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Short Communication

Isolation, Characterization, and *In Vitro* Evaluation of Bacteriophages for Controlling the Fish Pathogen *Aeromonas hydrophila*

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Abstract

Aquaculture plays a vital role in global protein supply, yet its increasing production faces disease-related challenges, particularly *A. hydrophila* infections. This pathogen causes *Motile Aeromonas Septicemia* (MAS), leading to mass mortality in catfish and significant economic losses. While antibiotics have been the primary solution, their use is now restricted due to antimicrobial resistance, necessitating safe and sustainable alternatives. Phage have emerged as environmentally friendly, specific biocontrol agents to combat such infections. This study aimed to evaluate the effectiveness of lytic phage in controlling the fish pathogen *A. hydrophila* through an *in vitro* approach. The research stages included phage isolation (density assay), characterization (plaque morphology and host range testing), and evaluation of phage against *A. hydrophila* (bacteriolytic efficacy assay). A completely randomized design was employed, with six treatments and three replicates, media control (K-), *A. hydrophila* control (K+), antibiotic control (enrofloxacin 0.1 mg/mL, KA), and phage treatments at *multiplicities of infection* (MOI) of 0.1, 1, and 10 (P0.1, P1, P10) for the bacteriolytic efficacy assay. Phage isolated from catfish ponds in Dramaga, Bogor, exhibited a high titer (5.68×10^9 PFU/mL) and formed clear, round plaques (0.17-0.35 cm diameter). They demonstrated a narrow host range, lysing only 1 of 10 *A. hydrophila* isolates. In efficacy assays, phage treatment at an MOI of 10 significantly reduced bacterial density ($p < 0.05$) at 24 hours post infection, decreasing the OD 600 nm by 38.7% compared to the positive control. These results indicate the potential of these phage as an antibiotic alternative for controlling *A. hydrophila* in aquaculture.

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1. Introduction

Aquaculture has emerged as a key sector in global food production, particularly in meeting the rising demand for high-quality protein for human consumption (Brye, 2023). With increasing demand for dietary protein and the stagnation or decline in wild fish catches, aquaculture production has expanded significantly (FAO, 2022). However, this growth is accompanied by various challenges, including disease outbreaks in farmed fish. Bacterial infections are a major constraint in aquaculture, causing substantial economic losses (Irshath *et al.*, 2023). One prevalent pathogen in freshwater fish is *A. hydrophila*. It causes *Motile Aeromonas Septicemia* (MAS), which can lead to mass mortality in cultured catfish (Semwal *et al.*, 2023).

A. hydrophila is a Gram-negative, facultatively anaerobic, rod-shaped bacterium commonly found in aquatic environments, muddy sediments, and various food sources (Liang *et al.*, 2025). This opportunistic pathogen can infect a wide range of animals, including mammals, reptiles, birds, fish, and amphibians (Tomás, 2012). In fish, *A. hydrophila* causes diseases such as red sore disease and ulcerative enteritis, resulting in significant economic losses in aquaculture (Zaheen *et al.*, 2022). Antibiotics remain the primary method for controlling *A. hydrophila* infections. However, inappropriate antibiotic use has led to the emergence of resistant strains (Pepi *et al*., 2021). Research by Nhinh *et al.* (2021) reported that *A. hydrophila* exhibits high resistance to oxacillin, amoxicillin, and vancomycin, as well as resistance to erythromycin, oxytetracycline, florfenicol, and sulfamethoxazole-trimethoprim combinations. Therefore, alternative strategies to antibiotics are needed to combat *A. hydrophila* infections (Liang *et al.*, 2025).

Phage present an environmentally friendly alternative to antibiotics. Phage are viruses that specifically target and kill bacteria and have proven effective in controlling bacterial infections (Youssef *et al.*, 2023). Their unique feature lies in their ability to remain active until a significant reduction in the host bacterial population occurs (Laguna-Castro and Lázaro, 2022). Generally, phage follow one of two life cycles, lytic or lysogenic. Phage with a lytic cycle are known as virulent phage, while those undergoing a lysogenic cycle are termed temperate phage (Makky *et al.*, 2023). Lytic phage offer several advantages as antimicrobial agents, including their natural and organic origin, effectiveness in bacterial control, environmental safety, lack of zoonotic risk, host specificity, and potential for synergistic action (D'accolti *et al.*, 2021).

Studies on phage use against *A. hydrophila* have been reported in Vietnam and Thailand (Dien *et al*., 2022; Le *et al.*, 2018). However, their scope is limited to specific fish species and local environmental conditions, such as striped catfish *Pangasius* spp. in Vietnam and Nile tilapia *O. niloticus* in Thailand. To date, no comprehensive study in Indonesia has investigated the isolation, characterization, and application of phage against *A. hydrophila* in African catfish *C. gariepinus*. This research gap includes the optimization of the *multiplicity of infection* (MOI), assessment of phage stability under tropical pond conditions, and determination of host range against local isolates (Maimaiti *et al.*, 2023). Furthermore, data on phage plaque morphology remain particularly limited, posing a significant challenge for optimizing phage therapy protocols.

Research on phages in Indonesia, particularly those targeting *A. hydrophila*, remains limited. Given these considerations, this study aims to isolate, characterize, and evaluate the potential of phage in controlling *A. hydrophila* growth *in vitro*.

2. Materials and Methods

2.1 Materials

2.1.1 The equipment

The primary equipment used in this study included a 0.22 µm syringe filter (Biosharp Life Sciences, China), 10 mL syringes (OneMed Health Care, Indonesia), 50 mL centrifuge tubes (OneMed Health Care, Indonesia), Duran bottles (DWK Life Science, Germany), microplates (OneMed Health Care, Indonesia), micropipettes (Axygen, USA), blue and yellow micropipette tips (OneMed Health Care, Indonesia), 1.5 mL microtubes (OneMed Health Care, Indonesia), sterile Petri dishes (OneMed Health Care, Indonesia), 250 mL Erlenmeyer flasks (Jaya Abadi Lab, Indonesia), a Multiskan Skyhigh microplate spectrophotometer (Thermo Fisher Scientific, USA), a microcentrifuge (Thermo Fisher Scientific, USA), an IN30 bacterial incubator (Memmert, Germany), a Stemi DV4 stereo microscope (Zeiss, Germany), a refrigerator (GEA Medical, Indonesia), and an autoclave (GEA Medical, Indonesia).

2.1.2 The materials

The materials used in this study included Tryptone Soya Agar (TSA) (Himedia, India), Tryptone Soya Broth (TSB) (Himedia, India), bacteriological-grade agar powder (Himedia, India), RS Medium Base (Himedia, India), ENRO (Enrofloxacin) antibiotic (Anugra PS, Indonesia), glycerol (Merck, USA), sodium chloride (NaCl) (Merck, USA), magnesium

sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (Merck, USA), and Tris-HCl (1st Base, Malaysia).

2.1.3 Ethical approval

This study did not require ethical approval as it did not involve animal experimentation.

2.2 Methods

2.2.1 Research design

This study employed a completely randomized design (CRD) with six treatments and three replicates, consisting of TSB medium control (K-), positive control (K+), antibiotic control with enrofloxacin (KA), and phage treatments at *multiplicity of infection* (MOI) 10 (P10), MOI 1 (P1), and MOI 0.1 (P0.1). The MOI values were selected based on the range commonly used in phage studies to evaluate the effects of varying infection doses, spanning sub-optimal (MOI 0.1), equivalent (MOI 1), and excessive (MOI 10) conditions, in order to assess bacterial lysis efficiency under different infection scenarios. The detailed experimental design used in this study is presented in Table 1. The experimental procedures included preparation of pathogenic test bacteria, phage isolation, purification and storage, plaque characterization, titer determination, host range testing, and bacteriolytic efficacy assay.

Table 1. Experimental design used in the *in vitro* assay

Treatment	Description
K-	300 µL TSB medium without *A. hydrophila* or bacteriophage
K+	200 µL TSB medium + 100 µL *A. hydrophila* (10^8 CFU mL^{-1})
KA	100 µL TSB medium + 100 µL *A. hydrophila* (10^8 CFU mL^{-1}) + 100 µL enrofloxacin antibiotic (0.1 mg mL^{-1})
P0.1	100 µL TSB medium + 100 µL *A. hydrophila* (10^8 CFU mL^{-1}) + 100 µL bacteriophage (10^7 PFU mL^{-1})
P1	100 µL TSB medium + 100 µL *A. hydrophila* (10^8 CFU mL^{-1}) + 100 µL bacteriophage (10^8 PFU mL^{-1})
P10	100 µL TSB medium + 100 µL *A. hydrophila* (10^8 CFU mL^{-1}) + 100 µL bacteriophage (10^9 PFU mL^{-1})

2.2.2 Preparation of test bacteria

The test bacterium used was *A. hydrophila* AH03 from Aquatic Animal Health Laboratory (AAHL), along with four *Aeromonas* sp. Aeromonas isolates were collected from catfish ponds in Sukabumi, Depok, and Bogor. These isolates were obtained from different sources using a selective Rimler Shotts medium as the base. On Rimler Shotts agar, the colonies appeared yellow with black centers. In contrast, when cultured on Tryptic Soy Agar (TSA), the colonies were circular, convex, and creamy white in color. The *A. hydrophila* AH03 isolate from the AAHL collection was cultured on *tryptic soy agar* (TSA) using the streak plate method and incubated at 28–30°C for 24 hours. Identification was performed via polymerase chain reaction (PCR) using the specific primers 685F (5'-GAA-AGG-TTG-CCT-AAT-ACG-TA-3') and 658R (5'-CGT-GCT-GGC-ACC-AAA-GGA-GAG-3'), which target a 685-bp fragment (Altinok *et al.*, 2008). The PCR amplification was carried out under the following conditions: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds; with a final extension step at 72°C for 5 minutes. Sequence analysis using BLAST (www.ncbi.nlm.nih.gov) revealed 99.27% similarity with *A. hydrophila* strain ATCC 7966 accession NR_118944. The obtained isolates were then stored in TSA medium at 4°C as stock cultures. For testing purposes, the isolates were recultured in *tryptic soy broth* (TSB) and incubated in a shaker at 150 rpm for 18–24 hours.

2.2.3 Phage isolation and purification

Phage isolation was performed using a mod ified method from Ture *et al.* (2022). Water samples from catfish ponds in Dramaga, Bogor Regency, were centrifuged at 10.000 rpm for 10 minutes, and the supernatant was filtered through a 0.22 µm *syringe filter* to obtain phage filtrate. The filtrate was then induced with *A. hydrophila* AH03 and subjected to the *double layer agar* (DLA) method using TSB + 0.7% *agar*. Formed plaques were purified by picking single plaques using a sterile pipette until uniform plaques

indicated a pure isolate. Propagation was conducted using *polyethylene glycol* (PEG) 8000 to concentrate phage from the TSB medium. The obtained phage were resuspended in *Saline Magnesium Buffer* (*SM buffer*) as pure isolates.

2.2.4 Phage plaque characterization

Plaque characterization was assessed by observing plaque morphology on the agar surface. Plaque diameter was measured using a caliper, while plaque shape (round, irregular, elongated, or pinpoint), margin type (smooth, indented, or wavy), and clarity (clear, turbid, or halo zone) were recorded (Sankappa *et al.*, 2024).

2.2.5 Phage titer assay

Phage titer was determined using serial dilution (Glonti and Pirnay, 2022). A mixture of 10 µL phage inoculum and 20 µL *A. hydrophila* culture was added to 900 µL *SM buffer*, followed by serial dilution (10^{-1} to 10^{-7}). Each dilution was mixed with 3 mL semi solid TSB (0.7% *agar*) and evenly spread on a TSA base layer in Petri dishes. After 24 hour incubation at 37°C, *plaque forming units* (PFU/mL) were calculated using the formula.

2.2.6 Host range test

The host range was determined to assess phage specificity (broad or narrow) using a modified method (Lyu *et al.*, 2024). Phage isolates were serially diluted (10^{-1} to 10^{-6}) and tested against 10 bacterial isolates (Table 3). A 20 µL log-phase bacterial culture was mixed with 10 µL phage suspension (10^{-4}, 10^{-5}, and 10^{-6} dilutions) and incubated at 37°C for 15 minutes. The mixture was then combined with 3 mL top *agar* (TSB + 0.7% *agar*) and poured onto TSA plates. After 24 hour incubation at 37°C, plaque formation was observed and classified as clear (complete lysis) or no plaque (no lytic activity).

2.2.7 Bacteriolytic efficacy

The bacterial inhibition assay evaluated phage efficacy against *A. hydrophila* (Choliq *et al.*, 2020). Six treatments were tested, and bacterial growth was monitored using a *spectrophotometer* (OD 600 nm) at hourly intervals for 24 hours at 37°C. Growth curves and inhibition percentages were calculated by comparing OD values between treatments and the positive control.

2.3 Analysis Data

This study employed a Completely Randomized Design (CRD). The research data were tabulated using Microsoft Excel and then analyzed descriptively and statistically using SPSS version 25 software. Prior to analysis of variance (ANOVA), normality and homogeneity tests were conducted. If the ANOVA results showed a significant difference ($P < 0.05$), a Tukey's test was performed at a 95% confidence level.

3. Results and Discussion

3.1 Results

3.1.1 Isolation and purification of bacteriophages

Bacteriophage isolation was successfully conducted from various aquatic sources, including aquaculture pond water, rivers, lakes, and public water bodies from several regions in Indonesia, such as Sukabumi, Depok, and Bogor. The isolation method employed was the *double layer agar* (DLA) technique, which proved effective in detecting the lytic activity of phage against the target host, *A. hydrophila* AH03 strain ATCC 7966 accession NR_118944. Among all collected samples, only water samples from Bogor Regency yielded phage with significant lytic activity against *A. hydrophila* AH03. The presence of

Table 3. Bacteriophage plaque characterization

Dilution	Morphological Characteristics			
	Shape	Edge Type	Clarity Level	Plaque Diameter (cm)
1-Oct	Round and *irregular*	Smooth and wavy	Clear	-
2-Oct	Round and *irregular*	Smooth and wavy	Clear	-
3-Oct	Round and *irregular*	Smooth and wavy	Clear	-
4-Oct	Round and *irregular*	Smooth and wavy	Clear	-
5-Oct	Round and *irregular*	Smooth and wavy	Clear	-
6-Oct	Round and *irregular*	Smooth and wavy	Clear	0.17 ± 0.02
7-Oct	Round and *irregular*	Smooth and wavy	Clear	0.35 ± 0.02

phage was indicated by the formation of clear zones (plaques) on the *agar* layer, demonstrating their ability to infect and lyse the target bacteria.

3.1.2 Phage density

Phage density was assessed using the DLA method with serial dilutions ranging from 10^{-1} to 10^{-7}. The observed plaque counts at each dilution are presented in Table 2. At a dilution of 10^{-6}, the plaque count ranged between 54–61 PFU (*plaque forming units*), with phage concentrations reaching 5.4×10^9 to 6.1×10^9 PFU/mL. Meanwhile, at a dilution of 10^{-7}, the plaque count stabilized at 5–6 PFU, corresponding to a concentration of 5.68×10^9 PFU/mL. For dilutions from 10^{-1} to 10^{-5}, the plaque count was too numerous to count (TNTC), indicating a high phage population in the isolate.

3.1.3 Characterization of phage plaques

The morphological characterization of phage plaques was conducted by observing their shape, edge type, clarity level, and diameter at various dilution levels. The observations revealed that the phage plaques exhibited round and *irregular* shapes with smooth to wavy edges and high clarity across all dilution levels. Plaque diameter measurements were performed at dilutions of 10^{-6} and 10^{-7}, yielding average diameters of 0.17 ± 0.02 cm and 0.35 ± 0.02 cm, respectively (Table 3). These morphological characteristics indicate strong lytic activity of the phage isolate (Figure 1).

3.1.4 Host range

The host range assay was performed against ten bacterial targets, including five *A. hydrophila* iso

Table 2. Density of *A. hydrophila* lytic bacteriophages

Dilution	Replicate			Bacteriophage Concentration (PFU/mL)			Average (PFU/ml)
	1	2	3	1	2	3	
1-Oct	TNTC	TNTC	TNTC	-	-	-	
2-Oct	TNTC	TNTC	TNTC	-	-	-	
3-Oct	TNTC	TNTC	TNTC	-	-	-	-
4-Oct	TNTC	TNTC	TNTC	-	-	-	
5-Oct	TNTC	TNTC	TNTC	-	-	-	
6-Oct	61	56	54	6.1×10^9	5.6×10^9	5.4×10^9	5.68×10^9

Note : TNTC (too numerous to count)

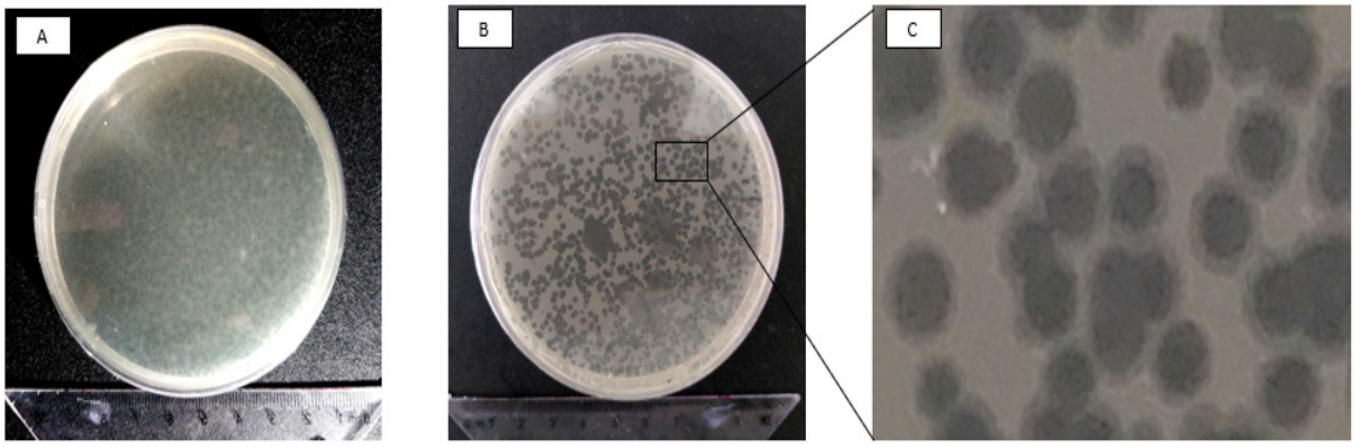

Figure 1. Characterization of lytic bacteriophage plaques isolated from *A. hydrophila* in Dramaga region, Bogor Regency. (A) High-density plaques that could not be counted individually, (B) Discrete plaques that were visually countable, (C) Magnified view of panel B.

lates (AH01–AH05) and five bacteria from different genera. The results demonstrated that the phage only formed plaques on the *A. hydrophila* isolate. The absence of plaques on the other nine isolates indicates that the isolated phage has a narrow host range and exhibits specificity toward *A. hydrophila* AH03. The host range test results are summarized in (Table 4), while plaque formation against different bacterial isolates is shown in (Figure 2).

3.1.5 Bacteriolytic effectiveness

The measurement of optical density at 600 nm (OD 600 nm) revealed distinct growth kinetics among the phage treatments (P0.1, P1, P10), the antibiotic control (KA), and the positive control (K+). The K+ group exhibited a steady increase in OD 600 nm over the 24 hour period, indicating unimpeded bacte rial growth. In contrast, growth was suppressed in all

Table 4. Host range test results

NO	Target Bacteria	Bacteriophage Plaque Formation
1	*A. hydrophila* AH01 (*Helostoma temminckii* isolate)	-
2	*A. hydrophila* AH02 (*Oreochromis niloticus* isolate)	-
3	*A. hydrophila* AH03 (AAHL collection isolate)	+
4	*A. hydrophila* AH04 (Sukabumi isolate)	-
5	*A. hydrophila* AH05 (IPB experimental pond isolate)	-
6	*Streptococcus agalactiae* (AAHL collection isolate)	-
7	*Citrobacter freundii* (AAHL collection isolate)	-
8	*Edwardsiella tarda* (AAHL collection isolate)	-
9	*Staphylococcus aureus* (AAHL collection isolate)	-
10	*Vibrio parahaemolitycus* (AAHL collection isolate)	-

Note : (+) Plaque formation observed, (–) No plaque formation

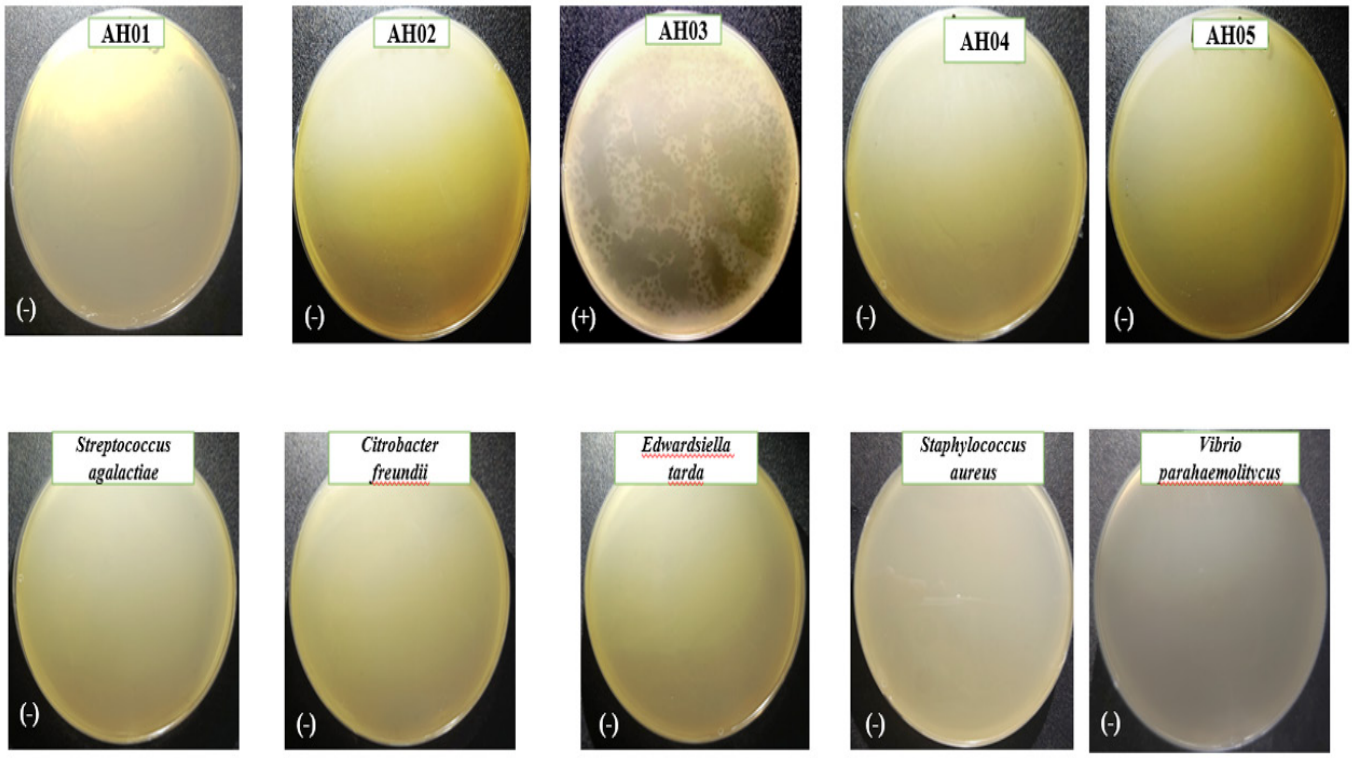

Figure 2. Results of the host range test of the bacteriophage against ten target bacteria.

phage treated groups and the KA group. Specifically, in the phage treated groups, an initial increase in OD 600 nm was observed within the first 7 hours, albeit at a significantly lower rate than in the K+ control. After this period, the OD 600 nm values in these groups plateaued and then declined by the 24 hour time point. This kinetic profile indicates that approximately 7 hours were required for the phage to reach peak lytic activity, leading to effective suppression of *A. hydrophila* growth under the tested *in vitro* conditions. The bacterial growth curve during 24 hours of incubation is illustrated in (Figure 3). The optical density values of each treatment after 24 hours are presented in (Figure 4).

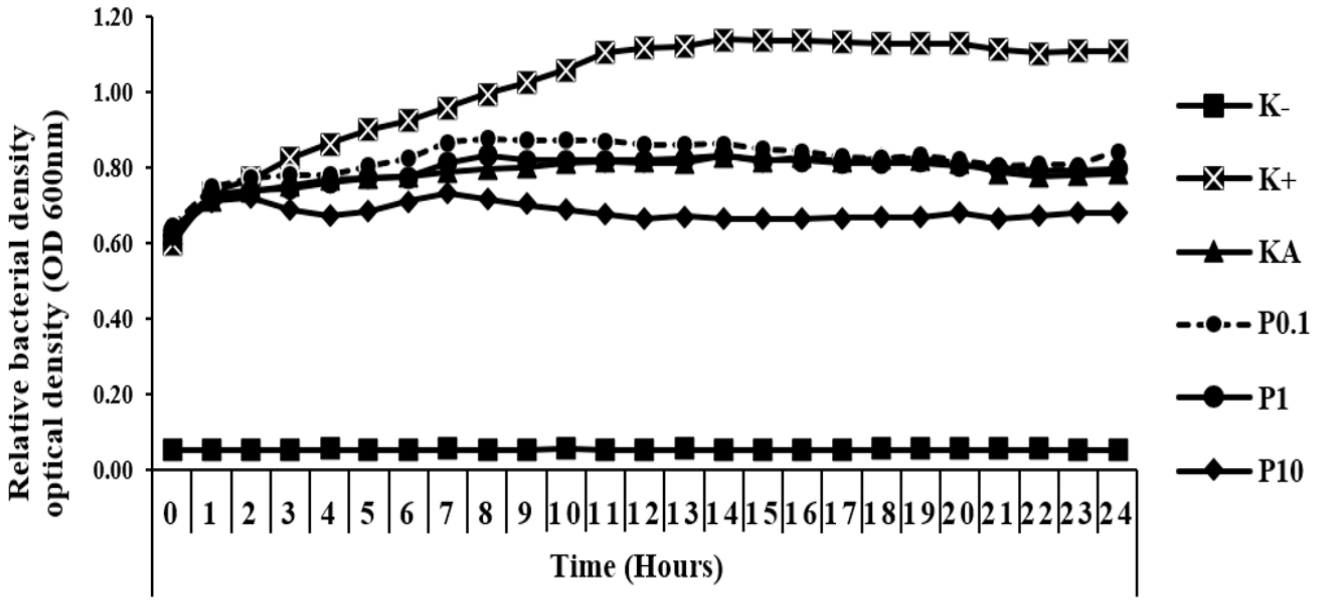

Figure 3. Bacterial growth curve over 24 hours measured via *spectrophotometer* at optical density 600 nm.

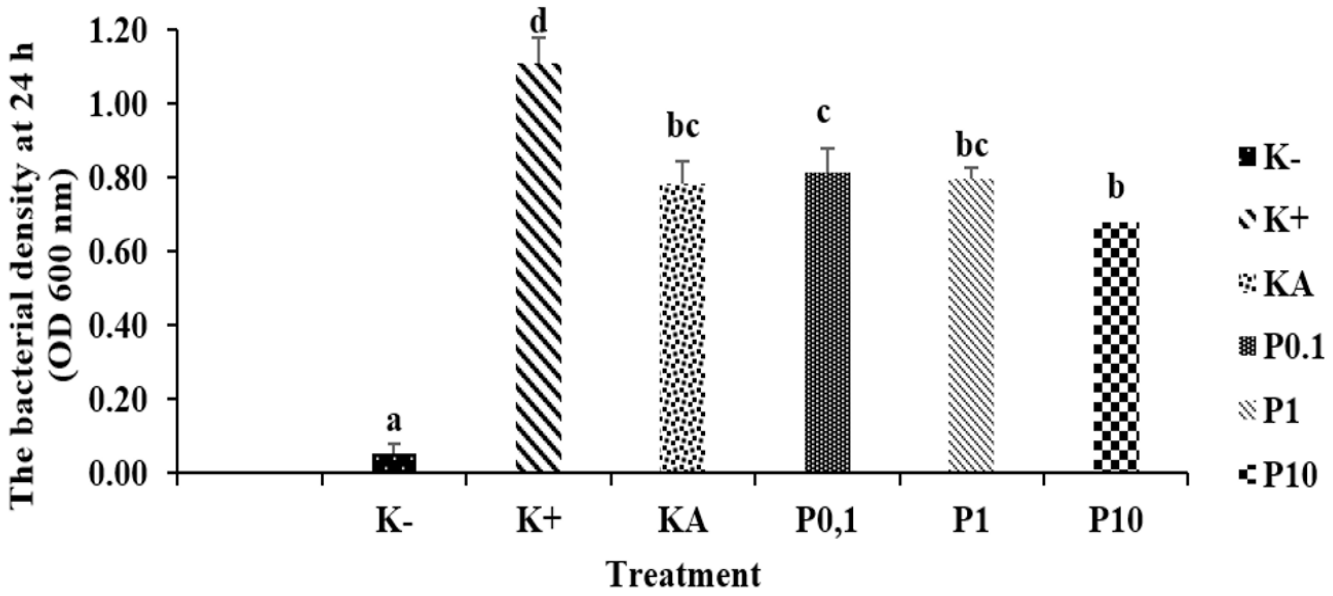

Figure 4. Bacterial growth values at 24 hours measured via *spectrophotometer* (OD 600 nm). Different letters denote statistically significant differences between treatments based on tukey's test ($P < 0.05$).

3.2 Discussion

3.2.1 Isolation and purification of phage

The emergence of antibiotic resistant pathogenic bacteria has become a serious challenge in aquaculture, particularly in managing *A. hydrophila* infections in catfish, as antimicrobial therapy options become increasingly limited (Akmal *et al.,* 2020). This antibiotic resistance phenomenon has driven the search for more sustainable infection control alternatives. Phage, as biological therapeutic agents, have long been utilized for bacterial infection control since their initial discovery. The clinical efficacy of phage therapy has been demonstrated in humans, animals, plants, and aquaculture practices (Jun, 2024). Their specific mechanism of action and lytic capability against target bacteria make phage a potential solution to antibiotic resistance. Several recent studies have successfully isolated phage targeting *A. hydrophila* and demonstrated their protective effects in preventing and controlling bacterial infections in fish (Feng *et al*., 2022). These findings reinforce the potential of phage therapy as an alternative approach in sustainable aquaculture health management.

Phage were successfully isolated from water samples collected from catfish aquaculture ponds in Bogor Regency, West Java. Lytic phage were identified by observing plaque formation on *double layer agar* (DLA) media. The results revealed clear zones (plaques), indicating lytic activity against the host bacteria. The formation of clear plaques on DLA media aligns with the findings of **Ács** *et al*. (2020), who reported that phage capable of infecting target bacteria produce clear lysis zones. This mechanism begins with the lysis of a single bacterial cell by one phage particle, followed by replication and spread to infect neighboring bacterial cells. According to Shaniyah (2023), the use of the DLA method with soft *aga*r as an overlay plays a crucial role in facilitating phage replication.

3.2.2 Phage density

Optimal isolation conditions allowed the phage to efficiently propagate within its host cells, yielding high quality isolates. The study results showed a phage density of 5.68×10^9 PFU/mL, which falls within the high range. High phage density is a key indicator of their effectiveness as biocontrol agents (Jo *et al.,* 2025). A study by Abedon (2017) emphasized that sufficient phage concentration is required for effective elimination of target bacteria, enabling rapid and efficient adsorption. The optimal phage density for bacterial infection and lysis ranges from 10^6 to 10^{11} PFU/mL (Clokie and Kropinski, 2009).

3.2.3 Phage plaque characterization

Morphological characterization of phage plaques at various dilution levels revealed that the plaques were round to *irregular* in shape, with smooth to wavy edges, and exhibited consistent clarity. The measured plaque diameters at 10^{-6} and 10^{-7} dilutions were 0.17 ± 0.02 cm and 0.35 ± 0.02 cm, respectively. According to Richards (2014), clear plaques indicate a virulent or lytic phage, which is essential for phage therapy applications. The clear plaque morphology reflects the lytic nature of the phage, leading to complete host cell lysis. This finding aligns with the observations of Valencia-Toxqui and Ramsey (2024), who reported that lytic phage produce clear plaques due to total destruction of bacterial host cells without genetic integration, unlike lysogenic phage.

3.2.4 Host range

The phage isolated in this study demonstrated a narrow host range. As presented in (Table 3), they formed plaques exclusively on a single strain, *A. hydrophila* AH03 (ATCC 7966), and exhibited no lytic activity against other tested strains from the same or different genera. This indicates a high degree of specificity for *A. hydrophila* AH03. This finding is consistent with the critical role of phage structural proteins in host recognition. For instance, He *et al.* (2024) demonstrated that mutations in tail tube proteins can alter host range, establishing receptor recognition as a primary determinant of phage specificity. The high selectivity observed in our isolates aligns with this mechanism.

3.2.5 Bacteriolytic efficacy

Growth inhibition assays of *A. hydrophila* using spectrophotometry at 600 nm demonstrated that phage treatment significantly suppressed bacterial growth. Among the tested treatments, a multiplicity of infection (MOI) of 10 (P10) exhibited the most optimal inhibitory effect. This finding is consistent with the study by Liu *et al.* (2020), which evaluated the bacteriolytic activity of five phage isolates against *A. hydrophila* at different MOI. Their study reported a faster and more significant reduction in optical density (OD 600 nm) at higher MOI, with the lowest absorbance observed at 7 hours post treatment, particularly at MOI 10.

4. Conclusion

The lytic phage isolated from Dramaga, Bogor Regency, demonstrated *in vitro* efficacy in controlling *A. hydrophila*, exhibiting a high density (5.68×10^9

PFU/mL), clear plaque morphology, and high specificity for the *A. hydrophila* AH03 isolate. Treatment with an MOI of 10 (P10) was the most effective in inhibiting bacterial growth. These findings suggest that phage hold promise as an environmentally friendly alternative to antibiotics in aquaculture disease management. Future studies should focus on developing phage cocktails that combine multiple phage with distinct host ranges to enhance the control efficacy against *A. hydrophila*. This approach may reduce the risk of phage resistance emergence, broaden the coverage against diverse pathogenic strains, and improve field application success. *In vivo* trials across various cultured fish species, along with stability testing of phage cocktails under different environmental conditions, represent critical steps to ensure safety and efficacy prior to commercial implementation.

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Authors' Contributions

All authors contributed to the final manuscript. The specific contributions were as follows: NJ conducted the research, collected data, and prepared the initial draft. DW, W, and S supervised the research, reviewed the findings, and performed manuscript editing and refinement.

Conflict of Interest

The authors declare that they have no competing interests.

Declaration of Artificial Intelligence (AI)

The authors acknowledge the use of ChatGPT and DeepSeek for language refinement in preparing this manuscript. All AI-generated content was rigorously reviewed, edited, and validated to ensure accuracy, originality, and scientific relevance. The authors take full responsibility for the final content.

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