

# Isolation and identification of *Vibrio* spp. and their potential as hosts for bacteriophage in Indonesian *Penaeus (Penaeus) monodon* Fabricius, 1798 and *Scylla* spp. Hatcheries

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**Abstract.** The hatchery environments of tiger shrimp (*Penaeus monodon* Fabricius, 1798), and mud crabs, *Scylla* spp., are critical to successful aquaculture but are often challenged by microbial contamination. Among the most significant threats are pathogenic bacteria, particularly *Vibrio* spp., which are associated with severe disease outbreaks. In light of this, bacteriophages, viruses that specifically infect bacteria, are emerging as a promising alternative for the biological control of *Vibrio* spp. This study investigated the presence of *Vibrio* spp. It assessed their suitability as hosts for bacteriophage isolation in the hatchery environments of *P. monodon* and *Scylla* spp. in Barru, South Sulawesi, Indonesia. *Vibrio* spp. were isolated and identified both morphologically and through 16S rRNA gene sequencing. Bacteriophages were subsequently isolated, purified, and characterized for host range using selected *Vibrio* spp. isolates. A total of 39 *Vibrio* spp. isolates were recovered from various sources, including *P. monodon* larvae, grow-out waters, *Scylla* spp. broodstock waters, and environmental samples. Molecular identification of seven isolates revealed four as *Vibrio parahaemolyticus* (99.09–99.72% similarity), with the remaining isolates identified as *Vibrio alginolyticus* (99.30%), *Vibrio sinaloensis* (98.26%), and *Photobacterium* sp. (95.07%). Bacteriophages were detected in 11 out of 28 environmental samples. Host range assays demonstrated that the *V. parahaemolyticus* phage (FSB28.T.3.1) exhibited lytic activity against eight *Vibrio* spp. isolates, while the *Vibrio harveyi* phage (F.MP1.T.3.1) lysed nine *Vibrio* spp. isolates. These results indicate that both phages possess a broad host range and that water and sediment from *P. monodon* and *Scylla* spp. hatcheries are potential reservoirs for bacteriophages. In conclusion, this study highlights the potential of the hatchery environment for sourcing bacteriophages as biological control agents against *Vibrio* spp., offering a promising strategy for managing bacterial infections in aquaculture.

**Key Words:** *Penaeus monodon* hatchery, *Scylla* spp. hatchery, shrimp disease, Vibriosis, bacteriophage, AHPND.

**Introduction.** The hatchery environments of tiger shrimp (*Penaeus monodon* Fabricius, 1798) and mud crabs, *Scylla* spp., are critical to successful aquaculture, but they are often challenged by microbial contamination. Among the most significant threats are pathogenic bacteria, particularly *Vibrio* spp., which are associated with severe disease outbreaks such as vibriosis. Vibriosis can lead to mass mortality in both prawns and crabs, ultimately causing significant losses to aquaculture industries. (de Souse Valente & Wan 2021).

*Vibrio* bacteria are gram-negative, motile, and facultative anaerobic bacteria, members of the Vibrionaceae family. These bacteria are the cause of vibriosis disease that attacks farmed animals (Gomathi et al 2013; Khamesipour et al 2014). Vibriosis is one type of disease that has a significant negative impact on the shrimp farming industry, including on a seed scale (Yu et al 2020; Ghosh et al 2023; Wang et al 2024). Vibriosis

has also been reported to attack several species of farmed fish (Sanches-Fernandes et al 2022; Aly et al 2023). In addition to shrimp and fish, vibriosis has also been reported to infect several types of mollusks (Sharma et al 2024) and corals (Munn 2015; Kemp et al 2018; Rubio-Portillo et al 2020).

*V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. campbellii* are some of the *Vibrio* bacterial species that are often found in disease events in hatcheries and shrimp ponds (Zhang et al 2020). These bacterial species are known to be the main cause of firefly disease in marine and brackish water organisms. These bacteria are found throughout the aquatic environment. They often act as opportunistic agents in secondary infections in addition to being the primary cause (Saulnier et al 2000).

It was recently discovered that *V. parahaemolyticus* can produce the toxins pirA and pirB (Ong et al 2021; Tan et al 2021). People's Republic of China (2010), Vietnam, Malaysia, Thailand (Flegel 2012), the Philippines (Dabu et al 2017), and Korea (Kim et al 2024) have all recorded cases of the disease in shrimp. Hepatopancreas pale to white, atrophy, mushy skin, intestines not filled/cut into pieces or even empty, and black patches or melanization on hepatopancreatic tubules are some of the clinical symptoms of shrimp infected with AHPND. Since the shrimp were stocked in the pond at 10 days old, a clinical gala can be observed. AHPND attacks are marked by their quick incidence, with up to 100% of individuals dying within 30-35 days after the virus's spread (Kumar et al 2020), while individuals older than that can also contract the virus (De La Peña et al 2015). Reverse gavage, food/cannibalism, and water/immersion are all ways that VpAHPND can propagate (Soto-Rodriguez et al 2018).

Although antibiotics for the prevention of shrimp disease have been banned by governments in almost all countries, antibiotics are still used to treat vibriosis, especially in hatcheries, antibiotics are still used to treat vibriosis, especially in hatcheries (Holmström et al 2003), because natural biodegradable compounds that can be used as alternatives to antibiotics are not yet available in sufficient quantities, types, and efficacy. This further exacerbates the problem in the field due to side effects in the form of antibiotic-resistant pathogenic bacteria, accumulation of chemicals, and environmental pollution that worsen cultivation conditions (Nachimuthu et al 2021; Ghosh et al 2023). Therefore, it is necessary to find alternative treatments for vibriosis in shrimp.

The use of bacteriophages, viruses that specifically target and kill bacteria, has emerged as a promising alternative to conventional antibiotics for controlling these pathogenic bacteria (Culot et al 2019). Bacteriophages offer several advantages, including their ability to specifically target harmful bacteria without disrupting the beneficial microbial communities essential for aquaculture health (Principi et al 2019). Additionally, they are seen as a more sustainable and eco-friendly solution, particularly in the face of rising antibiotic resistance. Despite their potential, research into the use of bacteriophages in aquaculture, especially in hatcheries, remains limited. This is partly due to the complex dynamics of the microbial environments in aquaculture systems, the challenges in identifying and selecting the right bacteriophages, and the need for more rigorous studies on their safety and effectiveness. Furthermore, there is a lack of awareness and funding in the research sector, which has contributed to the slower adoption of bacteriophage therapy in controlling vibriosis. Given the increasing threat of antibiotic resistance and the growing demand for sustainable aquaculture practices, further exploration and investment in bacteriophage research are urgently needed to optimize their use in preventing vibriosis in hatcheries (Hossain et al 2024).

The general objective of this study is to evaluate the presence and potential of *Vibrio* spp. and assess their suitability as hosts for bacteriophage isolation, aiming to explore their use as a biological control alternative for managing pathogenic bacterial infections in the hatchery environments of *P. monodon* and *Scylla* spp. in Barru, South Sulawesi, Indonesia.

This research aims to identify and isolate *Vibrio* spp. from environmental samples in the hatcheries, perform molecular identification to determine the bacterial species, and isolate, purify, and characterize bacteriophages from these samples. Furthermore, the study seeks to assess the potential of these bacteriophages in controlling *Vibrio* spp. infections and evaluate their host range against the identified *Vibrio* spp. isolates.

## Material and Method

**Sampling and culture media.** Sampling was conducted on April 8, 2021, at the *P. monodon* and *Scylla* spp. hatchery station in Barru, South Sulawesi, Indonesia (Figure 1). The sampling sites included the *P. monodon* hatchery, *Scylla* spp. hatchery, drainage channel, water intake pond, surrounding shrimp ponds, and seawater, which serves as the water source for both hatcheries. An exploratory sampling approach was employed, with no replication for each sample type and location.

Water samples (50 mL) and sediment samples (up to 20 g) were collected in sterile bottles and transported in a cool box to the Pathology Laboratory of the Research Institute for Brackish Water Aquaculture and Fisheries Extension (RIBAFE). *P. monodon* larvae and hemolymph samples were collected directly at the hatchery during the seeding process. Before homogenization using a vortex agitator, shrimp larvae were rinsed 2-3 times with sterile seawater to minimize external contamination.

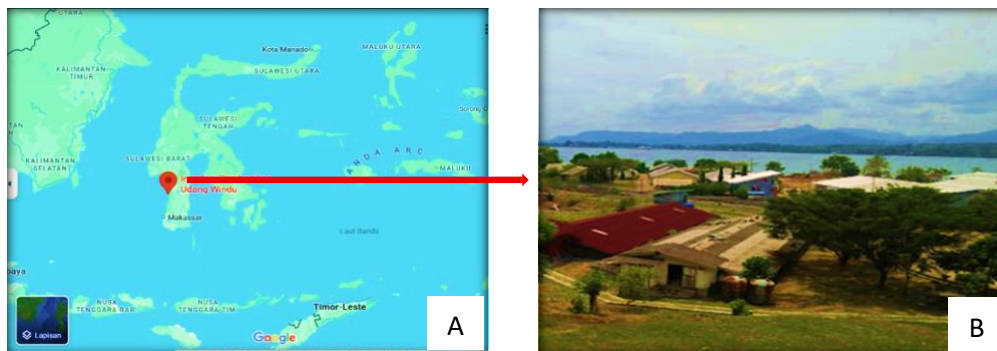


Figure 1. Sampling locations for the isolation of *Vibrio* spp. and bacteriophages. (A) Map of South Sulawesi, Indonesia. (B) *P. monodon* and *Scylla* spp. hatcheries in Barru, South Sulawesi, Indonesia.

**Isolation and morphological identification of *Vibrio* spp.** To obtain single colonies of *Vibrio* spp., serial dilutions ( $10^{-1}$  to  $10^{-5}$ ) were prepared using sterile saline solution (0.85% w/v NaCl). A volume of 100  $\mu$ L from each dilution was spread onto Thiosulfate Citrate Bile Salt (TCBS) agar plates and incubated at 28 °C for 48 hours. Colonies were morphologically identified based on shape, color, elevation, margin, and size. Pure cultures were obtained by streaking isolated colonies onto fresh TCBS agar plates (Azwai et al 2016), followed by incubation at 28°C for 48 hours. If necessary, further streaking was performed to ensure the isolation of single colonies. Representative colonies from TCBS agar were then subcultured on tryptic soy agar (TSA) slants in test tubes for subsequent analysis.

**Molecular identification of *Vibrio* spp. bacteria.** Molecular identification was performed on selected *Vibrio* isolates using 16S rRNA gene sequencing to distinguish between species (Azwai et al 2016; Regev et al 2020). Genomic DNA was extracted following the protocol provided by the Geneaid Presto™ Mini gDNA Bacteria Kit. The extraction process began by culturing *Vibrio* isolates in nutrient broth media. After incubation for 24 hours, bacterial cells were harvested by centrifugation at 14,000 rpm for 5 minutes at 4°C. The resulting pellet was then processed using the Presto™ Mini gDNA Bacteria Kit (Geneaid). The isolated DNA was subsequently amplified by PCR using a pair of universal bacterial 16S rRNA gene primers: forward SB0008 (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse SB1492Rh (5'-GHT ACC TTG TTA CGA CTT-3') (Chen et al., 2017). PCR amplification was conducted using the MyTaq kit with the following reaction mixture: 12.5  $\mu$ L MyTaq reaction buffer, 1  $\mu$ L forward primer (10 pmol), 1  $\mu$ L reverse primer (10 pmol), 3  $\mu$ L DNA template, and nuclease-free water up to a final volume of 25  $\mu$ L. The PCR conditions were as follows: initial denaturation at 95°C for 1 minute; 35 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 10 seconds; and a final extension at 72°C for 10 minutes. The PCR products were subsequently sent to Genetika Science for sequencing. The resulting nucleotide sequences were edited using BioEdit v7.2.5 to trim

low-quality regions at both the 5' and 3' ends. The edited sequences were then compared with existing data in the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (BLAST). The bacterial species were determined based on the highest sequence identity obtained from BLAST analysis, with a threshold of 95-99% similarity. In addition to sequence identity, species-level identification was confirmed by referencing the GenBank accession numbers of the closest matched sequences. This dual approach ensured accurate taxonomic assignment and traceability of the reference sequences used.

**Bacteriophage isolation.** Bacteriophages were isolated from sediments, water, *P. monodon* larvae, and natural food sources. Samples were collected in sterile bottles and brought to the RIBAFE Pathology Laboratory in a chilled state (in a cool box). Bacteriophages are identified using techniques known as the double agar overlay plaque assay, which has been introduced by several prior researchers with modest changes (Phumkhachorn & Rattanachaikunsopon, 2010; Chen et al 2019; Echeverría-Vega et al 2019).

In this study, three different pathogenic strains of *Vibrio* bacteria, *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*, were utilized as hosts in 50mL suspensions of sediment and water. It is then incubated for an additional 24 hours at room temperature while being shaken on a swaying shaker. Following a 24-hour incubation period, the culture is centrifuged at 4°C for 10 minutes at a velocity of 12,000 g to separate the supernatants from the bacterial cells and solid contaminants. The filtrate was incorporated into the 24-hour bacteria in a total volume of 100 microliters. The mixture is then placed so that the Tryptic Soy Broth is solid in a volume of 4.5 mL and distributed over the TSA plate media, and is then allowed to sit at room temperature for 30 minutes. The inoculants are cultured for 48-72 hours at a temperature of 28-30°C, and bacteriophage plug formation is then monitored by observing the clear zones (Hao et al 2023).

**Purification of bacteriophage.** Bacteriophage purification was carried out based on the protocol reported by several previous researchers with slight modifications (Echeverría-Vega et al 2019). One plaque of bacteriophage was taken using a sterile Pasteur pipette (Cao et al 2021), then inoculated into log phase cultures of *V. parahaemolyticus* and *V. harveyi*, and then incubated for 48 hours at 25°C. The phage-host mixture was centrifuged at 12,000 x g for 10 minutes, and the filtrate was then collected using a membrane filter with a pore size of 0.45 microns. The filtrate was then inoculated onto the media with a double layer, repeated three times for each plaque. The final plate diluted phage was prepared by adding 5mL of SM buffer (50 mM Tris-HCl, pH 7.5, 99 mM NaCl, 8 mM MgSO<sub>4</sub>, 0.01% gelatin) to the plate and shaking it for a four-hour incubation period at room temperature. After centrifugation at 12,000 x g for 10 min, the buffer containing the phages removed from the plate was filtered through a membrane filter with a pore size of 0.45 µm. The obtained phage suspension was collected for further testing.

**Determination of bacteriophage host range.** To ascertain how broad the host range of the collected phage was, the host range of the isolated bacteriophage was determined. The approach taken to ascertain the variety of hosts for bacteriophages that have been gathered is one that has been developed by earlier researchers and modified (Wong et al 2019; Liu et al 2020; Fu et al 2023).

A 100 mL of the log phase culture of each of the tested bacteria was mixed with 4.9 mL of soft gelatin at 50°C (agar-agar 0.5%), poured into a petri dish, and then mixed. In the middle of the plate that has been inoculated with *Vibrio* spp. bacteria, 10 L of purified phage suspension (10<sup>8</sup> PFU mL<sup>-1</sup>) is hatched. A clear zone that appeared on a petri dish after 12-48 hours of incubation demonstrated the phage's capability to infect the tested bacteria.

## Results

**Vibrio isolation and morphological identification.** *Vibrio* spp. bacteria have been isolated from *P. monodon* larvae rearing water, *P. monodon* larvae, *Scylla* sp broodstock rearing water, nucleus centers, water sources, *P. monodon* larva hatching wastewater, pond water, and seawater. These bacteria have been identified both morphologically by looking at the color and luminescence of the colonies and biomolecularly through sequencing 16S-rRNA. The bacteria that have been isolated are dominated by fluorescent bacteria, both green and yellow colonies with fluorescent properties. Following that, the bacteria serve as hosts to isolate the bacteriophage from their initial environment.

A total of 26 isolates of *Vibrio* spp. Bacteria were successfully isolated from the water used to maintain *P. monodon* larvae and *P. monodon* larvae. Of the 26 isolates, 10 (38.5%) came from the water used to maintain larvae, and 16 (61.5%) came from *P. monodon* larvae (Table 1). The 10 bacterial isolates isolated from the water used to maintain *P. monodon* larvae, with isolate codes IP70 to IP79, were dominated by bacteria with 30% fluorescent green colony color, 30% non-luminescent yellow, 20% luminescent yellow, and 20% non-luminescent green. Meanwhile, of the 16 bacterial isolates from *P. monodon* larvae, 31.25% were green luminescent bacteria, 31.25% were green non-luminescent bacteria, 25% were yellow luminescent bacteria, and 12.50% were yellow non-luminescent bacteria (Figure 2). The dominance of luminescent bacteria isolated from *P. monodon* and the rearing water is suspected to have caused their mortality. At the time of sampling, cases of mass larval death were observed, with vibriosis suspected as the underlying cause.

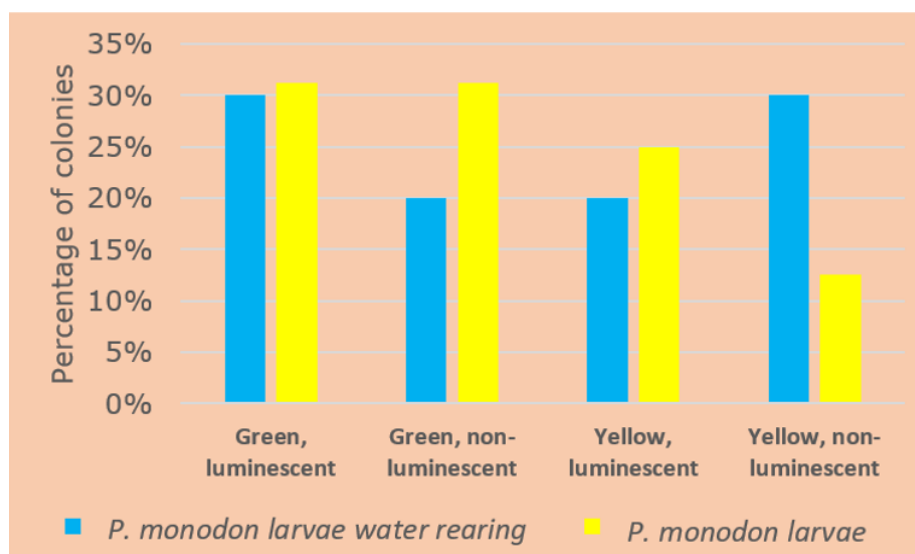


Figure 2. Colonies of *Vibrio* spp. isolated from (blue bar) rearing water and (yellow bar) *P. monodon* larvae during hatchery cultivation.

Table 1

Morphological identification of *Vibrio* isolated from water rearing and *P. monodon* larvae, Barru, South Sulawesi, Indonesia

No.	Code	Bacterial colony	Source	Cases of <i>P. monodon</i> death
1	IP70	Green Luminescent Green luminescent	<i>P. monodon</i> larvae rearing water	There are cases of shrimp death and AHPND-positive There are deaths and AHPND-positive
2	IP71	Green luminescent	<i>P. monodon</i> larvae rearing water	There are deaths and AHPND-positive
3	IP72	Yellow luminescent	<i>P. monodon</i> larvae rearing water	There are deaths and AHPND-positive
4	IP73	Green luminescent	<i>P. monodon</i> larvae rearing water	There are deaths and AHPND-positive
5	IP74	Yellow luminescent	<i>P. monodon</i> larvae rearing water	There are deaths and AHPND-positive
6	IP75	Yellow non-luminescent	<i>P. monodon</i> larvae rearing water	There are deaths and AHPND-positive
7	IP76	Yellow non-luminescent	<i>P. monodon</i> larvae rearing water	There are deaths and AHPND-positive
8	IP 77	Green non-luminescent	<i>P. monodon</i> larvae rearing water	There are deaths and AHPND-positive
9	IP78	Green non-luminescent	<i>P. monodon</i> larvae rearing water	There are deaths and AHPND-positive
10	IP79	Yellow non-luminescent	<i>P. monodon</i> larvae rearing water	There are deaths and AHPND-positive
11	IP80	Green non-luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
12	IP81	Green non-luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
13	IP82	Green non-luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
14	IP83	Green non-luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
15	IP84	Yellow non-luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
16	IP85	Green luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
17	IP86	Green non-luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
18	IP87	Yellow non-luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
19	IP88	Green luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
20	IP89	Green luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
21	IP90	Yellow luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
22	IP91	Yellow luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
23	IP92	Yellow luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
24	IP93	Yellow luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPsND-positive
25	IP94	Green luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive
26	IP95	Green luminescent	<i>P. monodon</i> larvae (PL11)	There are deaths and AHPND-positive

The isolates were cultured on TCBS agar, showing diverse colony morphologies indicative of different *Vibrio* strains. These bacterial colonies served as candidate hosts for bacteriophage screening. The visual distinction between colony types highlights the microbial diversity present in the hatchery environment, which is critical for evaluating phage specificity and host range. In addition to isolating *Vibrio* spp. Bacteria in *P. monodon* larvae and the rearing water, we also isolated *Vibrio* spp. from the environment around the *P. monodon* hatchery, such as the rearing water of the broodstock of *Scylla* spp., water sources, core centers, pond water around the hatchery, and seawater, which is the source of water for the *P. monodon* hatchery.

Table 2

Morphological identification of *Vibrio* isolated from the environment around the *P. monodon* hatchery, Barru, South Sulawesi, Indonesia

No.	Isolate code	Bacterial colony	Source	Notes
1	IP 108	Yellow non-luminescent	<i>Scylla</i> spp. broodstock water-rearing	No disease outbreaks
2	IP 109	Green non-luminescent	<i>Scylla</i> spp. broodstock water-rearing	No disease outbreaks
3	IP 110	Green non-luminescent	Clean water of Nucleus Centre	No disease outbreaks
4	IP 111	Green non-luminescent	Wastewater	No disease outbreaks
5	IP 112	Yellow non-luminescent	<i>Scylla</i> spp. broodstock water-rearing	No disease outbreaks
6	IP 113	Yellow non-luminescent	Pond water	No disease outbreaks
7	IP114	Green non-luminescent	Pond water	No disease outbreaks
8	IP115	Green non-luminescent	Seawater	No disease outbreaks
9	IP116	Yellow non-luminescent	Seawater	No disease outbreaks
10	IP117	Yellow non-luminescent	<i>P. monodon</i> broodstock hemolymph	No disease outbreaks
11	IP118	Yellow non-luminescent	<i>P. monodon</i> hemolymph	No disease outbreaks
12	IP119	Yellow non-luminescent	<i>Scylla</i> spp. broodstock hemolymph	No disease outbreaks
13	IP120	Yellow non-luminescent	<i>P. monodon</i> broodstock hemolymph	No disease outbreaks

Table 2 shows that of the 13 isolates of *Vibrio* bacteria that were successfully isolated from the environment, they were dominated by non-luminescent yellow colony bacteria, and no luminescent bacteria were found. This caused the condition of the shrimp, broodstock of *P. monodon*, larvae of mangrove crabs, and broodstock of mangrove crabs to be in a healthy condition, and there were no disease attacks or deaths, as in the sampling carried out on the maintenance water of *P. monodon* larvae and *P. monodon* larvae, where mass mortality occurred.

**Molecular identification of *Vibrio* spp.** Of the 39 isolates of *Vibrio* spp bacteria that have been isolated and identified morphologically, seven isolates were further identified molecularly using 16S-RNA sequencing (Table 3), and of the seven isolates, four isolates were identified as *V. parahaemolyticus* with a proximity index of 99.09-99.72%. The remaining three isolates were identified as *V. alginolyticus* (99.30%), *Vibrio sinaloensis* (98.26%), and *Photobacterium* sp. (95.07%).

Table 3

The results of sequencing bacterial isolates from the *P. monodon* and *Scylla* spp. hatchery, Barru, South Sulawesi, Indonesia

<i>Isolate code</i>	<i>Kinds of sample</i>	<i>Bacterial colony</i>	<i>Sequencing results</i>	<i>Identity (%)</i>	<i>Accession</i>
IP80	<i>P. monodon</i> Larvae (PL11)	Green non-luminescent	<i>V. parahaemolyticus</i>	99.24	NZ_CP031781.1
IP86	<i>P. monodon</i> Larvae (PL11)	Green non-luminescent	<i>V. parahaemolyticus</i>	99.09	NZ_CP031781.1
IP108	<i>Scylla</i> spp. broodstock water-rearing	Yellow non-luminescent	<i>Photobacterium</i>	95.07	NZ_DF093598.1
IP112	Wastewater	Green non-luminescent	<i>V. alginolyticus</i>	99.30	NZ_LOSN02000001.1
IP114	Pond Water	Green non-luminescent	<i>V. parahaemolyticus</i>	99.72	NZ_CP031781.1
IP115	Seawater	Green non-luminescent	<i>V. parahaemolyticus</i>	99.72	NZ_CP031781.1
IP120	<i>Scylla</i> spp. broodstock Hemolymph	Yellow non-luminescent	<i>V. sinaloensis</i>	98.26	NZ_JWLV01000018.1

**Bacteriophage isolation.** The results of bacteriophage isolation using *V. parahaemolyticus* and *V. harveyi* as hosts have obtained 11 positive samples containing bacteriophages from a total of 28 samples analyzed (Table 4). Of the 11 samples, seven were sediment samples, and four were water samples taken from the drainage channel of the *P. monodon* hatchery. This shows that sediment and water in the *P. monodon* and *Scylla* spp. hatcheries environment, Barru, South Sulawesi, Indonesia, are potential sources of bacteriophages with *V. parahaemolyticus* and *V. harveyi* as hosts.

In Table 4, it can be seen that the highest number of bacteriophage plaques was observed in sample T3.2, with 221 plaques, followed by sample T3.1 with 184 plaques, both using *V. harveyi* as the host (Figure 3). These two samples were sediment samples collected from *P. monodon* seed drainage channels. When *V. parahaemolyticus* was used as the host, the highest plaque count was found in sample A.3.1 (16 plaques), followed by sample T1.1 (10 plaques) (Table 4). It should be noted that this study was exploratory in nature and did not include experimental replicates. The plaque counts reported represent single observations for each sample, intended to provide preliminary insights into the presence and distribution of bacteriophages in different environmental matrices. Due to the absence of replication, error bars are not included, and variability was not assessed statistically. Nonetheless, these findings offer a valuable foundation for future studies involving quantitative validation and replication.

Table 4

Isolation of bacteriophages from the water outlet of the *P. monodon* hatchery, Barru, South Sulawesi, Indonesia

No.	Sample	Host	Kinds of sample	Results	Number of phage plug
1	A1	<i>V. harveyi</i>	Water	Positive	42
2	T1	<i>V. harveyi</i>	Sediment	Negative	0
3	A1	<i>V. parahaemolyticus</i>	Water	Negative	0
4	T1	<i>V. parahaemolyticus</i>	Sediment	Negative	0
5	A1.1	<i>V. harveyi</i>	Water	Negative	0
6	A1.2	<i>V. harveyi</i>	Water	Negative	0
7	A2.1	<i>V. harveyi</i>	Water	Negative	0
8	A2.2	<i>V. harveyi</i>	Water	Negative	0
9	A3.1	<i>V. harveyi</i>	Water	Negative	0
10	A3.2	<i>V. harveyi</i>	Water	Negative	0
11	T1.1	<i>V. harveyi</i>	Sediment	Negative	0
12	T1.2	<i>V. harveyi</i>	Sediment	Negative	0
13	T2.1	<i>V. harveyi</i>	Sediment	Positive	1
14	T2.2	<i>V. harveyi</i>	Sediment	Positive	1
15	T3.1	<i>V. harveyi</i>	Sediment	Positive	184
16	T3.2	<i>V. harveyi</i>	Sediment	Positive	221
17	A1.1	<i>V. parahaemolyticus</i>	Water	Negative	0
18	A1.2	<i>V. parahaemolyticus</i>	Water	Positive	3
19	A2.1	<i>V. parahaemolyticus</i>	Water	Negative	0
20	A2.2	<i>V. parahaemolyticus</i>	Water	Positive	4
21	A3.1	<i>V. parahaemolyticus</i>	Water	Positive	16
22	A3.2	<i>V. parahaemolyticus</i>	Water	Negative	0
23	T1.1	<i>V. parahaemolyticus</i>	Sediment	Positive	10
24	T1.2	<i>V. parahaemolyticus</i>	Sediment	Negative	0
25	T2.1	<i>V. parahaemolyticus</i>	Sediment	Positive	1
26	T2.2	<i>V. parahaemolyticus</i>	Sediment	Negative	0
27	T3.1	<i>V. parahaemolyticus</i>	Sediment	Positive	1
28	T3.2	<i>V. parahaemolyticus</i>	Sediment	Negative	0

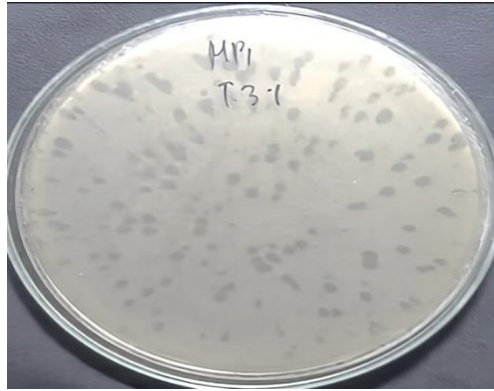


Figure 3. Bacteriophage plaques formed in sediment samples from *P. monodon* hatchery waste ponds in Barru, South Sulawesi, Indonesia (MP1: *V. harveyi* used as the host; T3.1: Sediment sample from the *P. monodon* hatchery drainage channel).

**Bacteriophage host range.** The host-range assay revealed that the bacteriophage isolated from sediment in the *P. monodon* hatchery pond using *V. harveyi* as the host - designated FMP1.T3.1 - was capable of lysing at least nine *Vibrio* spp. isolates. In comparison, the phage isolated from the same sediment sample using *V. parahaemolyticus* as the host - designated FSB28.T3.1 - lysed eight *Vibrio* spp. isolates out of the 20 tested. To visualize the lytic spectrum of both phages, a heatmap was constructed (Table 5), illustrating their activity against the 20 *Vibrio* isolates. Red cells indicate successful lysis, while green cells indicate resistance. FMP1.T3.1 exhibited a slightly broader host range than FSB28.T3.1, confirming its potential as a versatile biocontrol agent.

Table 5  
Lytic activity of FMP1.T3.1 and FSB28.T3.1 against 20 *Vibrio* isolates

Isolate code	<i>Vibrio</i> species	Phages	
		FMP1.T3.1	FSB28.T3.1
SB27	<i>V. alginolyticus</i>	0	0
SB28	<i>V. parahaemolyticus</i>	1	1
SB33	<i>V. parahaemolyticus</i>	0	1
SB53	Unidentified	0	0
SB58	Unidentified	1	0
SB59	<i>V. antiquarius</i>	1	1
BJ2	<i>V. campbellii</i>	0	0
BJ3	<i>V. campbellii</i>	1	1
BJ11	<i>V. campbellii</i>	1	1
BJ21	<i>V. antiquarius</i>	0	0
BJ23	<i>V. alginolyticus</i>	1	0
PR107	<i>V. vulnificus</i>	0	0
IP80	<i>V. parahaemolyticus</i>	1	1
IP84	unidentified	1	1
IP86	<i>V. parahaemolyticus</i>	0	0
IP108	<i>Photobacterium</i>	0	0
IP112	<i>V. alginolyticus</i>	0	0
IP114	<i>V. parahaemolyticus</i>	0	0
IP115	<i>V. parahaemolyticus</i>	1	1
IP120	<i>V. sinaloensis</i>	0	0

Notes: Red cells indicate lysis, while green cells indicate resistance. FMP1.T3.1 exhibited a broader host range, lysing 9 isolates, and FSB28.T3.1 lysing 8 isolates.

## Discussion

**Background and study objectives.** Vibriosis, caused by various *Vibrio* species, remains a leading cause of mortality in shrimp aquaculture, particularly at the seed stage, where it can result in up to 100% mortality in affected populations (Kumar et al 2021a; Benala et al 2023; Wang et al 2024). This study highlights the potential of using bacteriophages as an environmentally friendly solution to combat vibriosis, focusing on two *Vibrio* hosts, *V. parahaemolyticus* and *V. harveyi*, isolated from a *P. monodon* hatchery environment in Barru, South Sulawesi, Indonesia. To better understand the diversity and pathogenic potential of *Vibrio* species in this hatchery setting, we conducted a comprehensive characterization of bacterial isolates obtained during a mass mortality event.

***Vibrio* spp. associated with disease outbreaks.** Building on this context, several *Vibrio* species, including *V. harveyi* (Zhang et al 2020), *V. parahaemolyticus* (Ghenem et al 2017; Ding et al 2020), *V. alginolyticus*, and *V. campbellii* are frequently associated with disease outbreaks in hatcheries and shrimp ponds (Kumar et al 2021a; Aly et al 2023; Soto-Rodriguez et al 2024). These bacteria are widely distributed in aquatic environments and are known to cause luminescent diseases in marine and brackish water organisms. In addition to being primary pathogens, they often act as opportunistic agents in secondary infections (Saulnier et al 2000).

**Morphological and molecular characterization of *Vibrio* isolates.** To further characterize the bacterial community during the mortality event, green and yellow fluorescent colonies dominated the morphology of *Vibrio* spp. isolated from *P. monodon* larvae and larval maintenance water (Table 1). Sixteen isolates (IP80-IP95) were obtained, comprising 31.25% green luminescent, 31.25% green non-luminescent, 25% yellow luminescent, and 12.50% yellow non-luminescent bacteria (Figure 2). At the time of sampling, mass mortality of *P. monodon* larvae was observed. Laboratory analysis conducted at the RIBAFE confirmed the presence of *pirA* and *pirB* genes, indicating acute hepatopancreatic necrosis disease (AHPND) caused by *V. parahaemolyticus* (Ahmed et al 2021, 2023; Reyes et al 2022). This species has been widely reported as the causative agent of AHPND in both shrimp larvae and cultured shrimp (Duong et al 2023). AHPND is a highly virulent bacterial disease of penaeid shrimp, capable of reducing shrimp production by approximately 60%, with global economic losses reaching USD 43 billion (Kumar et al 2021b). Since its emergence in Asia in 2009, AHPND has caused significant declines in shrimp production (Caro et al 2020), with mortality rates ranging from 40 to 100% within 35 days of pond stocking (Hong et al 2016). By spring 2020, Liu et al. (2023) reported that AHPND had affected over 70-80% of coastal shrimp farms in China. In contrast, no fluorescent *Vibrio* colonies were isolated from seawater surrounding the hatchery, nucleic center source water, pond water, crab broodstock water, shrimp broodstock hemolymph, or crab hemolymph. The dominant colony colors were yellow and green (Table 3). The absence of luminescent bacteria in these samples suggests that the shrimp and crab broodstock were in healthy condition, with no signs of disease as observed in the previous sampling.

**Molecular identification and host selection for phage isolation.** To identify potential phage hosts, molecular identification of seven *Vibrio* isolates from the *P. monodon* hatchery environment, Barru, showed that four of them were identified as *V. parahaemolyticus* with a proximity index above 99.09-99.72%. The other three isolates were *V. alginolyticus* with a proximity index of 99.30%, *V. Sinaloensis* with a proximity index of 98.26%, and *Photobacterium* with a proximity index of 95.07% (Table 4). Two isolates out of four *Vibrio* bacteria identified as *V. parahaemolyticus* were isolated from shrimp larvae infected with AHPND, as evidenced by positive *PirA* and *PirB* analysis results. Further research was conducted by using *V. parahaemolyticus* as a host to detect the presence of bacteriophage in the environment of *P. monodon* and crab breeding in Barru, South Sulawesi, Indonesia. In addition to using *V. parahaemolyticus* as a host, this study also used *V. harveyi* obtained

from the Pathology Laboratory, RIBAFE, as a host to isolate bacteriophage from the environment.

**Phage isolation and host-range test.** As an environmentally acceptable biological control agent, bacteriophages offer a promising alternative to chemical drugs and antibiotics in aquaculture systems (Nachimuthu et al., 2021). One of their key advantages is host specificity, which allows phages to target pathogenic bacteria without harming beneficial or non-target microbial communities. Compared to broad-spectrum antibiotics, phages are considered safer for the environment and less likely to disrupt microbial balance (Sawa et al 2024). Building on this concept, the present study successfully isolated bacteriophages using *V. parahaemolyticus* and *V. harveyi* as hosts. Out of 28 environmental samples collected from the hatchery environment, 11 (39%) tested positive for phages (Table 4), a rate consistent with previous studies such as Phumkhachorn & Rattanachaiakunsopon (2010) and Cao et al (2021), who reported isolation rates of 32.6% and 33.3%, respectively. These findings reinforce the notion that *Vibrio*-specific phages are widespread in aquatic environments and may serve as effective biocontrol agents.

Among the 11 phage-positive samples, four originated from water and seven from sediment of the shrimp hatchery drainage channel. The highest number of plug phages was found in sediment sample T3.2 (221 plugs), followed by T3.1 (184 plugs), both isolated using *V. harveyi* as the host. In contrast, the highest plug count using *V. parahaemolyticus* as the host was observed in water sample A3.2 (16 plugs), followed by sediment sample T1.1 (10 plugs). Two representative phages, FMP1.T3.1 (from T3.1, using *V. harveyi*) and FSB28.T3.1 (from T1.1, using *V. parahaemolyticus*), were purified and subjected to host-range testing against 20 *Vibrio* isolates. FMP1.T3.1 lysed nine isolates, while FSB28.T3.1 lysed eight, demonstrating broad lytic activity and therapeutic potential. These results align with previous findings, such as the study by Cai et al (2023), which reported a lytic phage (vB\_VhaS-R18L) isolated using *V. harveyi* that was active against multiple *Vibrio* species, including *V. alginolyticus*, *V. cholerae*, *V. harveyi*, *V. parahaemolyticus*, and *V. proteolyticus*. Collectively, these data support the feasibility of using sediment- and water-derived phages as targeted, environmentally friendly solutions for controlling vibriosis in aquaculture.

**Mechanistic insights into host differences.** Interestingly, *V. harveyi* yielded a higher number of phage plaques compared to *V. parahaemolyticus*, suggesting that certain factors may contribute to this discrepancy. One possible explanation is the difference in receptor abundance on the bacterial cell surface, which could facilitate more effective phage binding and subsequent phage production. Additionally, *V. harveyi* is known for its ability to form biofilms (De Schryver et al 2014), which may provide an ideal environment for phage propagation. Biofilm-associated bacteria are often more resistant to external stresses, including bacteriophages, but they may also serve as more effective hosts in certain conditions due to the greater surface area available for phage attachment. Future studies investigating the molecular mechanisms of phage-host interactions, including receptor availability and biofilm formation, would help clarify the reasons behind the higher phage yields in *V. harveyi*.

Despite these promising findings, there are several limitations to this study. Firstly, only two *Vibrio* hosts (*V. parahaemolyticus* and *V. harveyi*) were tested for phage isolation. Future studies should consider including a broader range of *Vibrio* species to evaluate the host specificity and effectiveness of the isolated phages. Additionally, this study did not assess the seasonal variation in phage prevalence, which could influence phage concentrations due to changes in environmental conditions such as temperature and salinity. Seasonal variations in bacteriophage presence should be investigated to understand the broader ecological dynamics of phage populations.

To enhance the applicability of these findings, future research should focus on testing the efficacy of the isolated bacteriophages *in vivo*, under controlled aquaculture conditions. This would provide valuable insight into the practical use of phages as biocontrol agents in the prevention of vibriosis in shrimp farming. Furthermore, sequencing the genomes of the two broad-host phages (FSB28.T3.1, FMP1.T3.1) identified in this study

would help identify the key genetic factors responsible for their broad host range and potential for therapeutic use. Genome sequencing could also reveal insights into the evolutionary dynamics of *Vibrio* phages, aiding in the development of more effective and targeted phage therapies for aquaculture.

In light of these future directions, the findings of this study underscore the potential of bacteriophages as targeted, environmentally sustainable biocontrol agents against *Vibrio*-induced diseases in shrimp aquaculture. The successful isolation of phages from hatchery environments in Barru, South Sulawesi, combined with their broad lytic activity against pathogenic *Vibrio* strains, demonstrates a promising alternative to antibiotic-based treatments, particularly in Indonesian aquaculture systems where disease outbreaks remain a major challenge. These results not only highlight the ecological relevance of phages in tropical aquatic environments but also lay the groundwork for their practical application in hatchery-level disease prevention. Future research focusing on genomic characterization, seasonal dynamics, and *in vivo* efficacy will be essential to optimize phage-based interventions. Ultimately, this approach may support the development of more resilient and sustainable shrimp farming practices across Indonesia, reducing economic losses and enhancing biosecurity in coastal aquaculture.

**Conclusions.** This study identified a significant diversity of *Vibrio* spp. in the hatchery environments of *P. monodon* and *Scylla* spp. in Barru, South Sulawesi. A total of 39 *Vibrio* isolates were recovered from various sources, including *P. monodon* larvae, grow-out waters, *Scylla* spp. broodstock waters, and environmental samples. Molecular identification of seven isolates revealed that four were *V. parahaemolyticus* (99.09-99.72% similarity), while the remaining isolates were identified as *V. alginolyticus* (99.30%), *V. sinaloensis* (98.26%), and *Photobacterium* sp. (95.07%). Bacteriophages were successfully isolated from 11 out of 28 environmental samples. The isolated phages demonstrated potential as effective biological control agents against the *Vibrio* spp. present in the hatchery environments. Both the *Vibrio parahaemolyticus* phage (FSB28.T.3.1) and the *V. harveyi* phage (F.MP1.T.3.1) exhibited strong lytic activity against multiple *Vibrio* spp. isolates, confirming the successful isolation of phages with therapeutic potential. The isolated phages demonstrated a broad host range. The *V. parahaemolyticus* phage (FSB28.T.3.1) was able to lyse eight *Vibrio* spp. isolates, while the *V. harveyi* phage (F.MP1.T.3.1) lysed nine *Vibrio* spp. isolates. These findings indicate that both phages have the potential to target multiple *Vibrio* pathogens, making them versatile agents for biological control in aquaculture. The results of this study suggest that the sediment and water from Barru hatchery are promising sources of bacteriophages that can be used for biological control of *Vibrio* spp. pathogens. With the ability of these phages to infect a wide range of *Vibrio* species, they offer a sustainable, environmentally friendly alternative to antibiotics for managing bacterial infections in aquaculture. This opens up new opportunities for the development of more efficient and sustainable control strategies in the aquaculture industry.

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**Conflict of interest.** The authors declare that there is no conflict of interest.

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