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Infectious characteristics of some *Vibrio* spp. phages isolated in shrimp farming of the Mekong delta

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ABSTRACT

Vibrio spp. Were a cause of serious disease in aquaculture. This study aimed to develop a *Vibrio* spp. inhibition method that is safe and friendly for the environment, namely bacteriophage (phage). *Vibrio* spp. and their phages were isolated from shrimp, water, and mud from farms in Mekong Delta provinces (Kien Giang, Soc Trang, and Bac Lieu provinces). Then, the phage influence on the bacterial concentration and the *toxR* gene was investigated by comparing the gene sequences pre- and post-infection. The polymerase chain reaction identified 31 strains of *Vibrio* spp., including 9 of *Vibrio parahaemolyticus*. Then, using several isolated *Vibrio* spp. as hosts, 32 phage strains were isolated. Results found that each bacteriophage affected the *Vibrio toxR* gene in specific ways. Phage ST9 and phage ST10 did not change bacterial concentration, but they could change nucleotides at 4 positions compared to the *V. parahaemolyticus* sequence. Nevertheless, phage KG6 could reduce bacterial concentration but did not affect gene sequences. The bacteriophages can affect bacteria through several mechanisms, including reducing the bacterial concentration via the lytic process or affecting genes encoding bacterial virulence. These findings open a new direction in phage therapy research if such an effect can reduce or neutralize bacterial virulence.

1. INTRODUCTION

In shrimp, fish, and crustacean farming, several factors influence the disease's occurrence, including aquatic breed sources, environmental factors (water quality and agricultural management), and bacterial quantity or virulence. In the diseases caused by bacterial virulence, *Vibrio* spp., which belongs to the Gram-negative marine bacteria family, is one of the most serious [1]. Thus, in aquatic farming, bacteria, especially *Vibrio* spp., are strictly controlled by chemists, herbs, or biofactors.

ToxR is a *Vibrio* spp. virulence regulator gene, coding a protein in a group of transmembrane ones conserved throughout the bacteria. Several Vibrio spp. species use toxR to help them resist bile, and some strains choose toxR to cause infection [2] The protein helps bacteria survive and colonize by forming biofilms. However, in farm systems, the abuse of antibiotic usage in aquaculture leads to the emergence and increase of antibiotic-resistant bacteria [3]. Therefore, finding a biological therapy to replace antibiotics for bacterial disease control is urgently required.

One potential alternative therapy is using phages in farming, in which the bacterial resistance of phage expansion is nearly 10 times lower than that of antibiotics [4]. The phages still develop in harsh environments and constantly increase their concentration density until that of the infected host incredibly decreases [5]. Phages do not harm the environment's natural and beneficial microbial flora [6]. In aquaculture, Vibrio spp. can form biofilms, which decrease the effectiveness of antibiotics. Nevertheless, some studies have indicated that phages can resist bacterial biofilms [7,8].

Bacteriophages are viruses that parasitize bacteria and are categorized into two groups based on their life cycle: virulent phages that exhibit a lytic cycle and temperate ones that initiate either the lytic or lysogenic cycle [9]. During infection with bacterial cells, the reproduction of virulent phages always leads to host death, while in the lysogenic cycle of temperate phages, the main mechanism is the integration of phage nucleic acid into the host genome, which does not directly cause bacterial death [10]. Nevertheless, integration and excision from host chromosomes can also affect bacterial characteristics [9]. It has been widely observed that temperate phages increase bacterial virulence, the most important factor causing infection. However, there is also some research indicating that phages can reduce viral virulence [11].

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There have been many studies on isolating and applying lytic bacteriophages capable of inhibiting *Vibrio* spp. reported [12-14]. However, up to date, there are still very few studies on the effect of phage on the virulence genes of *Vibrio* spp. For the above reason, the study was conducted with the objective of isolating *Vibrio* spp. and their phages from shrimp and shrimp pond samples in some provinces of Viet Nam, the first step in determining the ability to infect bacteria by phages: directly reducing the number of bacteria or reducing the virulence of bacteria.

2. MATERIALS AND METHODS

2.1. Isolation and Identification of *Vibrio* spp.

Vibrio spp. Were isolated from 35 samples of pond water, button mud, and cultured shrimp from Kien Giang, Soc Trang, and Bac Lieu provinces. Ten cultured shrimp samples were collected for every five samples in Soc Trang and Kien Giang. Similarly, 10 samples of pond button mud were collected from Kien Giang and Bac Lieu, and 10 sick shrimp samples were collected from Bac Lieu and Soc Trang. The last five samples of pond siphon water were collected from Bac Lieu. Samples were diluted and inoculated on a thiosulfate-citrate-bile-salt-sucrose (TCBS) medium. The plates were incubated at room temperature for 24 h. Colonies specific to *Vibrio* spp. (large, blue, and yellow) were selected for domestication using the streak-plate culture method.

Bacterial cells were extracted through centrifugation at 12,000 rpm for 10 min at room temperature. Then, 800 µL of lysis buffer was added to 800 µL of chloroform: isoamyl (24:1 v/v), mixed well, and incubated at 28°C for 10 min. Then, the solution was centrifuged as described above to collect the supernatant. The mixture containing 700 µL of acquired and purified bacterial DNA supernatant and 700 µL of a 95% ethanol solution was mixed well and centrifuged at 12,000 rpm for 20 min to collect the precipitate. The precipitate was then washed with 500 µL of a 70% ethanol solution and centrifuged at 12,000 rpm for 10 min. Subsequently, the samples were vacuum dried at 45°C for 10 min. Afterward, DNA was dissolved in 100 µL of 0.1× TE solution and stored at 4°C. DNA quality was checked by 1% agarose gel electrophoresis.

Vibrio spp. and *Vibrio parahaemolyticus* were identified using polymerase chain reaction (PCR). The ToxR PCR contained 14 µL H_2O, 2 µL DNA, 8 µL My TaqMix 2×, 0.5 µL F-primer: GTCTTCTGACGCAATCGTTG, and 0.5 µL R-primer: ATACGAGTGGTTGCTGTCATG [15]. The amplifier was carried out at 94°C for 10 min, 20 cycles: –94°C (60 s), 63°C (90 s), 72°C (90 s), and 72°C (10 min) [16]. Then, products were examined by 2% agarose gel electrophoresis with 1× TBE (10 mM Tris; 5 mM borate; 0.1 mM ethylenediaminetetraacetic acid) to find the bank at the position of 368 bp. The 16S rRNA PCR was carried out similarly to those of ToxR, except for the F-primer: CAGGCCTAACACATGCAAGTC, R-primer: GCATCTGAGTGTCAGTATCTGTCC [17], carried out at 95°C for 10 min, 30 cycles –95°C (60 s), 55°C (60 s), 72°C (90 s), and 72°C (5 min) [16] and find the bank at the position of 700 bp. Then, the nucleotides were sequenced and compared to the Genbank using the basic local alignment search tool.

2.2. Isolation of *Vibrio* spp. Phage

Vibrio spp. Phage were isolated from 144 samples of pond water, healthy shrimp, and diseased shrimp from Kien Giang, Soc Trang, and Bac Lieu provinces. *Vibrio* spp. phages were isolated using the double-agar plaque assay [18]. Ten microlitters of the crude phage mixture and 100 µL of the isolated *V. parahaemolyticus* (isolated in the experiment) culture were aliquoted into 5 mL of a sterile soft nutrient medium (0.4% agar TSA), kept warm at 55°C, mixed, and spread on a hard nutrient medium (1.7% agar TSA). Plates were incubated at 35°C for 24 h to monitor plaque formation. Lastly, individual plaques were collected with sterile tips, added to 1 mL of a sterile SM buffer, and stored at 4°C.

2.3. Determination of the Host Range of Isolated Phages

The host range of the *Vibrio* spp. phages was determined by the double agar drop method [18]. Ten µL of each phage were dropped on a soft nutrient medium (TSA 0.4% agar) with *Vibrio* spp. Next, the plaques were classified into 3 categories of clarity: clear, turbid, and no reaction [19].

2.4. Comparison of the *toxR* gene of pre- and post-phage-infecting

A mixture of phages and bacteria, with an MOI of 100, was incubated for 24 h. The bacterial samples were then subjected to DNA extraction through PCR with a *toxR* primer. Next, the PCR products were electrophoresed using the Bio-Rad electrophoresis kit with PC and a 2% agarose gel × 1× TBE buffer. The product was then stained with SafeView, and the gel was photographed under UV light using a Bio-Rad Gel Doc system. Subsequently, the tape images were analyzed using Quantity One software. Following this, the GeneRuler™ 100-bp DNA ladder was used with a standard scale to estimate the size of the DNA fragment. The electrophoretic

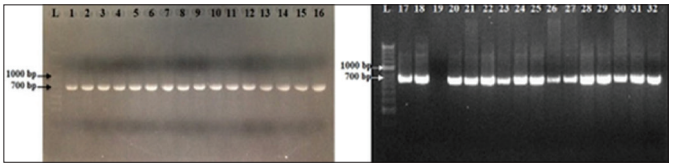

Figure 1: Polymerase chain reaction amplification of 16S rRNA gene region of 32 isolated strains.

Table 1: *Vibrio* spp. isolation from shrimp culture in areas.

Sampling location	Sample types	Samples	*Vibrio* spp.	*Vibrio parahaemolyticus*
Kien giang	Bottom mud	5	4	3
	Pond water	5	2	0
Bac lieu	Bottom mud	5	7	0
	Diseased shrimp	5	1	1
Soc trang	Pond water	5	0	6
	Pond siphon water	5	2	0
	Diseased shrimp	5	5	0

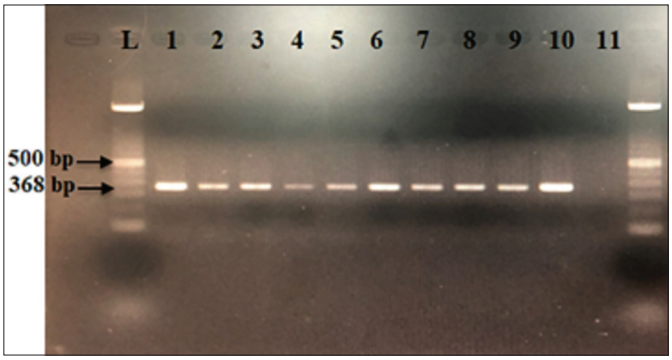

Figure 2: Polymerase chain reaction amplification of the *toxR* gene region of 9 isolated strains. L: Ladder 100 bp; 1: Positive control *Vibrio parahaemolyticus* ATCC 17802; 2: B32; 3: V4.1; 4: B42.2; 5: B42.3; 6: V4.2; 7: V4.3; 8: V4.6; 9: V4.7; 10: V4; 11: *Vibrio* spp. Computed tomography (negative control).

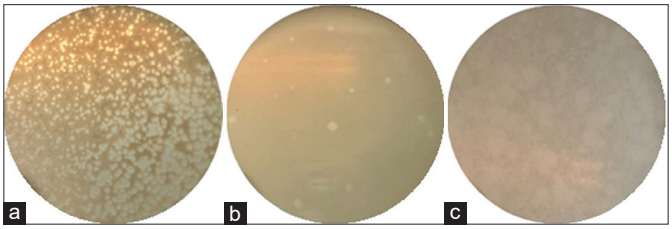

Figure 3: Plaque morphology of isolated phages on the double-layer agar plates. (a) phage ST2/*Vibrio* spp. B2.2; (b) phage ST8/*Vibrio* spp. VP5.1; (c) phage BL4/*Vibrio parahaemolyticus* B42.3.

PCR product was then sent for Sanger sequencing. Afterward, the bacterial *toxR* gene sequences were compared by BioEdit 6.0 and Multalin software.

2.5. Determination of Pre- and Post-phage Infecting *Vibrio* spp. V Concentration

A mixture of phages and bacteria, with an MOI of 100, was incubated for 24 h. Concentration of *Vibrio* spp. V before and after phage infection was determined by the spreading plate method on TCBS [20].

2.6. Statistical Analysis

Experiments were conducted with triplet replicates, the SD value was calculated by Excel software (Microsoft Corp., Redmond, WA, US), and statistics were analyzed by Statgraphics Centurion XV software (Statgraphics Technologies Inc., The Plains, Virginia, US).

3. RESULTS

3.1. Isolated *Vibrio* spp.

Thirty-two *Vibrio* spp. were isolated on a TCBS medium from the 35 collected samples with typical characteristics of *Vibrio* spp. colonies after 24 h of incubation as round, regular, blue-yellow colonies and 2 mm or more in diameter. To determine which strains belonged to *Vibrio*, a PCR technique targeting *Vibrio* spp. was used.

Through the use of a PCR with specific primers for *Vibrio* spp. [Figure 1], 31 bacterial strains all gave positive results. The strain at the 700 bp position shows that these bacterial strains were identified as *Vibrio* spp.

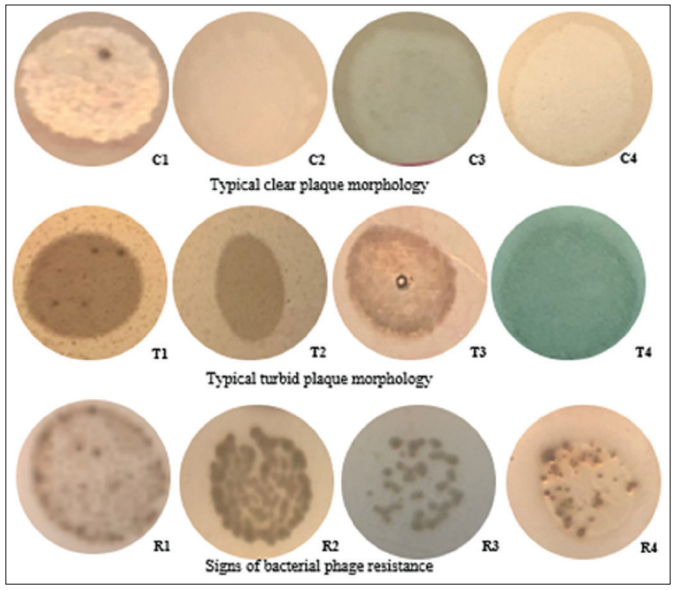

Figure 4: Diversity of phage plaque morphology. The results were accessed by double agar drop method.

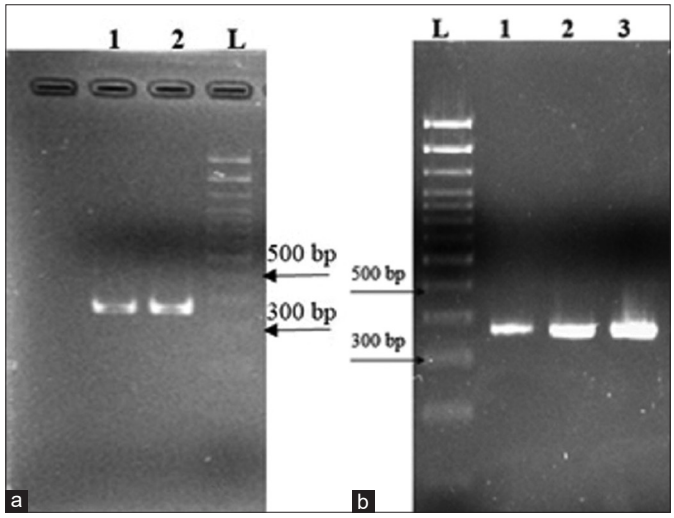

Figure 5: *ToxR* primer pairs for *Vibrio* spp. V in polymerase chain reaction electrophoresis. (a) (1) *Vibrio* spp. V (no phage infection), (2) *Vibrio* spp. V infected by KG6 phage; (b) (1) *Vibrio* spp. V (no phage infection), (2) *Vibrio* spp. V infected by ST9 phage, (3) *Vibrio* spp. V infected by ST10 phage.

The PCR results with primer pairs specific for the *V. parahaemolyticus* strain [Figure 2] showed that 10 samples were positive. The strains appeared at a position of 368 bp, in which Well 1 was a positive control of *V. parahaemolyticus* ATCC 17802. It means that nine suspected bacterial strains were identified as *V. parahaemolyticus*.

Thus, this result indicated that 31 strains of *Vibrio* spp. were isolated, including 9 strains of *V. parahaemolyticus*. The prevalence of *Vibrio* spp. and *V. parahaemolyticus* in shrimp cultures was very high. The prevalence of *Vibrio* spp. and *V. parahaemolyticus* in samples collected from shrimp culture in the 3 provinces, Bac Lieu, Soc Trang, and Kien Giang, is presented in Table 1.

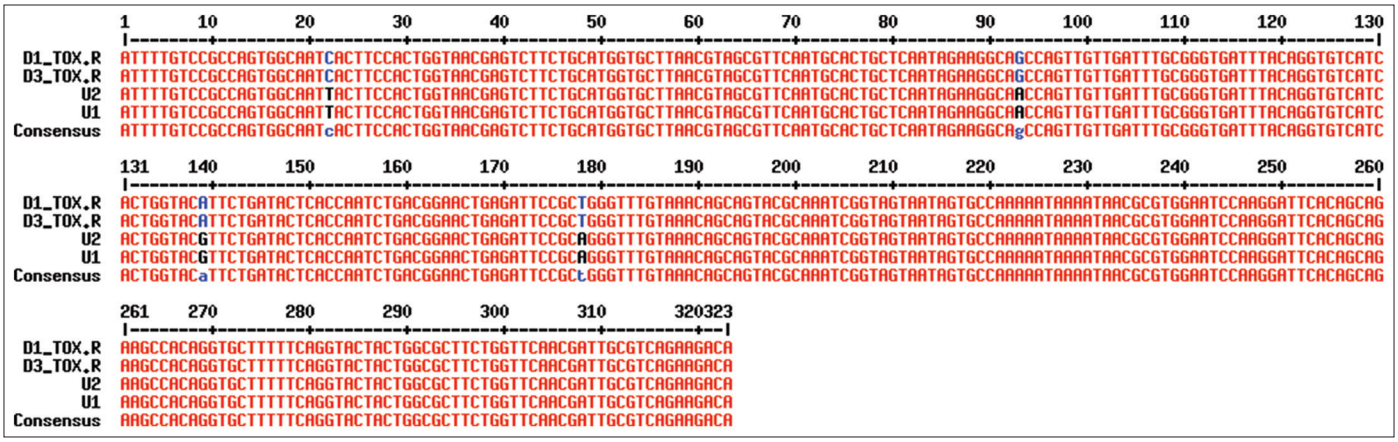

Figure 6: Results of alignment of the *Vibrio* spp. V *toxR* gene sequence with and without phage infection (D1_ *toxR*: *toxR* sequence of *Vibrio* spp. V (control); D3_ *toxR*: sequence of *Vibrio* spp. V infected by phage KG6, U2: *Vibrio* spp. V infected by phage ST10, and U1: *Vibrio* spp. V infected by phage ST9.

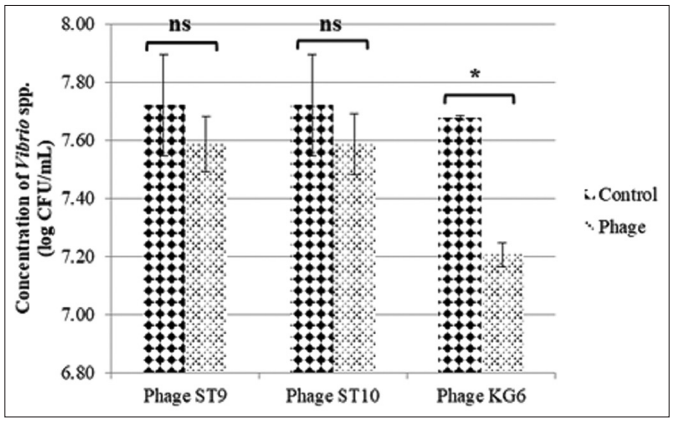

Figure 7: Concentration of *Vibrio* spp. V with (Phage) and without (Control) phage infection.

3.2. Isolated *Vibrio* spp. Bacteriophage

Bacteriophages were found in environments inhabited by bacterial hosts [Figure 3]. The abundance of phage populations in aquatic systems was estimated to be 10^4–10^8 virions/1 mL [21]. Indeed, 32 phages capable of infecting *Vibrio* spp. were isolated from 144 shrimp and shrimp pond samples in Soc Trang, Bac Lieu, and Kien Giang provinces. The list and origins of the phages are detailed in Table 2.

When conducting a host range survey of 32 isolated phages on 6 strains of *Vibrio* spp. [Table 3], there were many characteristic morphological patterns of the plaque, as shown in Figure 4. These morphologies were divided into the four groups below: Clear plaques representing toxic or lytic phages, turbid plaques, plaques resistant to host bacteria, and no plaque (the phage was not able to infect the bacteria selected as the host range) [22].

3.3. Bacterial *toxR* gene Sequence with and Without Phage Infection

The results indicated that the *tox*R nucleotide sequence of the bacterium infected by the phage KG6 remained unchanged as compared to that of the control, which was not infected by the bacteriophage [Figure 5]. However, in the case of infection by phages ST9 and ST10, the gene nucleotide sequence differed by 4 positions from that of the original bacterium. Phage ST9 caused the replacement of C with T, G with A, A with G, and T with A at 22, 93, 139, and 179 positions in the DNA sequence, respectively. Similarly, the replacement of C with T, G with A, A with G, and T with A was caused by phage ST10 at the specific positions of 25, 93, 142, and 181, respectively [Figure 6]. The Q values ranged from 26 to 62.

Table 2: *Vibrio* spp. phage isolation from shrimp culture in areas.

Sampling location	Sample types	Samples	*Vibrio* spp. phages
Kien giang	Shrimp pond water	16	1
	Healthy shrimp	16	14
	Diseased shrimp	16	1
Bac lieu	Shrimp pond water	16	6
	Healthy shrimp	16	0
	Diseased shrimp	16	0
Soc trang	Shrimp pond water	16	2
	Healthy shrimp	16	8
	Diseased shrimp	16	0

Then, the influence of phages on the bacterial concentration was carried out. Figure 7 showed that the concentration of phage ST9 and phage ST10 infected *Vibrio* spp. V and that of the control was not a statistically significant difference ($P > 0.05$). However, after being infected by phage KG6, *Vibrio* spp. V concentration significantly decreased as compared to that of the initial bacterium (control) ($P < 0.05$).

4. DISCUSSION

The situation of antibiotic resistance of bacteria in the current period is becoming more common and dangerous [23]. Faced with this situation, bacteriophage therapy has been focused on again. In order to use bacteriophage in therapy, there are two priority groups: Virulent phages (strictly lytic phages) have only a lytic cycle, in which the bacteriophages use the host cell to create new particles and lyse the host cell to release new phages. The second group is temperate phages (capable of integration into the bacterial genome during the lysogenic cycle) and therefore plays a more important role in the evolution of bacteria and bacterial pathogenicity [9].

Previous studies have demonstrated temperate phages' ability to increase host bacterial virulence [9]. In addition, many studies have identified the ability to reduce the bacterial virulence of temperate

Table 3: Host range of bacteriophages isolated from *Vibrio* spp.

Vibrio spp./Phages	17802	VPV4.1	VPB42.2	VPB42.3	VP5.1	B2.2
BL1						
BL2						
BL3						
BL4						
BL5						
BL6						
BL7						
BL8						
BL9						
BL10						
BL11						
BL12						
BL13						
BL14						
BL15						
BL16						
KG1						
KG2						
KG3						
KG4						
KG5						
KG6						
ST1						
ST2		✡			✡	✡
ST3	✡	✡	✡		✡	✡
ST4						✡
ST5			✡		✡	
ST6			✡			
ST7			✡			
ST8			✡		✡	
ST9						
ST10						

Host range of 33 phage strains isolated on 7 strains of *Vibrio spp.* Plaque morphology was classified into 3 categories: Clear (orange with symbol), turbid (orange), bacterial phage resistance (grey), and no reaction (no color).

phages, specifically P2, which is a prophage that can attach to the plasmid of *Klebsiella pneumoniae*. When infecting mice with *K. pneumoniae*, the mutant strain without P2 caused a mortality rate of 100%, while the bacterial strain carrying P2 had a mortality rate of only 70%, suggesting that the presence of P2 reduces the virulence of bacteria [24]. Similarly, for temperate phage PHB09, when the prophage integrated into the pilin gene of *Bordetella bronchiseptica*, it significantly reduced the virulence of the strain Bb01 in mice, most likely due to disruption of pilin gene expression [25]. Furthermore, prophages can also change the gene sequence of bacteria through integration and excision. For instance, integration of prophage A118 leads to the inactivation of comK, an important gene of *Listeria monocytogenes* for the infection of animal cells, thereby inhibiting the infection process [26]. Temperate phages are abundant in nature; nearly half of sequenced bacteria are lysogen [27], so temperate phages are a potential group in phage therapy research, although the effect of prophages on host bacteria still has many issues that need to be studied.

The bacterial *toxR* gene was sequenced with and without phage infection, and phage inhibition was measured via bacterial concentration (The *toxR* gene was discovered as the gene regulating the cholera toxin operon and many other genes in *Vibrio cholerae* [28]. For *V. parahaemolyticus* and *Vibrio alginolyticus*, toxR is required in biofilm formation [29,30]). The results showed that the phage ST9 and phage ST10 phages did not directly reduce the bacterial concentration, but they could change the *toxR* DNA sequence and virulence regulator gene in *Vibrio* spp. Preliminary results show that when infecting bacteria, some phages could change the nucleotide sequence of the *toxR*, the essential *Vibrio* spp. gene that regulates toxins and affects biofilm formation. In contrast, phage KG6 could reduce the bacterial concentration but ultimately did not affect the *toxR* gene.

These results are consistent with previous bacteriophage findings that the virulent phages, phage KG6, inject their genome into the cell after being absorbed by the bacterial cell. Bacterial DNA is fragmented

when phage nucleic acid replication and phage proteins are synthesized in the cell. The progeny virions are assembled and released from the cells at the end of a life cycle, leading to host death. Phage ST9 and phage ST10, in the lysogenic cycle, phage do not kill the host cell, but the viral genome attaches to the host cell genome, coexists for many generations, and could affect the host bacterial genome sequence [10]. It means that the phages could affect bacteria in several different pathways, such as directly killing bacteria, leading to a concentration decrease, and indirectly affecting the host bacteria by attachment to the gene sequence.

5. CONCLUSION

The prevalence and antibiotic resistance of *Vibrio* spp. are increasing, leading to the urgency of finding an alternative antibiotic therapy. One of them is the bacteriophage application method. In this study, the diversity between the isolated phages in terms of plaque characteristics, gene sequence-affected abilities, and the concentrations of host bacteria was found. The phage KG6 could reduce bacterial concentration; the others, phage ST9 and phage ST10 strains, did not significantly reduce bacterial concentration. However, they can attack and change *Vibrio* bacteria's virulence regulation gene sequence, toxR. Study results show that the first step to define a new direction in bacteriophage research is to identify phage trains capable of affecting the coding sequences of the virulent genes of bacteria and the potential application of phages for several purposes in aquaculture, mainly controlling *Vibrio* spp.

6. ACKNOWLEDGMENT

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7. AUTHORS' CONTRIBUTION

All authors contributed to conceptualization and design, data collection or data analysis and interpretation; pertaining to writing or reviewing important intellectual content.

8. FUNDING

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9. CONFLICTS OF INTEREST

The authors declared no financial or other conflicts of interest in this study.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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