

## ***In-vitro* characterization of lytic bacteriophage PhVh6 as potential biocontrol agent against pathogenic *Vibrio harveyi***

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**Abstract.** Bacterial diseases are the major threat to the shrimp aquaculture industry. However, the applications of antibiotics for treatment and prophylaxis are generally not permissible by law. The present study characterized a lytic bacteriophage strain PhVh6 for biocontrol potential against pathogenic *Vibrio* spp. PhVh6 was isolated from the water in shrimp pond encountered ongoing mortality using luminescent *Vibrio harveyi* strain Vh10 (GenBank: KJ700303) as enrichment host. Characterizations were conducted for morphology, titer (pfu mL<sup>-1</sup>), multiplicity of infection (MOI), one-step growth curve, physico-chemical sensitivities, host range susceptibility and bacteriolytic activities against *Vibrio* spp. Transmission electron microscopy revealed tailed icosahedral viral particles lacking of neck and collar, thus assigned PhVh6 to the order Caudovirales and family Siphoviridae. MOI of 1 was found optimum for PhVh6 and Vh10 interaction. One-step growth curve analysis revealed short latent period and high burst rate. PhVh6 was stable from 25 to 65°C, and optimum at 45°C. PhVh6 was able to tolerate low temperatures at 4, -20 and -75°C. The lytic ability was optimum at pH 6 to 8. PhVh6 was tolerant to salinities of 15, 30 and 45 ppt, 1-3% chloroform and 30-40% glycerol. Host range susceptibility test showed ability of PhVh6 to lyse *Vibrio parahaemolyticus*, *Vibrio alginolyticus* and *Vibrio harveyi* strain Vh8. The present study suggested the potential of PhVh6 as biocontrol agent against *Vibrio* spp. in shrimp aquaculture.

**Key Words:** Lytic bacteriophage, Caudovirales, biocontrol, *Vibrio harveyi*, shrimp aquaculture.

**Introduction.** Pacific white shrimp (*Penaeus vannamei*) is known as a high-demand seafood worldwide. However, bacterial infections in shrimp aquaculture cause disease outbreaks and massive losses to the farmers. Shrimp farming is mainly affected by vibriosis, especially luminescent vibriosis. The intensive culture of pacific white shrimp often leads to outbreaks of vibriosis. Hence, though illegal, some farmers have resorted to antibiotic treatment and prophylaxis to save the shrimp crops. The application of antibiotics in shrimp farming is generally not permissible by law. Shrimp consignments containing antibiotic residues will face rejection by the importing countries if detected. Besides, the misuses of antibiotics for prophylactic and treatment purposes have also led to the emergence of pathogenic bacteria with antibiotic-resistance (Uchiyama et al 2011). In addition, the biodiversity of coastal environment has also been disturbed by the misuse of antimicrobial, chemicals, fertilizers and pesticides in aquaculture industry (Uddin et al 2009).

These issues have prompted researchers to investigate for alternative methods for prevention of bacterial infections in farmed shrimp. Among the alternative methods being implemented against diseases in aquaculture are biofloc (Harini et al 2016; Cardona et al 2015) and medicinal plant extract (Lakshmi et al 2014; Wu et al 2015; Yeh et al 2009). Recently, bacteriophages (phages) have become another promising alternative for prevention of bacterial infection in shrimp aquaculture. Phages are viruses that infect bacteria. Lytic phages have been used as biocontrol agents to prevent or reduce pathogenic bacterial infections (Ly-Chatain 2014). Phages were first discovered prior to World War II, but later the studies stopped due to worldwide adoption of antibiotic therapy. However, the emergences multiple antibiotic-resistance in bacteria have prompted many researchers to re-examine phage therapy (Ly-Chatain 2014). Lytic phages can completely eliminate the target pathogenic bacteria without harming other beneficial microbiota (Zhang et al 2014) and thus deemed fit the purpose of therapy.

Prior to therapeutic application, it is necessary to understand in detail the phage and host interaction, which is affected by both biological and physical factors (Beke et al 2016). The biological aspect is related to bacterial resistance and fitness, whereas temperature and pH are the main physical factors affecting the phage adsorption and bacterial growth.

*In-vitro* analyses of phage–bacteria interaction kinetics are necessary to understand the course of infection, and for designing optimum protocol for subsequent experiments in aquaculture systems (Silva et al 2014). Hence, *in-vitro* studies of their growth patterns, stability against environmental factors (temperature, pH and salinity) and lysable bacterial species are vital. The wide host range of phages is considered to contribute to the effectiveness of phage therapy. Thus, the present *in-vitro* study was conducted to characterize a lytic phage, strain PhVh6 isolated from water in shrimp pond using *Vibrio harveyi* as enrichment host. We studied the one-step growth curve, multiplicity of infection (MOI), physico-chemical sensitivities, host range susceptibility and bacteriolytic activity of PhVh6 against pathogenic *Vibrio* spp. in order to assess its potential as biocontrol agent against *Vibrio* infections in shrimp aquaculture.

## Materials and Methods

**Enrichment host.** Luminescent *V. harveyi* strain Vh10 (GenBank: KJ700303) was used as enrichment host for bacteriophage isolation. Vh10 was previously isolated from haemolymph of diseased Pacific white shrimp *Penaeus vannamei* from a commercial shrimp farm in Setiu Wetland, Terengganu in June 2014. The exponential (log) phase of Vh10 was determined to be at 6 hr of incubation.

**Phage isolation, purification and titration.** Water samples from shrimp pond encountered ongoing mortality were collected in September 2014 for phage isolation using double-layer agar (DLA) method (Vinod et al 2006). Phage was purified three times (Kęsik-Szeloch et al 2013) and titrated to determine the concentration (pfu mL<sup>-1</sup>).

**Multiplicity of infection.** PhVh6 was tested for multiplicity of infection (MOI) of 0.01, 0.1, 1 and 10 following Shen et al (2012) using exponential phase of Vh10 (10<sup>8</sup> cfu mL<sup>-1</sup>; OD<sub>600</sub> 0.75) for ratio adjustment. Vh10 was treated with PhVh6 at 37°C for 20 min, then plated on DLA and incubated overnight at 35°C. The MOI resulted in the highest number of plaque was considered optimum for infection.

**One-step growth curve.** One-step growth curve of PhVh6 was determined according to Hyman & Abedon (2009). PhVh6 was added to Vh10 (10<sup>8</sup> cfu mL<sup>-1</sup>) at MOI 0.1, and incubated for 20 min at 37°C, then centrifuged (10,000 × *g*, 10 min) to pellet down the cells. The pellet containing the infected Vh10 cells were washed twice and re-suspended in tryptic soy broth (TSB) pre-warmed to 37°C, followed by incubation at 37°C. The TSB suspension was sampled at 10-min interval up to 60 min, and immediately diluted for phage titer determination using DLA as above.

**Transmission electron microscopic (TEM) characterization.** A high-titer bacterial lysate was filtered through 0.45 and 0.22  $\mu\text{m}$  polyvinylidene difluoride (PVDF) membranes to prevent host contamination (Surekhamol et al 2013). A drop (10  $\mu\text{L}$ ) of filtrate containing the phage was deposited on carbon-coated Formvar films and positively stained with 2% uranyl acetate (Keşik-Szeloch et al 2013), then examined under a JEM-100C transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV and a magnification of 66,000  $\times$ . The size of phage was determined from an average of 5 to 10 viral particles.

**Sensitivity assays.** All sensitivity assays (Basdew & Laing 2014) were conducted in triplicate with  $10^8$  pfu  $\text{mL}^{-1}$  of PhVh6 (predetermined to be optimum for plaque production) in SM buffer as follows:

**Heat sensitivity.** PhVh6 solution was subjected to different temperature treatments (25, 35, 45, 55 and 65°C) in water bath. Aliquots of 100  $\mu\text{L}$  were removed at 0, 20, 40 and 60 min of treatment, and plated on DLA (Vinod et al 2006), with an exponential Vh10 as the host. After 12 hr, the number of plaques resulted from each treatment was counted as the phage titer. The temperature resulted in the highest titer was considered optimum.

**Cold sensitivity.** This assay assessed the storage capability of PhVh6 at different temperatures and the storable duration. PhVh6 solutions stored at 4,  $-20$  and  $-75^\circ\text{C}$  for 0, 1, 2, 3 and 4 weeks were analyzed by DLA as above. The storage temperature resulted in the highest titer was considered optimum, whereas the titers resulted from each storage duration indicated the storage durability of PhVh6 at the respective temperature.

**pH sensitivity.** SM buffer was calibrated using 1 M HCl to pH 2, 4, 6, 8, 10, 12 and 14, and 900  $\mu\text{L}$  of each calibrated solution was aliquoted into 1.5 mL vials (Eppendorf, Germany). The stock of PhVh6 was added to the calibrated buffer solutions to the final titer of  $10^8$  pfu  $\text{mL}^{-1}$ . The vials were gently inverted to mix, and left to stand at room temperature (22°C) for 60 min. After 60 min, 100  $\mu\text{L}$  of each solution was sampled and mixed with 100  $\mu\text{L}$  of exponential Vh10. The mixtures were analyzed by DLA as above. After 12 hr incubation at 35°C, the pH resulted in the highest number of plaque (titer) was considered optimum.

**Salinity sensitivity.** SM buffer was calibrated with NaCl to salinities of 15, 30, 45 and 60 ppt, and used to prepare the PhVh6 test solutions. The test solutions were mixed with exponential Vh10, and incubated for 12 hr for DLA analysis as above. The resulting titers were used to determine the sensitivity of PhVh6 to each degree of salinity.

**Chloroform sensitivity.** PhVh6 test solutions were treated with 0, 1, 2 and 3% (v/v) chloroform at room temperature for 15 min. The control was treated with SM buffer. The treated PhVh6 solutions were centrifuged at 10,000 rpm for 10 min at 4°C. The aqueous phase was withdrawn for DLA analysis as above.

**Glycerol sensitivity.** This assay was adapted from Santos et al (2009). 0, 10, 20, 30, 40 and 50% (v/v) glycerol solutions were prepared using sterile distilled water. PhVh6 stock was added to 900  $\mu\text{L}$  of each glycerol solution to the final titer of  $10^8$  pfu  $\text{mL}^{-1}$ . SM buffer was used in the control. The mixed solutions were left at room temperature for 1 hr, then analyzed by DLA to determine the resulting titer.

**Host range susceptibility.** PhVh6 was assessed against *V. harveyi* strain Vh8, *V. parahaemolyticus*, *V. alginolyticus*, *V. mimicus* and *V. cholerae* by spot test on DLA (Surekhamol et al 2013). The lawns host bacteria on DLA were spotted with 10  $\mu\text{L}$  of PhVh6 test solution. The formation of clear plaques after overnight incubation indicated susceptibilities of the hosts to PhVh6.

**Bacteriolytic assay.** Lytic activities of PhVh6 against Vh10 and other *Vibrio* spp. (*V. harveyi* Vh8, *V. parahaemolyticus* and *V. alginolyticus*) was quantified by enzyme-linked immunosorbent assay (ELISA) (Zhang et al 2014). Briefly, PhVh6 was used to infect the

exponential cultures of the four *Vibrio* spp. at MOI 1. The mixtures were incubated at 37°C for 6 hr and aliquots were analyzed in an ELISA reader (Thermo Fisher Scientific, USA) at 0, 2, 4, 6, 8 and 10 hr post-infection. A preparation without phage was used as negative control.

**Statistical analyses.** The sensitivity data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 20 (IBM Corp., USA) to determine the significance of difference.

## Results and Discussion

Lytic phage PhVh6 was isolated from the water in a commercial shrimp pond encountered ongoing mortalities using luminescent *V. harveyi* Vh10 as enrichment host. The plaque characteristics of PhVh6 on DLA and TEM morphologies of the viral particles were summarized in Table 1. The clear zones indicated the lytic nature of PhVh6 (Figure 1a). PhVh6 showed icosahedral head, elongated and flexible tail, thus assigned to the order Caudovirales (tailed phages) (Figure 1b). The lack of neck and collar indicated the tail to be non-contractile, hence further classified PhVh6 into the family Siphoviridae (Shivu et al 2007).

It is known that 61% of the tailed phages belong to Siphoviridae (Ackermann 2007). A study by Shivu et al (2007) also showed that Siphoviridae are more commonly encountered, in which six out of seven tailed phages isolated belonged to Siphoviridae compared with the family Myoviridae. The genomes of Caudovirales are known to consist of double-stranded DNA packed within the capsid (Matsuzaki et al 2005; Yang et al 2010).

Table 1

The plaque characteristics of PhVh6 on DLA and morphologies of viral particles under TEM

<i>Plaque on DLA</i>	<i>Head</i>	<i>Tail</i>	<i>Order</i>	<i>Family</i>
Clear plaques, 10 mm diameter	Icosahedral, 53 ± 2 nm	Elongated, flexible, non- contractile (lack of neck and collar), 120 ± 26 nm	Caudovirales (tailed phage)	Siphoviridae

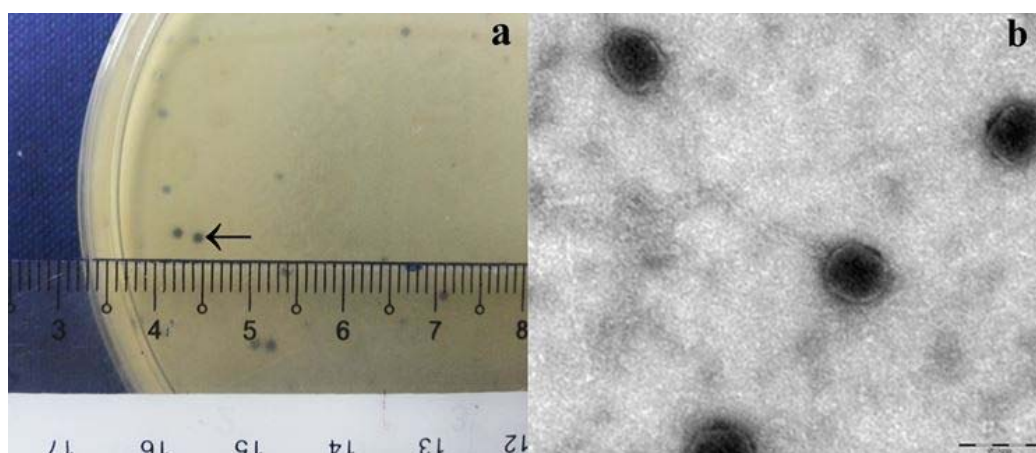


Figure 1. a - Clear plaques of PhVh6 on DLA; b - TEM morphologies of PhVh6 particles.

MOI is simply the ratio of phages to bacteria in the interaction. Determination of MOI is crucial for understanding the effects of phage therapy. MOI is related to the kinetic theory of phage therapy, and is critical for the efficiency of bacterial inhibition (Silva et al 2014). In the present study, the PhVh6-Vh10 interaction was optimum at MOI 1 which yielded the highest number of plaque at  $1.05 \times 10^{13}$  pfu mL<sup>-1</sup> (Figure 2).

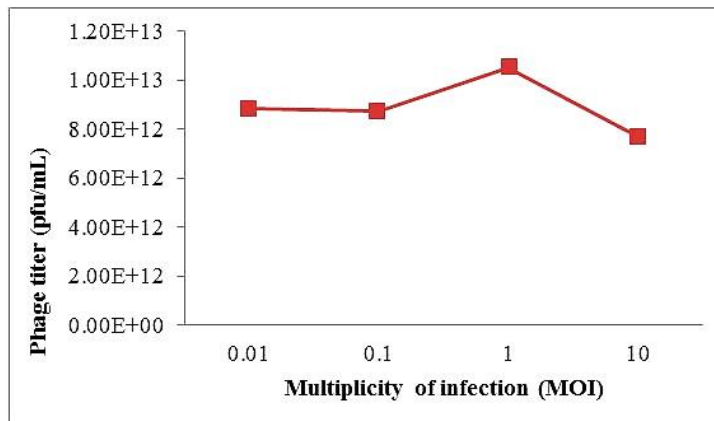


Figure 2. Different MOI of PhVh6 to Vh10.

A high MOI is preferred in any phage therapy program to achieve the complete suppression of bacterial growth (Goode et al 2003). In the present study, MOI 1 was found to be optimum for PhVh6 and Vh10. The highest MOI of 10 inversely resulted in the lowest plaque yield (Figure 2). This is in agreement with a previous research which showed maximum bacterial inactivation of 3.3 log after 24 hr of phage therapy, and increasing MOI to 10 did not significantly increase the inactivation (Silva et al 2014). Hence, MOI of 1 is deemed sufficient for inactivation of host. On the other hand, a study has reported that MOI 1 continuously decreased during the third hour of infection but MOI 10 showed no changes in value (Zhou et al 2015).

One-step growth curve showed a short latent period and generation time of PhVh6, which resulted in a high burst rate (Figure 3). A short latent period and high burst rate translates into faster phage replication and more efficient release of viral particles (Bao et al 2011). A previous study suggested that the short latent period of phage might relate to rapid production of the phage DNA polymerase (Melo et al 2014). This is a characteristic of a good candidate for phage therapy (Silva et al 2014).

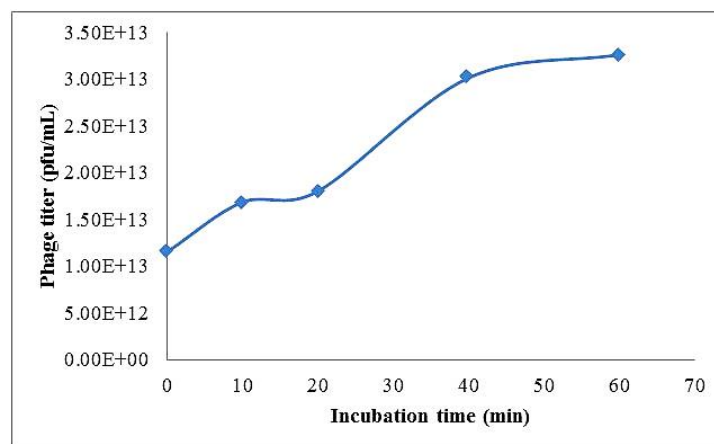


Figure 3. One-step growth curve of PhVh6 over 60 min.

The ability of phages to infect bacteria is influenced by various external physico-chemical factors such as temperature, acidity, salinity and ion concentrations, which could determine the occurrence and viability of phages during storage (Ackermann et al 2004). These factors may inactivate the phage through damage of its structural elements (head, tail and envelope), lipid loss or DNA structure (Ackermann et al 2004). Phages and other viruses are known for their rapid growths, high pH stabilities and high thermal resistances (Yang et al 2010). However, other parameters that may affect the stability of phage, such as various *in-vivo* stress conditions, must be considered (Lee et al 2014). In the present study, the sensitivities of PhVh6 to some of the parameters were significantly different ( $P < 0.05$ ). The heat sensitivity of PhVh6 was significantly different ( $P < 0.05$ ) and optimum at 45°C with the highest titer of  $8.69 \times 10^{12}$  pfu mL<sup>-1</sup> compared with other temperatures (Figure 4).

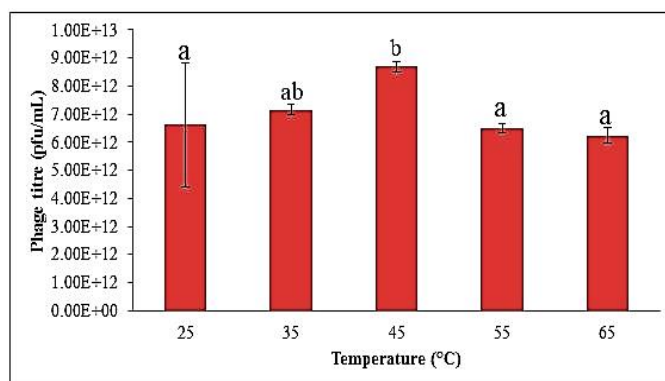


Figure 4. The sensitivity of PhVh6 to different temperatures ( $P < 0.05$ ).

Temperature is an important factor for phage survival (Olson et al 2004). It plays a significant role in phage attachment, penetration and multiplication (Jończyk et al 2011). Therefore, it is important to determine the workable range of temperature because *in-vivo* phage applications require tolerance to high temperature adaptation. PhVh6 in the present study was tested stable at 25-65°C, thus should have a good chance of survival when used in the natural environment, although the titer was decreased at the higher temperature. These observations are in agreement with a previous study which suggested that prolonged exposure to high temperature reduces the lytic ability of phages (Chandra et al 2011). This might also be attributed to the ineffective multiplication of host bacteria at high temperature.

It is also important to determine the suitable range of temperature for phage storage. In the present study, the cold sensitivity of PhVh6 declined over time in storage. The highest titer of PhVh6 ( $1.3 \times 10^{11}$  pfu mL<sup>-1</sup>) resulted from -75°C suggested the temperature to be optimum for storage, followed by -20°C ( $1.06 \times 10^{11}$  pfu mL<sup>-1</sup>). The titer declined drastically at 4°C ( $8 \times 10^9$  pfu mL<sup>-1</sup>) after 4 weeks of storage (Figure 5). Exposure to 4°C for a month also reduced the lytic ability of PhVh6 compared with -20 and -75°C. The result suggested possible destruction of the ultrastructure of the phage (Basdew & Laing 2014).

Determination of the optimum pH range is also important for *in-vivo* use of phage, so that the phage can retain its efficiency in the environment. The acidity and alkalinity of the environment can influence phage stability, attachment, infectivity, intracellular replication and multiplication (Silva et al 2014). PhVh6 was able to withstand pH 2-14, but the stability declined with increasing pH (alkaline conditions). PhVh6 was optimum at pH 6-8 with a titer of  $4.06 \times 10^{11}$  pfu mL<sup>-1</sup> at pH 6 and  $4.94 \times 10^{11}$  pfu mL<sup>-1</sup> at pH 8 (Figure 6).

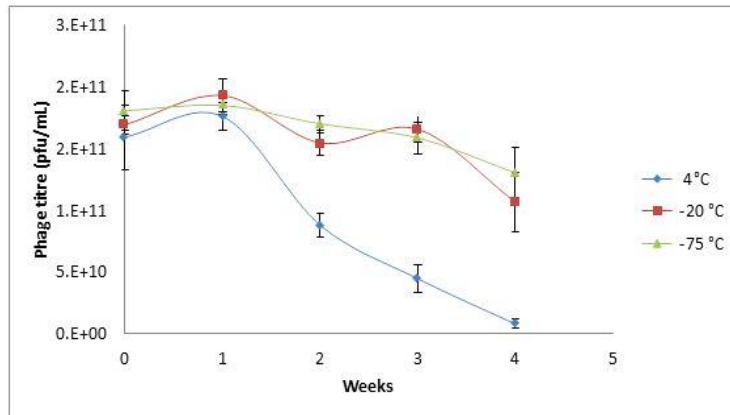


Figure 5. The effect of different storage temperatures on survival of PhVh6.

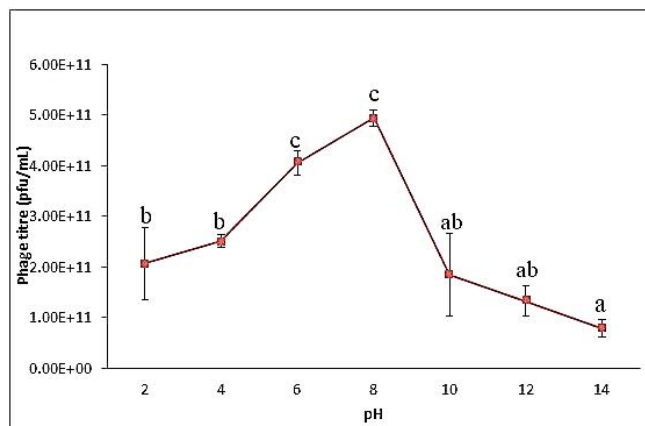


Figure 6. Sensitivity of PhVh6 to different pH ( $P < 0.05$ ).

The sensitivity of PhVh6 varied significantly at different pH, with pH 14 resulted in the lowest titer ( $7.83 \times 10^{10}$  pfu mL<sup>-1</sup>). Lysozyme is used during phage attachment to the receptor site of the host for subsequent weakening of the cell wall. However, pH can interfere with lysozyme and other capsid proteins, and prevent phage attachment to the receptor site (Leverentz et al 2004). Low pH reportedly affects phage aggregation and reduces their abilities to penetrate the host cells (Langlet et al 2007). In the present study, we observed stable lytic activities at pH 6–8, suggesting that PhVh6 is a good candidate for the *in-vivo* biocontrol of *Vibrio* spp. The pH range could also ensure optimum phage replication (Basdew & Laing 2014). Our findings that PhVh6 is able to withstand acid and alkaline conditions are in agreement with the previous studies that observed family Siphoviridae to be most resistant to adverse conditions (Lasobras et al 1997). Similarly, a previous study by Silva et al (2014) has also reported phage to be stable at neutral and near neutral pH from 6.5–7.4.

The titers of PhVh6 were significantly increased with increasing salinity from 15, 30 and 45 ppt, but drastically declined at 60 ppt ( $P < 0.05$ ). The optimum salinity of PhVh6 was 45 ppt with a titer of  $4.61 \times 10^{11}$  pfu mL<sup>-1</sup> (Figure 7).

A previous study has revealed that increase in salt concentration resulted in increment of inhibitory activity to a maximum of 5.2 log after 12-hr incubation (Silva et al 2014). The salinity sensitivity profile in the present study suggested that PhVh6 favors the marine salinity (usually between 32–35 ppt) more than the salinities of brackish water. This may have implications in phage application in shrimp farming in the brackish water environment. This also suggested that PhVh6 could possibly be originated from the marine environment that it favors more, but ended up in the brackish water in shrimp pond.

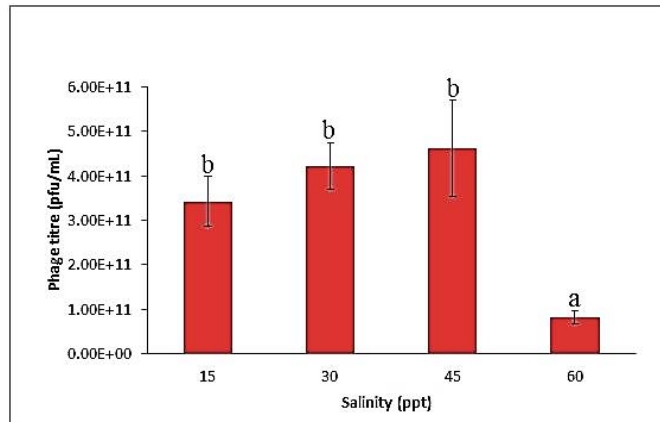


Figure 7. The effect of different salinities on PhVh6 ( $P < 0.05$ ).

Chloroform prevents bacterial contamination of phage during storage. The titers of PhVh6 with and without chloroform were not significantly different ( $P > 0.05$ ), and remained stable without chloroform. However, the addition of 1% chloroform was found to be optimum with a titer of  $4.43 \times 10^{11}$  pfu mL<sup>-1</sup> compared with other concentrations (Figure 8).

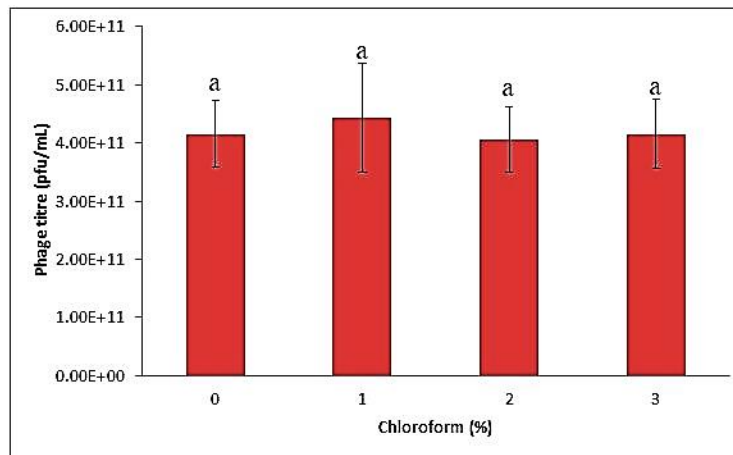


Figure 8. The effect of different concentrations of chloroform on PhVh6 ( $P > 0.05$ ).

It is necessary to add chloroform before phage storage to kill off any live bacterial cells that remained unlysed (Sambrook et al 1989) and to prevent from bacterial contamination. Thus, chloroform can serve as an alternative to the use of micro-filters. We observed that the addition of chloroform to phage lysates did not significantly affect the efficiency of PhVh6. This contradicts the previous study that found phages extremely sensitive to chloroform treatment (Basdew & Laing 2014).

Glycerol is needed for long-term storage of phages to ensure maintenance of their lytic efficiencies. PhVh6 stored without glycerol yielded significantly lower ( $P < 0.05$ ) titer (Figure 9). PhVh6 stored in SM buffer with glycerol showed higher viability. The addition of 30 and 40% glycerol for storage resulted in higher titer yields ( $4.94 \times 10^{11}$  and  $4.88 \times 10^{11}$  pfu mL<sup>-1</sup>, respectively). This could be due to the presence of Ca<sup>2+</sup> divalent ions (CaCl<sub>2</sub>) in the SM buffer, which the phages require for stable development. Previous study has mentioned the increased growth rate of phage aggregates with the increment of calcium salt concentration, possibly resulted from the neutralization of the negatively charged moieties on the phage surface by cation binding (Adams 1949).



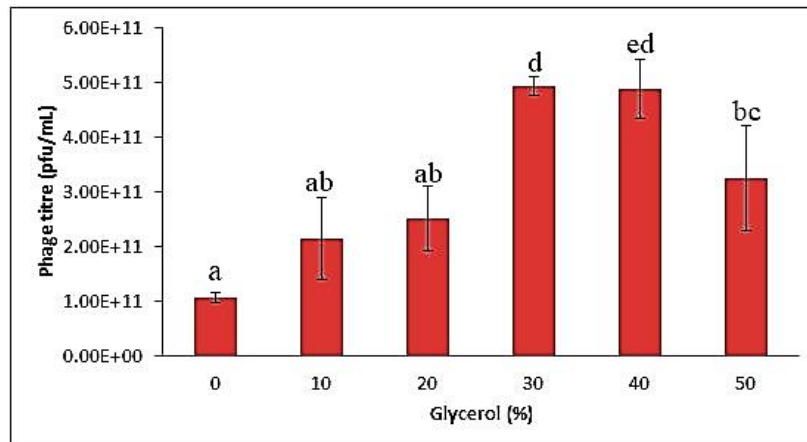


Figure 9. The effect of 0–50% glycerol on PhVh6 (P < 0.05).

PhVh6 was able to lyse other *Vibrio* spp., namely *V. parahaemolyticus* and *V. alginolyticus*, but not *V. mimicus* and *V. cholerae*. PhVh6 was also able to lyse another strain of *V. harveyi* (Vh8) (Table 2).

Table 2

Host range susceptibility of PhVh6

<i>Vibrio</i> spp.	Host susceptibility
<i>Vibrio harveyi</i> (Vh8)	+
<i>Vibrio parahaemolyticus</i>	+
<i>Vibrio alginolyticus</i>	+
<i>Vibrio mimicus</i>	–
<i>Vibrio cholerae</i>	–

+: positive –: negative

The susceptibility of bacteria to phage lysis may vary because of the difference in receptor molecules, restriction modification system in the host or superinfection exclusion (Duckworth 1976). Our present study contradicts previous studies which reported that phages lytic to *V. harveyi* were ineffective against other *Vibrio* spp. (Vinod et al 2006; Shivu et al 2007). Phages forming large clear plaques were suggested to have broad host range, and is regarded as one of the suitable characteristics for phage therapy (Shivu et al 2007; Surekhamol et al 2013).

In the bacteriolytic assay, the PhVh6-treated suspensions of Vh10, Vh8, *V. parahaemolyticus* and *V. alginolyticus* showed significantly lower absorbance (OD<sub>600</sub>) in ELISA compared with the negative control (without PhVh6), indicating effective reduction of cell densities due to strong bacteriolytic activities (Figure 10).

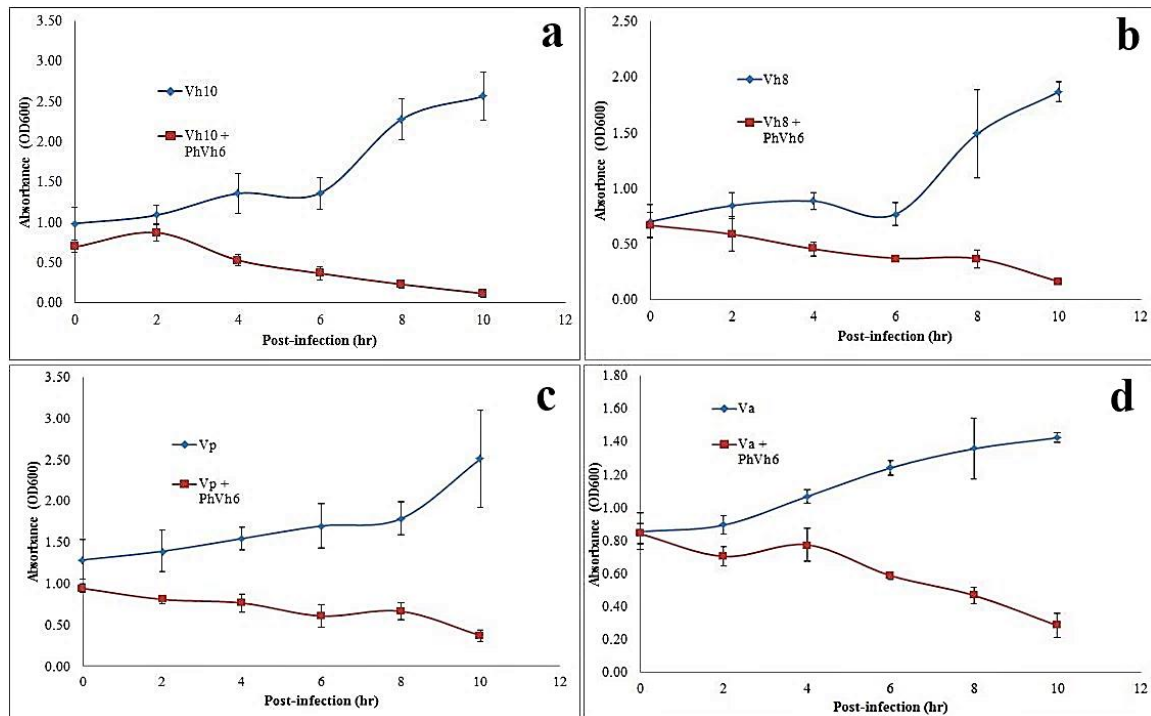


Figure 10. Bacteriolytic activities of PhVh6 against (a) *V. harveyi* Vh10, (b) *V. harveyi* Vh8, (c) *V. parahaemolyticus* (Vp), and (d) *V. alginolyticus* (Va) for 10 hr.

In recent years, phage preparations have been approved by Food and Drug Administration (FDA) as antimicrobial food additives (e.g., Listex, EcoShield) in poultry and ready-to-eat (RTE) products (FDA, 2006). The application of phage, if properly strategized according to the industry requirements, can potentially be the alternative to the banned antibiotic use in shrimp aquaculture as well as antimicrobial additive in seafood products against the life-threatening vibrio poisoning. Going the natural and environmental-friendly way, phage application helps ensure the environmental sustainability of aquaculture as well as the safety of aquaculture and seafood products.

**Conclusion.** We have characterized the MOI, one-step growth curve, physico-chemical sensitivity profiles and host range susceptibility of the lytic phage PhVh6. The MOI of 1, short latent period, high burst rate and wide host range indicate the potential of PhVh6 as a biocontrol agent against pathogenic *Vibrio* spp. in shrimp aquaculture.

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## References

- Ackermann H.W., 2007 5500 Phages examined in the electron microscope. Archives of Virology 152(2): 227–243.
- Ackermann H.-W., Tremblay D., Moineau S., 2004 Long-Term Bacteriophage Preservation. WFCC Newsletter 38: 35–40.
- Adams M. H., 1949 The Stability of Bacterial Viruses in Solutions of Salts. The Journal of General Physiology 579–594.
- Bao H., Zhang H., Wang R., 2011 Isolation and characterization of bacteriophages of

- Salmonella enterica* serovar Pullorum. Poultry Science 90(10): 2370–2377.
- Basdew I. H., Laing M. D., 2014 Stress sensitivity assays of bacteriophages associated with *Staphylococcus aureus*, causal organism of bovine mastitis. African Journal of Microbiology Research 8(2):200–210.
- Beke G., Stano M., Klucar L., 2016 Modelling the interaction between bacteriophages and their bacterial hosts. Mathematical Biosciences 279: 27–32.
- Cardona E., Saulnier D., Lorgeoux B., Chim L., Gueguen Y., 2015 Rearing effect of biofloc on antioxidant and antimicrobial transcriptional response in *Litopenaeus stylirostris* shrimp facing an experimental sub-lethal hydrogen peroxide stress. Fish & Shellfish Immunology 45(2): 933–939.
- Chandra M., Thakur S., Narang D., Saxena H. M., 2011 Isolation of a bacteriophage against *Salmonella Dublin* and determination of its physical resistance under varied *in vitro* conditions. African Journal of Microbiology Research 5(15): 2044–2047.
- Duckworth D. H., 1976 "Who discovered bacteriophage?" Bacteriological reviews 40(4): 793–802.
- FDA, 2006 Federal Register 47729 - Approval of Listeria-specific Bacteriophage Preparation on Ready-to-Eat (RTE) Meat and Poultry Products. Rockville, MD. Available online at: <http://www.fda.gov/OHRMS/DOCKETS/98fr/E6-13621.pdf>
- Goode D., Allen V. M., Barrow P. A., 2003 Reduction of Experimental *Salmonella* and *Campylobacter* Contamination of Chicken Skin by Application of Lytic Bacteriophages. Applied and Environmental Microbiology 69(8): 5032–5036.
- Harini C., Rajagopalasamy C. B. T., Stephen Sampath Kumar J., Santhakumar R., 2016 Role of Biofloc in the Growth and Survival of Blue morph, *Pseudotropheus saulosi*. Indian Journal of Science and Technology 9(8): 1–7.
- Hyman P., Abedon S. T., 2009 Practical Methods for Determining Phage Growth Parameters. In P. Hyman & S. T. Abedon, eds. *Bacteriophages: Methods and Protocols*. pp. 175–202.
- Jończyk E., Kłak M., Międzybrodzki R., Górski A., 2011 The influence of external factors on bacteriophages—review. Folia Microbiologica 56: 191–200.
- Kęsik-Szeloch A., Drulis-Kawa Z., Weber-Dąbrowska B., Kassner J., Majkowska-Skrobek G., Augustyniak D., Lusiak-Szelachowska M., Zaczek M., Górski A., Kropinski A. M. 2013 Characterising the biology of novel lytic bacteriophages infecting multidrug resistant *Klebsiella pneumoniae*. Virology Journal 10(100): 1–12.
- Lakshmi Y. S., Mala D., Gopalakrishnan S., Banu F., Brindha V., Gajendran N., 2014 Green synthesis and characterisation of Silver nanoparticles from the medicinal plant *Pithecellobium dulce*. Indian Journal of Nano Science 2(8): 4–9.
- Langlet J., Gaboriaud F., Gantzer C., 2007 Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage. Journal of Applied Microbiology 103:1632–1638.
- Lasobras J., Muniesa M., Frías J., Lucena F., Jofre J., 1997 Relationship between the morphology of bacteriophages and their persistence in the environment. Water Science and Technology 35(11–12): 129–132.
- Lee H. S. Choi S., Shin H., Lee J.-H., Choi S. H., 2014 *Vibrio vulnificus* Bacteriophage SSP002 as a Possible Biocontrol Agent. Applied and Environmental Microbiology 80(2): 515–524.
- Leverentz B., Conway W. S., Janisiewicz W., Camp M. J., 2004 Optimizing concentration and timing of a phage spray application to reduce *Listeria monocytogenes* on honeydew melon tissue. Journal of Food Protection 67(8): 1682–1686.
- Ly-Chatain M. H., 2014 The factors affecting effectiveness of treatment in phages therapy. Frontiers in microbiology 5(51): 1–7.
- Matsuzaki S., Rashel M., Uchiyama J., Sakurai S., Ujihara T., Kuroda M., Ikeuchi M., Tani T., Fujieda M., Wakiguchi H., Imai S., 2005 Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. Journal of Infection and Chemotherapy 11: 211–219.
- Melo L. D. R., Sillankorva S., Ackermann H.-W., Kropinski A. M., Azerodo J., Cerca N., 2014 Characterization of *Staphylococcus epidermidis* phage vB\_SepS\_SEP9 – a unique member of the Siphoviridae family. Research in Microbiology 165(8): 679–

685.

- Olson M. R., Axler R. P., Hicks R. E., 2004 Effects of freezing and storage temperature on MS2 viability. *Journal of Virological Methods* 122(2): 147–152.
- Sambrook J., Fritsch E. F., Maniatis T., 1989 *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory.
- Santos S. B., Carvalho C. M., Sillankorva S., Nicolau A., Ferreira E. C., Azeredo J., 2009 The use of antibiotics to improve phage detection and enumeration by the double-layer agar technique. *BMC Microbiology* 9(1): 148.
- Shen G. H., Wang, J. L., Wen F. S., Chang K. M., Kuo C. F., Lin C. H., Luo H. R., Hung C. H., 2012 Isolation and characterization of  $\phi$ km18p, a novel lytic phage with therapeutic potential against extensively drug resistant *Acinetobacter baumannii*. *PLoS One* 7(10): 1–8.
- Shivu M. M., Rajeeva B. C., Girisha S. K., Karunasagar I., Krohne G., Karunasagar I., 2007 Molecular characterization of *Vibrio harveyi* bacteriophages isolated from aquaculture environments along the coast of India. *Environmental Microbiology* 9(2): 322–331.
- Silva Y. J., Costa L., Pereira C., Cunha Ä., Calado R., Newton C. M. Gomes., Almeida A., 2014 Influence of environmental variables in the efficiency of phage therapy in aquaculture. *Microbial Biotechnology* 7(5): 401–413.
- Surekhamol I. S., Deepa G. D., Somnath Pai S., Sreelakshmi B., Varghese S., Singh B., 2013 Isolation and characterization of broad spectrum bacteriophages lytic to *Vibrio harveyi* from shrimp farms of Kerala, India. *Letters in Applied Microbiology* 58(1915): 197–204.
- Uchiyama J., Takemura I., Satoh, M., Kato S-I., Ujihara T., Akechi K., Matsuzaki S., Daibata M., 2011 Improved adsorption of an *Enterococcus faecalis* bacteriophage WEF24C with a spontaneous point mutation. *PLoS One* 6(10): 1–13.
- Uddin S. A., Kader A., Kamal A. M., Zafar M., 2009 Present status on the use of antibiotics and chemicals in shrimp hatcheries and grow-out ponds and their environmental Implications in Bangladesh. *AAFL Bioflux* 2(3): 369–379.
- Vinod M. G., Shivu M. M., Umesha K. R., Rajeeva B. C., Krohne G., Karunasagar I., Karunasagar I., 2006 Isolation of *Vibrio harveyi* bacteriophage with a potential for biocontrol of luminous vibriosis in hatchery environments. *Aquaculture* 255(1): 117–124.
- Wu C.-C., Chang Y.-P., Wang J.-J., Liu C.-H., Wong S.-L., Jiang C.-M., Hsieh S.-L., 2015 Dietary administration of *Gynura bicolor* (Roxb. Willd.) DC water extract enhances immune response and survival rate against *Vibrio alginolyticus* and white spot syndrome virus in white shrimp *Litopenaeus vannamei*. *Fish & Shellfish Immunology* 42(1): 25–33.
- Yang H., Liang L., Lin S., Jia S., 2010 Isolation and characterization of a virulent bacteriophage AB1 of *Acinetobacter baumannii*. *BMC Microbiology* 10(131): 1–10.
- Yeh R.-Y., Shiu Y.-L., Shei S.-C., Cheng S.-C., Huang S.-Y., Lin J.-C., Liu C.-H., 2009 Evaluation of the antibacterial activity of leaf and twig extracts of stout camphor tree, *Cinnamomum kanehirae*, and the effects on immunity and disease resistance of white shrimp, *Litopenaeus vannamei*. *Fish & Shellfish Immunology* 27(1): 26–32.
- Zhang L., Bao H., Wei C., Zhang H., Zhou Y., Wang R., 2014 Characterization and partial genomic analysis of a lytic Myoviridae bacteriophage against *Staphylococcus aureus* isolated from dairy cows with mastitis in Mid-east of China. *Virus Genes* 50(1):111–117.
- Zhou Y., Bao H., Wang R., 2015 Isolation and characterization of lytic phage vB\_EcoM\_JS09 against clinically isolated antibiotic-resistant avian pathogenic *Escherichia coli* and Enterotoxigenic *Escherichia coli*. *Intervirology* 58(4): 218–231.

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