Isolation and characterization of bacteriophages specific to *Vibrio cholerae*

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Abstract

Vibrio spp. the main bacteria found in marine shrimp can cause diseases either in shrimp or human. Using antibiotics is a first option to reduce this problem. Unfortunately, this treatment has no sufficient potential, due to antibiotic resistance can occur after long term of use. Therefore, bacteriophage has become better alternative to control *Vibrio* spp. in shrimp. In this study, 101 isolates of *Vibrio* spp. were screened and isolated from water and Pacific white shrimp samples *(Litopaeneus vannamei*) of shrimp hatchery and shrimp pond. The suspected *Vibrio* spp. were determined and classified into 5 species including *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus* and *V. fluvialis*. Concurrently, five bacteriophages including VC1, VC2, VC3, VC4 and VC5 were also isolated using double layer agar method and classified in the family *Siphoviridae*. In addition, those *Vibrio* spp. from the same sample sources were used as hosts. These bacteriophages are able to survive at the pH and temperature ranges of 6-10 and 30-60˚C, respectively with a non-significant difference on their titers. But, bacteriophage titer starts to decrease at 70 ˚C and completely loss at 90˚C. Moreover, bacteriophage infection was tested on *Vibrio* spp., *Aeromonas hydrophila, Bulkholderia gladioli* and other Gram negative bacteria. The results indicated that only *V. cholerae* host was infected specifically.

Keywords: *Vibrio*, bacteriophage, shrimp

Introduction

Shrimp has been considered as one of the worthy aquaculture product in Thailand because of its demand that extensively enlarged through worldwide including South Asia and Latin America (Yano et al., 2013). In addition, shrimp industry tends to be a sustainable aquaculture industry which has constant growth rate (Kongkeo, 1997) and able to create high income to Thailand from its transport. Since shrimp industry becomes a significant role in Thailand aquaculture industry, bacterial contamination have been an unavoidable major problem especially *Vibrio* spp. which have been concerned as a rampant outbreak of worldwide mortalities in shrimp hatchery and shrimp pond (Jayasree et al., 2006). Naturally, *Vibrio* spp. such as *V. alginolyticus*, *V. parahaemolyticus*. *V. harveyi*, *V. cholerae* and *V. vulnificus* are ubiquitous in aquaculture and involve with shrimp diseases call Vibriosis (Gopal et al., 2005).

Vibrio spp. not only characterized as a shrimp pathogen that cause loose shell disease and tail necrosis from *V. alginolyticus*, *V. parahaemolyticus* and *V. harveyi* (Jayasree et al., 2006) but also human pathogen which cause cholera, diarrhea from *V. cholerae* and gastroenteritis from *V. vulnificus* (Morris Jr., 2003). Using large quantities of antibiotics seem to be an initial solution to control this problem. Unfortunately, this method supports the emergence of antibiotic-resistant bacteria and provides chemical contamination in environment and shrimp tissue (Karunasagar et al., 2007; Holmström et al., 2002). Moreover, European Union

community denied Thai shrimp because the appearance of either chloramphenicol or nitrofuran was found (Alagappan et al., 2010). After unsuccessful chemical treatment, biological control was widely attended. Consequently, bacteriophage has been used as one candidate of the potential alternative process instead of antibiotics.

Bacteriophage or phage is the virus that highly specific and has an ability to lyse target bacteria after infected. Because of this, Bacteriophage therapy will not cause any harmful effect on other bacteria or microflora (Alagappan et al., 2010) and remain in low number due to self-restriction after lysing target bacteria (Ackermann, 2003). Furthermore, the success of using bacteriophage therapy on various animals were previously reported (Smith and Huggins, 1982; Smith and Huggins, 1983; Nakai and Park, 2002).Concurrently, diversity of bacteria has been occurred. Determination of new bacteriophage is the option to solve this problem. In addition, *V. cholerae* is a human pathogen that often found in shrimp pond and can effect on consumers' health (Gopal et al., 2005). In this study, isolation of bacteriophages specific to *V. cholerae* from different stages of shrimp larvae and cultured water from hatchery and ponds is one of our objectives. Their properties and structure of several isolates phages have been characterized and reported in this study.

Methodology

Sample preparation
Cultured water and shrimp samples including each stage of shrimp (nauplius, zoea, mysis and post-larvae) were collected from shrimp hatchery and shrimp pond of several places including Bangkok, Chachoengsao and Ratchaburi, Thailand. Shrimp larvae were collected by filtering using sterile Whatman No.4 filter paper.

Vibrio spp. isolation and identification
Vibrio spp. isolation from water sample: water sample was prepared by 10-fold dilution in sterile phosphate buffer saline (PBS) pH 7.4. All of the diluted samples were spread on thiosulfate citrate bile salt sucrose agar (TCBS) (Becton, Dickinson and Company, USA). After incubation at 37˚C for 24 hour, a total of yellow and green colonies were picked and restreaked on tryptic soy agar (TSA) or 2% NaCl tryptic soy agar (2% TSA) from Difco (Becton, Dickinson and Company, USA) and incubated at 37˚C for 24 h. The pure isolates were stored in TSB supplemented with 20% glycerol at -20˚C freezer for further use. These *Vibrio* spp. were used as bacteriophage hosts.

Vibrio spp. isolation from shrimp sample: shrimp larvae of nauplius, zoea and mysis each stage was rinsed twice with sterile normal saline solution (NSS) and centrifuged at 9,829 g for 15 min. One gram of sediment was homogenized with sterile glass homogenizer. For post-larvae samples, only intestine was rinsed thoroughly and cut into small pieces. Other processes are as same as water sample.

Vibrio spp. identification: pure isolates from both water and shrimp samples were primarily examined for Gram staining and oxidase test. Only Gram negative bacteria and gave positive on oxidase test were picked and identified later with biochemical test. Eight different medium including 1% peptone, MR-VP, nutrient broth (NB) supplemented with 0-10% NaCl, phenol red broth (PR) supplemented with 1% glucose, 1% lactose and 1% mannitol, lysine iron agar (LIA), motility indole ornithine (MIO), arginine glucose slant (AGS) and triple sugar iron (TSI) to distinguish tested bacteria. All chemicals are reagent grade purchased from Difco (Becton, Dickinson and Company, USA). All isolates were preliminarily characterized

circumspectly using biochemical test. To confirm *Vibrio* spp. identification all isolates were retested by using api® 20E test kit (bioMérieux, France) and antiserum agglutination by test kit (S&A Reagents lab, Thailand).

Isolation of bacteriophage

Bacteriophage isolation was performed by following the procedures with some modification as mentioned previously (Pringsulaka, 2004). Bacteriophages were isolated from water sample obtained from shrimp pond. All isolated *Vibrio* spp. were used as a host for bacteriophage isolation. Five milliliter from each host suspension (approximately 10^6 cfu/ml) was mixed with 50 ml TSB or 2% TSB depended on its suitable growth condition in any medium. After shaking at 200 rpm for 6 h, 50 ml of water sample was suddenly poured into bacterium-mixed media, this culture was shaken at 200 rpm and incubated at ambient temperature overnight. To screen for bacteriophage, this mixing suspension was centrifuged at 9,820 g for 15 min and the supernatant which was assumed as a phage-containing supernatant was filtered through 0.45 µm membrane pore size filter. To ensure this assumption, double layer method agar was used for inspection of plaque formation.

Multiplication of bacteriophage and examination of bacteriophage titer

Bacteriophage-mixed supernatant was collected and diluted for screening of plaque formation by using double layer agar method. To harvest bacteriophage, 5 ml fresh TSB was poured on the media which has proper phage titer. After 2 h of incubation, this TSB medium was collected and centrifuged at 9,820 g for 15 min. Then, this supernatant was examined its titer and calculated for plaque forming unit per milliliter (pfu/ml) by using double layer agar method and plaques detected were counted, respectively.

Physiology of bacteriophage

pH sensitivity: two hundred microliter bacteriophage suspension was inoculated in pH-adjusted TSB ranging from 6-10. After incubation at room temp for 24 h, 0.2 ml from each pH-adjusted TSB was mixed with 0.2 ml of its specific host at the concentration approximately 10^6 cfu/ml in TSA soft agar (0.5% agar of TSA). Double layer agar method was used to determine for phage titer.

Temperature sensitivity: preheated TSB until the temperature reached 30, 40, 50, 60, 70, 80 and 90 ˚C by using heat-controlling waterbath. Accurate temperatures were monitored by thermometer hooked inside the waterbath as well. Then, 0.2 ml bacteriophage suspension was mixed altogether with preheated TSB for 1 h. The culture was then examined for phage titer by double layer agar method.

Bacteriophage host range: All of the bacteria listed in Table 1 were used as a host against bacteriophages in this experiment. Two hundred microliter fresh bacterial suspension (a final concentration 10^6 CFU/ml) was mixed with TSA soft agar as a top agar and bacteriophage suspension were spotted on this top agar in order to search for plaque formation.

Bacteriophage examination under Electron microscope

Carbon-coated grid was pasted on bacteriophage–containing clear zone of double layer agar plate which has been already prepared in 24 h. After that, this grid was stained by 1% uranyl acetate and inspected for phage morphology by TEM-1010 transmission electron microscope operated at 120,000 V. This examination was performed at the Center of Nanoimaging, Mahidol University.

Results

Isolation and identification of *Vibrio* spp.

From 75 of water and shrimp samples collected from shrimp hatchery and shrimp pond, 101 bacterial isolates were selected. These isolates were distinguished based on biochemical test and confirmed by api® 20E test kit including antiserum agglutination. All bacteria isolates consist of 32 non O1/O139 *V. cholerae*, 15 *V. parahaemolyticus*, 12 *V. alginolyticus*, 19 *V. fluvialis*, 4 *V. vulnificus*, 18 *Aeromonas hydrophilla* and 1 *Bulkholderia gladioli*.

Isolation and characterization of bacteriophages

Five bacteriophages (coded as VC1, VC2, VC3, VC4 and VC5) specific to *V. cholerae* CWWREP5-3 and *V. cholerae* CWWREP5-4, were isolated from 3 of 75 samples. After propagation, these bacteriophages were stored at 4˚C in refrigerator. Five bacteriophages have the same morphology which is icosahedral head with non-contractile tail in the range 55-60 nm and 92-102 nm, respectively (Fig.1). According to Ackermann's taxonomy (Ackermann, 1984), all bacteriophages are Siphovirus and classified in the *family Siphoviridae*.

Physiology of bacteriophage
pH sensitivity: determination and analysis of plaque forming unit (pfu) and phage titer, all bacteriophages are stable from pH 6-10 with non-significant difference of log pfu/ml from triplicate trials ($p<0.05$) (Fig. 2).

Temperature sensitivity: after treating bacteriophages with various temperature, bacteriophage titer were shown non-significantly difference at 30-60˚C. Reduction of bacteriophage amount started from 70 to 80˚C and completely destroyed at 90˚C (Fig. 3).

Bacteriophage host range examination

Bacteriophages (VC1, VC2, VC3, VC 4 and VC5) were examined for their bacterial host specificity as indicated in Table 1. The results showed that our five bacteriophages possess infection only on their specific hosts *V. cholerae* CWWREP5-3 and *V. cholerae* CWWREP5-4 isolated from settle pond water sample.

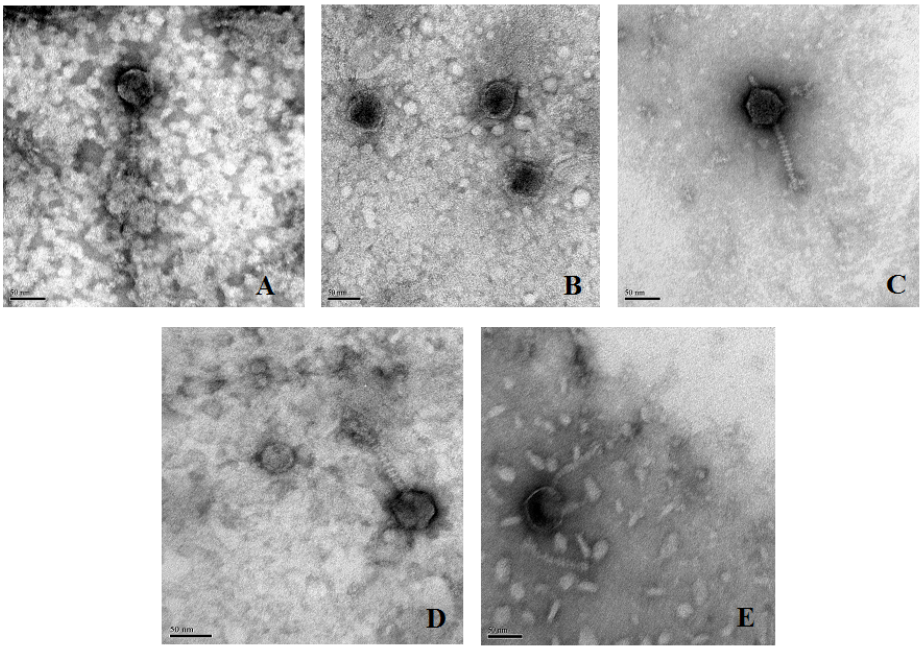

Figure 1 The morphology of Bacteriophages (A) VC1, (B) VC2, (C) VC3, (D) VC4 and (E) VC5 examined under TEM-1010 transmission electron microscope with the magnification of X 50,000. Bar represents 50 nm.

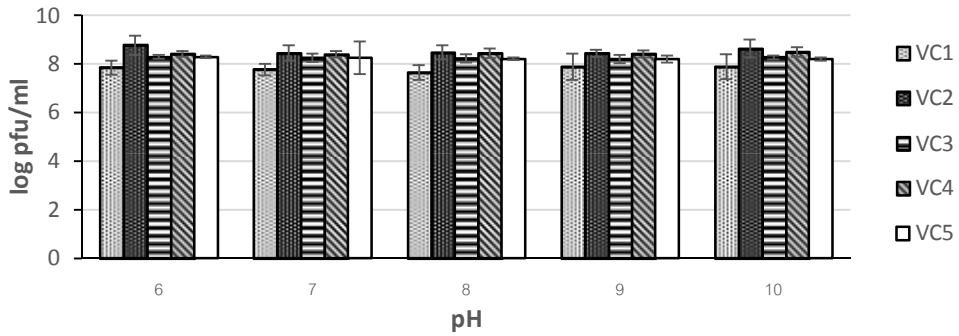

Figure 2 The stability of bacteriophages VC1, VC2, VC3, VC4 and VC5 at various pH.

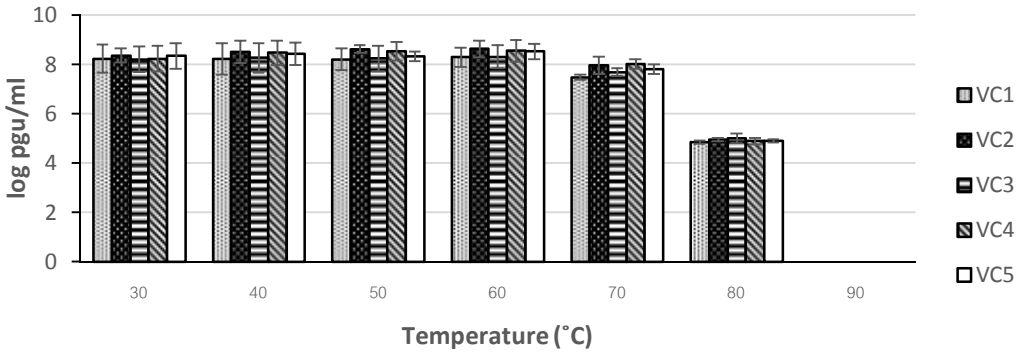

Figure 3 The stability of bacteriophages VC1, VC2, VC3, VC4 and VC5 at various temperatures.

Table 1 Bacteriophage host range

host	Bacteriophages				
	VC1	VC2	VC3	VC4	VC5
V. cholerae CWWREP5-3	+	+	+	+	+
V. cholerae CWWREP5-4	+	+	+	+	+
V. cholerae CWEPC	-	-	-	-	-
V. cholerae CWREP	-	-	-	-	-
V. cholerae CSEP	-	-	-	-	-
V. cholerae CWZ	-	-	-	-	-
V. cholerae CWTH	-	-	-	-	-
V. cholerae CWEPB	-	-	-	-	-
V. cholerae CWTPL	-	-	-	-	-
V. cholerae CWHP	-	-	-	-	-
V.parahaemolyticus CWDM	-	-	-	-	-
V.parahaemolyticus CSBF	-	-	-	-	-
V.parahaemolyticus CWEPC	-	-	-	-	-
V.parahaemolyticus CWZ	-	-	-	-	-
V.parahaemolyticus CSO	-	-	-	-	-
V.parahaemolyticus CWN	-	-	-	-	-
V.parahaemolyticus CWPM	-	-	-	-	-
V.parahaemolyticus DMST 5665	-	-	-	-	-
V. alginolyticus CSBF	-	-	-	-	-
V. alginolyticus CSEP	-	-	-	-	-
V. alginolyticus CSP	-	-	-	-	-
V. alginolyticus CSPL	-	-	-	-	-
V. alginolyticus CWZ	-	-	-	-	-
V. fluvialis CWEPB	-	-	-	-	-
V. fluvialis CWHPB	-	-	-	-	-
V. fluvialis CWREP	-	-	-	-	-
V. fluvialis CSPL	-	-	-	-	-
V. fluvialis CWTH	-	-	-	-	-
V. fluvialis CWPM	-	-	-	-	-
V. vulnificus CSBF	-	-	-	-	-
V. vulnificus CWDM	-	-	-	-	-
Aeromonas hydrophila	-	-	-	-	-
Bulkholderia gladioli	-	-	-	-	-
E. coli	-	-	-	-	-
Klebsiella sp.	-	-	-	-	-
Plesiomonas sp.	-	-	-	-	-

Discussion

Vibrio cholerae has been informed as one kind of bacteria contaminants in shrimp and associated with human disease. Since antibiotic-resistant bacteria and contaminated antibiotics in natural and shrimp residue occurred from excessively antibiotics used, bacteriophage have been widely attended as an alternative solution. Bacteriophage has plentiful amount and been found in every environment (Jończyk et al., 2011). Due to its specificity, using bacteriophages make more efficient to decrease *Vibrio* spp. in shrimp industry which is known as biocontrol. Five bacteriophages that only specific to non O1/O139 *Vibrio cholerae* CWWREP5-3 and non O1/O139 *Vibrio cholerae* CWWREP5-4 were isolated from water sample from shrimp pond. Although broad host specificity is more required, but highly specificity of bacteriophage has its advantage by not influence any impact to other bacteria including microflora. Five bacteriophages were classified based on their icosahedral head, non-enveloped and non-contractile tail of which are unique characteristic member of *Siphoviridae*. From 13 bacteriophage families, this family is the

most commonly found in environment about 61% (Ackermann, 2003). In addition, most bacteriophages specific to *Vibrio* spp. (vibriophage) classified in *Siphoviridae* were previously reported (Crothers-Stomps et al., 2009: Phumkhachorn and Rattabachaikunsopon, 2010: Yu et al., 2013). Concurrently, bacteriophage could be named by specifying its host though they belong to different family such as *Vibrio* phage (*Siphoviridae*) (Alagappan, 2010), *Staphylococcus* phage (*Myoviridae*) (O'Flaherty et al., 2004) and *Weissella* phage (*Podoviridae*) (Pringsulaka et al., 2011). All bacteriophages from this study are able to maintain their activity at the pH 6-10 and the temperature of 30-60˚C. This research confirms the results of many previous reports (Phumkhachorn and Rattabachaikunsopon, 2010: Yu et al., 2013). But they were completely loss at 90 ˚C which indicated that these phages could be easily eliminated by heat. Moreover, bacteriophages belong to *Siphoviridae* have been found to be used as a biocontrol of *Vibrio harveyi* successfully in black tiger shrimp (*Peneaus monodon*) hatcheries (Karunasagar et al., 2007). Therefore this may lead to the possibility of using bacteriophage in controlling *Vibrio* spp. in shrimp-cultured water. A model of using our bacteriophages for reducing of *Vibrio cholerae* during Pacific white shrimp (*Litopaeneus vannamei*) larvae culture will be conducted and reported in the future.

Conclusion

Five bacteriophages including VC1, VC2, VC3, VC4 and VC5 belong to *Siphoviridae* were isolated from water samples of shrimp (*Litopaeneus vannamei*) hatcheries of Chacheongsao province, Thailand. Our bacteriophages can tolerate in tryptic soy broth at the range of pH 6-10 and temperature 30-80˚C, with host specificity only on non O1/O139 *V. cholerae* CWWREP5-3 and non O1/O139 *V. cholerae* CWWREP5-4. These bacteriophages could be destroyed by heat over 90 ˚C.

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