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The potential of bacteriophage for controlling *Vibrio parahaemolyticus* as *in-vitro*

Potensi bakteriofage untuk pengendalian *Vibrio parahaemolyticus* secara *in-vitro*

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ABSTRACT

Vibrio parahaemolyticus infection is a major threat to Pacific white shrimp aquaculture, causing significant shrimp mortality. Bacteriophages (phages) provide a promising antibiotic alternative due to their specificity towards specific bacteria. This research includes essential steps isolating phages from shrimp pond water and characterizing them, including plaque morphology, spot tests, phage density evaluations, host range assessments, and in vitro studies targeting *V. parahaemolyticus*. In-vitro tests encompassed six treatments, each with three replicates: negative control (K-), positive control (K+), antibiotic control, phage density of 10^7 (FB7), phage density of 10^8 (FB8), and phage density of FB10^9 (FB9). Phages were successfully isolated from shrimp pond water in Banyuwangi, indicated by plaque formation on double-layer agar media. These phages exhibited morphological characteristics, featuring small, round plaques (d= 0.02-0.5 ± 0.08 cm) with flat, wavy edges and clear plaque. Phage plaques were amplified with an average density of 3.5×10^9 PFU/mL. Host range tests revealed that only *V. parahaemolyticus* Situbondo was susceptible to the isolated phages, while five other bacterial strains were not. In bacterial inhibition tests, treatments with different phage densities significantly outperformed the negative control, media control, and antibiotic control. However, the antibiotic control performed better than phage density treatments (0.61 ± 0.01). All phage density treatments effectively reduced *V. parahaemolyticus* density compared to the negative control, with the most favorable results observed in the FB9 treatment at a density of 10^9 PFU/mL.

Keywords: aquaculture, antibiotic, bacteriophage, vannamei shrimp, *Vibrio parahaemolyticus*

ABSTRAK

Penyakit akibat infeksi *Vibrio parahaemolyticus* menjadi perhatian utama dalam budidaya udang vaname saat ini karena dapat menyebabkan kematian massal pada udang. Penggunaan bakteriofage (fage) dapat menjadi alternatif solusi untuk menggantikan antibiotik, karena fage bersifat spesifik pada bakteri tertentu. Penelitian ini meliputi beberapa tahapan, yaitu isolasi fage dari beberapa sumber air tambak, karakterisasi fage meliputi morfologi plak fage, *spot test*, dan uji kisaran inang, serta uji *in vitro* penghambatan *V. parahaemolyticus* oleh fage. Uji *in vitro* dilakukan dengan enam perlakuan dan masing masing tiga ulangan yaitu kontrol negatif (K-), kontrol positif (K+), kontrol antibiotik, kepadatan fage 10^7 (FB7), kepadatan fage 10^8 (FB8), dan kepadatan fage 10^9 (FB9). Penelitian ini berhasil mengisolasi fage yang bersumber dari air tambak udang di Banyuwangi ditandai dengan terbentuknya plak pada media *double layer* agar, dengan ciri morfologisnya berbentuk titik-titik dan bulat besar (d= 0,02 ± 0,08 cm), tepian rata dan bergelombang serta memiliki warna plak yang bening. Plak fage dapat diperbanyak dengan densitas fage rata-rata $3{,}5\times10^9$ PFU/mL. Untuk uji kisaran inang menggunakan enam jenis bakteri dengan sumber dan strain yang berbeda, menunjukkan bahwa hanya bakteri *V. parahaemolyticus* Situbondo yang dapat lisis oleh fage hasil isolasi, sedangkan lima jenis bakteri lainnya tidak. Pada daya hambat bakteri, perlakuan dengan penambahan pada setiap densitas fage berbeda nyata dibandingkan dengan kontrol negatif, kontrol media dan kontrol antibiotik, walaupun kontrol antibiotik lebih baik dari perlakuan densitas fage (0,61 ± 0,01). Semua perlakuan kepadatan fage mampu mengurangi kepadatan *V. parahaemolyticus* dibandingkan dengan perlakuan kontrol negatif. Hasil terbaik terdapat pada perlakuan FB9 yaitu 10^9 PFU/mL.

Kata kunci: akuakultur, antibiotik, bakteriofage, udang vaname, *Vibrio parahaemolyticus*

INTRODUCTION

Aquaculture currently plays an important role in the global economy and is the fastest-growing supplier of animal protein in the world, increasing by approximately 10-20% annually (Ahmed *et al*., 2021). One of the prominent commodity in aquaculture sector is Pacific white shrimp. A significant problem that occurs during the production phase of shrimp is disease. The disease that frequently affects white shrimp is bacterial diseases. Vibriosis is a major concern in vannamei shrimp farming, caused by species of *Vibrio* sp., as these bacteria can lead to mass mortality in shrimp (Annam, 2008).

One of *Vibrio parahaemolyticus* strain that contains the toxins PirA and PirB causes Acute Hepatopancreatic Necrosis Disease (AHPND) (Tran *et al*., 2013). This disease typically appears around eight days after stocking and can result in up to 100% mortality within 20-30 days (Choi *et al*., 2017; Kumar *et al*., 2020). AHPND can cause atrophy, clinical symptoms such as anorexia, an empty digestive tract, pale soft tissues, and a pale and undeveloped hepatopancreas (Soto-Rodriguez *et al*., 2015; Kumar *et al*., 2021; Schofield *et al*., 2021; Valente & Wan, 2021). Additionally, shrimp infected with *V. parahaemolyticus* bacteria experience a weakened immune system (Tran *et al*., 2013; Ahmed *et al*., 2021). Recent treatments have involved using chemicals in the form of antibiotics to suppress bacterial infections.

However, this approach has created new problems, especially for the environment, hosts, and humans (Yin *et al*., 2014). Continuous use of antibiotics in aquaculture environments can pose dangers to aquatic organisms and humans who consume fish or shrimp, as it results in antibiotic residues and toxic effects (Okeke *et al*., 2022). According to Romano *et al*. (2015), Elmahdi *et al*. (2016), and Okeke *et al*. (2022), *Vibrio* species have developed resistance to tetracycline, cephalosporins, quinolones, aminoglycosides, erythromycin, sulphamethoxazole, rifampicin, doxycycline, tetracycline, and amoxicillin. The use of bacteriophages (phages) can be an alternative solution to antibiotics. Phages are specific killers of pathogenic bacteria and are known to be effective in controlling bacterial infections in aquaculture (Gxalo *et al*., 2021; Adesiyan *et al*., 2022).

The advantages of using lytic phages as antimicrobials are that they are considered natural and organic, easy to isolate and propagate because they can replicate, easy to apply either through feed or direct mixing with water, synergistic in cocktails (multi-component phage preparations), applicable for therapeutic or bioremediation purposes, ubiquitous, considered safe with minimal unwanted effects, and relatively inexpensive (Rao & Lalitha, 2015). Research by Alagappan *et al*. (2010) indicates that phages can only infect bacteria specifically. Therefore, phages can kill target bacteria without affecting the normal microflora of the environment. Research on phages in the field of aquaculture has been conducted by several researchers.

Some study reported that giant tiger shrimp infected with *V. parahaemolyticus* and fed with a phage cocktail showed a 70% increase in survival compared to the control group (Alagappan *et al*., 2016). Giant tiger shrimp larvae given phages and infected with *V. harveyi* had a 70% increase in survival compared to the control group (Vinod *et al*., 2006). The application of phages to white shrimp infected with *V. parahaemolyticus* was able to reduce mortality and inhibit the development of the infection (Lomelí-Ortega & Martínez-Díaz, 2014). Additionally, phages can reduce the population of *V. parahaemolyticus* in infected oysters (Rong *et al*., 2014). Therefore, this study aims to evaluate the potential of phages in controlling the growth of *V. parahaemolyticus* in vitro.

MATERIAL AND METHODS

Time and place

This research was conducted from June to October 2023 at the Aquatic Organism Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University.

Test materials

The test bacteria used were *V. parahaemolyticus* obtained from the collection of the Aquatic Organism Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University. Bacteriophages were obtained from water, sediment, and shrimp samples from ponds in various regions of Indonesia.

Experimental design

The research design for evaluating the effectiveness of phages in inhibiting the growth of *V. parahaemolyticus* in vitro used six treatments

with three repetitions. The experimental design can be seen in Table 1.

Methods

Preparation of *V. parahaemolyticus*

V. parahaemolyticus were obtained from thiosulfate citrate bile sucrose (TCBS) selective media stored at the Aquatic Organism Health Laboratory, IPB University. They were then isolated culture on sea water complete (SWC) broth and incubated at 28°C for 24 hours. The results were subjected to multiple isolations to obtain pure isolates. Pure isolates were identified by performing gram staining and biochemical tests and confirmed using conventional PCR with the AP4 primer (pirAVp and pirBVp).

The AP4 primer set for the first step PCR consists of AP4-F1 (5'- ATG-AGTAAC-AT-ATA-AAA-CAT-GAA-AC-3'), and for the nested PCR, it consists of AP4-F2 (5'-GTT-AGT-CAT-GTG-AGC-ACC-TTC-3') (Dangtip *et al*., 2015). First, bacterial extraction was performed using the heat method. Dba preparation for analysis involved isolating a single bacterial colony and suspending it in 200 µL TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) in a 1.5 mL centrifuge tube, followed by vortexing. The bacterial suspension was then lysed on a dry bath at 98°C for 10 minutes, followed by placement on ice for five minutes. The lysate was centrifuged at 10,000 rpm for two minutes, and the supernatant was used as the PCR DNA template.

Amplification for the first step consists of 30 cycles (pre-heat at 94°C for two minutes, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 90 seconds, final extension at 72°C for two minutes), while nested amplification involves 25 cycles (pre-heat at 94°C for two minutes, denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds, extension at 72°C for 20 seconds, final extension at 72°C for two minutes). The desired DNA amplicon size using this method is 230 bp (Dangtip *et al*., 2015). Confirmed isolates were cultured on sea water complete (SWC) agar media and stored at 4°C. Isolates used for phage isolation were cultured in SWC broth media and shaken for six hours. Subsequently, the total plate count (TPC) was calculated, resulting in a density of 10^8 CFU/mL (Dangtip *et al*., 2015).

Bacteriphage isolation

Phages isolation refers to Wang *et al*. (2017) with modifications. To isolate phages, 50 mL of water samples and 25 g of pond sediment samples were added to PBS (1:1, %w). The shrimp gut samples were ground until smooth, then added to PBS (1:1, %w), placed into 1 mL tubes, and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was filtered using a 0.45 µm syringe filter. 10 mL of the phage filtrate was taken and added to an Erlenmeyer flask containing 40 mL of SWC broth and 1 mL of *V. parahaemolyticus* liquid isolate. This mixture was shaken for 24 hours at a speed of 160 rpm. The result was placed into 1 mL tubes and centrifuged at 10,000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was collected and filtered again using a 0.22 µm syringe filter. This filtration process is to separate bacteria from the phages.

Table 1. In vitro experimental design.

Treatment	Annotation
KN	SM-buffer 300 µL without *V. parahaemolyticus*
KP	SM-buffer 100 µL and *V. parahaemolyticus* 200 µL (10^7 CFU/mL)
KA	Chlortetracycline antibiotics 100 µL (32 µg/mL) and *V. parahaemolyticus* 200 µL (10^7 CFU/mL)
FB7	Phages 100 µL (10^7 PFU/mL) and *V. parahaemolyticus* 200 µL (10^7 CFU/mL)
FB8	Phages 100 µL (10^8 PFU/mL) and *V. parahaemolyticus* 200 µL (10^7 CFU/mL)
FB9	Phages 100 µL (10^9 PFU/mL) and *V. parahaemolyticus* 200 µL (10^7 CFU/mL)

Table 2. Interpretation of Gram staining and biochemical tests on *V. parahaemolyticus* bacteria.

Isolate	Gram staining			Biochemical test			
	Colony color	Gram	Form	Catalase	Oxidase	Motile	O/F
Vibrio parahaemolyticus	Green	Negative	Bacil	+	+	+	F

Plaque assay

The plaque assay was conducted using two methods: co-culture and spot test. In the co-culture method, the phage filtrate samples were diluted up to 10^{-4} using an SM-buffer solution (200 mM NaCl, 10 mM MgSO4.7H$_2$O, 50 mM Tris HCl, pH 7.5). 100 µL of each dilution was added to 200 µL of *V. parahaemolyticus* bacterial isolate with a density of 10^8 CFU/mL, which had been cultured in SWC broth for six hours. The mixture of phage filtrate and *V. parahaemolyticus* was added to 3 mL of sterile top agar (SWC broth + 0.7% agar powder), then poured onto solid SWC agar media (as bottom agar).

The media was gently swirled in a figure-eight motion to evenly distribute the top agar. For the spot test method, *V. parahaemolyticus* bacteria were added to 3 mL of sterile top agar (SWC broth + 0.7% agar powder) and poured onto solid SWC agar media (bottom agar). After solidifying, 10 µL of phage filtrate was dropped onto the double-layer agar media. The solidified media was incubated at 37°C in an inverted position for 48 hours, followed by observation of plaque formation. Phage formation was observed every 24 hours. The number of plaques (lysis zones) against *V. parahaemolyticus* was analyzed using the double-layer agar method (Damayanti *et al*., 2016). Plaques formed on the media were counted using the formula from Damayanti *et al*. (2016). The resulting number is expressed in plaque-forming units (PFU/mL).

$$\text{Bacteriophage (PFU/ mL)} = \frac{\text{Number of Plaques (PFU)}}{\text{Dilution} \times \text{Inoculum Volume}}$$

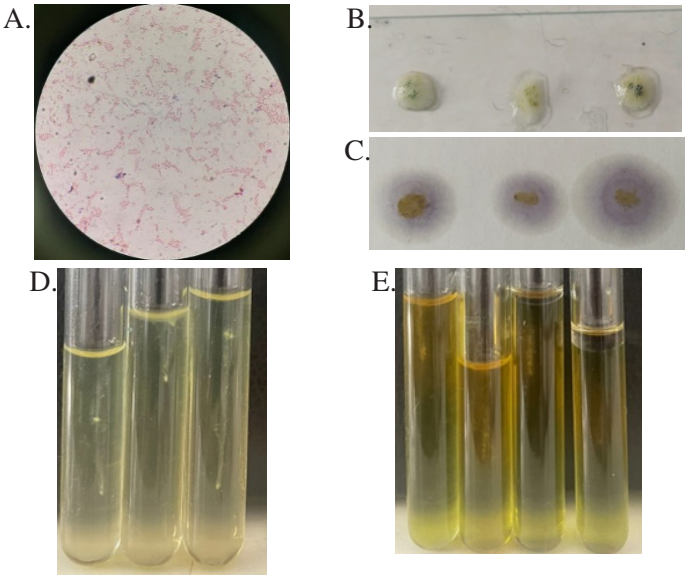

Figure 1. Biochemical tests are used to determine the characteristics and identify the strain of *Vibrio* bacteria by evaluating the reactions in the test media used.
Note: A. Gram staining; B. oxidase test; C. catalase test; Dd. motility test; E. O/F test (Haryati, 2020).

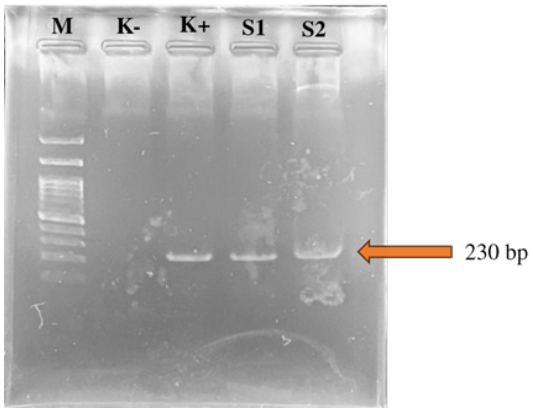

Figure 2. Confirmation of PCR results.
Note: M = marker; K- = negative control; K+ = positive control; S1 = purified *V. parahaemolyticus* sample; S2 = initial *V. parahaemolyticus* source sample used.

Purification and storage of bacteriophages

Purification of phages was performed using the method of Kropinski *et al*. (2009) with slight modifications. A single phage plaque was isolated and then diluted to a concentration of 10^{-4}. From each dilution, 100 µL was taken and added to 500 µL of *V. parahaemolyticus* culture (10^{8} CFU/mL). This mixture was incubated for 10 minutes at 37°C, followed by the addition of 3 mL of semi-solid SWC medium. The mixture was then poured onto solid SWC medium and incubated for 24 hours at 37°C. A single plaque with clear lysis was picked using a sterile loop and placed into 5 mL of SM buffer. The single lytic phage filtrate was centrifuged at 10,000 rpm for five minutes, the supernatant was collected, and then filtered using a 0.22 µm syringe filter.

The single lytic phage was amplified using the method of Bonilla *et al*. (2016) with modifications. A 1% *V. parahaemolyticus* culture was added to 100 mL of SWC broth, along with 200 µL of lytic phage lysate. This mixture was incubated at 37°C with shaking at 150 rpm until clear lysis was observed. The mixture was then centrifuged at 10,000 rpm at 4°C for five minutes. The supernatant was collected and filtered using a 0.22 µm syringe filter. The phage was then titrated to determine the infection dose, growth curve, bacteriolytic activity, and morphology.

Bacteriophage density test

The phage density test was analyzed using the dilution method according to Kauffman and Ploz (2018) and Jatmiko *et al*. (2018) with slight modifications. A 0.1 mL phage isolate and 0.5 mL *V. parahaemolyticus* isolate were added to 0.9 mL of SM buffer (dilution range 10^{-1} to 10^{-7}). After dilution, 0.1 mL of each dilution was placed into a test tube, 3 mL of semi-solid SWC was added, and the mixture was homogenized. This suspension was poured onto petri dishes and spread evenly, followed by incubation at 37°C for 24 hours. The resulting plaques were observed and counted using the PFU/mL formula.

Bacteriophage plaque characterization

The plaque characterization test was conducted by observing the plaques formed on the media surface and measuring their diameter using a ruler. The shape (round, irregular elongated, or small spots), edge type (smooth, lobed, or wavy), and plaque clarity (clear, cloudy, or halo zone) were also noted (Deshanda *et al*., 2018).

Host range test

The host range test aims to determine whether the phage has a narrow or broad host spectrum. This test refers to De Melo *et al*. (2019) with modifications. Six types of bacteria were tested with phage using the double-layer agar method. Each bacterium was first cultured for six hours (log phase). Then, 0.5 mL of the culture was taken into a test tube, and 0.1 mL of phage isolate was added. The mixture was incubated at 37°C for 15 minutes. After incubation, semi-solid SWC was added, poured onto solid SWC media, and incubated for up to 48 hours at 37°C. The formation of clear zones or plaques was then examined and classified as clear, cloudy, or absent. Media showing clear or cloudy inhibition zones indicated that the phage could lyse the tested bacteria.

Bacterial inhibition test

The bacterial inhibition test aims to determine the effectiveness of the phage in inhibiting the growth of pathogenic bacteria. The effectiveness test refers to Bicalho *et al*. (2010) and Choliq *et al*. (2020) with modifications. Log-phase *V. parahaemolyticus* bacteria (6-hour culture) were taken in 200 µL amounts into a microplate, and 100 µL of each treatment dose was added. The bacterial inhibition test was observed using a spectrophotometer by measuring the optical density (OD) at 600 nm every hour for 24 hours.

Data analysis

This study used a completely randomized design (CRD). Research data were tabulated using Microsoft Excel. The data were then analyzed descriptively and statistically using SPSS 20 software. If the ANOVA test results showed significant differences ($P<0.05$), Duncan's test was performed at a 95% confidence interval.

RESULT AND DISCUSSION

Result

Isolation and purification of bacteriophages

The phage isolation results were obtained by isolating several samples of pond water, sediment, and shrimp affected by AHPND from different pond locations such as Anyer, Banyuwangi, Lampung, Lebak, Lombok, Pandeglang, Ujung Kulon, and Yogyakarta using the double layer agar method and spot test. Banyuwangi phage was obtained. The isolation results yielded only 1-2

PFU/mL using the double layer method (Figures 3A-C) and the spot test method formed plaques with sizes of 2-3 cm (Figure 3D).

Bacteriophages density

From the observation results (Table 3), it was found that the Banyuwangi phage isolate plaques at a dilution of 10^{0}-10^{-5} produced too many plaques to be counted (TNTC). Meanwhile, at a dilution of 10^{-6}, there were 482 plaques, and at 10^{-7}, there were 22 plaques, with an average of 3.5×10^{9} PFU/mL and an average diameter of 0.02 ± 0.08 cm.

Characterisation of bacteriphages plaque

In the characterization of phage plaques, the shape, edges, and clarity of the plaques were observed. The characterization results of the Banyuwangi phage showed morphological features with two types of plaque shapes: pinpoint and large round. The edges were either smooth or wavy, and the clear color of the plaques indicated the presence of phage.

Host range

The host range used six types of bacteria from different sources and strains. Based on the host range test results, only *V. parahaemolyticus* from Situbondo tested positive, while the other five types of bacteria tested negative.

Bacterial inhibition activity in vitro

The inhibitory effect on *V. parahaemolyticus* using phages of different densities (FB7, FB8, and FB9) and KA was observed using a

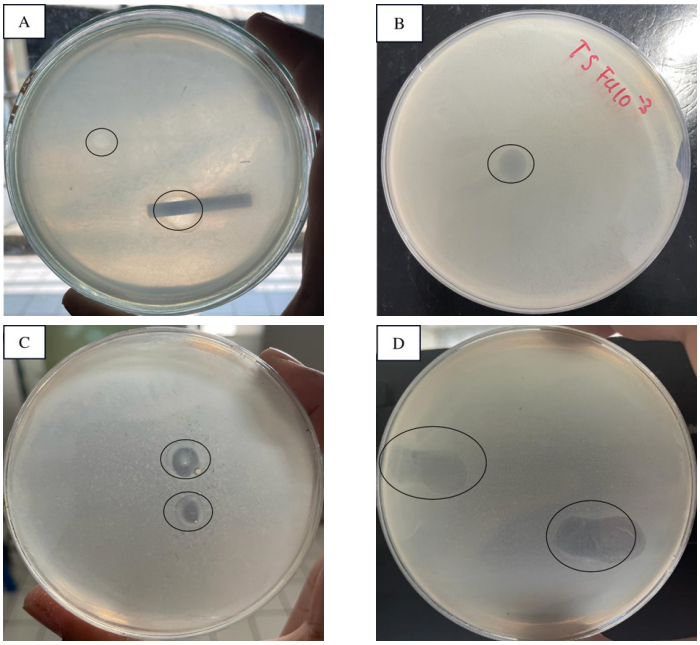

Figure 3. Inhibition zones of V. *parahaemolyticus* by phages forming clear zones or plaques. A-C = double-layer method and D = spot test method.

Table 3. Determination of phage density in inhibiting *V. parahaemolyticus*.

Dilution	Plaque number	Phage concentration (PFU/mL)	Average of plaque diameter (cm)
10^{0}	TNTC	-	0.008-0,5
10^{-1}	TNTC	-	0.008-0.01
10^{-2}	TNTC	-	0.008-0.01
10^{-3}	TNTC	-	0.008-0.01
10^{-4}	TNTC	-	0.008-0.01
10^{-5}	TNTC	-	0.008-0.01
10^{-6}	482	4.8×10^{9}	0.008-0.01
10^{-7}	22	2.2×10^{9}	0.008-0.01
Average	252	3.5×10^{9}	0.008-0.5

Note: TNTC (too numerous to count).

Tabel 4. Karakterisasi plak fage Banyuwangi.

Dilution	Morphological Characterisation		
	Shapes	Edges	Clarity level
10^{0}	Dotted and irregular	Smooth and wavy	Clear
10^{-1}	Dotted and irregular	Smooth and wavy	Clear
10^{-2}	Dotted and irregular	Smooth and wavy	Clear
10^{-3}	Dotted	Smooth	Clear
10^{-4}	Dotted	Smooth	Clear
10^{-5}	Dotted	Smooth	Clear
10^{-6}	Dotted	Smooth	Clear
10^{-7}	Dotted	Smooth	Clear

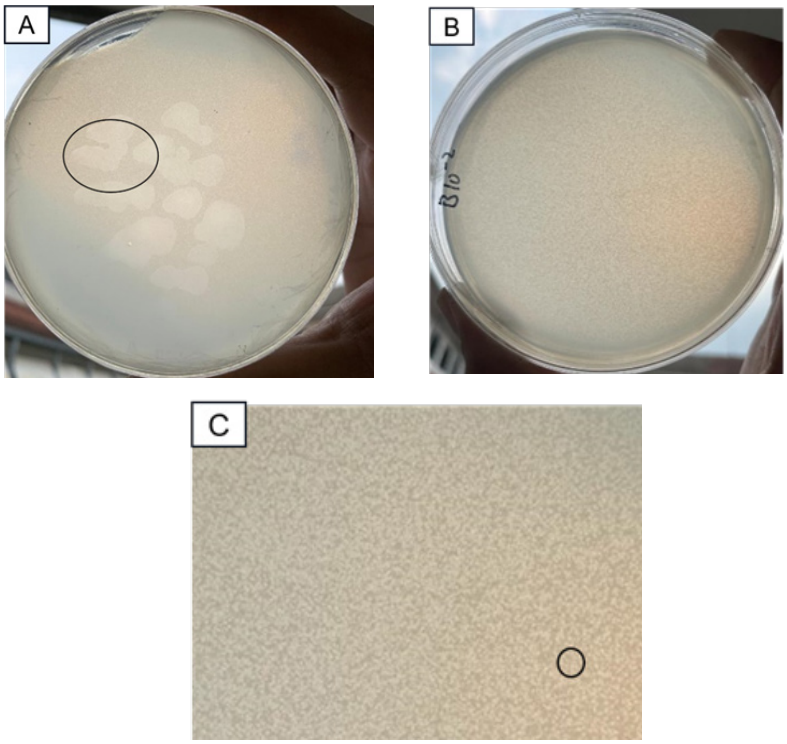

Figure 4. Characterization of lytic phage plaques of *V. parahaemolyticus*. A = irregular phage plaques; B = dotted phage plaques; C = enlarged phage plaques.

Table 5. Host range test of Banyuwangi phage with different bacterial strains.

No	Host	Phage Isolate
1	*V. parahaemolyticus* Situbondo	+
2	*V. parahaemolyticus* Anyer	-
3	*V. parahaemolyticus* Banyuwangi	-
4	*V. harveyi*	-
5	*Aeromonas hydrophilla*	-
6	*Aeromonas salmonicida*	-

Note: (+) plaques forming and (-) did not form plaques.

spectrophotometer by measuring the OD value at a wavelength of 600 nm. This observation was carried out for 24 hours, with measurements taken every hour. Based on the observation results, the addition of phages resulted in a decrease in OD value compared to the positive control (*V. parahaemolyticus* + SM-buffer), which increased over time. The KN treatment (SM-buffer) showed no increase in OD value. The KA treatment showed a lower OD value compared to the phage treatments (FB7, FB8, and FB9) as well as KA. In the phage treatments, there was an increase in OD value from the first hour until the 15th hour, but this increase was still lower compared to the KP treatment. Then, at the 16th hour, there was a decrease in OD value until the end of the observation.

At the 24-hour observation, treatments using different phage densities (FB7, FB8, and FB9) were able to reduce the density of *V. parahaemolyticus* compared to the KP treatment, with significant differences ($P<0.05$). The best results were found in the FB9 treatment. The KN treatment showed no increase because it only used SM-buffer without the addition of bacteria. The KA treatment produced the best results compared to the KP treatment and the phage treatments (FB7, FB8, and FB9).

Discussion

Vibrio cause mass mortality in shrimp in ponds, and the *V. parahaemolyticus* strain containing PirA and PirB toxins causes AHPND (Tran *et al*., 2013; Dangtip *et al*., 2015). This bacterium can kill

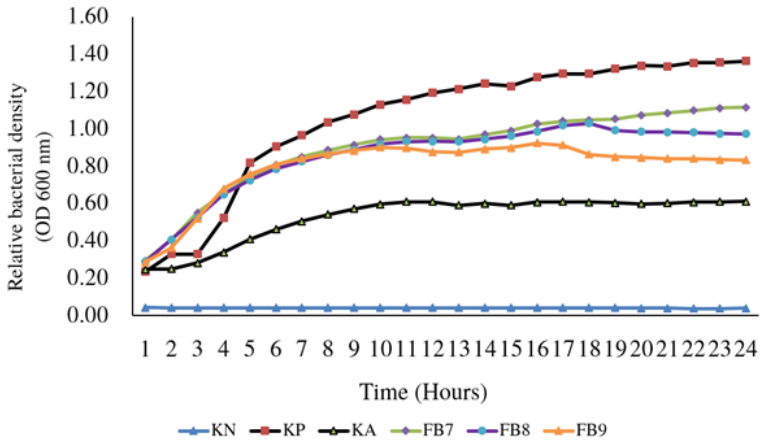

Figure 5. Relative bacterial density graph over 24 hours with inhibition test using Banyuwangi phage and antibiotics. Note: KN = Negatif control; KP = Positif control; KA = Antibiotic control; FB7 = Phages 10^7 PFU/mL, FB8 = Phages 10^8 PFU/mL; FB9 = Phages 10^9 PFU/mL.

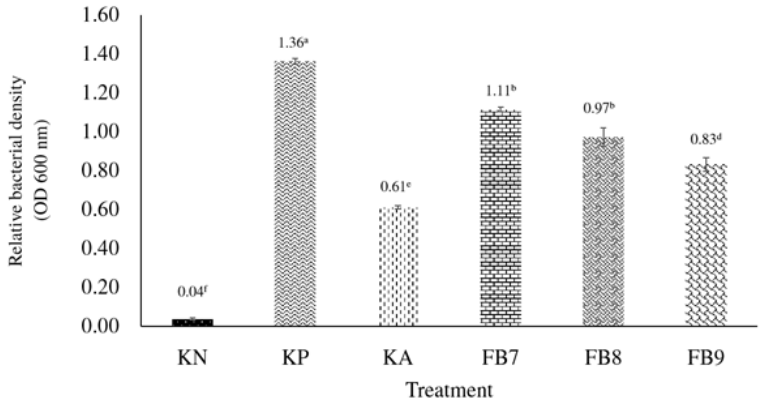

Figure 6. Relative bacterial density at the 24th hour after treatment with Banyuwangi phage. Different letters above each diagram indicate significantly different relative bacterial density values between treatments based on Duncan's test ($p<0.05$).
Note: KN = Negatif control; KP = Positif control; KA = Antibiotic control; FB7 = Phages 10^7 PFU/mL, FB8 = Phages 10^8 PFU/mL; FB9 = Phages 10^9 PFU/mL.

shrimp within one to several days after infection, with mortality rates exceeding 90% (Yang *et al*., 2022). This study discusses the potential of phages to combat *V. parahaemolyticus* bacteria observed in vitro. In this study, phage isolation was successfully conducted from pond water in Banyuwangi, indicated by the formation of plaques on double-layer agar media. The plaques formed signify that the phage has lytic properties.

This aligns with the statement by Ács *et al*. (2020), which notes that phages capable of infecting the tested bacteria form clear spots or plaques on double-layer agar media, indicating that the phages can lysis their host bacteria. Initially, a single plaque lyses one bacterium, then multiplies and kills other host bacterial cells. According to Shaniyah *et al*. (2023), the double-layer method with the addition of soft agar as the top media facilitates the propagation of phages present in the sample water, allowing them to reproduce on their host. The appearance of clear plaques on the incubated double-layer media indicates the presence of phages. The primary factor in phage isolation is highly dependent on the growth of its host (Taj *et al*., 2014).

If the host's growth is not optimal, phage growth will also affect the infection results from one cell to another. Therefore, this study used host bacteria in the exponential/log phase to ensure that the phages could replicate well. Additionally, several factors affecting phage quantity when isolated on media must be adjusted to the original environmental conditions of the community, considering salt concentration as a buffer (SM-buffer) or pH (Carroll-Portillo *et al*., 2021). Furthermore, Hu *et al*. (2021) state that as another prerequisite for their use as biological control agents, the survival conditions of phages at different pH and temperature values play a crucial role in phage attachment, penetration, and reproduction. In the phage characterization observation, the results showed morphological characteristics in the form of small dots and large circles with smooth and wavy edges, marked by the appearance of clear plaques.

According to Deshanda *et al*. (2018), factors influencing the size and shape of plaques include incubation time, agar media concentration, incubation conditions, and the log phase of bacterial cells. Additionally, the phage density obtained in this study was 10^9 PFU/mL, which is considered high. The optimal phage density that can infect and lyse bacteria ranges from 10^6 to 10^{11} PFU/mL (Clokie & Kropinski, 2009). In this study, the plaques formed had diameters ranging from 0.008 to 0.5 cm. In the study by Śliwka *et al*. (2023), the average diameter of phage BF9 *E. coli* plaques was 0.112 cm, whereas in the study by Chen *et al*. (2023), phage HS106 formed large plaques with a diameter of 0.68 cm, which is larger than the plaques obtained in this study. Gallet *et al*. (2011) stated that plaque diameter depends on phage virion morphology and the produced lysis time.

Different phage morphologies result in different plaque sizes. The plaque size formed on the media will be maximal if the lysis time is optimal. The optimal lysis time can vary, occurring with intermediate, fast, and sometimes not very fast times. The host range test using six bacterial isolates (*V. parahaemolyticus* from Situbondo, Banyuwangi and Anyer, *V. harveyi*, *A. hydrophila*, and *A. salmonicida*) showed that only *V. parahaemolyticus* from Situbondo formed plaques, while the others showed negative results (no plaques). This proves that the Banyuwangi phage has a narrow and highly specific spectrum, as it can only kill *V. parahaemolyticus* from Situbondo.

The phage mechanism in lysing host bacteria involves several stages: adsorption, penetration, synthesis, maturation, and lysis (Sabino *et al*., 2020). The process starts with the phage adsorbing to the surface of the host cell using the tail tip, followed by viral penetration into the host cell. The penetration process aims to inject genetic material into the host cell (the viral DNA enters the host cell). Next, the biosynthesis process occurs, where the viral DNA replicates, and proteins are made. The following stage is the maturation or assembly of phage particles.

Finally, the lysis process occurs, where the phage exits the host cell by creating holes in the host cell wall, causing bacterial cell lysis and forming plaques on double-layer media. The phage tail tip has lysozyme enzymes that can create small holes in the peptidoglycan of the bacterial cell wall during penetration (Hyman *et al*., 2019). The specificity of phages depends on the receptors they recognize. According to Dowah and Clokie (2018), some of the receptors that phages can recognize are proteins, polysaccharides, lipopolysaccharides (LPS), and carbohydrate groups (Silva *et al*., 2016). LPS is a common receptor that phages can recognize in gram-negative bacteria. Additionally, other receptors include outer membrane proteins, pili, and flagella (Sørensen *et al*., 2011).

Filik *et al*. (2022) stated that the phage tail shows high specificity and plays a crucial role in enabling infection. Phage tails and receptor binding proteins (RBPs) are essential for host recognition through specific interactions between the tail attachment sites and molecules on the bacterial surface. Phage tails use a set of tail fibers (TF) (possibly 3, 6, or 12 fibers) or a single TF located in the center of the baseplate to recognize receptors on the bacterial cell surface. The graph of the bacterial inhibition test observed hourly over 24 hours showed that phages could significantly inhibit the growth of *V. parahaemolyticus* compared to the KP treatment (*V. parahaemolyticus* + SM buffer).

This indicates that phages can lyse the host bacteria (*V. parahaemolyticus*) the higher the phage density, the greater the bacterial lysis produced (Figure 6). The ability of phages to lyse bacteria began to decrease after 15 hours. These results are consistent with those of Droubogiannis and Katharios (2022), who showed that in vitro bacterial inhibition tests could significantly kill host bacteria compared to the control treatment. Furthermore, Pallavali *et al*. (2017) found that phages observed over 24 hours showed a decrease at the 12th hour, followed by an increase, differing significantly from the control. Liang *et al*. (2022) observed that *V. parahaemolyticus* phages inhibited bacterial growth in vitro within eight hours, followed by an increase.

CONCLUSION

Based on the results of the conducted study, it can be concluded that the Banyuwangi-specific *V. parahaemolyticus* phage was successfully isolated and amplified. The phage exhibited plaque morphology characterized by small dots and circular shapes, with smooth and wavy edges, and the plaques formed were clear. Additionally, the phage was able to inhibit bacterial growth observed in vitro, with the best result being FB9 (Phages 100 µL 10^9 PFU/mL and *V. parahaemolyticus* 200 µL 10^7 CFU/mL).

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