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ISSN 0792 - 156X

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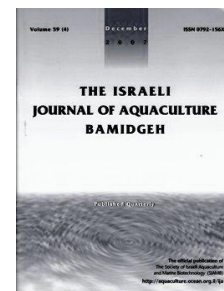
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## Effect of Total Dissolved Solids and Temperature on Bacteriophage Therapy against *Luminous vibriosis* in Shrimp

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(Received 22.9.11, Accepted 23.11.11)

Key words: *Luminous vibriosis*, *Vibrio harveyi*, phage therapy, total dissolved solids, temperature

### Abstract

Bacteriophage therapy is an efficient, eco-friendly, and scientifically demonstrable solution to antibiotic resistance in bacteria. Environmental factors have a profound influence on the growth and activity of phages. We evaluated the influence of two important parameters of the aquatic environment on phage activity, i.e., total dissolved solids (TDS) and temperature. Evaluated levels of TDS were 11.25, 22.63, and 38.43 mg/ml; studied temperatures were 20, 30, and 37°C. Levels were chosen from the ranges of values encountered during larvae rearing in a hatchery. Of seven *Vibrio harveyi* phages, phage V (earlier reported by us) was the best for bio-control of luminous vibriosis due to its broad host range (64%, n = 83). The optimum phage activity was obtained with a TDS value of 11.25 mg/ml and temperature of 30°C.

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## Introduction

Shrimp farming is one of the fastest growing food sectors in aquaculture. About 6% of the world's farmed shrimp production comes from India. In commercial aquaculture, improper farm management practices and stress may lead to incidences of disease or epidemics. Global *Penaeus monodon* production decreased worldwide by 131,090 tons from 2003 to 2007 due to various diseases (FAO, 2007). Luminous vibriosis caused by luminous *Vibrio harveyi* is one of the major bacterial diseases of shrimp larvae in hatcheries and juveniles in farms throughout Southeast Asia and South America (Karunasagar et al., 1994; Vandenberghe et al., 1998; Chrisolite et al., 2008). *Vibrio harveyi* is a gram-negative bioluminescent marine bacterium, ubiquitous in the marine environment, and part of the normal microflora of healthy shrimps and intestinal microbiota of marine animals (Makemson and Hermosa, 1999; Vandenberghe et al., 1999). This organism persists in hatchery environments by forming a biofilm on tank surfaces (Karunasagar et al., 1996).

Antibiotics have traditionally been used as prophylactic and therapeutic agents to control bacterial diseases in shrimp farms and hatcheries (Baticados and Paclibare, 1992). However, the indiscriminate use of antibiotics in aquaculture has led to the emergence and spread of antibiotic-resistant bacteria. In a study of a luminous vibriosis outbreak in a shrimp hatchery, isolates of *V. harveyi* were resistant to the antibiotics used; thus they were ineffective in controlling the disease among shrimp larvae (Karunasagar et al., 1994). Another concern associated with the use of antibiotics is the problem of residues, which has resulted in rejection by seafood importing countries of shrimp containing traces of antibiotics. Therefore, antibiotics are no longer the preferred treatment against vibriosis in shrimp culture and alternative methods are being sought.

Phage therapy is an efficient, eco-friendly, and scientifically demonstrable solution to bacterial infections. Phages are abundant in nature and probably contribute to controlling bacterial populations that cause disease in natural systems (Carlton, 1999; Imbeault et al., 2006). Thus, phage therapy can potentially control bacterial diseases in aquaculture, depending on the use of specific phages that lyse specific fish and shellfish pathogens (Nakai and Park, 2002; Park and Nakai, 2003; Skurnik et al., 2007). The use of bacteriophages to control pathogens in aquatic systems is gaining importance due to issues associated with drug residues and toxicity and consequent bans by importing countries (Wu and Chao, 1982; Nakai et al., 1999). Bacteriophage therapy appears to be a viable strategy for control of luminous vibriosis in shrimps (Vinod et al., 2006; Karunasagar et al., 2007).

Organisms require appropriate physicochemical parameters for optimal activity. Two important abiotic factors that affect the growth and survival of aquatic organisms are temperature (Prayitno et al., 1995; Boyd, 1999; Kumlu et al., 2000) and total dissolved solids (TDS; Boyd, 1990). Changes in environmental factors such as temperature, TDS, pH, and organic load can trigger outbreaks of luminous bacterial disease (Prayitno et al., 1995). In this study, the effect of two TDS and temperature on phage activity and their effect on controlling luminous vibriosis were evaluated.

## Materials and Methods

**Bacteria.** Eighty-three isolates of *Vibrio harveyi* stock cultures were maintained in T<sub>1</sub>N<sub>1</sub> (tryptone 1%, NaCl 1%) broth supplemented with 30% glycerol and stored at -80°C (Table 1). The cultures were activated overnight in tryptone soy broth (TSB; HiMedia, India) at 28°C in a shaker water bath, then subcultured on luminescent agar (LA; HiMedia, India) plates (West and Colwell, 1984). An isolated luminescent colony of each culture was picked up and maintained in T<sub>1</sub>N<sub>1</sub> semisolid agar butt for further work.

**Bacteriophage.** Seven *V. harveyi* phages with known potential for biocontrol of luminous vibriosis in the hatchery (Vinod et al., 2006) were isolated and maintained in a -80°C ultra-deep freezer in the Department of Microbiology, College of Fisheries, Mangalore. The phages were propagated in their respective hosts by mixing 4 ml host culture with 1 ml phage suspension and preparing a lawn on trypticase soya agar (TSA) containing 1% sodium chloride. After 5-6 h of incubation at ambient temperature

Table 1. *Vibrio harveyi* isolates with known potential for biocontrol of luminous vibriosis used in this study.

No.	Isolate	Isolated from:
1-18	Vh1-12, Vh34-37, Vh44, Vh45	Shrimp farm water (India)
19-24	Vh13, Vh14, Vh16, Vh21-23	Shrimp larvae (India)
25-27	Vh17, Vh42, Vh43	Dead shrimp larvae (India)
28-32	Vh24-28	Shrimp pond sediment (India)
33-37	Vh29-33	Water (India)
38-41	Vh38-41	Healthy shrimp larvae (India)
42-48	STD3-0942, STD3-0947, STD3-0949, STD3-0953 STD3-0983, STD3-0986, STD3-1007	Ecuador
49	STD3-0957	Shrimp hemolymph (Costa Azul Sinaloa, Mexico)
50	LMG04043	NCMB 24, National Collection of Marine Bacteria
51-55	LMG04044, LMG07890, LMG10948, LMG11659 LMG11660	USA
56	LMG10946	India
57	LMG10947	Seawater (Red Sea)
58	LMG11225	Seawater (Italy)
59	LMG11226	Sea water (Hawaii, USA)
60	LMG11755	Shark mouth (Bahamas)
61-62	LMG13949, LMG16874	Shrimp (Thailand)
63-67	LMG16828, LMG16830-16832, LMG16853	Black tiger prawn (Thailand)
68-69	LMG16862, LMG16863	Oyster (Spain)
70	LMG19643	Japanese horse mackerel ( <i>Trachurus japonicas</i> ; Japan)
71-78	STD3-0999, STD3-1000, STD3-1009, STD3-1011 STD31012, STD3-1020, STD3-1022, STD3-1024	Diseased <i>P. chinensis</i> (China)
79	ACMM20	Sea water (Australian Culture Collection of Marine Microorganisms)
80	VIB571	Sea bass ( <i>Dicentrarchus labrax</i> ; Spain)
81	VIB645	Sea bass ( <i>Dicentrarchus labrax</i> ; Tunisia)
82	BB120	Laboratory of Aquaculture and Artemia Reference Center
83	ACMM642	<i>Penaeus monodon</i> larvae (ACMM)

(28±1°C), the phages were harvested in 25 ml phage buffer (Vinod et al., 2006). Soft agar was prepared, dispensed in 3 ml volumes, autoclaved, and maintained in tubes at 48-50°C in a water bath. A soft agar overlay technique was used to determine phage titer (Adams, 1959). Phage suspensions were serially diluted in phage buffer. One hundred microliters of each phage dilution was mixed with 900 µl of an 8-h young host culture grown in TSB. This mixture was added to 3 ml molten soft agar, mixed by rolling between the palms, and overlaid on TSA plates. After solidification, the plates were incubated overnight at ambient temperature (28±1°C). The plaques that developed were counted and the titer expressed as plaque forming units/milliliter (pfu/ml).

**Determination of lytic activity of phage.** The lytic ability of seven phages of the 83 isolates was tested. A lawn of each *V. harveyi* isolate (n = 83) was prepared on TSA and 10 µl of each of the seven bacteriophages (10<sup>9</sup> pfu/ml) was spotted on each plate. The plates were incubated at ambient temperature (28±1°C) for 12 h and observed for clear zones.

**Phage activity under different TDS and temperatures.** Sea water with one of three concentrations of TDS (11.25, 22.63, 38.43 mg/l) and temperatures (20, 30, 37°C) was studied. Sea water was transported to the laboratory and TDS was estimated by the method outlined in EPA (1988), with minor modification. Briefly, 100 ml filtered sea water was taken in a clean, dry pre-weighed beaker. Samples were evaporated in a steam bath, transferred to an oven maintained at 105°C for 2 h, cooled, and weighed. TDS was calculated as the difference between the final and initial weights and expressed as mg/ml. Temperature was maintained by placing sterilized sea water in a water bath maintained at 20°C, 30°C, or 37°C.

**Experimental setup.** To test the effect of TDS on the bacterial phages, three groups of three troughs were stocked with 1 l autoclaved sea water. In each group, one trough

contained 11.25 mg/ml TDS, the second contained 22.63 mg/ml TDS, and the third contained 38.43 mg/l TDS. *Vibrio harveyi* was maintained at a concentration of  $10^6$  cfu/ml in all nine troughs. The first group served as a control and received *V. harveyi* but no phages. The second and third groups received phage suspensions (0.1 ml of phage titer  $10^9$  pfu/ml) at the beginning of the trial and 24 h later; the phage concentrations were maintained at  $10^5$  pfu/ml until the end of the trial at 72 h. Luminous bacteria count (LBC; cfu/ml) was determined at 0, 24, 48, and 72 h by spread plating on LA plates. To test the effect of temperature on phage activity, the setup was similar but the three troughs in each group were maintained at 20, 30, and 37°C. Temperature in all the TDS troughs was constant (30°C) and the TDS in all temperature troughs was constant (11.25 mg/ml).

**Statistical analysis.** The bacteria counts were subjected to analysis of variance (ANOVA). Duncan's multiple range tests were performed to determine significant differences between treatments. Data were statistically analyzed using the statistical package SPSS 15.0.

## Results

**Lytic activity of phage.** Of the seven phages tested on the 83 *V. harveyi* isolates, Phage V lysed the greatest percentage of isolates while phages K and R had limited activity and lysed only their specific hosts (Table 2). Based on its broad host range, phage V was used for subsequent study.

**Phage activity under different TDS levels and temperatures.** The lowest TDS (11.25 mg/ml) resulted in 3.3  $\log_{10}$  reduction in LBC, significantly lower than the other two TDS levels (Fig. 1). Duncan's multiple range test showed significant differences in LBC counts at different times. The best temperature for phage activity was 30°C, with a four  $\log_{10}$  reduction in LBC. ANOVA showed significant differences in LBC counts between temperatures while Duncan's multiple

Table 2. *Vibrio harveyi* bacteriophages used in this study.

Phage	Source	Specific host	Host range (n = 83)	Titer (pfu/ml)
V	Shrimp hatchery water	Vh17	64%	$1.41 \times 10^{11}$
J	Oyster	Vh36	61%	$2.61 \times 10^{10}$
M	Shrimp hatchery water	Vh36	49%	$1.17 \times 10^{10}$
N	Shrimp hatchery water	Vh10	53%	$2.91 \times 10^{11}$
A	Shrimp hatchery water	Vh25	43%	$9.6 \times 10^9$
K	Shrimp larvae	Vh39	1.15%	$2.71 \times 10^{11}$
R	Shrimp larvae	Vh39	1.15%	$1.12 \times 10^{11}$

range test showed significant differences at different times.

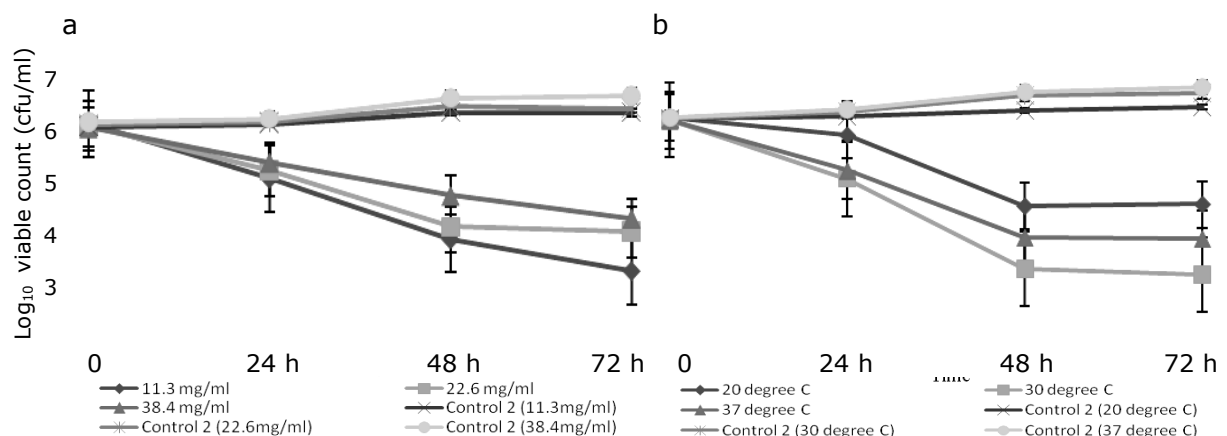


Fig. 2. Effect of (a) level of total dissolved solids and (b) temperature on luminous bacterial counts at different times after phage treatment.

## Discussion

Outbreaks of bacterial disease in shrimp aquaculture result in huge economic losses. *Vibrio harveyi* causes luminous vibriosis, resulting in rapid mortality in shrimp hatcheries.

Mass mortalities of black tiger shrimp (*P. monodon*) larvae in a shrimp hatchery were due to multiple antibiotic resistant *V. harveyi* (Karunasagar et al., 1994) that were able to form resistant biofilms on surfaces of rearing tanks (Karunasagar et al., 1996).

Bacteriophages are abundant in the aquatic environment and can be used for biocontrol of *V. harveyi* in shrimp hatcheries (Vinod et al., 2006; Karunasagar et al., 2007). For successful phage therapy, a phage must have a broad host range and be able to overcome phage resistance. Because of its high lytic activity against *V. harveyi*, one of the seven phages tested in this study, Phage V, was later used to study phage application. In similar studies, *V. harveyi* phages lysed different *Vibrio* species (Payne et al., 2004; Patil, 2005; Shivu et al., 2006) while bacteriophages of *V. harveyi* isolated from diverse environments had broad host ranges and lysed 55-70% of *V. harveyi* strains (Karunasagar et al., 2007). Thus, *V. harveyi* bacteriophages can be suitable for biocontrol of pathogens in shrimp hatcheries.

Seasonal variation impacts the virulence of luminous bacteria and, consequently, disease outbreaks occur during the rainy season (Sunaryanto and Mariam, 1986). Changes in environmental factors such as TDS and temperature can enhance the virulence of luminous bacteria though the mechanism of increased virulence is unknown (Prayitno et al., 1995). The results of our study show that when TDS is higher, *V. harveyi* phage activity is lower, possibly due to inactivation of the phage by soluble solids that prevent the phages from attaching to target cells. The best temperature for phage activity was 30°C, which is also the optimum temperature for growth of *V. harveyi*. Bacteriophages may dominate in environments where its optimum temperature is also the optimum temperature of its host. Statistical analyses using one-way ANOVA and Duncan's multiple range tests showed significant differences in LBC between different levels of TDS and temperature.

In conclusion, phage activity depends on environmental parameters and alterations in these parameters adversely affect results. Reduction in phage titers by such environmental changes necessitate the use of higher concentrations of phages to eliminate *V. harveyi*.

### Acknowledgements

We thank Prof. Patrick Sorgeloos and Prof. Peter Bossier, Laboratory of Aquaculture and Artemia Reference Center, University of Ghent, Belgium, for kindly providing *Vibrio harveyi* cultures from their collection for the study. The funding support received from the Department of Biotechnology, Government of India, through the program Support to Aquaculture and Biotechnology is gratefully acknowledged.

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