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## Phage Treatment of *Vibrio harveyi*: A General Concept of Protection against Bacterial Infection

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### ABSTRACT

The *Vibrio harveyi* (VH), called VH13-1, was isolated and collected from luminescent black tiger shrimp. To test the possibility for the use of permissive phage as a tool to treat the VH contamination in shrimp ponds, our new isolated temperate phage (VH-P) which is specific to the infection of VH13-1, was tested for its efficiency to kill the VH. Our study was conducted with the MOI (multiplicity of infection) of 1-100. The success of the treatment is determined if no VH bacterial colony is observed in the culture medium after the phage treatment. The MOI of 20 resulted in the killing of all the VH13-1 while MOI of 10 was inconsistent because two of the three experiments showed growth of bacterial colony and MOI of 1 resulted in significant survival of VH. These colonies were proved to become lysogenic infection of VH-P. The study shows that using temperate phage of VH-P to treat VH bacterium, it is necessary to treat with the sufficient amount of the VH-P phage at once. Insufficient amount of MOI will allow the VH to develop lysogenic infection and cause interference to super-infection to the same phage. Besides searching phage from natural sources as the subject to treat bacterial epidemic, genomic modification of phage genome is proposed to enhance the possibility for phage treatment to control the bacterium epidemic while drug resistance of the pathogenic bacteria is kept being developed but the new antibiotic can not be discovered.

**Key words:** Temperate phage, multiplicity of infection, lysogenic infection, super-infection, drug resistant pathogenic bacteria

### INTRODUCTION

*Vibrio harveyi* (VH) is one of the luminescent bacteria (Defoirdt *et al.*, 2008) which involve the luminescent disease in agricultural shrimp of the tiger prawn (*Panaeus* spp.) (Manefield *et al.*, 2000; Phuoc, 2008). The shrimp eventually die after performing luminescent for a few days (Ruangpan *et al.*, 1999). Based on the information from shrimp farmers the VH is reported to be seriously active in nursery shrimp ponds, much more than the grow-out ponds. Like other bacterium, *V. harveyi* can be infected by specific bacteriophage (Pasharawipas *et al.*, 2005; Khemayan *et al.*, 2006). Some of the bacteriophages were reported to be the partners of the bacterium infection to cause shrimp death due to the luminescent symptom, as called phage conversion (Ruangpan *et al.*, 1999). On the other hand, there are other bacteriophages of the VH that kill the bacterium, know as lytic phage infection (Srinivasan *et al.*, 2007).

Before the discovery of antibiotic, phage therapy had been studied to be a strategy to fight against bacterial infection (Kropinski, 2006; Bahador *et al.*, 2007; Dublanchet and Fruciano, 2008). After the discovery of antibiotic, however, the idea of phage therapy was overlooked in the western world. On the other hand, it has still been studied in Eastern Europe (Kropinski, 2006). Now, the concept of phage therapy has been investigated widely for advantage in the food industry (Leverentz *et al.*, 2001; Huff *et al.*, 2005; Atterbury *et al.*, 2007; Bielke *et al.*, 2007), animal farms (Wall *et al.*, 2010), environment (Abdulla *et al.*, 2007) and medical treatment (Bruttin and Brussow, 2005; Wang *et al.*, 2006; Kumari *et al.*, 2009; Sundar *et al.*, 2009; Santos *et al.*, 2010). However, the effectiveness of phage therapy is not certain according to different groups of investigators.

As informed by economic reports in the mass media, bacterial infection is the one of the greatest concerns in shrimp aquaculture industry. This forces many farmers to use excessive amounts of antibiotic with the hope to prevent the bacterial epidemic. Using astronomical amounts of antibiotic in shrimp farms cause various problems: e.g., the extravagant expense, environmental concern, lower shrimp quality with the excessive dose of antibiotics which becomes unacceptable in the high standard market. Also, this action increases the potential of antibiotic resistant pathogens to consumers (Duran and Marshall, 2005). The study of phage therapy is an interesting concept to pursue in the shrimp industry, especially with regard to the green environmental concept.

This study is to examine the possibility to use specific bacteriophage to eliminate the VH of infected shrimp by phage therapy. This allows us to prevent bacterial infection in aquaculture animals in a more natural strategy. The knowledge can be applied for further study in other models and should be useful in other economic animals and even in medical practice in the future.

## **MATERIALS AND METHODS**

**Media and reagents:** Mueller-Hinton Broth (MHB), Thiosulfate Citrate Bile Sucrose (TCBS) medium, were obtained from Oxford (Hampshire, England). The Mueller-Hinton Agar (MHA) was prepared by addition of Difco agar (Becton, Dickinson and Co., Franklin Lakes, NJ) at 1.5%. Artificial seawater (ASW) containing 400 mM NaCl, 100 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 20 mM KCl, 20 mM CaCl<sub>2</sub>·2H<sub>2</sub>O and other tract salts was prepared to be a final concentration of 3% in MHA and MHB. Restriction enzymes, S1 nuclease RNase and DNase were purchased from Promega (Medison, WI), digoxigenin nonradioactive DNA labeling and detection kits from Boeringer Mannheim and Roach (Mannheim, Germany). PCR reagents were acquired from Perkin-Elmer (New Jersey).

**Collection of pathogenic VHs:** The VH, called VH13-1, was originally isolated from the luminescent shrimp that was epidemic during 1998-1999 from shrimp ponds in Songkhla province which is in the Southern East of Thailand. The bacteria was isolated on the TCBS medium and confirmed to be *Vibrio* spp. by standard biochemical assay according to manufacturer.

The bacteria was identified as VH by an additional character to exhibit green colony and express luminescent in a dark room after incubation at 28°C or room temperature overnight (Ruangpan *et al.*, 1999; Khemayan *et al.*, 2006).

**Isolation of VH-phages:** VH-phages were screened and collected from the ponds of shrimp farms in Chantaburi province which is in the East of the gulf of Thailand in 2007. The specimens were treated with chloroform to kill and eliminate contaminated bacteria by shaking and centrifugation around 10,000 g for 10 min. The phage containing supernatant was tested for its existence by the

dot plaque assay with VH13-1 that was isolated from the infected shrimp pond as mentioned above (Khemayan *et al.*, 2006). The clear plaque was then isolated again to purify the phage by repeating the dot plaque assay. The specific phage of VH13-1 is then called VH-P. Besides, *Vibrio* phages of other *Vibrio* sp. were also collected but would not be included in this study.

**Morphology of the isolated VH-phages:** The isolated phage was enriched and stained with 2% phosphotungstic acid to identify their morphology by electron microscopy as mentioned in the previous publication (Pasharawipas *et al.*, 2005).

**Amplification of VH-phages particles:** The isolated VH 13-1 bacterium host was cultured in 3% NaCl-MHB for 24 h at 28°C and diluted to be  $10^{-8}$  before continuing incubation for 2 h. The VH-P was then infected the VH13-1 with the MOI (multiplicity of infection) of 1. The mixer was vigorously shaken at 28°C for 3-4 h until resulting to the complete lysis of the bacterial cell and then centrifuged approximately 10,000 g for 10 min to collect the supernatant. The phage titer was checked by the dot plaque assay to determine the phage concentration. If the phage concentration was sufficient at  $10^9$  PFU mL<sup>-1</sup>, the supernatant was then filtrated with 0.45 micron filtrated membrane to eliminate bacterium.

**Genomic restriction of the phages:** The phage genome was extracted and purified (Sambrook *et al.*, 1989). The genome was tested for its property by digestion with S1 nuclease, RNase and DNase enzymes according to the manufacturer. The VH-P genomes was also studied for its restriction characteristics by common restriction enzymes such as BamHI, DraI, EcoRI, HindIII, PstI and XbaI.

**PCR identification of the isolated VH-phage:** To search a molecular tool for identification the VH-P phage, the PCR technique is approached. In this case, a small part of the VH-P genome planned to be sequenced to design the PCR primers. Fortunately, the VH-P phage genome was found to be able to be digested with the common restriction enzymes of EcoRI and Hind III. Both enzymes were then used to operate double enzyme digestion in the same reaction tube because their requirement of the same condition to digest genomic DNA. Accordingly, EcoRI/HindIII digested fragments of VH-P were cloned into the EcoRI/Hind III digested pUC19 plasmid. The recombinant plasmids were selected from the white colony in X-gal/IPTG ampicillin plates according to the manufacturer (New England). The recombinant plasmids were proved to contain VH-P phage genome fragments by digestion with the double EcoRI/HindIII enzymes and run in 1% agarose gel electrophoresis. The recombinant fragment is confirmed to be derived from VH-P genome by Southern blot hybridization technique using digoxigenin labeling VH-P genome as a probe (Sambrook *et al.*, 1989). Eventually, the recombinant clone called VH-P15 was sent to Macrogen, Republic of Korea for nucleotide sequencing. The information of the nucleotide sequences was then designed for the PCR primers.

**PCR amplification:** The VH-P genome can be identified especially in lysogenic VH13-1 by PCR technique with the designed primers based on the nucleotide sequence of VH-P15. The forward primer, VHP15-F, is 5'GCAAAGCATTGTCTCTTG 3' and the reward primer, VHP15-R is 5'CCAACGACCAACTAACA3'. The amplification is operated based on the standard condition with the annealing temperature of 45°C. The PCR procedure was operated by standard method as mentioned by manufacturer (Perkin-Elmer, New Jersey).

**Phage treatment against VH:** The amplified VH-P phage was stored with the concentration of  $10^9$  PFU (Plaque forming unit)/mL in refrigerator at 4°C. Normally, the amount of the VH bacteria in shrimp pond is not higher than  $10^4$  CFU mL<sup>-1</sup>. So the  $10^4$  cfu mL<sup>-1</sup> of the VH13-1 is assumed to be the target amount for the treatment by the VH-P phage. The  $10^9$  pfu mL<sup>-1</sup> of stock VH-P phage was then diluted to be  $2 \times 10^6$ ,  $1 \times 10^8$ ,  $4 \times 10^5$ ,  $2 \times 10^5$  and  $2 \times 10^4$  pfu mL<sup>-1</sup>. The diluted solutions of the phage were added into 5 mL of the  $2 \times 10^4$  cfu mL<sup>-1</sup> of the VH13-1 suspension with the equal volume. After all, the final MOI are 100, 50, 20, 10 and 1, respectively. In the mean time, the VY16-1 with the concentration of  $2 \times 10^4$  cfu mL<sup>-1</sup> was also added in the same tube to be a control. The VH-P treated VH13-1 mixture was tested for phage treatment efficiency by sampling 50 mL of the mixture to culture the survival VH13-1 by the spreading plate technique in the 3% NaCl-TCBS agar medium. If the bacterium colonies were grown, they were randomly tested for its lysogenic property by the PCR technique as mentioned above. The survived VH13-1 was also tested for its susceptibility to be re-infected with the VH-P phage by dot plaque assay. The success of phage treatment was determined by the criteria that no bacterial colony was grown in the medium. If the bacterium colonies were grown, they were randomly tested for its lysogenic property by the PCR technique as mentioned above. The survived VH13-1 was also tested for its susceptibility to be re-infected with the VH-P phage by dot plaque assay to prove that they were lysogenized, not contaminated.

## RESULTS

**Characteristic of the isolated VH-phages (VH-P):** Based on an electron microscopy, the morphology of the VH-P particle is a Podoviridae-like particle with the short tail. The diameter of the head is 70-80 nm as shown in Fig. 1. The genomes become resistance to RNase and S1 nuclease but sensitive to DNase digestion which can be interpreted that the VH-P genome is a double stranded DNA phage. EcoRI and HindIII as mentioned including DraI and XbaI but not by BamHI and PstI enzymes can digest the VH-P genome. The nucleotide sequence of the VH-P15 recombinant plasmid which is selected to be a diagnostic tool for identification of VH-P by PCR

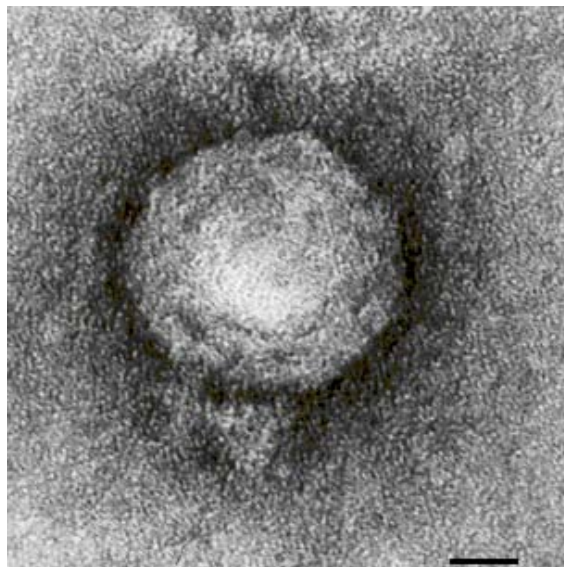


Fig. 1: Transmission electron micrograph of the VH-P phage. The solid bar indicates 20 nm

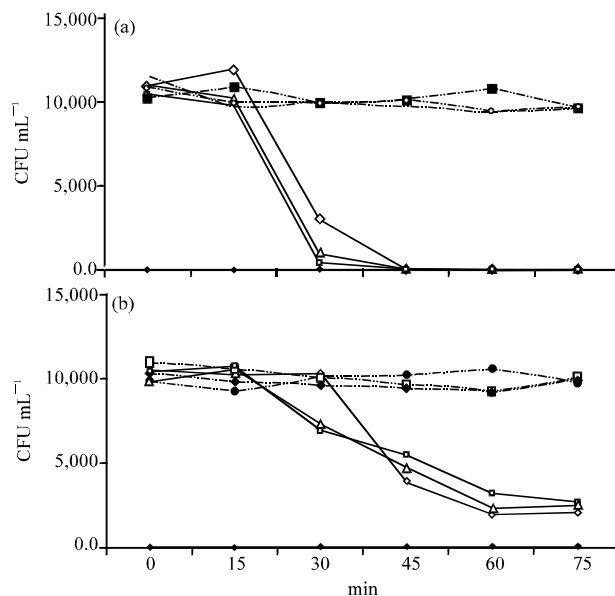


Fig. 2 (a-b): The VH-P phage treatments against VH13-1 with the MOI. 20 (a) and 1 (b), respectively. The solid lines represent VH13-1, the dash lines represent VY16-1. At, MOI 20 (a), showed no bacterial growth after the treatment by 45 min while MOI of 1 showed that the VH-P treated VH13-1 was still survived

technique, is analyzed to contain a conservative domain of terminase protein gene. The nucleotide sequence is deposited in Genbank and can be accessed with the number HQ533051.

**Phage treatment against VH:** The three repeated experiments have been tested for the phage treatment of VH-P against VH13-1 as shown in Fig. 2. We found that to kill the VH13-1 completely, the least MOI of the VH-P phage has to be 20 while the control VY16-1 was not affected as shown in Fig. 2a. The optimal time to completely kill all the VH can be within 45 min. This is determined by the result that there is no VH colony growth in TCBS after incubation for 24 h in 28°C. In case of the insufficient amount of VH-P treatment, at the MOI of 10, a few colonies were still observed in two of the three experiments while the MOI of 1, as shown in Fig. 2b, there are a lot more of VH13-1 survived. The bacterium colonies from both MOI of 10 and 1 were proved to be lysogenized by VH-P genome as tested by PCR technique and become resistance to the VH-P phage super-infection.

## DISCUSSION

In present study of the phage therapy by using the VH-P temperate phage to treat the pathogenic VH-13-1, we found that the bacterium can be killed completely without the development of lysogenic VH if the phage amount is sufficient to infect the target bacteria. The process can be completed within one hour. Longer incubation does not promote the phage to kill the bacterium any longer if the MOI is not sufficient initially. In our study, the experiment of using VH-P against VH13-1 was treated in ASW which is similar to their natural condition. This allows the maximum interaction between the phage particles to the VH bacteria. On the other hand, other studies did not find positive result in using temperate phage to treat bacterium infection especially *in vivo*

paper (Almeida *et al.*, 2009). Some others, who evaluate their results by comparison the survival rate of the experimental animals between the phage-treatment groups against the control group, can show the positive outcome (Almeida *et al.*, 2009). However, temperate phage might needed up till  $10^5$  to show the significant difference (Soothill, 1992; Almeida *et al.*, 2009). In present study, we believe that the success of phage treatment should be evaluated by the sense that the phage can kill or eliminate all the bacterium otherwise it cannot be counted to be practical to use the phage for the treatment. The reason that using temperate phage can be unsuccessful or not practical *in vivo* is mainly due to the immunity in the body which destroys the phage particle before having a chance to infect the targeting bacteria (Almeida *et al.*, 2009; Clark and March, 2006). This is the great obstruction for phage therapy especially in high-evolved-immune animals which can generate efficient immunity to eliminate the phage particles. At this moment, it is not promising to use temperate phage for therapy in the body.

On the other hand, using virulent or lytic phages the MOI can be as little as  $10^{-6}$  (Soothill, 1992). This is because the virulent phage can produce progeny to infect the bacteria again and again without the prevention of super-infection as it does by temperate phage (Dimmock *et al.*, 2001). Unfortunately, it is suggested that not less than a half of the phages in the world are temperate phage. However, some even mentioned that more than 90% of the known phages are temperate phages (Almeida *et al.*, 2009). Although the number cannot be counted exactly but it guilds us that it is not always possible to discover virulent phage which is an ideal phage for phage therapy for treatment any particular bacteria. In our experience, we can not find any virulent phage for *Vibrio harveyi*.

Our study suggests that in case of using temperate phage as a tool for phage therapy, it is essential to aware that the sufficient MOI of the phage suspension must be present at one time to kill the bacteria completely, otherwise the lysogenic bacteria can develop and thus, it becomes impossible to use the same phage to re-infect the bacteria because of the interference to super-infection. In our further investigation, it is not possible to eliminate the lysogenic bacteria completely although mitomycin-C and UV were treated (Jiang and Paul, 1996; Dimmock *et al.*, 2001). Ideally, it is more appropriate to use virulent phage for phage therapy. This will allow the progeny phages to re-infect bacterium again and again until all the bacterium in the certain condition is killed completely. If, however, we still desire to use temperate phage which is much easier to isolate from natural sources, as a tool for phage therapy we need to use the sufficient MOI at one time to avoid the development of lysogenic stage. Alternatively, modification of the temperate phage genome to exhibit only the lytic pathway by knockout or delete the lysogenic involving genes could be tried in case that the isolation of virulent phage is not possible for any particular pathogenic bacteria.

In addition, based on our experience, another requirement to make phage therapy successful is to use specific phage which is not only specie specific, but also strain specific. Thus, all the strains of the specific phages must be available for pathogenic bacteria. This means, we need to collect all the strain specific phages to be available for any pathogenic strains of the bacterial epidemic. This is quite difficult but may still be possible. However, there is no report that any organization has collected the all phages required for a specie specific bacterial infection. Alternatively, it is also of interest to identify the common molecule for the phage interaction to the pathogenic bacterium. This might lead us to modify the phage molecule that plays a role to infect the bacterium more broadly for the phage therapy in bacterium infection.

There are two main points for consideration in using phage as a tool to treat bacterial infection. The first one is to use virulent phage for treatment. This is very important especially in medical treatment. Secondly, sufficient MOI must be present at once if we desire to use the temperate phages which are much easier to isolate. In this case, it might be more appropriate in case of environmental bacteria in case that the lytic phage cannot be isolated.

In conclusion, although virulent phage is an ideal tool for phage therapy but the availability of the virulent phage is not always available for some particular problem-causing bacteria. If so, the available temperate phage can be used for the treatment especially in the environmental condition. However, we need to apply sufficient MOI of the phage for the treatment. Using temperate phage is not recommended inside the body. In this study, VH-P has been shown to have potential to treat the pathogenic bacteria, VH13-1, in the shrimp pond.

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