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Research Article

Isolation and Characterization of Bacteriophages Against *Aeromonas hydrophila* Bacteria Causing Hemorrhagic Septicemia from Striped Catfish (*Pangasianodon hypophthalmus*)

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Abstract

This study was conducted to isolate and characterize the bacteriophages capable of lysing *Aeromonas hydrophila* causing hemorrhagic septicemia in striped catfish (*Pangasianodon hypophthalmus*). The isolation and characterization were conducted in various samples of water, sludge, intestines and kidneys of animal. It was shown that, a total of 64 bacteriophages (58.2%) were isolated from 110 samples, with the highest value being from sludge samples (100%). When phages were tested in different pH levels (2-10), the above 64 phages were well adjusted to the pH 5-8 after 24 hours at 28°C, representing a rate of more than 60%. In addition, in one hour, 50% of the phages could survive at different temperatures (4°C, 20°C, 60°C, 80°C, 100°C, and 120°C) with no effects on phages observed at 4°C and 20°C. From the pH and temperature tests, six phages (B.N26, B.N16, B.R18, B.B8, B.R22, and B.N1) were selected to evaluate the lysis capacity against *A. hydrophila* in tap and river water for 21 days *in vitro.* The results revealed that the bacteriophages could survive better in river water than in tap water. All six phages could be utilized to investigate the effectiveness of treating *A. hydrophila*-caused hemorrhagic disease in catfish *in vivo*.

Keywords: *Aeromonas hydrophila*; Bacteriophage; Isolation; pH; Striped Catfish; Temperature

Introduction

Over the years, the striped catfish farming industry has grown significantly, becoming one of the main export aquaculture products of Vietnam. However, the increasing level of intensification to meet the rising market demand has led to frequent epidemics and serious consequences. Specifically, the hemorrhagic disease caused by *Aeromonas hydrophila* bacteria is one of the common diseases and causes significant damage to striped catfish farming in the Mekong Delta in Vietnam. In response to epidemics, antibiotic treatment has been a quick and effective method, but its improper and uncontrolled use increased drug resistance [1].

Many applications have been made to overcome these issues. Researchers have been looking for biological alternatives to antibiotics in disease treatment to prevent bacterial drug resistance and improve disease control. Among potential approaches, bacteriophage therapy is considered an effective alternative and provides a safe and sustainable method for farmers and consumers [2]. Previously, Jun., *et al.* [3] successfully isolated two bacteriophages that inhibited and treated diseases caused by *A. hydrophila* bacteria in fish. Easwaran., *et al.* [4] isolated a *Myoviridae* bacteriophage not only capable of lysing *A. hydrophila* causing disease in zebrafish but also having high stability when tested for temperature, pH, and ion resistance as well. Recently, Akmal., *et al.* [5] isolated and

characterized the Akh-2 bacteriophage, controlling Aeromonas in fish samples from Geoje Island in Korea. In Vietnam, Le., *et al*. [6] isolated two bacteriophages, namely *A. hydrophila*-phage 2 and *A. hydrophila*-phage 5, from water samples from the Saigon River in Ho Chi Minh city. However, until now, research on the use of bacteriophages in aquaculture in Vietnam has been limited. Therefore, the current study was undertaken to isolate and characterize bacteriophages against *A. hydrophila* causing hemorrhagic septicemia in striped catfish, which could be used to replace antibiotics in treating this disease.

Materials and Methods

Sample collection

A total of 110 samples were collected, including water (30 samples) and sludge (11 samples) in the culturing ponds, as well as intestines (23 samples), livers (23 samples), and kidneys (23 samples) from fish in Chau Thanh district, Dong Thap province. The samples were packed and stored in an insulated box containing crushed ice at 4°C. The samples were transferred quickly within 24 h to the laboratory of the Department of Veterinary Medicine, College of Agriculture, Can Tho University for further processing.

Water samples: All bottles and jars of sampling were thoroughly cleaned before being coated at least three times with the water sampled. The sterile glass bottles held around 250-300 mL of water. After that, the containers were carefully sealed with lids and refrigerated before being transported to the laboratory for isolating [7].

Sludge samples: The bottom sludge sample was collected using polyvinyl chloride pipes that had been disinfected with 70% alcohol. Sludge samples were taken from three locations: the beginning of the pond, the middle of the pond, and the pond end along a diagonal. At each site, approximately 100 g of sludge was collected [8].

Fish tissues samples**:** samples from striped catfish with typical symptoms such as hemorrhagic spots in the fin area, enlarged head, and abdomen were collected. Five to six infected fish were collected from each pond and stored on ice before being transported to the laboratory for further handling. The fish body was disinfected with 70°C alcohol and then cut with sterilized scalpels and scissors. Extra pathological signs of fish were also recorded, and the organs were then collected and ground for phage isolation [9].

Bacteriophage isolation capable of lysing A. hydrophila (ATCC® 7966™)

The bacteriophages were isolated according to the method of Kropinski., *et al*. [10]. Each sample (1 g or 1 mL) was mixed with 10 mL of TSB (tryptone soy broth) and incubated at 28°C for 24 h. After 24 h, the growth solution was supplemented with chloroform followed by incubation for 2 hours and centrifugation at 6000 rpm at 4°C for 15 min. Bacteria *A. hydrophila* (ATCC® 7966™) was used as the host to determine the presence of bacteriophages. In addition, the presence of phage was determined when plaque appeared on the surface of TSB + 0.6% agar plates containing *A. hydrophila* bacteria. The isolated bacteriophages were stored in 50% glycerol at -80°C.

pH stability test

The viability of bacteriophages at different pH was determined based on the study Verma., *et al*. [11] and Xuan., *et al*. [12]. The pH of the TSB medium was adjusted using either 1M HCl or 1M NaOH to attain a solution with pH levels 2, 3, 4, 5, 6, 7, 8, 9, and 10. A total of 0.1 mL bacteriophage suspension at a concentration of 10^8 PFU/mL or 10^9 PFU/mL were mixed with 1 mL of TSB and incubated at 28°C for 24 h. After incubation, the growth solution was supplemented with chloroform (incubated for 2 h) and centrifuged at 6000 rpm at 4°C for 15 min. The viability of bacteriophages on the surface of TSB+0.6% agar plates containing *A. hydrophila* bacteria was tested. Bacteriophage suspension maintained at pH 7 was used as a control. Each pH test was conducted in triplicate.

Temperature stability test

Screening the viability of bacteriophages was carried out using the methods described by Easwaran., *et al*. [4] and Xuan., *et al*. [12] at different temperatures of 4°C, 20°C, 60°C, 80°C, 100°C, and 120°C. The phage suspensions (concentrations 10^8 PFU/mL or 10^9 PFU/mL) were incubated at the respective temperatures for 1 h. Later, the viability test of the bacteriophages on the surface of TSB+0.6% agar plates containing *A. hydrophila* bacteria was performed. Each temperature treatment was conducted in triplicate.

Evaluation of bacteriophages capable of lysing A. hydrophila in water

Six bacteriophages were selected to lyse *A. hydrophila* (ATCC® 7966™) in tap and river water samples. Two experiments were conducted, in which tap and river water was used in experiment

1 and experiment 2, respectively. There were three treatments in each experiment. They included treatment 1-only bacteria, treatment 2-only bacteriophages, and treatment 3-both bacteria and bacteriophages. The experiments were conducted in 21-day test with triplicate in aquariums without fish to observe the interaction of *A. hydrophila* (ATCC® 7966™) and the bacteriophage. All water samples were autoclaved at 121°C for 15 minutes.

For the first experiment, 1 L of tap water was added to each aquarium. In treatment 1, 0.7 mL of bacteriophage culture 24 h (10^8 CFU/mL) was added to each aquarium. In treatment 2, 0.7 mL of a bacteriophage (10^9-10^{10} PFU/mL) was added to each aquarium. For treatment 3, bacteria and bacteriophages were added as above to each aquarium. The bacteria and bacteriophage were detected daily by colony and plaque count method. For the second experiment, the river water experiment was conducted like the first experiment and maintained at room temperature [13]. Bacterial counts were measured per day at OD = 600 nm and bacteriophage density.

Phage density

The concentration of phages was diluted by adding 0.1 mL of each phage solution into sterile Eppendorf containing 0.9 mL of distilled water, then shaking to homogenize the mixture. This step was repeated until the phage concentration reached 10^{-5}. Then, 0.1 mL of each diluted phage was spread on each sterile Petri dish together with 0.1 ml of *A. hydrophila* host bacteria suspension (10^8 CFU/mL). About 10-12 mL TSB medium with 0.6% agar were added, melted, and let cool in waterbath at 50°C for at least 30 minutes. The dish was shaken to homogenize the mixture, then let still until the agar cooled down at 37°C for 24 hours. After 24 hours, plaques were observed and counted [14].

The phage density was calculated using the following formula [15]: PFU/ mL = N × 1/DF × 1/V, in which: N is the number of countable melt; DF is the dilution factor; V is the phage volume (mL).

Results

Isolation of bacteriophages

From a total of 110 samples, 64 bacteriophages were isolated (58.2%) with the potential of lysing *A. hydrophila* bacteria (Table 1). Among them, 17 bacteriophages (56.7%) were isolated from water, and 11 bacteriophages (100%) were isolated from sludge samples. For striped catfish visceral samples, the percentages of isolated bacteriophages ranged from 43.5% to 65.2%, with the highest being in the intestine.

Type of sample	No. of samples	No. of bacteriophages (+)	Percentage (%)
Water	30	17	56.7
Sludge	11	11	100
Intestine	23	15	65.2
Liver	23	11	47.8
Kidney	23	10	43.5
Total	110	64	58.2

Table 1: The result of bacteriophage isolation.

Effects of pH on bacteriophage stability

The changes in bacteriophage viability under the influence of different pH conditions are shown in table 2. It gradually increased from acidic to neutral conditions (pH 2 to pH 7) and slightly decreased from neutral to alkaline or strongly alkaline conditions (pH 7 to pH 10). In detail, 39/64 bacteriophages survived at pH 5; 50/64 bacteriophages at pH 6; 64/64 bacteriophages at pH 7 and 51/64 bacteriophages at pH 8, which accounted for 60.9%, 78.1%, 100% and 79.7%, respectively. At the lowest and highest pH values (pH 2 and pH 10), fewer percentages of bacteriophages could survive (18.8% and 10.9%, respectively).

Type of sample 2		pH								
		3	4	5	6	7	8	9	10	
Water (n = 17)	No. of phages (+)	6	6	7	12	15	17	15	9	6
	Percentage (%)	35.3	35.3	41.2	70.6	88.2	100	88.2	52.9	35.3
Sludge (n = 11)	No. of phages (+)	1	1	2	3	7	11	7	3	1
	Percentage (%)	9.09	9.09	18.2	27.3	63.6	100	63.6	27.3	9.09
Intestine (n = 15)	No. of phages (+)	3	4	6	10	11	15	12	5	0
	Percentage (%)	20	26.7	40	66.7	73.3	100	80	33.3	0

Liver (n = 11)	No. of phages (+)	1	1	3	8	9	11	9	2	0
	Percentage (%)	9.09	9.09	27.3	72.7	81.8	100	81.8	18.2	0
Kidney (n = 10)	No. of phages (+)	1	2	4	6	8	10	8	2	0
	Percentage (%)	10	20	40	60	80	100	80	20	0
Total (n = 64)	No. of phages (+)	12	14	22	39	50	64	51	21	7
	Percentage (%)	18.8	21.9	34.4	60.9	78.1	100	79.7	32.8	10.9

Table 2: Stability of bacteriophage incubated at various pHs.

Effects of temperature on bacteriophage stability

Table 3 shows that bacteriophages had a high ability to survive in a wide range of temperature conditions (from 4°C to 120°C) within one hour, with a more than 50% survival rate. No effects of temperature at 4°C and 20°C on the stability of bacteriophage were found. At temperatures of 60°C, 80°C, 100°C, and 120°C, the adaptation of bacteriophages gradually decreased with the corresponding rate of 89.1%, 71.9%, 57.8%, and 51.6%.

Type of sample 4		**Temperature (°C)**					
		20	**60**	**80**	**100**	**120**	
Water (n = 17)	No. of phages (+)	17	17	14	13	12	12
	Percentage (%)	100	100	82.4	76.5	70.6	70.6
Sludge (n = 11)	No. of phages (+)	11	11	10	9	7	7
	Percentage (%)	100	100	90.9	81.8	63.6	63.6
Intestine (n = 15)	No. of phages (+)	15	15	14	11	10	9
	Percentage (%)	100	100	93.3	73.3	66.7	60
Liver (n = 11)	No. of phages (+)	11	11	9	8	5	2
	Percentage (%)	100	100	81.8	72.7	45.5	18.2
Kidney (n = 10)	No. of phages (+)	10	10	10	5	3	3
	Percentage (%)	100	100	100	50	30	30
Total (n = 64)	No. of phages (+)	64	64	57	46	37	33
	Percentage (%)	100	100	89.1	71.9	57.8	51.6

Table 3: Stability of bacteriophage incubated at various temperatures.

Evaluation of bacteriophages capable of lysing A. hydrophila in water

Through the investigation of the survival in different pH (2-10) and temperature (4, 20, 60, 80, 100, 120°C) conditions of 64 bacteriophages, six bacteriophages were selected to test the survival ability in tap water and river water conditions for 21 days. These bacteriophages were B.N26, B.N16, B.R18, B.B8, B.R22, and B.N1.

Bacteria density over 21 days in tap and river water

In tap and river water without bacteriophages, the bacterial density of *A. hydrophia* bacteria increased or decreased over time, as shown in figure 1. The results show that the bacterial density in both tap water and river water was of an upward trend from the 1st to 5th day and gradually decreased to the 21st of the experiment (Figure 1). Specifically, on the first day, the bacterial density in the tap water was 6.74 $\log_{10}$ CFU/mL and river water 7.08 $\log_{10}$ CFU/mL. On the 5th day, the bacterial density in the tap water increased by 7.16 $\log_{10}$ CFU/mL, and the bacterial density in the river water was 7.58 $\log_{10}$ CFU/mL. However, on the following days, the bacterial density in tap and river water started to drop until the 21st day. Yet, the bacterial density in the tap water experienced a more considerable decline than in the river water. On the 21st day of the study, the bacterial density in the tap water and river water fell at the rate of 3.30 $\log_{10}$ CFU/mL and 5.10 $\log_{10}$ CFU/mL, respectively.

Figure 1: Bacteria density in tap water and river water over 21 days.

Determination of bacteriophage density over 21 days in tap water and river water

In the tap and river water with bacteriophages, the bacteriophage density was tended to decrease gradually over 21 days of the experiment (Figure 2). Specifically, bacteriophage density on 1st day of tap water was 10.8 $\log_{10}$ PFU/mL and that of river water 10.8 $\log_{10}$ PFU/mL. Next, both started to go down slightly from the 2nd to the 4th day, to hit 10.7 $\log_{10}$ PFU/mL for the bacteriophage density in river water and 9.84 $\log_{10}$ PFU/mL for the bacteriophage density in tap water. In the following days until the 21st, the bacteriophage density witnessed a plunge, registering 4.17 $\log_{10}$ PFU/mL for the former and 3.13 $\log_{10}$ PFU/mL for the latter.

Figure 2: Bacteriophage density in clean water and river water over 21 days.

Determination of bacteria and bacteriophage density over 21 days in tap and river water

The results of figure 3 show that the density of bacteriophages and bacteria in tap and river water tended to decrease over the 21 days. The bacteriophage density in river water and tap water tended to increase early and drop later. Specifically, the former rose from the 1st to 8th day (from 10.6 $\log_{10}$ PFU/mL to 13.8 $\log_{10}$ PFU/mL), and then it fell until the 21st day (down to 9.12 $\log_{10}$ PFU/mL). Similarly, the increase from the 1st to 7th day from 10.6 $\log_{10}$ PFU/mL to 10.7 $\log_{10}$ PFU/mL, after which it registered a drop until the 21st day (reduced to 5.13 $\log_{10}$ PFU/mL).

Concerning bacteria, the densities in tap water and river water were unstable early and decreased later. Notably, the bacterial density in the river water fluctuated from the 1st to the 5th day with an overall upward trend (from 6.61 $\log_{10}$ CFU/mL to 7.56 $\log_{10}$ CFU/mL). By the 21st day, it fell to 1.30 $\log_{10}$ CFU/mL. Likewise, the bacterial density in tap water from the 1st to 5th day was seen, yet generally, it rose from 6.21 $\log_{10}$ CFU/mL to 7.19 $\log_{10}$ CFU/mL. After that, the figure was reduced to 0.63 $\log_{10}$ CFU/mL on the 21st day.

Figure 3: Bacteria and bacteriophage density in ctap water and river water over 21 days.

The density of six bacteriophages over 21 days in tap water and river water

The density of six bacteriophages (B.N26, B.N16, B.R18, B.B8, B.R22, B.N1) and *A. hydrophila* (ATCC ® 7966 ™) in tap and river water are shown in figure 4 and figure 5. For the tap water environment (Figure 4), the density of both six bacteriophages and bacteria experienced a downward trend. Still, the density of six bacteriophages increased from the 1st to the 7th day before seeing a fall to the 21st day. The figure for bacteriophages rose from 10.5-10.8 to 12.5-12.9 $\log_{10}$ PFU/mL, after which it went down to 4.98-5.22 $\log_{10}$ PFU/mL while that of bacteria decreased from 6.00-6.51 to 0.60-0.66 $\log_{10}$ CFU/mL over 21 days.

(a)

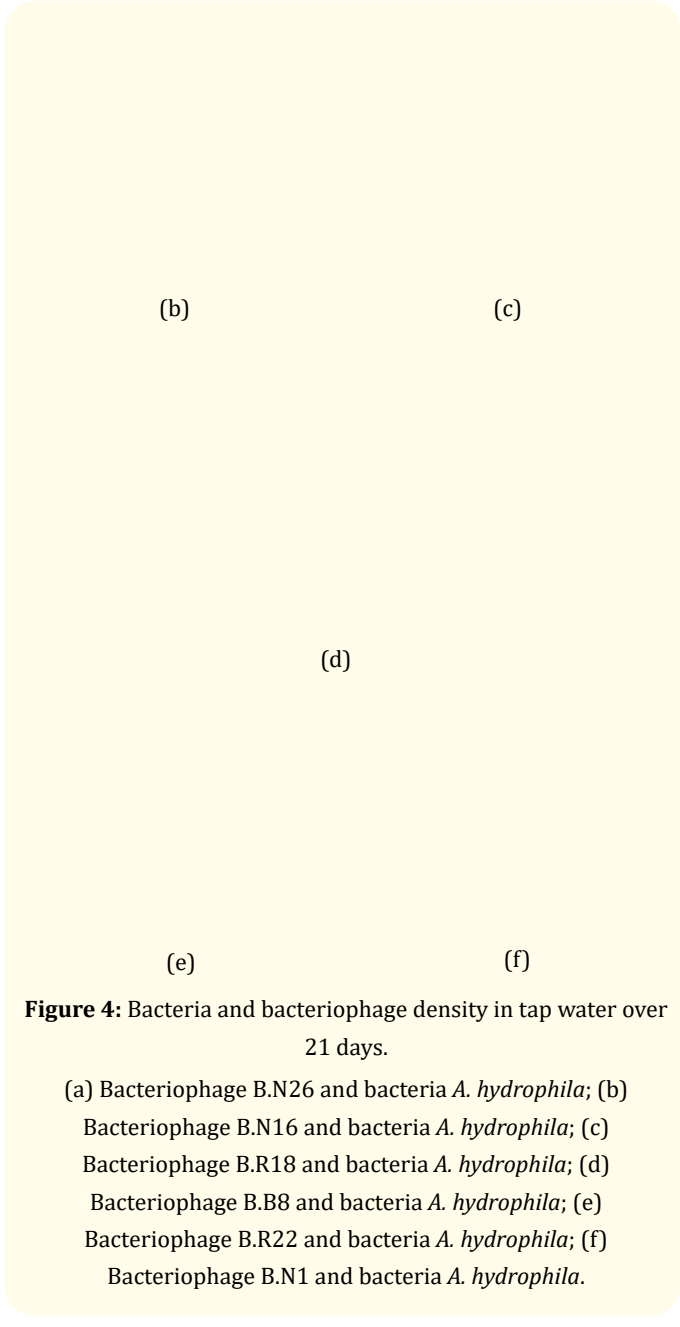
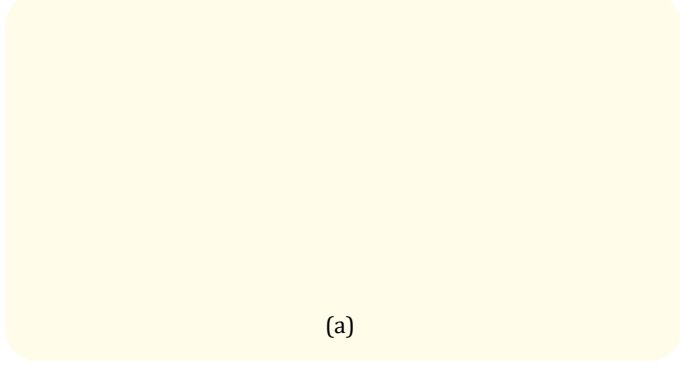


Figure 4: Bacteria and bacteriophage density in tap water over 21 days.

(a) Bacteriophage B.N26 and bacteria *A. hydrophila*; (b) Bacteriophage B.N16 and bacteria *A. hydrophila*; (c) Bacteriophage B.R18 and bacteria *A. hydrophila*; (d) Bacteriophage B.B8 and bacteria *A. hydrophila*; (e) Bacteriophage B.R22 and bacteria *A. hydrophila*; (f) Bacteriophage B.N1 and bacteria *A. hydrophila*.



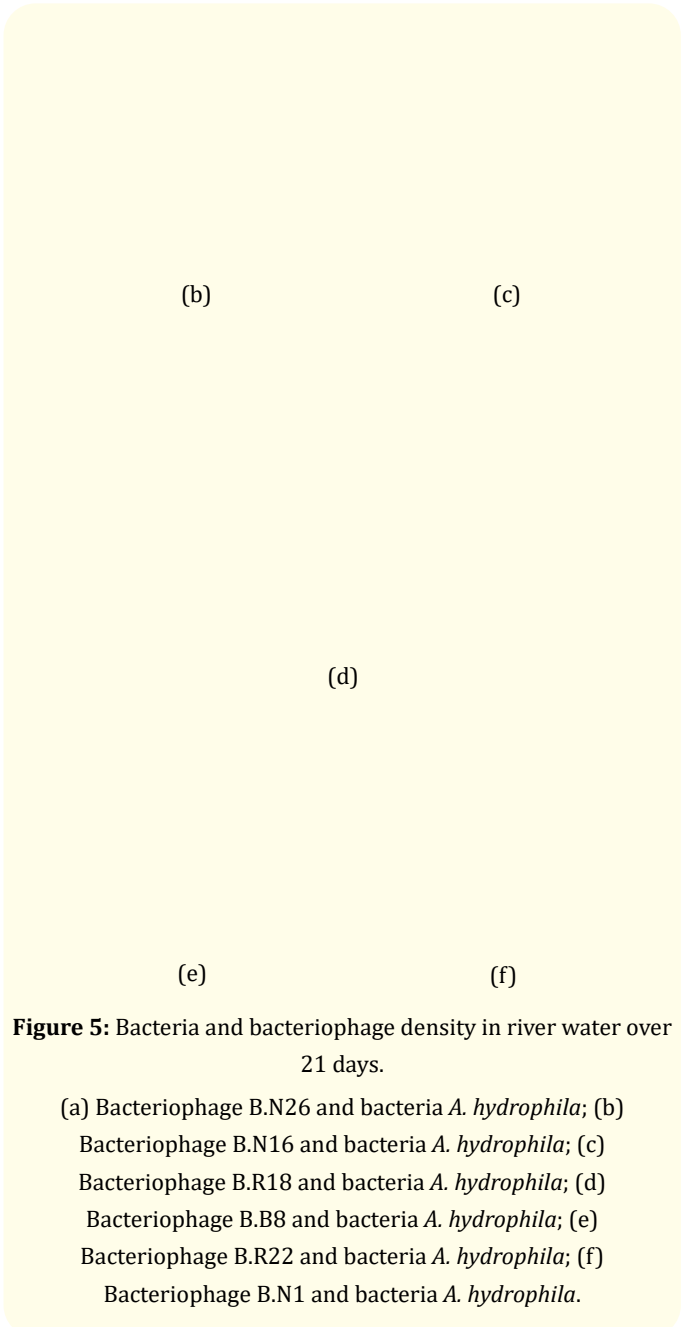


Figure 5: Bacteria and bacteriophage density in river water over 21 days.

(a) Bacteriophage B.N26 and bacteria *A. hydrophila*; (b) Bacteriophage B.N16 and bacteria *A. hydrophila*; (c) Bacteriophage B.R18 and bacteria *A. hydrophila*; (d) Bacteriophage B.B8 and bacteria *A. hydrophila*; (e) Bacteriophage B.R22 and bacteria *A. hydrophila*; (f) Bacteriophage B.N1 and bacteria *A. hydrophila*.

For the river water environment, it is shown in figure 5 that there was a drop in the density of both six bacteriophages and bacteria. However, the density of six bacteriophages increased from the 1st to the 7th day. It then decreased to the 21st while the bacterial density used by bacteriophages B.N26, B.N16, B.R22, and B.N1 rose slightly in the first seven days and then dropped until the end. By contrast, the bacterial density lysed by B.R18 and B.B8 decreased over 21 days. Specifically, from day 1 to 7, the density of six bacteriophages grew from 10.4-10.8 to 13.0-14.1 $\log_{10}$ PFU/mL and then

fell to 9.01-9.27 $\log_{10}$ PFU/mL). The bacterial density lysed by bacteriophages B.N26, B.N16, B.R22, and B.N1 increased slightly from the 1st to the 7th day (6.46-6.71 to 6.62-6.76 $\log_{10}$ CFU/mL) and then decreased to the 21st day (to 1.22-1.36 $\log_{10}$ CFU/mL). The bacterial density lysed by B.R18 and B.B8 decreased (from 6.56-6.77 $\log_{10}$ CFU/mL to 1.20-1.39 $\log_{10}$ CFU/mL).

Discussion

Isolation of bacteriophages

The results of the current work showed that 64 bacteriophages were isolated from water, sludge, and internal organs of a striped catfish farm. This indicated the existence of bacteriophages, as indicated by many authors. According to Jończyk., *et al*. [16], bacteriophages are detected in ground and surface water, soil, food (e.g., sauerkraut, wine), sewage, and sludge. Particularly, in the environment where bacteria exist, the probability of finding the desired bacteriophages is very high. In addition, Hyman [17] reported that not all bacteria are hosts of bacteriophages, and not all bacteriophages have the ability to lyse bacteria; phages are temperate bacteriophages. In 2020, Park., *et al*. [18] suggested that bacteriophages are the most abundant living entities on earth, and they play significant roles in bacterial ecology, adaptation, evolution, and pathogenesis. Bacteriophages are common in soils (approximately 10^7 to 10^9 virions/g) and highly abundant in freshwater and marine ecosystems (about 10^7 virions/ml), and their total number on earth was estimated at 10^{31} virions.

pH stability of bacteriophages

The various external physical and chemical factors as temperature, acidity, salinity, and ions, determine the occurrence, viability, and storage of bacteriophages. These factors can inactivate a phage through damage of its structural elements (head, tail, envelope), lipid loss, and/or DNA structural changes [19]. In this study, striped catfish were relatively vulnerable to pond water conditions. Two of the most key parameters were temperature and pH, with the suitable values range in pond water for striped catfish being 25-32°C and 5.5-9 [12]. In addition, according to Watanabe., *et al*. [20], gastric acid can negatively affect phage survival. Therefore, the present study was conducted to research the viability and selection of bacteriophages capable of lysing *A. hydrophila* from pH (2-10) to treat hemorrhagic disease on striped catfish. Easwaran., *et al*. [4] reported that phages are stable under different pH (1-13) and optimum pH of pAh-1 as 7.5 (1-h incubation at 28°C). The pAh-1 was unstable at highly acidic (≤ pH 3) levels accounting for under 20% as equally as phages isolated in the present report at highly acidic (≤ pH 3), accounting for about 20%. However, pH 5-11 showed more stable pAh-1 (>90%) and was higher than in the present study. Similarly, Chandrarathna., *et al*. [21] indicated that bacteriophages are active at pH 4-10 (about 80%) but inactive in highly acidic conditions (< pH 4) and highly alkaline conditions (>pH 10).

On the other hand, Gwak., *et al*. [22] indicated that KFS-YE isolated a lytic *Yersinia enterocolitica*-specific phage (KFS-YE) was stable at wide ranges of pH (4-11). According to Ma., *et al*. [23] and Ramirez., *et al*. [24], the bacteriophages can survive at low pH due to form microencapsulation. These encapsulation-forming bacteriophages maintained higher bacteria lysing-ability than did non-encapsulated phages in the pH range (3-7). Besides, Colom., *et al*. [25] recently demonstrated that encapsulation (alginate/CaCO3) could protect the bacteriophages against their destruction by the gastric juice, the low stomach pH, and the activities of bile and intestinal tract enzymes limit the efficacy of the phages.

Temperature stability of bacteriophages

Temperature is a crucial factor for bacteriophage survivability. It plays a fundamental role in attachment, penetration, multiplication, and the length of the latent period (in the case of lysogenic phages). At lower than optimal temperatures, fewer phage genetic materials penetrate bacterial host cells; therefore, fewer of them can be involved in the multiplication phase. Higher temperatures can prolong the length of the latent stage. Moreover, temperature determines the occurrence, viability, and storage of bacteriophages [16]. In the study of Liu., *et al*. [26], the five bacteriophages (W3, G65, Y81, N21, Y71) maintained almost 100% infectivity after being cultured at 4°C or 30°C for 1 day and remained relatively stable at 30°C and 40°C, but sensitive to higher temperatures. No more than 50% of phages remained alive after a 40-min incubation at 50°C. At 60°C, less than 1% of phages W3, G65, and Y81 survived for 20 min, and phages N21 and Y71 for 40 min. Besides, according to Easwaran., *et al*. [4], thermal stability results demonstrated that phage titer did not reduce when pAh-1 was incubated at 4, 20, and 40°C for 1 h. However, pAh-1 titre was significantly reduced at 60°C (18.9%), 80°C (97.2%) and 100°C (98.1%) at a specific temperature for 1 h. Similarly, Xuan., *et al*. [12] investigated the thermal stability of bacteriophages against mass mortality of the striped catfish caused by *A. hydrophila.* The authors also showed that the activity of TG25P retained to approximately 90% and 80% at 37°C and 50°C for 1 h, respectively.

Knowledge of phage stability is necessary when phages are subjected to industrial processes such as manufacturing medicated feed, where high temperatures and different pH are often used [21]. In 2018, Le., *et al*. [6] suggested that the survival of phages, together with their persistent survival on or in fish and phage-coated feed preparations, should be studied under different environmental factors (e.g., temperature, salt concentration) to determine whether phages can persist and effectively reduce *Aeromonas* spp. levels in fish farms. Additionally, Malik [27] showed that exploiting the potential of bacteriophages for phage therapy is an exciting prospect. However, to be successful, there is a pressing need for the safe manufacture and efficacious phage drug products to treat patients. The scalable manufacture of phage biologics as a stable solid dry powder form is highly desirable and achievable using spray drying. The process allows control over the final phage dose in the powder and the production of microparticles suitable for therapeutic uses. The activity of the phages in spray-dried powders is adversely affected during spray drying, and this is also an essential factor affecting the stability of the bacteriophages in the dry powder form.

Evaluation of bacteriophages capable of lysing *A. hydrophila* in water

The success of phage therapy to control fish pathogenic bacteria depends on viral survival and viability in culture water of fish-farming plants. Although there are some available data on the mechanisms and rates of mortality or loss of infectivity of phages in marine waters, little is known about their survival time in the marine environment [2].

Identification of bacteria density over 21 days in tap water and river water

The results of this study in the river water were higher than those of Imbeault., *et al*. [13] when comparing the viability of bacteria in open water and lower than the research of Imbeault., *et al*. [13] when investigating the viability of bacteria in interstitial water over 21 days. According to Imbeault., *et al*. [13], in open water, *A. salmonicida* concentration increased from 10^7 CFU/mL to 10^9 CFU/mL by two log units until day 8. It then decreased progressively to below detection level ($5x10^1$ CFU/mL) on day 16. In interstitial water, *A. salmonicida* concentration rose by four log units (from 10^7 CFU/mL to 10^{11} CFU/mL) between days 4 and 11 and then dropped slightly until day 15 (10^{10} CFU/mL), when a considerable increase in bacterial flora made *A. salmonicida* counts impossible.

Identification of bacteriophage density over 21 days in tap water and river water

In this work, parameters in the river water were similar to that of Imbeault., *et al*. [13]. The results showed that in aquariums with phages but no *A. salmonicida*, bacteriophages HER 110 decreased in number over time. However, HER 110 phage density in open water reduced less, and HER 110 phage density in interstitial water dropped more than in the present study. Their density fell below detection ($2x10^2$ PFU/mL) in open water on day 11 but remained constant at 500 PFU/mL in interstitial water between days 14 and 21.

Identification of bacteria and bacteriophage density over 21 days in tap and river water

The present study is similar to that of Imbeault., *et al*. [13] when using bacteriophages to prevent furunculosis caused by *Aeromonas salmonicida* in farmed Brook Trout. In the treatment supplied with bacteriophages and *A. salmonicida*, bacteriophage density remained high and stable throughout the test in both open and interstitial water. Bacteriophages density in interstitial water (10^{12} PFU/mL) was higher than in open water (10^9 PFU/mL). However, the bacterial density in the river water in this present study was higher than that in the research of Imbeault., *et al*. [13]. The concentration of *A. salmonicida* in open water increased to 10^8 CFU/mL before phages were added on day 4. It decreased rapidly to 10^3 CFU/mL on day 7 and below detection level on day 13. The concentration of *A. salmonicida* in interstitial water decreased by six log units (10^8 to 10^2 CFU/mL) from day 4 to day 9 and then remained detectable but low until day 21.

In addition, Pereira., *et al*. [2] reported that the pattern of bacteriophage survival in aquaculture water was different for the two bacteriophages tested. The abundance of AS-1 phage decreased by one order of magnitude in the first 15 days and, after reaching a plateau, that value remained constant for 45 days. Afterward, the bacteriophage titer decreased slightly until 91 days. In contrast, the abundance of VP-1 phage decreased strongly during the incubation period, showing a survival period of 16 days, much lower than the AS-1 phage. In this study, the bacteriophage density was increased in the first 8 days before reducing until day 21. According to Easwaran., *et al*. [4], the growth of the phage was directly proportional to the concentration of Ca^{2+}, and the divalent metal ions (Ca^{2+} and Mg^{2+}) can stabilize the activity of pAh-1. This characteristic is essential for applications of pAh-1 in the field level, where

it may have varying concentrations of Ca^{2+} and Mg^{2+}. That could be one of the reasons why phage density in river water was higher than in tap water.

In the present study, the bacteria density remained pretty high probably because the bacteria existed in mutant form. According to Silva., *et al*. [28], a fraction of the remained bacteria is phage-resistant. Still, it has been shown that virulent bacteria which become resistant to phage infection are less fit or lose their pathogenic properties. These occur mainly because the cell surface components, such as LPS and proteins, that act as receptors for phage adsorption also can act as virulence factors. Mutations in these receptors to develop resistance to the phage would reduce bacterial pathogenicity, and bacterial regrowth after phage therapy would result in few consequences.

Conclusion

The number of bacteriophages lysing *A. hydrophila* (ATCC® 7966™) isolated from 110 samples was 64, accounting for 58.2%. All isolated bacteriophages were in good adaptability at pH 5 to pH 8 (more than 60%) and 4°C and 20°C (constituting 100%). From the results of pH and temperature test, six bacteriophages have been selected and they could survive in both tap and river water. A cocktail of six bacteriophages can be further used to treat hemorrhagic septicemia caused by *A. hydrophila* in striped catfish.

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Conflict of Interest

No financial interest or any conflict of interest exists.

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