Vibrio harveyi phages: Isolation, characterization and evaluation of their potential as phage therapeutics on *Vibrio harveyi* in shrimp hatcheries

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Under the Faculty of Environmental Studies

by

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Certificate

This is to certify that research work presented in the thesis entitled **"*Vibrio harveyi phages*: Isolation, characterization and evaluation of their potential as phage therapeutics on *Vibrio harveyi* in shrimp hatcheries"** is based on the original work done by Mrs. Surekha Mol I.S. under my guidance, at the National Centre for Aquatic Animal Health, School of Environmental Studies, Cochin University of Science and Technology, Cochin-682 016, in partial fulfillment of the requirements for the degree of Doctor of philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Cochin – 682 016
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Declaration

I hereby do declare that the work presented in this thesis entitled **"*Vibrio harveyi* phages: Isolation, characterization and evaluation of their potential as phage therapeutics on *Vibrio harveyi* in shrimp hatcheries"** is based on the original work done by me under the guidance of Dr. I. S. Bright Singh, Professor, School of Environmental Studies and Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin-682 016, in partial fulfillment of the requirements for the degree of Doctor of philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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General Introduction

Marine products satisfy the nutritional requirements of about three billion people globally and generate employment opportunities and turns out to be the livelihood for more than 500 million people directly or indirectly. During 2004 the global marine fish production, through culture and capture fisheries, was about 106 million tonnes and provided an apparent per capita supply of 16.6 kg (FAO, 2006). Accordingly marine environment turns out to be the vital component of global food security.

1.1 Aquaculture

Aquaculture, cultivation of aquatic animals and plants in controlled environments all through the life cycle or part of it (FAO, 1997; Gunawardhana, 2009) has been gaining momentum for more than three decades. Farming implies a level of intervention in the rearing process to enhance production, such as regulated stocking, feeding, protection from predators, management of the environment etc. It also implies individual or corporate ownership of the stock being cultivated. World aquaculture has grown significantly during the past half-century (FAO, 2006) and the consumption of aquaculture products has more than doubled since 1973 (Washington and Ababouch, 2011). About 76 percent of aquaculture products are absorbed by developed nations of which European Union's share is 41 percent and the United States of America and Japan together account for 27.2 percent of the total. China plays a significant role as both exporter and importer (FAO, 2009).

Increasing demand for fish and seafood has been met by a robust increase in aquaculture production, with an estimated average annual growth rate of 8.5 percent in the period from 1990 to 2005. This represents an average annual

increase of 6.9 percent in quantity and 7.7 percent in value over reported figures for 2002 (Washington and Ababouch, 2011). As per another report of FAO (2006), production has been increasing from 1million tonnes to 59.4 million tonnes, from the early 1950s to 2004 with a value of US$ 70.3 billion. As a result, the contribution of aquaculture to fish food supply has increased significantly, reaching almost 47 percent in 2008 from a mere 8 percent in 1970. This trend is projected to continue, with the contribution of aquaculture to fish food supply, estimated to reach 60 percent by 2020 (Washington and Ababouch, 2011). In 2004, countries in Asia and Pacific region accounted for 91.5 percent of the production in quantity and 80.5 percent in value. Globally, China accounts for 69.6 percent of the total in quantity and 51.2 percent in value (FAO, 2006). As per FAO (2009), major producers and exporters of aquaculture products are none other than the developing nations. The top ten producers are China, India, Vietnam, Indonesia, Thailand, Bangladesh, Norway, Chile, Japan and Myanmar. Aquaculture production statistics of the year 2009 is shown in Table 1.1. Even though India's place in aquaculture production is second in the world the difference from that of China is huge.

Table1.1 Aquaculture production statistics of the year 2009. (FAO, 2009)

Country	**Production (Million Tonnes)**	**Country**	**Production (Million Tonnes)**
China	34.78	Bangladesh	1.06
India	3.79	Norway	0.96
Viet Nam	2.56	Chile	0.79
Indonesia	1.73	Japan	0.79
Thailand	1.4	Myanmar	0.78
Total = 48.64 Million Tonnes			

Major contribution of aquaculture production includes carp in freshwater and shrimps in brackish water. Among shrimps, *Penaeus monodon* dominates other species due to its worldwide demand (Kian *et al.*, 2004; FAO, 2009).

1.2 *Penaeus monodon*

Penaeus monodon is widely distributed throughout the Indo-Pacific region, ranging from South Africa to Southern Japan, and from Karachi to Northern New South Wales; it forms commercial fisheries only in the central part of the Indo-Pacific region (Rao, 2000). Shrimp farming has been practiced for more than a century for food and the livelihood of coastal people in the Asian countries, such as Indonesia, the Philippines, Taiwan, Province of China, Thailand and Viet Nam (FAO, 2010).

Total aquaculture production of *Penaeus monodon* increased gradually from 21,000 tonnes in 1981 to 200, 000 tonnes in 1988; then it sharply increased to nearly 500, 000 tonnes. Since then, production has been quite variable, ranging from as low as 480, 000 tonnes in 1997 to as high as 676, 000 tonnes in 2001 as shown in Fig. 1.1(Washington and Ababouch, 2011).

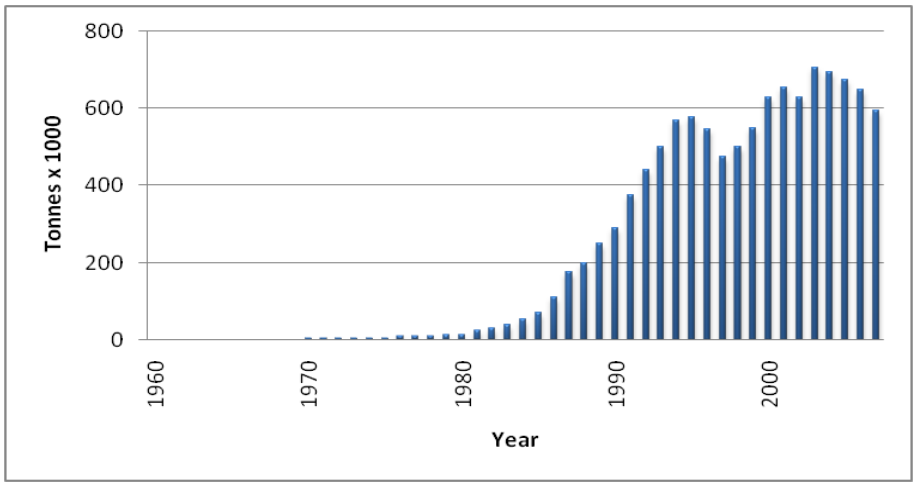


Fig. 1.1 Production of *Penaeus monodon.* (Washington and Ababouch, 2011)

Penaeus monodon forms an important marine fishery resource in India in view of its high demand in the export market. It forms the major component of the cultured prawn production in the country because of its fast growth, hardiness and high market price. This species is widely distributed in the Indian waters. It is cultured

in about one lakh hectares of prawn farms with an annual production of 82,850 tonnes. This species has been recorded in all the maritime states of India (Rao, 2000).

Penaeus monodon matures and breeds only in tropical marine habitats and spend their larval, juvenile, adolescent and sub-adult stages in coastal estuaries, lagoons or mangrove areas. In the wild, they show remarkable nocturnal activity, burrowing into bottom during the day and emerging at night to search for food as benthic feeders. Under natural conditions, the giant tiger prawn is more of a predator than an omnivorous scavenger or detritus feeder compared to other penaeid shrimps. The larval stages include, free swimming nauplii, protozoea which have feathery appendages and elongated bodies, mysis which have segmented bodies, eyestalk and tail characteristic of adult shrimp, and early postlarvae (PL) with similar characteristics to adult shrimp (FAO, 2010).

Fig. 1.2 *Penaeus monodon.*

The nauplius subsists on its own yolk and does not take any food. The protozoea feed on mixed diatom culture dominated by *Chaetoceros;* from mysis II onwards the larvae feed on *Artemia* nauplii until the larvae metamorphosed into the postlarvae. From postlarvae 1 to postlarvae 5 they depend either on *Artemia* nauplii

or egg and prawn custard or both as feed (Rao, 2000). The food of adult *Penaeus monodon* consists of crustaceans, molluscs, polychaetes, fishes and vegetable matter in the order of abundance. Crustaceans formed nearly 50% by volume of the stomach contents which consists of young penaeid prawns, mysids, isopods, crushed parts of prawns and shrimps which could not be identified and crab legs (Thomas, 1972).

1.3 Diseases in *Penaeus monodon*

Diseases are of great concern to aquaculture production and trade (Verschuere *et al.*, 2000) as they cause huge economic loss through mass mortality probably in the early stages, reduced growth and high cost of disease identification, prevention and treatment. The cultured organisms are affected by environmental fluctuations and management practices such as poor handling and transporting, reduced dissolved oxygen and excess ammonia via crowding, application of various drugs, under-nourishment, fluctuating temperature and pH, poor water quality, presence of toxic pollutants in water. All these factors can impose considerable stress on the homeostatic mechanisms of the animal rendering them susceptible to a wide variety of pathogens (Gunawardhana, 2009; Osman *et al.*, 2009). Supply of high quality brood stock is another major constraint in the production of good quality seed. Most hatcheries depend on wild stocks, whose supply and quality are variable (Kian *et al.*, 2004) which are the major source of diseases in shrimps. Among the aquaculture farmers every abnormality affecting the market value (mortality, loss of taste and loss of attractive appearance) is usually known as disease. Diseases are mainly caused by bacteria, fungi, viruses, macro parasites and non infectious agents (Lavilla-Pitogo *et al.*, 1998). Vibriosis caused by *Vibrio* spp. is the most prevalent bacterial disease. White spot Syndrome Virus (WSSV) belonging to the genus Whispovirus of the family Nimaviridae devastates the culture. Protozoan diseases include infections due to *Zoothamnium* sp., *Epistylis* sp., and *Vorticella* sp. etc. Non infectious agents may be nutritional diseases such

as shrimp scurvy, soft shell syndrome etc. or diseases caused due to environmental stress factors such as algal bloom, low dissolved oxygen, H_2S formation etc. (Ravichandran and Jalaluddin, 2003).

Opportunistic bacterial pathogens are present at low levels in most culture system under normal conditions. The diseases caused by them are of secondary nature, occurring as a result of other primary conditions including nutritional deficiency and extreme stresses (Vera *et al.*, 1992). Vibriosis is of that kind which is caused by luminescent vibrios mainly by *Vibrio harveyi*, the major pathogen behind mass mortality in shrimp hatcheries and farms (Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 2007).

1.4 Vibriosis

Vibriosis caused by *V. harveyi* is otherwise known as luminous bacteriosis (Karunasagar *et al.*, 2007) because the infected animals become luminescent in the dark (Gunawardhana, 2009). Vibriosis is caused by a number of *Vibrio* species including *Vibrio harveyi, Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio penaeicida and Vibrio splendidus* of which *Vibrio harveyi* is the major culprit (Rao, 1998). Vibriosis has been reported from throughout the shrimp farms of Asia and Latin America (Liu *et al.*, 1996; Oakey *et al.*, 2003). Mainly in countries such as Indonesia, Thailand (Ruangpan *et al.*, 1999), Australia, Philippines (Lavilla-Pitogo *et al.*, 1990) and Venezuela, vibriosis has been reported (Vidgen *et al.*, 2006). Vibriosis may be expressed in a number of ways such as oral and enteric vibriosis, appendage and cuticular vibriosis, localised vibriosis of wounds, shell disease, systemic vibriosis and septic hepatopancreatitis (Lightner, 1996).

Though most of the strains of *Vibrio* are opportunistic some are highly pathogenic to aquatic fauna, including penaeids (Oakey *et al.*, 2003), suggesting a great deal of

molecular and genetic variation. The pathogenic mechanism has been recently reported as bacteriophage mediated also (Rao, 1998). Exotoxins produced by *Vibrio harveyi* have been proved to be involved with diseases in shrimp larvae (Liu *et al.*, 1996; Harris and Owens, 2007).

1.5 *Vibrio harveyi* - Shrimp pathogen

Vibrio spp. are among the chitinoclastic bacteria, the causative agents of shell disease (Cook and Lofton, 1973) and are reported to enter the animal through wounds in the exoskeleton or pores (Alday-Sanz *et al.*, 2002) and are ingested with food (Lavilla-Pitogo *et al.*, 1990). They are responsible for five types of diseases such as tail necrosis, shell disease, red disease, loose shell syndrome (LSS) and white gut disease (WGD) in *Penaeus monodon*. This has been observed in the culture systems of all shrimp growing countries (Jayasree and Anakiram, 2006).

Vibrio harveyi is a waterborne bacterium, which is commonly found in tropical marine environments. The environmental parameters such as salinity, temperature and dissolved oxygen control its distribution and abundance. It is a rod shaped Gram-negative, luminous bacterium belonging to the family Vibrionaceae. *Vibrio harveyi* produces lateral flagella on solid media but, are motile by monotrichous or multitrichous polar flagella in liquid media. They are facultative anaerobs capable of both fermentative and respiratory metabolisms. Being chemoheterotroph, it depends on organic matter for energy and carbon. It also requires sodium ions for growth. Generally *Vibrio harveyi* emit blue-green bioluminescence catalysed by the enzyme luciferase. (Baumann and Schubert, 1984).

The primary source of *Vibrio harveyi* in hatcheries appears to be the midgut contents of female broodstock, which are shed during spawning. Juvenile shrimps are most susceptible to infection and it results in high mortality within short period. Concentration of *Vibrio harveyi* and period of exposure to the bacteria are important criteria of the onset of disease (Lavilla-Pitogo *et al.*, 1990). The infected

shrimp larvae show lack of appetite, sluggish swimming, reduced escape mechanism and lethargy. Lavilla-Pitogo *et al.* (1990) reported that moribund larvae settle to the bottom of the pond getting entrapped in pond sediment, often forming a luminescent mat.

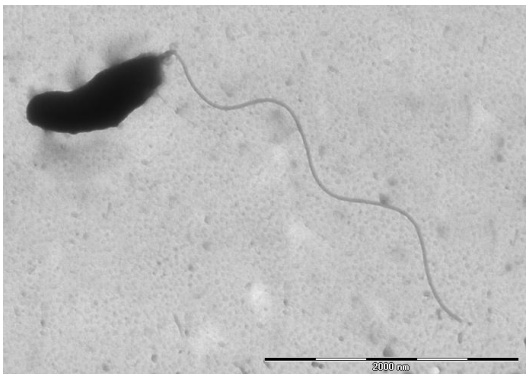


Fig. 1.3 *Vibrio harveyi* LB 19 used in the present study

Vibrio harveyi was originally named as *Achromobacter harveyi* (after E.N. Harvey, a pioneer in the systematics of bioluminescent bacteria (Johnson and Shunk, 1936). Subsequently at various stages, it has been named as *Lucibacterium harveyi*, and *Beneckea harveyi* arriving at its current taxonomic position as *V. harveyi* (Farmer *et al.*, 2005). *Vibrio trachuri* (Thompson *et al.*, 2002) and *Vibrio carchariae* (Grimes *et al.*, 1984) are other synonyms of *V. harveyi*. 16S rRNA sequence analysis revealed, *V. harveyi* and *Vibrio carchariae* as synonyms (Gauger and Gomez-Chiarri, 2002) and as one of the core species of the genus *Vibrio* (Dorsch *et al.*, 1992).

In addition to shrimps, *Vibrio harveyi* was found to cause disease in other organisms such as *Pinctada maxima* [Pearll Oyster] (Pass *et al.*, 1987); *Hippocampus kuda* [Sea horse] (Tendencia, 2004); phyllosoma larvae of *Jasus verreauxi* [Rock lobster] (Diggles *et al.*, 2000); in the larvae of *Holothuria scabra* [sea cucumber] (Becker *et al.*, 2004); *Chelonia mydas* [green turtles] (Work *et al.*, 2004) salmonids (Zhang and Austin, 2000); *Sciaenops ocellatus* [cultured red

drum] (Liu *et al.*, 2003); *Rachycentron canadum* [Farmed marine Cobia fish] (Liu *et al.*, 2004) etc. Hence *Vibrio harveyi* is considered as a serious aquaculture pathogen causing diseases in a variety of fishes and invertebrates.

1.6 Control of *Vibrio harveyi* in Aquaculture

Prevention, control and treatment of diseases are necessary for profitable aquaculture practice. Prevention is easier and cost effective than the treatment option (Mosig and Fallu, 2004). It has to be considered as essential during planning, site selection and establishment of aquaculture enterprises. Independent of high or low technology farming, good environmental conditions are important to maintain a healthy fish population (Sommerset *et al.*, 2005). Effective management is the best approach to avoid infections and diseases by addressing issues including water quality, water exchange, dissolved oxygen levels, balanced diet formulation and management of stress. Sanitation and disinfection are preventive measures for ensuring quality of sea water used for culturing as intake water happens to be the main source of pathogens (Lavilla-Pitogo *et al.*, 1990). Ozonization, filtration and ultraviolet irradiation are effective ways to reduce the level of pathogens in the intake water (Karunasagar *et al.*, 1994).

The most common practice in hatcheries to prevent bacterial infections is to have an elaborate water treatment system consisting of sand filters, cartridge filters and ultraviolet treatment. However, in spite of these water treatments, luminous bacteria are known to enter hatchery systems through brood stock (Lavilla-Pitogo *et al.*, 1990; Karunasagar *et al.*, 2007).

Recent studies on the application of vaccines revealed their potential to control bacterial and viral pathogens in Aquaculture (Sommerset *et al.*, 2005). According to Osman *et al.* (2009) vaccines, combined with good health management techniques, may result in substantial disease prevention and thereby profitable

production. Unlike antibiotics, vaccines stimulate the fish's immune system to produce antibodies that help and protect the fish from diseases. Even though vaccination is an effective method against bacterial infections, commercially available vaccines are very limited in the field of Aquaculture (Summers, 2001; Nakai and Park, 2002). Also it involves tough handling of animals especially with larger number of small animals (Gunawardhana, 2009) and found to be ineffective with invertebrates and early stages of vertebrates (Olafsen, 2001).

Although a wide range and number of chemo therapeutants have been developed and applied in aquaculture, chemo therapeutants for shrimp farming are limited. Though antibiotics are still used in some hatcheries, their application is banned or restricted due to severe negative side effects. Extreme use of antibiotics can cause several environmental disorders (contamination of the culture environment, damage to organisms, and creation of bacterial resistance that can be spread to the food chain (Kautsky *et al.*, 2000) as well as human health disorders (allergies, toxic effect, modification of the microflora of gastrointestinal tract and production of antimicrobial-resistant pathogenic bacteria) (Soltanian, 2007). More over the emergence of antibiotic resistant pathogens (Karunasagar *et al.*, 1994) and detection of antibiotic residues (Fiorentin *et al.*, 2004)) in shrimp tissues have led to the restriction of antibiotic treatment in aquaculture (Nakai and Park, 2002).

Studies on biological control such as probiotics have been reported in the field of fish disease management (Nogami and Maeda, 1992; Verschuere *et al.*, 2000). However, there involve substantial difficulties in scientific demonstration of the causal sequence, as mentioned in human use of probiotics (Tannock, 1999). In view of scientific demonstration, phage treatment can be definitively proven by confirming an increase in the number of phage particles or the presence of phages in the survivors, as a result of death of host bacterial cells. The feasibility of this

demonstration distinguishes phage treatment from other biological controls, which fail to utilize scientific methodology in demonstrating causal relationships (Nakai and Park, 2002). Under these circumstances, phages, as specific pathogen killers, could be attractive agents for controlling fish bacterial infections such as vibriosis (Park *et al.*, 2000; Vinod *et al.*, 2006).

1.7 Bacteriophages

Bacteriophages are viruses that infect bacteria and are widely distributed in the environment. Structure of bacteriophage includes a head, tail, tail fiber, and contractile sheath that are composed of protein as shown in Fig. 1.4. The nucleic acid can be either DNA or RNA linear or circular, double stranded or single stranded. The DNA molecule is packed in the head, which can be round or hexagonal. It needs a host cell for replication. Bacteriophage attaches on the surface of a host bacterial cell with their tails and then injects their nucleic acid in to the cell. If it is a lytic phage, the bacterial cells will produce a large number of copies of bacteriophage nucleic acid and the bacteriophage proteins. Following assembly of the protein and nucleic acid the bacterial cells lyse to produce mature bacteriophages and release the progenies in to the environment. They in turn attach to other bacterial cells. The bacteriophages are host specific (Ray, 1996).

Bacteriophages that lyse a variety of indigenous bacteria, including *Pseudomonas* spp., *Vibrio* spp., *Cytophaga* spp., *Agrobacterium* spp., *Photobacterium* spp., and various non marine bacteria particularly members of Enterobacteriaceae have been isolated from marine environment. Enrichments using seawater, sediments, fish and shellfish, have been successfully used as their source. (Baross *et al.*, 1978). Moreover, many of these bacteriophages are obligate marine phages that have a requirement for salt for infection and growth (Baross *et al.*, 1978).

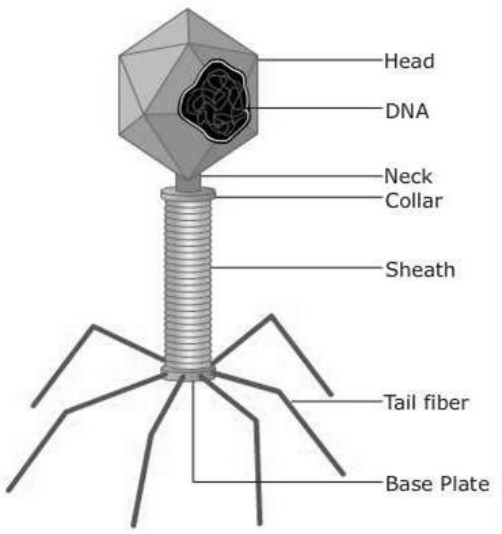


Fig. 1.4 Structure of bacteriophage. (Farabee, 2007)

1.8 Classification of Bacteriophages

There have been many attempts in classifying bacteriophages. The forerunner in phage classification was the great Australian Microbiologist, Sir Macfarlane Burnet, who could prove in 1937 that phages differed in size and resistance against physicochemical agents. In 1943, Ruska proposed a classification of viruses by electron microscopy (Ackermann, 2011). In 1948, Holmes classified viruses into three families based on host range and disease symptoms, of which phages belonged to the family Phagineae. Another approach was made by Adams (1953), who stated that bacteriophages should be classified outside any system used for other viruses, considering morphological and serological relationship. In 1962, Lwoff, Horne and Tournier stated that virus classification should be based on morphology and nucleic acid type and proposed a system with a latinised nomenclature (Lwoff *et al.*, 1962). Bradley (1965) favoured Adams' view because "it seemed much more logical that the major groups of viruses had separate origins." All events made, virus taxonomy a highly controversial subject.

Afterwards, a Provisional Committee on Nomenclature of Viruses (PCNV) was founded in 1965, later to become the International Committee on Taxonomy of Viruses (ICTV). The ICTV is the only international body concerned with virus taxonomy. In 1967, Bradley classified phages into six basic morphological types, corresponding to tailed phages (with contractile tails, long and non contractile tails, and short tails), small isometric single stranded DNA viruses, filamentous phages and small single stranded RNA phages (Bradley, 1967). This simple scheme, proposed by Bradley in 1967, is still the basis of the present edifice of phage classification (Calendar, 2006). This scheme was adopted by the ICTV. At that time, only 111 phages were known. In 1971, the International Committee on Taxonomy of Viruses (ICTV) classified phages into six genera corresponding to five of Bradley's basic types, namely the T4, λ, ΦX174, MS2, fd and newly described type PM2 (Wildy, 1971). This may be considered as the starting point of phage classification. In 1974, the tailed phages of the Bradley scheme were subdivided into morphotypes (Fig. 1.5), but this was purely for better identification by electron microscopy.

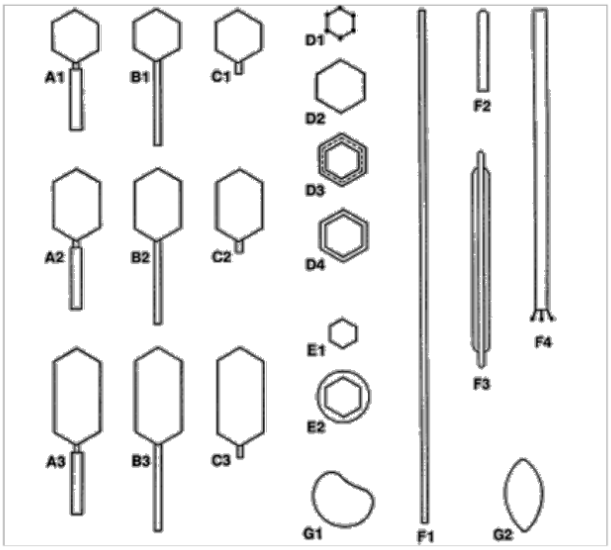


Fig. 1.5 Morphotypes of bacteriophages. (Ackermann, 2011)

Tailed phages constitute the order Caudovirales with three families, characterised by contractile, long and noncontractile, or short tails and named respectively Myoviridae, Siphoviridae, and Podoviridae. They represent over 96% of phages. Their heads are icosahedral or closely related bodies. The VIIIth ICTV Report includes 17 genera of tailed phages. The seven families of polyhedral, filamentous and pleomorphic families are separated by profound differences in nucleic acid content and structure (Ackermann, 2011).

1.8.1 Subdivision of Podoviridae and Myoviridae

The fully sequenced genomes of 55 Podoviridae and later 102 Myoviridae were compared and classified by the CoreGenes and CoreExtractor proteome techniques (Lavigne *et al.*, 2008; Lavigne *et al.*, 2009; Ackermann, 2011). Taxa were defined by the number of shared homologous/orthologous proteins. The results are summarised in Tables 1.2 and 1.3.

Table 1.2 Reclassification of Podoviridae phages. (Ackermann, 2011)

Subfamily	Genus	Example	Members	Host
Autographi-virinae	T7-like	T7	8	Enterics, *Pseudomonas, Vibrio*
	SP6-like	SP6	4	Enterics
	fKMV-like	fKMV	3	*Pseudomonas*
	P60-like	P60	3	*Prochlorococcus, Synechococcus*
Nanovirinae	Φ29	f29	4	*Bacillus*
	44AHJD	44AHJD	7	*Staphylococcus*
(P22-like)	P22-like	P22	7	Enterics
---	BPP-1-like	BPP1	4	*Bordetella, Burkholderia*
	e15-like	e15	2	Enterics
	N4-like	N4	1	Enterics
	119-like	119	2	*Pseudomonas*
	VP2-like	VP2	2	*Vibrio*

Table 1.3 Reclassification of Myoviridae phages. (Ackermann, 2011)

Sub family	Genus	Example	Members	Host
Teequatrovirinae	T4-like	T4	15	Enterics, *Aeromonas*
	KVP40-like	KVP40	5	*Aeromonas, Vibrio*
Peduovirinae	P2-like	P2	13	*Pseudomonas, Ralstonia*
	HP1-like	HP1	6	*Aeromonas, Haemophilus*
Spounavirinae	SPO1-like	SP01	1	*Bacillus*
---	Twort-like	Twort	7	*Staphylococcus, Listeria*
	Mu-like	Mu	2	Enterics
	P1-like	P1	2	Enterics
	Bcep781-like	Bcep781	5	*Burkholdera, Xanthomonas*
	BcepMu-like	BcepMu	2	*Burkholderia*
	Felix O1-like	Felix O1	3	Enterics
	HAP1-like	HAP1	2	*Halomonas, Vibrio*
	I3-like	I3	7	*Mycobacterium*
	ΦCD119-like	fCD119	3	*Clostridium*
	ΦKZ-like	fKZ	2	*Pseudomonas*
	PB1-like	PB1	7	*Pseudomonas*

Very large super groups of Podoviridae, T7 and f29 and of Myoviridae, P2, SPO1, and T4 were subdivided into subfamilies and many new "genera" were set up. In addition, both the Podoviridae and Myoviridae groups included some 20 viruses which, apparently stood alone, were unrelated to other phages, and represented

independent genera. Siphoviridae family has not yet been subdivided, but the attempts are going on (Ackermann, 2011).

Under podoviridae,T7-related phages and Φ29-related phages were subdivided into subfamilies named "*Autographivirinae*" and "*Nanovirinae*" respectively, which provided a straightforward tool for the molecular classification of new phage genomes (Lavigne *et al.*, 2008).

Three subfamilies were established under family myoviridae (*Peduovirinae*, *Teequatrovirinae*, the *Spounavirinae*) and eight new independent genera (Bcep781, BcepMu, FelixO1, HAP1, Bzx1, PB1, phiCD119, and phiKZ-like viruses). The *Peduovirinae* subfamily, derived from the P2-related phages, is composed of two distinct genera: the "P2-like viruses", and the "HP1-like viruses". At present, the more complex *Teequatrovirinae* subfamily has two genera, the "T4-like" and "KVP40-like viruses". The *Spounavirinae* contain the "SPO1-"and "Twortlike viruses" (Lavigne *et al.*, 2009).

1.9 Bacteriophage Replication Cycles

Phages dependent on their host bacteria for the production of progeny phages known as replication cycle (Madigan *et al.*, 2009). There exists a host density threshold for phage infection and replication, and that phage replication increases with host density (Weinbauer, 2004). Three main replication patterns occurring among bacteriophages are lytic cycle, lysogenic cycle and pseudolysogenic cycle (Weinbauer, 2004).

1.9.1 Lytic Cycle

The common steps in replication involve attachment to a specific receptor, penetration of phage nucleic acids into the host cell, synthesis of nucleic acid and

protein by cell metabolism, assembly of capsomeres, and release of mature virions from the cell (Madigan *et al.*, 2009). Phage infection involves three stages namely latent period, eclipse period and rise period. Burst size is the average number of virions released per infected host cell. Lysis occurs at the end of latent period and initiates the extracellular release of phage progenies (Abedon *et al.*, 2001). In tailed phages, the release is led by the action of an endolysin known as peptidoglycan hydrolase which break down peptidoglycan component of bacterial cell wall. Holin is another phage encoded enzyme which makes the plasma membrane permeable (Weinbauer, 2004). Abedon (1989) suggested that the rate of bacteria acquisition and killing by lytic phages should be favored by natural selection, particularly when bacteria are sufficiently large in number.

1.9.2 Lysogenic Cycle

In the lysogenic cycle, the genome of the (temperate or lysogenic) phage typically remains in the host in a dormant stage (prophage) and replicates along with the host, until the lytic cycle is induced by physical and chemical means which include UV radiation, temperature, pressure, mitomycin C, hydrogen peroxide or polyaromatic hydrocarbons (Williamson *et al.*, 2001; Weinbauer, 2004). A lysogenic decision whether or not to establish a prophage state is made by the temperate phage after infection with the suitable host bacteria (Weinbauer, 2004). It has been argued that lysogeny has evolved from lytic phages by co-adaptation as a result of interactions between phages and hosts. Lysogeny is a coincidental decision when the host abundance, i.e., the food resource, is below necessary to sustain lytic infection or when the destruction rate of free phages is too high to allow for lytic replication (Steward and Levin, 1984). Lysogeny is thought to occur with greater likelihood when phage multiplicities are greater than 1 (Wilson and Mann, 1997; Abedon *et al.*, 2001). According to Ackermann and DuBow (1987), establishing a lysogenic interaction always confers immunity to the lysogenic cell against infection with the same or related phage types.

1.9.3 Pseudolysogeny

Another frequent type of phage host interaction is termed as pseudolysogeny, in which the viral nucleic acid may remain within a host cell for some time, possibly for a few generations before lysis or cell destruction process. Pseudolysogeny may be related to host starvation, in which the virus adopts an inactive state, unable to initiate viral gene expression owing to the low energy state of the cell; normal viral activity returns when the cell is fed. Pseudolysogeny may be regarded as a transient state of host immunity, apparently induced by an immunizing agent (perhaps a polysaccharide depolymerase) released from infected cells, and helping to foster coexistence of host and virus (Fuhrman, 1999). Pseudolysogeny may be a chronic infection which could be seen as an intermediate evolutionary step (Weinbauer, 2004). One study found that ca. 45% of phages isolated with *Pseudomonas aeruginosa* as host were pseudolysogenic (Ogunseitan *et al.*, 1990).

1.10 Bacteriophage Therapy

Bacteriophages are viruses that only infect bacteria. It is a new breed of antimicrobials (Pirisi, 2000) that can be used for the prophylaxis and treatments of various bacterial infections. Bacteriophage therapy involves using phages or their products as bio-agents for the treatment or prophylaxis of bacterial infectious diseases (Matsuzaki *et al.*, 2005). Phage therapy is the recent development in the field of phage research. The emergence of multi-antibiotic resistant bacteria is one of the most critical problems of modern treatment methods. Prior to the discovery and widespread use of antibiotic, it was suggested that bacterial infections could be prevented and treated by the administration of bacteriophages. The ability of phages to replicate during the infection process make phages excellent potential diagnostic and therapeutic agents for fighting bacterial diseases (Mandeville *et al.*, 2003). The bacteriolytic activity of phages seems to be stronger than that of

bactericidal antibiotics such as vancomycin, oxacillin, and rifampicin (Matsuzaki *et al.*, 2005).

Phages are similar to antibiotics as they have remarkable antibacterial activity. All phages are specific some are even strain specific. They react to only their targeted bacterial host and not to human or other eukaryotic cells. For example, phages specific to *Vibrio cholerae,* always lyse *Vibrio cholerae* and will not lyse *Shigella, Salmonella* or *Escherichia coli.* This is a clear contrast to antibiotics, which target both pathogenic microorganisms and normal microflora.

1.11 Brief history of phage therapy

Bacteriophages were discovered independently by Frederick W. Twort in Great Britain in 1915 and Félix d'Hérelle in France in 1917 (Calendar, 2006). d'Hérelle coined the term bacteriophage, meaning "bacteria eater," to describe the agent's bacteriocidal ability. In 1922, he published a standard volume 'The Bacteriophage: Its Role in Immunity' with classical descriptions of different aspects of phages and their life cycles from where the research on bacteriophages took off worldwide. Between 1917 and 1956, around 800 publications were released in the area of medical applications of bacteriophages.

In 1921, it was reported for the first time that skin infections caused by *Staphylococcus* had been successfully treated with bacteriophages (Lorch, 1999). By the end of 1920s, France and the USA commercially produced phage preparations for a wide market. Meanwhile in the Soviet Union, the research on phage therapy had been flourishing. In 1923 the *Institute of Bacteriophage, Microbiology and Virology* was established in Tblisi, Georgia (ELIAVA, 2010).

During this period phage therapy was widely used to cure dysentery, typhus, paratyphus, cholera and infections of the urinal tract. The results of the treatment remained inconsistent because the trials were based more on euphoria than on

scientific knowledge of bacteriophages or microbiology. Phage therapy lost its popularity due to this uncontrolled administration (Alisky *et al.*, 1998; Barrow *et al.*, 1998). In 1931, the Council on Pharmacy and Chemistry of the American Medical Association came to the conclusion that "the use of bacteriophages in the treatment of infections is contradictory". This led to the reduced acceptance of phage therapy among the medical research community in the USA (Lorch, 1999). With the advent of new chemical antibiotics like penicillin, which became widely available in the 1940s, research on the potent but unpredictable phage therapy was abandoned by the western world (Hanlon, 2007).

1.12 Revival of phage therapy

During 1980's, there has been a renewed interest in phage therapy due to the increasing incidence of antibiotic-resistant bacteria and the lack of development of new types of antibiotics to control infections caused by these antibiotic-resistant organisms (Cerveny *et al.*, 2002). During this period a series of proven clinical usages of phages for drug-resistant infections in humans were described by Polish and Soviet groups (Slopek *et al.*, 1987; Alisky *et al.*, 1998). Thereafter, many successful results on phage therapy have been reported as given below:

1. Smith and colleagues used *Escherichia coli* models with mice and farm animals, to prove the potential of phages for the treatment and prophylaxis against bacterial infections (Smith and Huggins, 1982; Smith and Huggins, 1983; Smith *et al.*, 1987).

2. Soothill (1994) described the prophylactic application of bacteriophage BS2 lytic strain no. 37194 on the infection of split skin grafts by *Pseudomonas aeruginosa* 3719 in guinea-pigs.

3. A lytic bacteriophage (Barrow *et al.*, 1998), isolated from sewage was used to prevent septicemia and a meningitis-like infection in chickens caused by a $K1^+$ bacteremic strain of *Escherichia coli*. Protection was

obtained even when administration of the phage was delayed until the appearance of disease signs.

4. Alisky *et al.* (1998) evaluated Polish, British and Soviet modes of administration of phages and discovered that treatment of antibiotic resistant bacterial infections such as suppurative wound infections, gastroenteritis, sepsis, osteomyelitis, dermatitis, empyema and pneumonia, caused by pathogens such as *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Escherichia*, *Proteus*, *Pseudomonas*, *Shigella* and *Salmonella* species. In all cases phage therapy achieved 80-95% success.

5. In a report (Kudva *et al.*, 1999), virulent *Escherichia coli* O157 antigen-specific cocktail bacteriophages were isolated and tested to determine their ability to lyse laboratory cultures of *Escherichia coli* O157:H7 and could lyse the bacterial cells completely. These phages are promising to play a role in biocontrol of *E. coli* O157:H7 in animals and fresh foods without compromising the viability of other normal flora or food quality.

6. A bacteriophage, specific for *Clostridium difficile* was examined for its potential to prevent ileocecitis (Ramesh, 1999).

7. Burn wounds were found to be healed by phage therapy against staphylococci and streptococci infections (Lazareva *et al.*, 2001).

8. Bacteriophages were used to treat antibiotic-resistant infections in cancer patients and cure of infection was achieved in all cases indicating very high efficacy of phage therapy (Weber-Dabrowska *et al.*, 2001).

9. Ahmad (2002) reported 90% success in the treatment of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella pneumonia* infection using its specific phage. He also reported 80% success rate in phage therapy against *Enterococcus* infections.

10. Diseases due to Vancomycin-resistant *Enterococcus faecium* (VRE) which colonized the gastrointestinal tract could be treated successfully using phage treatment. The experiments conducted in mice could rescue 100% of the animals after 45 min of bacterial challenge (Biswas *et al.*, 2002).

11. Cerveny *et al.*, (2002) examined the potential use of bacteriophages as therapeutic agents against *V. vulnificus* in an iron dextran-treated mouse model. They found that two different phages were effective against three different *V. vulnificus* strains with various degree of virulence whereas a third phage that required the presence of seawater to lyse bacteria *in vitro* was ineffective in treating mice.

12. Human fatal sepsis by antibiotic resistant *Staphylococcus aureus* (O'Flaherty *et al*., 2005b), skin abscesses, caused by methicillin-resistant *Staphylococcus aureus* (Capparelli *et al*., 2007), infections caused by antibiotic resistant *Pseudomonas aeruginosa* (Heo *et al*., 2009), liver abscesses and bacteremia caused by multiple drug resistant *Klebsiella pneumonia* (Hung *et al*., 2011) etc., could be treated successfully by phage therapy.

13. Phage therapy using phage encoded products such as phage endolysins are also grouped under phage therapeutics. Murein hydrolases, the phage encoded lytic enzyme, used as therapeutics against infection caused by antibiotic resistant *Streptococcus pneumonia* was reported to bring rapid killing of the bacterial pathogen (Jado *et al.*, 2003; Lopez *et al.*, 2004). Some *Staphylococcus* phage lysins have been isolated and studied, including LysK, ClyS, MV-L, LysWMY, and ΦH5 etc. The novel lysin LysGH15 from staphylococcal phage, GH15 demonstrated strong lytic activity in rescuing bacteremia in a murine model of MRSA infection (Gu *et al.*, 2011).

Various animal models have been used to evaluate the efficiency of lytic phages against experimentally created bacterial infections. A bacteriophage, specific for

Clostridium difficile was proved to be efficient in a hamster model. Mice were used as models to evaluate phages like Vancomycin-Resistant *Enterococcus* phages (Biswas *et al.*, 2002), *Staphylococcus aureus* phages (Capparelli *et al.*, 2007), *Vibrio vulnificus* phages (Cerveny *et al.*, 2002) *Klebsiella pneumonia* phages (Hung *et al.*, 2011) etc. Chickens and calves were used as models for assessing the potential of lytic *Escherichia coli* phages (Barrow *et al.*, 1998). *Pseudomonas aeruginosa* phages were assessed for their biocontrol potential in mice and *Drosophila melanogaster* models (Heo *et al.*, 2009). Phages have also been used as successful therapeutics to prevent *Salmonella* infection in tomatoes (Flaherty *et al.*, 2000).

1.13 Phage Therapy in Aquaculture

Though bacteriophage therapy of other animal bacterial diseases has been the subject of countless studies, little attention has been given to phages as agents of therapy against bacterial diseases in aquaculture.

The protective effect of bacteriophages on experimental *Lactococcus garvieae* infection in Yellowtail fish *(Seliora quinqueradiata)* was reported by (Nakai *et al.*, 1999). Similarly oral administration of phage impregnated feed to Ayu fish (*Plecoglossus altivelis*) increased resistance to experimental infection with *Pseudomonas plecoglossicida* (Park *et al.*, 2000). Nakai and Park (2002) reviewed efficacy of phages on both the above pathogens, and also the potential of phages for controlling bacterial infections in aquaculture in general. Yuksel *et al.* (2001) reported that the fish pathogen *Piscirickettsia salmonis* showed the presence of phage particles attached to the cell wall and the phages appeared to eventually lyse the cells. *Aeromonas hydrophila* and *Edwardsiella tarda* are the two major pathogens of eel, *Anguilla japonica*. Control of these pathogens using bacteriophages were effective with a reduction of bacterial load by three order of magnitude within two hours (Hsu *et al.*, 2000).

In *Vibrio harveyi*, certain therapeutic phages have been reported. Vinod *et al.* (2006) introduced a novel bacteriophage in *Vibrio harveyi* having broad spectrum lytic potential. The phages reported by Shivu *et al.* (2007) were promising therapeutic agents for the control of *Vibrio harveyi.* Karunasagar *et al.* (2007) suggested that a cocktail of two Vibriophages could control the biofilms due to *Vibrio harveyi* in hatchery tanks and pipes. In the light of such successful trials in aquaculture, it can be concluded that phages, as specific pathogen killers, could be effective agents for controlling fish bacterial infections.

The potential advantages of phage treatment over other therapeutics (Smith and Huggins, 1982) are the following:

- Narrow host range of phages, indicating that the phages do not harm the normal intestinal microflora.
- Self-replicating nature of phages in the presence of susceptible bacteria, indicating the superfluousness of multiple administrations.

1.14 Challenges in Phage Therapy

Oakey and Owens (2000) reported that *V. harveyi* myovirus like bacteriophages (VHML) affected the virulence of *V. harveyi* ACMM # 642 and subsequently Munro *et al.* (2003) demonstrated that VHML bacteriophages were responsible for mediating virulence in *V. harveyi* ACMM # 642. This is due to the toxins coded by lysogenic conversion genes (LCG) or toxin genes present in the lysogenic bacteriophages. Therefore while selecting a phage system much care has to be given to obtain phages which are exclusively lytic but do not carry any virulent gene which can transform *Vibrio harveyi* more virulent. In the course of development of therapy care has to be given to two vital points.

1. The phages selected should be lytic and should not revert to lysogenic.

2. It should not carry any toxin gene which would make the host (*Vibrio harveyi*) more virulent to shrimp.

1.15 Lysogenic Conversion Genes/Toxin genes

Precisely, while phage can be used to treat bacterial infections they can also play a major role in bacterial pathogenesis. A number of phage genes have been discovered that encode toxins, or factors that enhance bacterial virulence. They may also contribute, through transduction to the transmission of antibiotic resistance genes. The horizontal transfer of virulence (toxin or toxin-enhancing protein or antibiotic resistance genes), has been characterized in several bacterial strains. Tailed phages are the most efficient gene-transfer entities developed in evolution. There is increasing evidence from bacterial pathogens that lysogeny is a cause of short term bacterial evolution. Temperate phages carry in their genomes extra genes that change the phenotype of the bacterial host [Lysogenic Conversion Genes (LCG)] (Canchaya *et al.*, 2003).

LCG were also identified in prophages from Gram-negative and Gram-positive bacteria (Canchaya *et al.*, 2003). Genes located in the lysogenic and early lytic operons of *Vibrio cholerae* temperate phage K139 (accessory replication genes, methyl transferase genes, exclusion and phage interference ORFs and other uncharacterized ORFs) seem to be totally unrelated or are highly divergent from each other (Kapfhammer *et al.*, 2002) which may likely to be adopted from temperate phage infection. To take examples from among Gram-positive bacteria, in phages of methicillin-resistant *S. aureus*, triple lysogenic conversion of enterotoxin A, staphylokinase and fl-lysin (Alisky *et al.*, 1998) do exist. Some of them are located at the prophage genome ends (Newton *et al.*, 2001; Allison *et al.*, 2002). Moreover, some of the LCG were shown to respond to environmental cues (Broudy *et al.*, 2001; Wagner and Waldor, 2002). Many bacteria were polylysogenic contained multiple prophages (Canchaya *et al.*, 2003).

The first virulence factor found encoded by bacteriophage genomes were bacterial exotoxins (Skurnik *et al.*, 2007). These compounds are among the most poisonous substances known and exotoxin production is the major pathogenic mechanism for several bacteria (Wagner and Waldor, 2002). Examples of such toxins are diphtheria toxin encoded by *Corynebcterium diphtheriae*-phage B (Freeman, 1951), botulinum toxin encoded by *Clostridium botulinum*-phage c-st (Sakaguchi *et al.*, 2005), cholera toxin encoded by *Vibrio cholerae*-phage CTXΦ (Waldor and Mekalanos, 1996) and shiga toxins encoded by several phages of enterohemorrhagic *Escherichia coli* strains (Plunkett *et al.*, 1999; Garcia-Aljaro *et al.*, 2006). Phospholipase A2 encoding slaA gene carried on by a phage genome enhances virulence of *Streptococci* (Sitkiewicz *et al.*, 2006). *Salmonella enterica* serovar Typhimurium offers a good example of phage-mediated evolution of pathogenic properties. In this species, temperate phages Gifsy-1, Gifsy-2, Gifsy-3, SopEΦ and Fels-1 are responsible for various virulence factors, such as superoxide dismutases, neuraminidase and multiple type III translocated effector proteins (Figueroa-Bossi *et al.*, 2001).

According to Oakey *et al.* (2002), the phenotypic changes occurred after lysogenic conversion of *V.harveyi* are the alteration of colony morphology, up-regulation of haemolysin, production of previously absent extracellular proteins (shown to be antigenically similar to the toxin components produced by *V. harveyi* 642) and also lethality to penaeid larvae. However, no conclusion could be made as to whether VHML transduced the toxin gene(s), as was the case with CTXΦ and cholera toxin (Waldor and Mekalanos, 1996) or whether the phage genome included a gene that up-regulated or altered existing chromosomal gene(s), increasing virulence in some manner.

Virulence mediated toxins of *Staphylococcus aureus, Bordetella avium, Pseudomonas aeroginosa, Shigella flexneri, Vibrio parahaemolyticus* are also

phage mediated (Calendar, 2006). The known phage-encoded toxin genes are summarized in Table 1.4 (Calendar, 2006).

It may be possible to reduce the occurrence of such adverse effects by sequencing the genome of phages of interest for therapeutic applications and select those phages which do not carry homologies with known toxin genes, islands of pathogenicity or genes that foster integration of DNA into the bacterial genome.

Tablse 1.4 Phages that carry toxin genes. (Calendar, 2006).

Bacteria	**Phage**	**Gene**	**Gene product/Phenotype**
Escherichia coli O157:H7	933,H-198	*Stx*	Shiga toxins
	ΦFC3208	*Hly2*	Enterohemolysin
	λ	*lom*	Serum resistance
	λ	*bor*	Host cell envelope protein
Shigella flexneri	Sfi6	*oac*	O-antigen acetylase
	sfII,sfV,sfX	*gtrII*	Glucosyl transferase
Salmonella enterica	SopEΦ	*sopE*	Type III effector
	Gifsy-2	*sodC-1*	Superoxide dismutase
	Gifsy-2	*nanH*	Neuraminidase
	Gifsy-1	*gipA*	Insertion element
	ϵ^{34}	*rfb*	Glusosylation
Pseudomonas aeruginosa	CTX Φ	*ctx*	Cytotoxin
Clostridium botulinum	C1	*C1*	Neurotoxi
Staphylococcus aureus	NA	*see,sel*	Enterotoxin
	Φ13	*entA, sak*	EnterotoxinA, staphylokinase
	TSST-1	*tst*	Toxic shock syndrome-1
Streptococcus pyrogenes	T12	*speA*	Erythrogenic toxin
Corynebacterium diphtheriae	β-phage	*tox*	Diphtheria toxin

1.16 Vibriophages

Chakrabarthi *et al.* (1993) described Vibriophage D 10, a myovirus with a 60 nm diameter head and a 100 nm tail. Kellogg *et al.* (1995) reported a myovirus vibriophage with head diameters up to 65 nm and tails up to 100 nm. Some reports of tailed vibriophages included that of Reidl and Mekalanos (1995) who obtained K 139, a myovirus with a 54 nm head and a 100 nm tail. Phages lytic to *V. vulnificus* were discovered in estuarine water samples collected from Lousiana (Pelon *et al.*, 1995). It has been found that a diverse group of *Vibrio vulnificus* phages are abundant in Gulf coast oysters from families Podoviridae and Myoviridae (DePaola *et al.*, 1998). According to Mathur *et al.* (2003) water from the Ganges (India) has been found to be a rich source of vibriophages. Filamentous phages have recently been reported in vibrios, particularly in *V.cholerae* (Waldor and Mekalanos, 1996; Jouravleva *et al.*, 1998). Matsuzaki *et al.* (1998) reported a broad spectrum vibriophage KVP40, a myovirus, with an elongated head of 140x70 nm. Nakasone *et al.* (1999) reported the presence of a filamentous phage (lvpf5) in *Vibrio parahaemolyticus* 03:K6 strain (LVP5) isolated from Laos.

1.17 *Vibrio harveyi* phages

A little attempt has been made to study about the therapeutic potential of *Vibrio harveyi* phages. Vinod *et al.* (2006) reported the bio control potential of *a* phage against *Vibrio harveyi* in comparison with antibiotic treatment. Shivu *et al.* (2007) characterized a number of broad spectrum phages and Karunasagar *et al*. (2007) proved the lytic efficiency of a phage and found application in removing biofilms caused due to *V. harveyi* in hatchery tanks. These are the only therapeutic *V. haryeyi* phages reported worldwide.

Ruangpan *et al*. (1999) observed a temperate bacteriophage in *P. monodon* tissues infected with *Vibio harveyi* (VH1039) and reported that the combined infection caused diseases in shrimp, while injection of either partner alone caused no

mortality. *Vibrio harveyi* 642 isolated from a shrimp rearing pond, Australia was found to contain the bacteriophage VHML (Oakey and Owens, 2000), a lysogenic bacteriophage which imparted more virulence to *Vibrio harveyi* 642. Pasharawipas *et al.* (2005) introduced a pseudolysogenic bacteriophage VHS1 of the family Siphoviridae, isolated together with *V. harveyi* (VH1114) from a black tiger shrimp cultivation pond in Thailand.

In the present study we tried to evaluate the therapeutic potential of a number of newly isolated *Vibrio harveyi* phages. The novelty of the current study includes primarily it's focus on diverse strains of *Vibrio harveyi* which may likely to be found in hatchery environment and accordingly, a phage cocktail has been developed with the phages having broad spectrum and diverse host range. The purpose is to control diverse strains of *V. harveyi* in hatcheries which may not be able to be identified at any point of time rapidly. During the course of the study a highly potent phage was accidentally found out which had the capability to lyse more than 70% of the *V. harveyi* isolates tested. Added advantage of the phage is that it did not allow resistant bacteria to emerge through out the experimental period. Other members of the cocktail were also broad spectrum not only against *Vibrio harveyi*, but also against other pathogenic bacteria such as vibrios and aeromonads confined to aquaculture.

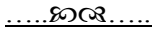
1.18 Objectives

A biological method of control for *Vibrio harveyi* is an absolute requirement for sustainable shrimp culture without environmental degradation. A suitable option is to develop a viable sustainable phage therapy. Accordingly a research programme was undertaken with the following objectives:

- Isolation, purification, screening and characterization of *Vibrio harveyi* phages for 'phage therapy'.

- Screening of *Vibrio harveyi* phages based on the presence of lysogenic/toxin genes and determination of phage infection properties.
- Evaluation of bio-control potential of *Vibrio harveyi* phages individually and in cocktail in *vitro* and *in vivo.*

Each of the objectives has been presented as each chapter in the thesis.



Chapter-2
Isolation, purification, screening and characterization of *Vibrio harveyi* phages for 'phage- therapy'

2.1 Introduction

Luminous *Vibrio harveyi*, the major pathogenic bacterium involved in the shrimp disease 'vibriosis', has been reported worldwide from aquaculture systems, mainly from shrimp farming areas where shrimp culture is a major industry (Harris and Owens, 1999; Ruangpan *et al.*, 1999; Oakey and Owens, 2000). As the infected larvae of shrimp emit blue-green luminescence, this disease is otherwise known as bacterial luminous disease or luminous bacteriosis (Karunasagar *et al.*, 2007). Even though *Vibrio harveyi* is a common aquaculture pathogen which causes diseases in a variety of aquatic animals, vibriosis in *Penaeus monodon*, the giant tiger shrimp, is of great concern as far as its high export value is concerned (Rao, 2000). The extensive use of antibiotics for eradication of *Vibrio harveyi* led to its ban in many sea food importing countries due to the presence of antibiotic residues in shrimp tissues and also the emergence of antibiotic resistant bacteria. Hence, the need of alternatives to antibiotics has gained considerable importance recently. From the very beginning of 20th century, among the alternatives, phages were proved to have excellent antibacterial properties. Even though, phages have lost its importance as therapeutics (Lorch, 1999) for a while, it has been rediscovered as an efficient and successful treatment option for those bacterial diseases associated with the aetiological agents having antibiotic resistance (Biswas *et al.*, 2002; O'Flaherty *et al.*, 2005a). Viruses are the most abundant biological entities and most marine viruses are bacteriophages (Fuhrman, 1999). Marine phages are very significant living entities as they not only maintain the bacterial density and

diversity, but also take a good part in influencing biogeochemical cycles and ecological nutrient cycling (Fuhrman, 1999; Suttle, 2007). As vibrios are the common microflora of the marine environment, vibriophages are diverse and abundant in the marine systems (Kellogg *et al.*, 1995) and hence studies of these diverse biological particles are relevant. Vibriophages, including phages of *Vibrio harveyi*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* etc., have been reported from several areas. *Vibrio harveyi* phages, both lytic and temperate, have been isolated from Australia, Thailand, and India (Oakey and Owens, 2000; Shivu *et al.*, 2007; Pasharawipas *et al.*, 2008). Ruangpan *et al*. (1999) first reported a temperate bacteriophage in *Vibrio harveyi,* which caused increase in bacterial virulence to *Penaeus monodon* by transferring a toxic gene to the host bacterium. Another temperate bacteriophage was isolated from a toxin producing *Vibrio harveyi* in Australia (Oakey and Owens, 2000) which was later found to increase virulence in avirulent strains of the same species (Austin *et al.*, 2003; Munro *et al.*, 2003). Pasharawipas *et al*. (2005) isolated and characterized another bacteriophage (VHS1) from tiger shrimp rearing pond in Thailand which showed pseusolysogeny in subsequent studies by Khemayan *et al.* (2006). Vinod *et al*. (2006) isolated a lytic bacteriophage which had broad spectrum biocontrol ability against *Vibrio harveyi*. They compared the lytic efficiency of bacteriophage with antibiotics and found to be very effective. Karunasagar *et al.* (2007) demonstrated the efficiency of *Vibrio harveyi* phages against biofilm formation of *Vibrio harveyi* in hatchery tanks.

2.1.1 Characterization

Characterization methods include total protein profiling (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), and nucleic acid-based methods such as whole genome sequencing, Ribotyping, Pulsed-Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD), Amplified-fragment length polymorphisms (AFLP) and Restriction Fragment Length Polymorphism (RFLP).

Some of the methods used elsewhere in phage characterization and the outcome are detailed below.

2.1.1.1 Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) is an inevitable technology in observing the morphological features of phages. Besides, TEM also has been used to examine the abundance and distribution of viruses or phages (Hara *et al.*, 1991).

2.1.1.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

This technique is a characterization method widely used to reveal the structural proteins of bacteriophages and can be used to compare the protein profiles of related and unrelated phage isolates. SDS-PAGE has been used to reveal the protein profile of individual (Phumkhachorn and Rattanachaikunsopon, 2010) and also group of *Vibrio harveyi* phages in diversity studies (Shivu *et al.*, 2007). Munro *et al*. (2003) used SDS-PAGE in order to study the protein expression of toxin in avirulent *Vibrio harveyi* strains as a result of infection with a toxin gene carrying temperate *Vibrio harveyi* phage, VHML.

2.1.1.3 Restriction Fragment Length Polymorphism (RFLP) Analyses

DNA restriction enzymes (Nathans and Smith, 1975) recognize specific sequences in DNA and catalyze endonucleolytic cleavages, yielding fragments of defined lengths. Restriction fragments may be displayed by electrophoresis in agarose gels, separating the fragments according to their molecular size. Differences among individuals in the lengths of a particular restriction fragment could result from genotypic differences: one or more individual bases could differ, resulting in loss of a cleavage site or formation of a new one; alternatively, insertion or deletion of blocks of DNA within a fragment could alter its size (Botstein *et al.*, 1980). These genotypic changes can all be recognized by the altered mobility of restriction fragments on agarose gel electrophoresis. Such a procedure would not require any

knowledge of the biochemical nature of the trait or of the nature of the alterations in the DNA responsible for the trait. No specific gene isolation is required (Botstein *et al.,* 1980). RFLP was first used as a tool for genetic analysis in 1974. Linkage of temperature-sensitive mutations of adenovirus to specific restriction fragment length differences was used to locate the mutations on a physical map of the restriction fragments (Grodzicker *et al.,* 1974). RFLP has widely been used in revealing genetic discriminations among bacteriophages (Shivu *et al.*,2007).

2.1.1.4 Random Amplified Poymorphic DNA (RAPD) profiling

Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited numbers of DNA markers. The utility of DNA-based markers is generally determined by the technology that used to reveal DNA-based polymorphism. Currently, the restriction fragment length polymorphism (RFLP) assay (Botstein *et al.,* 1980) has been the choice for many species to measure genetic diversity and construct a genetic linkage map. However, an RFLP assay which detects DNA polymorphism through restriction enzyme digestion, coupled with DNA hybridization is, in general, time consuming and laborious (Bardakci, 2001). The popularity of PCR is primarily due to its apparent simplicity and high probability of success. Unfortunately, because of the need for DNA sequence information, PCR assays are limited in their application. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes (Welsh and McClelland, 1990; Williams *et al.*, 1990). The simplicity and applicability of the Random Amplified Polymorphic DNA (RAPD) technique have captivated interests of many Scientists. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterization of the genome of the species in question (Bardakci, 2001). The standard RAPD technology (Williams *et al.*, 1990) utilizes

short synthetic decamer oligonucleotides of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR. Amplification products are generally separated on agarose gels and stained with ethidium bromide. Though species, subspecies and strain level discriminations (Johnson *et al.*, 1994) are possible by this technique, it is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous with a dominant RAPD marker (Williams *et al.*, 1990). RAPD markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with other methods (Bardakci, 2001). Limitations of RAPD include (1) the low reproducibility because of its high sensitivity to reaction conditions and Taq or DNA concentration and (2) primers of short length will amplify an unreasonably large number of sequences and that larger primers will amplify too few sequences to be routinely informative (Williams *et al.*, 1990; Hadrys *et al.*, 1992). RAPD profiling has been proved to be very effective in revealing intraspecific differentiation of wide variety of organisms like bacteria, phage and other organisms (Arias *et al.*, 1998; Pomper *et al.*, 1998; Shivu *et al.*, 2007). This technique has been used in diversity studies in discriminating strains *of Vibrio sp.* (Hernandez and Olmos, 2004; Maiti *et al.*, 2009). Apart from bacteria, it has been found application in characterizing phage isolates in determining genetic relatedness of six *Leuconostoc fallax* bacteriophages from California (Barrangou *et al.*, 2002), seven *Vibrio harveyi* phages from India (Shivu *et al.*, 2007) and related *Micromonas pusilla* viruses and *Chlorella* sp. viruses from Canada (Comeau *et al.*, 2006).

2.1.1.5 Pulsed-Field Gel Electrophoresis (PFGE)

Gel electrophoresis (Lai *et al.*, 1989) has found widespread use in biological

assays, and in the purification and separation of proteins and nucleic acids. DNA fragments from 100 to 200 base pairs (bp) up to 50 kilobase pairs (kb) are routinely separated by conventional gel electrophoresis techniques. Above 50 kb, because of the size of the molecules, the sieving action of the gel is lost, and fragments run as a broad, unresolved band with anomalously high mobility. In 1982, Schwartz *et al.* (1982) introduced the concept that DNA molecules larger than 50 kb can be separated by using two alternating electric fields (i.e. PFGE). The development of PFGE has increased by two orders of magnitude the size of DNA molecules that can be routinely fractionated and analyzed (Basim and Basim, 2001). Its range of application spans all organisms (Gardiner, 1991) from bacteria and viruses to mammals (Smith *et al.*, 1986). An important bonus of this technique is the ease with which the genome size can be measured, a parameter that was previously subjected to considerable error when measured by other techniques (Basim and Basim, 2001). One important outcome of the use of PFGE and restriction endonuclease digestion is the construction of a physical map. General applications of PFGE can be in the separation of whole chromosomes, the large scale restriction mapping of chromosome regions (Hernandez-Rivas *et al.*, 1997). PFGE and restriction endonuclease treatment is a molecular characterization method widely used in determination of genetic variability in phages (Schnabel and Jones, 2001; Chang *et al.*, 2005). This technique plays an important role in determining bacteriophage genome size (Talledo *et al.*, 2003; Seaman and Day, 2007), as conventional electrophoresis lacks the resolution necessary to distinguish most viral genomes (Steward and Azam, 1999). It also has been used to determine total community analysis of Chesapeake Bay virioplankton (Wommack *et al.*, 1999) and diversity of marine bacteriophage at a station of Pacific Ocean (Jiang *et al.*, 2003).

2.2 Materials and Methods

2.2.1 Bacterial isolates and Media

Eighty seven *Vibrio harveyi* isolates stocked at National Centre for Aquatic

Animal Health, Cochin University of Science and Technology, previously isolated from shrimp hatcheries of East and West coast of India during mass larval mortalities were used for the isolation of bacteriophages and for the determination of host range. The details of *Vibrio harveyi* isolates used in this study are given in Table 2.1. From among 33 phage isolates 6 were short listed having maximum host range. The respective host *V. harveyi* (6nos.) thus segregated were further used for subsequent studies. They included LB 6 (MCCB 153), LB 15 (MCCB 154), LB 19 (MCCB 155), LB 21 (MCCB 156), LB 32 (MCCB 157) and LB 68 (MCCB 158). The isolates identified based on phenotypic characterization following Alsina and Blanch (1994a, b) were confirmed of their identity based on sequence analyses of 16S rRNA gene. All the cultures were cryopreserved as 60% glycerol stocks at -80°C. Working cultures were maintained in ZoBell's marine agar slants (5 g peptone, 1 g yeast extract and 20 g Agar, 1000 mL 20 ppt sea water). *Vibrio harveyi* viable counts were determined using spread plate method in a selective medium of *Vibrio harveyi* known as *Vibrio harveyi* Agar (VHA) (Harris *et al.*, 1996) composed of D-cellobiose, 2 g., L-ornithine, 2 g., NaCl, 30 g., Tris[hydroxymethyl] aminomethane, 1.21 g., agar, 20 g., K_2HPO_4, 0.075 g., thymol blue, 0.04 g., bromothymol blue, 0.04 g., Bacto Peptone, 0.1 g., yeast extract, 0.1 g., and distilled water, 1,000 ml. The medium was heated to boiling, allowed to cool to 56 ^{0}C and pH adjusted to 9 by the addition of 1M NaOH. The medium was dispensed into sterile petri dishes and allowed to solidify to get an azure blue colored medium. TCBS agar was also used to compare the colony counts with that of VHA.

2.2.2 DNA extraction from host *Vibrio harveyi* isolates

To extract the DNA, a sample of 2 ml bacterial cell suspension (18 hr old bacterial cell suspension grown in Luria–Bertani broth) was centrifuged at 15,000 *g* for 10 min at 4°C. The pellet was collected and re-suspended in 500µL of TNE buffer (10mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.15 mM NaCl) and

centrifuged again at 15,000 *g* for 10 min at 4°C. Subsequently, the pellets were re-suspended in 500 µL lysis buffer (0.05 mM Tris-HCl, pH 8.0, 0.05 mM EDTA, 0.1 mM NaCl, 2%, SDS, 0.2 % PVP and 0.1% mercaptoethanol) (Lee *et al.*, 2003) and 10µL of proteinase K (20mg/ml) was added and incubated initially for 1 hr at 37°C and then for 2 h at 55°C. Further extraction was carried out by phenol-chloroform method (Sambrook and Russell, 2001). The sample was deproteinated by adding equal volume of phenol (Tris- equilibrated, pH 8.0), chloroform and isoamyl alcohol mixture (25:24:1). The phenol and the aqueous layers were separated by centrifugation at 15,000 *g* for 15 min at 4°C. The aqueous phase was carefully pipetted out into a fresh tube and the process was repeated once more. Following this, an equal volume of chloroform and isoamyl alcohol (24:1) mixture was added, mixed by gentle inversion and centrifuged at 15,000 *g* for 15 min at 4°C. The separated aqueous phase was then transferred to a fresh tube. Then the DNA was precipitated by incubation at -20°C overnight after adding equal volume of chilled absolute ethanol. The precipitated DNA was collected by centrifugation at 15,000 *g* for 15 min at 4°C and the pellet washed with 70% ice cold ethanol. Centrifugation was repeated once more and the supernatant decanted and the tubes left open until the pellet got dried. The DNA pellet was dissolved in 100µL MilliQ (Millipore) grade water. The isolated DNA was quantified spectrophotometrically (Abs_{260}) and the purity of DNA assessed by calculating the ratio of absorbance at 260 nm and 280 nm (Abs_{260}/Abs_{280}). Electrophoresis was done using 1% agarose gel.

Concentration of DNA (µg μL^{-1}) = $Abs_{260} \times 50 \times$dilution factor

2.2.3 PCR amplification of 16S rRNA gene of selected *V. harveyi* hosts

All the six *Vibrio harveyi* strains were subjected to 16S rRNA gene amplification. Amplification of 16S rRNA gene was carried out according to Reddy *et al*. (2000) using universal primers 16 S1 (GAG TTT GAT CCT GGC TCA) and 16 S2 (ACG

GCT ACC TTG TTA CGA CTT) using DNA thermal cycler (Eppendorf, Germany). The reaction mixture (final volume 25 µL) contained 2.5 µL 10 X buffer, 1 µL 10 pmol each of oligonucleotide primer, 1.5 µL DNA template, 2.5 µL 2.5 mM each deoxynucleoside triphosphate, 1 µL Taq polymerase, and the remaining volume made up with sterile Milli Q water. The amplification profile consisted of initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 94°C for 20s, annealing at 58°C for 30s and extension at 68°C for 2 min followed by a final extension at 68°C for 10 min. The PCR product was separated on 1 % agarose gel prepared in 1x TAE buffer and stained with ethidium bromide.

2.2.4 Gel purification of PCR products

Gel purification was carried out using GenEluteTM Gel Extraction kit (Sigma, USA). For purifying the gene products, the agarose gel that contained DNA fragments of appropriate size was excised and taken in a 1.5 mL tube, weighed and added 3 gel volumes (~450 µL) of gel solubilization solution and incubated at 60 °C for 10 min with repeated vortexing in every 2 min. After incubation, added 1 gel volume (~150 µL) of 100% isopropanol, mixed gently until it became homogenous. This solubilized gel solution was loaded into the binding column that was pre treated with column preparation solution, centrifuged at 12,000 x g for 1 min. Added 700 µL wash solution and centrifuged for 1 min at 12,000 x g, repeated the centrifugation, and residual wash solution was removed. The binding column was transferred to a fresh collection tube (2mL MCT) and added 50 µL of preheated (at 65 °C) 10 mM Tris-HCl (pH 9.0), centrifuged at 12,000 x g for 1 min, stored at -20 °C. The concentration of DNA was measured spectrophotometrically at 260/280 nm in a UV-VIS spectrometer (U2800, Hitachi, Japan). The purified PCR products of 16S rRNA gene were sequenced at SciGenom Labs Pvt. Ltd., Kochi, Kerala, India.

2.2.5 16S rRNA gene sequence similarity and Phylogenetic analysis

The sequences obtained were matched with Genbank database using the BLAST search algorithm (Altschul *et al.*, 1990). The 16S rRNA gene sequence of all the six isolates were multiple aligned using the ClustalW algorithm (Thompson *et al.*, 1994). A phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) using the software MEGA 5 (Tamura *et al.*, 2007).

2.2.6 Bacteriophage isolation

Vibrio harveyi bacteriophages were isolated from water and sediment samples collected from various shrimp farming ponds of Kerala. Phages were isolated following enrichment method (Cerveny *et al.*, 2002). This method involved two levels of enrichment, 1) Primary enrichment, followed by 2) Secondary enrichment.

The primary enrichment of bacteriophages was carried out by mixing 300 ml of water sample with autoclaved ZoBell's broth of 100 ml (5x strength). In the case of sediment samples, 400 ml sediment slurry was prepared by mixing 100g sediment with 200 ml estuarine water (water collected along with the sediment and 100 ml seawater of the same salinity. Then it was added to ZoBell's broth of 100 ml (5x strength). The samples were supplemented with 1 hr grown ten *Vibrio harveyi* isolates each of 10 ml volume at a time mixed together. Then the mixture was kept for overnight incubation at 28°C on a magnetic stirrer set at 120 rpm. The bacterial debris and mud were removed by centrifugation at 10,000 g for 15 min and the supernatant was filtered through GF/C membrane filter, followed by passing through PVDF membrane filters (Millipore) of 0.45µm and 0.22 µm pore-size. The resultant filtrate known as 'primary lysate' was stored at 4°C for further enrichment in individual *Vibrio harveyi* isolates.

During secondary enrichment, phage titre in the primary lysate was further

amplified by mixing the primary lysate with equal volume of overnight grown cultures of individual *Vibrio harveyi*. They were incubated on a shaking incubator at 28°C for 6-8 hrs until a visible clearance was observed. Then the cultures were centrifuged at 10,000 g and filtered as described above. For determining the presence of the phages, 10 µL each of the lysates were spot inoculated on respective bacterial lawn prepared by mixing 100 µL overnight grown broth culture with 4 mL molten soft agar and pouring in to plates on ZoBell's agar. The plaque formation after incubation confirmed the presence of phages in the lysate.

The bacteriophage titre in the lysates was determined by soft agar overlay method (Adams, 1959). Accordingly, 200 µL serially diluted phage lysates and 100 µL overnight grown corresponding host bacterial cultures were added together into an aliquot of 4 mL molten ZoBell's soft agar, mixed well and then poured onto the already prepared ZoBell's agar plates. Plates were kept for overnight incubation at 28°C. The dilutions which resulted in countable plaques (50-300) were chosen to determine the phage titres in the lysate. Plaques of *Vi ha* 21 in 7^{th} and 8^{th} serial dilutions on the bacterial lawn of *Vibrio harveyi* LB 21 are shown in Fig.2.1.a and 2.1.b respectively.

2.2.7 Purification of phages

For purification of bacteriophages, well isolated homogenous plaques were picked up with a sterile loop and dispensed into one hour grown respective host culture, incubated for 6 hrs under continuous agitation (120 rpm) on a shaker incubator at 28^0 C. The enriched content was centrifuged and filtered through 0.45 and 0.22 µm PVDF membrane filter as described earlier. The resultant filtrates were serially diluted and checked for the presence of plaques. All the above steps were repeated twice to result well purified homogenous lysates. Titres were determined, confluent plaques were harvested, labelled and stored at -80°C as 50% glycerol stocks and as such at 4°C.

2.2.8 Propagation of phages

Phage propagation was carried out as per the modified method described by Su *et al,* (1998). The plates with confluent lysis were selected and flooded with 5 mL of Salt-Magnesium (SM) buffer (50mM Tris HCl pH 7.5, 100mM NaCl, 10mM $MgSO_4$, 0.1% gelatin). The plates were placed on an orbital shaker to undergo continuous gentle agitation (60-80 rpm) for 6 hrs. The contents were decanted (without disturbing the bottom agar layer) and were then centrifuged and the supernatant filtered to get bacteria free phage lysates. The titres were determined by soft agar overlay method and the lysates were stored at 4°C and -80°C in 1 mL aliquots.

2.2.9 Host range Analyses

Host range analyses were conducted to segregate broad spectrum lytic phages out of 33 phage lysates generated. All the thirty three phages were allowed to infect the 87 isolates of *Vibrio harveyi* in the collection. The host range analyses were carried out by spotting 10 µL of purified phage lysate on respective bacterial hosts lawns prepared on ZoBell's agar plates. They were prepared in such a way that 100 µL overnight grown cultures of the *V. harveyi* were mixed with molten ZoBell's soft agar and plated on to the hard agar medium plates. After incubation at 28°C for 30 min, the lysates were spotted on to respective bacterial over lays. The presence of plaques after overnight incubation indicated the lytic efficiency of each phage lysate on the host bacterium. The plaques were categorized as very clear (glassy), clear and turbid. Based on the host range determination of 33 *Vibrio harveyi* phages against 87 isolates of *Vibrio harveyi*, 6 *Vibrio harveyi* phages which produced clear glassy plaques against maximum number of bacterial isolates were segregated as the 'highly broad spectrum phages'. Accordingly, a protocol was formulated to develop a phage cocktail which could be used for infecting

maximum number of *Vibrio harveyi* likely to be found in the hatchery environment.

2.2.10 Cross infectivity with other related bacteria

The segregated 6 *Vibrio harveyi* phages were examined for their infectivity on *Vibrio parahaemolyticus* VPa 6 (133), *Vibrio parahaemolyticus* V pa LMG, *Vibrio proteolyticus* V pr 4 (134), *Vibrio proteolyticus* V pr LMG, *Vibrio cholera* V c 23 (129), *Vibrio cholera* V c 26, *Vibrio cholera* V c 3906, *Vibrio splendidus* V sp 3 (135), *Vibrio splendidus* V sp LMG, *Vibrio alginolyticus* V al 3, *Vibrio alginolyticus* V al LMG, *Vibrio nereis* V Ne 30, *Vibrio nereis* V Ne LMG, *Vibrio mediterranei* V medi (131), *Vibrio mediterranei* V medi LMG, *Vibrio fluvialis* V f (130), *Vibrio fluvialis* V f LMG, *Vibrio vulnificus* V v LMG, *Aeromonas* sp. Ae TTB 1-6, *Aeromonas* sp. Ae TTB 2-2, *Aeromonas* sp. Ae CCL4, *Aeromonas* sp. Ae CCL6, *Aeromonas* sp. Ae G7, *Pseudomonas* sp. Ps 3, *Pseudomonas* sp. Ps 4, *Bacillus* MCCB101, *Micrococcus* sp. MCCB104 following the method described in section 2.2.9.

2.2.11 Cross infectivity to nitrifying bacterial consortia

Nitrification is a two step process, oxidation of ammonia to nitrite and nitrite to nitrate. Nitrifying bacterial consortia developed at National Centre for Aquatic Animal Health by Achuthan *et al*. (2006) were used to evaluate the impact of the segregated phages in nitrification and to assess whether phages could be safely applied in Recirculating Aquaculture System (RAS) integrated with nitrifying bioreactors. The concept is that as the phages are meant for the elimination of vibrios especially *Vibrio harveyi* in RAS they should not lead to the destruction of nitrifiers which could be gauged by assaying nitrification. The experiment was conducted as follows: Nitrifying bacterial consortium having the salinity optima of 15 ppt was inoculated in to mineral bases medium (composition : 10 ppm $(NH_4)_2 SO_4$ and 2 ppm $KH_2 PO_4$ in 15ppt seawater) in triplicate in 100ml aliquots in

250ml Erlenmeyer flasks . The cultures were supplemented with ammonium sulphate at a final concentration of 10ppm. The flasks were incubated under obscurity on a rotary shaker at 150 rpm and once in every 24hours the concentration of total ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were determined spectrophotometrically (Bendschneider and Robinson, 1952; Strickland and Parson, 1968; Solorzano, 1969). Ammonia-nitrogen was supplemented with its consumption till nitrite and nitrate were detected in each flask indicating stabilization of the culture. When both the conversions were stabilized in the culture, three flasks were inoculated with the cocktail of six bacteriophages and others were kept as control. Measurement of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were carried out on daily basis for 5 days in order to evaluate whether nitrification got disturbed in the presence of phages.

2.2.12 Characterization of phages

The six *Vibrio harveyi* phages selected based on the widest host range were further characterized employing Transmission Electron Microscopy (TEM), Random Amplified Polymorphic DNA (RAPD) finger printing and protein profiling by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.12.1 Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was used to characterize the phages morphologically. The purified phage lysates fixed with various fixatives such as 0.5% glutaraldehyde in 4% paraformaldehyde and Kernovsky's fixatives, were positively stained with 2 % uranyl acetate and viewed under TEM (Philips CM 10).

2.2.12.2 Random Amplified Polymorphic DNA (RAPD) Analyses

DNA extraction of six *Vibrio harveyi* phages were carried out and subjected to RAPD profiling.

2.2.12.2.1 DNA extraction of bacteriophages

Extraction of DNA of bacteriophages was carried out using the modified method of Su *et al*., (1998).

To the filtered phage lysates obtained from plates with confluent lysis, DNase I was added to a final concentration of 10 µg ml^{-1} and incubated at 37°C for 30 min to lyse any bacterial DNA. Filter sterilized 2 M ZnCl2 was added to the phage lysate at a ratio of 1:50 (v/v) and incubated at 37°C for 5 min. Then, the phage particles were pelleted (gray in colour) by centrifugation at 6000 *g* for 5 min at room temperature (28°C ± 1°C). The supernatant was discarded and the pellet dissolved in 700 µL TENS buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 0.3% Sodium dodecyl sulfate) per mL of phage lysate. Proteinase K was added to a final concentration of 100 µg ml^{-1} and incubated at 37°C for 2 hr followed by 65°C for 1 hr. The mixture was further deproteinated by extracting the solution with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) thrice. Then the DNA was precipitated by incubation at -20°C overnight after adding equal volume of chilled absolute ethanol. The precipitated DNA was collected by centrifugation at 15,000 *g* for 15 min at 4°C and the pellet washed with 70% ice cold ethanol. Centrifugation was repeated once more and the supernatant decanted and the tubes left open until the pellet got dried. The DNA pellet was dissolved in 100µL MilliQ (Millipore) grade water. The isolated DNA was quantified spectrophotometrically (Abs_{260}) and the purity of DNA assessed by calculating the ratio of absorbance at 260 nm and 280 nm (Abs_{260}/Abs_{280}). Electrophoresis was done using 1% agarose gel.

Concentration of DNA (µg μL^{-1}) = $Abs_{260}\times50\times$dilution factor

2.2.12.2.2 Nature of nucleic acid in phages

The extracted nucleic acid was digested with DNase free RNase (New England

Biolabs, USA) to determine whether the nucleic acid was DNA or RNA. Twenty U of the enzyme was used to digest 10 µg of the nucleic acid at 37°C for 1 hr, and the product was electrophoresed through 0.8% agarose gel. To determine whether the phage DNA was single/double stranded, 10 µg of DNA was incubated with 20 U of Moong Bean nuclease and 2 µL NEB buffer 1 (New England Biolabs, USA) at 37°C for 1 hr, and the resultant product was electrophoresed through 0.8% agarose gel.

2.2.12.2.3 RAPD profiling

RAPD profiling was carried out following the method described by Johansson *et al*. (1995) with a few modifications. Two decamer primers P-1 (5′-CCG CAG CCA A-3′) and P-2 (5′-AAC GGG CAG A-3′) reported by Shivu *et al*. (2007) were used in this study to amplify RAPD sequences. RAPD-PCR mixture contained 2.5 µL 10 X buffer, 2.0 mM each deoxynucleoside triphosphate, 1 µL 10 pmol each of decamer primer, 1 µL DNA template and 1 µL Taq polymerase and the remaining volume was made up with sterile Milli Q water to a final volume of 30 µL. DNA amplification was performed using a DNA thermal cycler (Eppendorf, Germany). Thermal cycler programmes were modified as given below: for primer (P-1): Initial denaturation of 94°C for 5 min; 35 cycles of 94°C for 20 S, 45°C for 30 S, 72°C for 1 min and a final extension of 7 min at 72°C: for primer (P-2): initial denaturation of 94°C for 3 min; 35 cycles of 94°C for 5 S, 36°C for 45 S, 72°C for 1.3 min and 5 min at 72°C. The amplified products were examined in 1.5% agarose gel electrophoresis (Amersham Bioscience GE Healthcare EPS 301). 1kb and 100 bp ladders (NEB, USA) were used as molecular size markers. The amplified products were analysed on 1.5% agarose gel electrophoresis carried out at a constant current of 60mA. Images of agarose gels were analysed and band size of random fragments were determined using the gel documentation system (Biorad Laboratories, Universal Hood II XR+, USA).

2.2.12.3 Protein profiling by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

2.2.12.3.1 Phage Purification

The harvested phage lysates were clarified by centrifugation at 8000 g for 15 min. The clarified supernatant was passed through 0.45 and 0.22 µm PVDF membrane filter. The phages in the supernatant were concentrated by polyethylene glycol (PEG) precipitation; to the filtered supernatant, NaCl and PEG 8000 were added at a concentration of 0.5 mol/L and 7% (w/v), respectively (Shukla *et al.*, 2009). When both were dissolved, the solution was centrifuged at 8000g for 30 min at 4°C. After removing the supernatant, phage pellet was dissolved in 1mL salt magnesium buffer (pH 7.5). For purification of the precipitated phages, discontinuous sucrose gradients (30% and 70%) were prepared in Salt Magnesium buffer (SM buffer). Initially, 1 mL of 70% sucrose solution was taken in a11.5 mL polyallomer tube in which 3 mL 30% sucrose solutionwas layered slowly avoiding intermixing of the layers. The 1 mL PEG pelleted phage suspension was carefully layered on to the top of the 30% sucrose cushion and the remaining volume was made up with SM buffer .Ultracentrifugation was carried out at 1, 00,000 g for 2 hr. The clear opaque band obtained at the interface was collected, dissolved in SM buffer and stored at 4°C.

2.2.12.3.2 SDS-PAGE

The profiles of phage structural proteins were analysed by SDS-PAGE. To 100 µL purified phage suspension obtained by sucrose gradient centrifugation, 5 µL reducing sample buffer was added and boiled for 5 min. The resultant solution was subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970) using 5% stacking gel and 15 % resolving gel at a constant current of 12mA using a gel electrophoresis unit (Amersham Bioscience GE Healthcare SE 660 & EPS 601). After electrophoresis,

gels were stained with 0.025 % Coomassie brilliant blue stain R-250 and then de-stained in a solution of 5% methanol and 7% acetic acid. Molecular weight of the unknown protein band was determined by comparing with the molecular weight standards (Bangalore Genei, India).

2.3 Results

2.3.1 Molecular Characterization of *Vibrio harveyi* as the host of phages

Good quality DNA (260/280 ratio ~1.8) was obtained ranging from 120-210 ng/µL.

The 16S rRNA genes of 6 *Vibrio harveyi* isolates were amplified as shown in Fig. 2.2. The 16S rRNA gene sequences were matched with Genbank *Vibrio harveyi* using the BLAST search algorithm. The sequences were submitted under the accession numbers JN990075 (MCCB 153-LB6), JN990076 (MCCB 154-LB 15), JQ920474 (MCCB 15-LB 19), JN990077 (MCCB 156-LB 21), JN990078 (MCCB 157-LB 32) and JQ920475 (MCCB 158-LB 68) (sequences are shown in Appendix Section 1.1–1.6). *Vibrio harveyi* MCCB 153, 154, 155, 156, 157 and 158 were showing 99, 100, 99, 99, 99 and 100% similarity to *V. harveyi* GenBank data base respectively. The phylogenetic tree constructed based on 16S rRNA is shown in Fig. 2.3.

2.3.2 Isolation of *Vibrio harveyi* phages

Altogether 33 *Vibrio harveyi* phages were isolated from 8 samples collected from different parts of Kerala. Sampling locations are shown in Fig. 2.4 and the details given in Table 2.2. Out of the 8 samples, 1 to 6 were water and 7 & 8 were sediment, collected from shrimp farms.

2.3.3 Host-range determination of the phages

Lytic potential of all the 33 phages are shown in the Table 2.3, as very clear glassy

plaques (+++), moderately clear plaques (++) and turbid plaques (+). Based on the spectrum of activity, *Vi ha* 32, *Vi ha* 21, *Vi ha* 15, *Vi ha* 68, *Vi ha* 6 and *Vi ha* 19 were segregated as the most potent phages among the group having a wide host range of more than 40%. (Phages were named as abbreviations of host name with code, *Vi ha* denotes *Vibrio harveyi*). The code number of phage lysates and percentage of *V. harveyi* isolates infected are given in Table 2.4.

2.3.4 Cross infectivity of phages

As far as cross infectivity is concerned, *Vi ha* 68 lysed 2 strains of *Vibrio parahaemolyticus*, 1 strain of *Vibrio proteolyticus*, 1 strain *of Vibrio cholera,* 2 strains of *Vibrio alginolyticus,* (in total 6 strains of *Vibrio* spp. other than *Vibrio harveyi*). *Vi ha* 32 lysed 1 strain of *Vibrio parahaemolyticus,* 1 strain of *Vibrio proteolyticus,* 1 strain of *Vibrio cholera,* 1 strain of *Vibrio alginolyticus* and 2 strains of *Aeromonas* spp.(Ae TTB 1-6, Ae G7) (In total 4 strains of *Vibrio* spp. and 2 strains of *Aeromonas* spp.). *Vi ha* 21 and *Vi ha* 19 lysed 1 strain of *Vibrio parahaemolyticus* and 1 strain of *Vibrio alginolyticus* (2 strains of *Vibrio* spp.). *Vi ha* 15 showed lytic potential against 1 strain of *Vibrio parahaemolyticus,* 1 strain of *Vibrio alginolyticus and 1* strain of *Aeromonas* sp. (2 strains of *Vibrio spp* and 1 strain of *Aeromonas* sp). *Vi ha* 6 lysed 1 strain of *Vibrio parahaemolyticus,* 1 strain of *Vibrio proteolyticus and* 1 strain of *Vibrio alginolyticus* (3 strains of *Vibrio* spp) other than *Vibrio harveyi*. At the same time they did not lyse *Bacillus* sp. (MCCB 101) and *Micrococcus* sp *(*MCCB 104) which are used as probiotics in aquaculture (Table 2.5).

2.3.5 Influence of phage cocktail on Nitrifying bacterial consortia

In the culture of nitrifying bacterial consortium NO_2 production was found to be stabilized on 4^{th} day and NO_3 on 5^{th} day of the experiment. Before and after addition of phage cocktail, the pattern of nitrification were the same in both test and control groups. The Fig. 2.5 and 2.6 represents setting up of nitrification by

nitrifying bacterial consortia and its stabilization over a period of 13 days. The cocktail phage lysate was added on 8th day which did not cause any negative impact on nitrification, which progressed exactly the same way as that of the control. From the figures it could be ascertained that both the conversions were not disturbed by the phage cocktail.

2.3.6 Characterization of phages

2.3.6.1 Transmission Electron Microscopy

Morphology of the phages was revealed by transmission electron microscopy (Fig 2.7.a-2.7.f). All of them were tailed and grouped under the order Caudovirales. Except *Vi ha* 68, all other phages had icosahedral head and contractile tail with defined base plates getting themselves grouped under the family Myoviridae. *Vi ha* 68 was having icosahedral head with long, flexible, non contractile tail and were accordingly placed under the family Siphoviridae. All myoviridae phages and the single siphoviridae phage *Vi ha* 68 had isometric head and hence were brought under A1 and B1 morphotypes respectively (Ackermann, 2011). The dimensions and features of the phages are given in the Table 2.6. Head diameter of phages ranged from 62.8 to 107.1 nm. The total length of the phages ranged from 175.61 to 284.16 nm. The largest phage with smallest head diameter is *Vi ha* 68. *Vi ha* 32 was the largest myoviridae member in the group. The transmission electron microscopy of narrow spectrum phage *Vi ha* 57 (Fig. 2.8) revealed a unique morphology having a long capsid (200 nm) and long and flexible tail with a total length of 376.3 nm. It can be proposed as a new morphotype in the bacteriophage classification.

2.3.6.2 Random Amplified Polymorphic DNA (RAPD) profiling

2.3.6.2.1 Nature of nucleic acid in phage

Digestion with RNase and Moog bean nuclease did not affect the phage nucleic

acids thus confirming that all the six phages in this study were double-stranded DNA phages.

2.3.6.2.2 RAPD

RAPD gel picture based on primer P-292 is shown in Fig.2.9. Upon comparing the RAPD pattern obtained in the respective dendrogram (Fig.2.10) clusters could be distinguished at ≥0.96r. Cluster-1 consists of *Vi ha* 6 & 32 which showed 97% S. This cluster joined at 96.5% to cluster-2 consisting of *Vi ha*19 & 21 which exhibited a correlation between themselves at 97.5%. Cluster-2 joined with cluster-3 at 96%, the highest correlation was exhibited by this cluster containing *Vi ha* 15 and *Vi ha* 68 at 0.98r.

The band pattern obtained by Primer P-293 is shown in Fig.2.11. In the dendrogram, three clusters were obtained of which Cluster-1 (*Vi ha* 6) joined with Clusters-2&3 at 0.89 & 0.86r respectively (Fig 2.12). Cluster-2 consisted of *Vi ha* 15, 19, 32 & 21 of which the highest similarity was exhibited by *Vi ha* 19 and *Vi ha* 32 at 0.96r. While *Vi ha* 15 and *Vi ha* 21 showed coefficient of similarity of about 0.93r. Cluster-3 was formed of single phage *Vi ha 68*, and the divergence pattern shown by this phage made its identification as the only siphoviridae member of the group. However, none of the phages examined with the primers used in this study could exhibit 100 % similarity amidst them or none showed a similarity coefficient of 1 based on the amplification pattern with both the primers. Hence it could be inferred that there existed divergence among phages based on the RAPD profiling using both the primers.

2.3.6.3 Protein profiling by Poly Acrylamide Gel Electrophoresis (PAGE)

To further characterize the phages, the structural protein composition was analyzed by SDS-PAGE as shown in the Fig. 2.13. Protein profile of the phages revealed that two major structural protein bands were common for all the five myoviridae

phages. One major band was found between 43 and 66 kDa and another between 66 and 97 kDa. The major component found between 43 and 66 kDa was the most abundant protein for *Vi ha* 6 and *Vi ha* 15. Besides, *Vi ha* 6 was found to have three extra major bands between 29 and 43 kDa. The least number of bands was found in *Vi ha* 68. Major bands of siphoviridae phage *Vi ha* 68 included two major bands between 29 and 43 kDa.

2.4 Discussion

Thirty three *Vibrio harveyi* phages isolated in this study formed a good representation of the State of Kerala, as the samples were collected from almost all major shrimp growing regions of the State. The enrichment method for the isolation was aimed at amplification of even low titre phages in the samples. From the 33 phages, six broad spectrum potent lytic phages were screened based on host range. Out of the 33, eight phages showed more than 40% lytic activity against 87 strains of *Vibrio harveyi* and a type strain. However 6 phages were selected for development of a cocktail, of which 5 phages could lyse more than 50% of the *V. harveyi* isolates. The phage *Vi ha* 19 showed more than 70% lytic efficiency which is the most potent phage brought out by this study. It was found to be a competitor to the phage cocktail in terms of survivability of test animals without any emergence of bacteriophage insensitive mutants (BIMs). On comparing the lytic activity, one of the phages of Shivu *et al.* (2007) showed 69% lytic activity. Karunasagar *et al*. (2007) presented 4 *Vibrio harveyi* phages, which showed 55-70% lysis against 100 *Vibrio harveyi* isolates. Vinod *et al*. (2006) reported a *Vibrio harveyi* phage which showed 100% lytic efficiency against 50 *V. harveyi* isolates.

For successful application of therapeutic phages in aquaculture system, it is necessary to assess whether the phages attack beneficial microbiota in the system. As per cross infectivity studies, nitrifying bacterial consortia used in Recirculating Aquaculture System (RAS) were proved not to be susceptible to cocktail phage

developed for the management of *Vibrio harveyi*. It is also proved that probiotic therapy can go hand in hand with phage therapy as the phages were not found to be infecting the probiotic bacteria such as *Bacillus cereus* sensu lato (MCCB 101) and *Micrococcus* sp. (MCCB 104). This entails phage therapy to control *Vibrio harveyi* in Recirculating Aquaculture System. Furthermore, all the six phages showed lytic potential to other vibrios and Aeromonads as well. Therefore it could be concluded that all the six phages used in this study were broad spectrum from the aquaculture point of view. However, none of the early reported *Vibrio harveyi* phages have been proved to have broad spectrum potential against other aquaculture pathogens.

All the six phages have been characterized morpholologically based on Transmission Electron Microscopy, and found to have unique structural features and dimensions. Among them five belong to family Myoviridae and one to Siphoviridae. This is contrary to Shivu *et al*. (2007) who observed that members of the family siphoviridae are more prevalent in aquaculture environment compared to myoviridae. However, it has been generalized that the therapeutic phages reported repeatedly belong to the members of either myoviridae or siphoviridae. Meanwhile, Park *et al*. (2000) reported a therapuetic phage belonging to podoviridae. All phages under myoviridae and the single siphoviridae phage *Vi ha* 68 had isometric head and hence were brought under A1 and B1morphotypes respectively (Ackermann (2011). Interestingly, the phage *Vi ha* 57 (which was not found to be therapeutic based on host range analyses) presented morphologically novel structure representing a new morphotype in the bacteriophage classification.

All the phages used in this study were dsDNA phages. RAPD profiling presented in this study resulted in unique and reproducible fingerprints generated from all the 6 *Vibrio harveyi* phages. In this case, two different clustering could be seen as a result of amplification with two decamer primers. When amplified with two different decamer primers, different patterns have been reported for *Vibrio harveyi*

phages by Shivu *et al.* (2007). According to Shivu *et al*. (2007), 2 phages belonged to their collection showed similarity between themselves not only based on RAPD pattern but also by protein profile and RFLP. Similarly, Barrangou *et al.* (2002) also presented RAPD profiling for six *Leuconostoc fallax* bacteriophages which showed phages of unique band pattern amplified with a primer and phages of similar pattern with another primer. They justified that bacteriophages with common morphotypes have common DNA bands. In this study, RAPD phage fingerprinting results suggested that even though all the phages were distinct from each other, some phages in the group showed significant phylogenetic relationship with one another. Comeau *et al.*(2004) demonstrated RAPD profiling in which genomes of *Micromonas pusilla* viruses and *Chlorella* sp. viruses were found to have closely related banding pattern. Subsequently, they ascertained that all strains were unique based on the relatedness of DNA pol gene sequence data where 77% similarity alone could be observed between the two *Chlorella* viruses, even though their DP-RAPD fingerprints were remarkably similar (98%). In the present study maximum similarity of 98% could be seen between *Vi ha* 15 and *Vi ha* 68 phages based on RAPD analyses using primer P-292. However, 100 % similarity with a similarity coefficient of 1 based on the amplification pattern was not observed with any of the primers.

Protein profiling by SDS-PAGE has been extensively used to discriminate bacteriphage isolates. Most of the phages in the present study showed the presence of 2 major bands probably head and tail proteins. However, *Vi ha* 6 was found to have 3 major bands, 2 of which were prominent. Meanwhile, *Vi ha* 19 showed a single major band. At the same time Phages *Vi ha* 6 and *Vi ha* 15 were found to have an array of minor bands apart from the major bands. T even phages were reported to have forty-two bands (Price and Rooyen, 2001) and in the case of giant 250 kb *Stenotrophomonas maltophilia* phage ΦSMA5 virion, which have 25 protein bands (Chang *et al.*, 2005) genome size of which was second to that of the

largest phage, ΦKZ of *Pseudomonas aeruginosa* (Mesyanzhinov *et al.*, 2002). Presence of a very few bands were also reported in a small phage *Burkholderia cepaciaphage* BcP15with short genome size of 11.9 kb (Hens *et al.*, 2005). In *Leuconostoc fallax* bacteriophages about 2-5 protein bands were detected in SDS-PAGE analyses (Barrangou *et al.*, 2002). Nasu *et al.* (2000) described about a filamentous phage of *Vibrio parahaemolyticus*, SDS PAGE of which revealed a single major 5 kDa protein band alone. However, in the present study, SDS-PAGE was found to be effective in discriminating the phages unlike the report of Shivu *et al*. (2007) where two *Vibrio harveyi* phages were showing quite identical pattern based on protein profiling and other molecular typing methods. According to Barrangou *et al*. (2002), the phages with similar protein profile might belong to same morphotype and hence SDS-PAGE could be used for identifying phage morphotypes. However, all the phages in this study are unique based on protein profiling. The minor bands present in the phages show variability among them. Accordingly, phages could be grouped into three, group 1: comprised of *Vi ha* 6 and *Vi ha* 15, group 2: included *Vi ha* 19, *Vi ha* 21 and *Vi ha* 32 and group 3: *Vi ha* 68 alone. This sort of grouping is found to be in agreement with the RAPD pattern revealed by Primer NP-293. In the case of RAPD profiling, *Vi ha* 6 was found to be another cluster which stood slightly distantly from rest of the myoviridae phages, but stood with *Vi ha* 15 in protein profiling existing as a group. It may be reasonably believed that some difference in the minor proteins found in this study are responsible for the diversity of host range, exhibited by these phages, as the same case was reported for T-even phages of *E. coli and Vibrio harveyi* phages (Hantke, 1978; Shivu *et al.*, 2007). However, no geographical resemblance among phages could be identified based on any of the molecular typing methods used in this study.

2.5 Conclusion

The therapeutic *Vibrio harveyi* phages presented in this chapter are very promising not only in managing *Vibrio harveyi* but also other aquaculture pathogens like vibrios and aeromonads based on host range and cross infectivity analyses. The phages are found to be refractory to lysis of nitrifying bacterial consortia and a few other beneficial bacteria in Aquaculture. All the six phages are found to be unique and distinct from each other based on Transmission Electron Microscopy, Random Amplified Polymorphic DNA analyses and Protein profiling, but show significant phylogenetic similarity

2.6 Tables

Table.2.1. *Vibrio harveyi* isolates used in the study and their sources

Code	Sample Type	Condition	Stage	Tank Details	Hatchery Location
LB1-LB 15	Rearing water	Mass Mortality	PL-10	-	Kakinada, Andhra Pradesh
LB 16-LB 33	PL	Moribund	PL-10	-	
LB 34	PL	Normal	PL-5	L-9	
LB 35-LB 40	Water	Drain out	-	-	
LB 41	Mysis	Normal	M-1	L-15	
LB 42-LB 51	Nauplii	Mass Mortality	N to Zoea	Quarantine Tank	
LB 52-LB 59	Raw Seawater	Intake	-	-	
LB 60-LB 62	Rearing Water	Normal	-	Crab Tank	
LB 63	Crab Carapace	Normal	-	Crab Tank	
LB 64-LB 70	Beach Sand	-	-	-	
LB 71-LB 85	PL	Mass Mortality	-	-	Azhikode, Kerala
LB 86-LB 87	PL	Mass Mortality	-	-	Kodungallore, Kerala

Table 2.2 Sampling points for the isolation of *Vibrio harveyi* phages

Sl No.	Location	No. of phages isolated	Sample type
1	Vypeen, Ernakulam	8	Aquaculture pond water
2	Kodungalloor,Thrissur	7	
3	Chellanam, Ernakulam	2	
4	Munrothuruth, Kollam	5	
5	Thiruvannur, Kozhikodu	3	
6	Vaikam, Kottayam	1	
7	Thuravur, Alapuzha	2	Aquaculture pond Sediment
8	Thrikaripur, Kasargod	5	

Table 2.3. Host Range of *Vibrio harveyi* phages
(First set of 11 phages versus 88 isolates of *Vibrio harveyi)*

Bacterial host	*Vi ha*										
	BL2	**BL4**	**BL5**	**BL6**	**BL8**	**BL9**	**BL11**	**BL12**	**BL13**	**BL15**	**BL16**
LB 1	-	-	-	-	-	-	-	-	-	-	-
LB 2	++	++	++	++	-	-	-	+++	-	-	-
LB 3	-	-	-	-	-	-	-	-	-	-	-
LB 4	-	-	-	-	-	-	-	-	-	-	-
LB 5	-	-	-	-	-	-	-	-	-	-	-
LB 6	+++	+++	+++	+++	-	-	-	+++	-	-	-
LB 7	+++	+++	+++	+++	-	-	-	+++	-	-	-
LB 8	+++	+++	+++	+++	-	-	-	+++	-	-	-
LB 9	-	-	-	-	-	-	-	-	-	-	-
LB 11	-	-	-	-	-	-	-	-	-	-	-
LB 12	+++	-	-	+++	-	-	-	+++	-	-	++
LB 13	-	-	-	-	-	-	-	-	-	-	-
LB 14	-	-	-	-	-	-	-	-	-	-	-
LB 15	+++	+++	+++	+++	-	-	-	+++	-	-	-
LB 16	-	-	-	-	-	-	-	-	-	-	-
LB 17	-	+	+	-	-	-	-	-	-	-	-
LB 18	-	-	-	-	-	-	-	-	-	-	-
LB 19	-	-	-	-	-	-	-	-	-	-	-
LB 20	+++	+++	+++	+++	-	-	-	+++	-	-	-
LB 21	+++	+++	+++	+++	-	-	-	+++	-	+	-
LB 22	-	-	-	-	-	-	-	-	-	-	-
LB 23	+++	-	+++	+++	-	-	-	+++	-	-	+++
LB 24	-	-	-	-	-	-	-	-	-	-	-
LB 25	+++	-	-	-	-	-	-	+++	-	-	-
LB 26	-	++	++	-	-	-	-	-	-	-	-
LB 28	++	++	++	++	-	-	-	++	-	++	-
LB 29	-	-	-	-	-	-	-	-	-	-	-
LB 30	-	-	-	-	-	-	-	-	-	-	-
LB 31	-	-	-	-	-	-	-	-	-	-	-
LB 32	+++	+++	+++	+++	-	-	-	+++	-	-	-
LB 33	+++	++	++	+++	-	-	-	+++	-	-	-

Bacterial host	*Vi ha*										
	BL2	**BL4**	**BL5**	**BL6**	**BL8**	**BL9**	**BL11**	**BL12**	**BL13**	**BL15**	**BL16**
LB 34	+++	+++	+++	+++	-	-	-	+++	-	-	-
LB 35	-	-	-	-	-	-	-	-	-	-	-
LB 36	-	-	-	-	-	-	-	-	-	-	-
LB 37	-	-	-	-	-	-	-	-	-	-	-
LB 38	-	-	-	-	-	-	-	-	-	-	-
LB 39	-	-	-	-	-	-	-	-	-	-	-
LB 40	-	-	-	-	-	-	-	-	-	-	+
LB 41	-	+	+	-	-	-	-	-	-	-	-
LB 42	-	+	-	-	-	-	-	-	+	-	-
LB 43	-	+	-	-	-	-	-	-	-	-	-
LB 44	-	-	-	-	-	-	-	-	-	-	-
LB 45	-	+	+	-	-	-	-	-	-	-	-
LB 46	-	+	+	-	-	-	-	-	-	-	-
LB 47	-	-	-	-	-	-	-	-	-	-	-
LB 48	-	+	+	-	-	-	-	-	-	-	-
LB 49	-	-	-	-	-	-	-	-	-	-	-
LB 50	-	-	-	-	-	-	-	-	-	-	+
LB 51	-	+	-	-	-	-	-	-	-	-	-
LB 52	-	+	+	+	-	+	-	-	+	+	-
LB 53	-	+	+	-	-	-	-	-	-	-	-
LB 54	+	-	+	-	-	-	-	+	-	-	-
LB 55	-	-	-	-	-	-	-	-	-	-	-
LB 56	-	+	+	-	-	-	++	-	-	-	-
LB 57	-	-	-	-	-	-	-	-	-	-	-
LB 58	-	-	-	-	-	-	-	-	-	-	-
LB 59	-	+	-	-	-	-	++	-	-	-	-
LB 60	-	-	-	-	-	-	-	-	-	-	-
LB 61	-	+	-	-	-	-	-	-	-	-	-
LB 62	-	++	++	+	++	-	++	-	-	-	-
LB 63	-	-	-	-	-	-	-	-	-	-	-
LB 64	-	-	-	-	-	-	-	-	-	-	-
LB 65	-	-	-	-	-	-	-	-	-	-	-
LB 66	-	-	-	-	-	-	-	-	-	-	-

Bacterial host	*Vi ha*										
	BL2	**BL4**	**BL5**	**BL6**	**BL8**	**BL9**	**BL11**	**BL12**	**BL13**	**BL15**	**BL16**
LB 67	-	-	-	-	-	-	-	-	-	-	-
LB 68	-	-	-	-	-	-	-	-	-	-	-
LB 69	-	-	-	-	-	-	-	-	-	-	-
LB 70	-	-	-	-	-	-	-	-	-	-	-
LB 71	-	-	-	-	-	-	-	-	-	-	-
LB 72	-	-	+	-	++	-	++	-	-	-	-
LB 73	+	+	+	-	-	-	-	-	-	-	-
LB 74	-	-	-	-	-	-	-	-	-	-	-
BL 2	-	-	-	-	-	-	-	-	-	-	-
BL 4	-	-	-	-	-	-	++	-	-	-	-
BL 5	-	+	+	-	-	-	-	-	-	-	-
BL 6	-	-	-	-	-	-	-	-	-	-	-
BL 7	-	+	+	-	-	-	-	-	+	-	-
BL 8	-	++	+	-	-	-	-	-	-	-	-
BL 9	-	+	+	-	++	-	++	-	+	-	-
BL 11	-	++	++	-	-	-	++	-	++	-	-
BL 12	-	-	-	-	-	-	-	-	-	-	-
BL 13	-	++	-	-	-	-	-	-	-	-	-
BL 14	-	+	-	-	-	-	-	-	-	-	-
BL 15	-	+	+	-	-	-	-	-	-	-	-
BL 16	-	-	-	-	-	-	-	-	-	-	-
BL 17	-	-	-	-	-	-	-	-	-	-	-
BL 18	-	-	-	-	-	-	-	-	-	-	++
BCCM LMG 4044 VH	-	-	-	-	-	-	-	-	-	-	-
Total (+ves)	**16**	**35**	**31**	**15**	**3**	**1**	**7**	**15**	**5**	**3**	**5**

Host range of second set of 11 phages versus 88 *Vibrio harveyi* isolates

Bacterial host	*Vi ha*										
	LB 2	LB 6	LB 7	LB 9	LB 15	LB 16	LB 17	LB 18	LB19	LB 21	LB 25
LB 1	-	++	-	-	-	-	-	-	+++	++	-
LB 2	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
LB 3	-	+	-	-	-	-	-	-	++	++	-
LB 4	-	+++	-	-	-	-	-	-	++	+	-
LB 5	-	+	-	-	-	-	-	-	++	-	-
LB 6	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
LB 7	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
LB 8	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
LB 9	-	-	-	-	++	-	-	-	++	-	-
LB 11	-	+	-	-	-	-	-	-	++	+	-
LB 12	++	+++	+	+	-	+++	+++	+	-	+	-
LB 13	-	++	-	-	-	-	-	-	+	+	-
LB 14	-	+++	-	-	-	-	-	-	+++	+++	-
LB 15	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
LB 16	++	-	-	-	-	-	-	-	-	-	-
LB 17	-	+	-	-	-	-	-	-	-	-	-
LB 18	-	+	-	-	-	-	-	-	+	-	-
LB 19	++	+++	-	-	-	-	-	-	+++	+++	-
LB 20	++	+++	+++	+	+++	+++	+++	+	+++	+++	+++
LB 21	-	+++	++	++	++	+++	+++	++	++	++	++
LB 22	-	+	-	-	-	-	-	-	+++	-	-
LB 23	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
LB 24	-	-	-	-	-	-	-	-	-	-	-
LB 25	-	++	-	++	++	+++	+++	-	++	+	-
LB 26	-	+	-	-	+	-	-	-	+	+	-
LB 28	+	+++	+++	+++	+++	++	++	+++	+++	+++	+++
LB 29	+	-	-	-	-	-	-	-	-	-	-
LB 30	-	++	-	-	-	-	-	-	+	+	-
LB 31	+	+++	+++	+++	+++	-	-	+++	+++	+++	+++
LB 32	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	-
LB 33	++	++	++	++	++	++	++	++	++	++	+
LB 34	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Bacterial host	*Vi ha*										
	LB 2	LB 6	LB 7	LB 9	LB 15	LB 16	LB 17	LB 18	LB19	LB 21	LB 25
LB 35	-	+	-	-	-	-	-	-	+	-	-
LB 36	-	-	-	-	-	-	-	-	++	-	-
LB 37	-	+++	-	-	-	-	-	-	++	++	-
LB 38	-	+	-	+	-	-	-	-	+	+	-
LB 39	-	-	-	-	-	-	-	-	-	-	-
LB 40	-	-	-	+	+	-	-	-	-	+	+
LB 41	-	-	-	-	+	-	-	-	+	-	-
LB 42	-	-	-	+	+	-	-	-	-	+	-
LB 43	-	+	-	-	-	-	-	++	-	-	-
LB 44	-	-	-	-	-	-	-	-	-	-	-
LB 45	-	-	-	-	++	-	-	-	++	-	-
LB 46	++	-	-	-	+	-	-	-	-	-	-
LB 47	-	-	-	-	-	-	-	-	+	-	-
LB 48	-	-	-	-	-	-	-	-	-	-	-
LB 49	-	+	-	+	-	-	-	-	++	-	-
LB 50	-	+	-	++	+++	-	-	+	-	++	+
LB 51	-	-	-	-	-	-	-	-	-	-	-
LB 52	-	+	-	+	+	+	+	-	+	+	-
LB 53	-	-	-	-	-	-	+++	-	-	-	-
LB 54	-	+	-	-	-	+	+	-	-	-	-
LB 55	-	-	-	-	+	-	-	+	-	-	-
LB 56	-	-	-	-	+	-	-	-	-	-	-
LB 57	-	-	-	-	-	-	-	-	-	-	-
LB 58	-	-	-	-	-	-	-	-	-	-	-
LB 59	-	+	-	-	+	-	-	-	+	-	-
LB 60	++	-	-	-	-	-	-	-	-	-	-
LB 61	-	+	-	-	+	-	-	-	+	-	+
LB 62	++	-	-	-	++	-	-	-	-	-	-
LB 63	++	-	-	-	-	-	-	-	-	-	-
LB 64	-	-	-	-	-	-	-	-	-	-	-
LB 65	++	+	-	-	-	-	-	-	++	-	-
LB 66	-	+	-	-	+	-	-	-	-	-	-
LB 67	++	-	-	-	-	-	-	-	++	+	-

Bacterial host	*Vi ha*										
	LB 2	LB 6	LB 7	LB 9	LB 15	LB 16	LB 17	LB 18	LB19	LB 21	LB 25
LB 68	-	++	-	+	+	-	-	-	++	+	-
LB 69	+	+++	-	-	-	-	-	-	+++	++	-
LB 70	-	+	-	-	+	-	-	-	+++	+	-
LB 71	++	+++	-	-	+	-	-	-	+++	++	-
LB 72	++	+++	-	-	+	-	-	-	+++	+	-
LB 73	++	+++	-	-	+	-	-	-	+++	++	-
LB 74	++	+++	-	-	+	-	-	-	+++	+	-
BL 2	-	+++	-	+++	+	-	-	-	+++	+++	-
BL 4	++	++	-	+	-	-	-	-	++	+	-
BL 5	-	-	-	-	-	-	-	-	++	-	-
BL 6	-	++	-	+	+	-	-	-	++	+	-
BL 7	-	+	-	-	++	-	-	-	+	-	-
BL 8	-	++	-	+	++	-	-	-	+	++	-
BL 9	++	++	-	-	-	-	-	-	++	-	-
BL 11	-	-	-	-	+	-	-	-	+	-	-
BL 12	-	++	-	-	-	-	-	-	++	-	-
BL 13	-	-	-	-	++	-	-	-	-	-	-
BL 14	-	++	-	-	+	-	-	-	++	-	-
BL 15	-	++	-	-	+	-	-	-	++	+	-
BL 16	-	+++	-	-	+	-	-	-	+++	-	-
BL 17	-	++	-	-	++	-	-	-	-	-	-
BL 18	-	-	++	++	++	-	-	++	++	++	++
BCCM LMG 4044 VH	-	-	-	-	-	-	-	-	-	-	-
Total (+ves)	**29**	**58**	**15**	**27**	**46**	**16**	**17**	**18**	**60**	**44**	**16**

Host range of third set of 11 phages versus 88 *Vibrio harveyi* isolates

Bacterial hosts	*Vi ha*										
	LB28	LB32	LB38	LB39	LB45	LB46	LB48	LB56	LB57	LB64	LB68
LB 1	+++	-	-	-	-	-	-	-	-	-	-
LB 2	+++	+++	++	++	++	++	-	++	-	++	++
LB 3	+	-	-	-	-	-	-	-	-	-	-
LB 4	+++	-	-	-	-	-	-	-	-	-	-
LB 5	++	-	-	-	-	-	-	-	-	-	-
LB 6	+++	+++	++	++	++	++	-	++	-	++	++
LB 7	+++	+++	++	++	++	++	-	++	-	++	++
LB 8	+++	+++	++	++	++	++	-	++	-	++	++
LB 9	-	-	-	-	-	-	+++	-	-	-	+++
LB 11	++	-	-	-	-	-	-	-	-	-	-
LB 12	+	+	++	++	++	++	-	++	-	++	++
LB 13	++	-	-	-	-	-	-	-	-	-	-
LB 14	+++	-	-	-	-	-	+	-	-	-	-
LB 15	+++	+++	++	++	++	++	-	++	-	++	++
LB 16	-	-	++	++	++	++	-	++	-	++	++
LB 17	-	-	-	-	-	-	-	-	-	-	++
LB 18	+	-	-	-	-	-	-	-	-	-	-
LB 19	+++	-	++	++	++	++	-	++	-	++	++
LB 20	+++	+	++	++	++	++	-	++	-	++	++
LB 21	+++	++	-	+	+	-	-	-	-	-	+++
LB 22	++	-	-	-	-	-	-	-	-	-	-
LB 23	+++	+++	++	++	++	++	-	++	-	-	++
LB 24	-	-	-	-	-	-	-	-	-	-	-
LB 25	+	++	-	-	-	-	-	-	-	-	+
LB 26	-	-	-	-	-	-	-	++	-	-	++
LB 28	+++	+++	++	++	-	-	-	+	-	-	++
LB 29	-	-	+	+	++	++	-	++	-	-	++
LB 30	-	+	-	-	-	-	-	-	-	-	-
LB 31	+++	+++	+	+	+	-	-	+	-	-	+
LB 32	+++	+++	++	+	+	+	-	+	-	-	++
LB 33	++	++	++	+++	++	++	-	++	-	-	+++
LB 34	+++	+++	++	++	++	++	-	++	-	++	++

Bacterial hosts	*Vi ha*										
	LB28	LB32	LB38	LB39	LB45	LB46	LB48	LB56	LB57	LB64	LB68
LB 35	+	-	-	-	-	-	-	-	-	-	-
LB 36	-	-	-	-	-	-	-	-	-	-	+
LB 37	+++	-	-	-	-	-	-	-	-	-	-
LB 38	-	+	-	-	-	-	-	-	-	-	++
LB 39	-	-	-	-	-	-	-	-	-	-	++
LB 40	-	+	-	-	-	-	+++	-	+++	-	-
LB 41	-	+	-	-	-	-	-	-	-	-	++
LB 42	-	+	-	-	-	-	-	-	-	-	+++
LB 43	-	-	-	-	-	-	-	-	-	-	+++
LB 44	-	-	-	-	-	-	-	-	-	-	++
LB 45	-	-	++	-	-	-	+++	-	+++	-	+++
LB 46	-	-	++	++	++	-	+	++	+	++	++
LB 47	-	+	-	-	-	-	+++	-	+++	-	-
LB 48	-	-	-	-	-	-	+++	-	+++	-	-
LB 49	-	+	-	-	-	-	+++	+	+++	-	-
LB 50	-	-	-	+	+	+	++	-	++	-	-
LB 51	-	-	-	-	-	-	+++	-	+++	-	-
LB 52	+	-	-	-	-	-	+++	-	+++	-	+
LB 53	-	-	-	-	-	-	-	-	+++	-	-
LB 54	-	++	-	-	-	-	+++	-	+++	-	+
LB 55	-	+	-	-	+	-	+++	-	+++	-	-
LB 56	-	-	-	-	-	-	+++	-	+++	-	++
LB 57	-	-	-	+	-	-	-	+	-	-	++
LB 58	-	-	-	-	-	-	-	-	-	-	+++
LB 59	-	+	-	-	-	-	+++	-	+++	-	-
LB 60	-	-	-	-	-	-	+++	-	+++	-	++
LB 61	-	+	-	-	-	-	+	-	++	-	-
LB 62	-	-	++	++	++	++	+++	++	+++	++	++
LB 63	-	-	++	++	++	++	-	++	-	-	+++
LB 64	-	-	-	-	-	-	-	-	+	-	++
LB 65	-	-	++	++	++	++	-	++	-	++	+++
LB 66	-	-	-	-	-	-	-	-	-	++	+++
LB 67	-	-	++	++	++	++	+	++	+++	++	+++

Bacterial hosts	*Vi ha*										
	LB28	**LB32**	**LB38**	**LB39**	**LB45**	**LB46**	**LB48**	**LB56**	**LB57**	**LB64**	**LB68**
LB 68	-	+	-	-	-	-	-	-	-	-	++
LB 69	+	+	+	+	++	-	+++	+	-	++	+++
LB 70	-	+	-	-	-	-	+++	-	-	-	+
LB 71	-	+	++	++	++	++	-	++	-	++	+++
LB 72	+	-	++	++	++	++	-	++	-	++	+++
LB 73	+	+	++	++	++	++	-	++	-	++	+++
LB 74	-	-	++	++	-	++	-	++	-	++	+++
BL 2	+	+++	+	-	-	+	-	-	-	+	+
BL 4	-	+	++	++	++	++	-	++	-	++	++
BL 5	-	-	-	-	-	-	-	-	-	-	++
BL 6	-	+	-	-	-	-	-	-	-	-	-
BL 7	-	-	-	-	-	-	-	-	-	-	+
BL 8	-	+	-	-	-	-	-	-	-	-	+
BL 9	-	-	++	++	++	++	-	++	-	++	++
BL 11	-	-	-	++	-	++	-	-	-	-	++
BL 12	-	-	-	-	-	-	-	-	-	-	-
BL 13	-	-	-	-	-	-	-	-	-	-	-
BL 14	+	+	-	-	-	-	-	-	-	-	-
BL 15	-	-	-	-	-	-	-	-	-	-	-
BL 16	+	+	-	-	-	-	-	-	-	-	+
BL 17	-	-	++	-	-	-	-	-	-	-	-
BL 18	++	++	-	-	-	-	-	-	-	-	++
BCCM LMG 4044 VH	-	-	-	-	-	-	-	-	-	-	-
Total (+ves)	35	38	31	32	29	27	21	31	19	23	58

Table 2.4. Phage lysates which have infected maximum number of isolates of *Vibrio harveyi* and the percentage of lysis

Phage isolates	**No of *V. harveyi* lysed**	**% lysis**
Vi ha LB 32	38	43.7
Vi ha LB 21	45	51.7
Vi ha LB 15	47	54.0
Vi ha LB 68	59	67.8
Vi ha LB 6	59	67.8
Vi ha LB 19	61	70.1

Table 2.5. Cross infectivity of segregated phages to other species of *Vibrio*, *Aeromonas* and probiotics.

Bacteria	***Vi ha***					
	6	**15**	**19**	**21**	**32**	**68**
Vibrio parahaemolyticus VPa 6 (133)	+++	++	+	+	+	+++
Vibrio parahaemolyticus V pa LMG	-	-	-	-	-	+++
Vibrio proteolyticus V pr 4 (134)	-	-	-	-	-	-
Vibrio proteolyticus V pr LMG	+	-	-	-	+	+
Vibrio cholera V c 23 (129)	-	-	-	-	-	-
Vibrio cholera V c 26	-	-	-	-	-	-
Vibrio cholera V c 3906	-	-	-	-	+	+
Vibrio splendidus V sp 3 (135)	-	-	-	-	-	-
Vibrio splendidus V sp LMG	-	-	-	-	-	-
Vibrio alginolyticus V al 3	+	+	+	+	+	+
Vibrio alginolyticus V al LMG	-	-	-	-	-	+++
Vibrio nereis V Ne 30	-	-	-	-	-	-
Vibrio nereis V Ne LMG	-	-	-	-	-	-
Vibrio mediterranei V medi (131)	-	-	-	-	-	-
Vibrio mediterranei V medi LMG	-	-	-	-	-	-

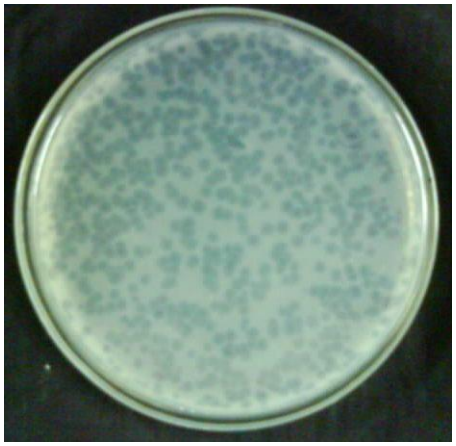
Continued…

Bacteria	*Vi ha*					
	6	**15**	**19**	**21**	**32**	**68**
V. fluvialis V f (130)	-	-	-	-	-	-
V. fluvialis V f LMG	-	-	-	-	-	-
Vibrio vulnificus V v LMG	-	-	-	-	-	-
Aeromonas sp. Ae TTB 1-6	-	-	-	-	++	-
Aeromonas sp. Ae TTB 2-2	-	++	-	-	-	-
Aeromonas sp. Ae CCL4	-	-	-	-	-	-
Aeromonas sp. Ae CCL6	-	-	-	-	-	-
Aeromonas sp. Ae G7	-	-	-	-	++	-
Pseudomonas sp. Ps 3	-	-	-	-	-	-
Pseudomonas sp. Ps 4	-	-	-	-	-	-
Bacillus sp. MCCB 101	-	-	-	-	-	-
Micrococcus sp. MCCB 104	-	-	-	-	-	-

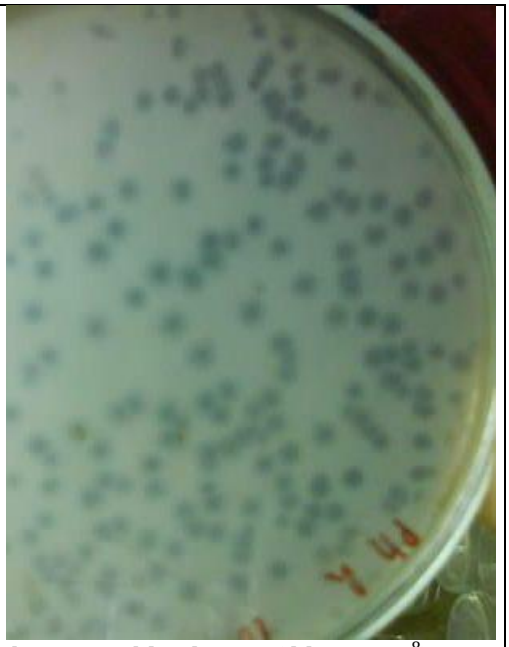
Table 2.6. Morphological features of phages revealed by transmission electron microscopy

Phages	**Characteristics**	**Morpho-type**	**Total Length (nm)**	**Head Diameter (nm)**	**Family**
Vi ha 6	Isometric head, contractile tail	A1	152.0	70.8	Myoviridae
Vi ha 15	Isometric head, contractile tail	A1	180.6	84.0	Myoviridae
Vi ha 19	Isometric head, contractile tail	A1	186.1	94.4	Myoviridae
Vi ha 21	Hexagonal head, contractile tail	A1	197.3	78.9	Myoviridae
Vi ha 32	Hexagonal head, contractile tail	A1	221.4	107.1	Myoviridae
Vi ha 68	Isometric head, non contractile tail	B1	277.1	62.8	Siphoviridae

2.7 Figures

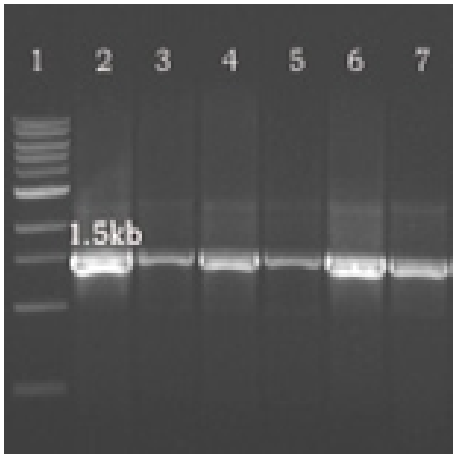


a. Confluent plaques (dilution 10^7)



b. Countable plaques (dilution 10^8)

Fig. 2.1Plaques formed by *Vi ha* 21on the bacterial lawn of *Vibrio harveyi* LB 21



Lane	Description
1	1 kb DNA marker
2	*Vibrio harveyi* LB 6
3	*Vibrio harveyi* LB 15
4	*Vibrio harveyi* LB 19
5	*Vibrio harveyi* LB 21
6	*Vibrio harveyi* LB 32
7	*Vibrio harveyi* LB 68

Fig. 2.2 Gene amplification of 16S rRNA from host bacteria

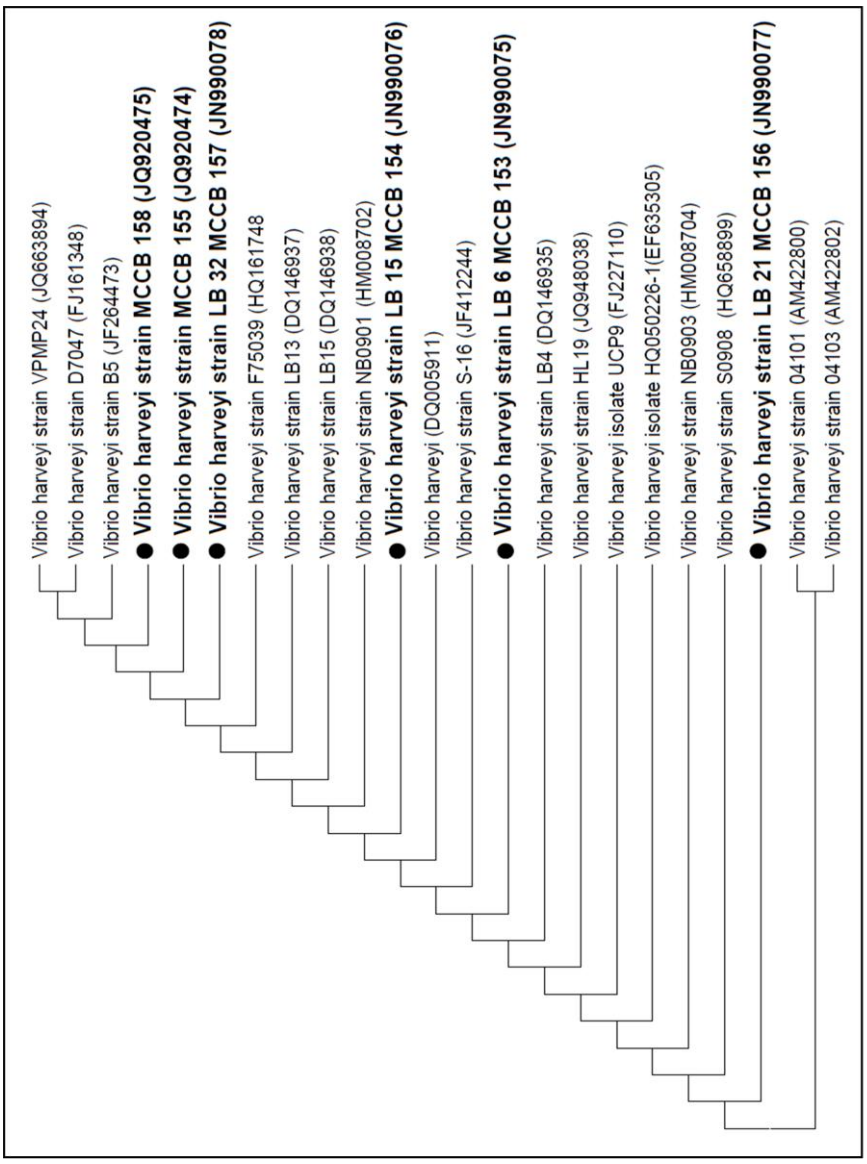


Fig 2.3 Phylogenetic tree based on 16S rRNA sequences of different isolates of *Vibrio harveyi* compared with the sequences available in GenBank

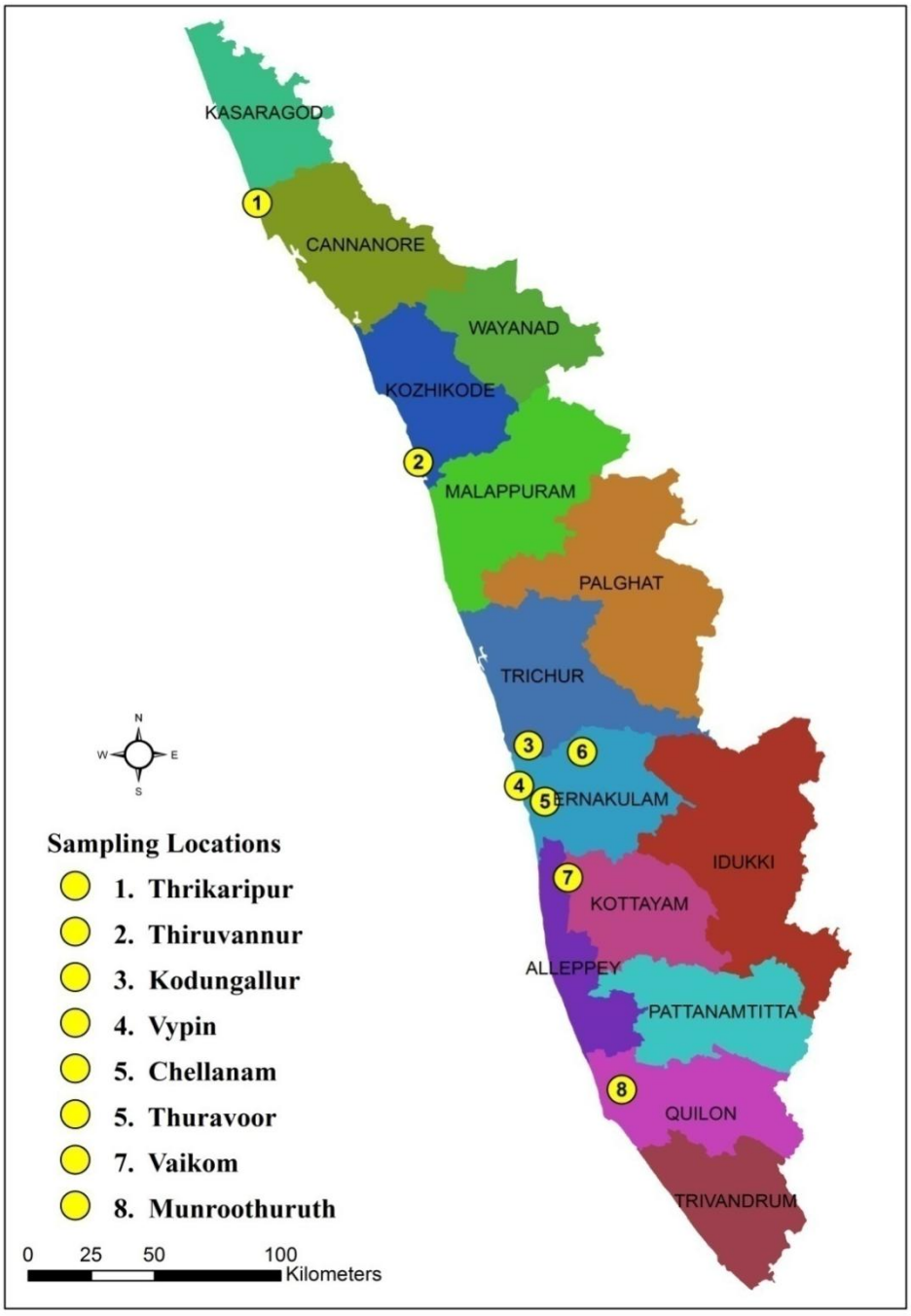


Fig.2.4 Spatial distribution of sampling locations for *Vibrio harveyi* phage isolation

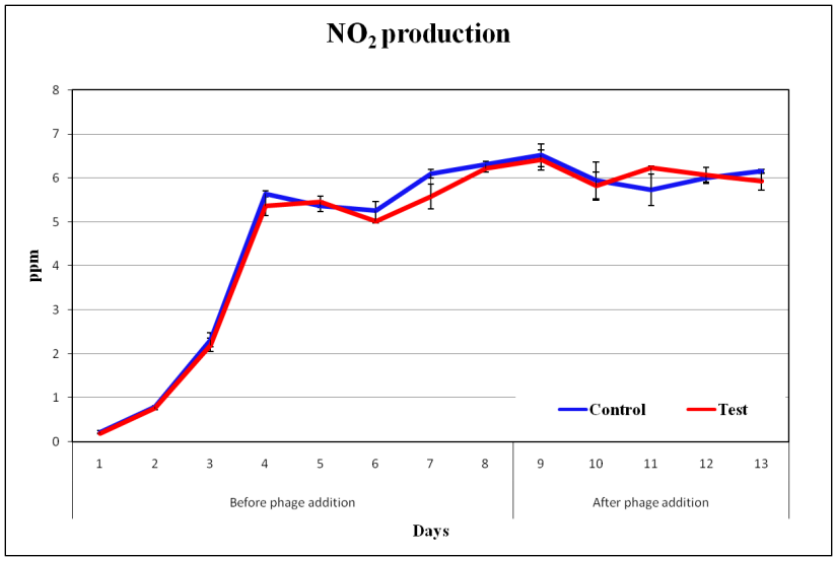


Fig. 2.5 Influence of *Vibrio harveyi* phage cocktail on NO_2 production

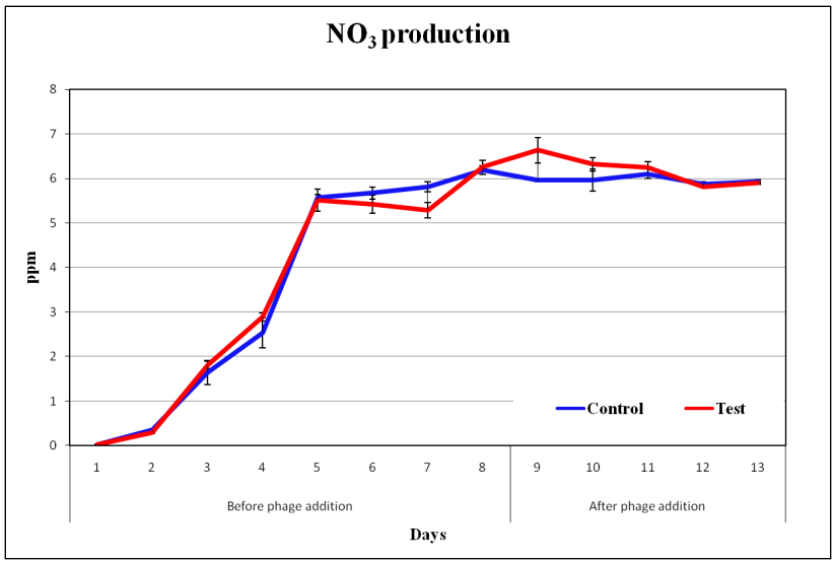


Fig. 2.6 Influence of *Vibrio harveyi* phage cocktail on NO_3 production

Transmission Electron Micrographs of *Vibrio harveyi* phages

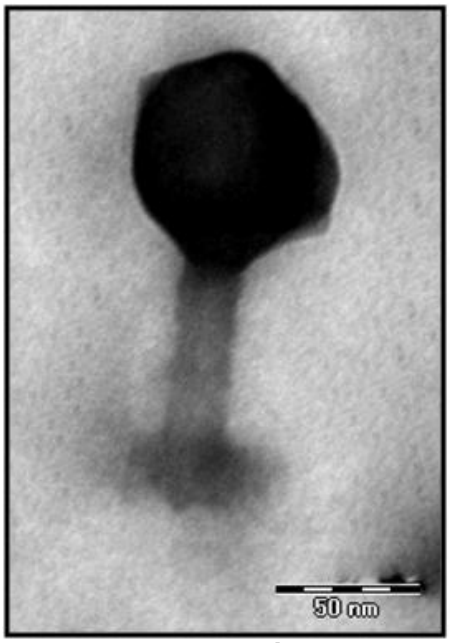


Fig. 2.7.a *Vi ha* 6

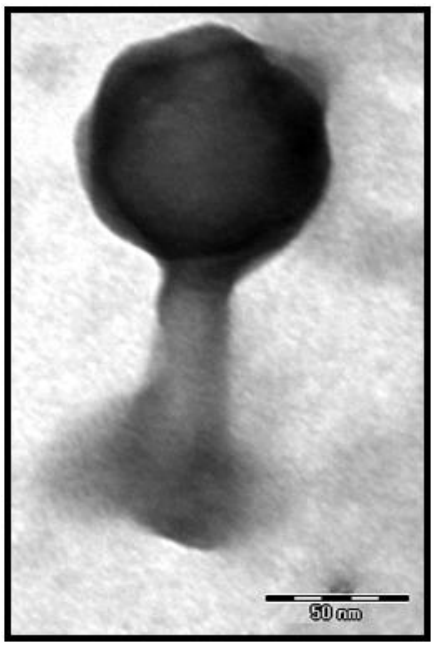


Fig. 2.7.b *Vi ha* 15

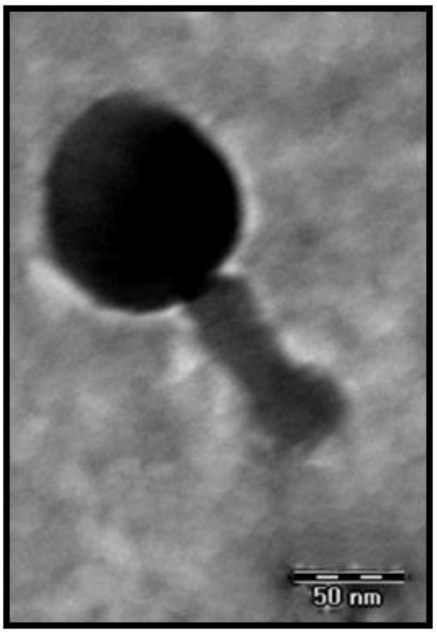


Fig. 2.7.c *Vi ha* 19

Fig. 2.7.d *Vi ha* 21

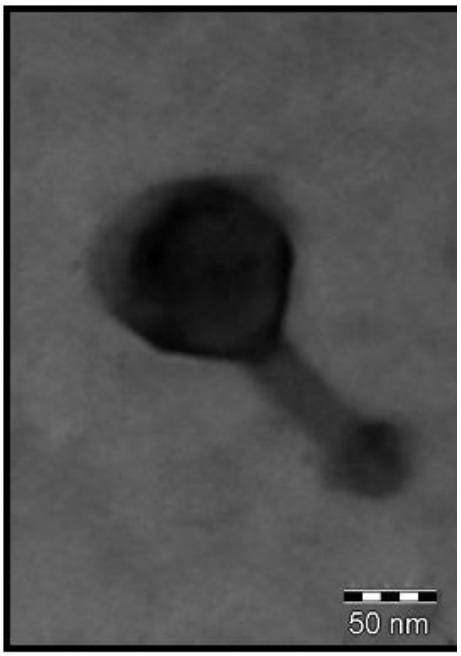


Fig. 2.7.e *Vi ha* 32

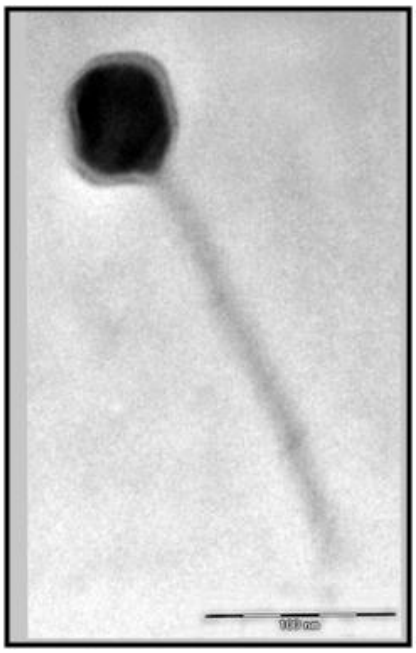


Fig. 2.7.f *Vi ha* 68

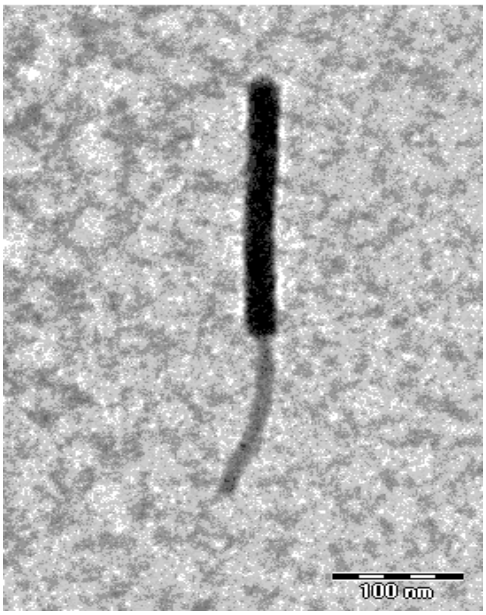
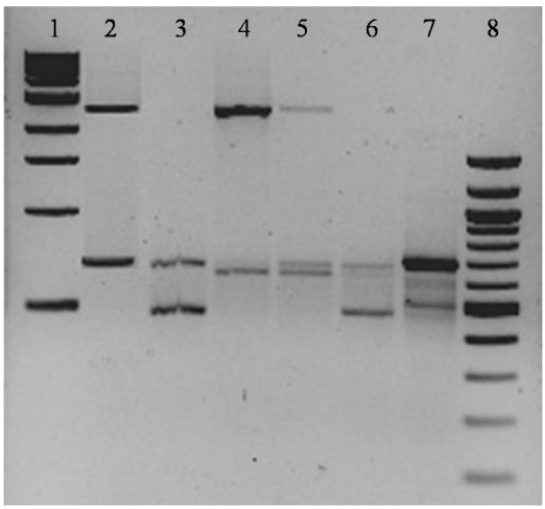
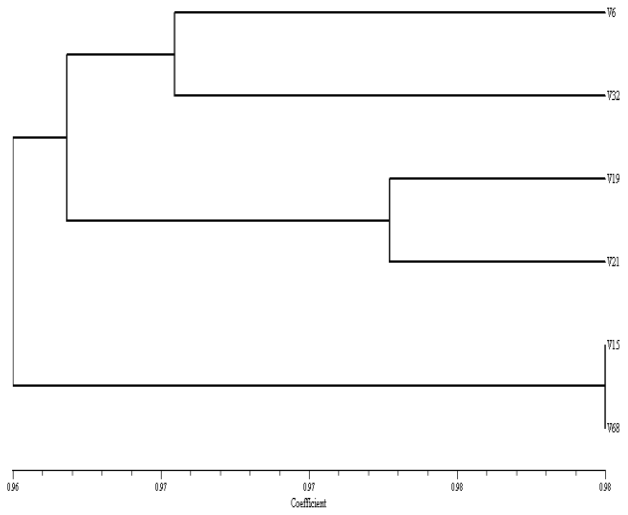


Fig.2.8 *Vi ha* 57



Lane	Description
1	1 kb DNA marker
2	*Vi ha* 6
3	*Vi ha* 15
4	*Vi ha* 19
5	*Vi ha* 21
6	*Vi ha* 32
7	*Vi ha* 68
8	100 bp DNA marker

Fig. 2.9 RAPD profile of *Vibrio harveyi* phages using Primer NP -292



V6 *Vi ha* 6

V15 *Vi ha* 15

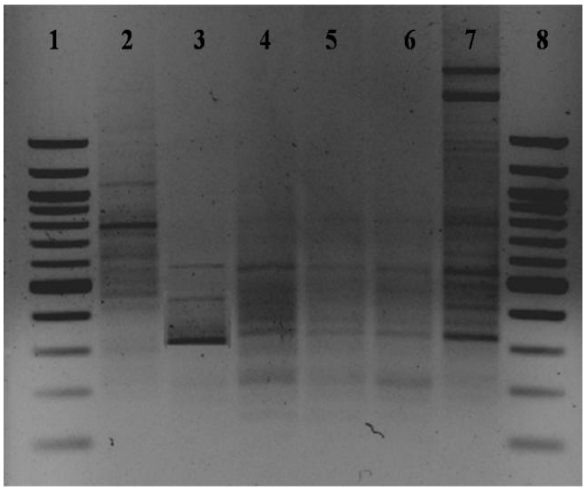
V19 *Vi ha* 19

V21 *Vi ha* 21

V32 *Vi ha* 32

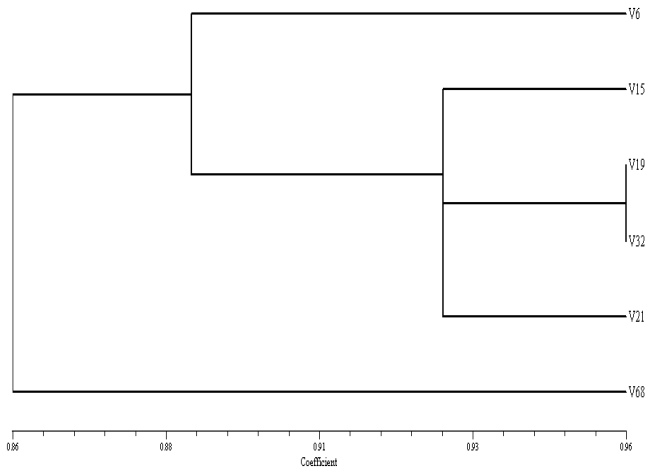
V68 *Vi ha* 68

Fig. 2.10 Dendrogram based on the RAPD profiling using Primer NP -292



Lane	Description
1	1 kb DNA marker
2	*Vi ha* 6
3	*Vi ha* 15
4	*Vi ha* 19
5	*Vi ha* 21
6	*Vi ha* 32
7	*Vi ha* 68
8	100 bp DNA marker

Fig. 2.11 RAPD profile of *Vibrio harveyi* phages using Primer NP -293



V6 *Vi ha* 6

V15 *Vi ha* 15

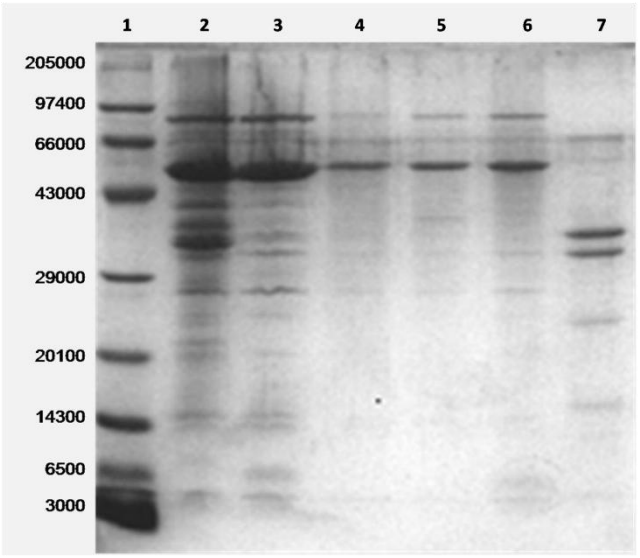
V19 *Vi ha* 19

V21 *Vi ha* 21

V32 *Vi ha* 32

V68 *Vi ha* 68

Fig. 2.12 Dendrogram based on the RAPD profiling using Primer NP -293



Lane	Description
1	Marker
2	*Vi ha* 6
3	*Vi ha* 15
4	*Vi ha* 19
5	*Vi ha* 21
6	*Vi ha* 32
7	*Vi ha* 68

Fig. 2.13 Protein profile of *Vibrio harveyi* phages

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Chapter-3
Screening of *Vibrio harveyi* phages based on the presence of lysogenic/toxin genes and determination of phage infection properties

3.1 Introduction

For successful phage therapy the biggest impediment is the presence of transferable toxin genes which can either be transferred to the host or the phage may enter in to lysogeny expressing the virulent or the toxin genes. This situation has to be addressed by selection of suitable phages that do not have either generalized or specialized transduction capabilities, do not have the toxin gene and do not enter in to lysogeny. Other than selection such phages can be genetically modified by inactivating genes responsible for lysogenicity and by knock out of toxin or virulent genes (Matsuzaki *et al.*, 2005).

3.1.1 Lysogeny

Lysogeny, by definition, is the consequence of an infection of a bacterium by a temperate bacteriophage (Miller, 2004), where the phage down regulates its gene expression to establish a quasi-stable long term relationship between the phage and its host. In such a relationship, prophage may transfer genomic fragments to the host which may alter the phenotypic characters of the host bacterium. As per Skurnik *et al.* (2007), the least requirement to ensure safety for phage therapy is the selection of lytic, but non transducing phages because therapeutic use of phages having virulence genes in their genomes constitutes a risk of transfer of the pathogenic properties to the bacteria. Many known notorious virulent bacteria got toxin genes from such genomic transfer. Bacterial virulence factors such as toxins are often encoded by accessory genetic elements such

as bacteriophages, plasmids, chromosomal islands, and transposons (Waldor and Mekalanos, 1996). In *Vibrio harveyi* also prophage induced virulence has been observed. Ruangpan and colleagues (1999) isolated such a prophage which mediated toxicity of *Vibrio harveyi* in *Penaeus monodon* by the transfer of toxin genes. Another lysogenic bacteriophage (VHML) was reported from Australia (Oakey and Owens, 2000) which imparted virulence to non virulent strains of *Vibrio harveyi*. In such a context, amplification of a number of known lysogeny or toxin related genes is an inevitable step in the selected therapeutic phages brought under the present study. The phage isolates from which amplification of these genes could not be accomplished were selected as therapeutic phages.

3.1.1.1 Integrase

The gene Integrase '*int*' is inevitable for lysogenization of lytic phages as it helps for integration of the phage genome to the host genome. Integration of phage genome involves a reciprocal recombination between specific (att) sites on both the phage and bacterial chromosomes; this is-carried out by a phage-encoded integrase, or int protein (Argos *et al.*, 1986). Once integrated into the host genome, a lysogenic phage must employ a phage repressor to prevent transcription and translation of lysis and other late genes (Oakey *et al.*, 2002). The filamentous bacteriophage, VPIΦ discovered in 1999 (Karaolis *et al.*, 1999) encoding toxin-coregulated pilus (TCP) is encoded on a pathogenicity island, which includes putative integrase and transposase genes and is flanked by att-like sequences (Kovach *et al.*, 1996). The putative *Vibrio cholerae* integrase shows significant amino acid similarity with the P4 family of integrases (Argos *et al.*, 1986).

3.1.1.2 Recombinases

The phage genes recombinases are classified into two families: the tyrosine recombinase and the serine recombinase. The tyrosine recombinase family serves

as the marker for the detection of temperate phages (Balding *et al.,* 2005). Over 300 members of this gene family are present in the databases. Members of the family have been isolated from Archaea, Bacteria and their phages and from yeast (Nunes-Duby *et al.,* 1998). Their functions include integration and excision of phage DNA into and out of the host chromosome, insertion and excision of conjugative transposons, resolution of dimers of bacterial and phage chromosomes, plasmid multimer resolution, driving of invertible gene switches, shufflon DNA rearrangement, and are associated with pathogenicity islands.

3.1.1.3 Transposase

The gene transposase codes for the enzyme transposase which binds to the ends of a transposon and catalyzes the movement of the transposon to another part of the genome by a cut and paste mechanism or a replicative transposition mechanism which is an essential step for the process of lysogenization. The temperate phage Mu is remarkably efficient at transposing its DNA into multiple sites in many bacterial genomes and mediating a variety of DNA rearrangements (Harshey *et al.,* 1985). Transposition requires two phage encoded proteins: the transposase and the transposition enhancer (Harshey *et al.,* 1985).

3.1.1.4 Dam toxin

In VHML, the most interesting link with virulence is the identification of the putative N6-Dam (DNA adenine methyltransferase) protein encoded by ORF 17. In *Salmonella typhimurium,* Dam positively regulates the pathogenicity island (Heithoff *et al.,* 1999; Portillo *et al.,* 1999). Virulence of *Yersinia pseudotuberculosis* and *Vibrio cholerae* was also reported to be regulated by Dam (Julio *et al.,* 2001). Apart from this, Kapfhammer *et al.* (2002) reported DNA methyl transferase gene in *Vibrio cholerae* Phage K139 in ORF 8 which contains the same N6 adenine-specific DNA methytransferase signature and it was found

similar to L7082 (*E. coli* O157:H7) and YdcA (*Shigella flexneri*). In addition to this potential role of Dam in activation of virulence genes, the translated sequence of VHML ORF 17 contained a site similar to the reported active site for an ADP-ribosylating toxin (ADPRT). Specifically, the active site resembled that of the group 4 ADPRTs that act upon actin filaments to produce a neurotoxic effect (Oakey *et al.*, 2002). From various instances regarding loss of pathogenicity due to Dam mutations, Bujnicki *et al.* (2001) speculates that Dam mutants of some pathogenic Proteobacteria would lose their virulence due to the disturbance of expression of Dam-regulated genes.

3.1.1.5 *Ctx A* gene for Cholera toxin

Waldor and Mekalanos (1996) first reported that the genes encoding the cholera toxin resided in a filamentous bacteriophage, the cholera toxin conversion bacteriophage (CTX Φ). *Vibrio cholerae*, the causative agent of cholera, requires two coordinately regulated factors for full virulence: cholera toxin (CT), a potent enterotoxin, and toxin-coregulated pili (TCP), surface organelles required for intestinal colonization. The genes encoding CT (including *CtxAB)* reside on the CTX genetic element, a 7- to 9.7-kb segment of DNA present in the chromosome of toxigenic strains but absent in nontoxigenic strains (Waldor and Mekalanos, 1996). Davis *et al.* (2000) reported that the virulence of *Vibrio cholerae* was dependent upon the secretion of cholera toxin (CT), which is encoded within the genome of the CTX Φ.

3.1.2 Phage Infection (One step growth) Properties

Determining phage infection properties such as Multiplicity of Infection (MOI) and one step growth properties such as latent period and burst size are helpful in evaluating the efficiency of phages in lytic infection in bacterial hosts. Multiplicity of infection is defined as the ratio of virus particles to infect host cells (Birge, 2000; Lu *et al.*, 2003). The optimal MOI needs to be worked out for maximum

number of progeny phages to be released during therapeutic assays. The optimal MOI plays another important role to maintain lytic cycle; high MOI has been shown to stimulate lysogenic cycle of replication (Weinbauer, 2004) especially when MOI is greater than one (Friedman *et al.*, 1984).

One step growth properties include latent period, burst time and burst size depending on which the proliferation of phage is determined. In 1938, Emory Ellis and Max Delbruck performed a classical experiment which revealed the fundamental nature of phage replication. Growth of phage was divided into three periods accordingly, such as adsorption, latent period and burst. The minimal time needed for the phage-controlled bacterial lysis is termed the latent period (Krylov, 2001). Lysis inhibition results in a delay of the latent period (Doermann, 1948) which may switch on to lysogenic cycle. Therapeutic phages should have shorter latent periods and high burst sizes. Length of the latent period depends on phage species, physiological condition, type of host, composition of medium and temperature (Weinbauer, 2004). The number of virions released per cell into the extracellular environment is called the burst size which increases with cell size and depends on phage size as well, i.e., larger phages may produce less progeny (Weinbauer, 2004).

This chapter discusses whether the selected therapeutic phages are likely to be lysogenic or carry any toxin or virulence genes which might cause phage therapy ineffective. This chapter also explains the basic infection characteristics like multiplicities of infection and one step growth characteristics such as latent period, burst time, burst size etc. which further prove the lytic infectivity of the phages.

3.2 Materials and Methods

3.2.1 VHML Prophage Induction Experiment

For amplification of lysogenic genes and Dam toxin gene, primers were designed from the genome of *Vibrio harveyi* myovirus like (VHML) bacteriophage. Amplification in positive control, VHML was essential to confirm the absence of genes in samples. VHML is a prophage present in the lysogen *Vibrio harveyi* ACMM 642 (Oakey *et al.*, 2002).

Vibrio harveyi ACMM 642, obtained in lyophilized form, from Dr. Jane Oakey, James Cook University, Australia was revived and cultured in ZoBell's broth in triplicates, incubated for 12 hours on an orbital shaker at 28°C. The culture was inducted to lytic cycle by the addition of 50ng mL^{-1} mitomycin C and further incubation for 10-12 hrs on an orbital shaker at 28°C. Optical density at 600 nm was measured intermittently. (A decrease in Abs_{600} or same Abs throughout the incubation indicates successful induction). After incubation, cells were pelleted by centrifugation at 5000 x g for 10 min. The supernatant was harvested and filtered through a syringe membrane PVDF filter of 0.22 µm and stored at 4°C. As there was no significant variation in the Abs_{600}, the supernatant was checked for the presence of VHML phages by plaque assay. A sample of the supernatant was sent for TEM to AIIMS, Delhi for confirming the presence of VHML. DNA of VHML bacteriophage was extracted for using as positive control for lysogenic and toxin gene amplification.

3.2.2 DNA extraction of bacteriophages

Refer Chapter 2: Isolation, purification, screening and characterization of *Vibrio harveyi* phages for phage therapy; Section: 2.2.12.2.1

3.2.3 PCR Amplification of genes associated with lysogeny/toxin genes

Oligonucleotide primers for lysogenic genes and toxin genes were synthesized by Sigma Aldrich, USA. The genes selected for detection of lysogenization included integrase, transposase, and recombinase, and genes for virulence included *N6-Dam* and *Ctx A*. Primers for all lysogenic genes were newly designed using the software, Genetool from the genome sequence of the phage VHML (Oakey *et al.*, 2002). The primers for N6-Dam toxin was adopted from Vinod *et al*. (2006) and the primers for Ctx gene was used from the collection of NCAAH, CUSAT. For *Ctx A* gene amplification in phages, *Vibrio cholera* type strain MTCC 3906 DNA was kept as positive control and for rest of all genes VHML DNA served as the positive control. For amplification of all genes, except that of *Ctx A* PCR was adopted with initial denaturation at 95°C for 1 min, 40 cycles of 95°C for 1 min, annealing temperature for 2 min, and a final extension of 72°C for 5 min. Annealing temperatures of all primers are given in Table 3.1. PCR mix included 15 µL deionized water, 2.5 µL 10X Thermopol buffer (New England Biolabs, USA), 1 µL 10 mM dNTPs, 2 µL Taq polymerase (New England Biolabs, USA), 1 µL 10 pmol primers and 120 ng DNA template in a final volume of 25 µL. The amplification of *Ctx A* gene was carried out in a thermal cycler (Eppendorf, Germany) which involved initial denaturation of 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. The PCR mix was consisted of 2.5 µL 10X Thermopol buffer (New England Biolabs, USA), 2µL 10 mM dNTPs, 2µL 10 picomol of each primer (10µM), 1.5 µL of template (50ng/µL) and 1µL Taq DNA Polymerase (0.5 U, New England Biolabs, USA) and Milli Q (to a final volume of 25 µL). Details of primer sequences, PCR conditions and product sizes are given in Table 3.1.

3.2.4 Cloning of '*N6-Dam'* gene into pGEM-T Easy vector

Fresh PCR product of N6-Dam toxin was cloned into pGEM-T Easy vector

(Promega, USA). The ligation mix (10 µL) consisted of 5 µL ligation buffer (2X), 0.5 µL of the vector (50 ng/ µL), 3 µL PCR product and 1 µL T4 DNA ligase (3U/ µL). The ligation mix was incubated overnight at 4°C. The entire ligated mix was used to transform *Escherichia coli* JM 109 competent cells, prepared using calcium chloride method. The ligation mix was added to 10 mL glass tube previously placed in ice to which 50 µL competent cells were added and incubated on ice for 20 min, a heat shock at 42°C was given for 90s, immediately after the tubes were placed on ice for 2 min and subsequently 600 µL SOC medium was added and incubated for 2 hr at 37° in an incubator shaker at 250 rpm. The transformation mixture (200 µL) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/mL), IPTG (100 mM), X-gal (800 µg/mL). The plates were incubated at 37°C overnight. The clones were selected using the blue/white screening. The white colonies were selected and streaked to purify on LB- Amp+X-gal+IPTG plates and incubated overnight at 37° C. To confirm the insert, colony PCR of the white colonies were carried out using the vector primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'- GATTTAGGTGA CACTATA G-3'). White colonies picked from the transformed plate were dispensed into the PCR mix (25 µL) containing 2.5 µL 10X PCR buffer, 2.0 µL 2.5 mM dNTPs, 1 µL 10 pmol/ µL of T7 and SP6 primers, 0.5 U of taq polymerase and the remaining volume was made up with Milli Q. The PCR conditions were as follows: hot start PCR 95 °C for 5 min; 35 cycles of 94°C for 15s, 57°C for 20 s, 72°C for 60 s, final extension of 72°C for 10 min, following which the temperature was brought down to 4°C.

3.2.5 Plasmid extraction & purification

Plasmids from the positive clones were extracted using the 'GenElute HP' plasmid miniprep kit (Sigma Aldrich, USA). An overnight grown culture of *E.coli* was harvested with centrifugation and subjected to a modified alkaline-SDS lysis procedure followed by adsorption of the plasmid DNA onto silica (column) in the

presence of high salts. Contaminants were removed by spin wash step. Finally, the bound plasmid DNA was eluted to 100 µL in 5 mM Tris-HCI at pH 8.0.

3.2.6 Sequencing

Nucleotide sequencing was performed at m/s Xcelris Genomics, Ahmedabad, India. The primers used were T7 and SP6. The sequences obtained were matched with Genbank database using the BLAST search algorithm. The sequences of Dam toxin gene fragments of both VHML and *Vi ha* 68 are given in Appendix (Section 2.1- 2.2).

3.2.7 Screening of *Vibrio harveyi* phages

Out of six broad spectrum lytic phages obtained based on the host range analyses, one was already eliminated due to the presence of 'N6-Dam toxin gene' partially. Rest of the five phages were selected to develop phage cocktail for further investigations on the therapeutic applications on *Vibrio harveyi*.

3.2.8 Evaluation of infection properties of individual phages

As infection characteristics of phages are to be evaluated for the phage therapeutic assays, properties such as multiplicity of infection, latent period, burst size and burst time were determined.

3.2.8.1 Determination of optimal multiplicity of infection (MOI)

Multiplicity of infection was defined as the ratio of virus particles to host cells (Birge, 2000). In other words, it is the average number of phages per bacterium and is determined by dividing the number of phages (mL added x PFU/mL) by the number of bacteria (mL added x CFU/mL) in a suspension. In the present study five MOI values were selected to find out the optimal MOI to evaluate their comparative therapeutic efficacy. Although the MOI tells us the average number of

phages per bacterium, the actual number of phage that infects any given bacterial cell is a statistical function. For example, if the MOI is 1, some cells will get infected with one phage and some may not be infected and other cells may be infected with two phages.

Overnight grown *Vibrio harveyi* cultures (MCCB 153 - LB6), (MCCB 154- LB 15), (MCCB 155 - LB 19), (MCCB 156 - LB 21), (MCCB 157 - LB 32) and (MCCB 158 - LB 68) were re inoculated into freshly prepared 100 mL ZoBell's broth in 250 mL flasks and incubated on a temperature controlled shaker (100 rpm) to attain an absorbance of 0.01 at Abs_{600} nm. At this point, the bacteria were infected with the respective phages at five ratios [Phage titre (PFU/mL)/ Bacterial count (CFU/mL)] such as 0.01, 0.1, 1, 10 and 100. The flasks were incubated on an orbital shaker for 6 hrs. Phage activity was monitored periodically until 6 hrs by measuring the absorbency at Abs_{600} nm. After incubation for 6 hrs at 30°C, the phage lysate was centrifuged at 9000 x g for 3 min. The supernatant was filtered (0.45-µm and 0.22-µm pore size PVDF syringe filter) and assayed to determine the phage titer following the soft agar overlay method of plaque assay in triplicates. MOI based on phage titre and Abs_{600} were compared to see if there is any variation between two methods. The MOI which resulted in the highest phage titre within 6 hrs was considered as the optimal MOI and used in subsequent bacterial challenge and phage therapy studies.

3.2.8.2 Determination of one-step growth properties

For one-step growth experiments, a modified method of Capra *et al.* (2006) was used. Aliquots of 10 mL each of fresh ZoBell's broth were inoculated with overnight grown cultures of *Vibrio harveyi* (MCCB 153 - LB6), (MCCB 154- LB 15), (MCCB 155 - LB 19), (MCCB 156 - LB 21), (MCCB 157 - LB 32) and (MCCB 158 - LB 68) as the hosts and incubated until the absorbance reached Abs 0.5 at 600 nm. Following centrifugation at 10,000 x g for 10 minutes, the

pellet containing host bacterial cells was re-suspended in 1/5 th volume (2 mL) of fresh ZoBell's broth. The cells were infected with 0.002 MOI of phage particles and allowed for adsorption for 10 minutes. The partially adsorbed host cells were centrifuged and the pellet was re-suspended in a fresh 10 mL ZoBell's broth. Samples were taken at 10 min-intervals from 0 min to 2 hr and immediately tittered by the the soft agar overlay method of plaque assay. The bacterial counts at the start of the experiment were determined using spread plate method. Assays were carried out in triplicates. Latent period was defined as the time interval between the adsorption (not including 10 min pre-incubation) and the beginning of the first burst, as indicated by the initial rise in phage titer (Ellis and Delbruck, 1939; Adams, 1959). Burst size was calculated as the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells during the latent period (Adams, 1959; Lu *et al.*, 2003).

3.3 Results

3.3.1 VHML prophage induction experiment

Vibrio harveyi ACMM 642 (Oakey *et al.*, 2002) which contained prophage VHML integrated to host genome was revived from the lyophilized culture. On incubation no significant reduction in absorbance was observed indicating of successful induction (Fig.3. 1). Plaque assay of induced VHML on the lawn of *Vibrio harveyi* ACMM 642 could not produce any plaque; might be due to the unavailability of naive *Vibrio harveyi* ACMM 642. However, the transmission electron microscopy of the supernatant which resulted after induction using mitomycin C could prove the presence of lytic VHML particles (Fig.3. 2) which were excised from the host genome.

DNA extracted was of good quality as the values were around 1.7-1.8 as per Abs 260/280 ratio.

3.3.2 Amplification of lysogenic genes in VHML (positive control)

The lysogenic genes such as integrase, transposase and recombinase could be amplified from VHML genome (Fig. 3.3). The amplicons of integrase had a product size of 1529 bp located at 297-1826 bp region of VHML genome. The gene recombinase having a product size of 929 bp located at the region 8889-9818bp and transposase having a product size of 911 located at the region 29054-29965. However, the gene for Dam toxin got amplified only partially with a product size of 182 bp instead of 1080 bp located at the region 12635-13714bp. (Fig.3.3). This was carried out as part of the standardization of the PCR conditions in the amplification of three lysogenic genes and one toxin gene.

3.3.3 Amplification of lysogenic genes in the phage lysates

3.3.3.1 Integrase

Amplification of integrase from phage lysates could not be accomplished following standardized PCR conditions. However, with VHML control DNA the gene could be amplified with a product size of 1529 bp (Fig.3. 4).

3.3.3.2 Recombinase

None of the *Vibrio harveyi* phages under study had the gene recombinase, as no amplicons could be produced following standardized PCR corresponding to the positive control (Fig.3. 5) having the product size 929 bp.

3.3.3.3 Transposase

None of the *Vibrio harveyi* phages under study had the gene transposase, as no amplicons could be produced following standardized PCR corresponding to the positive control (Fig.3. 6) having the product size 911bp.

3.3.3.4 Amplification of Dam toxin gene in the phage lysates

While amplifying the Dam toxin gene in *Vibrio harveyi* phages, a PCR product of 182bp could be generated from *Vi ha* 68 corresponding to the positive control. However, from none of the other phages such amplification could be obtained (Fig.3.7)

3.3.3.5 Cloning and Sequence Analysis

PCR products of Dam toxin gene of both *Vi ha* 68 and VHML positive control having product size 182 bp (Fig.3.8) were cloned into pGEMT easy vector, presence of insert confirmed by colony PCR using T7 and SP6 primers which generated a PCR product of 350bp (Fig.3.9 and Fig.3.10). The plasmid that contained Dam toxin gene in VHML and *Vi ha* 68 were linearized by restriction digestion as shown in Fig.3.11.

The sequences were multiple aligned and the nucleotide sequence of the cloned PCR product of Dam toxin gene fragment in Viha 68 was 99% identical to that of the VHML positive control (GenBank Accession no. AY133112).

3.3.3.6 Amplification of *Ctx A* in phage lysates

Ctx A gene could not be amplified from any of the phage DNA corresponding to the positive control *Vibrio cholerae* type strain MTCC 3906 from which the gene was amplified having a product size of 564 bp (Fig.3.12).

3.3.4 Evaluation of infection properties of the phages

The infection properties included optimum multiplicity of infection, latent period, burst time and burst size.

3.3.4.1 Determination of optimal multiplicity of infection (MOI)

All experiments towards the determination of MOI started with *V. harveyi* specific for each phage having Abs 0.1, except for *Vi ha* 32, for which commencement of the experiment was with Abs 0.15. Measurement of absorbency was continued up to 6 hrs at an interval of 1 hr. At the end of 6th hour, titres of all five phages were determined and MOI compared. For the phage *Vi ha* 19, Abs measurements were continued up to 10 hrs, as there was no significant rise of Abs throughout the experimental period. Optimal MOI obtained based on phage titre and the host Abs measurements were tabulated and variations determined (Table 3.2).

Optimal MOI for *Vi ha* 6 based on the phage titre was 0.1 (Fig.3.13.b) and that based on absorbance was 1.0 (Fig.3.13.a). As per the absorbance profile of MOI 100 and 1, no resistant bacteria or Bacteriophage Insensitive Mutants (BIMs) were emerged throughout the infection period. Absorbance profile of all other three MOIs, showed the emergence of resistant bacteria, showing highest for MOI 0.01

Optimal MOI for *Vi ha* 15 based on both titre and absorbance was 0.1 (Fig.3.14.a & 14.b). This is the only phage which has showed similar optimal MOI based on both titre and absorbance profile. For all the MOI, emergence of resistant bacteria was observed as per absorbance profile.

Optimal MOI for *Vi ha* 19 based on the titre was 0.01 (Fig.3.15.b) and that based on absorbance was 100 (Fig. 3.15.a). For MOI 0.01, 1 and 100, a rise in absorbance was observed between hrs 3 & 4. Subsequently the absorbance got declined at 4th hour beyond which no emergence of bacteria could be observed for any MOIs. This is the only phage which has overcome the Bacteriophage Insensitive Mutants in all the given MOIs.

Optimal MOI for *Vi ha* 21 based on the titre was 0.01 (Fig.3.16.b) and that based on absorbance 100 (Fig.3.16.a). The least emergence of resistant bacteria was

observed for the MOI 100 and 10. More Bacteriophage Insensitive Mutants were observed for MOI 0.01. But highest phage titre was shown by MOI 0.01

Optimal MOI for *Vi ha* 32 based on titre was 100 (Fig.3.17.b) and that based on absorbance was 1 (Fig.3.17 a). Absorbance profile for all MOIs showed a declining trend, without emergence of Bacteriophage Insensitive Mutants.

The MOI resulting in highest phage titre within 6 hrs was considered as an optimal MOI and used in subsequent large-scale phage production, as described in Lu *et al*. (2003).

3.3.4.2 One step growth properties of the phages

The infection cycles of phages with their respective host strains were characterized by their multiplication phage parameters such as latent period, burst time and burst size.

Latent period of *Vi ha* 6-LB 6 system was 50 minutes, burst time 80 minutes and burst size 127 PFU/infective cell. Burst occurred 30 minutes after the latent period (Fig.3.18).

Latent period exhibited by *Vi ha* 15-LB 15 system was 30 minutes, burst time 70 minutes. Burst occurred 40 minutes after the latent period and burst size was123 PFU/infective cell (Fig.3.19).

Latent period of *Vi ha* 19-LB 19 system was 60 minutes, burst occurred 30 minutes after the latent period, that is at 90^{th} minute. Burst size was 1135 PFU/infective cell. It is the highest burst size observed out of the five phages, a characteristic of highest lytic efficiency (Fig.3.20).

Latent period of *Vi ha* 21-LB 21 system was 50 minutes, burst occurred at 90th minute, 40 minutes after the latent period and burst size was 615 PFU/infective cell, the second highest lytic efficient phage (Fig.3.21).

Latent period of *Vi ha* 32-LB 32 system was 50 minutes, burst time 90 minutes 40 minutes after the latent period and burst size 223 PFU/infective cell (Fig.3.22). One step growth properties or multiplication parameters of phages are tabulated in Table 3.3.

3.4 Discussion

Amplification of lysogenic and virulence genes were carried out in 6 *Vibrio harveyi* phages which were segregated from a whole lot of 33 lysates isolated in order to evaluate the probability of lysogenization and lysogenic conversions in the host bacteria imparting them toxin/virulent genes. Phage therapy using such phages might result in impaired therapeutic effects. It might lead to the enhancement of virulence of the host pathogens as in the case of increased virulence in *Vibrio harveyi* ACMM 642 under the status of infection with the *Vibrio harveyi* Myovirus Like temperate bacteriophage (VHML) (Oakey and Owens, 2000). Out of the three lysogenic genes and two virulence genes examined in this study, only the virulence gene for Dam toxin could partially be amplified in one of the *Vibrio harveyi* phages (*Vi ha* 68) described in this study. None of the phage DNA was positive to lysogenic genes such as integrase, recombinase and transposase and to virulence related Ctx toxin gene. The phage with partial Dam toxin gene was excluded from further study considering the negative impact of such genes which might happen in phage therapy.

Vibrio harveyi phages under the present study were found to be safe for therapeutic use as they were negative for all the major lysogenic attributes such as integration, recombination etc. Substantiating this finding Chen and Lu (2002) stated that all

the fully sequenced lysogenic siphoviridae and myoviridae phages were reported to have the gene that codes for integrase. Balding *et al.* (2005) also stated that DNA integration is a required property of lysogeny. For instance, the lysogenic *Vibrio cholerae* phage K139 (Kapfhammer *et al.*, 2002) showed the presence of '*int'* gene in its genome. The phages in the present study were not likely to be pseudolysogenic as well because its expression required gene integrase along with lytic genes such as helicase, DNA polymerase, and thymidylate synthase as in the case of the pseudolysogenic phage ΦJL001 isolated from the marine sponge *Ircinia strobilina* (Lohr *et al.*, 2005). Not only the gene for integrase, but the genes for transposase and recombinase were also not amplified from the phage genomes under this study. *Vi ha* 68 alone showed the partial Dam toxin gene despite the absence of lysogenic genes. In a similar pattern two *V. parahaemolyticus* phages VP16T and VP16C had a virulence-associated protein VapE, even in the absence of lysogenic gene such as recombinase (Seguritan *et al.*, 2003). To make sure the absence of cholera toxin in test phage samples, *Ctx A* gene amplification was carried out. Payne *et al*. (2004) stated that even though *Ctx A* toxin is normally associated with *Vibrio cholera* phages, there is possibility for horizontal transfer of genes in other *Vibrio* phages. However none of the *Vibrio harveyi* phages investigated here contained *Ctx A* gene for cholera toxin in their genome.

Phage VHML ORF 17 encodes a complete N6-DNA adenine methyltransferase (Dam) gene and a site similar to an active site of ADPRT, which may be responsible for virulence conversion of *Vibrio harveyi* (Oakey *et al.*, 2002). The bacterial ADPRTs are involved in pathogenesis (Holbourn *et al.*, 2006) by ADP ribosylation of key regulatory proteins in the host cells. The 1080 bp long ORF 17 is located in 12635-13714 region in the VHML genome. To determine the presence of putative Dam toxin gene of VHML in the *Vibrio harveyi* phages in this study, the primers described by Vinod *et al*. (2006) were used. The primers amplified the 13285-13466 bp region of the VHML genome. The phage *Vi ha* 68 (which was

eliminated from the cocktail) was PCR positive for this region. The PCR product (182 bp) was 99% identical in base sequence to the PCR product of VHML genome. However, VHML is a Myoviridae phage and *Vi ha* 68 belongs to Siphoviridae. On the other hand, all the 5 Myoviridae phages in this study were PCR negative for the putative virulence gene of N6 Dam toxin gene of VHML. However, it has been reported that from a potent siphoviridae (Vinod *et al.*, 2006) and a myoviridae *V. harveyi* phages (Gunawardhana, 2009) isolated from geographically different areas the similar fragment of gene has been amplified to result a gene product of 182 bp instead of 1080 bp alike the amplification of the same in the positive control VHML genome (Gunawardhana, 2009) based on the same primers.

However, there is an ambiguity regarding the failure of amplification of Dam toxin gene with the product size of 1080bp even in the VHML positive control. The reason might be the inefficiency of the primer used or the incorrect excision of VHML genome from the host genome during induction to lytic cycle. In other words, as a result of specialized transduction (Canchaya *et al.*, 2003), induced VHML might have lost a part of its genome where a good portion of the Dam toxin gene was situated. However, the induction was successful as the presence of VHML phage particles were revealed by the Transmission Electron Micrograph in the lysate. In another instance also tail sheath gene of the VHML was partly amplified in *Vibrio harveyi* phages (Chrisolite *et al.*, 2008) where 350 bp fragment could be amplified instead of actual product size of 900 bp.

Multiplicity of Infection (MOI) of *Vibrio harveyi* phages were determined to assess the phage bacterium ratio to be applied in the phage therapeutic experiments. Normally, determination of optimal MOI represents the ratio in which maximum phage titre has been resulted after a period of infection of the host bacteria (Lu *et al.*, 2003). However, optimal MOI resulted after following the absorbance

profile was found to be different from that based on phage titre. Therefore an attempt has been made here to compare the optimal MOI based on phage titre and absorbance profile. In this context the phage *Vi ha* 15 alone exhibited the same optimal MOI based on both the criteria. As per the absorbance profile of *Vi ha* 19-LB19 system, absorbance reached below zero in all the ratios at various points of time period without the emergence of bacteriophage insensitive mutants (BIMs) or resistant bacterial forms. *Vi ha* 32 also showed no emergence of BIMs in any MOI. All other phages exhibited the emergence of BIMs. However, optimal MOI based on phage titre has been selected to be used in all experiments related to phage therapy as per the literature. It has to be pointed out that no information regarding the observation of optimal MOI based on absorbance has been reported so far.

On assessing the phage multiplication parameters by one step growth experiment, it was found that latent period ranged from 30 to 60 min, burst times from 70 to 90 min and burst sizes from 123 to 1135 phages per infected cell. *Vi ha* 19 was found to have the highest burst size (1135) with longest duration of latent time (60 min). It reconfirms the unusual potential of *Vi ha* 19 in terms of phage therapy. Similar burst size of 1000 PFU/mL has been reported by a mutant of lambda phage by Reader and Siminovitch (1971). The *Vibrio harveyi* phage of Shivu *et al*. (2007) reported a burst size of 10^5 PFU per cell. Very low burst size of 5 PFU/cell was reported by Miklic and Rogelj (2003) for a *Lactococcus* phage from Slovenia. In the present study, the lowest burst size (123) was shown by phage *Vi ha* 15 with shortest duration of latency (30 minutes). From the observations regarding phage multiplication parameters, it has been observed that in all the cases, there was a relationship between burst size and latent time. Greater burst sizes are usually associated with a longer latent period, whereas lesser burst sizes are associated with a shorter latent period as stated previously (Gnezda-Meijer *et al.*, 2006).

3.5 Conclusion

The highly potent siphoviridae phage *Vi ha* 68 was excluded from further studies on therapy as a result of partial amplification of Dam toxin gene. Optimal multiplicity of infection of phages was determined. Phage infection properties were evaluated and found that all the remaining five lytic phages were efficient enough to be used for phage therapy based on latent period and burst size.

3.6 Tables

Table 3.1 Primer sequence, PCR conditions and product size of lysogenic/toxin genes

Genes	**Primer sequence**	**Annealing Temp**	**Product Size**
Gene for 'integrase'	NP 156(F)-CGG TCG TGT CGC TAA GGA TGA NP 156(R)-CCT GCT ATT ACA TTT CCG CTG AT	54°C for 2 min	1529bp
Gene for 'recombinase'	NP 157(F)-TTC GGT GCT TTG TTC ATT TGG GA NP 157(R)-AACGGA TTG ACA GAG GGC TAT TT	53°C for 2 min	929 bp
Gene for 'transposase'	NP 158(F)-ATG CGT TCA CTG GGC TGC TCA NP 158(R)-GGC CAG CCA GGA TGA TGT TC	65°C for 2 min	9110bp
Gene for 'N6-Dam'	NP 159(F)-TGA TCA TGC CGA TGG TCT TA NP 159(R)-GGT CAA AAT CCC ACA CAT CC	55°C for 2 min	182 bp
Gene 'Ctx A'	NP 33(F)- CGG GCA GAT TCT AGA CCT CCT G NP 33(R)- CGA TGA TCT TGG AGC ATT CCC AC	60°C for 1 min	564 bp

Table 3.2 Comparison of optimal MOI determined based on absorbance and titre of phage

Phages	Optimal MOI determination based on	
	Titre	Absorbance
Vi ha 6	0.1	1
Vi ha 15	0.1	0.1
Vi ha 19	0.01	100
Vi ha 21	0.01	100
Vi ha 32	100	1

Table 3.3.Multiplication parameters for *Vibrio harveyi* phages

System phage/strain	Latent period (min)	Burst time (min)	Burst size (PFU/infective cell)
Vi ha 6- LB 6	50	80	127
Vi ha 15-LB15	30	70	123
Vi ha 19-LB19	60	90	1135
Vi ha 21-LB21	50	90	615
Vi ha 32-LB32	50	90	223

3.7 Figures

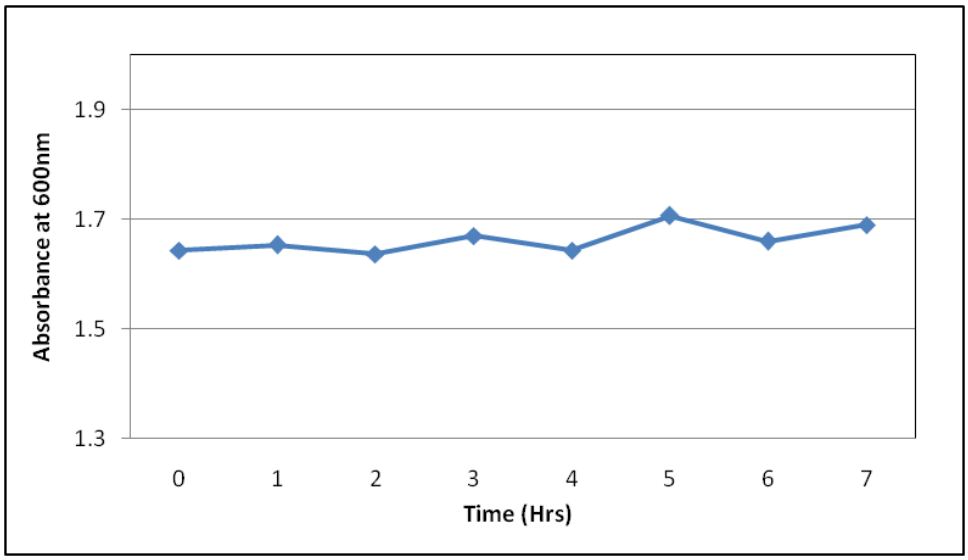


Fig. 3.1. Absorbance profile during induction of VHML prophage

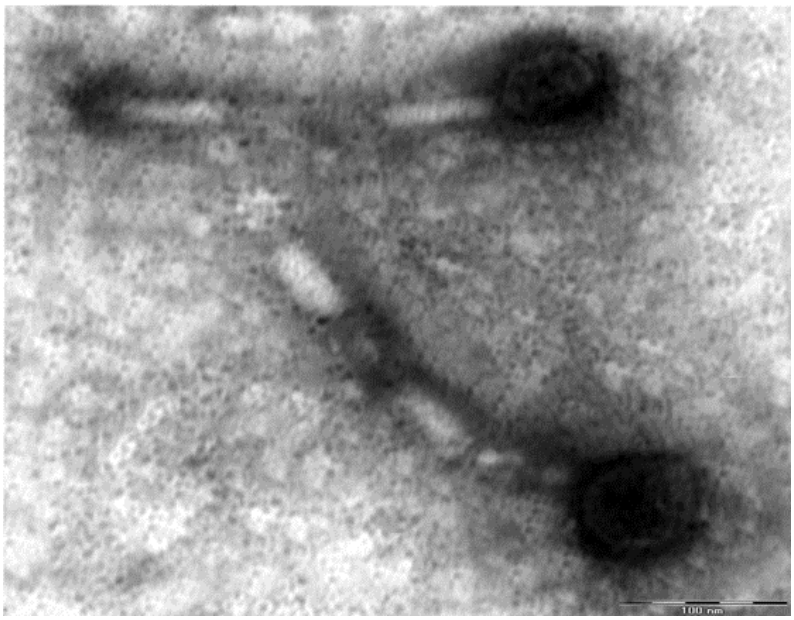
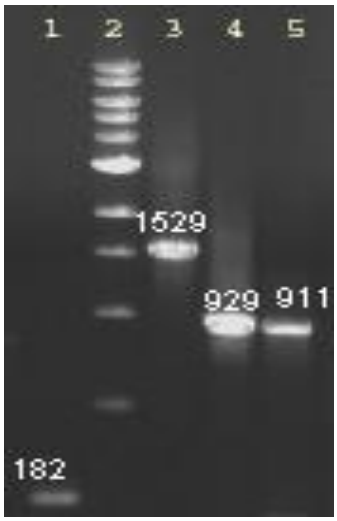
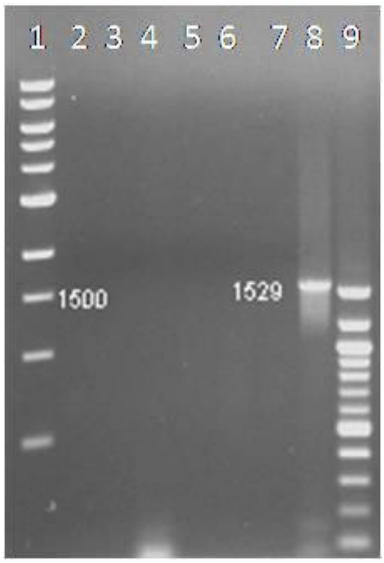


Fig. 3.2. Transmission Electron Micrographs of VHML phages



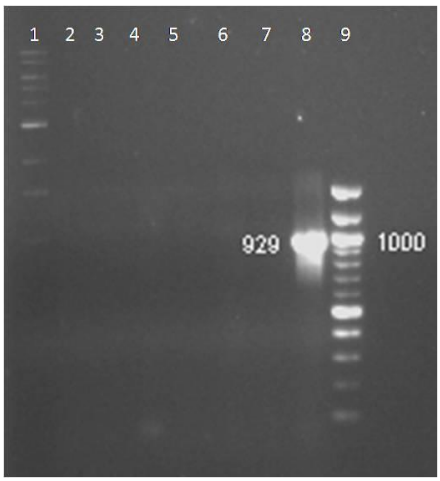
Lane	Description
1	Dam toxin
2	1kb DNA marker
3	Integrase
4	Recombinase
5	Transposase

Fig. 3.3. Genes amplified from VHML positive control



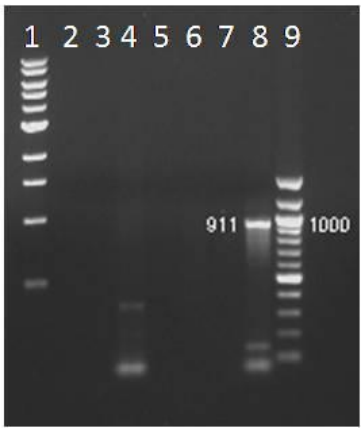
Lane	Description
1	1kb marker
2	Vi ha 6
3	Vi ha 15
4	Vi ha 19
5	Vi ha 21
6	Vi ha 32
7	Vi ha 68
8	VHML (positive control)
9	100 bp DNA marker

Fig. 3.4. Amplification of integrase gene from phage DNA



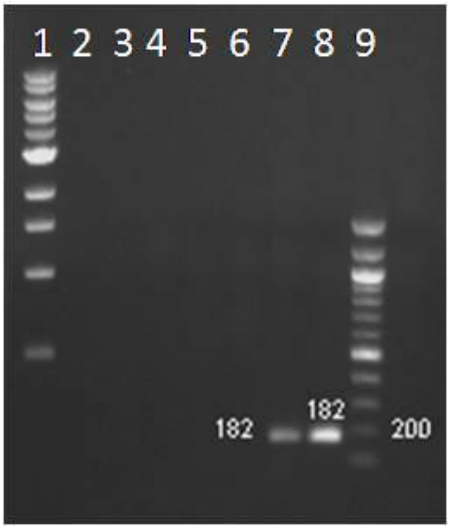
Lane	Description
1	1 kb DNA marker
2	*Vi ha* 6
3	*Vi ha* 15
4	*Vi ha* 19
5	*Vi ha* 21
6	*Vi ha* 32
7	*Vi ha* 68
8	VHML (positive control)
9	100 bp DNA marker

Fig. 3.5. Amplification of recombinase gene from phage DNA



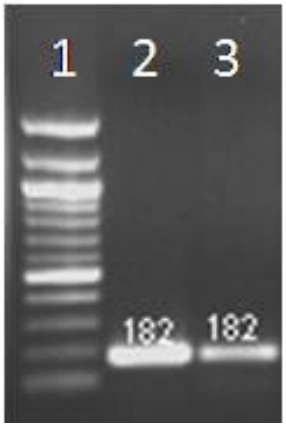
Lane	Description
1	1 kb DNA marker
2	*Vi ha* 6
3	*Vi ha* 15
4	*Vi ha* 19
5	*Vi ha* 21
6	*Vi ha* 32
7	*Vi ha* 68
8	VHML (positive control)
9	100 bp DNA marker

Fig. 3.6. Amplification of transposase gene from phage DNA



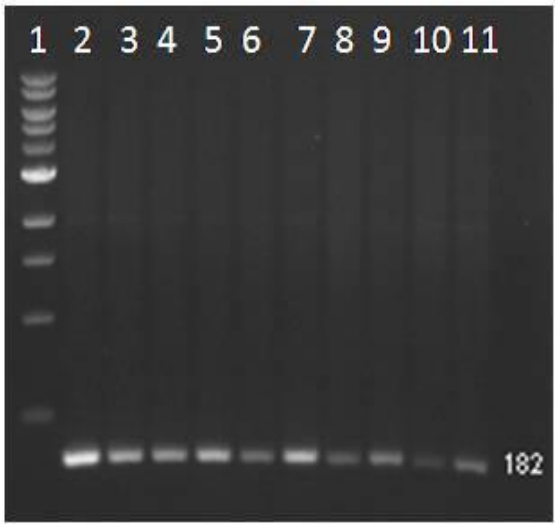
Lane	Description
1	1 kb DNA marker
2	*Vi ha* 6
3	*Vi ha* 15
4	*Vi ha* 19
5	*Vi ha* 21
6	*Vi ha* 32
7	*Vi ha* 68
8	VHML (positive control)
9	100 bp DNA marker

Fig. 3.7. Amplification of Dam toxin gene from phage DNA



Lane	Description
1	100 bp DNA marker
2	Dam toxin gene amplified from *Vi ha* 68
3	Dam toxin gene amplified from VHML

Fig. 3.8. Dam Toxin genes amplified from *Vi ha* 68 and VHML positive control

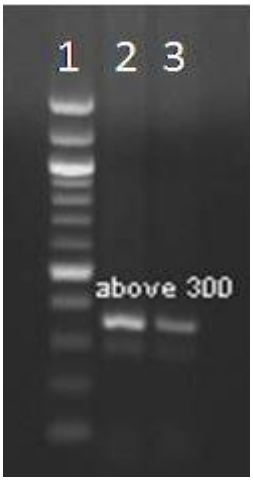


Lane 1: 1 kb marker

Lanes 2, 3, 4, 5, 6 :
Dam toxin gene amplified from *Vi ha* 68

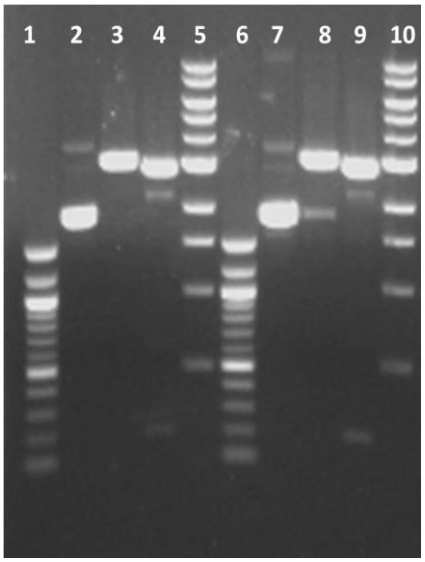
Lanes: 7, 8, 9, 10, 11:
Dam toxin gene amplified from VHML

Fig. 3.9. Colony PCR of cloned PCR product from *Vi ha* 68 and VHML



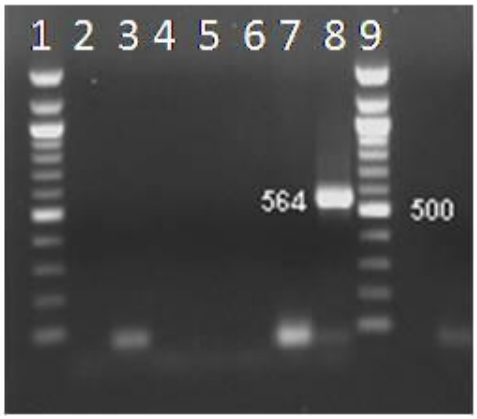
Lane	Description
1	1 kb marker
2	Dam toxin gene cloned in to pGEM-T Easy vector amplified from *Vi ha* 68
3	Dam toxin gene cloned in to pGEM-T Easy vector amplified from VHML

Fig. 3.10. Colony PCR of cloned PCR products from *Vi ha* 68 and VHML



Lane	Description
1	100 bp marker
2	plasmid in *Vi ha* 68
3	linearised plasmid (Sal 1 treated) in *Vi ha* 68
4	182 bp insert released from plasmid in *Vi ha* 68 (EcoR1 treated).
5	1 kb marker
6	100 bp marker
7	plasmid in VHML
8	linearised plasmid (Sal 1 treated) in VHML
9	182 bp insert released from plasmid in VHML EcoR1 treated).

Fig. 3.11. Dam toxin gene cloned in to pGEM T vector from *Vi ha* 68 and VHML



Lane	Description
1	1 kb DNA marker
2	*Vi h*a 6
3	*Vi ha* 15
4	*Vi ha* 19
5	*Vi ha* 21
6	*Vi ha* 32
7	*Vi ha* 68
8	*Vibrio cholera* MTCC 3906 (positive control)
9	100 bp DNA marker

Fig. 3.12. Amplification of *Ctx A* gene from phage DNA

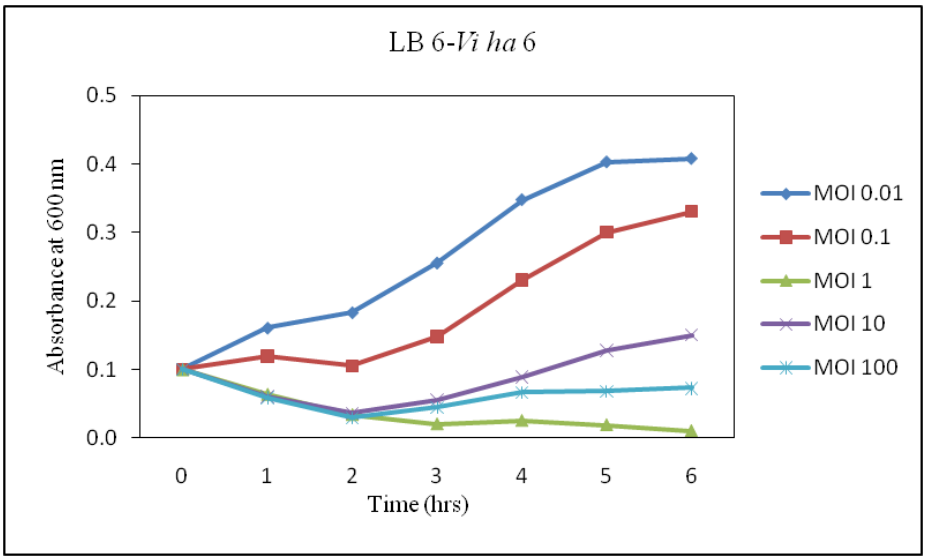


Fig. 3.13.a. Determination of optimal MOI based on absorbance in LB 6-*Vi ha* 6

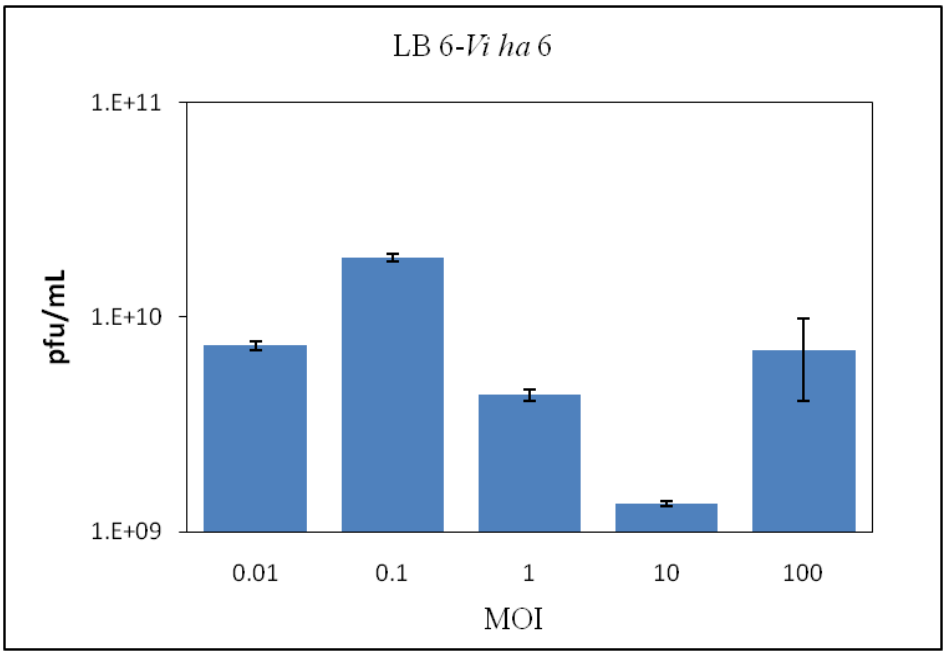


Fig. 3.13 b. Determination of optimal MOI based on phage titre in LB 6-*Vi ha* 6

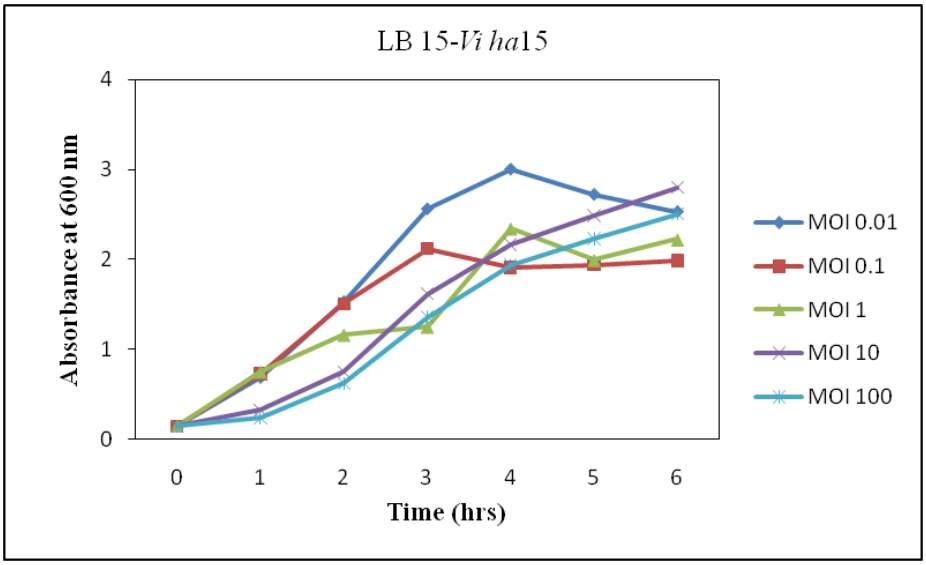


Fig. 3.14.a. Determination of optimal MOI based on absorbance in LB15-*Vi ha* 15

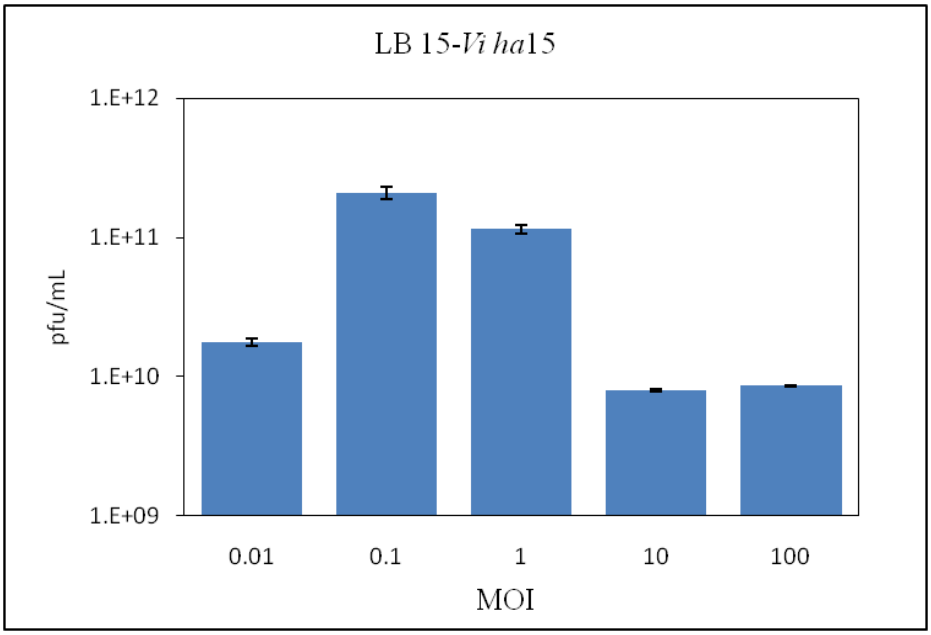


Fig.3.14.b. Determination of optimal MOI based on phage titre in LB 15-*Vi ha* 15

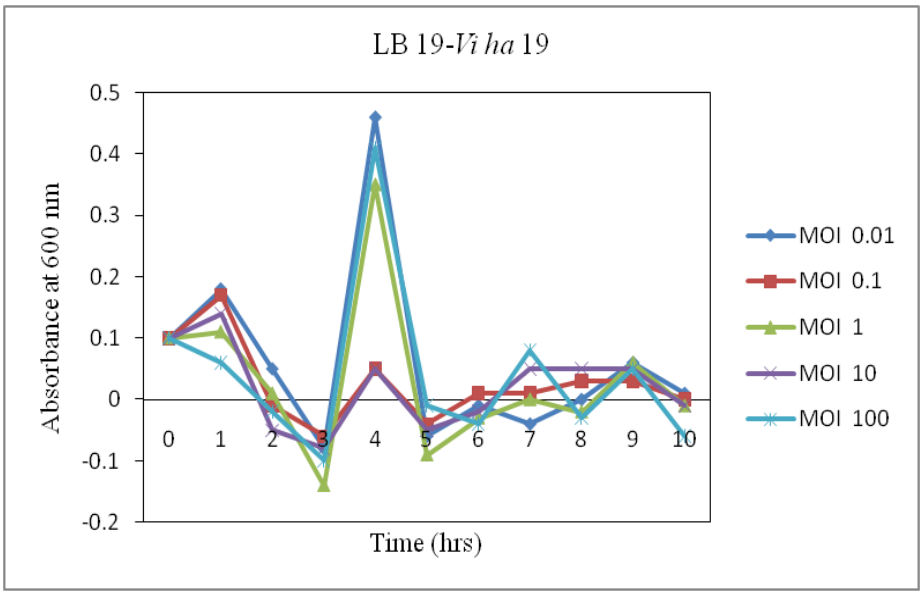


Fig. 3.15.a. Determination of optimal MOI based on absorbance in LB 19-*Vi ha* 19

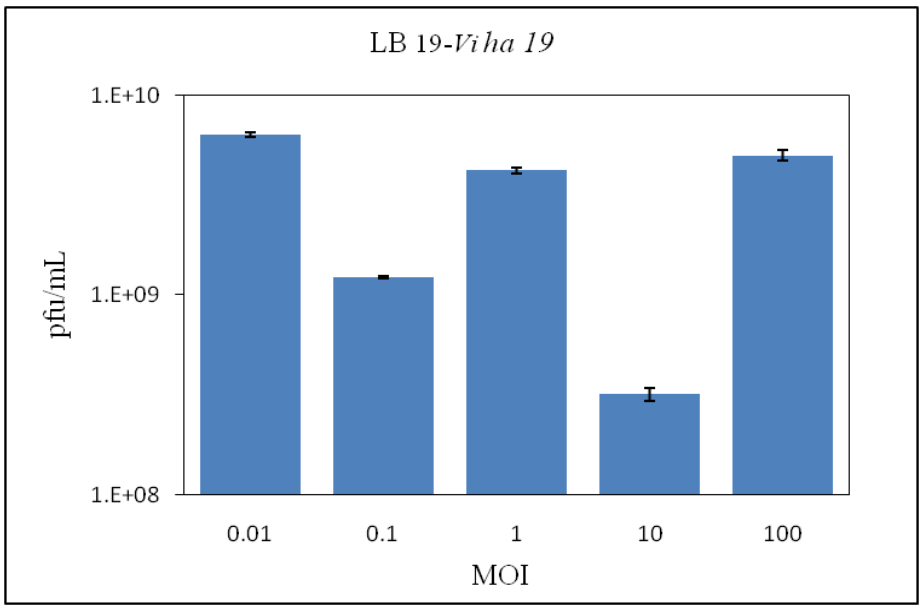


Fig.3.15.b. Determination of optimal MOI based on phage titre in LB 19-*Vi ha* 19

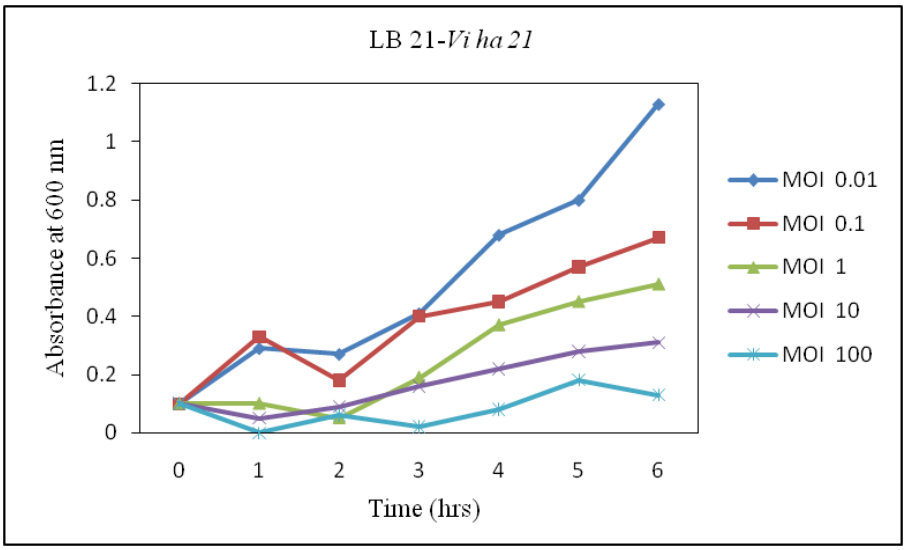


Fig. 3.16.a. Determination of optimal MOI based on absorbance in LB 21-*Vi ha* 21

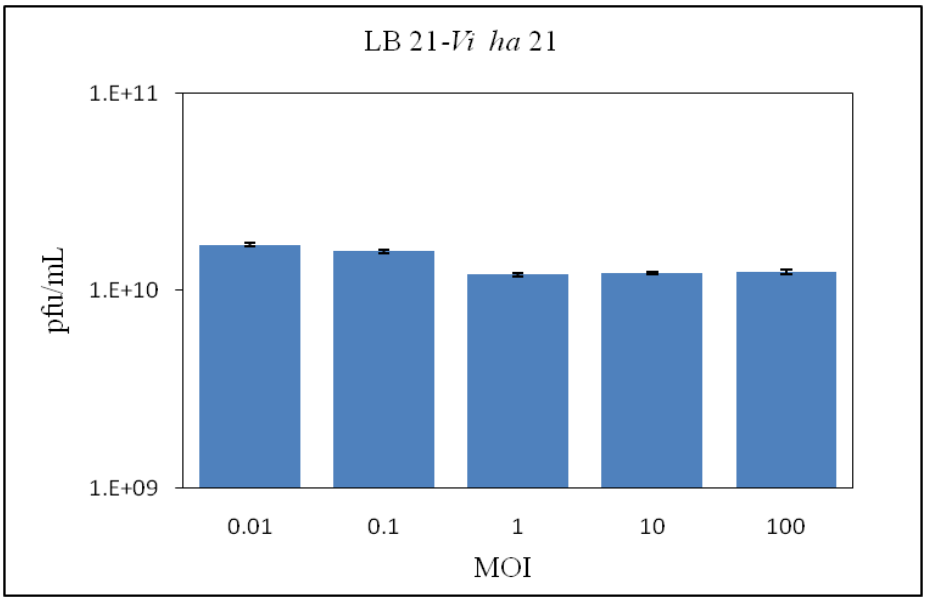


Fig. 3.16.b. Determination of optimal MOI based on absorbance in LB 21-*Vi ha* 21

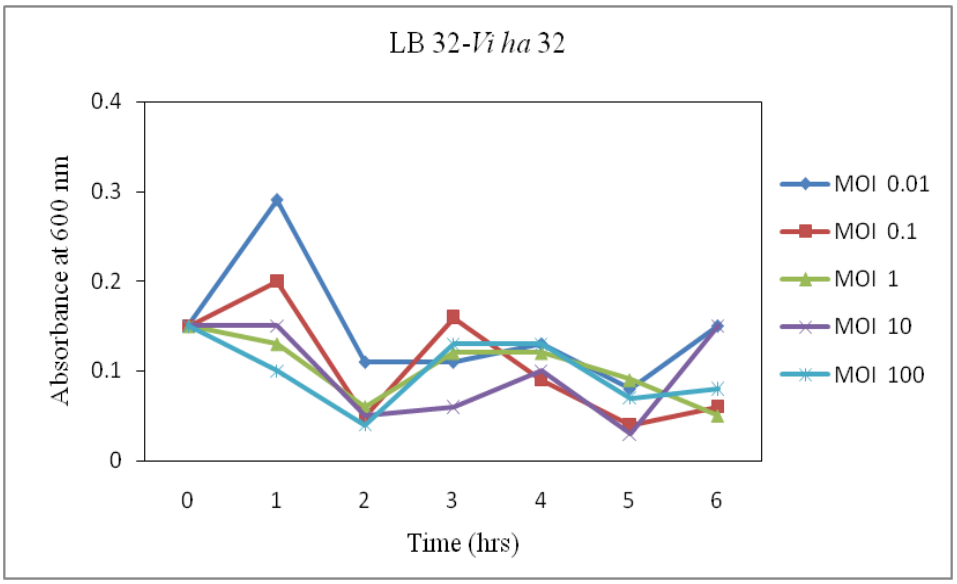


Fig. 3.17.a. Determination of optimal MOI based on absorbance in LB 32-*Vi ha* 32

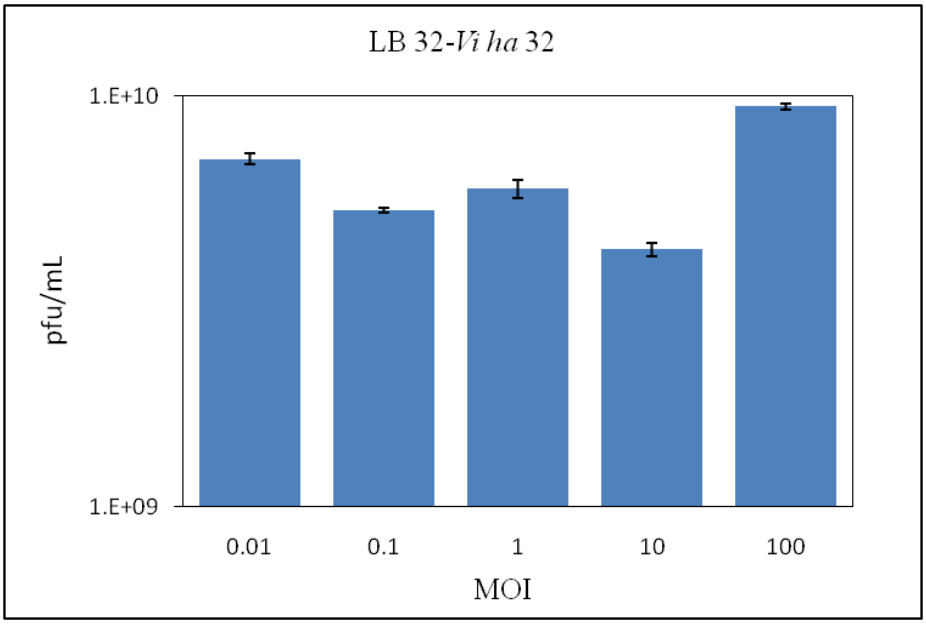


Fig.3.17.b. Determination of optimal MOI based on phage titre in LB 32-*Vi ha* 32

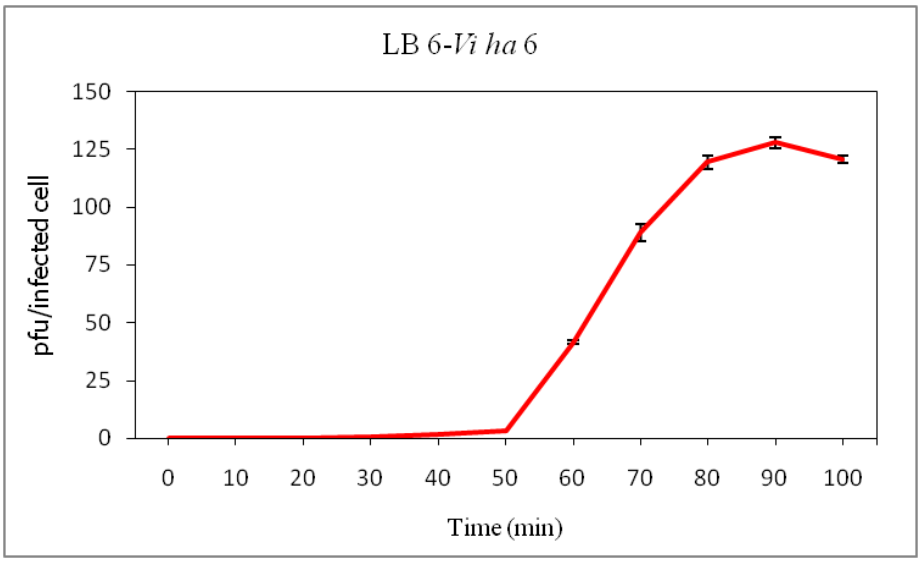


Fig. 3.18. One step growth curve – *Vi ha* 6

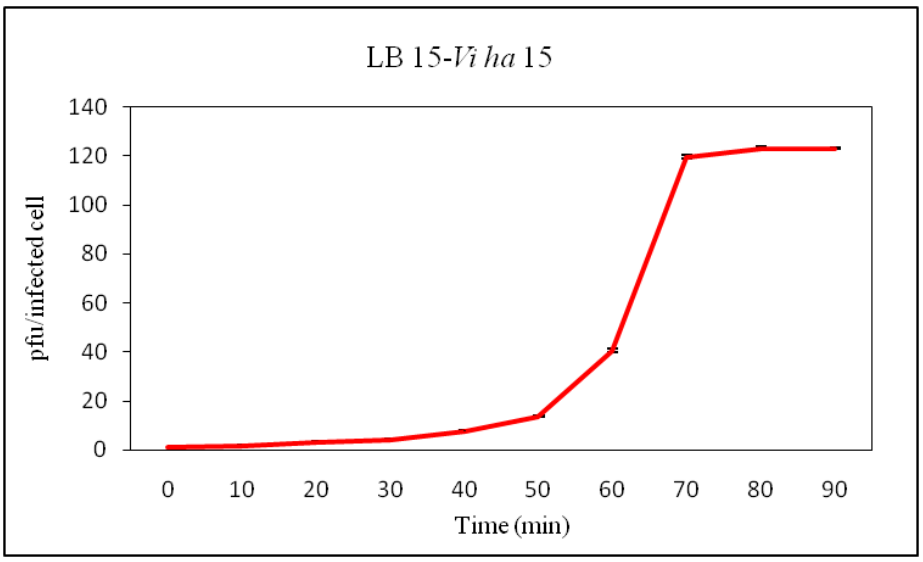


Fig. 3.19. One step growth curve – *Vi ha* 6

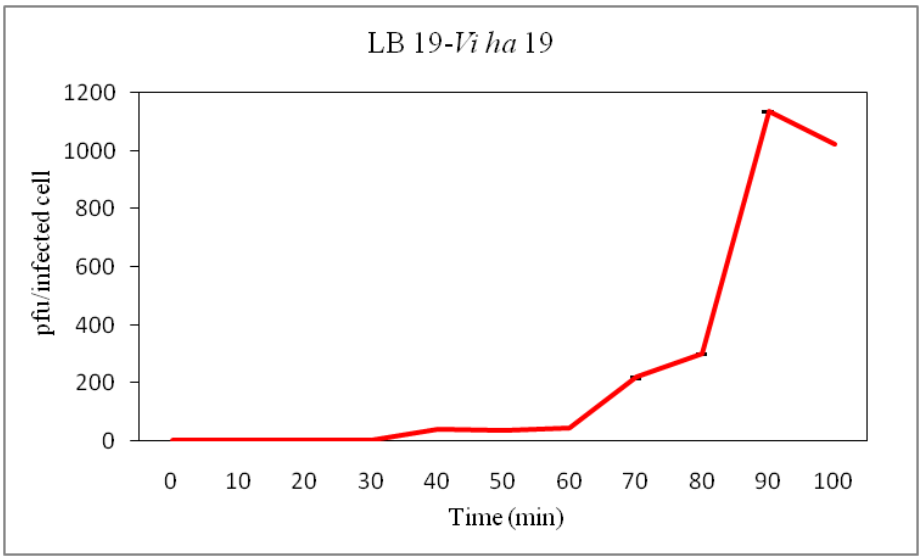


Fig. 3.20. One step growth curve – *Vi ha* 19

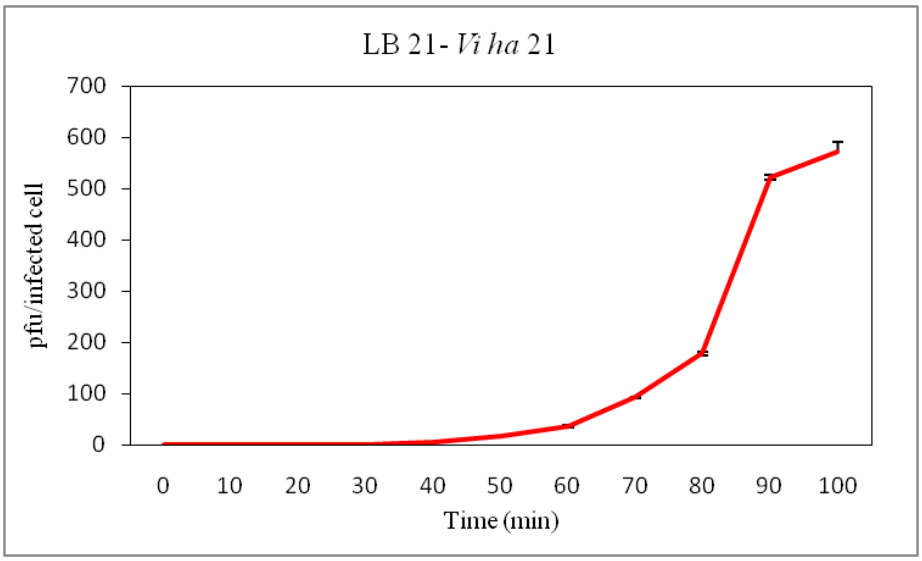


Fig. 3.21 One step growth curve – *Vi ha* 21

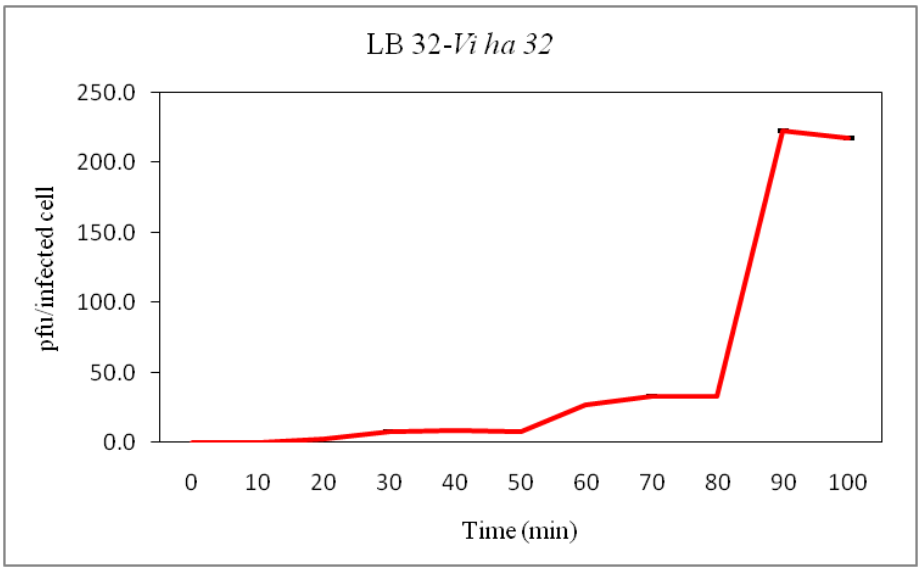


Fig. 3.22. One step growth curve – *Vi ha* 32

Chapter-4

Evaluation of biocontrol potential of therapeutic phages individually and as cocktail *in vitro* and *in vivo*

4.1 Introduction

Phages are the most abundant entities as there are more than 10^{30} phage particles estimated to be around the globe (Brussow and Hendrix, 2002). However, only a few strains prove to be effective as therapeutic antibacterial agents. There are a number of factors that affect the therapeutic efficacy of phages. Studies of phage multiplication in bacteria *in vitro* provide information as to which phage would be appropriate for the therapeutic application against a specific bacterial infection. Meanwhile, it has to be realized that, observation of phage multiplication in defined culture media need not necessarily be a reflection of the interaction of phages with bacteria *in vivo*. This is because, bacterial gene expression and their phenotype are influenced by innumerable variables *in vivo*, ranging from differences in the available basic nutrients to altered physical parameters including pH and ionic strength (Calendar, 2006). In addition, *in vivo* infections have the added complication of interactions between the innate and active immune system of the test animals (Calendar, 2006).

4.1.1 Therapeutic potential of the phages *in vitro*

Potential of bacteriophages as therapeutic agents invariably has to be evaluated thoroughly under *in vitro* conditions prior to bio assays. This includes dosage of therapeutic phages required for *in vivo* experiments, optimum temperature (O'Flynn *et al.*, 2004) salinity and pH (Capra et al., 2006; Ramesh, 1999).

4.1.2 Animal models used for *in vivo* experiments on phage therapy

Animal models used in phage therapy include mice (Biswas *et al.*, 2002), chicken, calves (Barrow *et al.*, 1998), hamster (Ramesh, 1999), *Drosophila melanogaster (*Heo *et al., 2009)* etc. Besides, *Artemia* has also been reported as a crustacean model system to determine the efficacy of phage therapy (Gunawardhana, 2009).

4.1.3 *Artemia* as animal model

An ideal *in vivo* model generally used to study crustacean survivability in terms of bacterial pathogenicity and demonstration of efficacy of therapeutics is the nauplii of gnotobiotic *Artemia* (brine shrimp). The advantage of using *Artemia* as animal model are several such as the availability of its cysts throughout the year, easiness of hatching the cysts following simple procedures and ease of their culturing and maintenance under gnotobiotic conditions. It has previously been reported as model system in the study of *Vibrio harveyi* as pathogen (Soto-Rodriguez *et al.*, 2003) and to screen effective antibiotics against infectious *Vibrio harveyi* (Roque and Gomez-Gil, 2003) and to demonstrate VHML mediated virulence in avirulent strains of *Vibrio harveyi* (Austin *et al*., 2003). Literature on this is exhaustive beyond the scope of this chapter.

4.1.4 *Penaeus monodon* as animal model

Penaeus monodon has been widely used in bio assay systems to determine lethal infectivity dosage of a number of viral and bacterial pathogens. Such studies have been supported by histopathology and PCR based detection of the pathogens. Abundant literature is available on the use of *P. monodon* in the study of White Spot Syndrom Virus (WSSV), Monodon Baculo Virus (MBV), Taura Syndrome Virus (TSV) (Mishra et al., 2005; Prior et al., 2003; Rajendran et al., 1999). For the in *vivo* study of bacterial pathogens such as *Vibrio harveyi* (Liuxy *et al.*, 2008), *Vibrio alginolyticus* (Lee *et al.*, 1996), *Vibrio parahaemolyticus* (Sudheesh and Xu, 2001) *P. monodon* has been used repeatedly. In terms of studies related to phage

therapy, *P. monodon* has been used to assess the therapeutic ability of phages against *Vibrio harveyi* (Vinod *et al.*, 2006).

4.2 Materials and Methods

4.2.1 *In vitro* lytic efficacy of *Vibrio harveyi* phages under nutrient rich conditions

Phage challenges were performed to investigate the ability of individual phages in nutrient rich condition to lyse their specific host bacterium under *in vitro* conditions. Phages were added at respective optimal MOI to log - phase ($1x10^8$ CFU/mL) cultures of the host bacterium (*Vibrio harveyi*) in ZoBell's broth and incubated for 8 hours in an orbital shaker (120 rpm) at room temperature. All the five bacterial hosts (LB 6, LB 15, LB 19, LB 21 and LB 32) were individually challenged with their specific phages (*Vi ha* 6, *Vi ha* 15, *Vi ha* 19, *Vi ha* 21 and *Vi ha* 32). Besides, phages as cocktail (phages in combination) and the most potent phage *Vi ha* 19 (phage that allowed no emergence of resistant bacteria when individually tested) were challenged to the consortium of 5 isolates of *Vibrio harveyi*. Samples were taken every hour to enumerate both phage particles and bacterial cells following overlay method of plaque assay for plaque forming units and spread plate method for colony forming units respectively in triplicate after serial dilutions (10^1-10^9). Representing all host bacterial isolates of *V. harveyi*, bacterial count of LB19 without phage was worked out using spread plate method and plotted (Fig. 4.6) along with phage treated bacterial count for revealing the growth pattern of untreated bacterial control.

4.2.2 *In vitro* lytic efficacy of *Vibrio harveyi* phages under nutrient limiting conditions (sea water)

In vitro bacterial challenge tests were carried out in nutrient limited conditions also. The mixed bacterial hosts, the consortium of 5 isolates of *Vibrio harveyi* (LB 6,LB 15, LB 19, LB 21and LB 32) were subjected to infection by the phage cocktail (*Vi ha* 6, *Vi ha* 15, *Vi ha* 19, *Vi ha* 21 and *Vi ha* 32) and the most potent

phage *Vi ha* 19 at their respective optimal MOI. The medium used for the experiment was sterilized sea water containing 0.01 g/100 mL peptone. The preparations were incubated with gentle agitation of 60 rpm at room temperature. Samples were drawn at an interval of 6 hours to enumerate both phage particles and bacterial cells from 0 to 72 hrs after inoculation. Serial dilutions were made and both phages and bacteria were plated in triplicates by overlay method and spread plate method respectively.

4.2.3 Evaluation of the potential of *Vibrio harveyi* phages for bio control of the pathogen in animal models

Nauplii of *Artemia salina* and post larvae of *Penaeus monodon* were used as animal models and challenged with pathogenic *Vibrio harveyi* at selected bacterial doses, which were further infected by respective *V. harveyi* specific phages, keeping the MOI as 10.

4.2.3.1 Therapeutic potential of *Vibrio harveyi* phages on gnotobiotic *Artemia salina* nauplii

Artemia nauplii were used as model crustacean to study the efficacy of *Vibrio harveyi* phages to control and / or prevent experimental infection by *Vibrio harveyi* (Payne and Jansen, 2001) prior to use them in shrimp. The use of *Artemia* nauplii as model organism has been reported previously in biological infections (Roque and Gomez-Gil, 2003; Soltanian, 2007) including the infection by *Vibrio harveyi* phages (Eduardo and Sergio, 2009; Gunawardhana, 2009).

4.2.3.1.1 Hatching of *Artemia* cysts

Artemia nauplii were reared according to the methodology proposed by Sorgeloos *et al*. (1977). About 2 g *Artemia salina* cysts were immersed in tap water for 30 minutes and then in 1L tap water containing 20 % sodium hypochlorite solution for decapsulation. Subsequently the hypochlorite content was removed by washing 3 times by passing through 500mL sterile sea water using a sieve of 112 µm. The

washed content was retained in the sieve and transferred to 1L 30 ppt sterile sea water with adequate aeration through an air sparger and temperature mainatained at around 30°C by lighting 60 W electric bulb nearby. The cysts normally hatch out after 12-18 hrs of incubation in seawater.

Healthy nauplii which hatched out of the cysts were attracted towards light and collected by a scoop net. They were disinfected by treating (immersion) with 2 mL of antibiotic mixture containing Pencillin and Streptomycin sulphate in 1000 mL sterile sea water for 6 hrs. Antibiotic residues were washed away using sterile sea water and a mesh and re-suspended in fresh sterile sea water. To evaluate the presence of antibiotic residue, 20 nauplii were homogenized in 3 mL 15 ppt sterile sea water, and placed 20 µL on a pack of 5 Whatman No. 1 filter paper discs, placed on ZoBell's agar plates swabbed with 18h grown *Vibrio harveyi* (LB 3) broth culture. The plates were observed for clearing zone after 24 hrs demonstrating the presence of antibiotic residue. Antibiotic free larvae were used for further study.

4.2.3.1.2 Pathogenicity of the consortium of *Vibrio harveyi* on *Artemia* nauplii

Pathogenicity of the consortium of 5 isolates of *Vibrio harveyi* (LB 6, LB 15, LB 19, LB 21and LB 32) was determined primarily by inoculating the bacterial consortium to the final concentration of 10^6, 10^7 and 10^8 CFU/mL in 10 mL volume of sterile sea water in test tubes. In total, 200 *Artemia* nauplii were introduced at a ratio of 20 nauplii per mL sterile seawater and incubated on a shaking water bath at room temperature (30 ^{0}C) for 48hours. Mortality of the larvae was determined and the inoculum size of the consortium of *V. harveyi* associated with the highest percentage mortality of larvae after 24 hours was accepted for further experimentation. In order to confirm the cause of death, the moribund nauplii (5 nos) were washed with sterile sea water, macerated and

serially diluted. Each dilution was spread plated on VHA. Colonies formed on the plates were counted after incubation.

4.2.3.1.3 Efficacy of the phages in protecting *Artemia nauplii* from *Vibrio harveyi*

This experiment was conducted with the inoculum size of the consortium of *V. harveyi* which resulted in the highest mortality of Artemia nauplii, after 24 hours. It was conducted in 30mL capacity test tubes with 10 mL sterile seawater and 20 *Artemia* nauplii per mL. Aliquots of freshly grown *V. harveyi* consortium were inoculated to obtain the size of $1x10^7$ CFU/mL required for the highest recorded mortality after 24 hours of incubation. Subsequently, the tubes were inoculated with the cocktail of *V. harveyi* phages and *Vi ha* 19 to attain the optimal MOI (10). The preparation was kept in shaking water bath to provide constant temperature of 30^0 C and aeration/agitation for 48 hours. From each tube phage count as PFU and bacterial count as CFU were determined for 48 hours at different intervals of time. Besides, the extent of survival of *Artemia* nauplii in each tube was also determined. In order to confirm the cause of death, the moribund nauplii (5 nos) were washed with sterile sea water, macerated, serially diluted and spread plated on VHA. Colonies formed on the plates were counted after incubation.

4.2.3.2 Therapeutic potential of *Vibrio harveyi* phages on post larvae (PL5) of *Penaeus monodon*

Post larvae of *Penaeus monodon* (PL5) were obtained from a commercial hatchery, Abad Hatchery, Kandakkadavu, Kochi. They were allowed to acclimatize, fed with gnotobiotic *Artemia* and provided with aeration and illumination. The larvae were tested for white spot syndrome virus (WSSV) by polymerase chain reaction (PCR) (Lo *et al.*, 1996). The presence of luminous *Vibrio* was confirmed by plating each serial dilution of 5 larvae macerated in 5 mL sterile seawater on VHA.

4.2.3.2.1 Pathogenicity of *Vibrio harveyi* on post larvae of *Penaeus monodon*

Pathogenicity of the consortium of *Vibrio harveyi* on the post larvae of *P. monodon* was determined by inoculating the bacterial cultures in consortium to the final concentration of 10^7, 10^8 and 10^9 CFU/mL in plastic transparent 2 L tubs where the larvae were maintained at 30 numbers per litre sterile seawater under constant aeration through cartridge filters of 0.45µm porosity. Mortality of the larvae was determined and the inoculum size of the consortium of *V. harveyi* associated with the highest percentage mortality with in a period of five days was considered for further experimentation. In order to confirm the cause of death, the moribund larvae were collected, washed in sterile sea water, homogenized, serially diluted and spread plated on VHA and incubated..

4.2.3.2.2 Efficacy of the phages in protecting post larvae of *P. monodon* from Vibrio *harveyi*

Post larvae of *P. monodon* were maintained at a density of 30 larvae per litre in plastic tubs of 2 L capacity having 1.5 L sterile sea water of 30 ppt salinity. They were fed on freshly hatched and gnotobiotic treated *Artemia* nauplii. After 24 hours of acclimation the post larvae were challenged with different strains of *V. harveyi* (LB 6, LB 15, LB 19, LB 21and LB 32) individually and as consortium, grown in ZoBell's broth to attain the predetermined inoculum size sufficient to cause mortality ($1x10^8$ CFU/mL). After 30 min of inoculation, phages and phage cocktail (*Vi ha* 6, *Vi ha* 15, *Vi ha* 19, *Vi ha* 21 and *Vi ha* 32) were added at MOI 10. The controls included experimental set up with the bacterial cultures and with phage lysates alone. From each tub *Vibrio harveyi* count was determined by plating on VHA for three days once in 24 hours. Survival of post larvae was recorded regularly till the completion of the experiment. In order to confirm the cause of death, the moribund larvae were collected, washed, homogenized and serially diluted. Each dilution was spread plated and confirmed as *Vibrio harveyi* after incubation based on the colony characteristics.

4.2.3.3 Statistical Analyses

All experiments were done in triplicate and the results presented as mean with standard deviation. Karl Pearson's coefficient of correlation was worked out to find the relationship between bacterial reduction and phage multiplication upon time in all *in vitro* experiments. Two way ANOVA was carried out to find the significant variations in the treatments in all *in vivo* phage therapeutic experiments.

4.3 Results

4.3.1 *In vitro* lytic efficacy of *V. harveyi* phages under nutrient rich conditions

On challenging LB6 with *Vi ha* 6 at optimal MOI (0.1), the bacterial count got reduced by two log within the first one hour and maintained the same population density until the fourth hour (Fig. 4.1) subsequent to which a gradual increase in cell count was observed till the termination of the experiment. Meanwhile, phage titre went up by 2 log with in the first two hours of incubation subsequent to which a gradual increase could be recorded. The effective bacterial reduction throughout the experiment was one log. Though phage count got increased and bacterial count showed an overall reduction, there did not exist significant Karl Pearson correlation between phage titre and bacterial count due to the emergence of resistant bacteria during the second half period of the experiment ($r = 0.027$).

When LB 15 was challenged with *Vi ha* 15 at optimal MOI of 0.1, phage reduced the host strain by four log within the first hour onwards until fourth hour of the experiment with a two log increment in phage titre (Fig. 4.2). Beyond the fourth hour bacterial load slowly began to rise due to the emergence of resistant forms and grown up to more than two log by the end of the experiment. The effective bacterial reduction is more than one log and phage titre increment by five log. Karl Pearson correlation tells that there is no significant correlation between the count of bacteria and phage at 0.01 level ($r = -0.142$).

When *Vi ha* 21 was treated against LB 21 as shown in Fig. 4.3, less than one log reduction in bacterial titre was found in the first hour of the experiment. Still bacterial count got reduced until 5th hour of the experiment by more than 1 log unit. A slight increase in bacterial count was observed beyond that time point until the end of the experiment due to the emergence of resistant forms. Effective bacterial reduction throughout the experiment resulted was by one log. Phage titre was found to be increased by four log within first two hours and maintained as such until the end of the experiment. Here, when bacterial count decreased, phage count increased and Karl Pearson correlation analysis revealed a significant negative correlation ($r = -0.70$) ($P < 0.01$) between bacterial count and phage count. It confirmed the enrichment of *Vi ha* 21 and simultaneous control of LB 21 in the experimental system.

In the trial against LB 32, *Vi ha* 32 reduced five log of the bacterial load within first 2 hours, which was most remarkable bacterial reduction within the shortest time period (Fig. 4.4). Beyond 2nd hour, bacterial load gradually increases till 7th hr, where resistant forms emerged. Effective bacterial reduction was more than two log by the end of the experiment. There was no significant increment in the phage titre, on the other hand it was more or less same throughout the experimental period. There is a positive correlation ($r = 0.45$) between phage and bacterial count.

In the case of system LB 19/*Vi ha* 19, more than two log reduction in bacterial load was found within the first two hours with more than three log increment in phage titre (Fig. 4.5). More than four log bacterial reduction was resulted throughout the experiment without the emergence of resistant bacterial forms. Compared to the bacterial count in untreated bacterial control, phage treatment brought down the bacterial load significantly about by about 6 log units at the end of the experiment. Phage titre was found increased within the first three hours and maintained steady until the end of the experiment. The bacterial count would have still been reduced had the experiment been continued beyond 8 hours. This observation could be

supported with the Abs $_{600}$ measurement of LB 19 (Chapter 3; Fig. 3.15.a) when treated with *Vi ha* 19, as Abs $_{600}$ was found to be zero at 10th hour. This is the only phage which did not allow the resistant bacteria to emerge. Karl Pearson correlation analysis indicated that there is significant negative correlation between bacterial count and phage count ($r = -0.66$) ($P < 0.01$).

When a consortium of *Vibrio harveyi* (LB 6, LB 15, LB 19, LB 21, LB 32) was challenged with a cocktail of *Vibrio* phages (*Vi ha* 6, *Vi ha* 15, *Vi ha* 19, *Vi ha* 21, and *Vi ha* 32) about two log unit reduction of bacterial count resulted within the first one hour of incubation (Fig. 4.6). Subsequently the bacterial counts started increasing and outnumbering the initial count at the end of 8 hours of incubation. Meanwhile, the phage titre remained almost stable throughout the experimental period. There was significant positive correlation ($r = 0.75$) ($P < 0.01$) between the phage and bacterial count as the bacterial load in the final hours has gone up due to resistant forms.

When the bacterial consortium was challenged with *Vi ha* 19 (Fig. 4.7) one log bacterial reduction was observed during the first two hours subsequent to which a gradual increase in the bacterial population was observed for 7 hours. However during the 8th hour the bacterial count declined by 1 log. Meanwhile, the titre of the bacteriophage *Vi ha* 19 registered a declining trend during the first two hours and exhibited sharp increase during rest of the hours of incubation. Over all, the Karl Pearson correlation ($r = 0.485$) between phage and bacterial counts was found to be positive as both phage titre and bacterial count showed a decline at 2nd hour of the experiment and a rise subsequently.

4.3.2 *In vitro* lytic efficacy of *V. harveyi* phages under nutrient limited conditions

Under nutrient limited conditions, the consortium of *V. harveyi* exhibited 4 log count increase with in the first 30 hours, then remained stationary for another 30

hrs and showed a declining trend subsequently. When the *V. harveyi* consortium was challenged with the cocktail of phages and *Vi ha* 19, 4 log and 2 log reduction in bacterial count as observed at 12th hr. At the 30th hr 1 log reduction was observed in both the phage treated systems. At 72 hr *Vi ha* 19 showed 1 log reduction compared to other two systems (Fig. 4.8). As given in Fig. 4.9, the phage titre under both the set of experiments exhibited a similar pattern of 4 log increase in titre within the first 36th hr, slight reduction till 42nd hr and a stationary phase till the end of the experiment (72nd hr). Karl Pearson correlation suggested that there was no significant correlation between phage and bacterial count in the case of challenge of the *V. harveyi* consortium with phage cocktail (r = 0.18) whereas there was positive correlation (r = 0.360) between *Vi ha* 19 and bacterial count.

4.3.3 Evaluation of the potential of *Vibrio harveyi* phages for bio control of the pathogen in animal models

Nauplii of *Artemia salina* and post larvae of *Penaeus monodon* were used as animal models and challenged with pathogenic *Vibrio harveyi* at selected doses, which were further infected by respective *V. harveyi* specific phages, keeping the MOI as 10.

4.3.3.1 Therapeutic potential of *Vibrio harveyi* phages on gnotobiotic *Artemia salina* nauplii

4.3.3.1.1 *Artemia* hatching

More than 90% cysts were found hatching to healthy nauplii which after antibiotic treatment and washing were found without any antibiotic activity.

4.3.3.1.2 Pathogenicity of consortium of *V. harveyi* on *Artemia* nauplii

Out of the three doses of consortium used such as 1x10^6, 1x10^7, and 1x10^8 CFU/mL, 10^6 CFU/mL could not cause even 50% mortality during the treatment period of 48 hours. Meanwhile the dosage of 10^8 CFU/mL produced an early mortality at 4th hour of treatment (Fig. 4.10). Meanwhile, the dosage

$1x10^7$CFU/mL resulted in 50% mortality between the incubation period of 28 and 32 hours and hence this dosage was chosen for further experimentation.

4.3.3.1.3 Efficacy of phages in protecting *Artemia* nauplii from *V. harveyi*.

When phage cocktail and *Vi ha* 19 were inoculated separately on the consortium of *V. harveyi*, higher survival of *Artemia* nauplii compared to untreated control could be obtained (Fig. 4.11). At 28th hour of the incubation, when 45 % of the nauplii were dead in untreated control, 82 and 83 % survival of larvae could be obtained in the systems which were inoculated with phage cocktail and *Vi ha* 19 respectively. At the end of 48 hour of experiment 80% mortality was recorded in the untreated control group, against 18 and 17 % mortality in the phage cocktail and *Vi ha* 19 treated systems respectively. Analysis of variance revealed that there was significant difference between treatments ($P<0.05$) in terms of survivility, whereas no significant difference in terms of bacterial count. However, both the phage treated systems show more than 1 log reduction in bacterial count (Fig. 4.12) compared to the untreated control especially towards the termination of the experiment. With respect to the phage titre in *Artemia* nauplii with the cocktail and *Vi ha*19 there was 4 log increase towards the end of 48hour experimental period (Fig. 4.13), the increase of the titre of *Vi ha* 19 was of higher magnitude than that of the cocktail. In Fig. 4.14 disappearance of live *Artemia nauplii* in untreated control due to mortality, and survival in both phage treated tubes are presented.

4.3.3.2 Therapeutic potential of *Vibrio harveyi* phages on post larvae (PL5) of *Penaeus monodon*

4.3.3.2.1 Pathogenicity of *Vibrio harveyi* on post larvae of *Penaeus monodon*

Out of the three dosages such as $1x10^7$, $1x10^8$ and $1x10^9$ CFU/mL, the dosage $1x10^8$ CFU/mL was chosen for further experimentation due to the fact that 50 % mortality occurred between day 2 and 3 out of the five day long experiment. Meanwhile at $1x10^7$ CFU/mL, percentage mortality of *P. monodon* PL was below

10. However, when the inoculum size was increased to 10^9 CFU/mL total mortality of the larvae occurred within a day (Fig. 4.15).

4.3.3.2.2 Efficacy of phages in protecting post larvae of *P. monodon* from *V. harvayi*

(a) LB 6 vs *Vi ha* 6

In case of LB 6/*Vi ha* 6 system survival was slightly higher in the untreated control (Fig. 4.16). Meanwhile, there was not much difference in the survival of PL in the system treated with *Vi ha* 6 (51%) and the one with *V. harveyi* LB 6 alone (53%). The larvae with phage alone were kept as phage control which showed 93% survival. *V. harveyi* count was higher in phage treated animal on the 1st day (Fig. 4.17), which declined sharply to the third day nearing zero, matching with the bacterial count of the untreated system.

(b) LB 15 vs *Vi ha* 15

When post larvae were challenged with *V. harveyi* (LB 15) and compared with the system having LB 15 and *Vi ha* 15, post larvae survived well in phage treated system by 58% (Fig. 4.18) at the end of fifth day experiment, while the untreated system exhibited16% survival. Meanwhile, the phage administered control larvae showed 95% survival. On considering the bacterial count, both the treatments showed more or less the same initially which on the second and third days declined sharply to undetectable levels (Fig. 4.19). But the bacterial count in phage treated systems started to decline on the second day onwards and on the third day, both the bacterial counts were found declined sharply to undetectable levels.

(c) LB 19 vs *Vi ha* 19

When *V. harveyi* (LB 19) challenged post larvae were treated with the phage *Vi ha* 19, 48% survival was observed against untreated control (18%) on the 5th day of the experiment. In the phage control system where phage was alone administered

93% survival could be observed (Fig. 4.20). *V. harveyi* count was very low in the system administered with the phage compared to the one with *Vibrio* alone (Fig. 4.21).

(d) LB 21 vs *Vi ha* 21

Survival of *P. monodon* post larvae challenged with *V.harveyi* (LB 21) and treated with the phage *Vi ha* 21 was lesser (38%) during the five day experiment than that of the one challenged with the pathogen and not treated with the phage (59%) registering 20% drop .However, the phage control (larvae treated with the phage alone) showed 93 % survival (Fig. 4.22). Corresponding to these results the *V. harveyi* count on treating with *Vi ha* 21 showed sharp increase on the first day and declined on the subsequent days. Meanwhile the untreated post larvae registered a much lesser count (Fig. 4.23) of *V. harveyi*.

(e) LB 32 vs *Vi ha* 32

On completion of five day experiment the larvae challenged with *V. harveyi* (LB 32) and treated with *Vi ha* 32 showed 58 % survival compared to the larvae treated with the LB 32 and untreated with the phage (15%). Meanwhile the larvae treated with the phage *Vi ha* 32 (Fig. 4.24) registered 92% survival at the end of five day experiment. The bacterial count in *P. monodon* larvae challenged with LB 32 and treated with *Vi ha* 32 was much lower (100 CFU/mL) than that of the larvae challenged with LB 32 and not treated with the phage (Fig. 4.25).

In all instances, survival of larvae challenged with *V.harveyi* and treated and untreated with the respective phages and the *Vibrio harveyi* count of the same were significantly ($P < 0.05$) different.

(f) Consortium of *Vibrio harveyi* vs *V. harveyi* phage cocktail and *Vi ha* 19

The pattern of survival of *P. monodon* post larvae challenged with *V.harveyi* consortium and treated with the phage cocktail and *Vi ha* 19 were more or less uniform registering a high survival of 60 and 64% compared to the batch of larvae challenged with *Vibrio harveyi* consortium alone (17%) (Fig. 4.26), which were significantly different ($P<0.05$) from each other.

Vibrio harveyi load (in water) of the three treatments were compared and plotted (Fig. 4.28). When bacterial count at day 1 got increased more than 1 log unit in the untreated control, both the phage treated groups showed 1 log unit reduction. Precisely, a difference of 2 log was brought about between untreated and treated experimental groups. This must have caused detrimental effect on larvae of the control group. However, on day 2 , untreated control bacteria showed a decreasing trend in the count which got enhanced on the 3rd day than both the treated groups. At the end of the experiment the one treated with *Vi ha* 19 exhibited lowest count of *V. harveyi*. Higher bacterial count was observed in the untreated post larvae compared to the treated ones as shown in Fig. 4.27. Both the treated larvae were found to have very less count of *V. harveyi.* which were similar in magnitude.

Vibrio harveyi from moribund post larvae of *P. monodon* and *Artemia* nauplii plated on VHA could be identified based on colony characteristics and the death was related to the pathogenicity caused of *Vibrio harveyi*.

4.4 Discussion

In vitro lytic efficacy of *V. harveyi* phages under nutrient rich conditions was assessed on 5 *V. harveyi* strains with 5 respective phages, out of which the highest lytic efficacy was demonstrated by *Vi ha* 19 and *Vi ha* 32. *Vi ha* 19 reduced bacterial load by four log unit without the emergence of resistant forms. Compared to the untreated control, about 6 log unit reduction was noticed in the *Vi ha* 19

treated test flask at the end of the experiment. Therefore the efficacy of *Vi ha* 19 treatment is evident against the target bacteria. *Vi ha* 32 showed five log unit reduction in host bacteria within first two hours, even though resistant forms emerged towards the later stage. On *V. harveyi* consortium also *Vi ha* 19 exhibited better lytic activity compared to the phage cocktail in terms of reduction in bacterial count and thereby reducing the emergence of resistant forms especially at final hours. In all the individually treated nutrient rich *in vitro* experiments, 1-5 log reduction in bacterial load resulted with in the first two hours. However, further significant reduction could not be seen beyond two hours of incubation in any case other than that with *Vi ha* 19, due to the emergence of resistant forms of *Vibrio* which emerged mostly after the 2^{nd} hour of incubation. As pointed out above an exception was the case with *Vi ha* 19 to LB 19 as well as to the consortium where emergence of resistance could be controlled successfully. Similarly, bacteriophage K could control the resistant bacterial forms in a therapeutic trial against methicillin-resistant *Staphylococcus aureus* (MRSA) strains (O'Flaherty et al., 2005a). The resistant bacteria are named as bacteriophage-insensitive mutants (BIMs) (Moineau, 1999) and were reported in majority of phage therapeutic experiments (Nakai et al., 1999; Smith and Huggins, 1982; Smith and Huggins, 1983; Smith et al., 1987). O'Flynn *et al*., 2004 observed that BIMs commonly reverted to be phage sensitive within 50 generations. According to them, the BIMs may not attain the level of turbidity of the parental strain even though both of them reach more or less same count. Documented evidence indicates that BIMs are generally not as virulent as the parent strain (Levin and Bull, 2004; O'Flaherty et al., 2005b; Smith and Huggins, 1982; Smith and Huggins, 1983). Resistance of bacteria to phages is often caused by changes in the phage-receptor molecules in Gram-negative bacteria (Matsuzaki *et al.*, 2005) as a result of mutation. In all experiments the phage titre increased by 3 to 5 log which indicated replication of phages during bacterial killing.

Under nutrient limited conditions, even though *Vi ha* 19 exhibited better lytic activity than the phage cocktail in terms of reduction in bacterial count, BIMs started to emerge at initial stage and was brought under control later on. The extent of reduction of bacterial count in this study was higher compared to the results obtained by Park *et al.* (2000) in nutrient limited conditions where phage therapy was tried against *Pseudomonas plecoglossicida*. In the latter there was only nominal difference in bacterial count between the bacterial control and phage treated experiment groups. Nevertheless, phage therapy did impart protection to Ayu fish (*Plecoglossus altivelis*) from pathogenic *Pseudomonas plecoglossicida in vivo*. This situation can be related to the observation in present study that the bacterial forms that emerge as resistant to phage might be avirulent BIMs which fail to cause an infection in the culture system as a result of phage – bacteria interaction.

Evaluation of the potential of *V. harveyi* phages for bio control of the pathogen in animal models such as *Artemia* nauplii and *P. monodon* post larvae was accomplished. In this study, dosage of consortium of *V. harveyi* was fixed as $1x10^7$ CFU/mL for further experimentation based on the pattern of mortality of *Artemia* nauplii obtained in pathogenicity assay. Mean while, Roque and Gomez-Gil (2003) infected *Artemia* nauplii with $3.8x\ 10^6$ and $6.15x10^6$ CFU mL^{-1} *V. harveyi* in two different experiments and reported 100 % mortality at 48^{th} hour. However, in the present study mortality at 48^{th} hour was just above 80 % with higher *V. harveyi* count indicating differences in the pathogenicity of the strains and the rearing conditions. In other attempts, *V. campbellii* LMG21363 (Soltanian, 2007) and *V. harveyi* (Soto-Rodriguez *et al.*, 2003) were applied at a dose of 5×10^6 and $1x10^5$-$1x10^6$ CFU/mL respectively to develop pathogenicity to *Artemia* nauplii.

The study of phage-host interactions in an animal model is essential to understand the potential role of phages in biological control. However, when the efficacy of phage cocktail and *Vi ha* 19 in protecting *Artemia* naupllii from infection of

V. harveyi consortium was evaluated in the present study, survival of *Artemia* nauplii was more than 80% in both the phage treated systems and 20% in untreated control. It has to be pointed out that the efficiency of the most potent phage, *Vi ha* 19 is more or less equal to the phage cocktail suggesting the easiness in handling a single phage preparation (*Vi ha* 19) against multiple strains of *V. harveyi* in hatchery environment compared to the requirement of the phage cocktail, where five individual phages have to be generated separately and mixed together. Another point to be mentioned here is the non emergence of resistant bacteria in administration of *Vi ha* 19 which is an added advantage to this phage to be recommended for successful management of *V. harveyi* in shrimp hatcheries. In another study, 78 to 95 % survival at the 96th has been reported in *Artemia* nauplii on phage treatment against *V. harveyi* infection (Gunawardhana, 2009).

Next to *Artemia* nauplii, *in vivo* biocontrol potential of *Vibrio harveyi* phages was studied in post larvae of *Penaeus monodon* individually and as cocktail. Phages may not behave identical *in vivo* and *in vitro* and the phages that act well *in vitro* in a given host may behave differently *in vivo*. Accordingly, LB 6 vs *Vi ha* 6 and LB 21 vs *Vi ha* 21 systems were not able to impart better survival to post llarvae in phage treated system, eventhough both the above mentioned phages showed marked biocontrol potential *in vitro*. Strikingly, *in vivo*, post larvae harboured higher *V. harveyi* count in phage (*Vi ha* 6 and *Vi ha* 21) treated groups compared to untreated control in the above mentioned systems, which reflected adversely to the survival of post larvae in LB 21 vs *Vi ha* 21 system alone. Cerveny *et al.* (2002) also reported that strains of *Vibrio vulnificus* which were phage susceptible *in vitro* turned out to be resistant to phages and the unsusceptible bacterial strains under *in vitro* transformed to susceptible strains to the phage when infected in mice models. Another interesting observation in the present study was that in LB 6 vs *Vi ha* 6 system, the enhanced bacterial count (LB 6) in phage (*Vi ha* 6) treated systems could not cause larval mortality. That might be due to the emergence of

bacteriophage-insensitive mutants (BIMs) and loss of virulence of host bacteria as part of phage infection in the treated group. Bacteriophage Insensitive mutants (BIMs) might have lost the ability to cause mortality in post larvae treated with *Vi ha* 6 as stated by Levin and Bull (2004) and O'Flaherty, (2005b). On the other hand, the *Acinetobacter baumanii* phage, which was very active *in vitro*, was found to be protective *in vivo* also at remarkably low MOI of 1 PFU to 10^6 CFU (Soothill, 1992). As per Smith & Huggins (1982) and Barrow and Soothil (1997), there are a lot many incidents of unsuccessful stories of phage therapy in controlling bacterial infections in man and animals.

However, alike the inability of *Vi ha* 6 and 21 to bring down the corresponding host bacteria, the other three phages could perform well to reduce the bacterial count significantly to bring out better survival to post larvae. On the other hand, on applying the cocktail of *V. harveyi* phages inclusive of *Vi ha* 6 and 21 to the system challenged with a consortium of *V. harveyi*, higher survival was obtained compared to control. However, the inefficacy of *Vi ha* 6 and *Vi ha* 21 to act in a biological system *in vivo* might have been compensated by the other three phages especially the most potent phage *Vi ha* 19 present in the cocktail. The cumulative effect of all the five phages in the cocktail was positive resulting in better survival of post larvae after the challenge. Meanwhile, the very high survival of larvae obtained in all the control groups (without bacterial challenge) administered with the phages including the cocktail was intriguing, suggesting that phages might be destroying the *Vibrio* already present in the larvae as normal flora performing a positive role in the percent survival.

Targeting a bacterial consortium to be managed by cocktail phage and most potent phage is a novel idea brought under this study. Even though the application of phage cocktail has been reported earlier (O'Flynn *et al.*, 2004; Tanji *et al.*, 2004), studies of phage therapy on bacterial consortium have not been reported yet. However the attempts were turned out to be successful in protecting *Artemia*

nauplii and post larvae of *P. monodon* from the multiple infection of *V. harveyi* consortium.

In the bioassay of post larvae of *P. monodon*, for individual phage-host systems, bacterial counts of post larvae were compared. Even though water contained bacterial load, mortality was effected directly by the load in the animal. Therefore it is very relevant to find the count in the animals too. Except in LB 6/*Vi ha* 6 and LB 21/*Vi ha* 21 systems, all other phage treated larvae were found to have lesser bacterial count compared to the untreated larvae. However, in case of *V. harveyi* consortium, bacterial counts of water as well as the animals were determined. Results in bacterial count had a direct influence on survival of post larvae.

The experimental use of phage therapy to prevent or treat infections by *V. harveyi* has been shown to be successful. The phage cocktail and most potent phage *Vi ha* 19 showed remarkable effectiveness against the *V. harveyi* consortium. Potential of *Vi ha* 19 to manage the consortium is highly evident as it alone could cover the host range of phage cocktail. Thus the management of bacterial consortium from hatchery point of view is possible either using phage cocktail or single potent phage developed through this investigation. Phage therapy against *Vibrio harveyi* in *Penaeus monodon* was found to be effective earlier also (Karunasagar et al., 2007; Vinod et al., 2006). In both the cases larval survival was found to be higher by 85% compared to that of the bacterial control.

Other successful reports include phage therapy in yellowtail fish against *Lactococcus garvieae* infection, which resulted 40-90 % survival in different experiements (Nakai *et al.*, 1999) whereas 80-100% survival was observed in Ayu fish against *Pseudomonas plecoglossicida* (Park *et al.*, 2000). More than one log reduction in bacterial count could be resulted due to phage therapy against *Campylobacter jejuni* in young chickens (Wagenaar *et al.*, 2005). In experimental

bacteremic infections of mice with vancomycin-resistant *Enterococcus faecium*, phage therapy reduced mortality by 100% (Biswas *et al.*, 2002).

4.5 Conclusion

Based on the *in vitro* and *in vivo* experiments conducted in this study, *Vi ha* 19 showed best performance compared to the other phages and phage cocktail based on larval survival without the emergence of bacteriophage insensitive mutants. In this context *Vi ha* 19 can be recommended to be used as the most ideal candidate for phage therapy for successful management of *Vibrio harveyi* in Aquaculture.

4.6 Figures

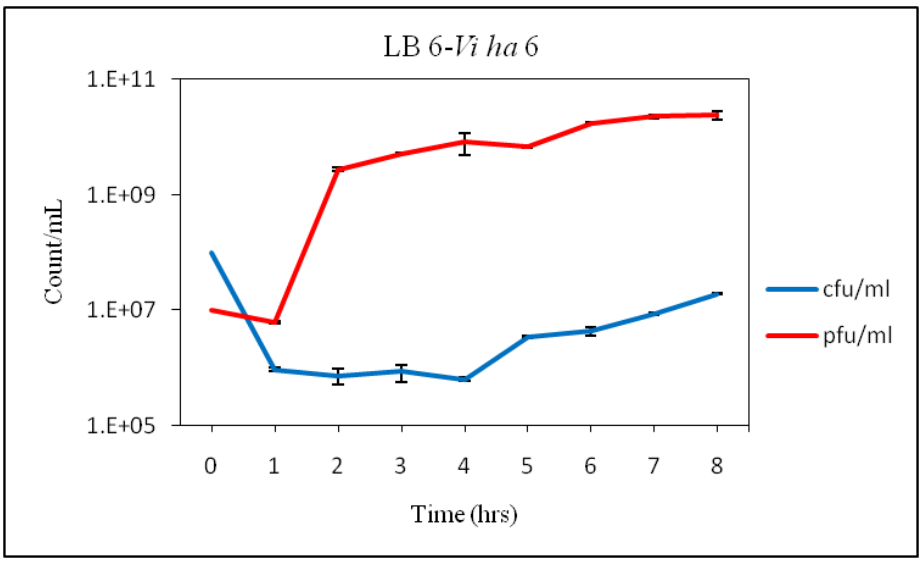


Fig. **Error! No text of specified style in document.**.1 Interaction of *Vibrio harveyi* LB 6 and its phage *Vi ha* 6 in ZoBell's broth.

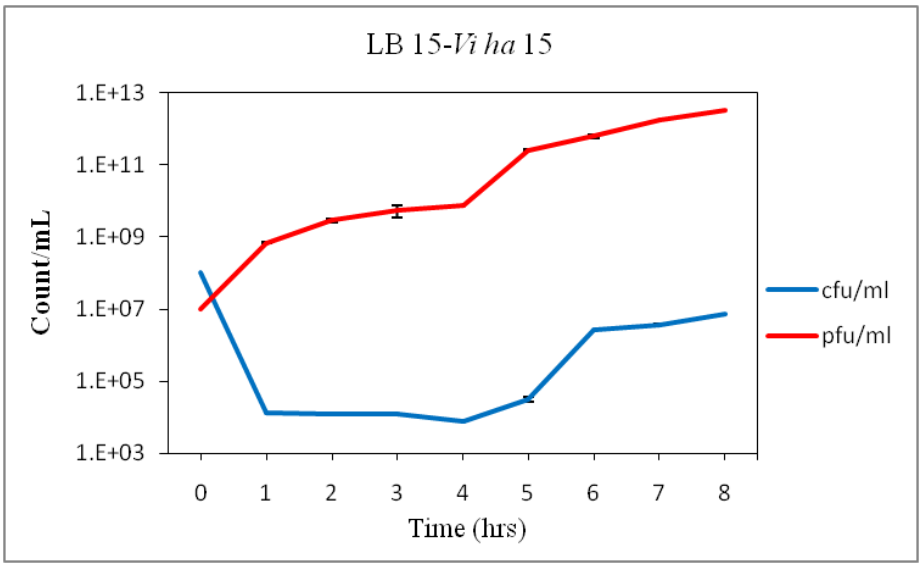


Fig. 4.2 Interaction of *Vibrio harveyi* LB 15 and its phage *Vi ha* 15 in ZoBell's broth.

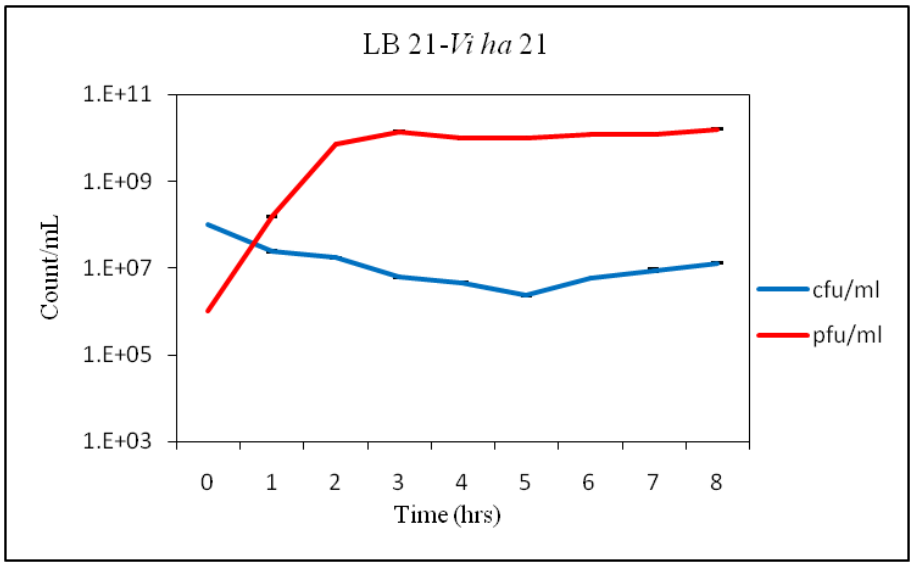


Fig.4.3 Interaction of *Vibrio harveyi* LB 21 and its phage *Vi ha* 21 in ZoBell's broth.

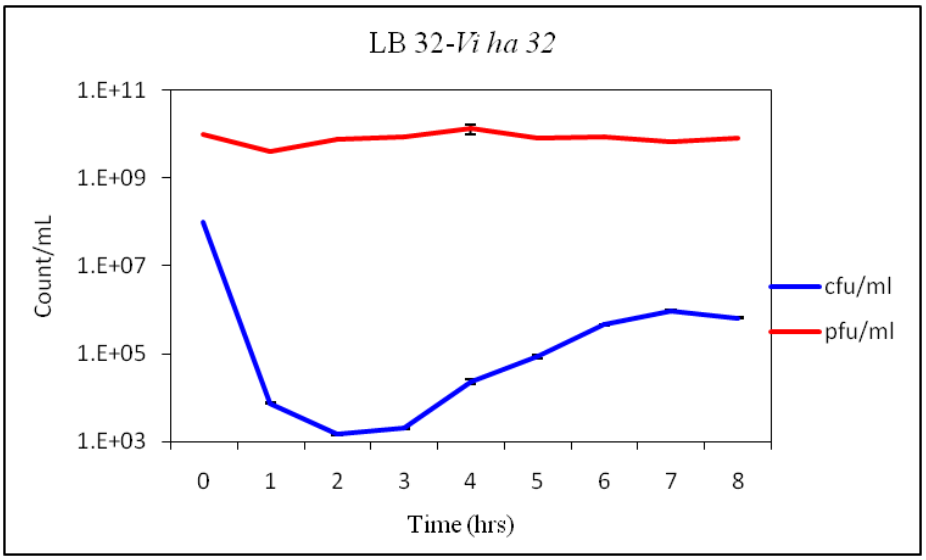


Fig. 4.4 Interaction of *Vibrio harveyi* LB 32 and its phage *Vi ha* 32 in ZoBell's broth.

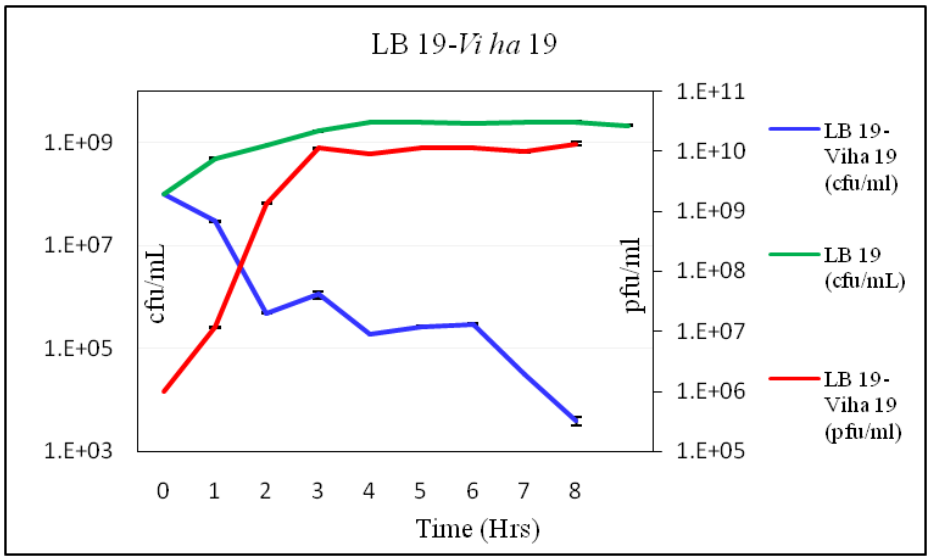


Fig. 4.5 Interaction of *Vibrio harveyi* LB 19 and its phage *Vi ha* 19 in ZoBell's broth.

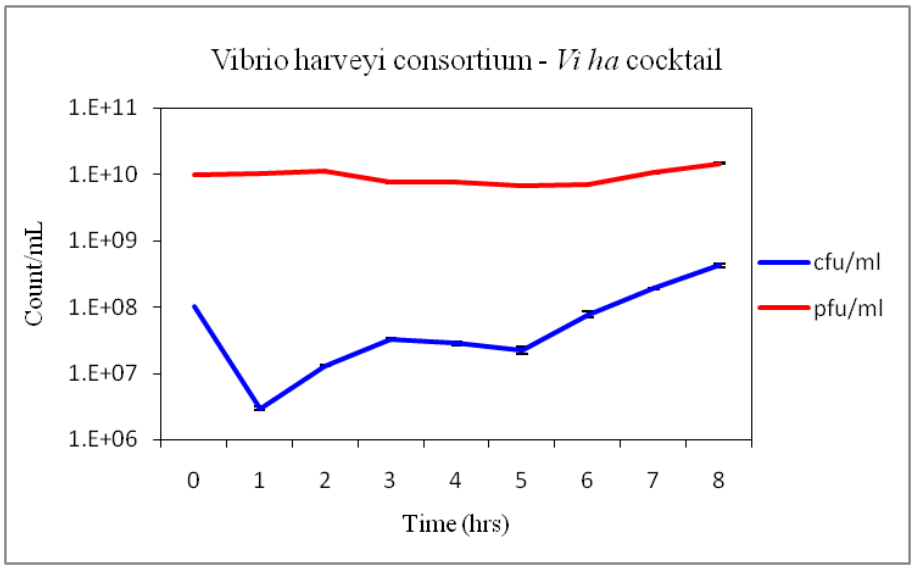


Fig. 4.6 Interaction of *Vibrio harveyi* consortium and phage cocktail in ZoBell's broth.

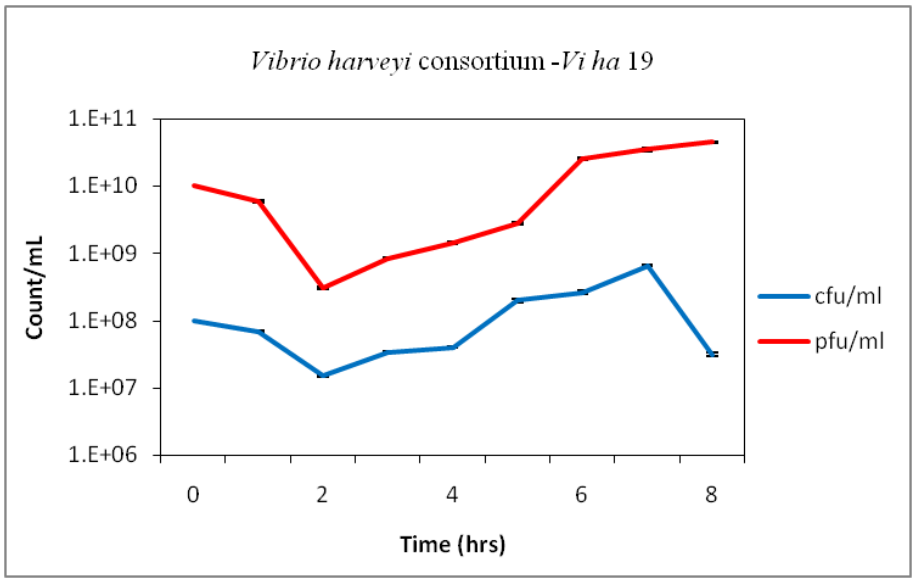


Fig. 4.7 Interaction of *Vibrio harveyi* consortium and *Vi ha* 19 in ZoBell's broth.

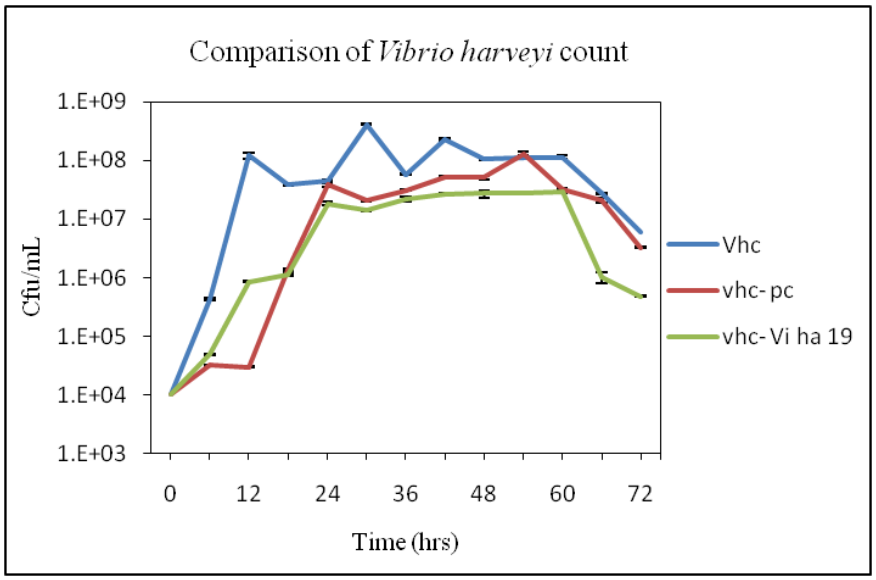


Fig. 4.8 Comparison of bacterial count [*Vibrio harveyi* consortium (vhc)] on treatment with phage cocktail (vhc-pc) and *Vi ha* 19 in sea water.

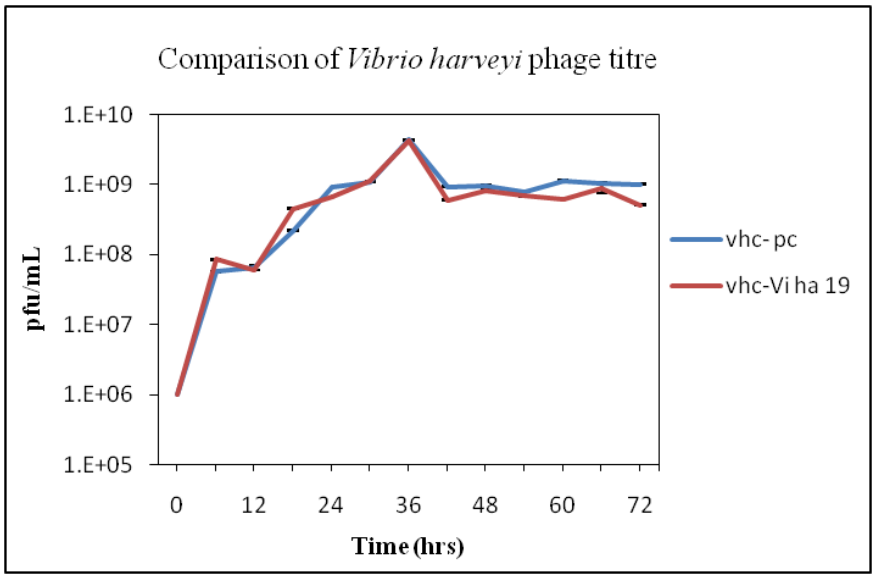


Fig. 4.9 Comparison of phage cocktail (vhc-pc) and *Vi ha* 19 titre on treatment with *Vibrio harveyi* consortium (vhc) in sea water.

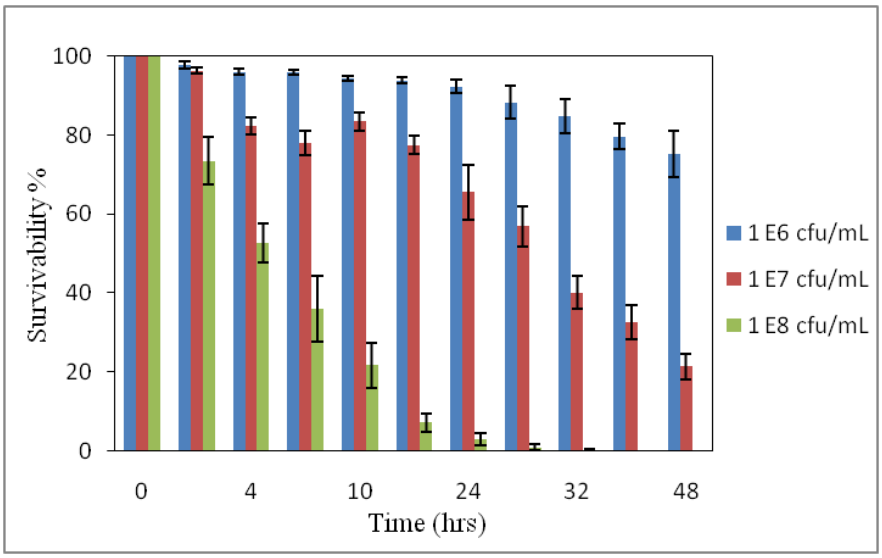


Fig. 4.10 Pathogenicity of *Vibrio harveyi* consortium on *Artemia* nauplii.

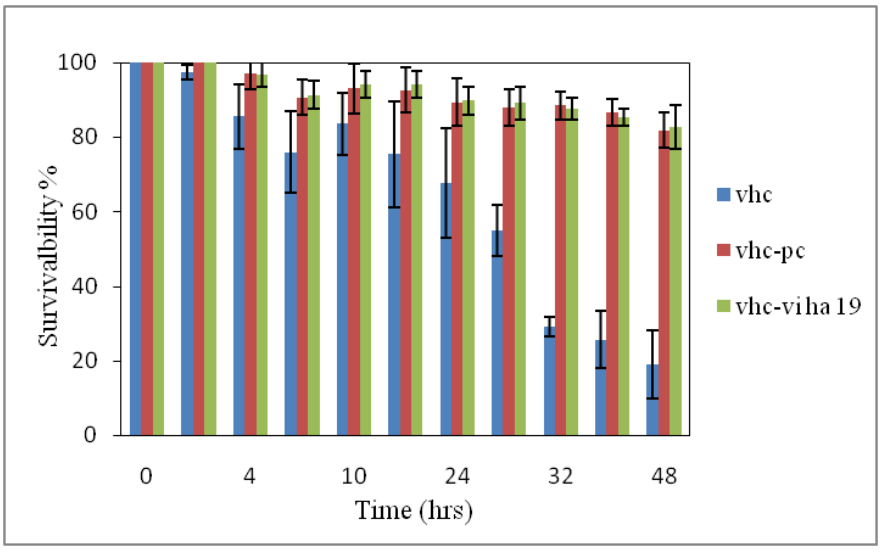


Fig. 4.11 Survivability of *Artemia* nauplii challenged with *Vibrio harveyi* consortium (vhc) and treated with phage cocktail (vhc-pc) and *Vi ha* 19.

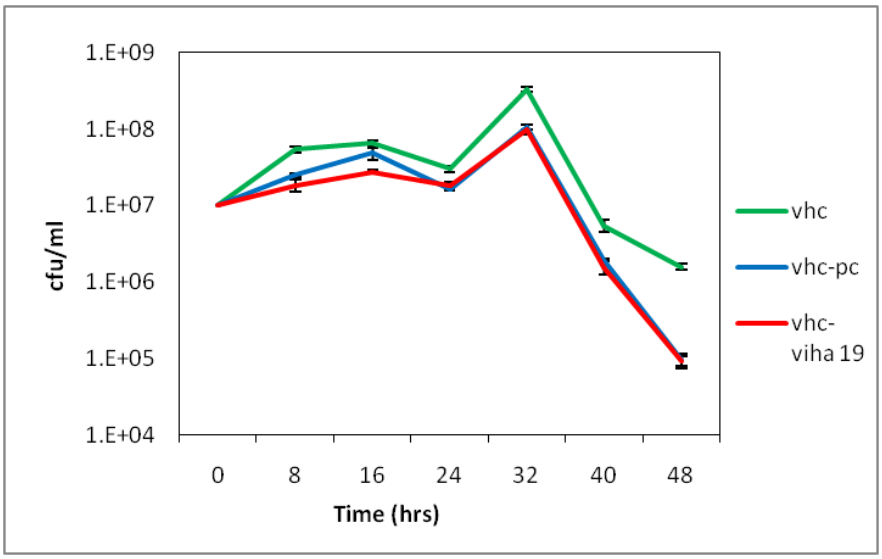


Fig. 4.12 Comparison of the count of *Vibrio harveyi* consortium (vhc) treated with phage cocktail (pc) and *Vi ha* 19 in *Artemia* nauplii.

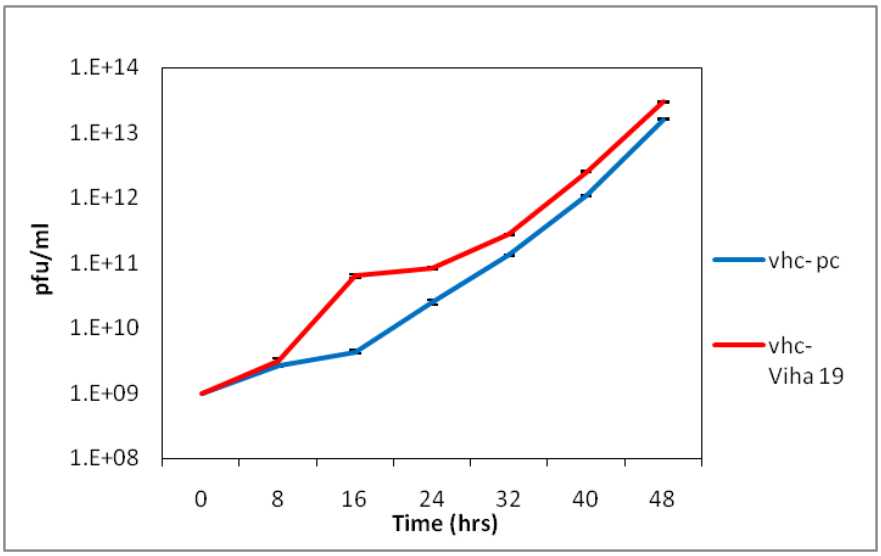


Fig. 4.13 Comparison of phage cocktail and *Vi ha* 19 titres in *Artemia* nauplii challenged with *Vibrio harveyi* consortium.

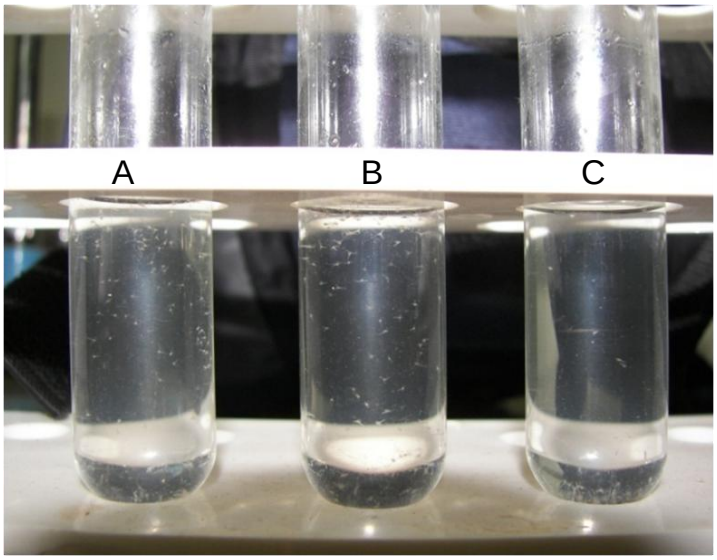


Fig. 4.14 Survival of *Artemia* nauplii challenged with *Vibrio harveyi* consortium and treated with (A) *Vi ha* 19, (B) Phage cocktail (vhc-pc) and (C) untreated (vhc) at 48th hour.

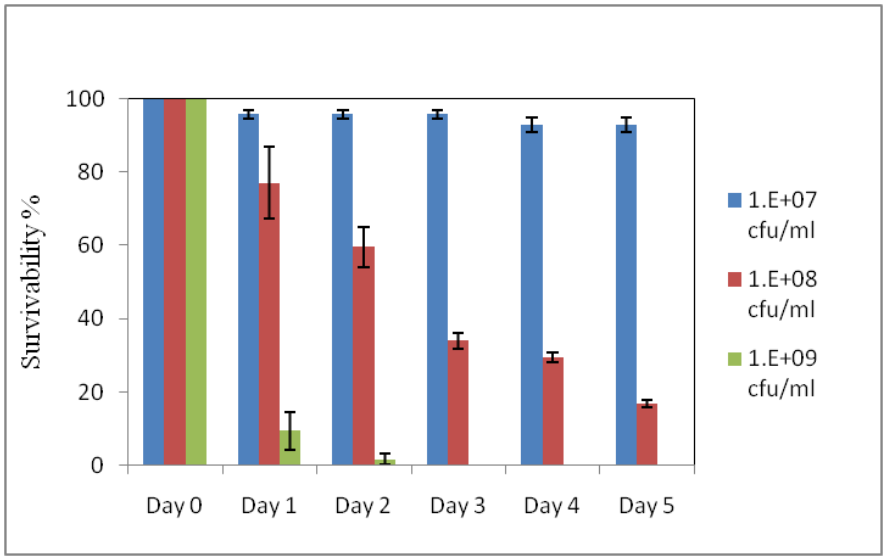


Fig. 4.15 Pathogenicity of *Vibrio harveyi* consortium on post larvae of *Penaeus monodon.*

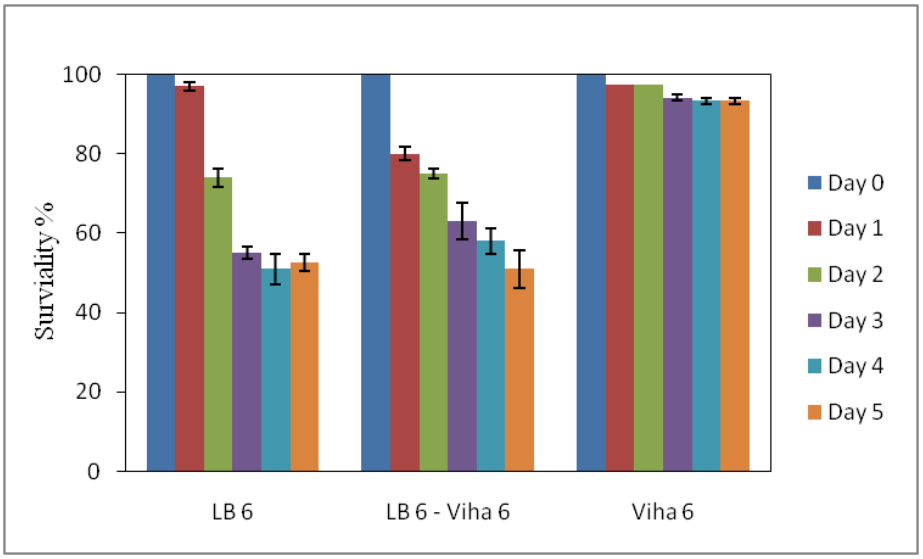


Fig.4.16 Comparison of survivability of *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB6) and treated with *Vi ha* 6.

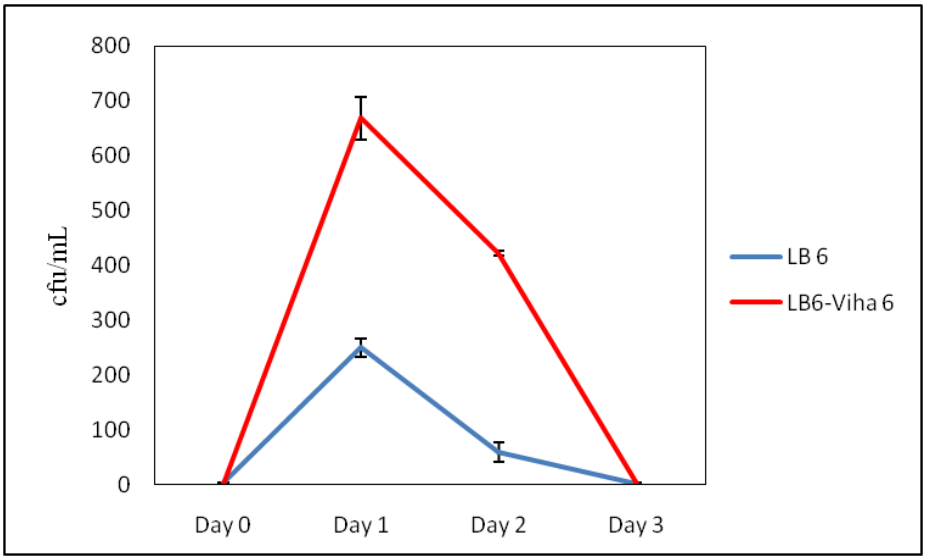


Fig. 4.17 Comparison of bacterial count in *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB6) and treated with *Vi ha* 6.

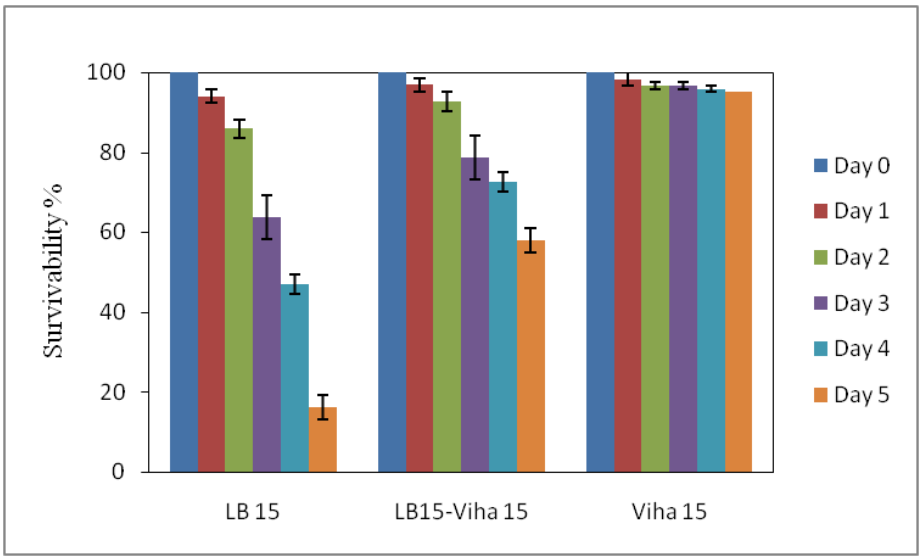


Fig. 4.18 Comparison of survivability of *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB15) and treated with *Vi ha* 15.

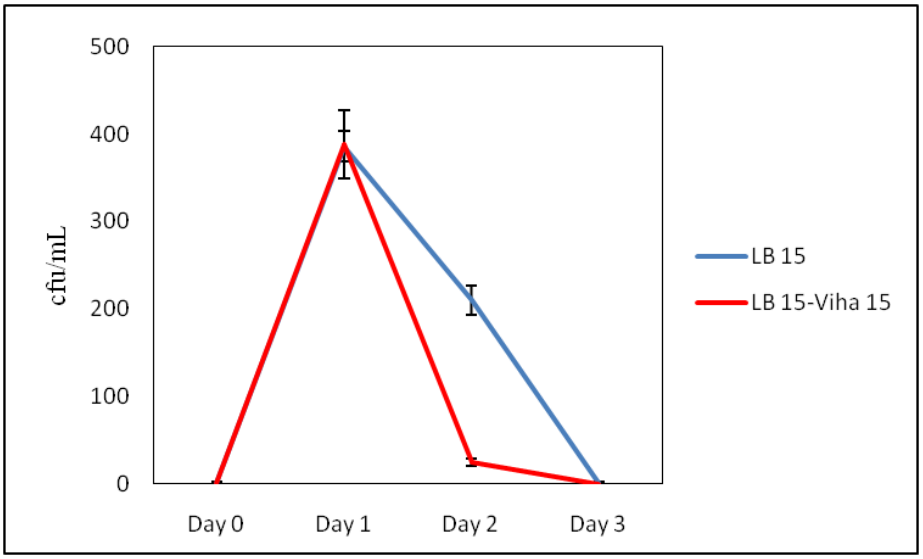


Fig. 4.19 Comparison of bacterial count in *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB15) and treated with *Vi ha* 15.

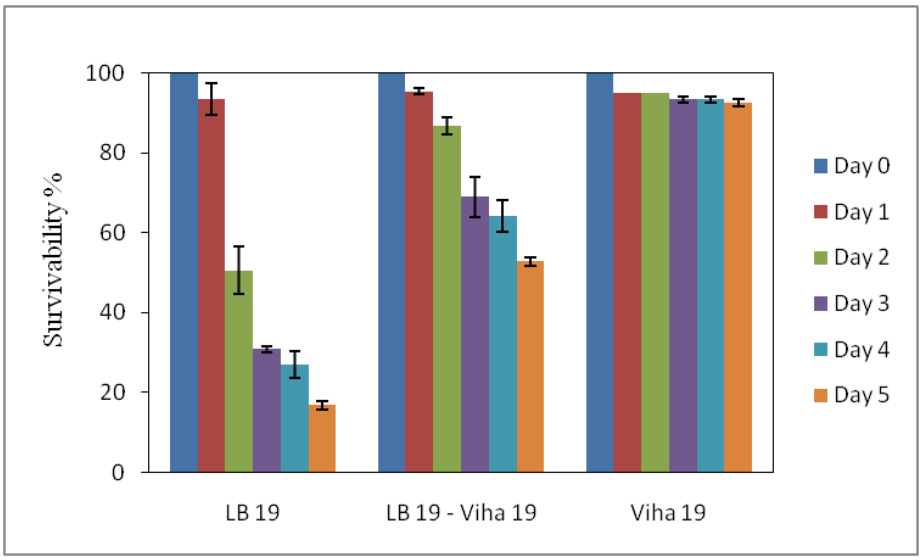


Fig. 4.20 Comparison of survivability of *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB19) and treated with *Vi ha* 19

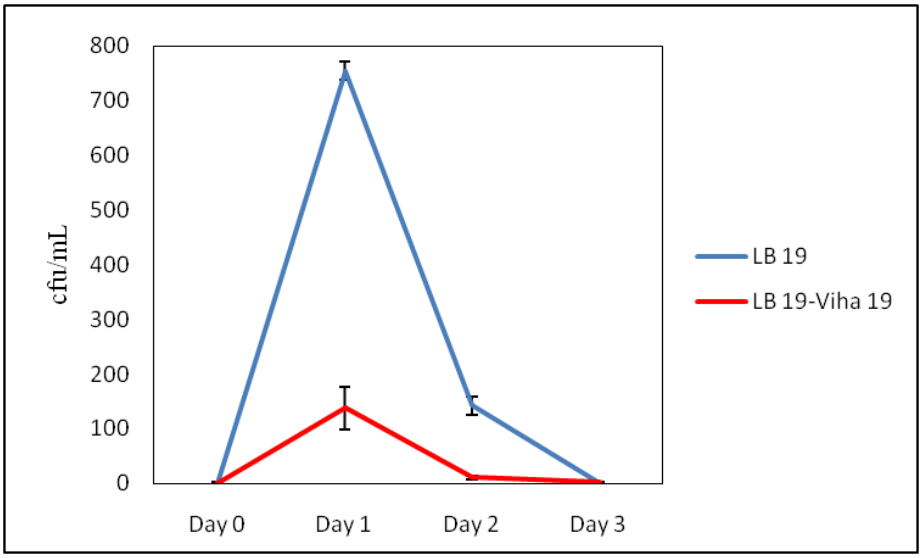


Fig. 4.21 Comparison of bacterial count in *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB19) and treated with *Vi ha* 19

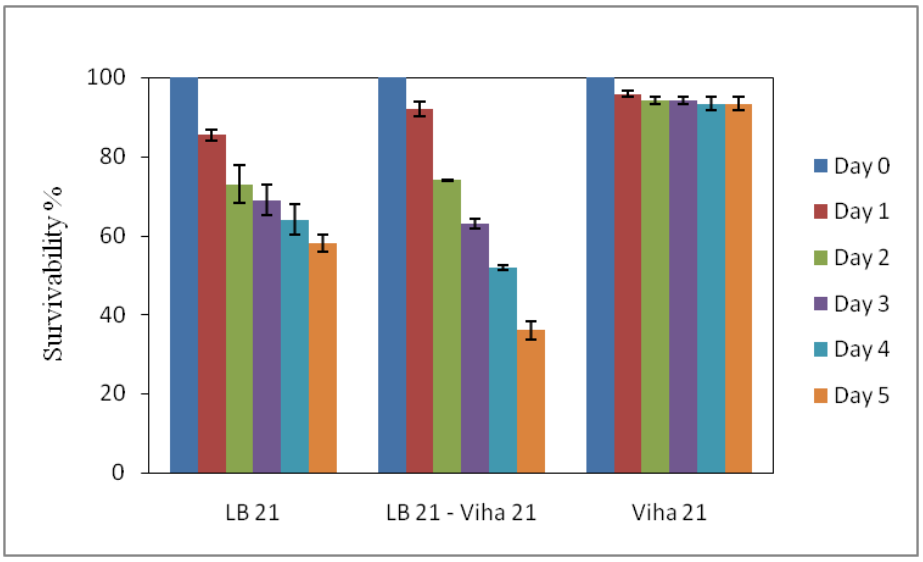


Fig. 4.22 Comparison of survivability of *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB21) and treated with *Vi ha* 21

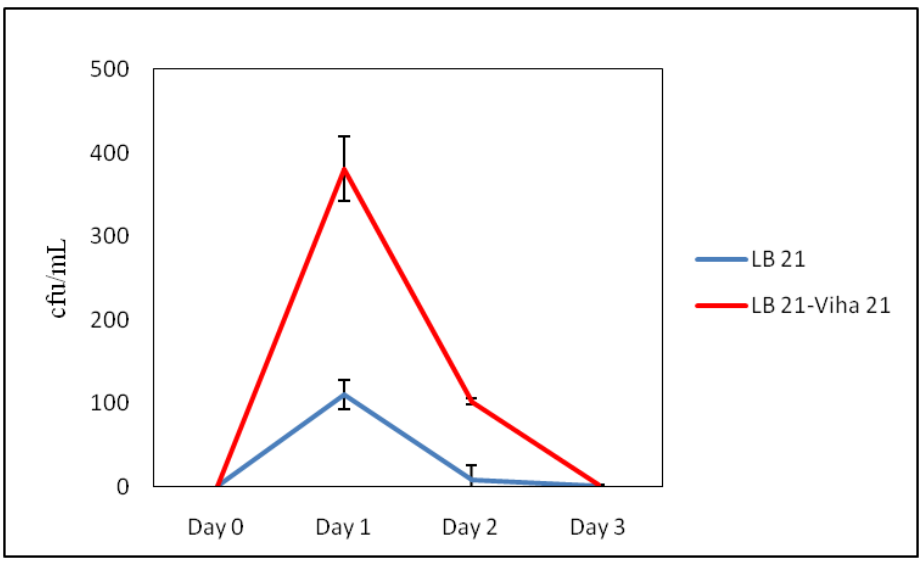


Fig. 4.23 Comparison of bacterial count in *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB21) and treated with *Vi ha* 21.

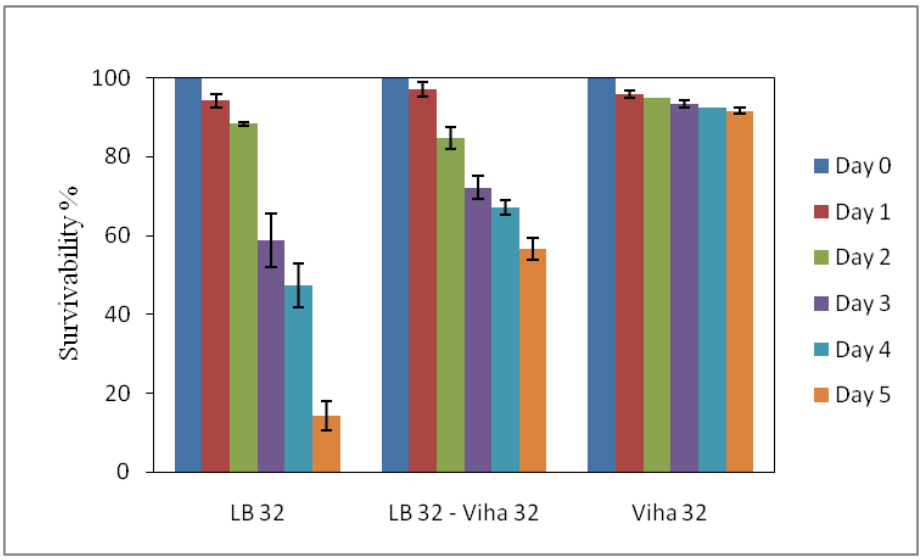


Fig. 4.24 Comparison of survivability of *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB32) and treated with *Vi ha* 32

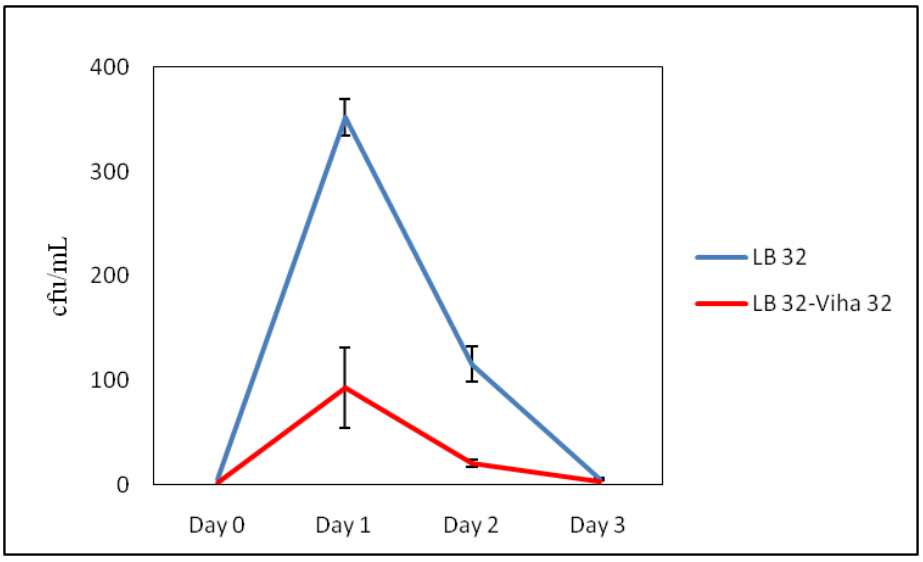


Fig. 4.25 Comparison of bacterial count in *Penaeus monodon* post larvae challenged with *Vibrio harveyi* (LB32) and treated with *Vi ha* 32.

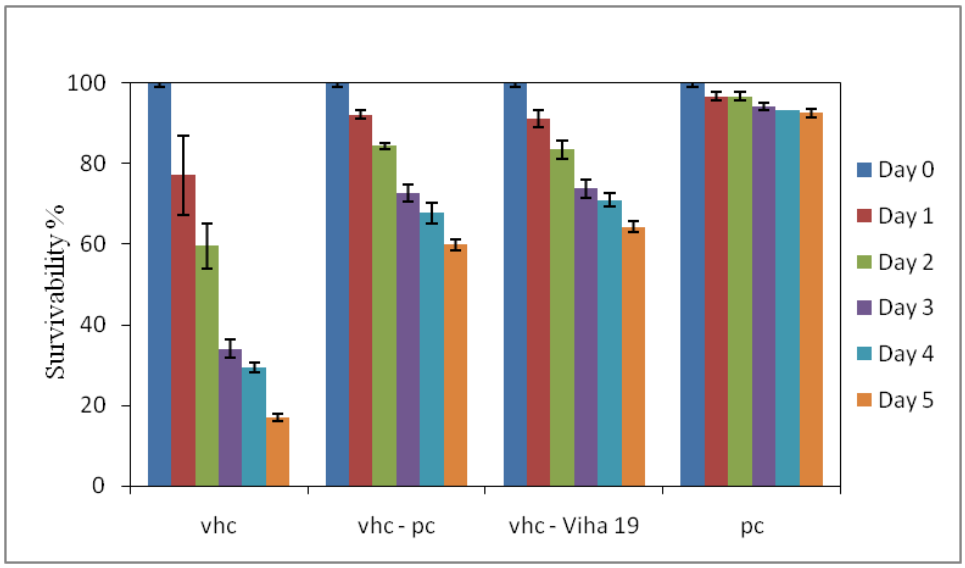


Fig. 4.26Comparison of the survivability of post larvae of *Penaeus monodon* challenged with *Vibrio harveyi* consortium (vhc) and treated with phage cocktail (pc) and *Vi ha* 19

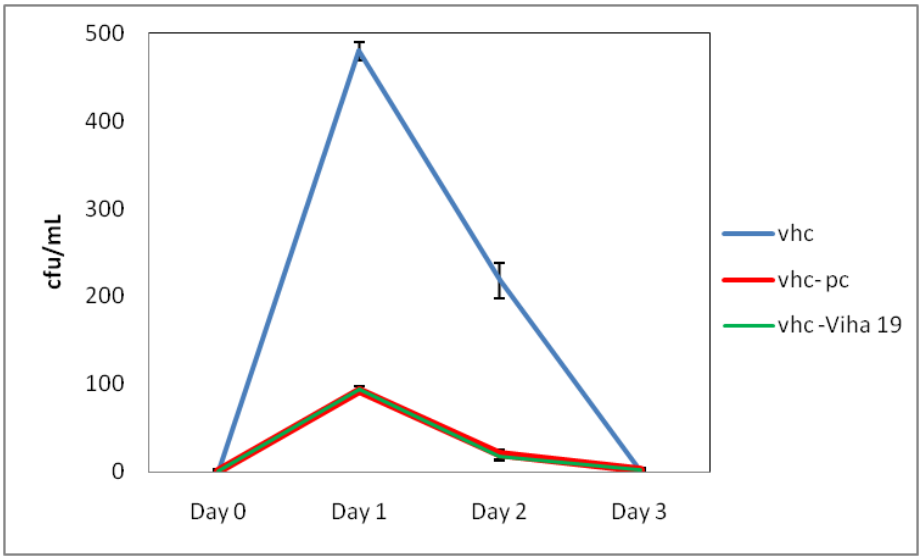


Fig. 4.27 Comparison of the count of *Vibrio harveyi* consortium (vhc) in post larvae of *Penaeus monodon* treated with phage cocktail (pc) and *Vi ha* 19

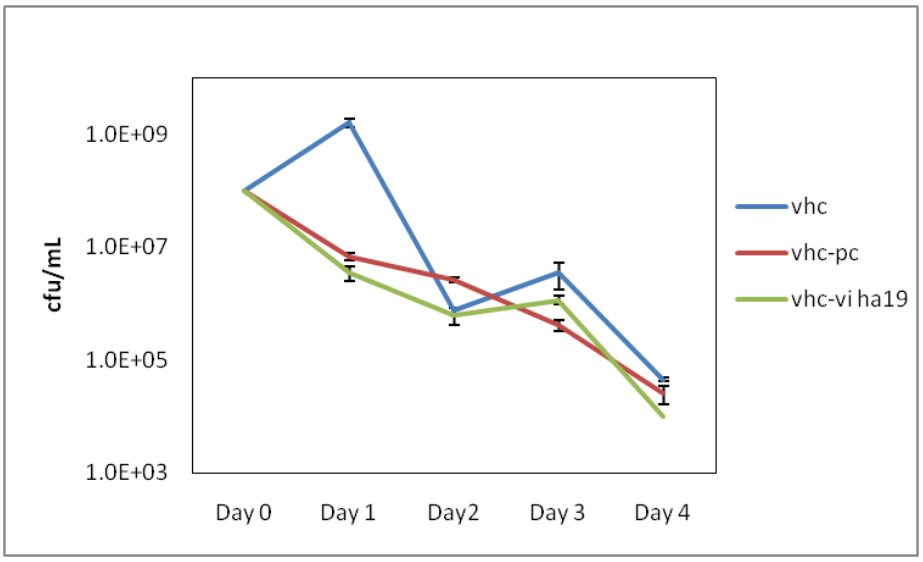


Fig. 4.28 Comparison of the count of *Vibrio harveyi* consortium in water treated with phage cocktail and *Vi ha* 19 in the *Penaeus monodon* post larvae rearing system

Chapter-5
Conclusions and scope for future research

5.1 Introduction

Aquaculture is a global industry providing food and employment thereby contributing to the economy. For the sustenance of aquaculture, disease management is a major requirement. Among the bacterial pathogens *Vibrio harveyi* remains to be the major one especially in shrimp culture systems. Rapid and mass mortality of shrimp larvae due to *Vibrio harveyi* infection is well known, and the pathogen causes serious economic losses in grow out systems as well. It suggests that a well defined management strategy has to be built up to protect the crop from *Vibrio harveyi* infection in aquaculture systems. Antibiotics have been the choice for quite some times which led to residues in meat and development of multidrug resistant bacteria which invited ban on their application. In this context several alternate options have been thought off such as probiotics, immunostimulants and vaccines. Phage therapy is yet another option. Phages being natural parasites of bacteria and are abundant in aquatic environments their application to control bacterial pathogens in aquaculture has commendable potential in lieu of antibiotics. For that matter the therapeutic effect of phages has been proven in several antibiotic resistant pathogens inclusive of *Vibrio harveyi.*

There are so many reports on phage therapy against aquaculture pathogens including *Lactococcus garvieae* infection in *Seliora quinqueradiata*, *Pseudomonas plecoglossicida* infection in *Plecoglossus altivelis, Piscirickettsia salmonis* infection in *Oncorhynchus kisutch, , Aeromonas hydrophila* and *Edwardsiella tarda* infection in *Anguilla japonica* etc. With respect to *Vibrio harveyi* phages two category of reports are available. A group of phages were found potential enough

for biocontrol of *V. harveyi* in hatcheries whereas another group was found to confer virulence to avirulent strains of *Vibrio harveyi*. This points to the relevance of sound understanding of phage-host relationship as evident from these findings before selecting phage candidates as therapeutics. Meanwhile, development of resistant bacteria or bacteriophage insensitive mutants is another potential limitation in using bacteriophages as biocontrol agents.

5.2 Significance of the present study

V. harveyi is a causative pathogen for luminous bacteriosis in shrimp culture of Asia and Latin America. Antibiotics and chemotherapeutics are found to be ineffective due to the emergence of antibiotic resistance in bacteria, and detection of residual antibiotic in shrimp meat resulting in the imposition of ban of antibiotic use in aquaculture. A biological method of control for *Vibrio harveyi* is an absolute requirement for sustainable shrimp culture. One of the options is phage therapy.

5.3 Objectives

1. Isolation, purification, screening and characterization of *Vibrio harveyi* phages for 'phage – therapy'
2. Screening of *Vibrio harveyi* phages based on the presence of lysogenic/toxin genes and determination of phage infection properties
3. Evaluation of biocontrol potential of therapeutic phages individually and as cocktail in *vitro* and *in vivo*

5.4 Over all achievements

- In total, 33 *Vibrio harveyi* phages were isolated from major aquaculture centres of Kerala following enrichment method using *Vibrio harveyi* isolates as hosts maintained at National Centre for Aquatic Animal Health, Cochin University of Science and Technology.

- All phages were subjected for host range analyses with 87 *V. harveyi* isolates in collection along with a type strain BCCM LMG 4044 VH. Based on the percentage of lysis of the host bacteria and the quality of plaques, 6 broad spectrum *V. harveyi* phages were segregated for further study. Out of 6 phages, 5 could lyse more than 50% of the host bacteria and *Vi ha* 19 was the most broad spectrum phage which could lyse 70.1 % of the *V. harvaey* isolates tested.

- Respective host bacteria of 6 broad spectrum lytic phages were subjected for molecular characterization using 16S rRNA gene sequencing besides phenotypic characterization and all of them were identified as *Vibrio harveyi*. *Vibrio harveyi* MCCB 153 (LB 6), 154 (LB 15), 155 (LB 19), 156 (LB 21), 157 (LB 32) and 158 (LB 68) were showing 99, 100, 99, 99, 99 and 100% similarity to *V. harveyi* GenBank data bases respectively. The phylogenetic tree based on 16S rRNA gene sequences has been constructed.

- The sequences were submitted under the accession numbers JN990075 (MCCB 153 - LB6), JN990076 (MCCB 154- LB 15), JQ920474 (MCCB 155 - LB 19), JN990077 (MCCB 156 - LB 21), JN990078 (MCCB 157 - LB 32) and JQ920475 (MCCB 158 - LB 68).

- Cross infectivity tests of the 6 selected phages revealed that all phages were having varied level of infectivity with other *Vibrio* sp. and *Aeromonas* sp., which is an added advantage to use the phages to target other aquaculture pathogenic bacteria as well, which may likely to be associated with *Vibrio harveyi*. However, the phages were not found to infect beneficial bacteria being used as probiotics in Recirculating Aquaculture system (RAS).

- The segregated phages were not found to disturb nitrification (production of nitrite - nitrogen and nitrate- nitrogen) on inoculating the nitrifying bacterial

consortia used in RAS with the phages suggesting that phages are fit to be used in RAS for successful management of *V. harveyi*.

- Transmission Electron Microscopy revealed unique morphology and dimensions for each phage member. They all were found to belong to the order Caudovirales and two families such as myoviridae and siphoviridae. All the 5 myoviridae members were designated as A1 morphotype and the single siphoviridae phage as B1 morphotype. The less therapeutic efficient phage *Vi ha* 57 revealed a unique morphology and could be proposed as a new morphotype in the bacteriophage classification.

- All the six phages are found to be double stranded DNA phages as DNase free RNase and Moong bean nuclease could not digest the genomes of the phages.

- RAPD profiling resulted in unique and reproducible fingerprints from all the 6 *Vibrio harveyi* phages. Two different clustering could be seen as a result of amplification with two decamer primers. Primer 292 exhibited similarity coefficient ranging from 96-98 % and primer 293 exhibited 86-96 %, suggesting certain degrees of phylogenetic relationship among the phages in this study. However, none of the phages examined could exhibit 100 % similarity amidst them or none showed a similarity coefficient of 1 based on the amplification pattern with both the primers.

- Characterizing phages by protein profiling proved that SDS-PAGE could discriminate the phages based on the protein banding pattern. Two major bands were common to all Myoviridae phages, which must be head and tail proteins. However, all the phages in this study were unique as per the variability shown by the minor bands. Accordingly, phages could be grouped into three, group 1: comprised of *Vi ha* 6 and *Vi ha* 15, group 2: included *Vi*

ha 19, *Vi ha* 21 and *Vi ha* 32 and group 3: *Vi ha* 68. This sort of grouping is found to be in agreement with the RAPD pattern revealed by Primer NP-293. In case of RAPD profiling, *Vi ha* 6 was found to be another cluster which stood slightly distant from rest of the myophages, but stood with *Vi ha* 15 in protein profiling existing as a group. It could be speculated that some difference in the minor proteins found in this study might be responsible for the diversity of host range and the minor difference in similarity coefficient resulted in RAPD clustering.

- The phages were found to be free of lysogenic genes such as integrase, recombinase and transposase and the virulence related *Ctx A* toxin gene. The phage *Vi ha* 68 alone showed the presence of partially amplified Dam toxin gene corresponding to the positive control VHML. In this context, the phage *Vi ha* 68 was excluded from further study considering the negative impact of such genes in phage therapy.

- On comparing the optimal multiplicity of infection based on phage titre and the absorbance profile, both the values were different except for the phage *Vi ha* 15. As per the absorbance profile of *Vi ha*19-LB19 system, absorbance reached below zero in all the MOI ratios at various points of time period. However, optimal MOI based on phage titre has been selected to be used in all experiments related to phage therapy following the literature.

- One step growth properties of all phage-host systems were found out. Accordingly, *Vi ha* 19 was found to have the highest burst size (1135) with longest duration of latent time (60 minutes). The lowest burst size (123) was shown by phage *Vi ha* 15 with shortest duration of latency (30 minutes). From the observations regarding phage multiplication parameters, it is observed that in all the cases, there was a relationship between burst size and

latent time. Greater burst sizes are usually associated with a longer latent period, whereas lesser burst sizes are associated with shorter latent periods.

- *In vitro* lytic efficacy of *V. harveyi* phages under nutrient rich conditions was assessed with 5 *V. harveyi* strains and 5 respective phages, out of which the highest lytic efficacy was demonstrated by *Vi ha* 19 and *Vi ha* 32. *Vi ha* 19 reduced bacterial load by four log unit without the emergence of bacteriophage insensitive mutants (BIMs) or resistant bacterial forms. *Vi ha* 32 showed five log unit reductions in host bacteria, even though resistant bacterial forms were observed. In all the individually treated nutrient rich *in vitro* experiments, 1-5 log reduction in bacterial load was resulted with in the first two hours.

- *Vi ha* 19 showed better activity against *V. harveyi* consortium compared to the phage cocktail in terms of reduction in bacterial count and the absence of emergence of resistant bacteria. *Vi ha* 19 was the only phage which could overcome the emergence of BIMs against its specific host and also against bacterial consortium. Even though rest of the other phages significantly reduced the bacterial load within the first two hours of challenge, beyond then BIMs started to emerge in the system.

- Under nutrient limited conditions *Vi ha* 19 exhibited better lytic activity than phage cocktail in terms of reduction in the count of the *V.harveyi* consortium. Compared to phage cocktail, *Vi ha* 19 showed reduction in bacterial count by 1 log unit.

- Evaluation of the potential of *Vibrio harveyi* phages for bio control of the *Vibrio harveyi* in *Artemia* nauplii was carried out. In the pathogenicity assay, mortality of *Artemia* nauplii occurred at a dosage of $1x\ 10^7$ cfu/mL of *Vibrio*

harveyi consortium. In this study, survival of *Artemia* nauplii was more than 80% in both the phage treated systems and 20% in untreated control.

- It has to be pointed out that the efficiency of most potent phage *Vi ha* 19, is more or less equal to the phage cocktail based on the reduction in *Vibrio* count and survivability of *Artemia* nauplii. It also suggests the easiness in handling the preparation (*Vi ha* 19) against multiple strains of *V. harveyi* in hatchery environment compared to the phage cocktail, where five phages have to be generated individually and mixed together.

- *In vivo* biocontrol potential of *Vibrio harveyi* phages was investigated in post larvae of *P. monodon.* Accordingly, LB 6 vs. *Vi ha* 6 and LB 21 vs *Vi ha* 21 systems were not able to impart better survival to phage treated post larvae, even though both the above mentioned phages showed marked bio-control potential *in vitro*. Strikingly, phage treated (*Vi ha* 6 and *Vi ha* 21) post larvae harboured higher *Vibrio harveyi* population compared to untreated control.

- Another interesting observation in the present study was the enhanced bacterial count (LB 6) in phage (*Vi ha* 6) treated systems which could not cause larval mortality. That might be due to the emergence of bacteriophage-insensitive mutants (BIMs) and loss of virulence of host bacteria as part of phage infection in the treated group.

- On the other hand, the other three phages (*Vi ha* 15, *Vi ha* 19 and *Vi ha* 32) individually performed well against their respective hosts such as LB 15, LB 19 and LB 32 to bring out significant survival in post larvae of *P.monodon* and reduction in bacterial count.

- However, in contrast to the inability of *Vi ha* 6 and 21 to bring down the corresponding host bacteria resulting in better survival of larvae, the cocktail of *V. harveyi* phages on applying to the system challenged with a consortium

of *V. harveyi* resulted in higher survival. This might be due to the fact that the inefficacy of *Vi ha* 6 and *Vi ha* 21 to act in a biological system *in vivo* might have been compensated by the other phages especially the most potent phage *Vi ha* 19 present in the cocktail. The cumulative effect of all the five phages in the cocktail was positive resulting in better survival of post larvae.

- The very high survival shown in all the control groups administered with phages including the cocktail indicated that phages appeared to have a positive role in the percent survival.

- In the present study an attempt was made to understand the efficacy of cocktail of *V. harveyi* phage as well as the phage *Vi ha* 19 to control *V. harveyi* consortium in *P.monodon* post larvae to protect them from vibriosis. The phage cocktail and the most potent phage *Viha* 19 showed remarkable effectiveness in the control of the consortium of *Vibrio*. Potential of *Vi ha* 19 to manage the consortium of *Vibrio harveyi* is highly evident as it alone could bring down the requirement of phage cocktail to manage vibrios in larval rearing systems. In both the cases larval survival was found to be over 85% compared to that with the bacterial control. Accordingly, the management of *Vibrio* in hatchery point of view happens to be possible either using phage cocktail or single potent phage brought out through this investigation.

- *In vivo* trials proved that under nutrient limited conditions there would not be any emergence of BIMs. Thus the management of vibrios in hatchery point of view appears to become a reality either using phage cocktail or the single potent phage *Vi ha* 19 isolated through this investigation.

Precisely, out of 33 *Vibrio harveyi* phages isolated from different parts of Kerala, 6 were selected as broad spectrum lytic phages, which were

characterized based on TEM, RAPD profiling and protein profiling by SDS-PAGE. Following the amplification of lysogenic/toxin gene using specific oligonucleotide primers, one phage was eliminated as it contained partial sequence of Dam toxin gene corresponding to positive control VHML. As a result, 5 *Vibrio harveyi* phages were selected to be used as therapeutics both *in vitro* and *in vivo* experiments. Therapy using phage cocktail against *Vibrio harveyi* consortium provided encouraging results in protecting shrimp larvae from vibriosis alike the most potent phage *Vi ha* 19 which was comparatively more efficient in controlling *Vibrio* without the emergence of BIMs. Accordingly, it could be concluded that *Vi ha* 19 alone would serve the purpose of controlling *Vibrio* in shrimp larval rearing systems in the place of phage cocktail, which transforms the whole process simple and economically viable.

5.5 New areas of research emerged from the study

- Further studies are required to be carried out on the most potent lytic phage *Vi ha* 19 which includes extensive field level application, designing the phage application as a technology package and whole genome sequencing.

- Cloning the phage encoded lysin and holin genes in an expression system and their expression and mass production, to be used as drugs to control the pathogens.

- *Vi ha* 68, from which partial amplification of Dam toxin gene obtained has to be subjected for further investigations to evaluate its efficacy to transform an avirulent *Vibrio* to virulent and the development of lysogeny.

- TEM analyses of the novel morphotype brought out through this study, its whole genome sequencing, and positioning it in the phylogenetic tree.

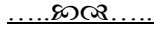
- Examination of BIMs of the phage *Vi ha* 32 to evaluate the loss of virulence of *Vibrio* as a result of phage infection.

5.6 Future Prospects for Phage Therapy

- Recent advance in genomics and phylogenic studies make it possible to understand gene flow among phages and hosts by which potentially harmful bacteriophages could be avoided or re-designed without undesirable traits.

- Comparative genomics of phage tail fibre genes involved in the recognition of specific host receptors will lead to approaches to expand the host range. A detailed analysis of the bacterial receptors will also help understand and predict the development of bacteria insensitive mutants often due to the loss or mutation of these molecules**.**

- The co-administration of phages and antibiotics may help prevent the emergence of bacterial resistance to antibiotics, thereby greatly prolonging their clinical usefulness.



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Appendix
Sequences of Amplified Genes

1 16S r-RNA gene sequences

1.1 *Vibrio harveyi* LB 6 (MCCB 153)

AACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAA
TTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTACG
GGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCT
AGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATC
AGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGG
TTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACGT
TAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGA
TGTGAAAGCCCGGGGCTCAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTG
TAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACC
GGTGGCGAARGCGGCCCCCTGGACAGATAMTGACAYTCAGATGCGAAAGCGTGGGGAG
CAAACAGGRWTTAGATACCCTGGTAGTWCACGCCGTAAACGATGTCTACTTGGAGGTTG
TGKCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGG
TCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGA
GATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTG
TTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGA
GTAATGTCGGGAACTCCAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGA
CGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACA
GAGGGCAGCCAACTTGCGAGAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATCG
GAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCA
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGC
AAATAGAAGCT

1.2 *Vibrio harveyi* LB 15 (MCCB 154)

TGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGA
AATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTA

CGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAG
CTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA
TCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGG
GTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACG
TTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGA
TGTGAAAGCCCGGGGCTCAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTG
TAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACC
GGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGT
GGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGT
CGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAG
ATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
TGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAG
TAATGTCGGGAACTCCAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGAC
GTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAG
AGGGCGGCCAACTTGCGAGAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATCGG
AGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCAC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCA
AATAG

1.3 *Vibrio harveyi* LB 19 (MCCB 155)

GAGCGGAACGAGTTATCTGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGG
GTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTA
ATACCGCATAATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATA
TGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAG
CTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT
GTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAA
TAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC
CGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAG
GTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATTGCATTTGAAACT
GGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAG

AGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGAT
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACC
GCCTGGGGAGTACGGTCGCAAGATTAAAACTCAATGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGATGCAACGCGAGAACCTTACCTACTCTTGACATCCAG
AGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCT
GTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCC
TTGTTTGCCAGCGAGTAATGTCGGGAACTCCAGGGAGACTGCCGGTGATAAACCGGAGG
AAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTAC
AATGGCGCATACAGAGGGCGGCCAACTTGCGAGAGTGAGCGAATCCCAAAAAGTGCGT
CGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGT
GGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
TGGGAGTGGGCTGCAAAAGAAGTAGGTAGTTTAACCTTCG

1.4 *Vibrio harveyi* LB 21 (MCCB 156)

TAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGG
ATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGACC
TTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAAGGG
CTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA
GCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAG
TCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGC
ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCT
CAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATT
TCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAAATACCCGGTGGGCGAAAGGC
GGCCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACTGGATTA
GATACCC

1.5 *Vibrio harveyi* LB 32 (MCCB 157)

TGAACCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGA
AATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTA
CGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAG
CTAGTTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGA

TCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
ATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGG
GTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACG
TTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGT
GCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGA
TGTGAAAGCCCGGGGCTCAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTG
TAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACC
GGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGT
GGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGT
CGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAG
ATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
TGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAG
TAATGTCGGGAACTCCAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGAC
GTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAG
AGGGCGGCCAACTTGCGAGAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATCGG
AGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCAC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGC

1.6 *Vibrio harveyi* LB 68 (MCCB 158)

CCTTCGGGGAACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTG
CCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGC
CAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGT
TGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGC
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
ACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGT
AAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTAATAGCTGCATTATTTGACGTTAGC
GACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGA
GCGTTAATCGGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTG
AAAGCCCGGGGCTCAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTGTAGA
GGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTG
GCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCT
TGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCGCAA

GATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGG
ATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG
AAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAGTAAT
GTCGGGAACTCCAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCA
AGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAGAGGG
CGGCCAACTTGCGAGAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATCGGAGTC
TGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTG
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGG

2 N6 Dam toxin genes

2.1 Dam Toxin gene amplified from *Vi ha* 68

TGAGCATGCCGATGGTCTTAACAAGGACCACCAGCAATTAACAGAGGAATACGGCCAGC
CTCACAGGACGTATGTAGAGCTTCGCCGGGACTATGATGATTTGCGTGTTCAGTATGAAC
AGTTGCGCAGACCGTTTGGTGTTACGGCTGATGTGCCATATACGGATGTGTGGGATTTTG
ACC

2.2 Dam Toxin gene amplified from VHML

TGATCATGCCGATGGTCTTAACAAGGACCACCAGCAATTAACAGAGGAATACGGCCAGC
TTCACAGGACGTATGTAGAGCTTCGCCGGGACTATGATGATTTGCGTGTTCAGTATGAAC
AGTTGCGCAGACCGTTTGGTGTTACGGCTGATGTGCCATATACGGATGTGTGGGATTTTG
ACC

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