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Research Article

Isolation of Lytic Bacteriophages infected Indonesian-strain *Vibrio parahaemolyticus* and its Protective Effects on Brine Shrimp (*Artemia* sp.)

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Abstract

Acute Hepatopancreatic Necrosis Disease (AHPND) caused by *V. parahaemolyticus* infection was one of the major diseases in shrimp culture in recent years. The *Vibrio* could also affect the survival of *Artemia* as the shrimp's main live feed in the hatchery and they become the possible carrier for the AHPND. Phage therapy in shrimp aquaculture could reduce the application of the antibiotic as an antibacterial agent for the AHPND. The present study aimed to isolate the specific lytic phage for the Indonesian strain of *V. parahaemolyticus* (*Vp*) and evaluate the phage therapy for the brine shrimp *Artemia* infected with the *Vp*. The Vp-specific phage was isolated from the shrimp farm's water at Tasikmalaya, and North Jakarta City, Indonesia. After isolation and plaque assay, brine shrimp were used as a model to evaluate the phages' anti-Vibrio activity The Vp-lytic phage was successfully isolated from shrimp culture water at North Jakarta and Tasikmalaya (Vb_Vp_TSK01 and Vb_Vp_JKT01, respectively) and the results showed that both isolated phages and their cocktails were capable to inhibit the growth of *Vp* with the highest inhibition shown at the cocktail treatment ($p<0.05$). The survival of *Artemia* was higher in the phage treatments ($p<0.005$) compared to the infected control. Infected control had 68.33% of brine shrimp survival, and the Vb_Vp_TSK01, Vb_Vp_JKT01, and their cocktail had similar average brine shrimp survival of 91.11%. In conclusion, phage therapy proved effective in preventing vibriosis in brine shrimp under the conditions tested.

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1. Introduction

Vannamei shrimp *Litopenaeus vannamei* is one of the main cultured-aquaculture species in the world (FAO, 2020). However, disease infection, especially Acute Hepatopancreatic Necrosis Disease (AHPND) has hindered shrimp aquaculture in recent years (Bondad-Reantaso and Arthur, 2018; Munaeni *et al*., 2020). The disease is caused by a strain of *V. parahaemolyticus* that generates *Photorhabdus insect-related* (Pir) toxins that lead to shrimp mass mortality, especially at the larva and the early days of culture (Kumar *et al*., 2019; Nakamura *et al*., 2019; Cao *et al*., 2021). This bacterial disease has consistently impacted shrimp aquaculture, causing significant economic loss (Raja *et al*., 2017; Bondad-Reantaso and Arthur 2018). Antibiotics have generally been effective in controlling bacterial infections in shrimp farming (Holmström *et al*., 2003; Thornber *et al*., 2020). However, the overuse of antibiotics has resulted in antibiotic resistance in many bacterial species (Yasin *et al*., 2022). Furthermore, antibiotic residues can accumulate in the environment and animal tissues, causing concerns to consumers (Thornber *et al*., 2020).

Several alternative strategies have been proposed to eliminate the use of antibiotics in shrimp farming, including the use of probiotics, prebiotics, and synbiotics (Hamsah *et al*., 2019; Ramadhani *et al*., 2019; Hong *et al*., 2022), immunostimulants (Javahery *et al*., 2019; Munaeni *et al*., 2020), and phage treatment (Jun *et al*., 2018; Quiroz-Guzmán *et al*., 2018; Cao *et al*., 2021). Several recent investigations have demonstrated that phage treatment has significant potential for preventing and managing AHPND-causing *Vibrio* (Jun *et al*., 2018; Cao *et al*., 2021; Xue *et al*., 2021).

The use of phages as a therapeutic agent (phage treatment) is advantageous since they are natural antibacterial agents that only multiply in their host bacterium (Tian *et al*., 2022), and might not negatively affect the host or the consumers (Jun *et al*., 2018; Gazeev, 2018). The phage specificity to the host's strain is important for phage therapy in shrimp, especially for the antibiotic-resistance strain (Cao *et al*., 2021). In addition, for phage therapy in larval cultures, phages are not effectively administered orally through artificial feed, intraperitoneally, intramuscularly, or topically (Martínez-Díaz and Hipólito-Morales, 2013).

The bio-encapsulated phage inside the live feed of Artemia nauplii could be the phage's possible carrier of the infection site inside the shrimp larva or post-larva (Quiroz-Guzmán *et al*., 2018; Nikapitiya *et al*., 2020). For further development, the phage should be able to protect *Artemia* against the targeted pathogen. The study related to the phage isolation for the Indonesian strain of *V. parahaemolyticus* is scarce. This is important since host-specificity is an important thing in phage therapy.

The use of phage therapy is tightly correlated with the host's strain since phages have a specific lytic activity for the certain host. Here we isolated the specific *V. parahaemolyticus* lytic phage from shrimp farms in Indonesia. The lytic ability was also evaluated using the plaque method and in vivo by measuring the *V. parahaemolyticus* growth curve after phage addition.

The study related to the phage treatment using the Indonesian strain of *V. parahaemolyticus* is still scarce. Hence, the objective of the present study is to isolate *V. parahaemolyticus*-specific lytic phages from shrimp farms in Indonesia and evaluate their effectiveness in phage therapy for brine shrimp Artemia infected with *V. parahaemolyticus.*

2. Materials and Methods

2.1 Materials

The main equipment and tools used in this research included: a 0.45 µm and 0.22 µm syringe filter (Merck Millipore, USA), Duran® laboratory bottles (DWK Life Science, Germany), micropipettes (Axygen, USA), microtips (Axygen, USA), microtubes (Axygen, USA), microplate spectrophotometer (Multiskan SkyHigh, Thermoscientific, USA), laboratory glassware (Pyrex, USA), disposable sterile petri dish (SPL Life Sciences, Korea), bacterial incubator (IN30, Memmert, Germany), stereo microscope (Stemi DV4, Zeiss, Germany), and autoclave (GEA medical, Indonesia). The materials used in this study included: Thiosulfate-citrate-bile salts-sucrose (TCBS) agar media (Himedia, India), peptone (Himedia, India), yeast extract (1st Base, Malaysia), glycerol (Merck, USA), NaCl (Merck, USA), $MgSO_4 \cdot 7H_2O_2$ (Merck, USA), Tris-HCl (1st Base, Malaysia), agarose (1st Base, Malaysia), and brine shrimp (*Artemia* sp., Golden West, USA).

2.1.1 Ethical approval

This study does not require approval because it does not use experimental animals.

2.2 Method

2.2.1 Bacteria

V. parahaemolyticus was isolated and characterized from our previous study, obtained from the infected shrimp on a commercial farm (Lampung, Indonesia) (Kurniawinata *et al*., 2021). The pirA and pirB plasmids were detected from the bacteria using the polymerase chain reaction method (OIE, 2019). The bacteria were cultured in Thiosulfate-citrate-bile salts-sucrose (TCBS) agar media (Himedia, India) at 37°C for 24 hours and subsequently transferred to seawater-complete (SWC) (0.5% peptone, 0.3% yeast extract, 0.3% glycerol) broth and cultured for another 24 hours. The pure isolate was tested for antibiotic resistance: enrofloxacin, tetracycline, rifampicin, and oxytetracycline using the Kirby-Bauer method (Hudzicki, 2012). This test was conducted to use the antibiotic-resistance isolate of *Vp* for phage screening. Thus, in the future practical application, the isolated lytic phage possessed the ability to prevent or treat shrimp diseases caused by antibiotic-resistant *Vibrio* infection. The *Vp* isolate showed to be resistance to the tested antibiotics.

2.2.2 Phage isolation

The bacteriophage was isolated separately from the shrimp farm in two different locations. The First was a commercial shrimp farm located in Tasikmalaya, West Java, Indonesia, and the second was located on the shrimp farm in Kepulauan Seribu, North Jakarta, Indonesia. The water was collected from the rearing net aseptically. The water was transported on ice to our laboratory in Bogor, West Java, Indonesia, and stored at 4°C overnight. The water was centrifuged, and the supernatant was filtered sequentially through a 0.45 µm and 0.22 µm syringe filter (Merck Millipore, USA). The filtered supernatant (20 mL) was enriched with 1 mL of the cultured *Vp* bacteria inside 50 mL of SWC broth media and incubated at room temperature overnight. After centrifugation, the supernatant was filtered through a 0.22 µm syringe filter. Specific *Vp* phages were then isolated using the double-layer agar assay (DLAA) (Tian *et al*., 2022). A total of 5 mL of soft agar containing 100 µL of phage lysate and 200 µL of *V. parahaemolyticus* was added onto the base agar (TCBS with 2% agar) when the bacteria cells were in their exponential growth phase (OD_{600nm} = ~0.5). The plates were gently swirled and dried for 10 min at room temperature. After overnight incubation at 37 °C, the plates were examined for the phage's presence in the form of plaques. A single isolated clear plaque was picked using microtips and then dissolved in 1 mL of SM buffer (100 mM NaCl, 8 mM $MgSO_4 \bullet 7H_2O_2$, 50 mM Tris-Cl 1 M, 1 L of sterile distilled water). Subsequently, the *Vp* phage was purified by performing two rounds of DLAA from the single isolated plaque. Purified phages were kept at 4°C inside the 1 mL SM buffer after the addition of 200 µL chloroform as the phages stock. The purified phage concentration as the plaque-forming units (PFU mL^{-1}) was then counted using the DLAA method with the serially diluted phage lysate (10^0, 10^{-1}, 10^{-2}, 10^{-3} from the stock). The formed plaque's diameters were analyzed from all agars. For each phage, 65 plaques were measured.

2.2.3 In vitro lytic activity

The inhibition of the *Vp* bacteria growth curve was conducted only using one phage titer of 103 PFU mL-1, because of the low concentration of the obtained phage. The *Vp* growth curve analysis was conducted using the spectrophotometry method at OD= 600 nm. The experimental treatments were 200 µL *Vp* bacterial suspension (10^7 CFU mL^{-1}) mixed with 100 µL of phage suspension (10^3 PFU mL^{-1}) either vB_Vp_JKT01 or vB_Vp_TSK01; or their cocktail (1:1). For positive control, bacterial suspension was mixed with SM buffer without phage addition. 300 µL of SM buffer without *Vp.* and phage suspension was used as a negative control. Experimental units were inoculated in triplicates. The absorbance value was recorded every hour for 24 hours. The lytic activity was measured by comparing the *Vp* growth relative to the bacterial concentration at 0 h (density at 0 h =1).

2.2.4 Brine shrimp infection

The experimental model was brine shrimp *Artemia* sp. The *Artemia* nauplii were hatched, and the instar-2 *Artemia* (24 hours after hatch) was used in the infection experiment since the *Artemia* enrichment procedure generally started at instar-2 for the shrimp and fish larva (Panah *et al*., 2021; Morshedi *et al*., 2022). The dose of *Vp.* was adjusted to 10^6 CFU mL^{-1} which resulted in 50% mortality at approximately 48 hours after infection based on our previous study. Instar-2 of *Artemia* (n= 30) was placed inside the petri dish with 30 mL of seawater. 100 µL of *Vp* bacterial suspension was homogenized with 1 mL of vB_Vp_JKT01, vB_Vp_TSK01, or their cocktail (1:1) (10^3 PFU mL^{-1}) inside a sterile microtube. The mixture was then poured into the petri dish by pipetting gently. A total of 1.1 mL of phosphate-buffered saline (PBS, pH 8) without *Vp* and phage suspension was used as a negative control. Each treatment was triplicate,

and the *Artemia* survival was observed 48 hours after infection. The *Artemia* was kept at 27-29°C without aeration or feed. The number of live organisms was counted under a stereomicroscope from each replication.

2.3 Analysis Data

The plaque diameter, bacterial relative growth, and *Artemia* survival were analyzed statistically using one-way ANOVA and continued with Duncan's test with a confidence level of 95% (α= 0.05).

3. Results and Discussion

3.1 Result

In this study, the *Vp* lytic phages were successfully isolated: vB_Vp_JKT01 (Jakarta), and vB_Vp_TSK01 (Tasikmalaya), referred to their source. The results were indicated by the lytic plaques formed on the agar (Figure 1). The plaque's diameter was then recorded from the isolated phage. The results indicated that the vB_Vp_TSK01 had larger lytic plaques ($p<0.05$) against *V. parahaemolyticus* (0.83±0.75 mm) compared to vB_Vp_JKT01 (0.33±0.29 mm) with the same initial phage concentration (Figure 2).

Application of the vB_Vp_TSK01, vB_Vp_JKT01, and their cocktail against *Vp* infections was evaluated in-vitro and in vivo. The results showed that both isolated phages and their cocktails were capable of inhibiting the growth of *Vp* (Figure 3). The inhibition was mainly started at 8-12 hours for vB_Vp_TSK01 and vB_Vp_JKT01 treatment. For the phage cocktails, the inhibition had already started at 4-6 h of culture compared to the control.

At the end of the experiment, Vp concentration in control was increased by 43.11±2.87% (by fold change) compared to the initial hour. At vB_Vp_JKT01 treatment, the growth was only 29.72±5.81% higher and 27.61±3.12% in vB_Vp_TSK01. Meanwhile, their cocktail showed the lowest growth into the initial our ($p<0.05$), the Vp concentration was increased only by 4.8±3.73%. Compared to the control, the cocktail treatment had 30.92±3.89% lower bacterial concentration ($p<0.05$). Single phage treatment also showed a similar pattern, the vB_Vp_TSK01 had 11.46±1.35% lower concentration and 9.86±5.92% at vb_Vp_JKT01.

3.2 Discussion

Currently, *V. parahaemolyticus* is the most common pathogen causing AHPND or Early Mortality Syndrome in the majority of shrimp-producer countries, including Indonesia (Raja *et al*., 2017; Sarjito *et al*., 2018; Abdel-Latif *et al*., 2022). Because of its safety, non-pollution, and low negative effects on aquaculture and consumers, phage treatment was seen as a promising strategy for bacterial disease management (Jun *et al*., 2018; Abdel-Latif *et al*., 2022). The phage's specificity to the host strain is critical for phage treatment in shrimp, especially for antibiotic-resistant strains (Cao *et al*., 2021).

Brine shrimp (*Artemia sp*.) is the most utilized live feed in shrimp hatcheries due to their size, nutritional quality, and flexibility in enrichment methods (Quiroz-Guzmán *et al*., 2018; Sorgeloos and Roubach, 2021). It is anticipated that Vibrio bacteria could enter the shrimp hatcheries through brine shrimp. However, the bio-encapsulated phage inside the live

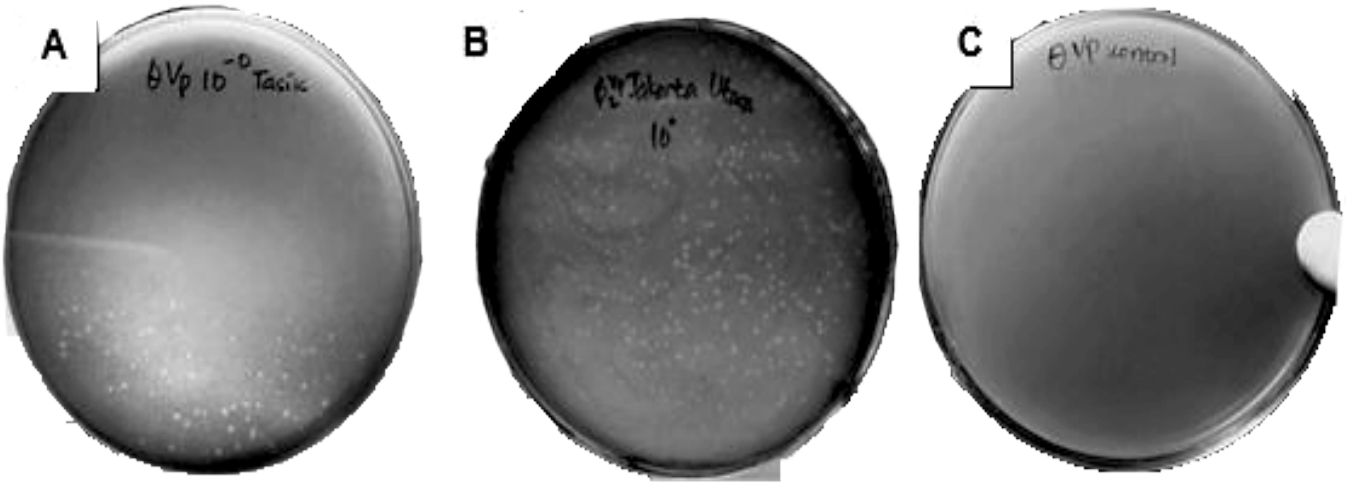


Figure 1. Plaques of phage isolated from shrimp aquaculture water on the antibiotic-resistance strain of *V. parahaemolyticus* lawn. (a) vB_Vp_TSK01 isolated from Tasikmalaya; (b) vB_Vp_JKT01 isolated from North Jakarta; (c) *V. parahaemolyticus* lawn without phage suspension (control).

feed of Artemia nauplii could be the phage's possible carrier to the infection site inside the shrimp larva or post-larva (Quiroz-Guzmán *et al*., 2018; Nikapitiya *et al*., 2020). For the development of phage therapy in shrimp aquaculture, the phage should be able to protect the brine shrimp against the targeted pathogen. Brine shrimp also could be a model for phage therapy and other pathological studies for shrimp (Quiroz-Guzmán *et al*., 2018; Kumar *et al*., 2019; Srinivasan *et al*., 2020). The effect of the phage therapy during experimental infection of brine shrimp nauplii with the Indonesian strain of Vibrio parahaemolyticus was found (Figure 4). In this experiment, the survival of Artemia was higher by 28.57% in the phage treatments ($p<0.005$) compared to the infected control. Infected control had 68.33% of brine shrimp survival, and the vB_Vp_TSK01, vB_Vp_JKT01, and their cocktail had similar average brine shrimp survival of 91.11%.

The results indicate that the JKT01, TSK01, and their cocktail could inhibit the growth of antibiotic-resistance *V. parahaemolyticus* strain although the concentration used in the experiment was relatively low (103 PFU mL-1). Generally, the application of phage therapy in shrimp used more than 106 PFU mL-1 in bath immersion or feeding treatment (Jun *et al*., 2018; Quiroz-Guzmán *et al*., 2018; Chen *et al*., 2019). Moreover, the results from the in-vitro inhibition of *Vp* showed that the use of a single phage had a lower inhibition compared to the cocktail. The improved efficacy of the phage cocktail can be attributed to the fact that the diverse pathways likely result in phage synergy in killing the hosts (Chen *et al*., 2019). The phage characteristics such as strong host specificity, production of anti-bacteria substances, self-propagation, and self-restriction in the presence of the bacterial target are responsible for the anti-bacterial activities (Kokkari *et al*., 2018; Srinivasan *et al*., 2020). In contrast, the cocktail application was not superior to the single phage application in the brine shrimp after infection, although still higher than the control. This might correlate to the microbial compositions of the seawater used as the brine shrimp culture media and the complexity inside the brine shrimp body. Both the environment microbiota and the shrimp gut microbiota can interfere with the lytic activity of the phage (Chen *et al*., 2019). To understandthe lytic mechanisms of vB_Vp_TSK01 and vB_Vp_JKT02, a full characterization of morphological and phage genomes is required to confirm their obligate lytic nature (Kokkari *et al*., 2018).

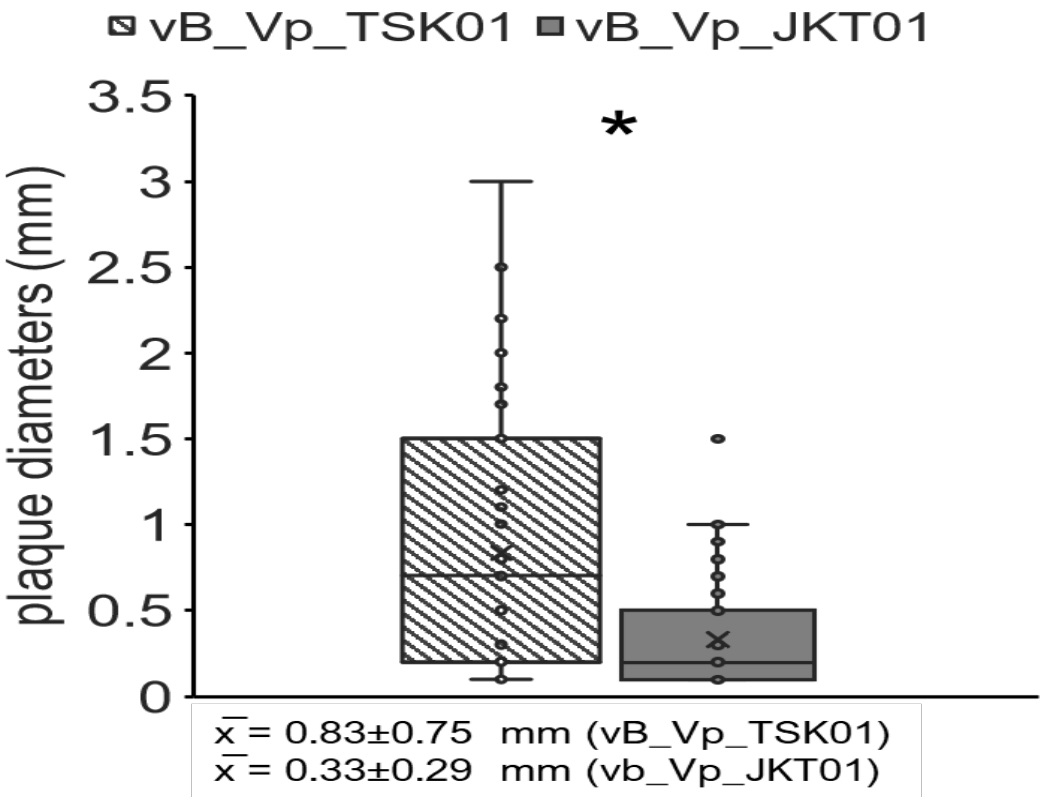


Figure 2. The plaque diameter from vB_Vp_TSK01 isolated from Tasikmalaya and vB_Vp_JKT01 isolated from Jakarta. Both plaques were formed under the phage titer of 103 PFU mL-1. The value was presented from 65 plaques formed on the layer of agars. Asterisk indicates a significant difference between phage isolates ($p<0.05$). The average diameter ($\bar{x}$) is presented below the chart.

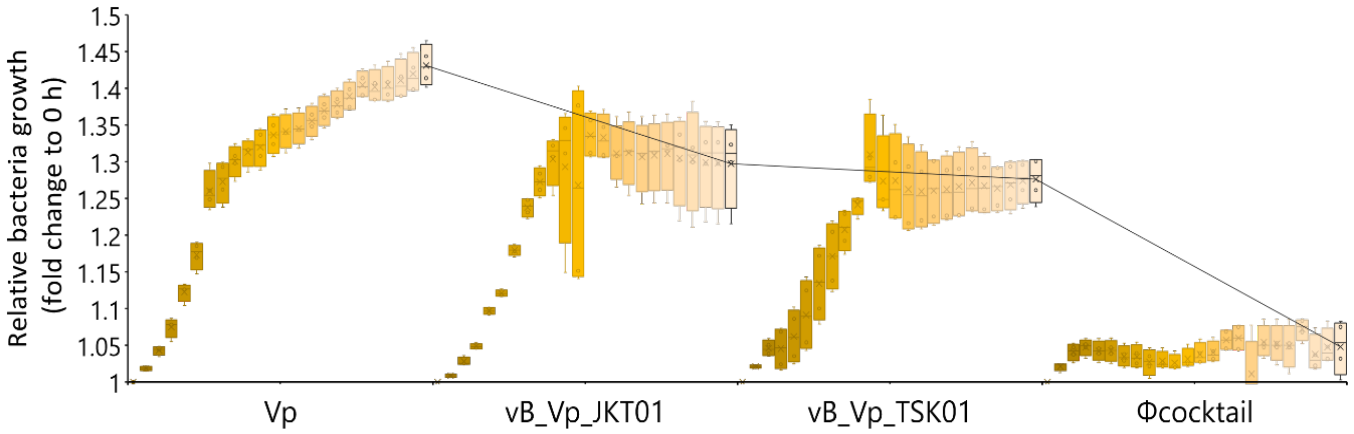
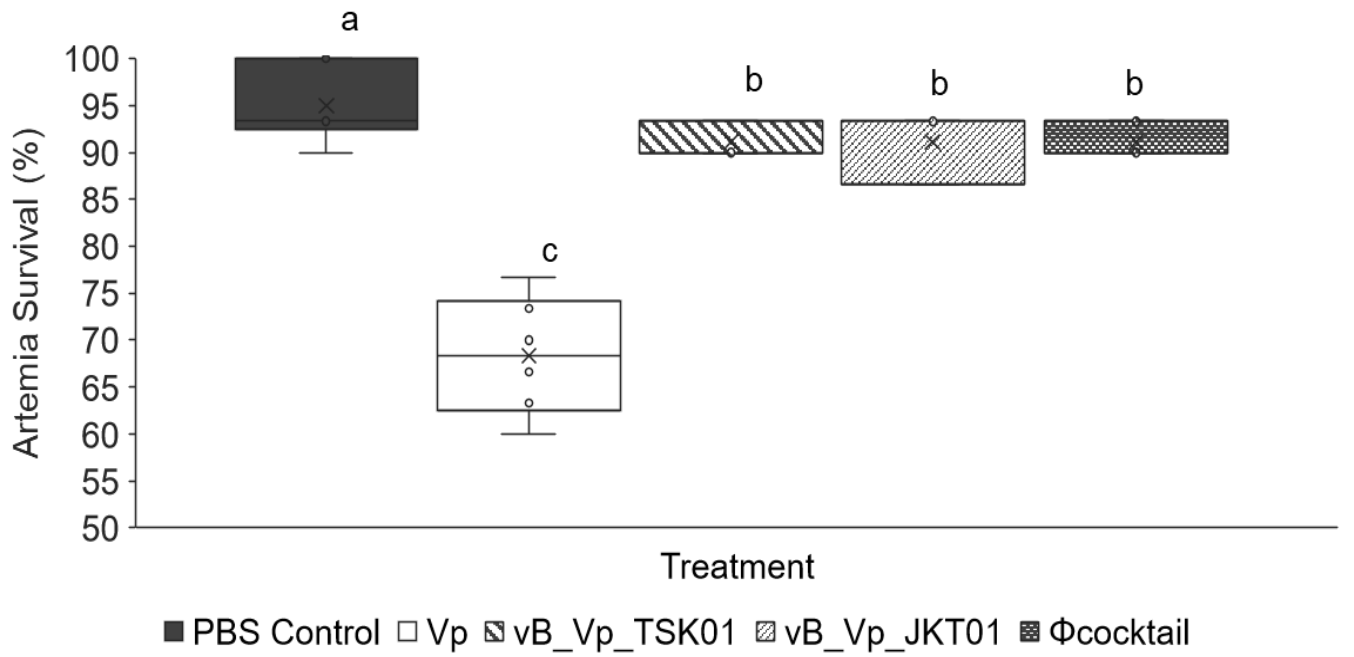


Figure 3. Growth inhibition of *V. parahaemolyticus*. Vp= Positive growth control; vB_Vp_JKT01 isolated from North Jakarta, vB_Vp_TSK01 isolated from Tasikmalaya, and Φcocktail was their cocktail mixture (1:1). The concentration of the phages and their cocktails was 10^3 PFU mL^{-1}. Each bar represents the concentration for 1 h of observation for a total of 24 h (n=4 for each treatment). The line showed the value of average relative growth after 24 h.



Treatment	PBS Control	VP	vB_Vp_TSK01	vB_Vp_JKT01	Φcocktail
Survival (%)	95±3.73	68.33±5.69	91.11±1.57	91.11±3.14	91.11±1.57

Figure 4. Comparison of the survival rates of the *Artemia* in different treatment groups in vivo after 48 hours of *V. parahaemolyticus* challenge. PBS control is not infected with *V. parahaemolyticus*; Vp is the infection control without phage addition, vB_Vp_JKT01 isolated from North Jakarta and vB_Vp_TSK01 isolated from Tasikmalaya and the Φcocktail was their cocktail mixture (1:1). The concentration of the phages and their cocktails were 10^3 PFU mL^{-1}. Data presented as the percentage of *Artemia* survival from 3 replicates. Different letters above the box indicate the significant difference between treatments ($p<0.05$).

4. Conclusion

The specific lytic phage for the Indonesian strain of *V. parahaemolyticus* was successfully isolated, namely, vB_Vp_TSK01 and vB_Vp_JKT02. Both the single-phage application and the cocktail application could inhibit the growth of antibiotic resistance *V. parahaemolyticus* and increase the survival of brine shrimp after infection.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author is as follows, DW & HN; conceived and designed the analysis, verified the data, and wrote the original draft. PSR & LN; conducted the research, collected the data, prepared the original draft. SS & MY; supervised the research, reviewed and edited the manuscript, and verified the data.

Conflict of Interest

The authors declare that they have no conflict of interest.

Declaration of Artificial Intelligence (AI)

The author(s) affirm that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement of this manuscript. All content presented is the result of the independent intellectual efforts of the author(s), ensuring originality and integrity.

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