΦALPVC3 was consistently induced with mitomycin C and produced high phage titre on the indicator strain MAK 757. These considerations prompted the examination of ΦALPVC3 phage using transduction assays with emphasis on its possible role in cholera outbreaks.

Cells cured of ΦALPVC3 (designated *ALPVC3) but which can support the growth of ΦALPVC3, were identified after irradiation with UV and curing with mitomycin C from populations of *V. cholerae* strain ALPVC3. Phage cured cells were incapable of producing zones of lysis on the lawn of MAK 757 indicator strain. A number of procedures are reported in curing *V. cholerae* classical biotype strain, 569B of defective phages dVcA-1 and dVcA-2 (Gerdes and Romig, 1975). These methods included UV, mitomycin C or acridine orange treatment, and thymine starvation of thymine auxotrophs. Phage curing using UV and mitomycin C was also successfully in *Streptococcus cremoris* (Georghiou *et al*., 1981), *Lactobacillus casei* (Shimizu and Sakurai, 1982) and *Lactococcus lactis* (Wiederholt and Steele 1993). The *zot* gene was absent in *ALPVC3 indicating that the *zot* gene is a part of the temperate phage and was positive for *tcp*A gene (toxin corregulated pili) making it a strong recipient of CT^+ phage.

ctxA^+ *ctx*B^+ (CT^+) phage with ominous potential to cause cholera was induced and isolated from *V. cholerae* strain O139, in accordance with the studies on the inducible phages by Faruque and collegues (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 (Faruque *et al*., 1998a). The propagation of CTXΦ in its natural habitat involves the excision and replication of the lysogenic phage followed by infection of recipient *V. cholerae* strains (Mitra *et al*., 2000).

The isolated CT^+ phages were *zot*$^+$ in accordance to the earlier studies that it codes for zonula occludens toxin, a membrane protein with transmembrane topology, essential for CTXΦ morphogenesis and found immediately upstream of *ctx*A and *ctx*B genes (Koonin, 1992). The zot gene, along with the adjacent CTX operon encoding the two subunits of the classical cholera toxin, belongs to a site-specific transposable element (Baudry *et al.*, 1992). 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 occurrence of cholera.

Transduction study was conducted on a purely genetic approach to investigate whether *ctx*AB is transmissible by CT^+ phages to nontoxigenic *V. cholerae* O1 recipient strains(*ALPVC3). Boyd and Waldor (1999) similarly reported generalized transduction in *V. cholerae* that transferred CTX prophage into non-toxigenic *V. cholerae* strains *in vitro*. The putative lysogens after transduction are identified as the bacterial colonies in the opaque region at the centre of the translucent plaques. Lysogens of *Acinetobacter* strains were similarly obtained by picking viable cells from the centre of a turbid plaque (Herman and Juni, 1974).

PCR analysis of these transductants proved that the phage lysate of CT^+ bacteria lysogenic for CTXΦ was able to transduce the *ctx* gene in to phage cured non-O1 environmental strain *ALPVC3. The genes *ctx*A, *ctx*B and *zot* were successfully transduced to the environmental non-O1 strain *ALPVC3 which is tcp^+, that is in accordance to the observation that CTXΦ DNA generally integrates at either one (El Tor) or two (Classical) loci within the *V. cholerae* genome (Mekalanos, 1983; Pearson *et al.,* 1993; Waldor and Mekalanos, 1996). 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). Present study accentuates the chances of lysogenic conversion of non-toxigenic *V. cholerae* by CT^+ transducing phage in natural conditions.

The tcp^- non-O1 strains do not produced any transductants. The *tcp*A gene product is proven to be the most likely receptor for CTXΦ phage-encoded virulence determinant (Karaolis and Kaper, 1999). Bacteriophage infections within bacterial strains appear to be decidedly selective. The bacteriophage uses the Toxin Co-regulated Pilin (TCP) as a receptor and therefore expression of TCP

by the bacterium is a requirement for its susceptibility to the phage, demonstrating a co-evolution of genetic elements mediating the transfer of virulence genes with the pathogenic bacterial species they infect (Faruque *et al*., 1998a).

The transduction frequency per phage decreased drastically at higher M.O.I. The choice between lysis and non-lysis of host bacterium after phage infection, affect the recovery of transductional clones (Bushman, 2001). It is estimated that phage mediated gene transfer in the oceans takes place at the incredible rate (Paul *et al*., 2002). Ogg *et al.*, (1981) reported that phage infection of *Vibrio cholerae* results in antigenic changes and they identified that a strain of biotype *cholerae* serotype Ogawa was converted into serotype Hikojima. Incomplete transfer of biotype characters was previously demonstrated (Ogg *et al.*, 1981). They proposed that a pandemic strain of one biotype may arise from other biotypes of cholera *Vibrio* by processes mediated by transducing phages or by mutations.

The induction of CTXΦ in naturally occurring strains of toxigenic *V. cholerae* may also occur in the environmental habitat. Our efforts are at present directed toward understanding the role of possible environmental factors in the induction and propagation of the CTX phage. Little is known about the environmental reservoir of *V. cholerae*, although present studies suggest that it can interact with a various genomic sources in the environment to acquire the missing genes in the virulence cassette. Environmental non-O1 strain ALPVC3 is a potent strain which can undergo lysogenic conversion and can accept the virulence genes through Horizontal Gene Transfer mediated by phages. It can provide a model for understanding the genetic changes that environmental non-O1/non-O139 strains undergo to become virulent from their precursor state.

Chapter 6

SUMMARY AND CONCLUSION

The resurgence of the enteric pathogen *Vibrio cholerae*, the causative organism of epidemic cholera, remains a major health problem in many developing countries like India. The southern Indian state of Kerala is endemic to cholera. The outbreaks of cholera follow a seasonal pattern in regions of endemicity. Marine aquaculture settings and mangrove environments of Kerala serve as reservoirs for *V. cholerae*. The non-O1/non-O139 environmental isolates of *V. cholerae* with incomplete 'virulence casette' 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 genes coding cholera toxin are borne on, and can be infectiously transmitted by CTXΦ, a filamentous lysogenic vibriophages. Temperate phages can provide crucial virulence and fitness factors affecting cell metabolism, bacterial adhesion, colonization, immunity, antibiotic resistance and serum resistance. The present study was an attempt to screen the marine environments like aquafarms and mangroves of coastal areas of Alappuzha and Cochin, Kerala for the presence of lysogenic *V. cholerae*, to study their pathogenicity and also gene transfer potential.

Phenotypic and molecular methods were used for identification of isolates as *V. cholerae.* The thirty one isolates which were Gram negative, oxidase positive, fermentative, with or without gas production on MOF media and which showed yellow coloured colonies on TCBS (Thiosulfate Citrate Bile salt Sucrose) agar were segregated as vibrios. The genetic marker, *omp*W gene was targeted for the species-specific identification of strains as *V. cholerae.* In addition, partial 16S rDNA sequence analysis was also utilized for species level identification of the

isolates. Molecular serotyping used PCR to screen for the presence O1 *rfb* and O139 *rfb* marker genes whereby these 31 strains of *V. cholerae* were typed to be non O1/ non O139 serogroups.

Twenty two environmental *V. cholerae* strains of both O1 and non-O1/non-O139 serogroups on induction with mitomycin C showed the presence of lysogenic phages. They produced characteristic turbid plaques in double agar overlay assay using the indicator strain *V. cholerae* El Tor MAK 757. PCR based molecular typing with primers targeting specific conserved sequences in the bacterial genome, demonstrated genetic diversity among these lysogen containing non-O1 *V. cholerae* . Molecular fingerprinting using ERIC PCR and BOX PCR revealed genetic heterogeneity within the environmental isolates of *V. cholerae*. The analysis of the banding pattern obtained in the two methods aided clustering of the strains and highlighted the intraspecies relatedness and variability, as also their phylogenetic lineages.

Polymerase chain reaction was also employed as a rapid screening method to verify the presence of 9 virulence genes namely, *ctx*A, *ctx*B, *ace*, *hly*A, *tox*R, *zot*, *tcp*A, *nin*T and *nan*H, using gene specific primers. The environmental non-toxigenic isolates lacked the prophage encoded the cholera toxin genes (*ctx*A and *ctx*B), identifying CTXΦ prophage as a major determinant of cholera pathogenesis. All the strains, except MVN7 and EKM10, harbored at least one or a combination of the tested genes, while *V. cholerae* strain ALPVC3 hosted the highest number of virulence genes. The *tox*R gene was detected in 40% and *ace* in 14% of the environmental strains. The genes *nan*H and *int* were present in 72% of the tested strains, whereas *hly*A gene was observed in 50%.The frequency of *zot* and *tcp*A was only 9% and 5% respectively. It is important to mention at the outset that the high prevalence rate of various virulence genes in 22 non-O1/non-O139 strains of *V. cholerae* strains studied confirm the natural incidence of these genes in the environmental pool. Despite this, the presence of *tcpA* gene in ALPVC3 was

alarming, as it indicates the possibility of an epidemic by accepting the cholera toxin gene from CTXΦ, as Tcp pilin is the receptor for this filamentous phage.

The *tcp*A gene in strain ALPVC3 of *V. cholerae* was amplified and sequenced for genetic analysis. The 152 aminoacid protein sequence was identified as TcpA protein, and was established to have an amino acid sequence with a mosaic pattern of the *tcp*A allele in *V. cholerae* El Tor, as well as that reported in non-O1 environmental isolates. Structure prediction of the TcpA protein of *V. Cholera* strain ALPVC3 was done using PHYRE^2 software. Despite the nucleotide and amino acid diversity, the deduced template based homology model of TcpA protein of environmental strain ALPVC3 showed a high similarity to the *V. cholerae* TcpA-like pilin in pilin superfamily. Secondary structure of TcpA was predicted based on protein data bank (PDB) template d1oqva which contained very long N-terminal helix, with its end packed against beta-sheets, unique to pili subunits.

Seasonal outbreak of cholera in endemic areas is highly correlated to the induction of bacteriophages infecting vibrios. Differential induction studies used ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14, underlining the possibility of prophage induction in natural ecosystems, due to abiotic factors like antibiotics, pollutants, temperature and UV. The efficiency of induction of prophages varied considerably in response to the different induction agents. Mitomycin C produced maximum induction while UV and nalidixic acid were also recognised as potent inducing agent. Prophage induction with temperature and H_2O_2 were not as evident as with antibiotics and UV. High concentration NaCl did not prove to be an effective phage inducer. The latent period of the phages was low in the presence of strong induction agents. ΦALPVC3 and ΦALPVC11 were effectively induced in various inducing conditions.

The growth curve of lysogenic *V. cholerae* used in the study drastically varied in the presence of strong prophage inducers like antibiotics and UV. Bacterial cell lysis was directly proportional to increase in phage number due to induction. The optimum conditions for maximum phage induction using MMC was obtained when cultures at OD_{600} =1.0 was induced using 1 µg/mL mitomycin C and incubated at 37°C for 6 h, and when plated on base plate containing 1 µg/mL amplicillin.

Morphological characterization of vibriophages by Transmission Electron Microscopy revealed hexagonal heads for all the four phages. Vibriophage ΦALPVC3 exhibited isometric and contractile tails characteristic of family Myoviridae, while phages ΦALPVC11 and ΦALPVC12 demonstrated the typical hexagonal head and non-contractile tail of family *Siphoviridae.* ΦEKM14, the podophage was distinguished by short non-contractile tail and icosahedral head.

The basic growth properties of phage necessary to deduce a productive phage- host interaction is the optimal MOI and time taken for phage to adsorb on bacterial. The MOI under standard conditions was three phages per bacterium for vibriophages ΦALPVC3, ΦALPVC11 and ΦEKM14, and four phages per bacterium for ΦALPVC12. ΦALPVC3 had a larger burst size and a shorter generation period than any other phage studied. The calculated burst size was ~60 phages per bacterial cell for ΦALPVC3. A significantly longer lag phase was evident for ΦALPVC12 and ΦEKM14. The latent period of ΦALPVC3 and ΦALPVC11 was ~20 minutes, while that of ΦALPVC12 and ΦEKM14 was ~30 minutes.

This work demonstrated that environmental parameters can influence the viability and cell adsorption rates of *V. cholerae* phages. Adsorption studies showed 100% adsorption of ΦALPVC3 ΦALPVC11, ΦALPVC12 and ΦEKM14 after 25, 30, 40 and 35 minutes respectively. Exposure to high temperatures ranging

from 50ºC to 100ºC drastically reduced phage viability. The optimum concentration of NaCl required for survival of vibriophages except ΦEKM14 was 0.5 M and that for ΦEKM14 was 1M NaCl. Survival of phage particles was maximum at pH 7-8.

The adsorption rates increased between 0 ºC and 40 ºC, with maximum adsorption of all the four vibriophages at 37 ºC – 40 ºC. The vibriophages under study showed very low percentage of adsorption at pH above and below 7. The optimum NaCl concentration for maximum adsorption of all the phages isolated from Alappuzha was 0.5 M and the efficacy of adsorption beyond 0.5 M – 0.75 M was very low for ΦEKM14 isolated from Ernakulam. The results of the study demonstrated that no visible or very small plaques were produced in the absence of calcium ions and minimum $CaCl_2$ concentration of 10 mM was required for phage propagation. Protein profiles of ΦALPVC3, ΦALPVC11, ΦALPVC12 and ΦEKM14 showed a total of 6, 8, 7 and 5 bands respectively in the silver stained SDS-PAGE gel, which can be a signature tag of each phage, helping in identifying as well as differentiating it from the others. A well distinguishable restriction digestion pattern was obtained by digestion with restriction endonucleases *Bam*H1 and the four vibriophages with double-stranded DNA genome placed the vibriophages under the order *Caudovirales*.

Temperate phage ΦALPVC3 harboured the gene encoding accessory cholera enterotoxin (*ace*) and zonula occludens toxin (*zot*); both genes were previously identified as part of the core region CTX element in *V. cholerae* virulence cassette. The analysis of the *ace* and *zot* gene sequences using bioinformatic tools revealed 99% similarity with the corresponding gene in strain ALPVC3, *V. cholerae* O139 genome as well as the CTXΦ genome. This element could have evolved from the temperate phage ΦALPVC3 by generalised transduction, as *zot* gene together with the adjacent *ace* gene is known to belong to a site-specific transposable element.

The chances of phage mediated lateral gene transfer in the environment were studied with transduction assays. Cells cured of ΦALPVC3 (designated *ALPVC3) incapable of producing zones of lysis on the lawn of MAK757 indicator strain and that which can still support the growth of ΦALPVC3, were identified after irradiation with UV and curing with mitomycin C from populations of *V. cholerae* strain ALPVC3. The *zot*$^{+}$*ctx*A^{+} *ctx*B^{+} (CT^{+}) phage was isolated from *V. cholerae* strain O139. Transduction was successfully performed with a phage-cured *ALPVC3 strain of *V. cholerae* using the CT positive phage isolated from O139 standard strain. PCR analysis of these transductants proved that the genes *ctx*A, *ctx*B and *zot* were successfully transduced to the environmental non-O1 strain *ALPVC3 which is *tcp*$^{+}$. The *tcp*A gene product was proved to be the most likely receptor for CT^{+} phage-encoded virulence determinant, as none of the *tcp*$^{-}$ non-O1 strains produced transductants. The frequency of transduction was approximately 20% and transductant colonies were able to produce high frequency transducing lysates upon induction with mitomycin C.

Conclusion

V. cholerae is assumed to have existed long before their human host and so the pathogenic clones may have evolved from aquatic forms which later colonized the human intestine by progressive acquisition of genes. This is supported by the fact that the vast majority of *V. cholerae* strains are still part of the natural aquatic environment. CTXΦ has played a critical role in the evolution of the pathogenicity of *V. cholerae* as it can transmit the *ctx*AB gene. The unusual transformation of *V. cholerae* strains associated with epidemics and the emergence of *V. cholerae* O139 demonstrates the evolutionary success of the organism in attaining greater fitness. Genetic changes in pathogenic *V. cholerae* constitute a natural process for developing immunity within an endemically infected population.

The alternative hosts and lysogenic environmental *V. cholerae* strains may potentially act as cofactors in promoting cholera phage ‘‘blooms’’ within aquatic environments, thereby influencing transmission of phage sensitive, pathogenic *V. cholerae* strains by aquatic vehicles. Differential induction of the phages is a clear indication of the impact of environmental pollution and global changes on phage induction. The development of molecular biology techniques offered 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, revealed that the marine environment harbours potentially pathogenic group of bacteria with genetic diversity. The distribution of virulence associated genes in the environmental isolates of *V. cholerae* provides tangible material for further investigation. Nucleotide and protein sequence analysis along with protein structure prediction aids in better understanding of the variation in alleles of same gene in different ecological niche and its impact on the protein structure for attaining greater fitness of pathogens.

The evidences of the co-evolution of virulence genes in toxigenic *V. cholerae* O1 from different lineages of environmental non-O1 strains is alarming. Transduction studies would indicate that the phenomenon of acquisition of these virulence genes by lateral gene transfer, although rare, is not quite uncommon amongst non-O1/non-O139 *V. cholerae* and it has a key role in diversification. All these considerations justify the need for an integrated approach towards the development of an effective surveillance system to monitor evolution of *V. cholerae* strains with epidemic potential.

Results presented in this study, if considered together with the mechanism proposed as above, would strongly suggest that the bacteriophage also intervenes as a variable in shaping the cholera bacterium, which cannot be ignored and hinting at imminent future epidemics.

REFERENCES

Abedon, S. T. (1989). Selection for bacteriophage latent period length by bacterial density: a theoretical examination. *Microbial Ecology*, *18*(2), 79-88.

Abedon, S. T. (1990). Selection for lysis inhibition in bacteriophage. *Journal of theoretical biology*, *146*(4), 501-511.

Abedon, S. T. (2011). Lysis from without. *Bacteriophage*, *1*(1), 46.

Ackermann, H. W. (2007). 5500 Phages examined in the electron microscope.*Archives of virology*, *152*(2), 227-243.

Ackermann, H. W. (2009). Basic phage electron microscopy. In *Bacteriophages* (pp. 113-126). Humana Press.

Ackermann, H. W. (2009). Phage classification and characterization. In *Bacteriophages* (pp. 127-140). Humana Press.

Ackermann, H. W., & DuBow, M. S. (1987). Viruses of prokaryotes, vol. 2. *Natural groups of bacteriophages*. (pp. 145-167). Humana Press.

Adams, M. H. (1959): Bacteriophages, Interscience publishers, Inc, Newyork, NY.

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.

Albert, M. J. (1994). *Vibrio cholerae* O139 Bengal. *Journal of Clinical Microbiology*, *32*(10), 2345.

Albert, M. J., Siddique, A. K., Islam, M. S., Faruque, A. S. G., Ansaruzzaman, M., Faruque, S. M., & Bradley Sack, R. (1993). Large outbreak of clinical cholera due to *Vibrio cholerae* non-01 in Bangladesh. *The Lancet*, *341*(8846), 704.

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.

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.

Amitsur, M., Levitz, R., & Kaufmann, G. (1987). Bacteriophage T4 anticodon nuclease, polynucleotide kinase and RNA ligase reprocess the host lysine tRNA. *The EMBO Journal*, *6*(8), 2499.

Andersson, J. O. (2005). Lateral gene transfer in eukaryotes. *Cellular and Molecular Life Sciences CMLS*, *62*(11), 1182-1197.

Andersson, J. O., Sjögren, Å. M., Davis, L. A., Embley, T. M., & Roger, A. J. (2003). Phylogenetic analyses of diplomonad genes reveal frequent lateral gene transfers affecting eukaryotes. *Current Biology*, *13*(2), 94-104.

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.

Ashelford, K. E., Day, M. J., & Fry, J. C. (2003). Elevated abundance of bacteriophage infecting bacteria in soil. *Applied and environmental microbiology*, *69*(1), 285-289.

Atterbury, R. J., Connerton, P. L., Dodd, C. E., Rees, C. E., & Connerton, I. F. (2003). Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of Campylobacter jejuni. *Applied and environmental microbiology*, *69*(10), 6302-6306.

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.

Augustine, J., Varghese, S. M., & Bhat, S. G. (2013). ΦSP-3, a *Salmonella*-specific lytic phage capable of infecting its host under nutrient-deprived states.*Annals of Microbiology*, *63*(1), 381-386.

Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., & Struhl, K. (1987): Current Protocols in Molecular Biology, Green Publishing Associates & Wiley Intersciences.

Avitabile, A., Carlomagno-Cerillo, M. S., Favre, R., & Blasi, F. (1972). Isolation of transducing bacteriophages for the histidine and isoleucine-valine operons in *Escherichia coli* K-12. *Journal of bacteriology*, *112*(1), 40-47.

Azman, A. S., Rudolph, K. E., Cummings, D. A., & Lessler, J. (2013). The incubation period of cholera: A systematic review. *Journal of Infection*, *66*(5), 432-438.

Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K. I., Oguchi, A., & Hiramatsu, K. (2002). Genome and virulence determinants of high virulence community-acquired MRSA. *The Lancet*, *359*(9320), 1819-1827.

Bachellier, S., Clement, J. M., & Hofnung, M. (1999). Short palindromic repetitive DNA elements in *Enterobacteria*: a survey. *Research in Microbiology*, 150(9), 627-639.

Baine, W., Mazzotti, M., Greco, D., Izzo, E., Zampieri, A., Angioni, G., & Pocchiari, F. (1974). Epidemiology of cholera in Italy in 1973. *The Lancet*, *304*(7893), 1370-1374.

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.

Barksdale, L., Garmise, L., & Horibata, K. (1960). Virulence, toxinogeny, and lysogeny in *Corynebacterium diphtheriae*. *Annals of the New York Academy of Sciences*, *88*(5), 1093-1108.

Barnes, J. K., & Woodworth, J. M. (1875). The Cholera Epidemic of 1873 in the United States (No. 95). US Government Printing Office.

Baross, J. A., Liston, J., & Morita, R. Y. (1978). Incidence of *Vibrio parahaemolyticus* bacteriophages and other Vibrio bacteriophages in marine samples. *Applied and environmental microbiology*, *36*(3), 492-499.

Barua, D. (1992). History of cholera. In *Cholera* (pp. 1-36). Springer US.

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.

Basu, S., & Mukerjee, S. (1968). Bacteriophage typing of *Vibrio eltor*. *Experientia*, *24*(3), 299-300.

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.

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). Genus I. Vibrio. *Bergey's manual of systematic bacteriology*, *1*, 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.

Bennish, M. L. (1994). Cholera: pathophysiology, clinical features, and treatment. *Vibrio cholerae and cholera: molecular to global perspectives.* ASM Press, Washington, *DC*, 229-255.

Beres, S. B., Sylva, G. L., Barbian, K. D., Lei, B., Hoff, J. S., Mammarella, N. D., & Musser, J. M. (2002). Genome sequence of a serotype M3 strain of group A *Streptococcus*: phage-encoded toxins, the high-virulence phenotype, and

clone emergence. *Proceedings of the National Academy of Sciences*,*99*(15), 10078-10083.

Bergh, O., Borsheim, K. Y., Bratbak, G., & Heldal, M. (1989). High abundance of viruses found in aquatic environments. *Nature*, *340*(6233), 467-468.

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.

Bhattacharya, S. K. (2003). An evaluation of current cholera treatment. *Expert opinion on pharmacotherapy*, *4*(2), 141-146.

Bhowmick, T. S., Das, M., Roy, N., & Sarkar, B. L. (2007). Phenotypic and molecular typing of *Vibrio cholerae* O1 and O139 isolates from India.*Journal of Infection*, *54*(5), 475-482.

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.

Bik, E. M., Bunschoten, A. E., Gouw, R. D., & Mooi, F. R. (1995). Genesis of the novel epidemic *Vibrio cholerae* O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis. *The EMBO journal*,*14*(2), 209.

Bishai, W. R., & Murphy, J. R. (1988). Bacteriophage gene products that cause human disease. *The bacteriophages*, *2*, 683-724.

Black, R. E. (1982). The prophylaxis and therapy of secretory diarrhea. *The Medical clinics of North America*, *66*(3), 611.

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. (1993). Epidemiology of cholera in the Americas. *Gastroenterology Clinics of North America*, *22*(3), 639-660.

Blake, P. A. (1994). Historical perspectives on pandemic cholera. *Vibrio cholerae*, 293-295.

Blake, P. A., Allegra, D. T., Snyder, J. D., Barrett, T. J., McFarland, L., Caraway, C. T., & Feldman, R. A. (1980). Cholera-a possible endemic focus in the United States. *New England journal of medicine*, *302*(6), 305-309.

Boucher, Y., Douady, C. J., Papke, R. T., Walsh, D. A., Boudreau, M. E. R., Nesbø, C. L., & Doolittle, W. F. (2003). Lateral gene transfer and the origins of prokaryotic groups. *Annual review of genetics*, *37*(1), 283-328.

Boulanger, P. (2009). Purification of bacteriophages and SDS-PAGE analysis of phage structural proteins from ghost particles. In *Bacteriophages* (pp. 227-238). Humana Press.

Bourguignon, G. J., Levitt, M., & Sternglanz, R. (1973). Studies on the mechanism of action of nalidixic acid. *Antimicrobial agents and chemotherapy*,*4*(4), 479-486.

Boyd, E. F., & Brüssow, H. (2002). Common themes among bacteriophage-encoded virulence factors and diversity among the bacteriophages involved.*TRENDS in Microbiology*, *10*(11), 521-529.

Boyd, E. F., & Waldor, M. K. (1999). Alternative Mechanism of Cholera Toxin Acquisition by*Vibrio cholerae*: Generalized Transduction of CTXΦ by Bacteriophage CP-T1. *Infection and immunity*, *67*(11), 5898-5905.

Boyd, E. F., & Waldor, M. K. (2002). Evolutionary and functional analyses of variants of the toxin-coregulated pilus protein TcpA from toxigenic *Vibrio cholerae* non-O1/non-O139 serogroup isolates. *Microbiology*, *148*(6), 1655-1666.

Boyd, E. F., Davis, B. M., & Hochhut, B. (2001). Bacteriophage-bacteriophage interactions in the evolution of pathogenic bacteria. *Trends in microbiology*,*9*(3), 137-144.

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.

Bradley, D. E. (1967). Ultrastructure of bacteriophage and bacteriocins.*Bacteriological reviews*, *31*(4), 230.

Brodetsky, A. M., & Romig, W. R. (1965). Characterization of *Bacillus subtilis* bacteriophages. *Journal of bacteriology*, *90*(6), 1655-1663.

Brunel, F., & Davison, J. (1979). Restriction insensitivity in bacteriophage T5: III. Characterization of EcoRI-sensitive mutants by restriction analysis.*Journal of molecular biology*, *128*(4), 527-543.

Brüssow, H., & Desiere, F. (2001). Comparative phage genomics and the evolution of *Siphoviridae*: insights from dairy phages. *Molecular microbiology*,*39*(2), 213-223.

Brüssow, H., & Hendrix, R. W. (2002). Phage genomics: small is beautiful. *Cell*,*108*(1), 13-16.

Brüssow, H., Canchaya, C., & Hardt, W. D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion.*Microbiology and Molecular Biology Reviews*, *68*(3), 560-602.

Buchanan, R. E., & Gibbons, N. E., (1974). Bergey's Manual of Determinative Bacteriology, pp. 1246, The Williams and Wilkins Co. Baltimore.

Buchholtz, F., & Schneider, F. W. (1987). Computer simulation of T3/T7 phage infection using lag times. *Biophysical chemistry*, *26*(2), 171-179.

Bushman, F. (2001). Lateral DNA transfer: mechanisms and consequences. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press

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.

Cameron, D. N., Khambaty, F. M., Wachsmuth, I. K., Tauxe, R. V., & Barrett, T. J. (1994). Molecular characterization of *Vibrio cholerae* O1 strains by pulsed-field gel electrophoresis. *Journal of clinical microbiology*, *32*(7), 1685-1690.

Campos, J., Fando, R., Silva, A., Rodriguez, B. L., & Benitez, J. A. (1998). Replicating function of the RS1 element associated with *Vibrio cholerae* CTXΦ prophage. *FEMS microbiology letters*, *164*(1), 141-147.

Campoy, S., Aranda, J., Àlvarez, G., Barbé, J., & Llagostera, M. (2006). Isolation and sequencing of a *Pasteurell amultocida* temperate phage. *Applied and environmental microbiology*, *72*(5), 3154-3160.

Capra, M. L., Mercanti, D. J., Reinheimer, J. A., & Quiberoni, A. L. (2010). Characterisation of three temperate phages released from the same Lactobacillus paracasei commercial strain. *International journal of dairy technology*, *63*(3), 396-405.

Capra, M. L., Quiberoni, A., & Reinheimer, J. (2006). Phages of *Lactobacillus casei/paracasei*: response to environmental factors and interaction with collection and commercial strains. *Journal of applied microbiology*, *100*(2), 334-342.

Carlos, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M. L., & Brüssow, H. (2003). Phage as agents of lateral gene transfer. *Current opinion in microbiology*, *6*(4), 417-424.

Carroll, D., Kehoe, M. A., Cavanagh, D., & Coleman, D. C. (1995). Novel organization of the site-specific integration and excision recombination functions of the *Staphylococcus aureus* serotype F virulence-converting phages φ13 and φ42. *Molecular microbiology*, *16*(5), 877-893.

Cash, R. A., Music, S. I., Libonati, J. P., Snyder, M. J., Wenzel, R. P., & Hornick, R. B. (1974). Response of man to infection with *Vibrio cholerae*. I. Clinical,

serologic, and bacteriologic responses to a known inoculum. *Journal of infectious diseases*, *129*(1), 45-52.

Cattoir, V., Poirel, L., Aubert, C., Soussy, C. J., & Nordmann, P. (2008). Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas spp*. *Emerging infectious diseases*,*14*(2), 231.

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) (1993). Imported cholera associated with a newly described toxigenic *Vibrio cholerae* O139 strain--California, 1993. *MMWR. Morbidity and mortality weekly report*, *42*(26), 501.

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., & Nair, G. B. (2000). Virulence genes in environmental Strains of *Vibrio cholerae*. *Applied and environmental microbiology*, *66*(9), 4022-4028.

Chambers, J. S. (1938). *The Conquest of Cholera: America's Greatest Scourge*. Macmillan Company.

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.

Chattopadhyay, D. J., Sarkar, B. L., Ansari, M. Q., Chakrabarti, B. K., Roy, M. K., Ghosh, A. N., & Pal, S. C. (1993). New phage typing scheme for *Vibrio cholerae* O1 biotype El Tor strains. *Journal of clinical microbiology*, *31*(6), 1579-1585

Cheasty, T., Saif, B., & Threlfall, E. J. (1999). *V cholerae* non-01: implications for man? *The Lancet*, 354(9173), 89-90.

Chen, I., & Dubnau, D. (2004). DNA uptake during bacterial transformation. *Nature Reviews Microbiology*, *2*(3), 241-249.

Chiang, S. L., Taylor, R. K., Koomey, M., & Mekalanos, J. J. (1995). Single amino acid substitutions in the N-terminus of *Vibrio cholerae* TcpA affect colonization, autoagglutination, and serum resistance. *Molecular microbiology*,*17* (6), 1133-1142.

Chibani-Chennoufi, S., Sidoti, J., Bruttin, A., Kutter, E., Sarker, S., & Brüssow, H. (2004). *In vitro* and *in vivo* bacteriolytic activities of Coliphages: implications

for phage therapy. *Antimicrobial agents and chemotherapy*, *48*(7), 2558-2569.

Chiura, H. X., & Takagi, J. (1994). Phage-like particles production and gene transfer by marine bacteria. *Bull. Jpn. Soc. Microb. Ecol*, *9*, 75-90.

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.

Citarella, R. V., & Colwell, R. R. (1970). Polyphasic taxonomy of the genus Vibrio: polynucleotide sequence relationships among selected Vibrio species.*Journal of bacteriology*, *104*(1), 434-442.

Clark, A. J., Inwood, W., Cloutier, T., & Dhillon, T. S. (2001). Nucleotide sequence of coliphage HK620 and the evolution of lambdoid phages. *Journal of molecular biology*, *311*(4), 657-679.

Cochran, P. K., Kellogg, C. A., & Paul, J. H. (1998). Prophage induction of indigenous marine lysogenic bacteria by environmental pollutants. *Marine Ecology-Progress Series*, *164*, 125.

Collier, R. J. (2001). Understanding the mode of action of diphtheria toxin: a perspective during the 20th century. *Toxicon*, *39*(11), 1793-1803

Collier, R. J., & Mekalanos, J. J. (1980) *Multifunctional Proteins*. Bisswanger, H. & Schmincke-Ott, E. (Eds.), Wiley, New York, pp. 261-291.

Collins, J., Linz, S., & Semple, C. (2011). Quantifying hybridization in realistic time. *Journal of Computational Biology*, *18*(10), 1305-1318

Colombo, M. M., Mastrandrea, S., Santona, A., de Andrade, A. P., Uzzau, S., Rubino, S., & Cappuccinelli, P. (1994). Distribution of the Ace, Zot, and CtxA toxin genes In clinical and environmental *Vibrio cholerae*. *Journal of Infectious Diseases*, *170*(3), 750-751.

Colwell, R. R. (1996). Global climate and infectious disease: the cholera paradigm. *Science*, *274*(5295), 2025-2031.

Colwell, R. R., & Spira, W. M. (1992). The ecology of *Vibrio cholerae*. In *Cholera* (pp. 107-127). Springer US.

Colwell, R. R., Brayton, P., Herrington, D., Tall, B., Huq, A., & Levine, M. M. (1996). Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. *World Journal of Microbiology and biotechnology*, *12*(1), 28-31.

Colwell, R. R., Kaper, J., & Joseph, S. W. (1977). *Vibrio cholerae*, *Vibrio parahaemolyticus*, and other vibrios: occurrence and distribution in Chesapeake Bay. *Science*, *198*(4315), 394-396.

Comstock, L. E., Johnson, J. A., Michalski, J. M., Morris Jr, J. G., & Kaper, J. B. (1996). Cloning and sequence of a region encoding a surface polysaccharide of *Vibrio cholerae* O139 and characterization of the insertion site in the chromosome of *Vibrio cholerae* O1. *Molecular microbiology*, *19*(4), 815-826.

Cooper, K. L. F., Luey, C. K. Y., Bird, M., Terajima, J., Nair, G. B., Kam, K. M., & Ribot, E. M. (2006). Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae*. *Foodbourne Pathogens & Disease*, *3*(1), 51-58.

Cowlishaw, J., & Ginoza, W. (1970). Induction of λ prophage by nalidixic acid.*Virology*, *41*(2), 244-255.

Crawford, J. A., Kaper, J. B., & DiRita, V. J. (1998). Analysis of ToxR-dependent transcription activation of ompU, the gene encoding a major envelope protein in *Vibrio cholerae*. *Molecular microbiology*, *29*(1), 235-246.

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.

Currat, M., & Excoffier, L. (2011). Strong reproductive isolation between humans and Neanderthals inferred from observed patterns of introgression.*Proceedings of the National Academy of Sciences*, *108*(37), 15129-15134.

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.

d'Herelle, F. (1917). Sur un microbe invisible antagoniste des bacilles dysentériques. *CR Acad. Sci. Paris*, *165*, 373-375.

Das, P., Mukherjee, D., & Sarkar, A. K. (2005). Study of a carrier dependent infectious disease-cholera. *Journal of Biological Systems*, *13*(03), 233-244.

Datta-Roy, K., Banerjee, K., De, S. P., & Ghose, A. C. (1986). Comparative study of expression of hemagglutinins, hemolysins, and enterotoxins by clinical and environmental isolates of non-O1 *Vibrio cholerae* in relation to their enteropathogenicity. *Applied and environmental microbiology*, *52*(4), 875-879.

Davis, B. M., & Waldor, M. K. (2000). CTXφ contains a hybrid genome derived from tandemly integrated elements. *Proceedings of the National Academy of Sciences*, *97*(15), 8572-8577.

Davis, B. M., & Waldor, M. K. (2003). Filamentous phages linked to virulence of *Vibrio cholerae*. *Current opinion in microbiology*, *6*(1), 35-42.

Davis, B. M., Kimsey, H. H., Chang, W., & Waldor, M. K. (1999). The *Vibrio cholerae* O139 Calcutta bacteriophage CTXφ is infectious and encodes a novel repressor. *Journal of bacteriology*, *181*(21), 6779-6787

Davis, B. M., Lawson, E. H., Sandkvist, M., Ali, A., Sozhamannan, S., & Waldor, M. K. (2000). Convergence of the secretory pathways for cholera toxin and the filamentous phage, CTXϕ. *Science*, *288*(5464), 333-335.

Davis, B. M., Moyer, K. E., Boyd, E. F., & Waldor, M. K. (2000a). CTX prophages in classical biotype *Vibrio cholerae*: functional phage genes but dysfunctional phage genomes. *Journal of bacteriology*, *182*(24), 6992-6998.

Davis, B. R., Fanning, G. R., Madden, J. M., Steigerwalt, A. G., Bradford, H. B., Smith, H. L., & Brenner, D. J. (1981). Characterization of biochemically atypical *Vibrio cholerae* strains and designation of a new pathogenic species, *Vibrio mimicus*. *Journal of clinical microbiology*, *14*(6), 631-639.

De la Cruz, F., & Davies, J. (2000). Horizontal gene transfer and the origin of species: lessons from bacteria. *Trends in microbiology*, *8*(3), 128-133.

De Lappe, N., Doran, G., O'Connor, J., O'Hare, C., & Cormican, M. (2009). Characterization of bacteriophages used in the Salmonella enterica serovar Enteritidis phage-typing scheme. *Journal of medical microbiology*, *58*(1), 86-93.

Desiere, F., McShan, W. M., van Sinderen, D., Ferretti, J. J., & Brüssow, H. (2001). Comparative genomics reveals close genetic relationships between phages from dairy bacteria and pathogenic streptococci: evolutionary implications for prophage-host interactions. *Virology*, *288*(2), 325-341.

Deveau, H., Garneau, J. E., & Moineau, S. (2010). CRISPR/Cas system and its role in phage-bacteria interactions. *Annual review of microbiology*, *64*, 475-493.

d'Hérelle, F., & Smith, G. H. (1926). *The bacteriophage and its behavior* (pp. 490-497). Baltimore, MD:: Williams & Wilkins.

Di Pierro, M., Lu, R., Uzzau, S., Wang, W., Margaretten, K., Pazzani, C., & 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.

DiRita, V. J. (1992). Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. *Molecular Microbiology*, 6(4), 451-458

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.

DiRuggiero, J., Dunn, D., Maeder, D. L., Holley-Shanks, R., Chatard, J., Horlacher, R., & Weiss, R. B. (2000). Evidence of recent lateral gene transfer among hyperthermophilic archaea. *Molecular microbiology*, *38*(4), 684-693..

Dodin, A. (1978). Le cholera. *Bull. Sect. Geogr*, *83*, 11-18.

Doolittle, W. F. (1999). Lateral genomics. *Trends in cell biology*, *9*(12), M5-M8.

Dowell, S. F., & Braden, C. R. (2011). Implications of the introduction of cholera to Haiti. *Emerging infectious diseases*, *17*(7), 1299-1300.

Dubnau, D. (1999). DNA uptake in bacteria. *Annual Reviews in Microbiology*,*53*(1), 217-244.

Durmaz, E., Higgins, D. L., & Klaenhammer, T. R. (1992). Molecular characterization of a second abortive phage resistance gene present in *Lactococcus lactis* ME2. *Journal of bacteriology*, *174*(22), 7463-7469.

Dutta, C., & Pan, A. (2002). Horizontal gene transfer and bacterial diversity.*Journal of biosciences*, *27*(1), 27-33.

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.

Echols, H. (1972). Developmental pathways for the temperate phage: lysis vs lysogeny. *Annual review of genetics*, *6*(1), 157-190.

Edgar, R. S., & Lielausis, I. (1964). Temperature-sensitive mutants of T4D: their isolation and genetic characterization. *Genetics*, *49*(4), 649.

Elliott, H. L., Carpenter, C. C., Sack, R. B., & Yardley, J. H. (1970). Small bowel morphology in experimental canine cholera. A light and electron microscopic study. *Laboratory investigation; a journal of technical methods and pathology*,*22* (2), 112-120.

Ellis, E. L., & Delbrück, M. (1939). The growth of bacteriophage. *The Journal of general physiology*, *22*(3), 365-384.

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

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., Alim, A. A., Albert, M. J., Islam, K. N., & Mekalanos, J. J. (1998b). Induction of the lysogenic phage encoding cholera toxin in naturally occurring strains of toxigenic *Vibrio cholerae* O1 and O139. *Infection and immunity*, *66*(8), 3752-3757.

Faruque, S. M., Chowdhury, N., Kamruzzaman, M., Dziejman, M., Rahman, M. H., Sack, D. A., & Mekalanos, J. J. (2000). 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.

Faruque, S. M., Kamruzzaman, M., Nandi, R. K., Ghosh, A. N., Nair, G. B., Mekalanos, J. J., & Sack, D. A. (2002). RS1 element of *Vibrio cholerae* can propagate horizontally as a filamentous phage exploiting the morphogenesis genes of CTXΦ. *Infection and immunity*, *70*(1), 163-170.

Faruque, S. M., Naser, I. B., Fujihara, K., Diraphat, P., Chowdhury, N., Kamruzzaman, M., & Mekalanos, J. J. (2005a). Genomic sequence and receptor for the Vibrio cholerae phage KSF-1Φ: evolutionary divergence among filamentous vibriophages mediating lateral gene transfer. *Journal of bacteriology*, *187*(12), 4095-4103.

Faruque, S. M., Naser, I. B., Islam, M. J., Faruque, A. S. G., Ghosh, A. N., Nair, G. B., & Mekalanos, J. J. (2005b). Seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phages. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(5), 1702-1707.

Faruque, S. M., Rahman, M. M., Islam, K. N., & Mekalanos, J. J. (1999). Lysogenic conversion of environmental *Vibrio mimicus* strains by CTXΦ.*Infection and immunity*, *67*(11), 5723-5729.

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

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.

Feeley, J. C. (1965). Classification of *Vibrio cholerae* by intra subspecific characteristics. *Journal of bacteriology*, *89*(3), 665-670.

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.

Felsenfeld, O. (1974). The survival of cholera vibrios. *Cholera. The WB Saunders Co. Philadelphia, PA, United States of America*, 359-366.

Ferretti, J. J., McShan, W. M., Ajdic, D., Savic, D. J., Savic, G., Lyon, K., & McLaughlin, R. (2001). Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proceedings of the National Academy of Sciences*, *98*(8), 4658-4663.

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.

Figueroa, N., & Bossi, L. (1999). Inducible prophages contribute to Salmonella virulence in mice. *Molecular microbiology*, *33*(1), 167-176.

Fineran, P. C., Blower, T. R., Foulds, I. J., Humphreys, D. P., Lilley, K. S., & Salmond, G. P. (2009). The phage abortive infection system, ToxIN, functions as a toxin antitoxin pair. *Proceedings of the National Academy of Sciences*, *106*(3), 894-899.

Fischetti, V. A., & Zabriskie, J. B. (1968). Studies on *streptococcal* bacteriophages II. Adsorption studies on group A and group C *streptococcal* bacteriophages. *The Journal of experimental medicine*, *127*(3), 489-505.

Fischetti, V. A., Nelson, D., & Schuch, R. (2006). Reinventing phage therapy: are the parts greater than the sum?. *Nature biotechnology*, *24*(12), 1508-1511.

Fitzgerald, J. R., & Musser, J. M. (2001). Evolutionary genomics of pathogenic bacteria. *Trends in microbiology*, *9*(11), 547-553.

Fitzpatrick, D. A., Logue, M. E., & Butler, G. (2008). Evidence of recent interkingdom horizontal gene transfer between bacteria and Candida parapsilosis. *BMC evolutionary biology*, *8*(1), 181.

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.

Freifelder, D. (1987). Molecular biology. *Jones and Bartlett, Inc*., Boston, Mass.

Friedman, S. D., Genthner, F. J., Gentry, J., Sobsey, M. D., & Vinjé, J. (2009). Gene mapping and phylogenetic analysis of the complete genome from 30

single-stranded RNA male-specific coliphages (family Leviviridae). *Journal of virology*, *83*(21), 11233-11243.

Frobisher, M., & Brown, J. H. (1927). Transmissible toxicogenicity of streptococci. *Bull. Johns Hopkins Hosp*, *41*, 167-173.

Fuhrman, J. A. 1999. Marine viruses and their biogeochemical and ecological effects. Nature 399:541–548.

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.

Gangarosa, E. J., & Mosley, W. H. (1974). Epidemiology and surveillance of cholera. *Cholera. Philadelphia: WB Saunders*, 381-403.

Gardner, A. D., & Venkatraman, K. V. (1935). The antigens of the cholera group of vibrios. *Journal of Hygiene*, *35*(02), 262-282.

Gáspár, S., Rontó, G. Y., & Müller, G. (1979). Determination of the biological parameters of bacterium-phage complexes. *Zeitschrift für allgemeine Mikrobiologie*, *19*(3), 163-169.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., & Bairoch, A. (2003). ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic acids research*, *31*(13), 3784-3788.

Geeta, M. G., & Krishnakumar, P. (2005). Cholera in Kerala. *Indian Pediatrics Journal*, 42, 89.

Georghiou, D., Phua, S. H., & Terzaghi, E. (1981). Curing of a lysogenic strain of *Streptococcus cremoris* and characterization of the temperate bacteriophage. *Journal of General Microbiology*, *122*(2), 295-303.

Gerdes, J. C., & Romig, W. R. (1975). Genetic basis of toxin production and pathogenesis in *Vibrio cholerae*: evidence against phage conversion. *Infection and immunity*, *11*(3), 445-452.

Ghosh, A. N., Ansari, M. Q., & Datta, G. C. (1989). Isolation and morphological characterization of E1 Tor cholera phages. *Journal of general virology*, *70*(8), 2241-2243.

Gilbert, C., Schaack, S., Pace II, J. K., Brindley, P. J., & Feschotte, C. (2010). A role for host-parasite interactions in the horizontal transfer of transposons across phyla. *Nature*, *464*(7293), 1347-1350

Glass, R. I., & Black, R. E. (1992). The epidemiology of cholera. In *Cholera* (pp. 129-154). Springer US.

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*, *2011*.ERIC

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., Ponmariappan, S., Kamboj, D. V., & Singh, L. (2007). Single multiplex polymerase chain reaction for environmental surveillance of toxigenic O1 and Non-O1*Vibrio cholerae*. *Folia microbiologica*, *52*(1), 81-85.

Goerlich, O., Quillardet, P., & Hofnung, M. (1989). Induction of the SOS response by hydrogen peroxide in various *Escherichia coli* mutants with altered protection against oxidative DNA damage. *Journal of bacteriology*, *171*(11), 6141-6147.

Goldberg, I., & Mekalanos, J. J. (1986). Effect of a recA mutation on cholera toxin gene amplification and deletion events. *Journal of bacteriology*, *165*(3), 723-731.

Goodridge, L., Gallaccio, A., & Griffiths, M. W. (2003). Morphological, host range, and genetic characterization of two coliphages. *Applied and environmental microbiology*, *69*(9), 5364-5371.

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.

Gotuzzo, E., Seas, C., Echevarría, J., Carrillo, C., Mostorino, R., & Ruiz, R. (1995). Ciprofloxacin for the treatment of cholera: a randomized, double-blind, controlled clinical trial of a single daily dose in Peruvian adults. *Clinical infectious diseases*, *20*(6), 1485-1490.

Goyal, S. M., Gerba, C. P., & Bitton, G. (1987). Phage ecology. *Wiley series in ecological and applied microbiology (USA*

Graham, J. (1862). Cholera in India. *The Lancet*, *80*(2033), 192.

Greenough III, W. B., Rosenberg, I. S., Gordon Jr, R. S., Davies, B. I., & Benenson, A. S. (1964). Tetracycline in the treatment of cholera. *The Lancet*,*283*(7329), 355-357.

Griffith, D. C., Kelly-Hope, L. A., & Miller, M. A. (2006). Review of reported cholera outbreaks worldwide, 1995–2005. *The American journal of tropical medicine and hygiene*, *75*(5), 973-977.

Groth, A. C., Olivares, E. C., Thyagarajan, B., & Calos, M. P. (2000). A phage integrase directs efficient site-specific integration in human cells. *Proceedings of the National Academy of Sciences*, *97*(11), 5995-6000.

Hacker, J., & Kaper, J. B. (2000). Pathogenicity islands and the evolution of microbes. *Annual Reviews in Microbiology*, *54*(1), 641-679.

Hacker, J., Hacker, J. H., & Kaper, J. (Eds.). (2002). *Pathogenicity islands and the evolution of pathogenic microbes* (Vol. 1). Springer.

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.

Hall, C., & Dietrich, F. S. (2007). The reacquisition of biotin prototrophy in Saccharomyces cerevisiae involved horizontal gene transfer, gene duplication and gene clustering. *Genetics*, *177*(4), 2293-2307.

Hall, R. H., & Drasar, B. S. (1990). *Vibrio cholerae* HlyA hemolysin is processed by proteolysis. *Infection and Immunity*, 58(10), 3375-3379.

Hankin, E. H. (1896). L'action bactericide des eaux de la Jumna et du Gange sur le vibrion du cholera. *Ann. Inst. Pasteur*, *10*, 511.

Hantke, K. (1978). Major outer membrane proteins of E. coli K12 serve as receptors for the phages T2 and 434 (protein Ib). *Molecular and General Genetics MGG*, *164*(2), 131-135.

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.

Häse, 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.

Hatfull, G. F. (2008). Bacteriophage genomics. *Current opinion in microbiology*,*11*(5), 447-453

Hatfull, G. F., & Hendrix, R. W. (2011). Bacteriophages and their genomes.*Current opinion in virology*, *1*(4), 298-303

Hatfull, G. F., Jacobs-Sera, D., Lawrence, J. G., Pope, W. H., Russell, D. A., Ko, C. C., & Hendrix, R. W. (2010). Comparative genomic analysis of 60 mycobacteriophage genomes: genome clustering, gene acquisition, and gene size. *Journal of molecular biology*, *397*(1), 119-143.

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.

Hendrix, R. W. (2003). Bacteriophage genomics. *Current opinion in microbiology*, *6*(5), 506-511.

Hendrix, R. W., Smith, M. C., Burns, R. N., Ford, M. E., & Hatfull, G. F. (1999). Evolutionary relationships among diverse bacteriophages and prophages: all the world'sa phage. *Proceedings of the National Academy of Sciences*, *96*(5), 2192-2197.

Henrici, A. T. (1925). A Statistical Study of the Form and Growth of the Cholera *Vibrio* Three Plates. *Journal of Infectious Diseases*, *37*(1), 75-81.

Hens, D. K., Ghosh, A. N., & Kumar, R. (2005). A new small temperate DNA phage BcP15 isolated from Burkholderia cepacia DR11. *Archives of virology*,*150*(12), 2421-2428.

Herman, N. J., & Juni, E. (1974). Isolation and characterization of a generalized transducing bacteriophage for *Acinetobacter*. *Journal of virology*, *13*(1), 46-52.

Herold, S., Karch, H., & Schmidt, H. (2004). Shiga toxin-encoding bacteriophages–genomes in motion. *International journal of medical microbiology*, *294*(2), 115-121.

Herrington, D. A., Hall, R. H., Losonsky, G., Mekalanos, J. J., Taylor, R. K., & Levine, M. M. (1988). Toxin, toxin-coregulated pili, and the toxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. *The Journal of experimental medicine*, *168*(4), 1487-1492.

Herskowitz, I., & Hagen, D. (1980). The lysis-lysogeny decision of phage lambda: explicit programming and responsiveness. *Annual review of genetics*,*14*(1), 399-445.

Higgins, D. E., & DiRita, V. J. (1994). Transcriptional control of toxT, a regulatory gene in the ToxR regulon of *Vibrio cholerae*. *Molecular microbiology*,*14*(1), 17-29.

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

Hill, K. K., Smith, T. J., Helma, C. H., Ticknor, L. O., Foley, B. T., Svensson, R. T., ... & Marks, J. D. (2007). Genetic diversity among botulinum neurotoxin-producing clostridial strains. *Journal of bacteriology*, *189*(3), 818-832.

Hoshino, K., Yamasaki, S., Mukhopadhyay, A. K., Chakraborty, S., Basu, A., Bhattacharya, S. K., & Takeda, Y. (1998). Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. *FEMS Immunology & Medical Microbiology*, *20*(3), 201-207.

Howard-Jones, N. (1984). Robert Koch and the cholera vibrio: a centenary.*British medical journal (Clinical research ed.)*, *288*(6414), 379.

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.

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., 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.

Huson, D. H., & Scornavacca, C. (2011). A survey of combinatorial methods for phylogenetic networks. *Genome biology and evolution*, *3*, 23-35.

Hynes, W. L., Hancock, L., & Ferretti, J. J. (1995). Analysis of a second bacteriophage hyaluronidase gene from *Streptococcus pyogenes*: evidence for a third hyaluronidase involved in extracellular enzymatic activity. *Infection and immunity*, *63*(8), 3015-3020.

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.

Iida, T., & Honda, T. (1997). Hemolysins produced by *Vibrios*. *Toxin Reviews*, 16(4), 215-227.

Imlay, J. A., Chin, S. M., & Linn, S. (1988). Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science*, *240*(4852), 640-642.

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.

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). Foodborne gastroenteritis caused by *Vibrio*, *Yersinia*, and *Campylobacter* species. *Modern Food Microbiology*, 657-678.

Jensen, E. C., Schrader, H. S., Rieland, B., Thompson, T. L., Lee, K. W., Nickerson, K. W., & Kokjohn, T. A. (1998). Prevalence of Broad-Host-Range of Lytic Bacteriophages of *Sphaerotilus natans*, *Escherichia coli*, and *Pseudomonas aeruginosa*. *Applied and environmental microbiology*, *64*(2), 575-580.

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.

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, S. C., & Paul, J. H. (1994). Seasonal and diel abundance of viruses and occurrence of lysogeny/bacteriocinogeny in the marine environment. *Marine Ecology-Progress Series*, *104*, 163.

Jiang, S. C., & Paul, J. H. (1996). Occurrence of lysogenic bacteria in marine microbial communities as determined by prophage induction. *Marine Ecology-Progress Series*, *142*, 27-48.

Jiang, S. C., & Paul, J. H. (1998). Gene transfer by in marine environment. *Applied and Environmental Microbiology*, *64*(8), 2780-2787.

Jiang, S. C., Louis, V., Choopun, N., Sharma, A., Huq, A., & Colwell, R. R. (2000a). Genetic diversity of *Vibrio cholerae* in Chesapeake Bay determined by amplified fragment length polymorphism fingerprinting. *Applied and environmental microbiology*, *66*(1), 140-147.

Jiang, S. C., Matte, M., Matte, G., Huq, A., & Colwell, R. R. (2000). Genetic diversity of clinical and environmental isolates of *Vibrio cholerae* determined by amplified fragment length polymorphism fingerprinting. *Applied and environmental microbiology*, *66*(1), 148-153.

Johansen, L. K., Albrechtsen, B., Andersen, H. W., & Engberg, J. (1995). pFab60: a new, efficient vector for expression of antibody Fab fragments displayed on phage. *Protein engineering*, *8*(10), 1063-1067

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, 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.

Juhala, R. J., Ford, M. E., Duda, R. L., Youlton, A., Hatfull, G. F., & Hendrix, R. W. (2000). Genomic sequences of bacteriophages HK97 and HK022:

pervasive genetic mosaicism in the lambdoid bacteriophages. *Journal of molecular biology*, *299*(1), 27-51.

Kanamaru, S., Leiman, P. G., Kostyuchenko, V. A., Chipman, P. R., Mesyanzhinov, V. V., Arisaka, F., & Rossmann, M. G. (2002). Structure of the cell-puncturing device of bacteriophage T4. *Nature*, *415*(6871), 553-557.

Kane, N. C., King, M. G., Barker, M. S., Raduski, A., Karrenberg, S., Yatabe, Y. & Rieseberg, L. H. (2009). Comparative genomic and population genetic analyses indicate highly porous genomes and high levels of gene flow between divergent Helianthus species. *Evolution*, *63*(8), 2061-2075

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.

Kaper, J. B., Lockman, H., Colwell, R. R., & Joseph, S. W. (1979). Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. *Applied and Environmental Microbiology*, *37*(1), 91-103.

Kaper, J. B., Morris, J. G., & Levine, M. M. (1995). Cholera. *Clinical Microbiology Reviews*, 8(1), 48-86.

Kar, S., Ghosh, R. K., Ghosh, A. N., & Ghosh, A. (1996). Integration of the DNA of a novel filamentous bacteriophage VSK from *Vibrio cholerae* 0139 into the host chromosomal DNA. *FEMS microbiology letters*, *145*(1), 17-22.

Karaolis, D. K., & Kaper, J. B. (1999). *Vibrio cholerae* TCP, a trifunctional virulence factor. *Trends in microbiology*, *7*(10), 393.

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., Lan, R., & Reeves, P. R. (1995). The sixth and seventh cholera pandemics are due to independent clones separately derived from environmental, nontoxigenic, non-O1 *Vibrio cholerae*. *Journal of bacteriology*,*177* (11), 3191-3198.

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.

Karaolis, D. K., Somara, S., Maneval, D. R., Johnson, J. A., & Kaper, J. B. (1999). A bacteriophage encoding a pathogenicity island, type-IV pilus and a phage receptor. *Nature*, *399*(6734), 375-379.

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.

Kaufman, M. R., & Taylor, R. K. (1994). The toxin-coregulated pilus: biogenesis and function. *Vibrio cholerae and Cholera: Molecular to Global Perspectives*, ASM Press, Washington, DC,187-202.

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.

Keasler, S., & Hall, R. (1993). Detecting and biotyping *Vibrio cholerae* O1 with multiplex PCR. *The Lancet*, *341*(8861), 1661.

Kelley, L. A., & Sternberg, M. J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nature protocols*, *4*(3), 363-371.

Kelley, W. L. (2006). Lex marks the spot: the virulent side of SOS and a closer look at the LexA regulon. *Molecular microbiology*, *62*(5), 1228-1238.

Khan, W., Bennish, M. L., Seas, C., Khan, E. H., Ronan, A., Dhar, U. & Salam, M. A. (1996). Randomised controlled comparison of single-dose ciprofloxacin and doxycycline for cholera caused by *Vibrio cholerae* O1 or O139. *The Lancet*, *348*(9023), 296-300.

Kidwell, M. G. (1993). Horizontal transfer of P elements and other short inverted repeat transposons. In *Transposable Elements and Evolution* (pp. 158-172). Springer Netherlands.

Kim, J. W., & Kathariou, S. (2009). Temperature-dependent phage resistance of Listeria monocytogenes epidemic clone II. *Applied and environmental microbiology*, *75*(8), 2433-2438.

Kimberg, D. V., Field, M., Johnson, J., Henderson, A., & Gershon, E. (1971). Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin. *Journal of Clinical Investigation*, *50*(6), 1218.

Kimsey, H. H., & Waldor, M. K. (1998). *Vibrio cholerae* hemagglutinin/protease inactivates CTXφ. *Infection and immunity*, *66*(9), 4025-4029.

King, G., & Murray, N. E. (1994). Restriction enzymes in cells, not eppendorfs. *Trends in microbiology*, *2*(12), 465-469.

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.

Koch, R. (1884). An address on cholera and its *Bacillus*. *British medical journal*,*2* (1236), 453.

Koonin, E. V. (1992). The second cholera toxin, Zot, and its plasmid-encoded and phage-encoded homologues constitute a group of putative ATPases with an altered purine NTP-binding motif. *FEBS letters*, *312*(1), 3-6.

Kotetishvili, M., Stine, O. C., Chen, Y., Kreger, A., Sulakvelidze, A., Sozhamannan, S., & Morris Jr, J. G. (2003). Multilocus sequence typing has

better discriminatory ability for typing *Vibrio cholerae* than does pulsed-field gel electrophoresis and provides a measure of phylogenetic relatedness. *Journal of clinical microbiology*, *41*(5), 2191-2196.

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*(8), 2165-2174.

Kraus, R. H., Kerstens, H. H., van Hooft, P., Megens, H. J., Elmberg, J., Tsvey, A. & Prins, H. H. (2012). Widespread horizontal genomic exchange does not erode species barriers among sympatric ducks. *BMC evolutionary biology*,*12*(1), 45.

Krishnan, H. H., Ghosh, A., Paul, K., & Chowdhury, R. (2004). Effect of anaerobiosis on expression of virulence factors in *Vibrio cholerae*. *Infection and immunity*, *72*(7), 3961-3967.

Kropinski, A. M., Sulakvelidze, A., Konczy, P., & Poppe, C. (2007). Salmonella phages and prophages-genomics and practical aspects. In *Salmonella* (pp. 133-175). Humana Press.

Krueger, A. P., & Fong, J. (1937). The relationship between bacterial growth and phage production. *The Journal of general physiology*, *21*(2), 137.

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, P., Thulaseedharan, A., Chowdhury, G., Ramamurthy, T., & Thomas, S. (2011). Characterization of novel alleles of toxin co-regulated pilus A gene (*tcp*A) from environmental isolates of *Vibrio cholerae*. *Current microbiology*,*62*(3), 758-763.

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.

Kurazono, H., Pal, A., Bag, P. K., Balakrish Nair, G., Karasawa, T., Mihara, T., & Takeda, Y. (1995). Distribution of genes encoding cholera toxin, zonula occludens toxin, accessory cholera toxin, and El Tor hemolysin *Vibrio cholerae* of diverse origins. *Microbial pathogenesis*, *18*(3), 231-235.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature*, *227*(5259), 680-685.

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.

Lang, H., & Palva, E.T. *(*1993*)* The *ompS* gene of *Vibrio cholerae* encodes a growth-phase-dependent maltoporin. *Molecular Microbiology*,10, 891–901.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0.*Bioinformatics*, *23*(21), 2947-2948.

Laval, E. (2003). El cólera en Chile (1886-1888). *Revista chilena de infectología*, *20*, 86-88.

Lawrence, J. G. (1997). Selfish operons and speciation by gene transfer.*Trends in microbiology*, *5*(9), 355-359.

Lawrence, J. G. (1999). Gene transfer, speciation, and the evolution of bacterial enomes. *Current opinion in microbiology*, *2*(5), 519-523.

Lawrence, J. G., & Ochman, H. (1997). Amelioration of bacterial genomes: rates of change and exchange. *Journal of molecular evolution*, *44*(4), 383-397.

Lawrence, J. G., Hatfull, G. F., & Hendrix, R. W. (2002). Imbroglios of viral taxonomy: genetic exchange and failings of phenetic approaches. *Journal of Bacteriology*, *184*(17), 4891-4905.

Lederberg, J., & Tatum, E. L. (1946). Gene recombination in Escherichia coli.*Nature*, *158*(4016), 558-558.

Lee, J. H., Han, K. H., Choi, S. Y., Lucas, M. E., Mondlane, C., Ansaruzzaman, M., ... & Kim, D. W. (2006). Multilocus sequence typing (MLST) analysis of *Vibrio cholerae* O1 El Tor isolates from Mozambique that harbour the classical CTX prophage. *Journal of medical microbiology*, *55*(2), 165-170.

Lerat, E., Daubin, V., & Moran, N. A. (2003). From gene trees to organismal phylogeny in prokaryotes: the case of the γ-Proteobacteria. *PLoS biology*, *1*(1), e19.

Leverentz, B., Conway, W. S., Alavidze, Z., Janisiewicz, W. J., Fuchs, Y., Camp, M. J., & Sulakvelidze, A. (2001). Examination of bacteriophage as a biocontrol method for Salmonella on fresh-cut fruit: a model study. *Journal of Food Protection®*, *64*(8), 1116-1121.

Leverentz, B., Conway, W. S., Janisiewicz, W., & Camp, M. J. (2004). Optimizing concentration and timing of a phage spray application to reduce *Listeria monocytogenes* on honeydew melon tissue. *Journal of Food Protection*, *67*(8), 1682-1686.

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.

Lindberg, A. A. (1973). Bacteriophage receptors. *Annual Reviews in Microbiology*, *27*(1), 205-241.

Liu, C. H., Cheng, W., Hsu, J. P., & Chen, J. C. (2004). *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.

Livny, J., & Friedman, D. I. (2004). Characterizing spontaneous induction of Stx encoding phages using a selectable reporter system. *Molecular microbiology*,*51*(6), 1691-1704.

Łoś, J. M., Łoś, M., Węgrzyn, G., & Węgrzyn, A. (2009). Differential efficiency of induction of various lambdoid prophages responsible for production of Shiga toxins in response to different induction agents. *Microbial pathogenesis*, *47*(6), 289-298.

Los, M., Los, J. M., & Wegrzyn, G. (2008). Rapid identification of Shiga toxin-producing *Escherichia coli* (STEC) using electric biochips. *Diagnostic Molecular Pathology*, *17*(3), 179-184.

Lu, Z., Breidt Jr, F., Fleming, H. P., Altermann, E., & Klaenhammer, T. R. (2003). Isolation and characterization of a *Lactobacillus plantarum* bacteriophage. *International journal of food microbiology*, *84*(2), 225-235.

Lubbers, M. W., Waterfield, N. R., Beresford, T. P., Le Page, R. W., & Jarvis, A. W. (1995). Sequencing and analysis of the prolate-headed lactococcal bacteriophage c2 genome and identification of the structural genes. *Applied and environmental microbiology*, *61*(12), 4348-4356.

Ludwig, A., & Goebel, M. (1997). Hemolysins of *Escherichia coli*. In: Sussman, M. (Ed.) *Escherichia coli*: *Mechanisms of virulence*. Cambridge University Press, pp. 281-329.

Luria, S. E., Delbrück, M., & Anderson, T. F. (1943). Electron microscope studies of bacterial viruses. *Journal of bacteriology*, *46*(1), 57.

Lwoff, A., Jacob, F., Ritz, E., & Gagé, M. (1952). Induction de la production de bactériophages et d'une colicine par les peroxydes, les éthylèneimines et les halogénoalcoylamines. *CR Hebd Seances Acad Sci*, *234*(23), 2308-2310.

MacDonell, M. T., & Colwell, R. R. (1984a). Identical 5S rRNA nucleotide sequence of *Vibrio cholerae* strains representing temporal, geographical, and ecological diversity. *Applied and environmental microbiology*, *48*(1), 119-121.

MacDonell, M. T., & Colwell, R. R. (1984b). Nucleotide base sequence of *vibrionaceae* 5 S rRNA. *FEBS letters*, *175*(1), 183-188.

Mahalingam, S., Cheong, Y. M., Kan, S., Yassin, R. M., Vadivelu, J., & Pang, T. (1994). Molecular epidemiologic analysis of *Vibrio cholerae* O1 isolates by pulsed-field gel electrophoresis. *Journal of clinical microbiology*, *32*(12), 2975-2979.

Mahony, D. E., Bell, P. D., & Easterbrook, K. B. (1985). Two bacteriophages of *Clostridium difficile*. *Journal of clinical microbiology*, *21*(2), 251-254.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., & Spratt, B. G. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms.*Proceedings of the National Academy of Sciences*, *95*(6), 3140-3145.

Maiti, M., & Chatterjee, S. N. (1971). Characteristics of a group IV cholera phage. *Journal of General Virology*, *13*(2), 327-330.

Manning, P. A. (1997). The *tcp* gene cluster of *Vibrio cholerae*. *Gene*, *192*(1), 63-70.

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.

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.

McDaniel, L., & Paul, J. H. (2005). Effect of nutrient addition and environmental factors on prophage induction in natural populations of marine *Synechococcus* species. *Applied and environmental microbiology*, *71*(2), 842-850

McLaughlin, J. C. (1995). Vibrio. *Manual of clinical microbiology*, 6th ed. Washington, DC: ASM Press; 465-476.

Medhekar, B., & Miller, J. F. (2007). Diversity-generating retroelements. *Current opinion in microbiology*, *10*(4), 388-395.

Mekalanos, J. J. (1983). Duplication and amplification of toxin genes in *Vibrio cholerae*. *Cell*, *35*(1), 253-263.

Mekalanos, J. J., Rubin, E. J., & Waldor, M. K. (1997). Cholera: molecular basis for emergence and pathogenesis. *FEMS Immunology & Medical Microbiology*, *18*(4), 241-248.

Miller, R. V., & Kokjohn, T. A. (1990). General microbiology of recA: environmental and evolutionary significance. *Annual Reviews in Microbiology*,*44*(1), 365-394.

Miller, V. L., & Mekalanos, J. J. (1984). Synthesis of cholera toxin is positively regulated at the transcriptional level by toxR. *Proceedings of the National Academy of Sciences*, *81*(11), 3471-3475.

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.

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.

Mirold, S., Rabsch, W., Tschäpe, H., & Hardt, W. D. (2001). Transfer of the *Salmonella* type III effector sopE between unrelated phage families.*Journal of molecular biology*, *312*(1), 7-16.

Mitra, S. N., Kar, S., Ghosh, R. K., Pajn, S., & Ghosh, A. (1995). Presence of lysogenic phage in the outbreak strains of *Vibrio cholerae* O139. *Journal of medical microbiology*, *42*(6), 399-403..

Mitra, S. N., Mukhopadhyay, R., Ghosh, A. N., & Ghosh, R. K. (2000). Conversion of *Vibrio* El Tor MAK757 to Classical Biotype: Role of Phage PS166. *Virology*, *273*(1), 36-43.

Mizuta, M., Satoh, E., Katoh, C., Tanaka, K., Moriguchi, K., & Suzuki, K. (2012). Screening for yeast mutants defective in recipient ability for transkingdom conjugation with *Escherichia coli* revealed importance of vacuolar ATPase activity in the horizontal DNA transfer phenomenon. *Microbiological research*, *167*(5), 311-316.

Moineau, S., Durmaz, E., Pandian, S., & Klaenhammer, T. R. (1993). Differentiation of two abortive mechanisms by using monoclonal antibodies directed toward lactococcal bacteriophage capsid proteins. *Applied and environmental microbiology*, *59*(1), 208-212.

Molineux, I. J. (2001). No syringes please, ejection of phage T7 DNA from the virion is enzyme driven. *Molecular microbiology*, *40*(1), 1-8.

Mordacq, J. C., & Ellington, R. W. (1998). Bacterial Gene Transfer. *Trends in Genetics*, *14*(8), 307-311.

Morris JG Jr (1990). Non-O group 1 *Vibrio cholerae*: a look at the epidemiology of an occasional pathogen. *Epidemiological Reviews*, 12:179–191.

Morris Jr, J. G., & Black, R. E. (1985). Cholera and other vibrioses in the United States. *The New England journal of medicine*, *312*(6), 343-350.

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.

Morris, J. G., Tenney, J. H., & Drusano, G. L. (1985). *In vitro* susceptibility of pathogenic *Vibrio* species to norfloxacin. *Antimicrobial agents and chemotherapy*, *28*(3), 442-445.

Mukerjee, S. (1963). The bacteriophage-susceptibility test in differentiating *Vibrio cholerae* and *Vibrio* El Tor. *Bulletin of the World Health Organization*,*28*(3), 333.

Mukhopadhyay, R., & Ghosh, R. K. (2000). Mechanism of Phage PS166-Mediated Biotype Conversion in *Vibrio cholerae*. Role of the *hly*A Locus. *Virology*, *273*(1), 44-51.

Mutreja, A., Kim, D. W., Thomson, N. R., Connor, T. R., Lee, J. H., Kariuki, S., ... & Dougan, G. (2011). Evidence for several waves of global transmission in the seventh cholera pandemic. *Nature*, *477*(7365), 462-465.

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. (2008). The new emerging strain of cholera: One step ahead of genomics. *International Journal of Infectious Diseases*, 12, e5-e6.

Nair, G. B., Ramamurthy, T., Bhattacharya, S. K., Mukhopadhyay, A. K., Garg, S., Bhattacharya, M. K., & Deb, B. C. (1994). Spread of *Vibrio cholerae* 0139 Bengal in India. *Journal of Infectious Diseases*, *169*(5), 1029-1034.

Nair, M. A., & Sreedharan, C. (1986). Agroforestry farming systems in the homesteads of Kerala, southern India. *Agroforestry Systems*, *4*(4), 339-363.

Nalin, D. R., Daya, V., Reid, A., Levine, M. M., & Cisneros, L. (1979). Adsorption and growth of *Vibrio cholerae* on chitin. *Infection and Immunity*,*25*(2), 768-770.

Nandi, B., Nandy, R. K., Mukhopadhyay, S., Nair, G. B., Shimada, T., & Ghose, A. C. (2000a). 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.

Nandi, B., Nandy, R. K., Sarkar, A., & Ghose, A. C. (2005). Structural features, properties and regulation of the outer-membrane protein W (OmpW) of *Vibrio cholerae*. *Microbiology*, *151*(9), 2975-2986.

Nandi, B., Nandy, R. K., Vicente, A. C., & Ghose, A. C. (2000b). Molecular Characterization of a New Variant of Toxin-Coregulated Pilus Protein (TcpA) in a Toxigenic Non-O1/Non-O139 Strain of*Vibrio cholerae*. *Infection and immunity*,*68*(2), 948-952.

Nelson, E. J., Harris, J. B., Morris, J. G., Calderwood, S. B., & Camilli, A. (2009). Cholera transmission: the host, pathogen and bacteriophage dynamic. *Nature Reviews Microbiology*, *7*(10), 693-702.

Nelson, K. E., Clayton, R. A., Gill, S. R., Gwinn, M. L., Dodson, R. J., Haft, D. H., & Fraser, C. M. (1999). Evidence for lateral gene transfer between *Archaea* and bacteria from genome sequence of *Thermotoga maritima*. *Nature*, *399*(6734), 323-329.

Novais, R. C., Coelho, A., Salles, C. A., & Vicente, A. C. (1999). Toxin-co-regulated pilus cluster in non-O1, non-toxigenic *Vibrio cholerae*: evidence of a third allele of pilin gene. *FEMS microbiology letters*, *171*(1), 49-55.

Ochman, H., Lawrence, J. G., & Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature*, *405*(6784), 299-304.

Ogg, J. E., Timme, T. L., & Alemohammad, M. M. (1981). General transduction in *Vibrio cholerae*. *Infection and immunity*, *31*(2), 737-741.

Ogierman, M. A., & Manning, P. A. (1992). Genetic organization and sequence of the promoter-distal region of the *tcp* gene cluster of *Vibrio cholerae*. *Gene*, 126(1), 51-60.

Oliver, J. D. (2005). The viable but nonculturable state in bacteria. *Journal of Microbiology*, 43(1), 93-100.

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'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.

Pal, B. B., Khuntia, H. K., Samal, S. K., Das, S. S., & Chhotray, G. P. (2006). Emergence of *Vibrio cholerae* O1 Biotype E1 Tor Serotype Inaba Causing Outbreaks of Cholera in Orissa, India. *Japanese journal of infectious diseases*,*59*(4), 266.

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.

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.

Pattee, P. A. (1966). Use of tetrazolium for improved resolution of bacteriophage plaques. *Journal of bacteriology*, *92*(3), 787.

Paul, J. H., Sullivan, M. B., Segall, A. M., & Rohwer, F. (2002). Marine phage genomics. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *133*(4), 463-476.

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), 3750-37

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.

Pierce, N. F., Greenough 3rd, W. B., & Carpenter Jr, C. C. (1971). *Vibrio cholerae* enterotoxin and its mode of action. *Bacteriological reviews*, *35*(1), 1.

Pierce, N. F., Sack, R. B., Mitra, R. C., Banwell, J. G., Brigham, K. L., Fedson, D. S., & Mondal, A. (1969). Replacement of Water and Electrolyte Losses in Cholera by an Oral Glucose-Electrolyte Solution. *Annals of Internal Medicine*, *70*(6), 1173-1181.

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.

Pietilä, M. K., Demina, T. A., Atanasova, N. S., Oksanen, H. M., & Bamford, D. H. (2014). Archaeal viruses and bacteriophages: comparisons and contrasts.*Trends in microbiology*.

Pollitzer, R. (1954). Cholera studies: 1. History of the disease. *Bulletin of the World Health Organization*, *10*(3), 421.

Pollitzer, R. (1959). Cholera, monograph series, no. 43. *World Health Organization, Geneva*, 1019.

Pope, W. H., Jacobs-Sera, D., Russell, D. A., Peebles, C. L., Al-Atrache, Z., Alcoser, T. A., & Filliger, L. Z. (2011). Expanding the diversity of mycobacteriophages: insights into genome architecture and evolution. *PloS one*, *6*(1), e16329.

Popovic, T., Fields, P. I., Olsvik, O., Wells, J. G., Evins, G. M., Cameron, D. N. & Feeley, J. C. (1995). Molecular subtyping of toxigenic *Vibrio cholerae* O139 causing epidemic cholera in India and Bangladesh, 1992–1993. *Journal of Infectious Diseases*, *171*(1), 122-127.

Potter, N. N., & Nelson, F. E. (1952). Studies on Plaque Formation with a Modified Plating Technique1, 2. *Journal of bacteriology*, *64*(1), 105

Prevots, F., Mata, M., & Ritzenthaler, P. A. U. L. (1990). Taxonomic differentiation of 101 bacteriophages and characterization of bacteriophages with unusually large genomes. *Applied and environmental microbiology*, *56*(7), 2180-2185

Pyle, G. F. (1969). The diffusion of cholera in the United States in the nineteenth century. *Geographical Analysis*, *1*(1), 59-75.

Quinones, M., Kimsey, H. H., & Waldor, M. K. (2005). LexA cleavage is required for CTX prophage induction. *Molecular cell*, *17*(2), 291-300.

Rabinovitch, A., Hadas, H., Einav, M., Melamed, Z., & Zaritsky, A. (1999). Model for Bacteriophage T4 Development in *Escherichia coli*. *Journal of bacteriology*, *181*(5), 1677-1683

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.

Rahman, M., Sack, D. A., Wadood, A., Yasmin, M., & Latif, A. (1989). Rapid identification of *Vibrio cholerae* serotype O1 from primary isolation plates by a coagglutination test. *Journal of medical microbiology*, *28*(1), 39-41.

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. P. K. B., Bag, P. K., Pal, A., Bhattacharya, S. K., Bhattacharya, M. K., Shimada, T., & Nair, G. B. (1993a). Virulence patterns of *Vibrio cholerae* non-01 strains isolated from hospitalised patients with acute diarrhoea in Calcutta, India. *Journal of medical microbiology*, *39*(4), 310-317.

Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya, S. K., Balakrish Nair, G., Shimada, T., & Takeda, Y. (1993b). Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. *The Lancet*, *341*(8846), 703-704.

Ravin, V., Ravin, N., Casjens, S., Ford, M. E., Hatfull, G. F., & Hendrix, R. W. (2000). Genomic sequence and analysis of the atypical temperate bacteriophage N15. *Journal of molecular biology*, *299*(1), 53-73.

Rawlings, N. D., & Barrett, A. J. (1993). Evolutionary families of peptidases. *Biochem. J*, *290*, 205-218

Reeve, J. N., & Shaw, J. E. (1979). Lambda encodes an outer membrane protein: the lom gene. *Molecular and General Genetics MGG*, *172*(3), 243-248.

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.

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.

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., and Colwell, R. R. (2001): Genotypes associated with virulence in environmental isolates of *Vibrio cholerae*. *Applied and Environmental Microbiology*, **67**, 2421-2429.

Rodo, X., Pascual, M., Fuchs, G., & Faruque, A. S. G. (2002). ENSO and cholera: A nonstationary link related to climate change? *Proceedings of the national Academy of Sciences*, *99*(20), 12901-12906.

Rodrigue, D. C., Popovic, T., & Wachsmuth, I. K. (1994). Nontoxigenic *Vibrio cholerae* O1 infections in the United States. *Vibrio cholerae and cholera: molecular to global perspectives. ASM Press, Washington, DC*, 69-76.

Rooijakkers, S. H., Ruyken, M., Van Roon, J., Van Kessel, K. P., Van Strijp, J. A., & Van Wamel, W. J. (2006). Early expression of SCIN and CHIPS drives instant immune evasion by Staphylococcus aureus. *Cellular microbiology*, *8*(8), 1282-1293.

Roszak, D. B., & Colwell, R. R. (1987). Survival strategies of bacteria in the natural environment. *Microbiological Reviews*, 51(3), 365.

Rowe, B., & Frost, J. A. (1992). Vibriophages and phage-typing. In *Cholera* (pp. 95-105). Springer US.

Rubin, E. J., Lin, W., Mekalanos, J. J., & Waldor, M. K. (1998). Replication and integration of a *Vibrio cholerae* cryptic plasmid linked to the CTX prophage.*Molecular microbiology*, *28*(6), 1247-1254.

Safa, A., Nair, G. B., & Kong, R. Y. (2010). Evolution of new variants of *Vibrio cholerae* O1. *Trends in microbiology*, *18*(1), 46-54.

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.

Said, B., Smith, H. R., Scotland, S. M., & Rowe, B. (1995). Detection and differentiation of the gene for toxin co-regulated pili (*tcp*A) in *Vibrio cholerae* non-O1 using the polymerase chain reaction. *FEMS microbiology letters*, *125*(2-3), 205-210.

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.

Sails, A. D., Wareing, D. R. A., Bolton, F. J., Fox, A. J., & Curry, A. (1998). Characterisation of 16 *Campylobacter jejuni* and *E. coli* typing bacteriophages.*Journal of medical microbiology*, *47*(2), 123-128

Sakazaki, R. (1992). Bacteriology of *Vibrio* and related organisms. In *Cholera*(pp. 37-55). Springer US.

Sambrook, J., Fritish, E., and Maniatis, I (2000): Molecular Cloning, a Laboratory manual. In 3 (Ed.), C.S.H. Laboratory, Ed.

Schmidt, H. (2001). Shiga-toxin-converting bacteriophages. *Research in microbiology*, *152*(8), 687-695.

Schuster, H., Beyersmann, D., Mikolajczyk, M., & Schlicht, M. (1973). Prophage induction by high temperature in thermosensitive DNA mutants lysogenic for bacteriophage lambda. *Journal of virology*, *11*(6), 879-885.

Seas, C., DuPont, H. L., Valdez, L. M., & Gotuzzo, E. (1996). Practical guidelines for the treatment of cholera. *Drugs*, *51*(6), 966-973.

Sen, A., & Ghosh, A. N. (2005a). New *Vibrio cholerae* O1 biotype ElTor bacteriophages. *Virology journal*, *2*(1), 28.

Sen, A., & Ghosh, A. N. (2005b). Physicochemical characterization of vibriophage N5. *Virology journal*, *2*(1), 27.

Shafia, F., & Thompson, T. L. (1964). Calcium ion requirement for proliferation of bacteriophage φμ-4. *Journal of bacteriology*, *88*(2), 293-296.

Shao, Y., & Wang, N. (2008). Bacteriophage adsorption rate and optimal lysis time. *Genetics*, *180*(1), 471-482.

Sharma, A., & Chaturvedi, A. N. (2006). Prevalence of virulence genes (ctxA, *stn*, *OmpW* and *tcpA*) among non-O1 *Vibrio cholerae* isolated from fresh water environment. *International journal of hygiene and environmental health*, *209*(6), 521-526.

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.

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. (1998). 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.

Shimada, T., Arakawa, E., Itoh, K., Okitsu, T., Matsushima, A., Asai, Y., & Takeda, Y. (1994). Extended serotyping scheme for *Vibrio cholerae*. *Current Microbiology*, *28*(3), 175-178.

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.

Shimizu-Kadota, M., & Sakurai, T. (1982). Prophage curing in *Lactobacillus casei* by isolation of a thermoinducible mutant. *Applied and environmental microbiology*, *43*(6), 1284-1287.

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.

Shivu, M. M., Rajeeva, B. C., Girisha, S. K., Karunasagar, I., Krohne, G., & Karunasagar, I. (2007). Molecular characterization of *Vibrio harveyi* bacteriophages isolated from aquaculture environments along the coast of India. *Environmental microbiology*, *9*(2), 322-331.

Shousha, A. T. (1948). Cholera Epidemic in Egypt (1947): A Preliminary Report.*Bulletin of the World Health Organization*, *1*(2), 353.

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.

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.

Singh, D. V., Matte, M. H., Matte, G. R., Jiang, S., Sabeena, F., Shukla, B. N., & 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.

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, H. O., Gwinn, M. L., & Salzberg, S. L. (1999). DNA uptake signal sequences in naturally transformable bacteria. *Research in microbiology*,*150*(9), 603-616.

Smoot, L. M., Smoot, J. C., Graham, M. R., Somerville, G. A., Sturdevant, D. E., Migliaccio, C. A. L., & Musser, J. M. (2001). Global differential gene expression in response to growth temperature alteration in group A *Streptococcus*. *Proceedings of the National Academy of Sciences*, *98*(18), 10416-10421.

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.

Snow, J. (1855). *On the mode of communication of cholera*. John Churchill.

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*.

Spira, W. M., & Ahmed, Q. S. (1981). Gauze filtration and enrichment procedures for recovery of *Vibrio cholerae* from contaminated waters. *Applied and environmental microbiology*, *42*(4), 730.

Spira, W. M., Huq, A., Ahmed, Q. S., & Saeed, Y. A. (1981). Uptake of *Vibrio cholerae* biotype eltor from contaminated water by water hyacinth (*Eichornia crassipes*). *Applied and environmental microbiology*, *42*(3), 550-553.

Srinivasan, S. K., & Rangan, A. (1970). Stochastic models for phage reproduction. *Mathematical Biosciences*, *8*(3), 295-305

Staerk, J., Ronneberger, H. J., & Wiegandt, H. (1974). Neuraminidase, a virulence factor in *Vibrio cholerae* infection. *Behring Inst Mitt*, *55*, 145-146.

Stanley, T. L., Ellermeier, C. D., & Slauch, J. M. (2000). Tissue-specific gene expression identifies a gene in the lysogenic phage Gifsy-1 that affects *Salmonella enterica* serovar *Typhimurium* survival in Peyer's patches. *Journal of bacteriology*, *182*(16), 4406-4413

Stegemann, S., Keuthe, M., Greiner, S., & Bock, R. (2012). Horizontal transfer of chloroplast genomes between plant species. *Proceedings of the National Academy of Sciences*, *109*(7), 2434-2438

Stent, G. S. (1963). Molecular biology of bacterial viruses. *Molecular biology of bacterial viruses.*

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.

Stickler, D. J., Tucker, R. G., & Kay, D. (1965). Bacteriophage-like particles released from *Bacillus subtilis* after induction with hydrogen peroxide. *Virology*, *26*(1), 142-145.

Strom, M. S., & Lory, S. (1993). Structure-function and biogenesis of the type IV pili. *Annual Reviews in Microbiology*, *47*(1), 565-596.

Struhl, K., Cameron, J. R., & Davis, R. W. (1976). Functional genetic expression of eukaryotic DNA in *Escherichia coli*. *Proceedings of the National Academy of Sciences*, *73*(5), 1471-1475.

Sullivan, M. B., Coleman, M. L., Weigele, P., Rohwer, F., & Chisholm, S. W. (2005). Three Prochlorococcus cyanophage genomes: signature features and ecological interpretations. *PLoS biology*, *3*(5), e144

Sun, D., Lafferty, M. J., Peek, J. A., & Taylor, R. K. (1997). Domains within the *Vibrio cholerae* toxin coregulated pilin subunit that mediate bacterial colonization. *Gene*, *192*(1), 79-85.

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.

Suttle, C. A. (1994). The significance of viruses to mortality in aquatic microbial communities. *Microbial Ecology*, *28*(2), 237-243.

Swerdlow, D. L., & Ries, A. A. (1992). Cholera in the Americas: guidelines for the clinician. *Jama*, *267*(11), 1495-1499.

Syvanen, M. (1985). Cross-species gene transfer; implications for a new theory of evolution. *Journal of theoretical Biology*, *112*(2), 333-343.

Syvanen, M. (1994). Horizontal gene transfer: evidence and possible consequences. *Annual review of genetics*, *28*(1), 237-261.

Syvanen, M. (2012). Evolutionary implications of horizontal gene transfer.*Annual review of genetics*, *46*, 341-358.

Talkington, D., Bopp, C., Tarr, C., Parsons, M. B., Dahourou, G., Freeman, M., ... & Gerner-Smidt, P. (2011). Characterization of toxigenic *Vibrio cholerae* from Haiti, 2010–2011. *Emerging infectious diseases*, *17*(11), 2122

Tamrakar, A. K., Goel, A. K., Kamboj, D. V., & Singh, L. (2006). Surveillance methodology for *Vibrio cholerae* in environmental samples. *International journal of environmental health research*, *16*(4), 305-312.

Tapchaisri, P., Mathukorn, N., Jirayut, J., Potjanee, S., Manas, Yamasaki, S., Hayashi, H., Nair, G. B., Kurazono, H., and Wanpen, C. (2007): Virulence genes of clinical *Vibrio cholerae* O1 isolates in Thailand and their ribotypes *Journal of Infection* **55**, 557-565

Tapper, M. A., & Hicks, R. E. (1994). Ultraviolet light and chemical induction of temperate bacteriophage from a Great Lake ecosystem. In *94th General Meeting of the American Society for Microbiology* (Vol. 23, p. 353).

Taylor, R. K., Miller, V. L., Furlong, D. B., & Mekalanos, J. J. (1987). Use of phoA gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proceedings of the National Academy of Sciences*, *84*(9), 2833-2837

Thomas, S., Ramachandran, D., Remani, B., & Singh, D. V. (2008). Detection and molecular characterization of *Vibrio cholerae* O1 Inaba biotype El Tor strain in Kerala, S. India. *World Journal of Microbiology and Biotechnology*, *24*(3), 433-434.

Toranzo, A. E., & Barja, J. L. (1993). Virulence factors of bacteria pathogenic for coldwater fish. *Annual Review of Fish Diseases*, 3, 5-36.

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., 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.

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.

Twort, F. W. (1915). An investigation on the nature of ultra-microscopic viruses. *The Lancet*, *186*(4814), 1241-1243.

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.

Urwin, R., & Maiden, M. C. (2003). Multi-locus sequence typing: a tool for global epidemiology. *Trends in microbiology*, *11*(10), 479-487.

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.

Vaca Pacheco, S., Garcıća González, O., & Paniagua Contreras, G. L. (1997). The lom gene of bacteriophage λ is involved in *Escherichia coli* K12 adhesion to human buccal epithelial cells. *FEMS microbiology letters*, *156*(1), 129-132.

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.

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.

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.

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.

Wachsmuth, K., Olsvik, O., Evins, G. M., & Popovic, T. (1994). Molecular epidemiology of cholera. *Vibrio cholerae and cholera: molecular to global perspectives. ASM Press, Washington, DC*, 357-370.

Wagner, P. L., & Waldor, M. K. (2002). Bacteriophage control of bacterial virulence. *Infection and immunity*, *70*(8), 3985-3993.

Wagner, P. L., Acheson, D. W., & Waldor, M. K. (2001). Human neutrophils and their products induce Shiga toxin production by enterohemorrhagic Escherichia coli. *Infection and immunity*, *69*(3), 1934-1937.

Waldor, M. K., & Mekalanos, J. J. (1994). Emergence of a new cholera pandemic: molecular analysis of virulence determinants in *Vibrio cholerae* 0139 and development of a live vaccine prototype. *Journal of Infectious Diseases,170*(2), 278-283

Waldor, M. K., & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science*, *272*(5270), 1910-1914.

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), 917-92

Walker, G. C. (1978). Inducible reactivation and mutagenesis of UV-irradiated bacteriophage P22 in *Salmonella typhimurium* LT2 containing the plasmid pKM101. *Journal of bacteriology*, *135*(2), 415-421.

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.

Watanabe, K., & Takesue, S. (1972). The requirement for calcium in infection with *Lactobacillus* phage. *Journal of General Virology*, *17*(1), 19-30.

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.

Weinbauer, M. G. (2004). Ecology of prokaryotic viruses. *FEMS microbiology reviews*, *28*(2), 127-181.

Weinbauer, M. G., & Suttle, C. A. (1996). Potential significance of lysogeny to bacteriophage production and bacterial sertality in coastal waters of the gulf of Mexico. *Applied and environmental microbiology*, *62*(12), 4374-4380.

Weinbauer, M. G., & Suttle, C. A. (1999). Lysogeny and prophage induction in coastal and offshore bacterial communities. *Aquatic microbial ecology*, *18*(3), 217-225.

West, B., & Kelly, F. C. (1962). Relationship of sodium chloride tolerance and serological group of *staphylococcal* phages. *Journal of general microbiology*,*28*(2), 367-370.

West, P. A. (1984). Identification and classification of *Vibrionaceae*-an overview. *Vibrios in the Environment*, 285-363.

Wheby, M. S. (2014). Oral Rehydration Therapy in Cholera. *Clinical Infectious Diseases*, *59*(11), 1654-1655

Wheeler, D. L., Church, D. M., Federhen, S., Lash, A. E., Madden, T. L., Pontius, J. U., & Wagner, L. (2003). Database resources of the National Center for Biotechnology. *Nucleic acids research*, *31*(1), 28-33.

WHO, (1995). Cholera, Fact sheet No. 268, July 1995.

Wiederholt, K. M., & Steele, J. L. (1993). Prophage Curing and Partial Characterization of Temperate Bacteriophages from Thermolytic Strains of *Lactococcus lactis*. *Journal of dairy science*, *76*(4), 921-930.

Wilhelm, S. W., Brigden, S. M., & Suttle, C. A. (2002). A dilution technique for the direct measurement of viral production: a comparison in stratified and tidally mixed coastal waters. *Microbial Ecology*, *43*(1), 168-173.

Williamson, S. J., Houchin, L. A., McDaniel, L., & Paul, J. H. (2002). Seasonal variation in lysogeny as depicted by prophage induction in Tampa Bay. *Applied and environmental microbiology*, *68*(9), 4307-4314.

Wommack, K. E., & Colwell, R. R. (2000). Virioplankton: viruses in aquatic ecosystems. *Microbiology and molecular biology reviews*, *64*(1), 69-114.

World Health Organization. (2011). Cholera, Fact Sheet 107, August 2011.

Yamai S et al. (1997). Distribution of serogroups of *Vibrio cholerae* non-O1 non-O139 with specific reference to their ability to produce cholera toxin and addition of novel serogroups. *Journal of the Japanese Association for Infections Diseases*, 71:1037–1045.

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.

Yee, A. J., De Grandis, S., & Gyles, C. L. (1993). Mitomycin-induced synthesis of a Shiga-like toxin from enteropathogenic *Escherichia coli* HI 8. *Infection and immunity*, *61*(10), 4510-4513.

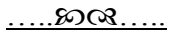
Yildiz, F. H., & Visick, K. L. (2009). *Vibrio* biofilms: so much the same yet so different. *Trends in Microbiology*, 17(3), 109-118.

You, L., Suthers, P. F., & Yin, J. (2002). Effects of *Escherichia coli* physiology on growth of phage T7 in vivo and in silico. *Journal of bacteriology*, *184*(7), 1888-1894.

Zhang, Z., Greene, B., Thuman-Commike, P. A., Jakana, J., Prevelige Jr, P. E., King, J., & Chiu, W. (2000). Visualization of the maturation transition in bacteriophage P22 by electron cryomicroscopy. *Journal of molecular biology*,*297*(3), 615-626.

Zinder, N. D., & Lederberg, J. (1952). Genetic exchange in Salmonella. *Journal of bacteriology*, *64*(5), 679.

Zupan, J. R., & Zambryski, P. (1995). Transfer of T-DNA from Agrobacterium to the plant cell. *Plant Physiology*, *107*(4), 1041.



APPENDIX – 1

MEDIA

Alkaline Peptone Water (APW)

Peptone	1 g
NaCl	0.17 M
Distilled water	100 mL

Dissolved the peptone and NaCl in distilled water and adjusted the pH to 8.5. Mixed well and autoclaved at 15 lbs pressure for 15 minutes and cooled to room temperature before use.

Nutrient Medium

Peptone	5 g
Sodium chloride	5 g
Beef extract	1 g
Yeast extract	2 g

Suspended 1.3 g of Nutrient Broth Medium (Himedia, India) in 100 mL of distilled water. Mixed well and autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55°C. When used as solid agar medium, 2.0 % agar (w/v) was 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

Suspended 25 grams of LB Broth medium (Himedia, India) in 1000 mL distilled water. The medium was heated to dissolve completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Final pH: 7.5±0.2.

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

Suspended 89.08 grams in 1000 mL distilled water. Heated to boiling to dissolve the medium completely. (Do not autoclave) Cooled to 50°C and poured into sterile petri plates.

MOF Medium (Marine Oxidation Fermentation Medium)

Casein enzymic hydrolysate	1 g
Yeast extract	0.1 g
Tris-hydroxymethylaminomethane	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	

Suspended 22.14 grams in 1000 mL distilled water. Heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooled to 55-60°C and aseptically added sterile dextrose solution (or other carbohydrate of choice) to a final concentration of 1%. Mixed well and dispensed into sterile test tubes.

APPENDIX – 2

Buffer systems in characterization of Bacteriophages

SM buffer

NaCl	-	5.8 g
$MgSO_4.7H_2O$	-	2.0 g
1 M Tris HCl (pH 7.5)	-	50 mL
2% gelatin	-	5.0 mL

Ingredients were dissolved and were made up to 1 litre with milliQ water and autoclaved at 15 lbs for 20 minutes and stored at 4°C until use.

Phosphate Buffered Saline (PBS)

NaCl	-	8.0 g
KCl	-	0.2 g
Na_2HPO_4	-	1.44 g
KH_2PO_4	-	0.24 g

Ingredients were dissolved in 800 mL of distilled water, pH adjusted to 7 with 1N HCl. The volume was made up to 1 litre with distilled water, autoclaved at 15lbs for 20 minutes and stored at room temperature until use.

Hydrochloric acid- potassium chloride buffer (pH 2)

Solution A: 0.2 M KCl
Solution B: 0.2 M HCl

Mixed 50 mLof solution A with 10.6 mLof solution B and made up to 200mL with distilled water.

Citrate buffer (pH 3 - 6)

Solution A: 0.1 M Citric acid

Solution B: 0.1 M sodium citrate

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 mL and filter sterilized.

Desired pH	Solution A (mL)	Solution B (mL)
3	46.5	3.5
4	33.0	17
5	20.5	29.5
6	9.5	41.5

Phosphate buffer (pH 7)

Solution A: 0.2 M NaH2PO4

Solution B: 0.2 M Na2HPO4

Mixed 39 mL of solution A with 61 mL of solution B and the volume was made up to 200mL with distilled water, followed by filter sterilization.

Tris (hydroxymethylamino methane buffer system (pH 8 and 9)

Solution A: 0.2 M Tris buffer

Solution B: 0.2 M HCl

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to make up volume to 200 mLand then filter sterilized.

Desired pH	Solution A (mL)	Solution B (mL)
8	50	26.8
9	50	5

Carbonate – bicarbonate buffer (pH 10 and 11)

Solution A: 0.2 M Na_2CO_3

Solution B: 0.2M $NaHCO_3$

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 mL and filter sterilized.

Desired pH	Solution A (mL)	Solution B (L
10	27.5	22.5
10.7	45.0	5

Sodium hydroxide - Potassium chloride buffer (pH 12 and 13)

Solution A: 0.2 M KCl

Solution B: 0.2M NaOH

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and then filter sterilized.

Desired pH	Solution A (mL	Solution B (mL)
12	50	12
13	50	132

APPENDIX – 3

Reagents for Molecular analysis

TE buffer

1M Tris-Cl	-	10 mL
500mM EDTA (pH 8.0)	-	2 mL

1M Tris-HCl

Tris base	-	60.57 g
Deionised water	-	500 mL

Adjusted to desired pH using concentrated HCl

0.5M EDTA

EDTA	-	18.6 g
Deionised water	-	100 mL

50X TAE Buffer

Tris base	-	121 g
Glacial acetic acid	-	28.6 mL
0.5M EDTA pH 8.0	-	50 mL

Deionised water added to make volume to 500 mL.

1X TAE Buffer

50X TAE buffer	-	10 mL
Deionised water	-	490 mL

10X TBE

Tris base	-	108g
Boric acid	-	55g
0.5M EDTA (pH 8.0)	-	20 mL

Deionised water added to make volume to 1000 mL.

0.5X TBE Buffer

10X TBE buffer	-	50 mL
Deionised water	-	1000 mL

3M Sodium acetate (pH 5.2, 7.0)

Sodium acetate.3H2O	-	408.3 g
Distilled water	-	800 mL

pH adjusted to 5.2 with glacial acetic acid. Dilute acetic acid was used to adjust the pH to 7.0. The volume was adjusted to 1 liter using distilled water and sterilized by autoclaving.

APPENDIX – 4

Polyacrylamide Gel Electrophoresis

Stock acrylamide solution (30:0.8)

Acrylamide (30%)	-	60.0 g
Bis-acrylamide (0.8%)	-	1.6 g
Distilled water	-	200.0 mL

Filtered through Whatman No. 1 filter paper and stored in amber colored bottle at 4°C.

Stacking gel buffer stock (0.5M Tris-HCl, pH 6.8)

Tris buffer	-	6 g in 40 mL distilled water

Titrated to pH 6.8 with 1M HCl (~ 48 mL) and made up to 100 mL with distilled water. Filtered in Whatman filter paper No.1 and stored at 4°C.

Resolving gel buffer stock (3M Tris-HCl, pH 8.8)

Tris buffer	-	36.3 g

Titrated to pH 8.8 with 1M HCl (~ 48 mL) and made up to 100 mL with distilled water. Filtered through Whatman No. 1 filter paper and stored at 4°C.

Reservoir buffer for SDS-PAGE (pH 8.3)

Tris buffer	-	3.0 g
Glycine	-	14.4 g
SDS	-	1.0 g

Dissolved and made up to 1L with distilled water. Prepared as 10X concentration and stored at 4°C.

Sample buffer for Reductive SDS-PAGE (1 X)

SDS-PAGE sample buffer (2X)	-	1.0 mL
50% Sucrose in distilled water	-	0.6 mL
Distilled water	-	0.4 mL

SDS-PAGE sample buffer (2X)

Tris-HCl (pH 6.8)	-	0.0625 M

Glycerol (optional)	-	10% (v/v)
SDS	-	2%
Dithiothreitol	-	0.1M
Bromophenol blue	-	0.01%

Sample buffer for Non-reductive SDS-PAGE(1X)

Sample buffer for Non-reductive		
SDS-PAGE (2X)	-	1.0 mL
50% Sucrose	-	0.4 mL
Distilled water	-	0.6 mL

Sample buffer for Non-reductive SDS-PAGE (2X)

Tris-HCl (pH 6.8)	-	0.0625 M
Glycerol (optional)	-	10% (v/v)
SDS	-	2%
Bromophenol blue	-	0.01%

SDS (10%) - 1 g in 10 mL distilled water

Sucrose (50%) - 5 g in 10 ml distilled water

Autoclaved at 121° C for 15 minutes and stored at 4°C until use.

Protein Markers for SDS-PAGE

Low molecular weight marker mix (BioRad, USA) was used. The standards were diluted at a ratio of 1: 20 in SDS reducing sample buffer and heated at 95^{o}C for 5 minutes. 5 µL of the marker was loaded on to the gel. The composition of the marker mix is as given below.

Components MW (daltons)

Phosphorylase b	-	97,000
Serum Albumin	-	66,200
Ovalbumin	-	45,000

Carbonic anhydrase	-	31,000
Trypsin inhibitor	-	21,500
Lysozyme	-	14,400

SDS Reducing Sample Buffer (prepared immediately before use)

ß-mercaptoethanol	-	25 µL
Stock Sample Buffer	-	475 µL

Stock Sample Buffer (stored at room temperature)

Distilled water	-	4.8 mL
0.5M Tris-HCl (pH 6.8)	-	1.2 mL
Glycerol	-	1.0 mL
10% (w/v) SDS	-	2.0 mL
0.1% (w/v) Bromophenol blue	-	0.5 mL

Silver staining

Fixing solution 1 – 50 mL methanol and 5 mL acetic acid in 45 mL water

Fixing soution 2 - 50 mL methanol in 50 mL water

Sensitizer - Sodium thiosulfate (20 mg/100 mL

Staining solution* - Silver nitrate (200 mg/100 mL)

Developing solution*

Sodium carbonate (anhydrous)	-	3 g/100 mL
Formaldehyde	-	25 µL/100 mL
Sodium EDTA solution	-	1.4 g/100 mL

* Mixed and prepared fresh before use.

APPENDIX – 4

Vibrio cholerae strains used for Host range studies

1. ALPVC1
2. ALPVC2
3. ALPVC3
4. ALPVC4
5. ALPVC5
6. ALPVC6
7. ALPVC7
8. ALPVC8
9. ALPVC9
10. ALPVC10
11. ALPVC11
12. ALPVC11
13. ALPVC12
14. ALPVC13
15. ALPVC14
16. ALPVC15
17. CO336
18. EKM1
19. EKM2
20. EKM4
21. EKM6
22. EKM8
23. EKM9
24. EKM10
25. EKM11
26. EKM12
27. EKM14
28. EKM16
29. MAK757
30. MUS2
31. MUS4
32. MUS5
33. MUS6
34. MUS7
35. MUS8
36. MUS9
37. MUS10
38. MUS11
39. MUS12
40. MUS14
41. MUS15
42. O139
43. P8
44. P10

LIST OF PUBLICATIONS

Full paper in peer-reviewed journals

1. **Linda Louis**, Jeena Augustine and Sarita G Bhat (2014). Induction of temperate vibriophage ΦKNM4 from the environmental non-O1 *Vibrio cholerae* by various biotic and abiotic agents. *International Journal of Scientific Research* **3(6)**: 24-26

2. **Linda Louis,** Siju M Varghese and Sarita. G. Bhat (2015). Clonal relationships among *Vibrio cholerae* isolates determined by Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR. Discourse **3(1)**: 13-19

3. Jeena Augustine, **Linda Louis**, Siju M Varghese, Sarita G Bhat and Archana Kishore (2012) Isolation and partial characterization of ΦSP-1, a *Salmonella* specific lytic phage from intestinal content of broiler chicken. *Journal of Basic Microbiology*, DOI 10.1002/jobm.201100319.

4. S. Shivashankar,V. Ravindra and **L. Louis** (2007) Biochemical changes in seed and mesocarp of mango (*Mangifera indica* L.) cv. 'Alphonso' and their significance during the development of spongy tissue. *Journal of Horticultural Science & Biotechnology* **82 (1)**: 35–40

Full paper in proceedings of national/international symposium/conferences/seminars

1. **Linda Louis**, Siju M Varghese and Sarita G Bhat (2014). Molecular characterization of environmental isolates of *Vibrio cholerae* with temperate phages using ERIC-PCR. Proceedings of 2 day UGC sponsored National seminar on "Translational biotechnology for a better tomorrow" organized by St. Peter's College, Kolenchery.

2. **Linda Louis**, Jeena Augustine and Sarita G Bhat (2011) "Prevalence of virulence genes among non-01 *Vibrio cholerae* isolated from marine environment". Proceedings of 2 day National symposium on "Emerging trends in Biotechnology" conducted by Department of Biotechnology, CUSAT, 1st and 2nd September, 2011. **ISBN number : 978-93-80095-30-1**

3. Jeena Augustine, **Linda Louis**, Mridula V G. and Sarita G Bhat (2011). *"Salmonella phages* as biocontrol agents to reduce Salmonella in experimentally contaminated chicken cuts" Proceedings. Proceedings of 2 day UGC sponsored two day national seminar on " Emerging trends in biopharmaceuticals" organized by St. Mary's College ,Thrissur.

4. **Linda Louis**, Jeena Augustine and Sarita G Bhat (2011). "Genetic diversity and virulence potential of environmental *Vibrio cholerae*" UGC sponsored seminar on "Advances of biotechnology in agriculture and medicine and the possible ethical issues" organized by St. Xavier's College for Women, Aluva.

Posters/ abstracts in national /international symposia

1. **Linda Louis**, Jeena Augustine and Sarita G Bhat (2009). Differential efficiency of induction of vibriophage (ΦALPVC3) encoding virulent genes in naturally occurring non o1 *Vibrio cholerae*. *In proceedings of vibrio 2010 conference.*
2. **Linda Louis**, Jeena Augustine and Sarita G Bhat (2009) "Isolation, purification and partial characterization of vibrio phages from mangrove ecosystem". Book of abstracts of MECOS 09, Cochin, Kerala. International symposium on Marine Ecosystems challenges and Opportunities, Cochin from 9-12 Feb, 2009

GENBANK SUBMISSIONS

1. GenBank Accession No KJ734981 *Vibrio cholerae* strain AR9 16S ribosomal RNA gene, partial sequence. **Linda,L**., Harisree,P.N., Jeena,A., Sarita,G.B. and Helvin,V
2. GenBank Accession No KJ734982 *Vibrio cholerae* strain KNM4 16S ribosomal RNA gene, partial sequence. **Linda,L**., Harisree,P.N., Jeena,A., Sarita,G.B. and Helvin,V.
3. GenBank Accession No KJ734983 *Vibrio cholerae* strain KNM12 16S ribosomal RNA gene, partial sequence. **Linda,L**., Harisree,P.N., Jeena,A., Sarita,G.B. and Helvin,V
4. GenBank Accession No KJ734984 *Vibrio cholerae* strain KNM15 16S ribosomal RNA gene, partial sequence. **Linda,L**., Harisree,P.N., Jeena,A., Sarita,G.B. and Helvin,V

5. GenBank Accession No KJ734985 *Vibrio cholerae* strain MVN15 16S ribosomal RNA gene, partial sequence. **Linda,L**., Harisree,P.N., Jeena,A., Sarita,G.B. and Helvin,V
6. GenBank Accession No KJ734986 *Vibrio cholerae* strain MVN7 16S ribosomal RNA gene, partial sequence. **Linda,L**., Harisree,P.N., Jeena,A., Sarita,G.B. and Helvin,V
7. GenBank Accession No KJ734987 *Vibrio cholerae* strain ALP(VC)3 toxin coregulated pilus major subunit (tcpA) gene, partial cds. **Linda,L**., Jeena,A., Sarita,G.B., Siju,M.V. and Vijaya Joseph,A.
8. GenBank Accession No. JQ936982.1 *Vibrio*sp. strain BTOS10 16S ribosomal RNA gene, partial sequence AlphonsaVijaya Joseph, **Linda,L**. and Sarita,B.G.
9. GenBank Accession No. JQ936983.1*Vibrio* sp. strain BTPF5 16S ribosomal RNA gene, partial sequence AlphonsaVijaya Joseph, **Linda,L**. and Sarita,B.G.
10. GenBank Accession No. JQ936984.1*Vibrio* sp. strain BTPR5 16S ribosomal RNA gene, partial sequence AlphonsaVijaya Joseph, **Linda,L**. and Sarita,B.G.
11. GenBank Accession No. JQ936985.1*Vibrio* sp. strain BTPS6 16S ribosomal RNA gene, partial sequence AlphonsaVijaya Joseph, **Linda,L**. and Sarita,B.G.
12. GenBank Accession No. HM030820 *Bacillus licheniformis* strain BTKM4 16S ribosomal RNA gene, partial sequence. Smitha, S., Helvin, V., **Linda, L**. and Sarita, B.G.

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Research Paper

Induction of Temperate Vibriophage Φ Knm4 From the Environmental Non O1 Vibrio Cholerae by Various Biotic and Abiotic Agents.

Biology

KEYWORDS : Vibrio cholerae, Vibriophage, Phage induction, Mitomycin

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ABSTRACT

Bacteriophages are viruses infecting bacterial hosts. Phages are metabolically inert in their extra-cellular form, reproducing only after infecting suitable host. While providing a valuable resource to the development of modern biotechnology, their ability to mobilize and transfer toxin genes in the environment is viewed with concern. Environmental samples were collected from mangrove ecosystems of Kannamally track off Cochin. Vibrios isolated from the water samples on TCBS plates and characterized using biochemical and molecular tools, were used as host for phage isolation by the enrichment technique. Prophage was induced by means of different biotic and abiotic agents viz. mitomycin C, nalidixic acid, NaCl, H2O2, ultraviolet radiation and temperature (60°C). The results presented in this report clearly indicate that different prophages may be induced with different efficiency and their development may be modulated by environmental factors.

Introduction

Vibriophages are viruses infecting the family Vibrionaceae. Phages serve as agents of gene transfer in the marine environment (Fuhrman, 1999). The production of temperate phages is dependent on the number of lysogenic bacteria and the presence of an inducing agent (Weinbauer and Suttle, 1996). Release of mature phage requires removal of the repressor, a process called induction. The SOS response due to bacterial DNA damage can lead to significantly higher levels of prophage induction (Ogg et al., 1978). One of the most effective and widely used inducing agents is mitomycin C. More than 40% of marine bacterial isolates contained inducible phages. Some of these phages are capable of changing various host characteristics (Canchaya et al., 2003). Present study compares efficiency of prophage induction and further lytic development of vibriophage Φ KNM4 from an environmental non O1 V. cholerae by various induction agents.

Materials and Methods:

Isolation and Identification of vibrios

Vibrio cholerae strain KNM4 was isolated from water samples collected from coastal mangrove ecosystems of Kannamally (09°52′43.3″N 76°15′50.6″E), off Cochin in Kerala, India. The serially diluted samples were plated onto Thiosulphate Citrate Bile salt Sucrose (TCBS) agar (HiMedia, Mumbai, India) plates by the spread plate method and incubated at 37°C for 24 hours. Isolated single colonies were picked, purified on nutrient agar (NA) plates and stored as 20% glycerol stock.

The isolate was characterized upto genus level as outlined by Buchanan and Gibbons (1974). Genomic DNA was isolated as described by Esteban et al. (1993). 16S rRNA gene (1.5kb) was amplified by PCR using the primer pair: 16SF5'AGTTTGATCCTGGCTCA3' and 16SR 5' ACGGCTACCTTGTTACGACTT 3' (Shivaji et al., 2000). The products were sequenced by Sanger's dideoxy method using ABI 3730 Excel (SciGenom Labs Pvt Ltd, Cochin, Kerala). Homology was analysed using BLAST software (http://www.ncbi.nlm.nih.gov/blast) and the identity of the sequences was established (Altschul et al., 1990).

Isolation of temperate phages

V. cholerae strain KMN4 was grown in Luria broth (LB) (HiMedia, Mumbai) at 37°C to get O.D600 =0.2. Mitomycin C (Sigma Chemical Co, USA) was added at 1µg/mL and incubated overnight at 37°C. The culture supernatant was sterilized by filtration through 0.22 µm membrane (Millipore, USA). The filtrate was used as putative phage lysate (Faruque et al., 1998).

The filtrates were tested for phages by soft agar overlay method (Adams, 1959). Tetrazolium staining was done to visualize the phages more clearly (Pattee, 1966).

Purification and partial characterization of phage

The phage was purified and concentrated using polyethylene glycol (PEG) 8000 as described by Boulanger (2009). Optimal MOI was determined according to Lu et al. (2003).

The phage DNA was extracted as described by Sambrook et al. (2000). The DNA was visualized by 1% agarose gel electrophoresis, stained by ethidium bromide (Sigma Chemical Co., USA). Bacteriophage coat proteins were analyzed by SDS- PAGE under denaturing conditions (Laemmli, 1970)

Prophage induction studies

Vibrio cholerae strain KNM4 at mid log phase was treated with following agents: 1 mg/mL mitomycin C (Sigma Chemical Co, USA), 0.2 mg/mL nalidixic acid (HiMedia, India), 200 mM NaCl and 3 mM H_2O_2, Ultraviolet radiation and temperature (60°C).

Analogous experiments but without addition of induction agents were performed. Relative phage titer is the ratio of phage titres in induced and non induced cultures.

Results:

Isolation and Identification of Vibrios

Vibrio- like 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.

Strain KNM4 was identified as non O1Vibrio cholerae by 16S rRNA gene partial sequence analysis. The sequence was submitted in Genbank and the accession number obtained as KJ734982.

Isolation of temperate phages

Phage induction of the environmental Vibrio cholerae strain KNM4 with Mitomycin C produced translucent plaques with bull's eye morphology. The vibriophage hence forth is referred as ΦKNM4.

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Purification and partial characterization of phage

The lysogenic phage ΦKNM4was purified by repeated plating. The optimal MOI of ΦKNM4 was four phages per bacterium. Protein profiling by denaturing SDS-PAGE and silver staining indicated that the phage ΦKNM4 had more than one kind of capsid protein (Fig 1). The results indicated that coat protein possessed 4 major proteins and many minor proteins of very low molecular weight. The four major bands were ~97 KDa, ~55KDa, ~40KDa and ~21 KDa. Phage DNA was isolated successfully and was found to be more than 21.2 kb (Fig 2).

Fig 1 and Fig 2 about here

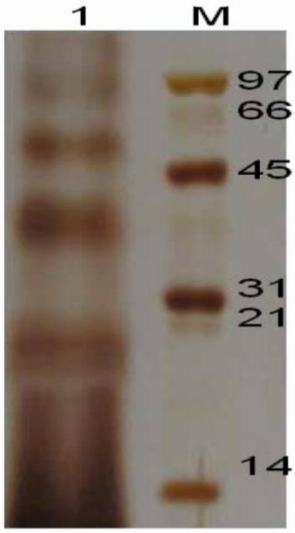


Fig 1.SDS-PAGE :Lane M-marker, Lane 1 -ΦKNM4

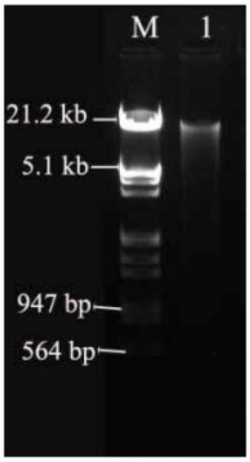


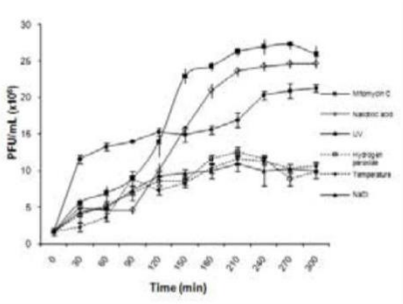
Fig 2: Agarose gel (1%) electrophoresis of phage DNA. Lane M-Lamba DNA/EcoR 1/Hind III Double Digest, Lane 1 - Phage DNA

Prophage induction studies

Efficiencies of induction of the prophages and their further development varied considerably in response to different inducing agents (Fig 3). Mitomycin was observed to be most efficient in induction of the lysogen under study. Nalidixic acid which was not reported earlier as an inducing agent, also proved to be a potent inducer. UV was also shown to induce phages significantly. Temperature (60°C), 200 mM NaCl and 3 mM H_2O_2 were not found to be effective in inducing the prophages. The induction of ΦKNM4 was highest in presence of antibiotics and lowest in 200 mM NaCl.

Fig 3 about here

Fig.3: Differential efficiency of induction of ΦKNM4 by various induction agents



Discussion:

Bacteriophages are natural viral pathogens of bacteria and coexist with their hosts in the same ecological niches (Fuhrman, 1999). V. cholerae is known to be primarily a human pathogen which can persist in the aquatic environment in unexplained ecological associations (Canchaya et al., 2003). The induction, isolation and characterization of the temperate phages in environmental Vibrio cholerae was part of the study on vibriophages to understand the causes of seasonal outbreaks of cholera along the west coast of Kerala, India. The bull's eye morphology of the plaques, not as clear as that of the typical lytic plaques is a unique character of the lysogenic plaques (Faruque et al., 1998).

Concentration and purification of viral particles are prerequisites for structural and functional characterization of phages. Concentration of phages was by PEG - NaCl precipitation as the efficiency of this method is independent of phage concentration (Boulanger 2009). The method allows a 100-fold phage concentration, even after low speed centrifugation with negligible loss of infectivity. Multiplicity of infection is the ratio of the phage particles to the infected bacteria. MOI giving maximum yield per infection is considered as the optimal MOI (Adams and Wassermann, 1956).

The induction studies with various inducing agents were a test of the biotic and abiotic factors which can cause changes in the bacterial genome. Any agent that can provoke the bacterial SOS response is a prophage induction agent (Kimmitt et al., 2000). The potential of nalidixic acid, a quinolone antibiotic as an induction agent is a novel report from our study. Since the antibiotics can challenge microbial populations they are considered as environmental pollutants. The use of mitomycin as induction agent is well studied in various prophages (Mandal and Chatterjee, 1987). Prophage induction is possible in natural ecosystem due to abiotic factors. Release of mature phage requires removal of the repressor (Takeya et al., 1965). Phage induction in the absence of external factors is called spontaneous induction which implies expression of the prophage genes. This can be a major driving force in bacterial population dynamics (Bossi et al., 2003).

Conclusions

Lysogenic phages encoding virulence factors can convert their bacterial host from a non pathogenic strain to a virulent strain or a strain with increased virulence. The presence of inducible lysogenic phages in environmental isolates of V. cholera is viewed with concern for their ability to mobilize and transfer toxin genes in the environment.

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REFERENCE

1) Adams MH (1959): Bacteriophages. N. Y. Wiley- Interscience. | 2) Adams, M. H., & Wassermann, F. E. (1956). Frequency distribution of phage release in the one-step growth experiment. Virology, 2(1), 96-108. | 3) 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. | 4) Bossi, L., Fuentes, J. A., Mora, G., & Figueroa-Bossi, N. (2003). Prophage contribution to bacterial population dynamics. Journal of bacteriology, 185(21), 6467-6471. | 5) Boulanger, P. (2009). Purification of bacteriophages and SDS-PAGE analysis of phage structural proteins from ghost particles. In Bacteriophages (pp. 227-238). Humana Press. | 6) Buchanan, R. E., & Gibbons, N. E. (1974). Bergey's Manual of Determinative Bacteriology 8ed. | 7) Canchaya, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M. L., & Brüssow, H. (2003). Phage as agents of lateral gene transfer. Current opinion in microbiology, 6(4), 417-424. | 8) 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. | 9) Faruque, S. M., Alim, A. A., Albert, M. J., Islam, K. N., & Mekalanos, J. J. (1998). Induction of the lysogenic phage encoding cholera toxin in naturally occurring strains of toxigenic Vibrio cholerae O1 and O139. Infection and immunity, 66(8), 3752-3757. | 10) Fuhrman, J. A. (1999). Marine viruses and their biogeochemical and ecological effects. Nature, 399(6736), 541-548. | 11) Kimmitt, P. T., Harwood, C. R., & Barer, M. R. (2000). Toxin gene expression by shiga toxin-producing Escherichia coli: the role of antibiotics and the bacterial SOS response. Emerging infectious diseases, 6(5), 458. | 12) Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259), 680-685. | 13) Lu, Z., Breidt Jr, F., Fleming, H. P., Altermann, E., & Klaenhammer, T. R. (2003). Isolation and characterization of a Lactobacillus plantarum bacteriophage, ΦJL-1, from a cucumber fermentation. International journal of food microbiology, 84(2), 225-235. | 14) Mandal, D., & Chatterjee, S. N. (1987). Mitomycin-induced prophage induction in Vibrio cholerae cells. Indian journal of biochemistry & biophysics, 24(6), 305-307. | 15) Ogg, J. E., Shrestha, M. B., & Poudayl, L. (1978). Phage-induced changes in Vibrio cholerae: serotype and biotype conversions. Infection and immunity, 19(1), 231-238. | 16) Pattee, P. A. (1966). Use of tetrazolium for improved resolution of bacteriophage plaques. Journal of bacteriology, 92(3), 787 | 17) Sambrook, J., Fritsch, E. F., & Maniatis, T. (2000). Molecular cloning (Vol. 2, pp. 14-9). New York: Cold spring harbor laboratory press. | 18) 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. | 19) Weinbauer, M. G., & Suttle, C. A. (1996). Potential significance of lysogeny to bacteriophage production and bacterial mortality in coastal waters of the Gulf of Mexico. Applied and environmental microbiology, 62(12), 4374-4380. |