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Simulated hatchery system to assess bacteriophage efficacy against *Vibrio harveyi*

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ABSTRACT: Vibriosis caused by luminous *Vibrio harveyi* commonly contributes to poor survival in shrimp hatcheries and aquaculture ponds. Lytic bacteriophages pathogenic for *V. harveyi* are currently being investigated as an alternative to antibiotics to prevent vibriosis. Here, 8 bacteriophages were isolated from oysters and clams using *V. harveyi* strains as baiting hosts. Among these bacteriophages, 1 strain (VHP6b) identified as broadly pathogenic for 27 *V. harveyi* strains examined was further characterized by electron microscopy and genome sequence analysis. Phage VHP6b possessed a tail and morphology consistent with it being a member of the family *Siphoviridae*, and its genome and proteome were most closely related to the *Vibrio* phages SSP02 and MAR10. An integrase gene essential for lysogeny was not evident. The ability of bacteriophage VHP6b to protect shrimp postlarvae against vibriosis caused by *V. harveyi* strain VH6 was demonstrated in a model system designed to simulate typical hatchery conditions. Bacteriophage treatment improved survival of postlarvae by 40 to 60% under these conditions, so therapies based on this or other bacteriophages may be useful in shrimp hatcheries.

KEY WORDS: Vibriosis · Shrimp hatchery · Bacteriophages · Simulated hatchery model · Phage efficacy

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INTRODUCTION

Increased export demand and high profits have intensified penaeid shrimp aquaculture in India. However, regional and international movements of shrimp for aquaculture have resulted in pathogens being translocated and increased incidences of diseases caused by various viruses, bacteria, and protozoa. Of the bacterial diseases, luminous vibriosis caused by *Vibrio harveyi* is commonly responsible for mortality of larval black tiger shrimp *Litopenaeus monodon* and Pacific white shrimp *L. vannamei* in hatcheries and to a lesser extent culture systems in India (Karunasagar et al. 1994, Otta et al. 1999, Chatterjee & Haldar 2012) and other countries throughout Southeast Asia (Lavilla-Pitogo et al. 1990, Song & Lee 1993, Jiravanichpaisal et al. 1994, Karunasagar et al. 1994, Pizzutto & Hirst 1995, Liu et al. 1996, Robertson et al. 1998). Attempts to mitigate the hatchery impacts of luminous vibriosis have resulted in the indiscriminate use of chemicals and antimicrobial drugs that have contributed to the emergence of resistant bacteria (Plumb 1995, Tendencia & de la Pena 2001). As an alternative, virulent bacteriophages are being investigated intensively for their ability to control *V. harveyi* (Phumkhachorn & Rattanachaikunsopon 2010, Pereira et al. 2011, Surekhamol et al. 2014).

Prophylactic therapies employing bacteriophages to reduce bacterial loads to safe levels have many benefits, including their ability to be applied through water without affecting other microbial flora, or as a topical treatment to disinfect equipment (Barrow &

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Soothill 1997). Upon isolation and characterization of their lytic properties and host ranges, cocktails of various bacteriophages have proved to be particularly valuable in controlling vibriosis in shrimp hatcheries (Crothers-Stomps et al. 2010). However, care is needed in selecting the composition of such cocktails to avoid lysogenic bacteriophages that can carry undesirable genetic elements with the potential to enhance bacterial virulence (Flegel et al. 2005), and in defining doses applicable to commercial hatchery conditions. While hatchery-based trials of bacteriophages have proved valuable (Takahashi et al. 1985, Vinod et al. 2006), such studies could be streamlined by a reproducible and standardized experimental system that simulates hatchery conditions. To examine this, here we report a simulated shrimp hatchery model system and its use to assess the efficacy of newly-isolated bacteriophages to kill *V. harveyi* isolates.

MATERIALS AND METHODS

Bacterial strains, growth media, and larval feed

To isolate *Vibrio* spp., seawater and samples from oysters, moribund juvenile shrimp, and shrimp larvae were collected from different regions of coastal Karnataka, India. Selective media TCBS (Hi-media), Luria–Bertani (LB) broth, and agar containing 1% NaCl were used to select and grow bacteria that were then characterized using standard biochemical methods (Gomez-Gil et al. 2004, Ransangan et al. 2012). The Hi-*Vibrio* kit (Hi-media) was used to identify *Vibrio* spp., and 7 additional characterized *V. harveyi* strains were procured (Table 1) from repositories. The standard strains were used as control strains during biochemical methods for strain characterization and phage host range studies. Stock *Vibrio* cultures were maintained in 20% glycerol at −80°C. *Artemia* cysts, Gold Coin artificial hatchery feed, and black tiger shrimp *Litopenaeus monodon* postlarvae (PL2 stage) were procured from Priya Hatchery, Mangalore, India.

Table 1. *Vibrio harveyi* isolates from various environmental sources and characterized strains

Source	Isolate designation
Seawater	VH1, VH2, VH7, VH8, VH13, VH14, VH16, VH17
Oyster	VH3, VH5, VH19, VH20
Shrimp	VH4, VH6, VH15
Shrimp postlarvae	VH9, VH12, VH18
Shrimp pond water	VH10, VH11
Germany[a]	DSMZ2165
LMG, Belgium	LMG16828
Mexico[b]	CAIM29, CAIM151, CAIM223, CAIM1793, CAIM1794

[a]Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany
[b]Collection of Aquatic Important Microorganisms, Mexico

Bacteriophage isolation, purification, and concentration

Soft agar overlays employing various luminescent *V. harveyi* isolates were used to isolate bacteriophages from oyster, clam, shrimp, and seawater samples. Briefly, 1 ml of each liquid sample was passed through 0.2 µm membrane filter and mixed with 100 µl 1.0 OD_{600} *V. harveyi* host culture and 5 ml molten soft 0.75% agar before being overlaid immediately on 1.5% agar plates. After incubation overnight at 30°C, lytic phages were detected as clear plaques. Phages isolated from representative single plaques were plaque-purified 3 times as described by Adams (1959). Bacteriophages were then propagated at 30°C for 4 h by infecting log stage bacterial culture at a multiplicity of infection (MOI) of 0.1 plaque-forming unit (PFU) CFU^{-1}. Growth media were clarified by centrifugation, and supernatant was passed through a 0.2 µm membrane filter. Phage was titrated using agar plates as described previously (Sambrook et al. 1989), and lysates were stored at 4°C. To increase phage titers and remove nutrient media components, VHP6b phage lysate was concentrated using ammonium sulfate precipitation (Robert 2005). In brief, 78 g ammonium sulfate (calculated for 0 to 60% ammonium sulfate cut off) was added to 200 ml of phage lysate. The lysate was kept at 4°C for 1 h, to allow for precipitation to occur. This was further centrifuged at 6000 × *g* for 30 min. The pellet was resuspended in 5 ml physiological saline and dialyzed overnight in saline. The dialyzed lysate was filter sterilized through 0.2 µm syringe filters and phage titer was determined.

Bacteriophage characterization

Serial 10-fold dilutions of bacteriophage strain VHP6b (10^{10} PFU ml^{-1}) were prepared in saline, and 100 µl of each dilution was mixed with 100 µl *V. har-*

veyi host strain VH4 propagating to 1.0 OD_{600}. This was mixed with 5 ml of 0.75% molten soft agar overlaid on an agar plate to observe plaque morphology. The host range of 8 bacteriophages isolated from different environments was determined by spotting 5 µl of each phage lysate onto lawns of *V. harveyi* isolates prepared by spreading 1.0 OD_{600} culture onto LB agar (with 1% NaCl) plates and incubation at 30°C overnight.

To examine bacteriophage morphology, lysate of VHP6b (10^{10} PFU ml^{-1}) was centrifuged at 25 000 × *g* for 1 h (Beckman JA-18.1 fixed-angle rotor in a Beckman J2–21 centrifuge). The phage-containing pellet was resuspended in a small volume of 1 M ammonium acetate and a drop was deposited onto a copper grid with a carbon-coated film to stain bacteriophage particles with 2% potassium phosphotungstate for visualization using a 60 kV Philips EM300 transmission electron microscope.

To examine for virulence-inducing mobile genetic elements, the genome of phage VHP6b was sequenced using a 454 Roche FLX Genome Sequencer system at Microsynth, Switzerland. Sequences were assembled using GS De Novo Assembler (Newbler) V2.6 using standard settings, and open reading frames (ORFs) in the genome were predicted using GeneMark.hmm (Lukashin & Borodovsky 1998) and Glimmer (Delcher et al. 2007) and annotated using a combination of BLASTP (Altschul et al. 1990) of the non-redundant GenBank protein and Pfam databases (Punta et al. 2012).

Bacteriophage compatibility with artificial feed and *Artemia* nauplii

To assess bacteriophage compatibility with artificial shrimp larval feed and *Artemia* nauplii, phage lysate (10^{10} PFU ml^{-1}) was incubated in 250 ml filter-sterilized artificial seawater alone or seawater containing 100 to 200 *Artemia* nauplii per shrimp postlarva as well as artificial feed. Over at 24 h period, 1 ml aliquots of seawater were collected at 0, 3, 6, and 24 h to quantify bacteriophage numbers.

To assess bacteriophage lysis efficiency in an *in vitro* system simulating commercial hatchery conditions, triplicate 250 ml beakers containing 100 ml continuously aerated seawater, feed, *Artemia*, and 10^8 CFU ml^{-1} *Vibrio* spp. were prepared. Aliquots containing 2 × 10^{10} PFU ml^{-1} of bacteriophage VHP6b were added to 2 of the 3 beakers, and 1 ml samples were collected at 0, 3, and 5 h time points. To quantify (CFU ml^{-1}) the number of viable bacteria, a 1 ml sample was pelleted, washed twice with saline to remove free phages, and resuspended in saline. Serial 10-fold dilutions were prepared and spread onto 1% NaCl LB agar plates that were incubated overnight at 30°C.

Simulated shrimp hatchery system

The simulated shrimp hatchery system was designed to consider factors including seawater filtration, feed, temperature, pH, aeration and tank disinfection methods. Shrimp postlarvae (PL2) procured from a commercial hatchery were acclimated to laboratory conditions for 2 to 3 d before transfer to 2.5 l glass conical flasks containing 1.25 l filtered sterilized seawater. Flasks were sealed with a rubber stopper containing air inlet and outlet ports including 0.2 µm filters to avoid contamination. Aeration was maintained constantly and each day, 20 to 30% water was exchanged aseptically for fresh sterile seawater, and fecal matter and other debris was siphoned off to maintain water quality. Postlarvae were stocked at a density of 20 l^{-1} and fed with 100 to 200 *Artemia* $postlarva^{-1}$ 4 times d^{-1} consistent with commercial hatchery practices. Various combinations of *Vibrio* spp., bacteriophage, and other agents were added depending upon what was being investigated. Survival and behaviors of the shrimp postlarvae were recorded at regular intervals.

Pathogenicity of *V. harveyi* for shrimp postlarvae

The simulated shrimp hatchery system was used to assess the pathogenicity of *V. harveyi* VH4 and VH6 strains for shrimp postlarvae procured from a hatchery. Postlarvae were acclimated for 3 d, during which time the absence of *V. harveyi* was confirmed by macerating groups of 30 postlarvae and incubating 0.1 g macerated larvae in 0.9 ml of alkaline peptone water for 4 to 6 h at 30°C. Following incubation, loops of media were streaked onto LB agar containing 1% NaCl that were then incubated at 30°C to identify evidence of luminescent *V. harveyi* colonies. Postlarvae were stocked and fed as described earlier, and 10^7 CFU ml^{-1} *V. harveyi* was added to some flasks. Dead larvae were removed from the flasks and counted daily over a period of 10 d. Numbers of surviving larvae were deduced accordingly based on numbers of dead larvae recorded.

Bacteriophage lysis efficiency

The simulated shrimp hatchery system was used to assess the efficacy of bacteriophage VHP6b lysis of *V. harveyi* strain VH6. Shrimp postlarvae (PL2) were procured, acclimated for 3 d, and confirmed to be free of *V. harveyi.* Postlarvae were stocked and fed as described earlier, and to some flasks, either 10^7 CFU ml^{-1} *V. harveyi* strain VH6, 10^{10} PFU ml^{-1} bacteriophage VHP6b, or 10^7 CFU ml^{-1} *V. harveyi* strain VH6 together with 10^{10} PFU ml^{-1} bacteriophage VHP6b was added. Numbers of surviving postlarvae were recorded daily over a period of 10 d as described above.

Statistics

For data obtained on *V. harveyi* pathogenicity and bacteriophage lysis efficiency trials, 1-way ANOVA and Tukey-Kramer multiple comparison tests were performed using GraphPad InStat software, with $p \leq 0.05$ considered statistically significant.

RESULTS

Vibrio harveyi strains

In total, 20 luminescent *V. harveyi* were isolated from the various samples of oysters (n = 4), seawater (n = 8), water from shrimp ponds (n = 2), moribund shrimp (n = 3), and shrimp postlarvae (n = 3) and designated VH1 to VH20 (Table 1).

Bacteriophage isolation and characterization

In total, 8 bacteriophages were isolated from the oyster and clam samples (Table 2) and plaques purified 3 times. All bacteriophages formed clear plaques of uniform size (2–3 mm diameter). Bacteriophage grew to titers up to 10^{10} PFU ml^{-1} when inoculated at 1 PFU per 10 CFU (0.1 MOI) using the broth growth and lysis method. Use of ammonium sulfate precipitation aided in bacteriophage VHP6b concentration along with removal of nutrient media. From 200 ml of VHP6b phage lysate (ca. 3.9×10^9 PFU ml^{-1}), a final volume of 6 ml was obtained upon concentration. Phage recovery after ammonium sulfate precipitation was 83% with phage concentration titers to 1×10^{11} PFU ml^{-1}.

Host challenge experiments showed that bacteriophage strains VHP1, VHP2, VHP5, and VHP6b were lytic for varying numbers of *V. harveyi* strains examined, with strain VHP6b having the broadest activity for 22 of the 27 (85%) stains challenged (Table 3).

Electron microscopy of bacteriophage VHP6b showed it to possess an elongated icosahedral head (96 × 54 nm) with a non-contractile tail (145 × 8 nm; Fig. 1). The phage was consistent with *Vibrio* VP5 species and with tailed phages that appear to be the dominant type infecting *Vibrio* spp. (Ackermann 2001). Based on the morphological features, bacteriophage VHP6b is a member of the family *Siphoviridae*.

Bacteriophage VHP6b genome sequence

The double-stranded DNA genome of bacteriophage VHP6b was determined to be 78 081 bp in length, with 49% G+C content and 104 ORFs. The nucleotide sequence of VHP6b is highly similar to bacteriophages of *V. vulnificus* (Phage SSP002; Lee et al. 2012) and *V. parahaemolyticus* (Bacteriophage vB_VpaS_MAR10; Alanis Villa et al. 2012). Genome comparisons using the Core-Genes algorithm (Zafar et al. 2002) revealed that the VHP6b proteome shows 92.2% homology to SSP002 and 87.9% homology to

Table 2. Hosts and *Vibrio harveyi* baiting strains from which bacteriophages were isolated

Host	Baiting strain	Phage designation
Oyster	VH5	VHP1, VHP2
	VH7	VHP3, VHP7
	VH4	VHP5, VHP6a, VHP6b
Clam	VH7	VHP4

Table 3. *Vibrio harveyi* strains lysed by bacteriophages VHP1, VHP2, VHP5, and VHP6b

Bacteriophage	Sensitive *V. harveyi* isolate
VHP1	VH1, VH3, VH4, VH5, VH6, VH7, VH8, VH10, VH12, DSMZ2165, CAIM29
VHP2	VH5, VH6
VHP5	VH1, VH3, VH4, VH6, VH10, VH12, VH13, VH14, VH15, VH16, VH17, VH18, VH19, VH20, DSMZ2165
VHP6b	VH1, VH3, VH4, VH5, VH6, VH7, VH8, VH10, VH11, VH12, VH13, VH14, VH15, VH16, VH17, VH18, VH19, VH20, DSMZ2165, CAIM 29, CAIM151, CAIM1794

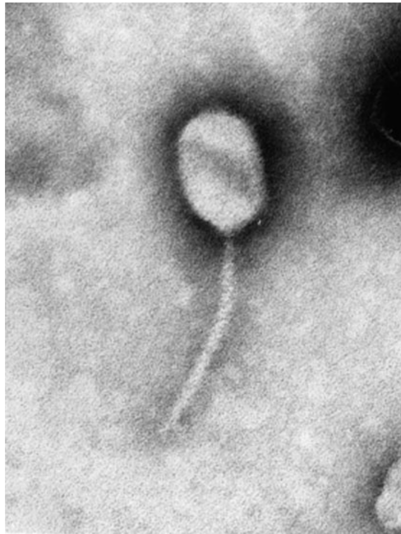

Fig 1. Transmission electron micrograph of bacteriophage strain VHP6b

MAR10. Of the predicted 104 ORFs, 24 could be assigned to proteins with, for example, DNA replication and modification, endolysin, head morphogenesis, and tail assembly functions. As in bacteriophages SSP002 and MAR10, genes encoding integrases were not identified, but an ORF containing a ParB-like nuclease domain involved in DNA partitioning was identified.

Phage compatibility with artificial feed and *Artemia* nauplii

When maintained in seawater or seawater containing larval shrimp feed and *Artemia* nauplii, bacteriophage VHP6b infectivity did not drop significantly compared to saline over a 24 h period (Fig. 2). Considering possible phage intervention points in a commercial hatchery, this finding indicated that phages could be added to tanks at any time to control vibriosis.

Bacteriophage lysis efficiency

The efficiency at which bacteriophage VHP6b could lyse the *V. harveyi* isolate VH4 was assessed over a 24 h period in seawater and seawater containing artificial feed and *Artemia* nauplii (Fig. 3). By 24 h post-challenge, VH4 CFU ml^{-1} titers were reduced significantly (99.99%; $p < 0.05$) within 5 h irrespective of whether artificial feed and *Artemia*

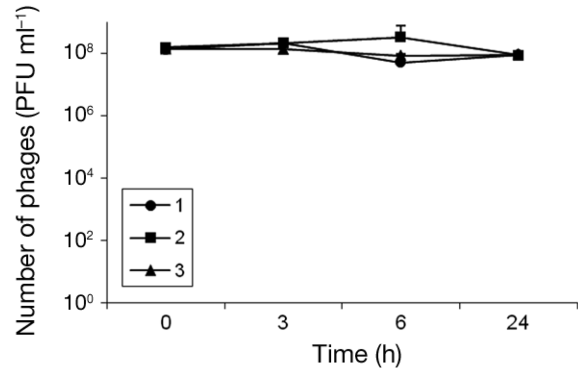

Fig. 2. Effect of artificial feed and *Artemia* nauplii on bacteriophage VHP6b infectivity over a 24 h period. Infectivity was assessed for 10^8 PFU ml^{-1} bacteriophage maintained in (1) seawater (●), (2) seawater containing artificial feed and *Artemia* nauplii (■), or (3) saline (▲)

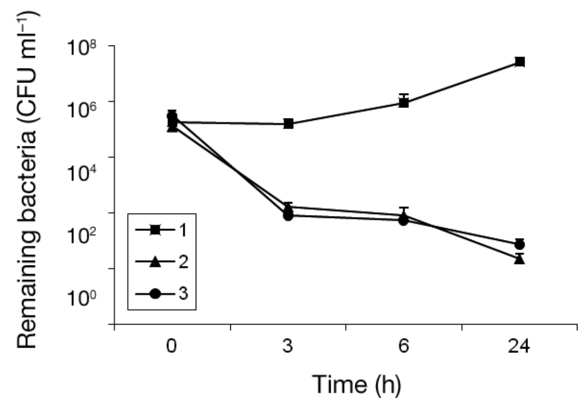

Fig. 3. Bacteriophage VHP6b lysis efficiency for *Vibrio harveyi* isolate VH4 in the presence of artificial feed and *Artemia* nauplii. VH4 infectivity (CFU ml^{-1}) was assessed over a 24 h period either in (1) seawater containing artificial feed and *Artemia* nauplii (■), (2) the same with added bacteriophage VHP6b (▲), or (3) seawater alone with added bacteriophage VHP6b (●)

nauplii were present and were further reduced at 24 h post-challenge.

Simulated shrimp hatchery system

A simulated shrimp hatchery system was established using 2.5 l glass conical flasks. Growth performance of batches of *Litopenaeus monodon* postlarvae procured at the PL2 stage and reared to the PL15 stage was comparable to postlarvae reared at commercial hatcheries as assessed by daily monitoring for molting and size increases.

The pathogenicity of *V. harveyi* isolates VH4 and VH6 and strain CAIM29 was determined with *P. mo-*

nodon PL2 using a 2.5 l glass conical flask system. By Day 5 post-challenge, VH4 and VH6 caused 34% and 65% mortality, respectively. Based on these data, the simulated hatchery system and *V. harveyi* VH6 were used to assess bacteriophage protective effects. After stocking shrimp postlarvae into flasks, bacteriophage VHP6b was added, and 30 min later, *V. harveyi* VH6 was added. Over the following 10 d, >70% mortality of postlarvae occurred in the flask challenged with *V. harveyi* VH6 alone compared to 20% mortality in the flask in which bacteriophage VHP6b was added just prior to challenge with *V. harveyi* VH6. The reduced mortality was highly significant ($p < 0.001$). Only low-level and non-specific mortality (<3%) occurred among postlarvae in an untreated flask or a flask treated with bacteriophage VHP6b alone.

DISCUSSION

Increasing levels of antibiotic resistance among common pathogenic bacteria, problems with release of antibiotics into aquatic environments, and contamination of seafood produced through aquaculture are driving research into alternative means of pathogen control. The use of lytic bacteriophages offers promise as an alternative means of controlling pathogenic *Vibrio harveyi* strains that regularly cause production losses in shrimp hatcheries. Here various strains of *V. harveyi* were isolated from seawater, shrimp, shrimp postlarvae, shrimp pond water and oysters, and bacteriophages were isolated from the strains infecting oysters and clams. Phage VHP6b showed broad-spectrum lytic activity for 86% of the *V. harveyi* strains examined, including various characterized strains as well as some isolated here from various sources including moribund shrimp and shrimp postlarvae. The lytic activity of bacteriophage VHP6b was evident from it generating clear plaques in lawns of *V. harveyi*. Bacteriophage VHP6b was also demonstrated to suppress *V. harveyi* growth for 10 to 12 h even at concentrations far in excess for those likely to occur naturally in shrimp hatcheries. Moreover, when added to water prior to challenge of *Litopenaeus monodon* postlarvae with pathogenic *V. harveyi* isolate VH6, bacteriophage VHP6b was able to reduce mortality by 60% over a 10 d period.

A prerequisite for applying bacteriophages in a commercial shrimp hatchery is their compatibility and stability in typical tank water conditions. In this regard, physical and chemical factors including seawater pH, temperature, salinity and organic matter content have been examined for their affects on bacteriophage stability and virulence (Silva et al. 2014). In addition, *in vivo* evaluations of the efficacy of therapeutic agents have generally been conducted using hatchery tanks or farm ponds (Takahashi et al. 1985b, Vinod et al. 2006). However, such studies possess limited abilities to control for variables such as the presence of additional pathogens that might impact data interpretation (Saulnier et al. 2000). To circumvent such variables, here we designed a simulated hatchery system based on 2.5 l glass flasks to assess the stability and virulence of bacteriophage isolates in the presence of live and artificial shrimp larval feeds that need to be accommodated in commercial hatcheries. The system also prevents escape of pathogens in aerosols, avoids influences of other pathogens, and allows accurate control of water variables including salinity, temperature, and pH. The system will allow doses and application timings of pathogenic bacteria, bacteriophages, and anti-bacterial drugs to be assessed with small numbers of shrimp larvae under highly controllable conditions prior to undertaking more complex and expensive field trials. The simulated hatchery system allowed us to assess the ability of a bacteriophage to protect *P. monodon* postlarvae against vibriosis similar to how this has been examined for highly pathogenic *V. harveyi* and *V. parahaemolyticus* strains in shrimp hatcheries (Vinod et al. 2006, Ortega & Martinez 2014). The system is currently being used to isolate additional bacteriophages and to assess the efficacy of bacteriophage cocktails and delivery doses and methods to optimize their therapeutic impact in commercial applications.

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