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Evaluation of bacteriophage therapy of *Aeromonas hydrophila* infection in a freshwater fish, *Pangasius buchanani*

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Introduction: The present study aimed to optimize the doses and schedule of specific bacteriophage cocktails in freshwater fish infections as prophylactic and therapeutic measures.

Methods: The three most active phages against *Aeromonas hydrophila (A. hydrophila*) (φAHBHU12, φAHBHU16, and φAHBHU19) were characterized phenotypically and genotypically. Intramuscular and water immersion routes were used to calculate the absolute lethal dose of *A. hydrophila* in *Pangasius buchanani*. Phage therapy was given simultaneously and after 6, 12, and 24 h of bacterial challenge through intramuscular and water immersion routes.

Results: The prophylactic and early phage administration could save the fish. Furthermore, the dose of intramuscular 1.0×10^4 plaque-forming unit (PFU)/fish and water immersion 1.0×10^6 PFU mL^{-1} of the phage cocktail was optimal.

Discussion: The efficacy of bacteriophage therapy as preventive or curative measures practical when administered simultaneously or early hours of *A. hydrophila* infection in aquaculture systems. Phage-based approaches may be used as an alternative to antibiotics in aquaculture to reduce antibiotic use as a part of the "One Health" approach.

KEYWORDS

***Aeromonas hydrophila*, *Pangasius buchanani*, bacteriophage therapy, aquaculture, lethal**

1 Introduction

Antibiotic-resistant bacteria are becoming more common as a result of antimicrobial resistance (AMR). The root cause of this problem is the indiscriminate use of antibiotics, including last-resort antibiotics such as colistin, in aquaculture, agriculture, and healthcare (Das et al., 2022). According to projections, AMR will cause 4.95 million deaths in 2019, with 1.3 million deaths directly attributable to treatment-resistant infections (Frei et al., 2023). The Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2021 (2021) reported that this figure is expected to rise to 10 million deaths by 2050, and according to the World Bank AMR could result in a 3.8% economic loss by 2050. This demonstrates the urgency with which we must act (O'Neill, 2016). As aquaculture activity expands and intensifies, the problem of microbial infections and the widespread utilization of antibiotics worsens.

Aquaculture is a thriving sector for developing countries in terms of growth and food security. Fisheries are one of India's fastest-growing industries. Globally, edible fish stocks are declining, which is concerning given that fish is a good source of omega-3 fatty acids and protein (Tahar et al., 2018). According to Yue and Shen (2022), more fish for human consumption are produced by aquaculture than caught in the wild. In 2020, the commercial value of fisheries increased considerably, outpacing the average production rate in the 1990s by more than 60%. This remarkable increase in output is primarily due to the thriving aquaculture industry, which has grown faster than the global population. The report *The State of World Fisheries and Aquaculture: Towards Blue Transformation*, published in 2022, provides compelling evidence of fisheries' and aquaculture's growing importance as sources of food, nutrition, and employment. In 2020, production in these sectors reached a new high of 214 million tons, worth approximately US $424 billion (Of, 2022).

The growth of aquaculture has resulted in high fish mortality rates due to disease outbreaks (Stentiford, 2012). Globally, it is estimated that fish diseases lead to a loss of revenue amounting to US$6 billion annually (Stentiford et al., 2017), and reasons for this may be improper animal care and insufficient methods of protecting against diseases. Poor environmental conditions can lead to increased animal deaths and decreased productivity (Huicab-Pech et al., 2016). Fish production systems globally utilize biocide and antibiotic treatments to prevent infectious pathogens that can lead to diseases (Wanja et al., 2020). These pathogens are frequently present in the aquatic environment where the fish are reared (Kumar et al., 2018).

Aeromonas hydrophila is one of the major bacterial pathogens present in aquatic environments. *A. hydrophila* is a causative agent of tail and fin rot, hemorrhagic septicemia, also known as motile *Aeromonas* septicemia (MAS), hemorrhagic septicemia, ulcer disease, and red-sore disease (a disease in freshwater and, to a lesser extent, marine fish). The signs of hemorrhagic septicemia include erosion of the fins, loss of scales, hemorrhages of the gills and vents, abscesses and ulcers, abdominal distension, accumulation of ascitic fluid, anemia, and damage to internal organs and musculature, with generalized liquefaction in the infected fish. In the aquaculture sector, research on phage therapy has already begun, focusing on various pathogenic bacteria (e.g., *Vibrio* spp., *Aeromonas* spp.) (Mateus et al., 2014; Laanto et al., 2015; Kalatzis et al., 2016; Hockett and Baltrus, 2017; Duarte et al., 2018; Almeida et al., 2019; Kazimierczak et al., 2019). Research has focused less on phage administration doses and routes and has instead concentrated on the isolation and characterization of virulent phage cocktail formulations.

To reduce the economic loss, bacteriophage treatment of water bodies and fish infections may be one of the potential alternatives to antibiotics under the "One Health" concept. Therefore, the present study planned to evaluate the efficacy of phage therapy in preventing and establishing infections caused by a known fish pathogen, *A. hydrophila*, in freshwater fish, *Pangasius buchanani*, in terms of safe doses and timing of phage administration.

2 Material and methods

2.1 Isolation and characterization of *A. hydrophila* isolates

2.1.1 Isolation and biochemical identification of *Aeromonas* species

Bacteria were isolated from pond water and diseased fish (12–15 cm in length) near Varanasi, Uttar Pradesh, India. A total of 65 diseased fish were identified by swimming abnormality, pale gills, skin ulceration, and wounds on the body and tail. Specimens for isolation of bacteria were taken from the pond water sample, organs (gills, liver, kidney, ovary, and skin) of diseased fish during postmortem, and swabs from wounds. The culture was performed using blood agar (HiMedia Laboratories, Maharashtra, India) and MacConkey agar (HiMedia Laboratories) and incubated at 37°C overnight. The next day, the plates were examined for growth.

The circular off-gray colonies from blood agar and non-lactose fermenting colonies from MacConkey were subjected to Gram staining and biochemical testing for such things as motility, oxidase production, sugars fermentation, gas production, susceptibility to vibriostatic agent O/129 (decarboxylase testing), and esculin hydrolysis for the identification of *A. hydrophila* (Samal et al., 2014).

2.1.2 Characterization of the *Aeromonas* species

Biochemically identified *Aeromonas* species were subcultured on Mueller–Hinton agar (MHA; HiMedia Laboratories), and then DNA extraction was performed using the phenol–chloroform–isoamyl alcohol (25:24:1) method (Sambrook and Russell, 2006). The isolated genomic DNA was subjected to amplification using 16S rDNA-specific primers targeting genus-specific sequences of *Aeromonas* species (forward 5′-GGG AGT GCC TTC GGG AAT CAG A-3′ and reverse 5′-TCA CCG CAA CAT TCT GAT TTG-3′) (Hussain et al., 2014). The positive strains for *Aeromonas* species were further amplified by using *A. hydrophila* species-specific primers Ahh1 (forward 5′-GCC GAG CGC CCA GAA GGT

GAG-3′ and reverse 5′-GAG CGG CTG GAT GCG GTT GT-3′) (Hussain et al., 2014). The master mix (Sigma-Aldrich, St. Louis, MO, USA) consisted of 50 ng of bacterial genomic DNA. The reaction mixture (25 µL) was subjected to 35 cycles on the following program: an initial melting temperature of 95°C for 5 min, with denaturation at 94°C for 45 s, annealing at 59°C for 45 s, and extension at 72°C for 1 s. The final extension was carried out at 72°C for 7 min in a thermal cycler (Bio-Rad Universal Hood II, Hercules, CA, USA). Then, PCR products were visualized on 1% agarose gel electrophoresis.

2.2 Isolation and characterization of *A. hydrophila*-specific bacteriophages

2.2.1 Bacteriophage isolation and purification

Phages were isolated from the pond, river, and sewage water by using the soft agar (0.8%) overlay method, following the method described by Kutter (2009), with some modifications. *A. hydrophila* was plated in a lawn culture (1.5×10^8 CFU mL^{-1}) to isolate bacteriophages on MHA and incubated for 6 h to reach the log phase. Water specimens from different sources were collected and treated with 1% chloroform (Sigma-Aldrich) for 10 min and centrifuged three times for 15 min at 10,778 × *g*. One milliliter of the supernatant was flooded on the 6-h bacterial lawn of the *A. hydrophila* on an MHA plate and incubated overnight at 37°C. The next day's lawn was washed with Tris-HCl magnesium gelatin (TMG, pH 7.4) buffer and centrifuged at 10,778 × *g* for 15 min.

Isolated phages were purified by using plaque counting by applying the 0.8% soft agar overlay method. The single isolated plaque was picked up for further processing (Orlova, 2012). The phage count was increased by lawn and spot methods (Marcó et al., 2012). For purification (toxin-free), the harvested fluid was subjected to membrane dialysis (membrane pore size 20 nm, HiMedia Laboratories) against poly ethylene glycol (PEG) 20% in 2.5 M sodium chloride (NaCl) solution (HiMedia Laboratories) overnight at 4°C and then washed with phosphate buffer saline. The dialysis and washing process was repeated twice (Gangwar et al., 2021).

2.2.2 Bacterial lytic activity of purified phages

For their bacteriolytic activity, different isolated phages were subjected against different isolates of *A. hydrophila*. In addition, the lawn culture of *A. hydrophila* (1.5×10^8 CFU mL^{-1}, 0.5 McFarland) was prepared on MHA. Ten microliters of each phage with the concentration of 1×10^9 PFU mL^{-1} was spotted on the MHA (Montso et al., 2019). The MHA plates were observed for the lysis of bacteria (clear zone) after incubation at 37°C overnight. Then, the three most active phages (i.e., φAHBHU12, φAHBHU16, and φAHBHU19) were selected for further characterization and experiments.

2.2.3 Host range determination

To see the spectrum of activity of these three phages (φAHBHU12, φAHBHU16, and φAHBHU19), we used different American Type Culture Collection strains of bacteria. The lawn culture of *Pseudomonas aeruginosa, Aeromonas sobria, Escherichia coli, Plesiomonas shigelloides, Enterococcus faecalis, Salmonella* Typhi, *Acinetobacter lwoffii, Enterobacter cloacae*, and *Staphylococcus aureus* (1.5 × 108 CFU mL^{-1}) were made on MHA. The lytic activity of phages against all the bacterial species was determined by spot assay. Spot assay was used to determine the lytic spectrum activity of phage isolates, as described previously.

2.2.4 Effect of different pH and temperature on phage activity

The bacteriophage strains were screened for their lytic activity at different pH ranges (3-12). Bacteriophages (1×10^9 PFU mL^{-1} final concentrations) were incubated in equal amounts of TMG buffer (1:1) of different pH at 4°C for 2 h. After incubation, the lytic activity of bacteriophages was checked by the spot assay method.

Activities of bacteriophages were screened at different temperatures. For this screening, phages (1mL; 1×10^9 PFU mL^{-1} final concentrations) were incubated at different temperatures (–80° C, –20°C, 4°C, 28°C, 37°C, 45°C, and 55°C) for 48 h, and then spot assay was performed to check its activity.

2.2.5 Phage morphology

The most virulent phages (i.e., φAHBHU12, φAHBHU16, and φAHBHU19) with titer 1.0×10^{11} PFU mL^{-1} were filtered through a 0.22-µm syringe filter. The phage suspension was centrifuged at 21,124 × *g* for 90 min, and the supernatant was decanted. The pellet was washed three times with 0.1 M ammonium acetate (HiMedia Laboratories, pH 7.0). At the final step, the pellet was resuspended in ammonium acetate. The samples were treated with 2% uranyl acetate for negative staining and carbon-coated formvar films and examined under transmission electron microscopy (TALOS, Thermo Scientific, AIIMS, New Delhi, India). The diameter of the head and tail of these phages was determined by ImageJ software.

2.2.6 Restriction digestion analysis

Bacteriophage DNA was extracted and purified using a Norgen Biotek Corp. kit (cat. no 46800). Further phage DNA was subjected to restriction digestion using *AluI* (10 U μL^{-1}) in accordance with the manufacturer's instructions (Thermo Fisher Scientific) for 60 min at 37°C in a 20-µL reaction mixture containing 1 µL of DNA (concentration 1 µg μL^{-1}), 2 µL of 10 × buffer, 1 µL of restriction enzyme, and 16 µL of sterile water. The digested products were electrophoresed with 1% agarose gel at 80 V for 1 h. The gel images were captured under ultraviolet light using a gel documentation system (BioRad Universal Hood II). The sizes of the DNA bands were estimated using 1 kb and 100 bp DNA ladders (Invitrogen, Thermo Fisher Scientific).

2.2.7 Genotyping of bacteriophage by enterobacterial repetitive intergenic consensus PCR

The genomic DNA of the phages against *A. hydrophila* was subjected to enterobacterial repetitive intergenic consensus (ERIC)

PCR. The master mix contained 50 ng of phage genomic DNA. The primers were used to amplify phage genomic DNA (forward 5′-ATG TAA GCT CCT GGG GAT TCA-3′ and reverse 5′-AAG TAA GTG ACT GGG GTG AGC G-3′) (Ranjbar et al., 2017). The PCR mixture was subjected to the following program for amplification: melting temperature at 94°C for 7 min with denaturation for 45 s at 92°C, annealing at 31.8°C for 45 s, and extension at 72°C for 1 s. A final extension step at 72°C for 7 min was performed at the end of the 34 cycles. The PCR product was run on 1% agarose gel electrophoresis with 100 bp and 1 kb DNA ladder.

2.3 Fish infection and phage therapy

2.3.1 Experimental fish rearing

The experimental fish, *P. buchanani*, weighing 12–16 g, 12–15 cm in size, and 6–8 weeks old, were procured from commercial fish farms in Varanasi. The fish were reared in well-aerated 40-L rectangular glass aquariums in the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India. The permission of the institutional ethics committee was obtained (reference number Dean/2016/CAEC/70/ dated 30 March 2017). Before the experiment, the fish were acclimatized for 1 week. Then, the fish were immersed in 0.01% potassium permanganate (HiMedia Laboratories) for 10 min to remove the parasitic infection (Prasad et al., 2011). The aquatic environment of the tank was maintained with dissolved oxygen 8.0 ± 0.5 mg^{-1}, ammonia 0.6 ± 0.05 mg^{-1}, pH 7.2 ± 0.2, and temperature 27°C ± 2°C without chlorination. Each aquarium was aerated with an air pump, and one-third of the water was replaced daily, dead fish were removed, and debris was siphoned from the bottom of the aquarium.

2.3.2 Determination of lethal doses

Two different routes of administration [i.e., intramuscular (IM) and water immersion] were used to establish *A. hydrophila* infection in the fish.

2.3.2.1 Determination of lethal dose 100 by intramuscular route

A. hydrophila was injected through the IM route in four groups containing 10 fish each group. In each tank (containing 2 L of water), add 0.1% Luria-Bertani broth (Himedia) for the given organic stress. The log phase of bacterial suspension was given to different groups of fish at doses of 8.0×10^2, 8.0×10^3, 8.0×10^4, and 8.0×10^5 CFU/fish. One group was injected with 100µL of 0.85% NaCl as a negative control. Then, we looked for lesions, sickness, and mortality. The lethality was observed for 7 days. After death, the fish were subjected to postmortem examination to ascertain the cause of death. Fish organs (liver, kidney, intestine, and stomach) were cultured on MHA and blood agar media. The whole experiment was repeated three times independently.

2.3.2.2 Determination of lethal dose 100 by water immersion

Forty fish were divided into four groups (10 fish in each group) in 2 L of water with 10 mL of Luria Bertani (LB) broth (HiMedia Laboratories). The first group was not infected. The other three groups were subjected to bacterial inoculation by putting them in a 2-L water tank with different concentrations of *A. hydrophila* (i.e., 1.0×10^5, 1.0×10^6, and 1.0×10^7 CFU mL^{-1}), and in each tank 10 mL of LB broth was added. The fish were observed for 7 days for any morbidity and mortality (Sarker and Faruk, 2016).

2.3.3 Assessment of the efficacy of the phage cocktail on fish infection

The phage cocktail was used for prophylactic and therapeutic purposes. The fish experiments were set up according to the following plan:

- The first control group was given only 0.85% NaCl.
- The second control group was infected with bacteria only.
- The third control group was given only phage.

All the experiments were repeated three times for robustness of data.

2.3.4 Bacteriophage cocktail given through IM simultaneously, 6, 12, and 24 h after *A. hydrophila* challenge given through IM

Experimental groups were challenged with 100 µL of *A. hydrophila* by injecting 8.0×10^5 CFU/fish IM. The bacteriophage cocktail at the doses of 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, and 1.0×10^8 PFU/fish were given IM simultaneously at a different site from that of the bacterial injection.

Each group of 10 fish was placed in the 10-L aquarium. Fish were challenged with 100 µL of *A. hydrophila* at the dose of 8.0×10^5 CFU/fish through the IM route, and phage cocktails at the quantity of 1.0×10^4 PFU/fish were given simultaneously after 6, 12, and 24 h of bacterial challenge IM. The water was changed daily, as described elsewhere, and the experiment was monitored for 7 days.

2.3.5 Bacteriophage cocktail added into the water simultaneously, 6, 12, and 24 h after *A. hydrophila* challenge given through IM

Ten groups of *P. buchanani* were placed in separate aquaria. The fish were challenged with 100µL of *A. hydrophila* at a concentration 8.0×10^5 CFU/fish through the IM. The phage cocktail at different concentrations (i.e., 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, 1.0×10^8, and 1×10^9 PFU mL^{-1}) was administered through water immersion simultaneously.

The groups, comprising 10 fish each, were challenged with 100 µL of *A. hydrophila* (8.0×10^5 CFU/fish) through the IM route. However, phage cocktails containing 1.0×10^8 PFU mL^{-1} were added into the water tank after 6, 12, and 24 of bacterial challenge.

2.3.6 Bacteriophage cocktail added into the water simultaneously, 6, 12, and 24 after *A. hydrophila* challenge given through water immersion

In water immersion, 1.0×10^7 CFU mL^{-1} bacterial suspension was added to the 2-L water aquaria with 10 mL of LB broth, and phages cocktails were given simultaneously at a concentration of 1.0×10^3, 1.0×10^4, 1.0×10^5, 1.0×10^6, 1.0×10^7, 1.0×10^8, and 1.0×10^9 PFU mL^{-1}, with 10 mL of LB broth in 2 L by adding in water. Furthermore, the phage cocktail (1.0×10^6 PFU mL^{-1}) was given at 6, 12, and 24 after adding bacteria into the water.

3 Statistical method

One-way ANOVA was applied to check the quality of the data before making different comparisons. When we found that there was a statistically significant difference between the means of three or more independent groups by using an ANOVA test, we used the *post hoc* test to compare the groups in pairs to obtain the significance levels. Survival at 0 h with survival at 6, 12, and 24 h were compared using *t*-test. The Student's *t*-test was applied to compare the means of paired groups using SPSS package.

4 Results

4.1 Identification of *A. hydrophila* isolates

A total of 38 *Aeromonas* spp. were isolated from diseased fish and pond water. From a biochemical and molecular basis, 18 isolates could be confirmed as *A. hydrophila*, Gram-negative, motile, and oxidase-positive bacteria. It could ferment sugars with gas production and resistance to vibriostatic agent O/129 and hydrolyze esculin hydrolysis. Confirmation of *Aeromonas* spp. and *A. hydrophila* was obtained by using primers that are specific to their genus and species. This was determined by the amplicon sizes of 356 bp and 130 bp, respectively (see Figures 1A, B). The AhBHU111 strain of *A. hydrophila* was used for further experiment.

4.2 Characterization of *A. hydrophila*-specific bacteriophages

4.2.1 Bacterial lytic activity of isolated bacteriophages

A total of 18 strains of *A. hydrophila* were tested against 23 bacteriophages by spot assay. In detail, the most virulent bacteriophages, φAHBHU12 (72.2%), φAHBHU16 (66.6%), and φAHBHU19 (83.3%), were used for further experiment (see Table 1).

4.2.2 Host range determination

The most virulent bacteriophages [φAHBHU12 (lysing 72.2%), φAHBHU16 (66.6%), and φAHBHU19 (83.3%)] were selected for further characterization. The three phages could not lyse *Pseudomonas aeruginosa*, *Aeromonas sobria*, *Escherichia coli*, *Salmonella* Typhi, *Acinetobacter lwoffii*, *Enterobacter cloacae*, *Plesiomonas shigelloides*, *Enterococcus faecalis*, and *Staphylococcus aureus*.

4.2.3 Effect of different pH and temperature on phage activity

Good lytic activity of the bacteriophages against *A. hydrophila* was observed at pH 3–12 (see Figure 2A), and the three phages could survive well at temperatures –80°C, –20°C, 4°C, 28°C, and 37°C. However, φAHBHU12 and φAHBHU19 had satisfactory activity (approximately 50%) at 45°C (see Figure 2B).

4.2.4 Morphological characterization

The bacteriophages φAHBHU12, φAHBHU16, and φAHBHU19 were examined under transmission electron microscopy cryo-TEM (Talos, Thermo Fisher Scientific,

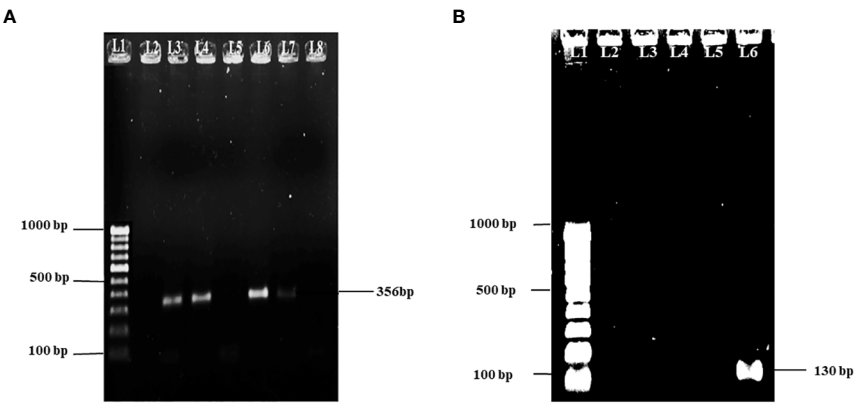

FIGURE 1
(A) Gel picture showing 100 bp molecular marker in lane 1 and 356 bp amplicon specific for *Aeromonas* spp. in lanes 3, 4, 6, and 7. **(B)** Gel picture showing 100 bp molecular marker in lane 1 and 130 bp amplicon specific for *A. hydrophila* in lane 6.

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TABLE 1 Lytic activity of 23 bacteriophages against 18 different strains of *A. hydrophila*.

Serial number	Bacteriophage	Susceptibility	Percentage of the host lysed by individual bacteriophage
1.	φAHBHU1	8/18	44.4
2.	φAHBHU2	2/18	11.1
3.	φAHBHU3	4/18	22.2
4.	φAHBHU4	9/18	50
5.	φAHBHU5	3/18	16.6
6.	φAHBHU6	11/18	61.1
7.	φAHBHU7	5/18	27.7
8.	φAHBHU8	7/18	38.8
9.	φAHBHU9	3/18	16.6
10.	φAHBHU10	12/18	66.6
11.	φAHBHU11	7/18	38.8
12.	**φAHBHU12**	**13/18**	**72.2**
13.	φAHBHU13	2/18	11.1
14.	φAHBHU14	9/18	50
15.	φAHBHU15	8/18	44.4
16.	**φAHBHU16**	**12/18**	**66.6**
17.	φAHBHU17	6/18	33.3
18.	φAHBHU18	4/18	22.2
19.	**φAHBHU19**	**15/18**	**83.3**
20.	φAHBHU20	10/18	55.5
21.	φAHBHU21	9/18	50
22.	φAHBHU22	2/18	11.1
23.	φAHBHU23	4/18	22.2

Bold values show the most active phages after the bacteriolytic activity test against 18 strains of *A. hydrophila*.

Waltham, MA, USA). Based on previous classification (Dion et al., 2020), the φAHBHU12 could be placed in the Podoviridae family, as the diameter of the isometric head was 52.47 nm, and a non-contractile short tail could not be visualized (see Figure 3A). In contrast, bacteriophage φAHBHU16 belongs to the Siphoviridae family, having an icosahedral head (47.82 nm in width) and a long non-contractile tail ranging from 93.55 to 117.30 nm in length (see Figure 3B). The φAHBHU19 could be classified as belonging to the

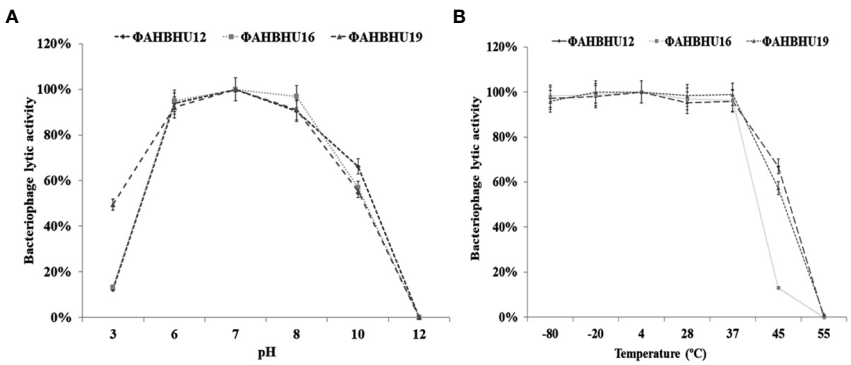

FIGURE 2
(A) Graph showing effect of pH on *Aeromonas hydrophila*-specific bacteriophages. **(B)** Graph showing effect of temperature on *A. hydrophila*-specific bacteriophages.

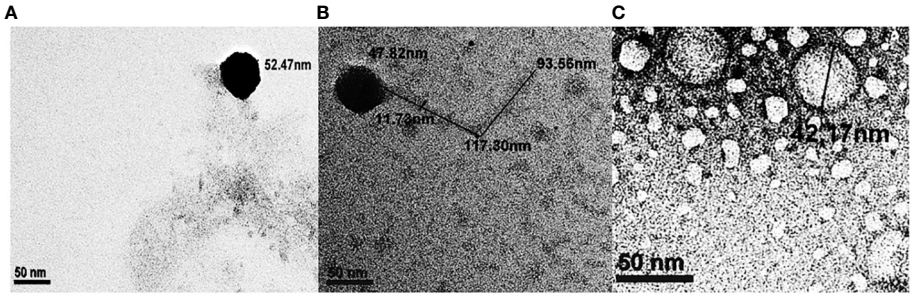

FIGURE 3
Transmission electron micrograph of bacteriophages, **(A)** φAHBHU12 belongs to the Podoviridiae family, **(B)** φAHBHU16 belongs to the Siphoviridae family, and **(C)** φAHBHU19 belongs to the Corticoviridae family.

Corticoviridae family, having an icosahedral head (42.17 nm in width) and no tail (see Figure 3C).

4.2.5 The whole-genome fingerprinting by using restriction enzyme digestion (*AluI*) and enterobacterial repetitive intergenic consensus PCR

The isolated three phages (φAHBHU12, φAHBHU16, and φAHBHU19) were further characterized at the level of whole-genome fingerprinting by restriction digestion and ERIC PCR of their genomic DNA. Different banding patterns were indicated in the restriction digestion with *AluI* (Figure 4A). Also, in ERIC PCR, distinct band patterns were observed in all three phages (Figure 4B). The results delineate that these three phages have different genetic characteristics.

The isolated potent phages were further characterized at the genomic level using RAPD-PCR and restriction digestion by isolating their genomic DNA. Different banding patterns were observed in the RAPD-PCR analysis (Figure 3A). Also, the restriction digestion with EcoRI shows a distinct band pattern in all three phages (Figure 3B). The results delineate that the isolated potent phages have different genetic makeup.

4.3 Fish infection and phages therapy

4.3.1 Lethal dose 100 of *A. hydrophila* on intramuscular administration and water immersion

The lethal dose of *A. hydrophila* killing the *P. buchanani* fish weighing 12–16 g on intramuscular injection in 7 days was 8.0×10^5CFU/fish. However, the lethal dose through the water immersion route was 1.0×10^7 CFU mL^{-1} within 7 days when mixed with 10 mL of LB broth containing 2 L of the water tank as organic contamination. Interestingly, no death could be observed in the absence of LB broth (organic matter).

4.3.2 Protection by phage cocktail at different routes of phage administration at different time intervals after *A. hydrophila* infection

4.3.2.1 Intramuscularly administered varying phage cocktail dose

Figure 5A (IM) shows that when a simultaneous lethal dose of *A. hydrophila* and different concentrations of phage cocktails were injected through the intramuscular route, 1.0×10^4 PFU/fish could provide 93% protection, which was comparable to the dose of

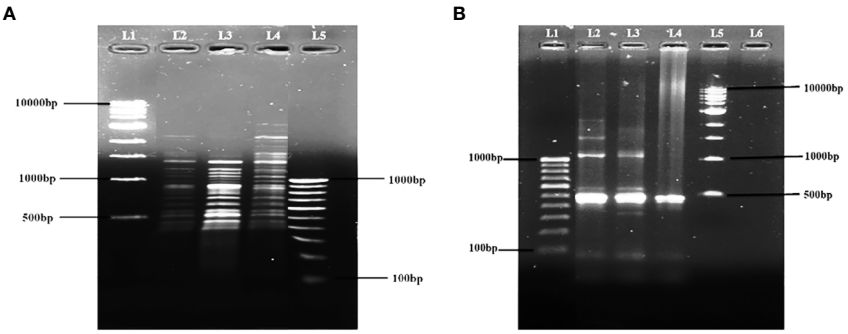

FIGURE 4
Fingerprinting of φAHBHU12, φAHBHU16, and φAHBHU19 by **(A)** Restriction digestion with *AluI* [L1- molecular marker (1 kb), L2- φAHBHU12, L3- φAHBHU16, L4- φAHBHU19, and L5 molecular marker (100 bp)], and **(B)** ERIC PCR [L1 molecular marker (100 bp), L2- φAHBHU12, L3- φAHBHU16, L4- φAHBHU19, and L5- molecular marker (1 kb marker)].

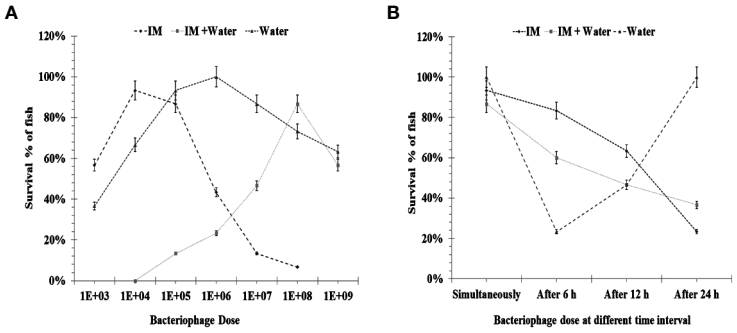

FIGURE 5
(A) Showing efficacy of phage administration through intramuscular and different doses of bacteriophage when given simultaneously through water immersion. IM: Effect of bacteriophage therapy on *Aeromonas hydrophila* (8.0×10^5 CFU/fish) infection in *Pangasius buchanani*. Bacteria and different doses of bacteriophage were given simultaneously through the intramuscular route. IM + water: Showing efficacy of simultaneous addition of different doses of bacteriophage cocktail in water and *A. hydrophila* (8.0×10^5 CFU/fish) challenge intramuscular in *P. buchanani*. Water immersion: Effect of bacteriophage therapy on *A. hydrophila* (1.0×10^7 CFU mL^{-1}) infection in *P. buchanani*. Bacteria and different doses of bacteriophage were given simultaneously through water immersion. **(B)** Showing efficacy of phage administration through intramuscular and water immersion at different time intervals after bacterial challenge. IM: Effect of bacteriophage (1.0×10^4PFU/fish) therapy given simultaneously, after 6 h, 12 h, and 24 h of *A. hydrophila* infection (8.0×10^5CFU/fish) in *P. buchanani*. Both doses were given through the IM route. IM + water immersion: Effect of bacteriophage (1.0×10^8 PFU mL^{-1}) added into the water (simultaneously, after 6 h, 12 h, and 24 h) with *A. hydrophila* infection (8.0×10^5CFU/fish) intramuscularly in *P. buchanani*. Water: Effect of bacteriophage (1.0×10^8 PFU mL^{-1}) added into the water (simultaneously, after 6 h, 12 h, and 24 h) with *A. hydrophila* infection (8.0×10^5CFU/fish) intramuscularly in *P. buchanani*..

1.0×10^5 PFU/fish. However, a dose of 1.0×10^6 PFU/fish led to the significantly lower protection of only 43% of the reared fish. When the doses were further reduced to 1.0×10^3 PFU/fish, 57% of the *P. buchanani* were protected (see Table 2). Better protection (83% and 63%, respectively) could be observed with the intervention at 6 and 12 h at the dose of 1.0×10^5 PFU/fish (see Figure 5B; IM). However, the intervention with phage therapy carried out 24 h after the *A. hydrophila* challenge resulted in significantly lower protection than when it was carried out simultaneously (see Table 3).

4.3.2.2 Route of bacterial dose intramuscular and phages administration challenge through water immersion at different time intervals

The highest protection (87%) through the water immersion route could be observed with the dose of 1.0×10^8 PFU mL^{-1} in simultaneous addition to aquarium water after IM injection of *A. hydrophila* 8.0×10^5 CFU/fish. However, comparable protection could also be achieved by mixing the phage cocktail at a concentration of 1.0×10^9 PFU mL^{-1}. However, lowering the phage concentration to 1.0×10^7 PFU mL^{-1} and 1.0×10^6 PFUmL^{-1} resulted in a significant decrease in the protection level to 47% and 23%, respectively (see Figure 5A; IM + water). Interestingly, the phage cocktail at the dose of 1.0×10^8 PFU mL^{-1} added 6 h after the bacterial challenge provided comparable protection with that given simultaneously at a similar concentration of phage cocktail (see Figure 5B; IM + water).

4.3.2.3 Bacterial dose and varying phage cocktail dose at different time intervals through water immersion

Figure 5A (water) shows that the bacteriophage cocktail concentrations of 1.0×10^5 PFU mL^{-1} and 1.0×10^6 PFU mL^{-1} gave comparable protection (93% and 100%, respectively) when both bacterial challenge and phage therapy were given water immersion simultaneously; however, a lower dose of 1.0×10^4 PFU mL^{-1} gave significantly lower (67%) protection to *P. buchanani* (see Table 2). However, a 6-h delay in administering a phage cocktail of 1.0×10^6 PFU mL^{-1} led to only 23% protection, while a delay of 12 h protected 47% of the fish. The other notable finding was that no mortality was observed when the phage cocktail of 1.0×10^6 PFU mL^{-1} was added to the water 24 h after the *P. buchanani* infection by *A. hydrophila* (8.0×10^7 CFU mL^{-1}) (see Figure 5B; water).

5 Discussion

Fisheries and aquaculture production are vital protein sources for human beings, and a growth in aquaculture production is required to meet the high demand for fish and seafood worldwide. However, despite an upsurge in freshwater aquaculture, various microbial infections, including bacteria, pose a big challenge. Therefore, bacteriophage therapy is becoming increasingly popular as a promising alternative to treat/prevent bacterial infection in livestock, including in fish rearing.

However, several conditions must be fulfilled to make the bacteriophages effective in aquaculture. Therefore, the present study aimed to see bacteriophages as preventive/curative tools in aquaculture systems. For this purpose, we reared the *P. buchanani* fish and determined the lethal dose of *A. hydrophilia* bacteria by intramuscular and water immersion routes. Furthermore, we evaluated the amount, timing, and mode of delivery of bacteriophages as the prophylactic and therapeutic measures in freshwater aquaculture systems against known fish pathogens (i.e., *A. hydrophila*).

TABLE 2 Showing efficacy of phage treatment given to the fish by administering simultaneously through intramuscular and water immersion routes at different doses (number of fish in each group = 30).

Serial number	Dose of the bacteriophage per fish	Both bacterial challenge and bacteriophage given through the intramuscular route			Bacterial challenge given through the intramuscular route, while bacteriophage by water immersion			Bacterial challenge and bacteriophage were given to fish through water immersion.		
		Mean of the surviving fish	Comparison made between	*p*-value	Mean of the surviving fish	Comparison made between	*p*-value	Mean of the surviving fish	Comparison made between	*p*-value
1	1.0×10^3	0.5667			0.0000			0.3667		
2	1.0×10^4	**0.9333**	2:1	0.000	0.1333	5:1	0.000	0.6667	4:1	0.000
3	1.0×10^5	0.8667	2:3	0.500	0.2333	5:2	0.001	0.9333	4:2	0.001
4	1.0×10^6	0.4333	2:4	0.000	0.4667	5:3	0.023	**1.0000**	4:3	0.515
5	1.0×10^7	0.1333	2:5	0.000	**0.8667**	5:4	0.000	0.8667	4:5	0.193
6	1.0×10^8	0.0667	2:6	0.000	0.5667	5:6	0.328	0.7333	4:6	0.010
7	1.0×10^9	NA	–	–	NA	–	–	0.6333	4:7	0.000

Bold values show the maximum survival of fish at 1×10^4, 1×10^5, 1×10^7 and 1×10^6 of bacteriophages.

TABLE 3 Showing efficacy of phage treatment given to the fish by administering simultaneously and at different time intervals after bacterial challenge through intramuscular and water immersion routes at effective doses (number of fish in each group = 30).

Serial number	Fixed dose of phage cocktail at different time interval	Both bacterial challenge and bacteriophage given through the intramuscular route (1.0×10^6 PFU mL^{-1})			Bacterial challenge given through the intramuscular route, while bacteriophage through water immersion (1.0×10^4 PFU mL^{-1})			Bacterial challenge and bacteriophage were given to fish through water immersion (1.0×10^8 PFU mL^{-1})		
		Mean of the surviving fish	Comparison made between	*p*-value Significance (two-tailed)	Mean of the surviving fish	Comparison made between	*p*-value Significance (two-tailed)	Mean of the surviving fish	Comparison made between	*p*-value Significance (two-tailed)
0	At 0 h without interference	1.00	-					1.00	1.00	
1	Simultaneously	**0.93**	-		**0.8667**			**1.00**	1.00	
2	After 6 h	0.83	1:2	0.264	0.300	1:2	0.03	0.23	1:2	0.000
3	After 12 h	0.767	1:3	0.005	0.533	1:3	0.01	0.47	1:3	0.000
4	After 24 h	0.267	1:4	0.000	0.400	1:4	0.00	**1.00**	1:4	≥ 0.05

Bold values show maximum survival at different time points.
NA, Not applicable.

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When *P. buchanani* was injected intramuscularly, a dose of 8.0×10^5 CFU/fish resulted in the death of all fish within 7 days. However, on simultaneous administration of phage cocktails through the IM route, the doses of 1.0×10^4 and 1.0×10^5 PFU/fish gave the best protection (93% and 87%, respectively). However, decreasing or increasing doses failed to prevent death. A phage cocktail given within the first 6 h protected 83% of the fish. However, high mortality rates (63% and 23%, respectively) could be observed when the injection of the phage cocktail was delayed by 12 and 24 h through the IM route. This observation indicates that the sooner the phage therapy is instituted, the better the results.

In the second experiment, we tried to determine the effect of variation in the route of administration of phages in fish having induced infection by *A. hydrophila* through the IM route. Interestingly, a higher dose (i.e., 8.0×10^8 PFU mL^{-1}) provided the best protection when the phages were given in water simultaneously. However, the protection rate was significantly reduced when the water immersion phage cocktail was delayed by 24 h in the aquarium containing fish infected intramuscularly.

We induced the lethal infection in the third experiment by adding 1.0×10^7 CFU mL^{-1} *A. hydrophila* to the water tank. The lethal dose for *P. buchanani* could be 1.0×10^7 CFU mL^{-1} when organic material was added to induce the stress. This observation of organic matter contamination indicates that stress is essential to initiate and establish the infection. We evaluated the different doses of bacteriophage cocktails at different time intervals in the aquaria. Intriguingly, while unprotected fish were dying within 7 days with 1.0×10^7 CFU mL^{-1}, the simultaneous addition of phage cocktail in the water body at concentrations of 1.0×10^5 and 1.0×10^6 PFU mL^{-1} resulted in 93% and 100% protection, respectively. However, a lower dose of 1.0×10^4 PFU mL^{-1} could protect only a few of the fish. It is worth noting that when the addition of phage cocktail (1.0×10^6 PFU mL^{-1}) in the water tank was delayed by 6 and 12 h, the protection rates were significantly lower. Surprisingly, when the same dose was delayed by 24 h the mortality was reduced to zero (i.e., 100% protection). Therefore, the amount of bacteriophage cocktail given at a particular time of infection seems extremely important. This varying protection is explained based on the zone phenomenon, an optimum number of bacteriophages in the cocktail leading to sudden lysis of all the bacteria and releasing a considerable amount of endotoxin to which fish succumb. At an early stage of infection, the bacterial count was less, while at a later stage, the bacterial count was higher than the bacteriophages. Even when bacteria are lysed slowly because of their small/large number, tolerable endotoxin is produced. In the latter case, because of a low multiplicity of infection of phages, sudden lysis of the bacteria did not occur, leading to a gradual release of endotoxin. A similar phenomenon has already been reported in treating septicemia with bacteriophages in animal models (Patel et al., 2021; Singh et al., 2022). Consistent with our findings, Zhang et al. (2015) and Karunasagar et al. (2007) have shown the protection of sea cucumber against *Vibrio alginolyticus* by a spectrum of bacteriophages.

Contrary to this, a few studies have mentioned that lower doses yielded adequate protection, and a difference in treatment efficacy with different quantities of phage cocktails was not reported (Li et al., 2016). In one study, small doses worked because the bacteriophages were self-perpetuating (Lomelí-Ortega and Martínez-Díaz, 2014). A study published in 2017 reported 70% protection when small doses of water immersion administration were carried out shortly after the infection (Wang et al., 2017). Therefore, the phage cocktail given at a particular point in time and the severity of illness may give variable protection. Thus, we can see that prophylactic or early phage therapy provides better protection in freshwater aquaculture systems.

Earlier reports had stated that prophylactic use of bacteriophages 24, 12, and 6 h before bacterial challenge gave significant protection irrespective of the route of administration. Lomelí-Ortega and Martínez-Díaz (2014) have also reported better protection using prophylactic doses and unsatisfactory outcomes when prophylactic use of bacteriophages started 24 h post infection, which agrees with our observation. However, when a phage cocktail was administered in water through immersion, significantly higher (100 to 1,000 times) doses per unit volume were required. A possible explanation for this may be that phages get adsorbed to specific receptors on phage-sensitive bacteria and dead and phage-resistant bacteria. It has already been reported that the immunoglobulin-like domain on the surface of phages makes them more susceptible to getting trapped in the intestinal mucosa (Fraser et al., 2006; Lepage et al., 2008). In addition, the gut is the most diversified, crowded organ of fish and animals, comprising eukaryotic cells, bacteria, fungi, and viruses (Sausset et al., 2020). Therefore, competitive inhibition may also play a role in the efficiency of lysing the target bacteria.

There are several issues to resolve while planning for phage therapy in aquaculture. First, the observations made in this study should not be generalized to all situations in freshwater aquaculture. Lower and repeated doses may reduce mortality when the infection is of longer duration, although this needs to be explored further. Last, if there is an increase in the density of the known bacterial pathogen in the aquaculture system, adding their respective phage cocktail in anticipation at adequate doses as prophylaxis may prevent infection and unnecessary use of antibiotics.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the institutional Ethics Committee for animals permitted the protocol for the study, which was carried out during Dean/2016/CAEC/70 dated 30.03.2017at the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India.

Author contributions

GN, DK, and RC conceived the idea. RK, RY, GN, DK, and RC executed the experimental work and analyzed the data. GN, RK, and DK wrote the initial manuscript. GN, DK, and RC completed the final editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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