

# ajava

Asian Journal of Animal and Veterinary Advances



Academic  
Journals Inc.

[www.academicjournals.com](http://www.academicjournals.com)

## **Inhibitory Effects of Bacteriophages on the Growth of *Vibrio* sp., Pathogens of Shrimp in the Indian Aquaculture Environment**

<sup>1</sup>P. Srinivasan, <sup>1</sup>P. Ramasamy, <sup>2</sup>G. P. Brennan and <sup>3</sup>R.E.B. Hanna

<sup>1</sup>Department of Biotechnology, Life Sciences Building, University of Madras,  
Guindy Campus, Chennai-600 025, Tamil Nadu, India

<sup>2</sup>School of Biology and Biochemistry, Medical Biology Centre,  
The Queens University of Belfast, Belfast, BT9 7BL, Northern Ireland, UK

<sup>3</sup>Pathology Department, Veterinary Sciences Division, Agri-Food and Biosciences,  
Stormont, Belfast Bt43SD, Northern Ireland, UK

---

**Abstract:** Bacteriophages of *Vibrio harveyi* from a shrimp farm located at Tuticorin, Tamilnadu, India, were isolated, characterized and identified. The phages, when examined by electron microscopy, were found to have icosahedral morphology. The phages belong to the Siphoviridae family and were designated VH1 to VH8. They produced circular clear plaques of growth inhibition on *Vibrio* sp., plate cultures. The growth inhibitory effects of the bacteriophages on isolates of *V. harveyi* were screened by a one-step agar bioassay method and the results suggest that the infectivity of the *V. harveyi* phages was not affected by ribonuclease, protease, trypsin, chloroform, acetone, ethyl alcohol, methyl alcohol and Triton-X100 treatment at 37°C. Even after their storage in chloroform or DMSO (dimethyl sulfoxide, 7%) at -40°C. They showed 100% infectivity and plaque-forming ability. In contrast, Infectivity of the *V. harveyi* phages was affected by lysozyme and SDS and in these cases; plaques were not formed on agar plate assays. The phages were found to be stable over the pH range 6-11 and exhibited optimal survival at 37°C while complete inactivation occurred at temperatures exceeding 55°C. All the isolates of bacteriophage (VH1-VH8) initiated an infection cycle and caused lysis of the host bacterial cells within 2 h from the start of incubation. The propagation curve for each phage shows a burst time started from 1-10 h. The results suggest that bacteriophages of *Vibrio* sp., might be effectively used *in vivo* as biological agents to control these pathogenic bacteria in aquaculture systems.

**Key words:** *Vibrio harveyi*, bacteriophage, siphoviridae, infectivity, growth inhibition

---

### **INTRODUCTION**

Bacteriophages represent a potential alternative to the use of antibiotics in the control of pathogenic bacteria. Bacteriophages have been administered to infected animals and humans in an effort to reduce populations of pathogenic bacteria through the natural ability of the phages to target and destroy bacterial cells. Interest in the ability of phages to control bacterial populations has extended from medical applications into the fields of agriculture, aquaculture and the food industry (Fuhrman and Suttle, 1993; Nakai and Park, 2002; Withey *et al.*, 2005). Viruses of bacteria have been hypothesised to play a key role in controlling host bacterial population density and diversity, nutrient

---

**Corresponding Author:** Dr. P. Ramasamy, Department of Biotechnology, Life Sciences Building, University of adras, Guindy Campus, Chennai-600025, Tamil Nadu, India  
Tel: +91-44-22351269/229 Fax: + 91-44-24916316

flow in marine food webs and the transfer of genes between hosts (Heldal and Bratbak, 1991; Fuhrman and Suttle, 1993). Due to their antibacterial activities, interest in the possibilities of bacteriophage therapy, as an alternative to antibiotics, is re-emerging (d'Herelle, 1917). Bacteriophage-typing schemes have been developed for most groups of pathogenic bacteria and more recently their host specificity has been applied in bacterial detection and diagnostic strategies (Marks and Sharp, 2000).

The present study was undertaken to investigate the antibacterial potential of bacteriophages isolated from cultures of *Vibrio* spp. found in shrimp aquaculture systems and in the marine environment of South India. This information may help in the development of specific phage therapy applicable to local aquaculture systems, which is considered to represent an interesting emerging alternative to antibiotic therapy and vaccination.

## MATERIALS AND METHODS

### Bacteria, Phages, Media and Culture Methods

*V. harveyi* isolates were obtained from shrimp farms located along the coast of the Bay of Bengal at Thulunkenkulam (30 kms from Tuticorin, 08°40' 05 to 08° 48'05 N; 78° 10' 05 to 78° 14' 10 E), Chennai (Tamil Nadu) and Nellore (Andhra Pradesh), India (Fig. 1) during 2001-2002 and grown on TCBS (Thio-sulphate Citrate Bile Salts) agar. The bacteria were cultured in LB liquid media for 24-48 h at 37°C and the cultures were then centrifuged at 10000 rpm for 5 minutes to sediment the bacterial cells. The supernatant was decanted and saved. Four drops of chloroform were added to the supernatant to kill any residual bacteria.

### Phage Isolation and Purification

*V. harveyi* phages ( $3 \times 10^3$  plaque forming units/mL) were mixed with an equal volume of overnight *Vibrio* bacterial cell culture and incubated at room temperature for 30 min to allow the phage particles

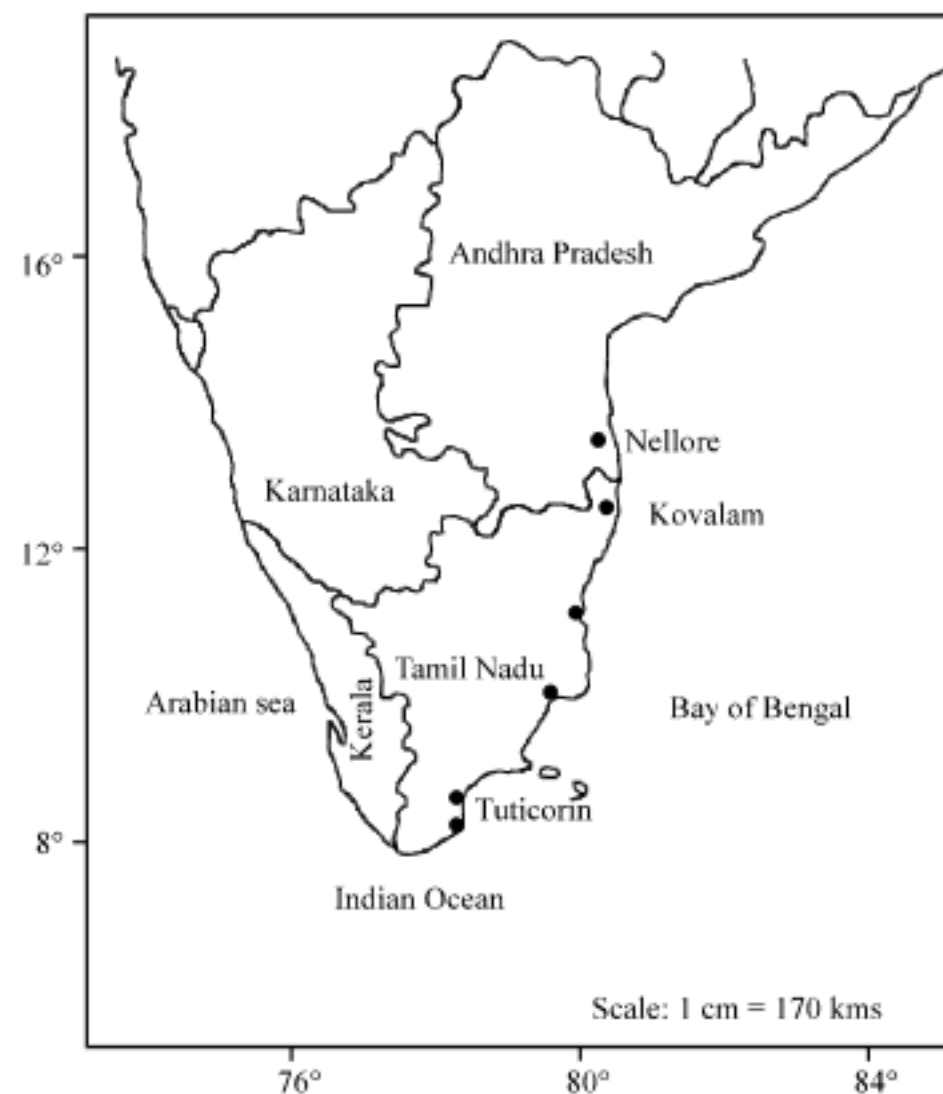


Fig. 1: South India map showing location of (collection sites) shrimp farms and hatcheries

to adsorb to the bacteria. The incubated sample was mixed with pre-cooled (45°C) 0.6% Luria Bertaini top agar and evenly poured over pre-set 1.5% (w/v) Luria Bertaini agar. The top agar mixture was allowed to set for 5-15 min at bench temperature (25°C) and the plate was incubated at 37°C for 24-48 h. When confluent lysis occurred, bacteriophages were harvested by adding 5 ml of SM solution [NaCl (5.8 g), MgSO<sub>4</sub> (2 g), 1 M Tris.Cl (pH 7.5 - 50 mL), 2% (w/v) Gelatin solution (5 mL) and Milli Q water (made up to 1000 mL)] and leaving the plate at room temperature (25°C) for 30 min with gentle shaking. This allowed the phages to diffuse out of the agar and the resulting suspension of phages was termed the 'lysate'. The lysate was removed from the culture plate and treated with chloroform at ratio [3 Lysate: 1 chloroform] for 30 min at room temperature. It was then centrifuged at 4°C for 15 min at 5000 rpm to remove the pellet containing unlysed, uninfected and infected bacterial cells and the supernatant containing phages was recovered. The supernatant was further filtered through a 0.22-µm pore-size syringe filter to remove any remaining unlysed, bacterial cells and the filtrate contained only phages and thus the phage purification was carried out repeatedly by following the method of Moebus and Nattkember (1981) to ensure the purity of the phage stock. The phage suspension was stored at -20°C prior to further use.

#### **Long-term Storage of *V. harveyi* Bacteriophage**

The following procedure was used for long-term storage of *Vibrio* bacteriophages in the archival collections. The phage isolate was treated with 7% DMSO (Dimethyl sulfoxide) (v/v) or with an equal volume of chloroform and frozen at -40°C (30 days). The stocks of each phage isolate were subdivided into 100 mL prior to storage. For use, the phage stocks were removed from storage and thawed. The remaining stock if any was recapped and returned immediately to -40°C. The contents of tubes containing aliquots of phages were mixed with the appropriate host bacterial cell isolates and streaked on to Nutrient/Luria Bertaini agar medium Petri dishes and in each case a single phage plaque was isolated and used subsequently.

#### **Infectivity and Inhibitory Effects of Bacteriophages on the growth of Isolates of *Vibrio* sp.**

The agar bioassay method and one step growth experiments were carried out to determine the infectivity and inhibitory effect of *Vibrio* bacteriophages.

#### **Agar Bioassay**

Inhibitory effects of eight bacteriophages of *V. harveyi* were determined against 40 different isolates of *Vibrio* sp., Sterilised Nutrient/Luria Bertaini agar medium was poured into Petri dishes and after solidification, two 3 mm diameter wells were punched in each plate with a gel puncher. One hundred µL of target *Vibrio* cell culture was spread on each agar plate and 100 µL aliquots of phages was pipetted into one of the wells. The remaining empty well was inoculated with sterile Nutrient broth/Luria Bertaini broth alone (without phages) to serve as a negative control. A zone of clearance around the well indicated phage infectivity and lysis of the cells of *Vibrio* sp.

#### **One-step Bacteriophage Growth Experiment**

The one-step bacteriophage growth experiment was developed by Ellis and Delbruck (1939) and was later described by Adams (1959). It was used to determine the lytic burst size and burst time for *Vibrio* bacteriophages. One ml of cells of the *Vibrio* isolate in LB broth was mixed with 1 ml of *Vibrio* bacteriophage suspension (containing  $3 \times 10^3$  plaque-forming units). Optical density readings of the mixture of bacteriophage and *Vibrio* host cells were taken every 1 h up to 10 h at 600 nm (OD<sub>600</sub>) using a UV1601 visible spectrophotometer (Shimadzu corporation, Japan).

#### **Electron Microscopy**

A suspension of *V. harveyi* phage infected *V. damsela* was centrifuged at 5000 rpm for 30 min and the resulting pellet was fixed with 2% EM grade glutaraldehyde buffered with 0.1 M sodium

cacodylate containing 3% sodium chloride and 5% sucrose. After 24 h fixation, the pellet of *V. harveyi* phage infected *V. damsela* was washed repeatedly with sodium cacodylate buffer, post fixed with 0.1% OsO<sub>4</sub> for 1 h at 4°C, dehydrated through a graded ethanol series (from 30% ethanol with 30 min. in each change) and embedded in Epon 812. Sixty to ninety-nanometer (i.e., gold-and silver-colored) sections were cut with a diamond or glass knife using a reichert Ultracut E ultramicrotome, stained with aqueous uranyl acetate and concentrated lead citrate and viewed using a Philips E10 JEOL 100B transmission electron microscope at an accelerating voltage of 80 kV. Electron micrographs of random bacterial cell clusters were taken.

#### **Effects of Enzymes, Organic Solvents and Detergents on Infectivity, Survival and Multiplication of *V. harveyi* Bacteriophages**

Purified *Vibrio* bacteriophage lysate samples ( $3 \times 10^3$  PFU mL<sup>-1</sup>) were treated with enzymes (RNase, protease, lysozyme and trypsin at a final concentration each of 100 µg mL<sup>-1</sup>) organic solvents (chloroform, acetone, ethyl alcohol and methyl alcohol at a final concentration each of 50%) and detergents (SDS and Triton-X 100 at a final concentration each of 50%) and were incubated at 37°C for 1 h. A purified *Vibrio* bacteriophage lysate sample incubated without enzymes, organic solvents or detergents served as control. The contents of tubes containing aliquots of treated and untreated phages were mixed with the appropriate host bacterial cell isolates and streaked on to Nutrient/Luria Bertaini agar medium. The samples were incubated for 16-24 h at 37°C and the number of plaques formed on a lawn of *Vibrio* cells in the treated and untreated samples were recorded and compared.

#### **Effect of pH on the Stability and Infectivity of *V. harveyi* Phages**

Growth media (LB broth/Nutrient broth) with a range of different pH values (4-12) were prepared using 5 N NaOH or 1 N HCl and were used to test their effect on the stability and infectivity of *V. harveyi* phages. Reaction mixtures incubated without the *V. harveyi* bacteriophage served as control. Samples of *V. harveyi* phage lysate were used to infect uninfected cells of *Vibrio* sp., by mixing and incubating for 1 h and subsequently the suspension of phage adsorbed/infected cells was spread on the agar plates and incubated for 16-24 h at 37°C and the plaques formed were counted and compared.

#### **Effect of Temperature on the Survival and Infectivity of *V. harveyi* Phages**

Samples of *V. harveyi* phage lysate ( $3 \times 10^3$  PFU mL<sup>-1</sup>) were incubated at temperatures ranging from 20°C to 90°C for 1 h and were used to test their effect on the stability and infectivity of *Vibrio* phages. Samples of treated and untreated *Vibrio* phage lysate were used to infect cells of *Vibrio* sp., by mixing and incubating them for 1 h and subsequently the suspension of phage adsorbed/infected cells was spread on the agar plates and incubated for 16-24 h at 37°C and the plaques formed were counted and compared.

## **RESULTS**

#### **Detection of *V. harveyi* Bacteriophages**

Phages of *V. harveyi* designated as VH1-VH8 were isolated from aquaculture environment in India. They produced circular plaques on isolates of *V. harveyi*, *V. parahaemolyticus*, *V. anguillarum* and *V. mimicus*. The plaques formed were about 1.9 mm in diameter after 24 h of incubation at 37°C. Isolates of phages of *V. harveyi* were collected from *P. monodon* egg sample, *Artemia nauplii*, *Artemia nauplii* rearing tank water; MBV-infected post-larval *P. monodon*, source sea water and from WSSV-infected shrimp tissues viz., hepatopancreas, intestine and gills of *P. monodon* (Table 1 and Fig. 2).

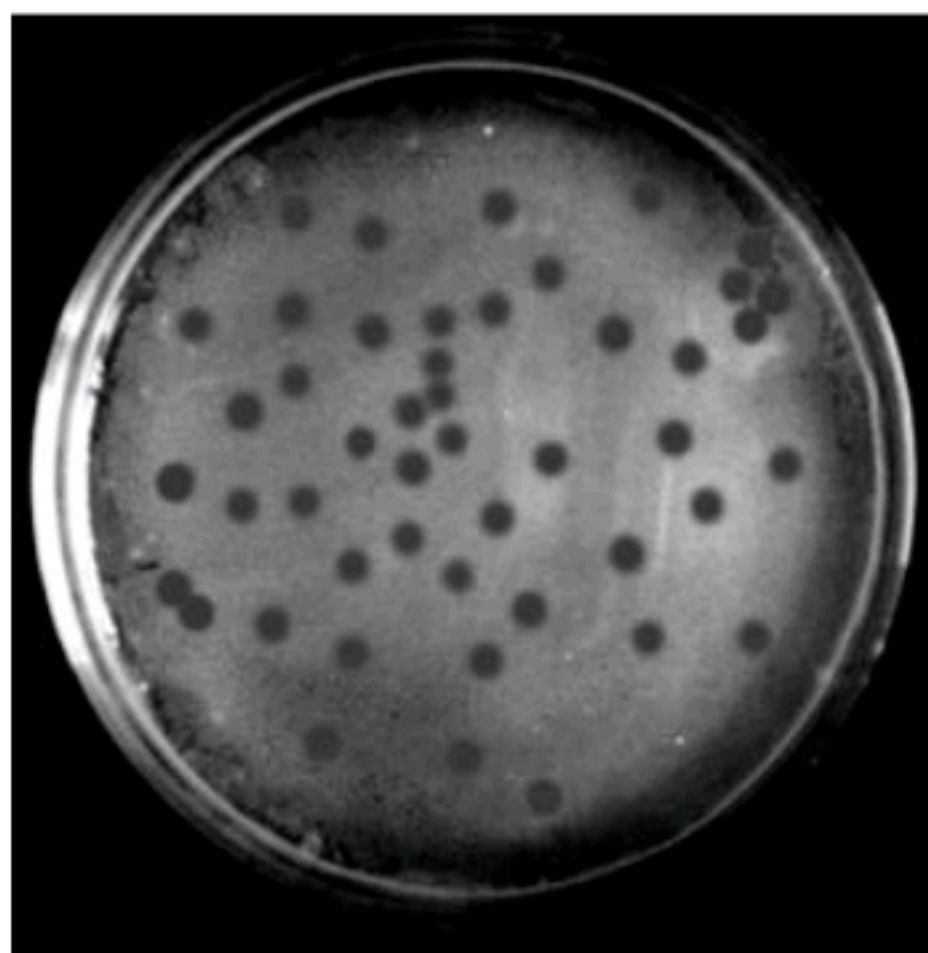


Fig. 2: Photographs showing clear circular plaques (1.9 mm) produced by bacteriophages of *Vibrio harveyi*

Table 1: Sources and total number of *Vibrio* sp., isolates recovered from the aquaculture environments

Name of the bacteriophages	Sources of isolation	Place of collection	Size of plaque (mm)
<i>Vibrio harveyi</i> (VH-1)	Egg samples	Tuticorin	1.9
<i>Vibrio harveyi</i> (VH-2)	Egg samples	Tuticorin	1.9
<i>Vibrio harveyi</i> (VH-3)	<i>Artemia</i> nauplii	Tuticorin	1.9
<i>Vibrio harveyi</i> (VH-4)	<i>Artemia</i> nauplii	Tuticorin	1.9
<i>Vibrio harveyi</i> (VH-5)	Hatchery tank water	Tuticorin	1.9
<i>Vibrio harveyi</i> (VH-6)	Uninfected post larvae	Chennai	1.9
<i>Vibrio harveyi</i> (VH-7)	MBV infected post larvae	Tuticorin	1.9
<i>Vibrio harveyi</i> (VH-8)	WSSV infected gills	Nellore	1.9

#### Long-term Storage of *V. harveyi* Bacteriophages

The plaque-forming experiments revealed that the ability of infectivity of *V. harveyi* bacteriophages was not affected even after their storage in chloroform or DMSO (dimethyl sulfoxide, 7%) for 30 days at -40°C. All the stored samples of bacteriophages in chloroform or DMSO (dimethyl sulfoxide, 7%) for 30 days at -40°C showed 100% infectivity and plaque-forming ability.

#### Growth Inhibitory Effects of *V. harveyi* Bacteriophages on Isolates of *Vibrio* spp. Determined by the Agar Bioassay Method

All the isolates of bacteriophages were found to inhibit the growth of several different isolates of *Vibrio* spp. when they were assayed in LB/Nutrient agar medium. The inhibition of growth of *Vibrio* isolates by *V. harveyi* bacteriophages was found to be a maximum at a temperature of 37°C. Three of the *V. harveyi* phage isolates namely VH-1, VH-3 and VH-6 showed 62.5% of inhibition of growth of *Vibrio* sp., while the other bacteriophage isolates showed inhibition of growth in the order VH-2, VH-4 and VH-7 (60%), VH-8 (57.5%) and VH-5 (55%) (Table 2 and Fig. 3a-d).

#### One-step Bacteriophage Growth Experiment

The propagation curve for each phage shows a burst time started from 1-10 h. The rate (percentage) of absorption of phages indicated that more than 80% of the bacteriophages adsorbed to

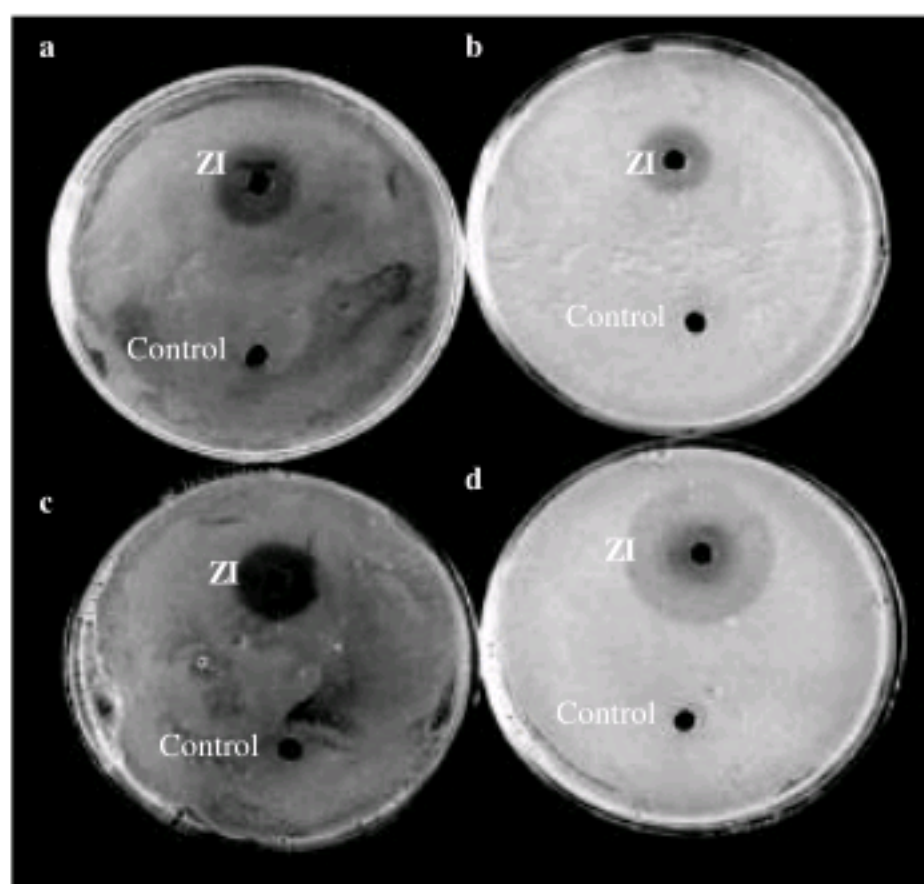


Fig. 3a-d: Petriplates consisting of *Vibrio* isolates exhibiting a zone of inhibition around the well of *Vibrio harveyi* bacteriophage against (a) *Vibrio mimicus*, (b) *Vibrio furnissi*, (c) *Vibrio parahaemolyticus* and (d) *Vibrio damsela*. ZI- Zone of Inhibition

Table 2: Infectivity and inhibitory effects of *Vibrio* bacteriophages on the growth of *Vibrio* isolates recovered from aquaculture environments using agar bioassay

Name of the Bacteriophages	Total No. of the <i>Vibrio</i> isolates tested	Inhibitory effect		Percentage infectivity of <i>Vibrio</i> phages (%)
		No. of <i>Vibrio</i> isolates showing inhibitory zone	No. of <i>Vibrio</i> isolates showing no inhibitory zone	
<i>Vibrio harveyi</i> (VH-1)	40	25 isolates	15 isolates	62.5
<i>Vibrio harveyi</i> (VH-2)	40	24 isolates	16 isolates	60.0
<i>Vibrio harveyi</i> (VH-3)	40	25 isolates	15 isolates	62.5
<i>Vibrio harveyi</i> (VH-4)	40	24 isolates	16 isolates	60.0
<i>Vibrio harveyi</i> (VH-5)	40	22 isolates	18 isolates	55.0
<i>Vibrio harveyi</i> (VH-6)	40	25 isolates	15 isolates	62.5
<i>Vibrio harveyi</i> (VH-7)	40	24 isolates	16 isolates	60.0
<i>Vibrio harveyi</i> (VH-8)	40	23 isolates	17 isolates	57.5

the host cells during 10 h incubation. All the isolates of bacteriophage (VH-1-VH-8) initiated an infection cycle and caused lysis of the host bacterial cells within 2 h from the start of incubation. The absorbance ( $A_{600}$ ) for all the phage-treated experimental samples of the isolates of *Vibrio* spp. was 0.05 at the beginning and reached a maximum value of 0.168 (VH-1), 0.062 (VH-2), 0.168 (VH-3), 0.76 (VH-4), 0.117 (VH-5), 0.076 (VH-6), 0.07 (VH-7) and 0.201 (VH-8) after 2 h of incubation and then, in all cases, the absorbance declined during the subsequent period, reaching values of 0.125 (VH-1), 0.028 (VH-2), 0.011 (VH-3), 0.024 (VH-4), 0.011 (VH-5), 0.024 (VH-6), 0.008 (VH-7) and 0.12 (VH-8) after 10 h of incubation. In contrast, the absorbance value of the control samples, incubated in the absence of bacteriophage, was 0.05 at the beginning of incubation and subsequently increased to reach a maximum value of 2.08 (VH-1), 2.0 (VH-2), 2.078 (VH-3), 0.321 (VH-4), 0.458 (VH-5) and (VH-6), 0.278 (VH-7) and 9.1 (VH-8) after 10 h of incubation at pH 7 indicating the proliferation and growth of uninfected bacterial cells (Fig. 4a-h).

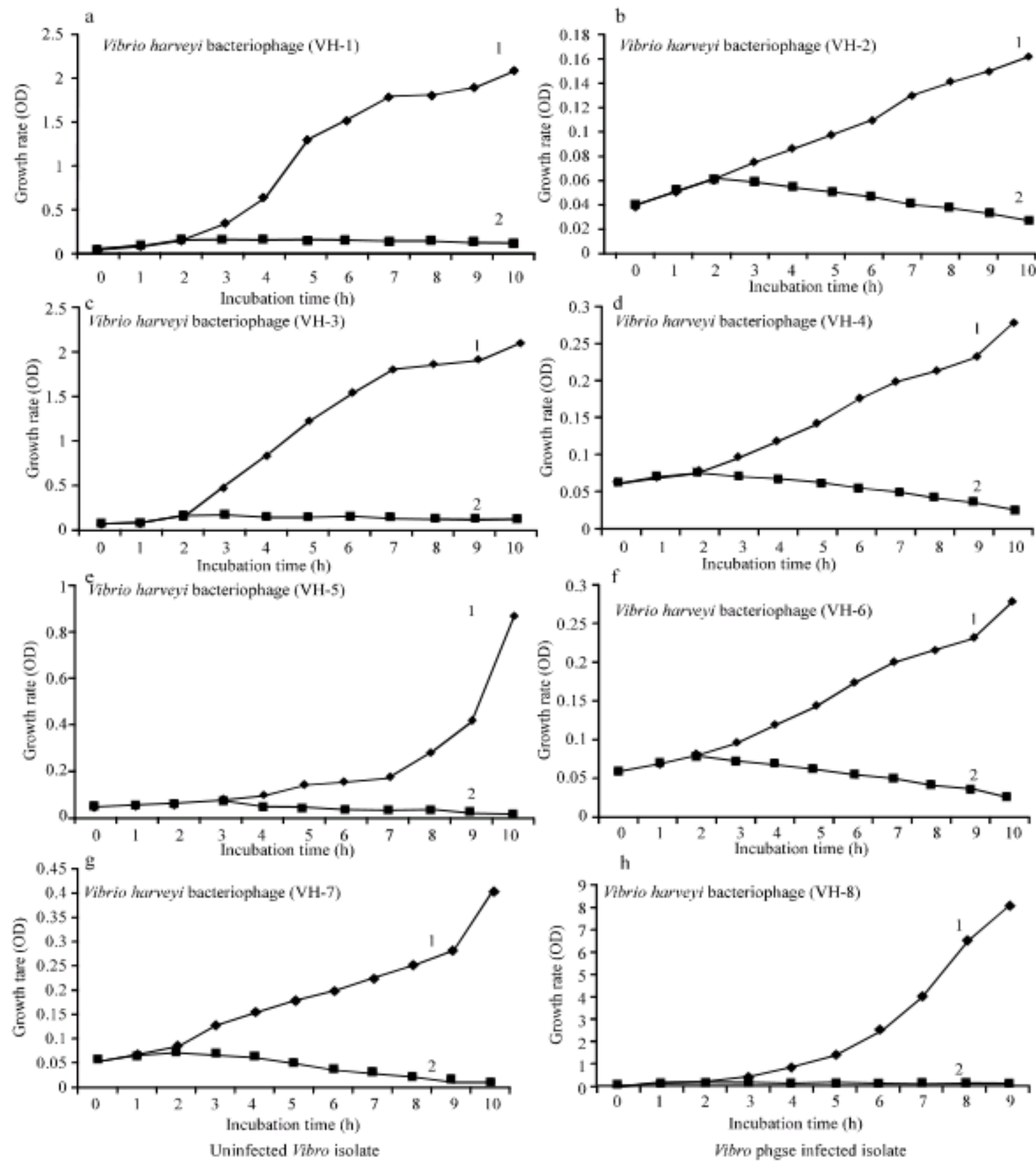


Fig. 4: One step growth experiment of *Vibrio* isolates infected with *Vibrio harveyi* bacteriophages (VH1-VH8, a-h, respectively) compared with uninfected controls. The incubation time was 0-10 h at 37°C; 1 = Uninfected *Vibrio* isolate; 2 = *Vibrio harveyi* phage-infected isolate

#### Transmission Electron Microscopy of *V. harveyi* Bacteriophages

Transmission electron microscopy of bacteriophages and infected bacteria was carried out to determine the occurrence, structure, site and mode of infection of the phages. The eight distinct *V. harveyi* phage isolates were examined. Phages were commonly encountered on the cell wall of the infected *V. harveyi* cells examined. Each phage consisted of a double envelope enclosing the nucleic acid and proteins and also featured a tail. The head displayed icosahedral symmetry and the tail, when visible on the electron micrographs, was seen to be short. Considering the morphological features and the host genus, the phages can be classified as belonging to the order Caudovirales of the family Siphoviridae. Phages from each and every isolate examined (VH-1-8) were found to conform to this description and identity (Fig. 5a-h).



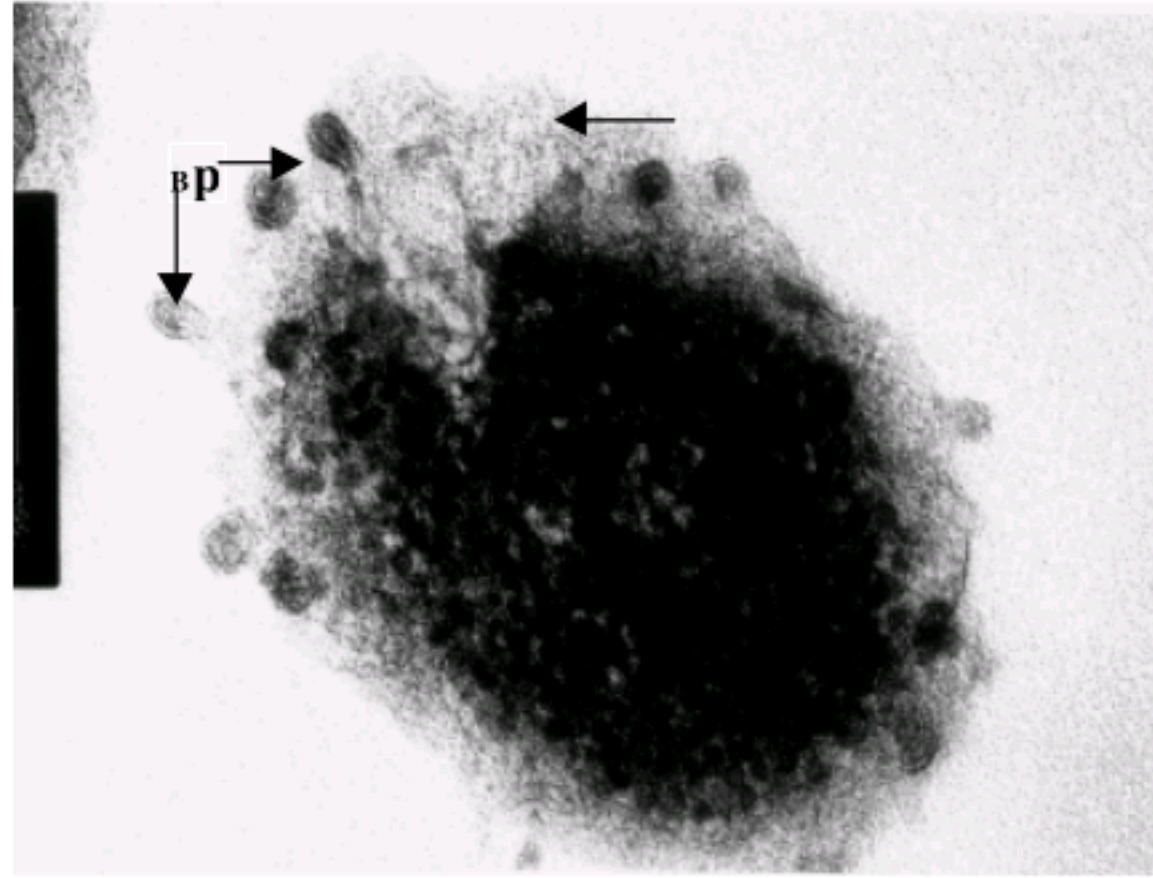


Fig. 5a: Transmission electron micrograph showing the surfacing of *Vibrio harveyi* (VH-1) phage from the cell wall of the host cell bacterium *Vibrio* sp., Note the presence of partially assembled phage (Bp) particles within the cytoplasm while those completely assembled particles are seen on the ruptured cell surface (unlabelled arrow)

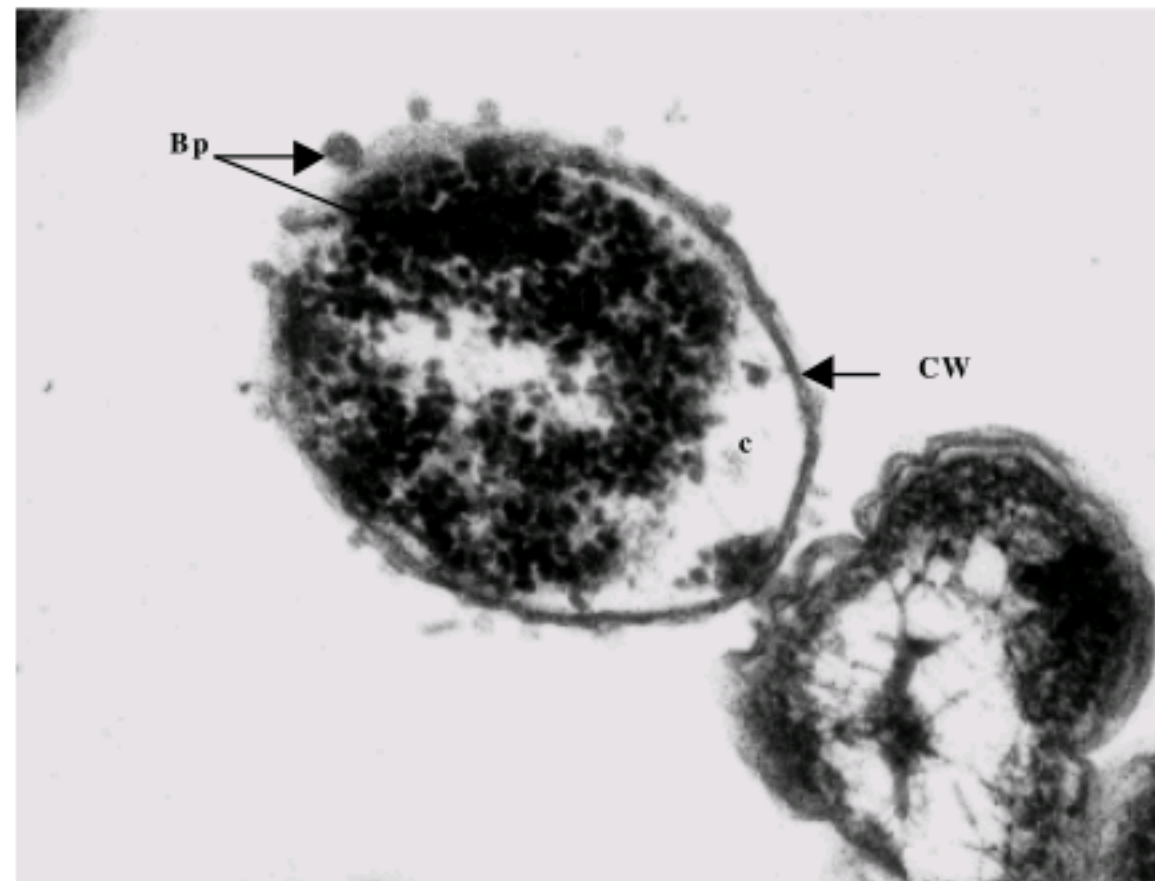


Fig. 5b: Transmission electron micrograph showing the surfacing of *Vibrio harveyi* (VH-2) phages (Bp) from the Cell Wall (CW) of the host cell bacterium *Vibrio* sp., Note the presence of partially assembled phage particles within the cytoplasm (c) while those completely assembled particles are seen on the cell wall

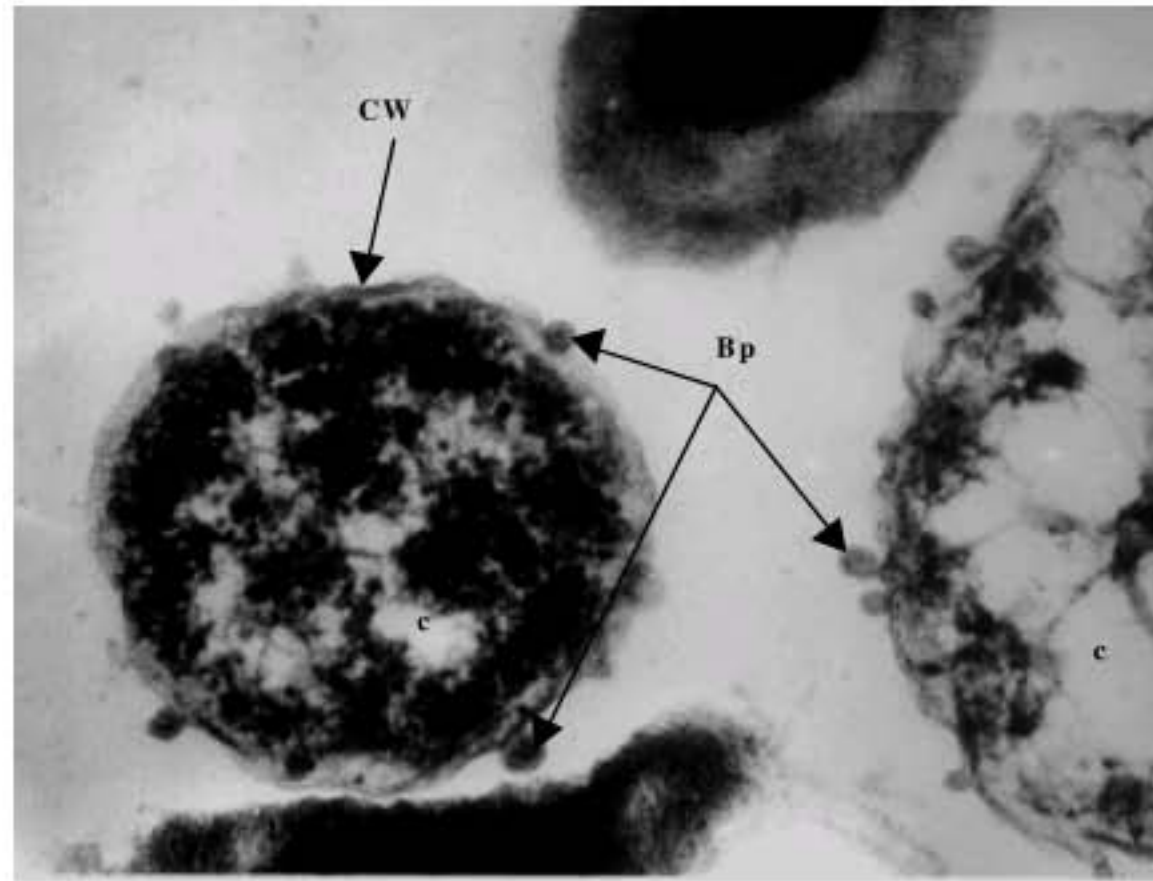


Fig. 5c: Transmission electron micrograph showing the surfacing of *Vibrio harveyi* (VH-3) phages (Bp) from the Cell Wall (CW) of the host cell bacterium *Vibrio* sp., Note the presence of partially assembled phage particles within the cytoplasm (c) while those completely assembled particles are seen on the cell wall

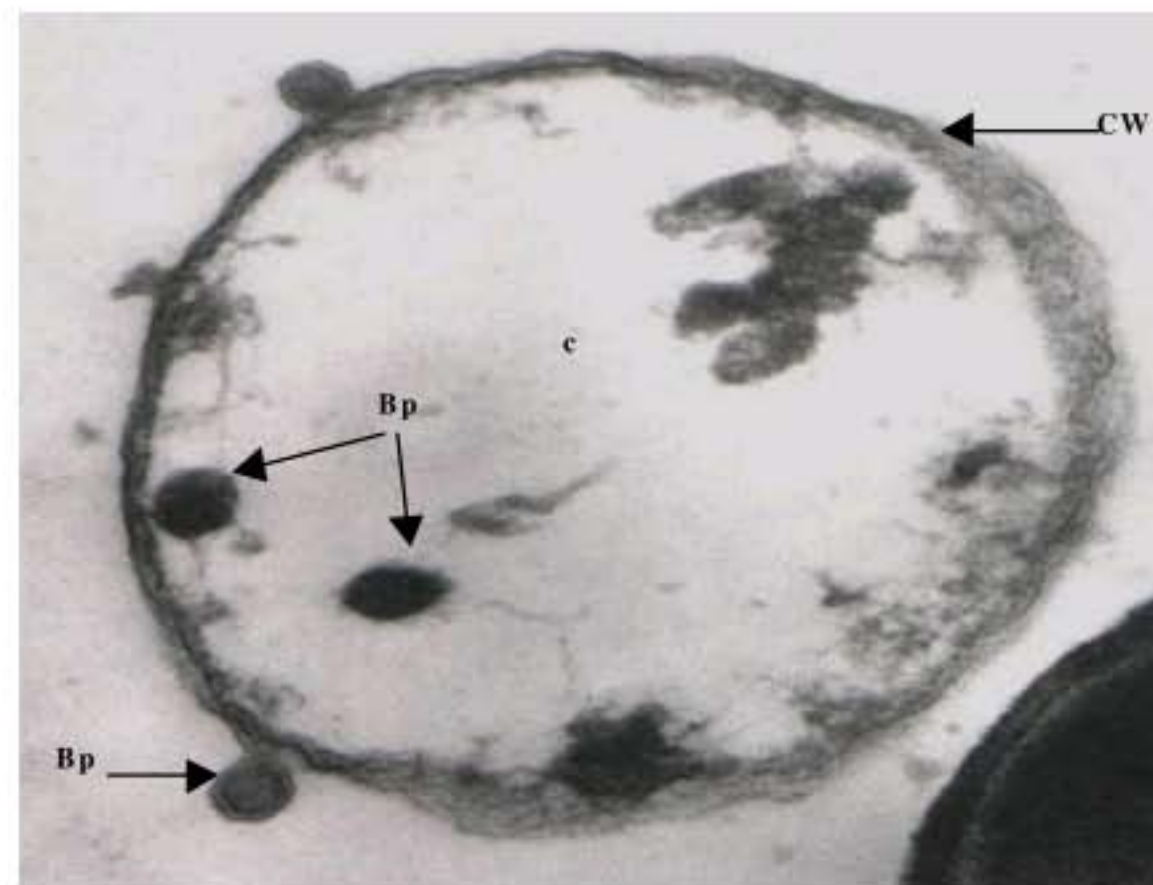


Fig. 5d: Transmission electron micrograph showing the surfacing of *Vibrio harveyi* (VH-4) phages (Bp) from the Cell Wall (CW) of the host cell bacterium *Vibrio* sp., Note the nature of the lysed cytoplasm (c)

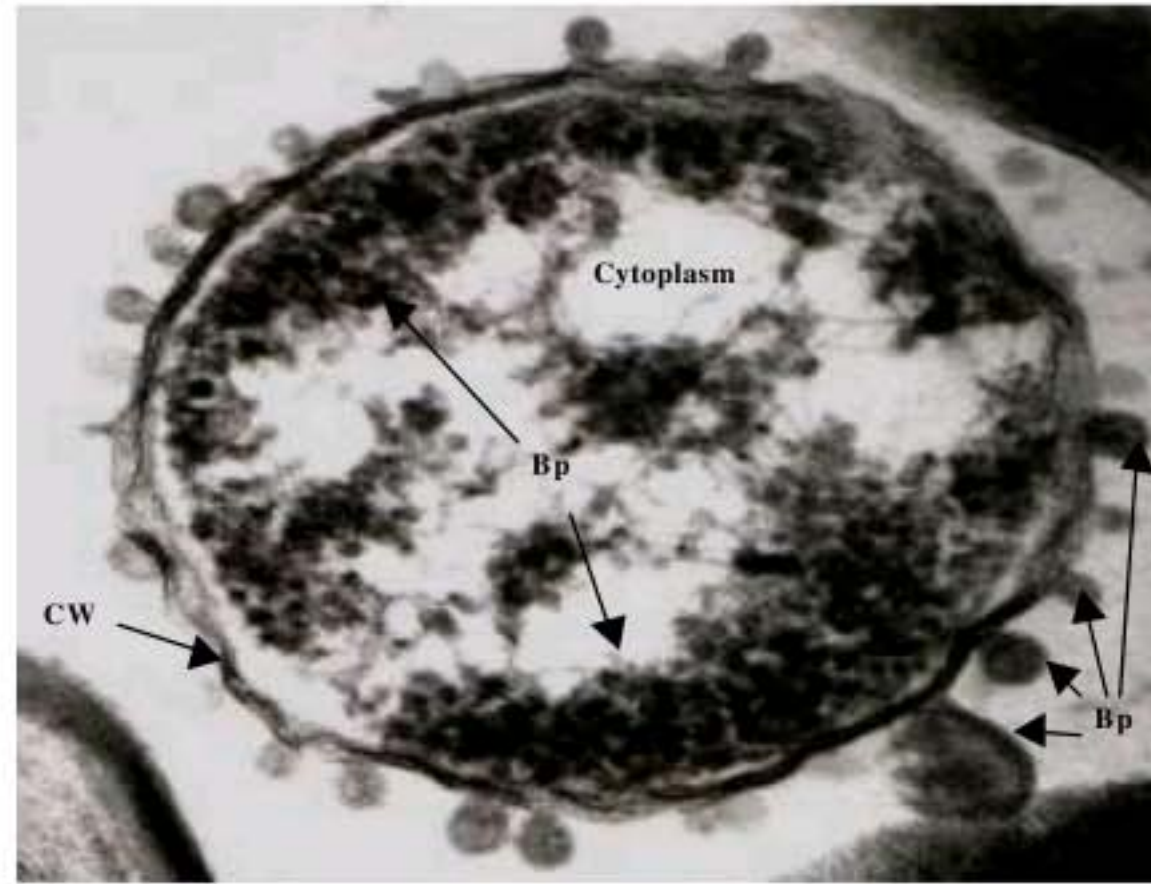


Fig. 5 e: Transmission electron micrograph showing the surfacing of *Vibrio harveyi* (VH-5) phages (Bp) from the Cell Wall (CW) of the host cell bacterium *Vibrio* sp., Note the presence of partially assembled phage particles within the cytoplasm while those completely assembled particles are seen on the cell

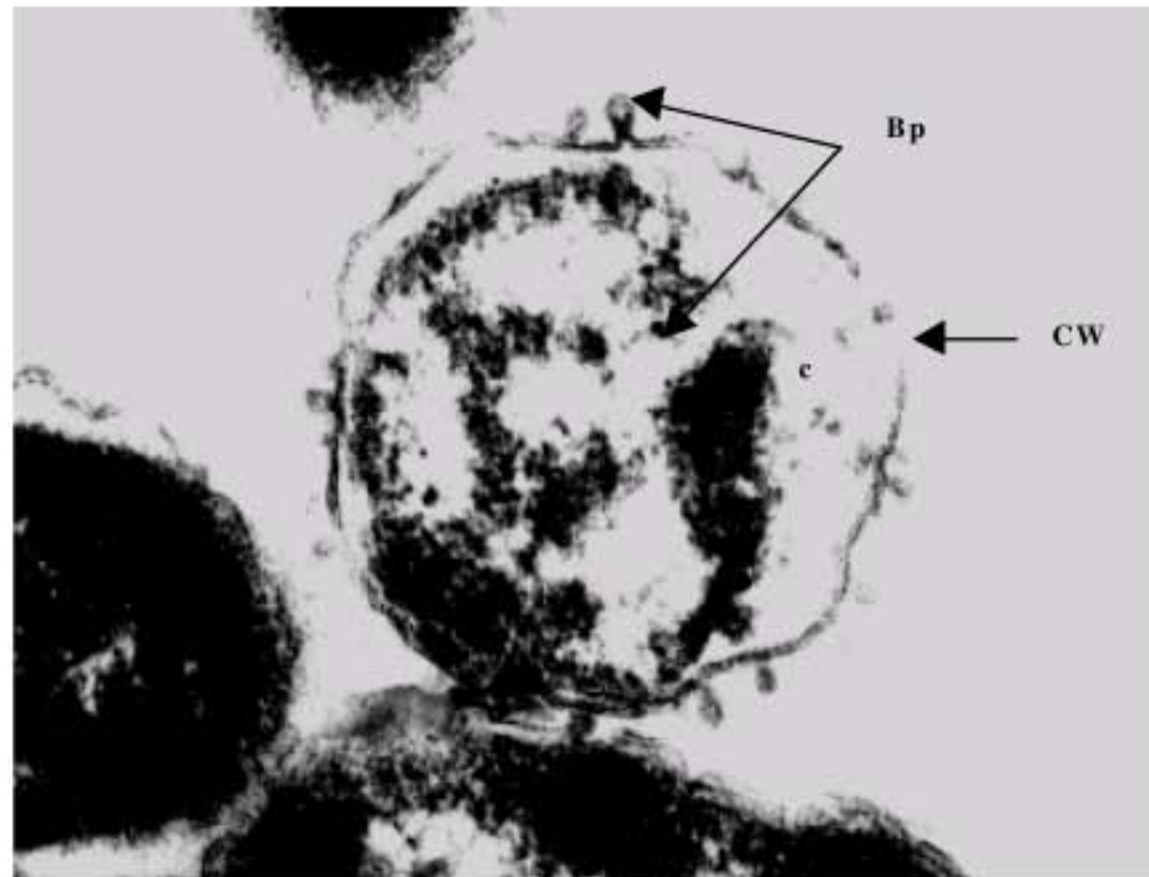


Fig. 5 f: Transmission electron micrograph showing the surfacing of *Vibrio harveyi* (VH-6) phages (Bp) from the Cell Wall (CW) of the host cell bacterium *Vibrio* sp., Note the presence of partially assembled phage particles within the cytoplasm (c) while those completely assembled particles are seen on the cell

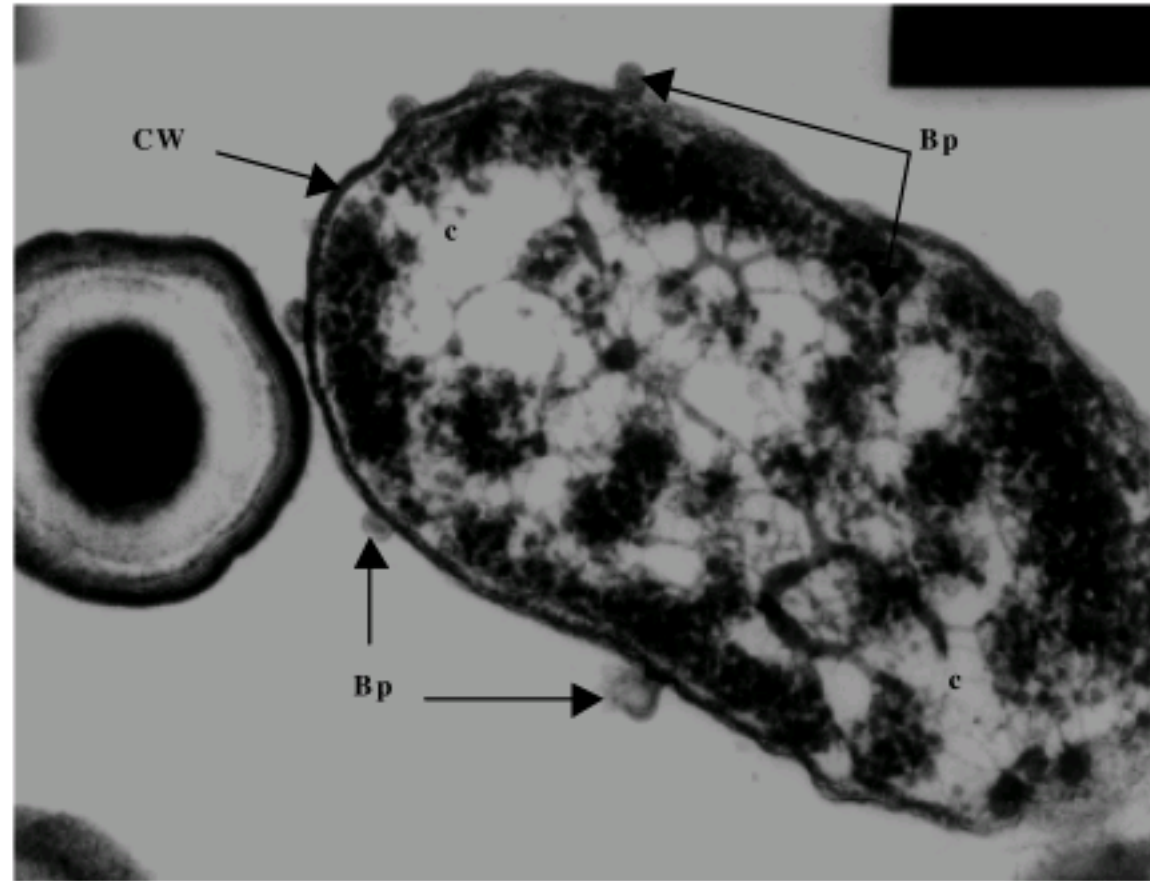


Fig. 5 g: Transmission electron micrograph showing the surfacing of *Vibrio harveyi* (VH-7) phages (Bp) from the Cell Wall (CW) of the host cell bacterium *Vibrio* sp., Note the presence of partially assembled phage particles within the cytoplasm (c) while those completely assembled particles are seen on the cell



Fig. 5 h: Transmission electron micrograph showing the surfacing of *Vibrio harveyi* (VH-8) phage (Bp) from the Cell Wall (CW) of the host cell bacterium *Vibrio* sp., Note the presence of partially assembled phage particles within the cytoplasm (c) while those completely assembled particles are seen on the cell

Table 3: Effects of enzymes viz., RNase, protease, lysozyme, trypsin (100 µg mL<sup>-1</sup>); organic solvents viz., chloroform, acetone, ethyl alcohol and methyl alcohol (50%); and detergents viz., SDS and triton-X 100 (1%) treated and untreated *Vibrio* bacteriophages

Name of the <i>Vibrio</i> bacteriophages	Phage infectivity (%)										
	Control	RNase	Protease	Lysozyme	Trypsin	Chloroform	Acetone	Ethyl alcohol	Methyl alcohol	SDS	Triton-x100
<i>Vibrio harveyi</i> (VH-1)	100	100	98.33	0	100	100	100	100	100	0	100
<i>Vibrio harveyi</i> (VH-2)	100	100	98.33	0	100	100	100	100	100	0	100
<i>Vibrio harveyi</i> (VH-3)	100	100	98.33	0	100	100	100	100	100	0	100
<i>Vibrio harveyi</i> (VH-4)	100	100	98.33	0	100	100	100	100	100	0	100
<i>Vibrio harveyi</i> (VH-5)	100	100	98.33	0	100	100	100	95.47	95.47	0	100
<i>Vibrio harveyi</i> (VH-6)	100	100	98.33	0	100	100	100	100	100	0	100
<i>Vibrio harveyi</i> (VH-7)	100	100	98.33	0	100	100	100	97.36	97.36	0	100
<i>Vibrio harveyi</i> (VH-8)	100	100	98.33	0	100	100	100	100	100	0	100

Experiments (treated with following)

Table 4: Effect of pH on the stability and infectivity of *Vibrio* bacteriophages with different pH ranges 3-12

Name of the <i>Vibrio</i> bacteriophages	Phage infectivity (%)											
	Controls (pH)		Experiments (pH ranges)									
	7	3	4	5	6	7	8	9	10	11	12	
<i>Vibrio harveyi</i> (VH-1)	100	-	56.66	78	87	100	96.66	84.16	71.66	53.33	-	-
<i>Vibrio harveyi</i> (VH-2)	100	-	56.66	78	87	100	96.66	84.16	71.66	53.33	-	-
<i>Vibrio harveyi</i> (VH-3)	100	-	56.66	78	87	100	96.66	84.16	71.66	53.33	-	-
<i>Vibrio harveyi</i> (VH-4)	100	-	56.66	78	87	100	96.66	84.16	71.66	53.33	-	-
<i>Vibrio harveyi</i> (VH-5)	100	-	56.66	78	87	100	96.66	84.16	71.66	53.33	-	-
<i>Vibrio harveyi</i> (VH-6)	100	-	56.66	78	87	100	96.66	84.16	71.66	53.33	-	-
<i>Vibrio harveyi</i> (VH-7)	100	-	56.66	78	87	100	96.66	84.16	71.66	53.33	-	-
<i>Vibrio harveyi</i> (VH-8)	100	-	56.66	78	87	100	96.66	84.16	71.66	53.33	-	-

Table 5: Effect of temperature on the stability and infectivity of *Vibrio* bacteriophages at different temperature from 20-90°C

Name of the Bacteriophages	Controls Temp. 37°C	Phage infectivity (%)										
		Experiments (Temperature ranges in°C)										
		20	30	37	40	50	55	60	65	70	80	90
<i>Vibrio harveyi</i> (VH-1)	100	66.66	80	100	95.33	58.66	28	-	-	-	-	-
<i>Vibrio harveyi</i> (VH-2)	100	66.66	80	100	95.33	58.66	28	-	-	-	-	-
<i>Vibrio harveyi</i> (VH-3)	100	66.66	80	100	95.33	58.66	28	-	-	-	-	-
<i>Vibrio harveyi</i> (VH-4)	100	66.66	80	100	95.33	58.66	28	-	-	-	-	-
<i>Vibrio harveyi</i> (VH-5)	100	66.66	80	100	95.33	58.66	28	-	-	-	-	-
<i>Vibrio harveyi</i> (VH-6)	100	66.66	80	100	95.33	58.66	28	-	-	-	-	-
<i>Vibrio harveyi</i> (VH-7)	100	66.66	80	100	95.33	58.66	28	-	-	-	-	-
<i>Vibrio harveyi</i> (VH-8)	100	66.66	80	100	95.33	58.66	28	-	-	-	-	-

The cytoplasm of phage-infected bacterial cells was less electrons dense than that of uninfected cells. Within the cytoplasm of some bacterial cells, various stages in the multiplication and assembly of viral particles were identified. This was apparently associated with 'clearing' of the cytoplasm, presumably the result of lytic activity induced by phage infection (Fig. 5d).

#### Effects of Enzymes, Organic Solvents and Detergents on the Phages of *V. harveyi*

The effects of enzymes (ribonuclease, protease, lysozyme and trypsin) organic solvents (chloroform, acetone, ethyl alcohol and methyl alcohol) and detergents (SDS and Triton-X 100) on the survival, infectivity, growth and multiplication of the *V. harveyi* phages (VH1-VH8) were determined. Infectivity of the *V. harveyi* phages was not affected by ribonuclease, protease, trypsin, chloroform, acetone, ethyl alcohol, methyl alcohol and Triton-X100 treatment at 37°C as determined by the ability of the phages to form plaques similar to those formed by untreated control phages on agar plate assays. Infectivity of the *V. harveyi* phages was however affected by lysozyme and SDS. In these cases, plaques were not formed on agar plate assays.

The percentage survival and infectivity of phages of *V. harveyi* (VH1 to VH8) after treatment with ribonuclease, trypsin, chloroform, acetone, ethyl alcohol and methyl alcohol (except VH5 and VH7) and Triton-X 100 were seen to be relatively higher (100%) when compared to treatment with other chemicals and the phages infectivity were found to be complete (100%). In contrast, the percentage infectivity of *V. harveyi* phages (VH1 to VH8) was observed to be 98.33% after protease treatment. 97.36 and 95.47% infectivity of *V. harveyi* phages (VH5 and VH7) was observed after ethyl alcohol and methyl alcohol treatment (Table 3).

#### Effect of pH and Temperature on the Stability and Infectivity of *V. harveyi* Bacteriophages

*V. harveyi* bacteriophages were stable and exhibited infectivity over the pH range 4-11, but there was complete loss of bacteriophages infectivity at pH 3 and 12. All the isolates of bacteriophages showed 100% survival and infectivity at pH 7, with uniform reduction of survival and infectivity in the ranges pH 6-4 and pH 8-11 (Table 4). *V. harveyi* bacteriophages were found to be stable at temperatures in the range 20-50°C but were inactivated at temperatures above 55°C (Table 4 and 5).

## DISCUSSION

*V. harveyi* bacteriophages, designated VH1-VH8, were isolated from diverse components of the aquaculture environment and from various tissues of infected shrimp. Experimental infectivity studies, plaque assays and transmission electron microscopy have conclusively demonstrated the ubiquitous occurrence and distribution of *Vibrio*-specific phages in *Vibrio* sp., from the Indian aquaculture environment. This is the first report of marine *Vibrio*-specific phages from India. A bacteriophage of

*V. parahaemolyticus* isolated from seawater is known to have a broad host range infecting various *Vibrio* species (Paul *et al.*, 1997; Matsuzaki *et al.*, 2000; Miller *et al.*, 2003) and nine phages of *V. vulnificus* have been isolated from estuarine waters of Louisiana (Pelon *et al.*, 1995). Four marine phage-host systems have been described from Mamala Bay, Hawaii Jiang *et al.* (1998). In the present study it was found that phages of *V. harveyi* formed clear plaques 1.9 mm in diameter in agar assays. Matsuzaki *et al.* (1998a, b) showed that vibriophage KVP20 exhibited a much narrower host range than KVP40 and formed plaques in only some *V. parahaemolyticus* strains. Bacteriophage VPP97 infects many strains of *V. parahaemolyticus* isolated from marine environments and forms clear plaques 0.6-1.0 mm in diameter (Pirisi, 2000). *V. alginolyticus* phages form plaques 0.5-0.9 mm in diameter (Ju *et al.*, 1997).

Whilst the present account deals specifically with the inhibitory effects of bacteriophages on the growth of shrimp-pathogenic bacteria, other agents that inhibit bacterial growth in the aquaculture environment have been described in the literature. More than 400 bacterial isolates from the intestine and the external surface of farmed *Scophtalmus maximus* as well as from fish food and hatchery water were screened for inhibitory effects against the fish pathogen *V. anguillarum* HI 11345 and seven other fish pathogens (Westerdahl *et al.*, 1991). Of the total number of isolates from the intestine, 28% were found to be inhibitory against *V. anguillarum* H 11345 and 60% had an inhibitory effect on five other fish-pathogenic serotypes of *V. anguillarum*. *Pseudomonas fluorescens* AH2 has been reported to reduce the growth of *V. anguillarum* (Gram *et al.*, 1999). A marine bacterial strain, *Pseudomonas* I-2 is reported to produce inhibitory compounds against shrimp pathogenic *Vibrio* spp. in aquaculture systems especially *V. harveyi*, *V. fluvialis*, *V. parahaemolyticus*, *V. damsela* and *V. vulnificus* (Chythanya *et al.*, 2001).

The one step growth studies carried out in the current investigation revealed relatively slow lytic activity for most of the vibriophages tested. Wu *et al.* (1981) demonstrated the formation of AH1 (*Aeromonas hydrophila*) phage particles after 50 min of infection and bursting occurred after 100 min when approximately 160 phages were present in each cell. Phage pA1 (*P. aeruginosa*) has been shown to exhibit a latent period of 60 min, a rise period of 60 min and a burst size of 130 particles per cell (Hirayama *et al.*, 1993). Jiang *et al.* (1998) reported that four phages viz., T- $\phi$ D0, T- $\phi$ D2S, T- $\phi$ HSIC and T- $\phi$ D1B exhibited a latent period ranging from 90 to 180 min. Eisenstark (1967) investigated the duration of the phage replication cycle and the relative amounts of progeny phage released using one-step replication experiments.

In the present study it has been shown that *V. harveyi* phage isolates were stable in long-term storage and they showed undiminished survival and infectivity even after their long-term storage. Similarly, Catherine *et al.* (2004) found no decrease in titre over a 6-month period storage at 4°C and -70°C and phage stability remained unaffected by freeze/thawing. In contrast, Pelon *et al.* (1995) reported that whilst phage isolates were found to be stable on storage at 4°C they exhibited a rapid loss of infectivity with repeated freezing and thawing at -70°C and similar results were observed by Gantzer *et al.* (1998).

Transmission electron microscopy revealed the presence of bacteriophages on the surface of infected bacterial cells. Attachment was by means of the short tail structure. Intra-cellular multiplication of the phages and lysis of the host cell following the phage multiplication were also visualized, providing evidence of the mode of infection and multiplication of phage particles in the host cell. These vibriophages may be unique to the Indian aquaculture environment and have not yet been reported elsewhere. Pelon *et al.* (1995) described nine phage isolates infectious for *V. vulnificus*. These were detailed as follows: (A) small, tailless icosahedral capsids (S1); (B) icosahedral heads with thin flexible tails (P13, P38, P53, P65, P108 and P111); (C) prolate icosahedral heads with relatively short (130 nm) rigid contractile tails (P68); and (D) icosahedral heads with long (233 nm) rigid tails consisting of contractile sheaths surrounding rigid tubes (P147). Morphological groups of Vibriophages similar

to those isolated in the study of Pelon *et al.* (1995) were also seen in our study. Shaffer *et al.* (1999) have shown that the morphology of the UNL-1 virion (*P. aeruginosa*) resembles most closely of that of members of the *Myoviridae* family, which are large and structurally complex bacterial viruses that possess contractile tails with fibers.

Bacteriophage VPP97 infects strains of *V. parahaemolyticus* and electron microscopy studies have revealed the presence of a hexagonal head and a relatively long tail (Yoon *et al.*, 1999). A vibriophage, KVP 241 and six of its relatives were isolated independently from seawater using *V. parahaemolyticus* as the host. Each of these phages has a hexagonal head and a tail with a contractile sheath. The host range is limited to some strains of *V. parahaemolyticus* (Matsuzaki, *et al.*, 2000). Vibriophages from *V. alginolyticus*, *V. fluvialis* and *V. furnissii* were found to possess a hexagonal head and a relatively long tail (Pirisi, 2000). Ackermann (1996) reported that over 4500 bacterial viruses have been examined by electron microscopy since 1959 and 4400 of these (96%) have been found to be tailed whilst only 162 phages (4%) are cubic, filamentous, or pleomorphic. They are classified by morphotype and host genus. Siphoviridae is one of the families that contains phages with long, non-contractile tails and includes about 60% of tailed phages. On the bases of morphology and host genus, the *V. harveyi* bacteriophages isolated in the present study are considered to fit best in the order Caudovirales of the Family Siphoviridae.

The present study has shown that the infectivity of *V. harveyi* phages was not inhibited by ribonuclease, trypsin and protease. In contrast, *V. harveyi* phages infectivity was inhibited by lysozyme perhaps indicating that the enzyme interfered with adsorptions of the phages to the bacterial cell wall. The effects of enzymatic treatment on several bacteriophages have been reported by Valyasevi *et al.* (1990) and Callegari *et al.* (1998). Binetti *et al.* (2002) reported that proteinase-K treatment did not change the adsorption ability of phage particles. MAV1 phage infectivity was found to be unaffected by treatment with RNAase, trypsin, or proteinase K (Voelker and Dybvig, 1998).

It has been shown here that the infectivity of *Vibrio* bacteriophages was unaffected by the treatment with chloroform, acetone, methyl alcohol and ethyl alcohol at 37°C for 1 hr. Kakimoto and Nagatomi (1972) reported that all six bacteriophages from the host bacteria *Aeromonas*, *Vibrio* and related organisms isolated from Kinko Bay, Japan, were fairly resistant to chloroform. One of the isolates was found to be stable in saturated chloroform, whilst the ability of another of the isolates to form plaques was reduced to 10% and two further isolates were completely inactivated by the treatment (Hidaka and Tokushige, 1978; Nakamura *et al.*, 2003). Bacteriophage BCJA1 (*Bacillus clarkii*) was found to be stable to exposure to chloroform indicating that it probably does not contain lipid (Jarrell *et al.*, 1997). In contrast, polyhedral, filamentous and pleomorphic (PFP) phages, comprising about 190 of the 4500 viruses studied by Ackermann (2003), were shown to be highly sensitive to chloroform and ether.

The results of the present study suggest that the *Vibrio* bacteriophages survived treatment with Triton X 100 whereas they did not survive treatment with 1% SDS. SDS might have hydrolyzed peptide bonds and removed membrane-bound proteins, thus affecting the infectivity, growth, survival and multiplication of the bacteriophages. These studies suggest that the phages contain proteins and that some detergents such as SDS present in the environment can affect bacteriophage adsorption and infectivity. They might therefore have a role in the control of phage infectivity. On the other hand, Binetti *et al.* (2002) found that SDS 1% treatment did not affect the adsorption ability of phage particles. Further studies on the proteins and lipids of vibriophages may help us to understand their roles in the life cycle and infectivity of bacteriophages.

pH has been shown to be an important control factor, influencing attachment, infectivity, intracellular replication and multiplication of phages. Generally, studies on the lytic activity of bacteriophages of *Vibrio* spp. have shown them to be most sensitive to pH values less than 5 and over 10 (Hirayama *et al.*, 1993; Jarrell *et al.*, 1997; Nakai *et al.*, 1999; Pirisi, 2000; Jepson and March, 2004).



In the present study, the phages of *V. harveyi* were found to be inactivated at pH values of 3 or less and at pH 12 and greater. In the range pH 4 to 11 the lytic activity of the phages varied, with the maximum at pH 7. Lytic activity was preserved for a greater range of pH values above pH 7 than below pH 7.

All the vibriophage isolates in the present study exhibited optimal survival at 37°C while complete inactivation occurred at a temperatures exceeding 55°C. Thus the present study has shown that temperature may be an important regulating factor on the infectivity of *Vibrio* bacteriophages. The VPP97 phage of *V. parahaemolyticus*, has been shown to be stable up to a temperature of 65°C but was totally inactivated at 70°C (Pirisi, 2000). Jepson and March (2004) reported that the phages were highly stable at 42°C, 37°C and at 4°C for up to 181 days. The current study has clearly underlined the fact that physico-chemical parameters are very important for the survival and infectivity of bacteriophages of *Vibrio* sp., in the aquaculture environment.

In conclusion, the present study highlights the occurrence of bacteriophages of *Vibrio* sp., in the shrimp aquaculture environment of South India. The study may assist in the early diagnosis of pathogenic *Vibrio* sp., that infect the crop and may point the way to non-chemotherapeutic control and preventative measures based on bacteriophage therapy.

#### ACKNOWLEDGMENTS

PR is thankful for DST-FIST. PS thanks the State Government, Tamilnadu, for the award fellowship.

#### REFERENCES

- Ackermann, H.W., 1996. Frequency of morphological phage descriptions in 1995. Arch. Virol., 141: 209-218.
- Ackermann, H.W., 2003. Bacteriophage observations and evolution. Res. Microbiol., 154: 245-251.
- Adams, M.H., 1959. *Bacteriophages*. Interscience, New York.
- Binetti, A.G., A. Quiberoni and J.A. Reinheimer, 2002. Phage adsorption to *S. thermophilus*. Influence of environmental factors and characterization of cell-receptors. Food Res. Int., 35: 73-83.
- Callegari, M.L., B. Riboli, J.W. Sanders, P.S. Cocconcelli, J. Kok, G. Venema and L. Morelli, 1998. The S-layer genes of *Lactobacillus helveticus* CNRZ 892: Cloning, sequence and heterologous expression. Microbiology, 144: 719-726.
- Catherine, S.B. and M.D. Susan, 2004. Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Developmental Cell, 6: 7-28.
- Chythanya, R., I. Karunasagar and I. Karunasagar, 2001. Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* I-2 strain. Aquaculture 208 (1-2), pp: 1-10.
- d'Herelle, F.H., 1917. Sur un microbe invisible antagoniste des bacilles dysenteriques. Paris. C. R. Acad. Sci., 165: 373-375.
- Eisenstark, A., 1967. Bacteriophage techniques. Methods Virol., 1: 449-529.
- Ellis, E.L. and M. Delbruck, 1939. The growth of bacteriophage. J. Gen. Physiol., 22: 365-356.
- Fuhrman, J.A. and C.A. Suttle, 1993. Viruses in marine planktonic systems. Oceanography, 6: 51-63.
- Gantzer, C., A. Maul, M. Audic and L. Schwartzbrod, 1998. Detection of infectious enteroviruses, Enterovirus genomes, somatic coliphages and *Bacteroides fragilis* phages in treated wastewater. Applied Environ. Microbiol., 64: 4307-4312.
- Gram, L.M., B. Spanggaard, I. Huber and F.T. Nielsen, 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Applied Environ. Microbiol., 65: 969-973.

- Heldal, M. and G. Bratbak, 1991. Production and decay of viruses in aquatic environments. Mar. Ecol. Prog. Ser., 72: 205-212.
- Hidaka, T. and A. Tokushige, 1978. Isolation and characterization of *Vibrio parahaemolyticus* bacteriophages in sea water. Mem. Fac. Fish. Kagoshima Univ., 27: 79-90.
- Hirayama, S., R. Ueda, K. Sugata and H. Kamiyoshi, 1993. Production of bacteriolytic enzyme by bacteriophage from seawater. Biosci. Biotechnol. Biochem., 57: 2166-2167.
- Jarrell, K.F., T. Vidykhan, P. Lee, M.D. Agnew and N.A. Thomas, 1997. Isolation and characterization of bacteriophage BCJA1, a novel temperate bacteriophage active against the alkaliphilic bacterium, *Bacillus clarkii*. Extremophiles, 1: 199-206.
- Jepson, C.D. and J.B. March, 2004. Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. Vaccine, 22: 2413-2419.
- Jiang, S.C., C.A. Kellogg and J.H. Paul, 1998. Characterization of marine temperate phage-host systems isolated from Mamala Bay, Oahu, Hawaii. Applied Environ. Microbiol., 64: 535-542.
- Ju, J.W., B.G. Moon, S.J. Ju, M.S. Heo, J.A. Ok and S.G. Yoon, 1997. Electron microscopic characterization about *Vibrio alginolyticus* phage isolated from marine products. J. Korean Soc. Microbiol., 32: 91-101.
- Kakimoto, D. and H. Nagatomi, 1972. Study of bacteriophages in Kinko Bay. Bull. Jap. Soc. Sci. Fish. 38: 271-278.
- Marks, Y. and R. Sharp, 2000. Bacteriophages and biotechnology: A review. J. Chem. Tech Biotech., 75: 6-17.
- Matsuzaki, S., T. Inoue and S. Tanaka, 1998a. A vibriophage, KVP40, with major capsid protein homologous to gp23 of coliphage T4. Virology, 242: 314-318.
- Matsuzaki, S., T. Inoue, M. Kuroda, S. Kimura and S. Tanaka, 1998b. Cloning and sequencing of major capsid protein (*mcp*) gene of a vibriophage, KVP20, possibly related to T-even coliphages. Gene, 222: 25-30.
- Matsuzaki, S., T. Inoue, S. Tanaka, T. Koga, M. Kuroda, S. Kimura and S. Imai, 2000. Characterization of a novel *Vibrio parahaemolyticus* phage, KVP241 and its relatives frequently isolated from seawater. Microbiol. Immunol., 44: 953-956.
- Miller, E.S., J.F. Heidelberg, J.A. Eisen, W.C. Nelson, A.S. Durkin, A. Ciecko, T.V. Feldblyum, O. White, I.T. Paulsen, W.C. Nierman, J. Lee, B. Szczypinski and C.M. Fraser, 2003. Complete genome sequence of the broad-host-range vibriophage KVP40: Comparative genomics of a T4-related bacteriophage. J. Bacteriol., 185: 5220-5233.
- Moebus, K. and H. Nattkemper, 1981. Bacteriophage sensitivity patterns among bacteria isolated from marine waters. Helgol. Wiss. Meeresunters. 34: 375-385.
- Nakai, T., R. Sugimoto, K.H. Park, S. Matsuoka, K. Mori, T. Nishioka and K. Maruyama, 1999. Protective effects of bacteriophage on experimental *Lactococcus garvieae* infection in yellow tail. Dis. Aquat. Organ., 37: 33-41.
- Nakai, T. and S.C. Park, 2002. Bacteriophage therapy of infectious diseases in aquaculture. Res. Microbiol., 153: 13-18.
- Nakamura, M., K. Tsumoto, I. Kumagai and K. Ishimura, 2003. A morphologic study of filamentous phage infection of *Escherichia coli* using biotinylated phages. FEBS Lett., 536: 167-172.
- Paul, J.H., J.B. Rose, S.C. Jiang, P. London, X. Xhou and C. Kellogg, 1997. Coliphage and indigenous phage in Mamala Bay, Oahu, Hawaii. Applied Environ. Microbiol., 63: 133-8.
- Pelon, W., R.J. Siebeling, J. Simonson and R.B. Luftwig, 1995. Isolation of bacteriophage infections for *Vibrio vulnificus*. Current Microbiol., 30: 331-336.
- Pirisi, A., 2000. Phage therapy-advantages over antibiotics? Lancet, 356:1418.

- Shaffer, J.J., L.M. Jacobsen, J.O. Schrader, K.W. Lee, E.L. Martin and T.A. Kokjohn, 1999. Characterization of *Pseudomonas aeruginosa* bacteriophage UNL-1, a bacterial virus with a novel UV-A inducible DNA damage reactivation phenotype. *Applied Environ. Microbiol.*, 65: 2606-2613.
- Valyasevi, R., W.E. Sandine and B.L. Geller, 1990. The bacteriophage kh receptor of *Lactococcus lactis* subsp. cremoris KH is the hamnose of the extracellular wall polysaccharide. *Applied Environ. Microbiol.*, 56: 1882-1889.
- Voelker, L.L. and K. Dybvig, 1998. Characterization of the Lysogenic Bacteriophage MAV1 from *Mycoplasma arthritidis*. *J. Bacteriol.*, 180: 5928-5931.
- Westerdahl, A., J.C. Olsson, S. Kjelleberg and P.L. Conway, 1991. Isolation and characterization of turbot (*Scophthalmus maximus*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. *Applied Environ. Microbiol.*, 57: 2223-2228.
- Withey, S., E. Cartmell, L.M. Avery and T. Stephenson, 2005. Bacteriophages-potential for application in wastewater treatment processes. *Sci. Total Environ.*, 339: 1-18
- Wu, J.L., H.M. Lin, L. Jan, Y.L. Hsu and L.H. Chang, 1981. Biological control of fish bacterial pathogen, *Aeromonas hydrophila* by bacteriophage AH 1. *Fish Pathol.*, 15: 271-276.
- Yoon, S.O., S.A. Ju, M.S. Heo, C.R. Jung and J.W. Ju, 1999. Characterization of a *Vibrio parahaemolyticus* phage isolated from marine. *J. Korean Sci. Microbiol.*, 34: 423-433.