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Phage diversity from shrimp ponds underpinning multi-host lytic activity in *Vibrio* pathogens

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Abstract. *Kinanthi AS, Prihanto AA, Jatmiko YD, Hemmatzadeh F, Nurdiani R. 2025. Phage diversity from shrimp ponds underpinning multi-host lytic activity in* Vibrio *pathogens. Biodiversitas 26: 4457-4464. Vibrio parahaemolyticus* is a major cause of seafood-related foodborne illness that has developed significant antibiotic resistance, creating an urgent need for effective and safer control management. This study aims to isolate and characterize lytic bacteriophages capable of inhibiting a broad spectrum of foodborne pathogenic bacteria. Bacteriophages were isolated from shrimp shell and pond wastewater samples, following a methodical approach that included bacteriophage isolation, density testing, confirmation, and host range testing. The results indicate that *V. parahaemolyticus* was successfully isolated from dead shrimp. The lytic bacteriophages, designated ΦALT (from pond wastewater) and ΦKU (from shrimp shells), were lysed their bacterial hosts, with activity detectable up to a dilution of 10^{-6}. This suggests that even at low concentrations, the phages could effectively lyse their bacterial targets. Both bacteriophages were found to exhibit a multi-host range, effectively lysing several pathogenic species such as *Vibrio alginolyticus*, *Vibrio cholerae*, *Vibrio harveyi*, and *Aeromonas hydrophila*, while not affecting *Salmonella* Typhi, *Pseudomonas aeruginosa*, or *Acinetobacter baumannii*. Transmission Electron Microscopy (TEM) revealed that ΦALT has a hexagonal shape, while ΦKU has an icosahedral shape. These findings highlight the potential of these bacteriophages as effective biocontrol agents in managing foodborne pathogens, offering a safer and more targeted alternative to antibiotics. Future molecular characterization is required to further understand these bacteriophages mechanism of action and improve their application in food safety.

Keywords: Aquaculture microbiome, broad host range, food safety, ICTV taxonomy

INTRODUCTION

Foodborne diseases, or food poisoning, are a major cause of illness and death worldwide. It is estimated that approximately 600 million people are affected by foodborne diseases annually, with 420,000 reported deaths linked to contaminated food consumption (Pires and Devleesschauwer 2021). Bacteria, viruses, and parasites can cause foodborne illnesses resulting from the consumption of contaminated food or beverages. Pathogen-related deaths are most often attributed to various bacterial agents such as *Clostridium perfringens*, *Clostridium botulinum*, *Listeria monocytogenes*, *Vibrio* spp., *Shigella* spp., *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp. (Almaary 2023). *Vibrio* species, which are opportunistic pathogens, are considered one of the primary causes of foodborne illnesses originating from seafood worldwide. Recent studies revealed that seafood-related disease outbreaks, especially those linked to *Vibrio* bacteria, are becoming more common in coastal areas, raising serious concerns for public health around the world (Brauge et al. 2024; Elbarbary et al. 2024).

While advanced technologies such as ultraviolet (UV) (UV) light, irradiation, and High-Pressure Processing (HPP) have been adopted to eliminate harmful bacteria, they can alter the sensory qualities of food and lead to nutrient loss. Furthermore, their high operational costs make them less accessible, particularly for small-scale producers (Khalid et al. 2023). This underscores the growing need for safer, cost-effective, and efficient antibacterial agents that can control foodborne pathogens while preserving food quality. The use of lytic bacteriophages is one of the promising alternatives (Irani et al. 2025).

Bacteriophages are viruses that naturally target and infect bacteria, specifically the pathogens responsible for foodborne illnesses. Unlike antibiotics, which can have broad-spectrum effects on both harmful and beneficial bacteria, lytic bacteriophages are highly specific and efficient in targeting only the pathogenic bacteria of interest. This specificity makes them an ideal biocontrol agent in the food industry. According to Śliwka et al. (2022), lytic bacteriophages work by lysing bacterial cells, which leads to the destruction of the pathogen without affecting human cells. Importantly, bacteriophages are not pathogenic to humans, making them a safe and viable option for use in food safety. In 2006, the U.S. Food and Drug Administration (FDA) also recognized the safety of using bacteriophages in food and classified them as Generally Recognized as Safe (GRAS) (Emilia 2020).

Several studies have explored the potential of bacteriophages as biocontrol agents to combat foodborne pathogens. Bhandare and Goodridge (2021) and Kinanti et al. (2024) suggest that bacteriophages could serve as bio-sanitizers and antibiofilm, providing effective antimicrobial action to support healthcare facilities and the food industry in ensuring food safety. The scientific investigations of De Miguel et al. (2020) also found that bacteriophages could serve as an alternative to antibiotics in the prevention and treatment of bacterial infections, particularly in the food supply chain. A significant breakthrough in this area is the discovery of *Vibrio parahaemolyticus* phages, which possess a broad host range, making them a valuable tool for the control of *Vibrio* species in food (Brauge et al. 2024).

The high density of phages in the aquaculture wastewater and shrimp carapace samples is attributed to the greater abundance of the host bacteria, *V. parahaemolyticus*, in that environment, which is the main reason for the exploration of these samples. Recent studies highlight a significant knowledge gap in the diversity of bacteriophages from Indonesian shrimp systems and their multi-host activity against different bacterial genera. While studies by Takwin et al. (2024) and Wahjuningrum et al. (2025) have successfully isolated phages from Indonesian shrimp ponds, their findings reveal limitations, such as narrow host specificity and limited cross-genus lytic activity. These results underscore the urgent need for further exploration to isolate and identify new, more effective potential phages.

In this study, we set out to find and examine lytic bacteriophages from shrimp waste and wastewater ponds that can attack *V. parahaemolyticus* and other harmful bacteria linked to foodborne illnesses. Our goal is to explore their potential as natural and effective solutions to improve seafood safety.

MATERIALS AND METHODS

Materials

The dead shrimp *Litopenaeus vannamei* (Boone, 1931) measuring approximately 16.9-17.2 cm and wastewater from ponds were obtained from shrimp farms in Probolinggo, East Java, Indonesia. *Vibrio* sp. was isolated from the hepatopancreas of the dead shrimp. Bacteriophage was isolated from the dead shrimp shell and pond wastewater.

Isolation and identification of *Vibrio* sp.

The isolation of *Vibrio* sp. was performed by following the method as described by Li et al. (2021) with slight modifications to the sample preparation. *Vibrio* sp. was isolated from the hepatopancreas of deceased *L. vannamei* shrimp using Thiosulfate-Citrate-Bile-Sucrose (TCBS) media composed of sodium citrate, 10.0 g; sodium thiosulfate, 10.0 g; sodium chloride, 10.0 g; yeast extract, 5.0 g. For species identification, the obtained isolates underwent molecular identification through PCR and 16S rDNA sequencing, beginning with DNA extraction using the Zymo Spin™ Kit. Bacterial cultures (24 hours) were mixed with sterile water, homogenized in ZR BashingBead™ and centrifuged. The supernatant was transferred to a Zymo-Spin™ IV Filter, followed by the addition of Binding Buffer, and then centrifuged. The DNA was washed with pre-wash buffer and wash buffer, then eluted with elution buffer. The purified DNA was filtered and analyzed using NanoDrop spectrophotometer with the A260/280 ratio used to assess its purity. The PCR protocol was set for 30 cycles, beginning with an initial denaturation at 94°C for 5 minutes, followed by denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 45 seconds. A final extension was carried out at 72°C. The resulting sequences were analyzed using Crustal X, BioEdit, and MEGA 5 software.

Bacteriophage isolation

The bacteriophage isolation process refers to the study by Tan et al. (2021), with modifications. In this process, 25 g of shrimp shell samples and 25 mL of pond wastewater were placed into separate Erlenmeyer flasks, and then 225 mL of SM Buffer was added. The mixture was centrifuged at 10,000 rpm at 4°C for 20 minutes. Subsequently, 20 mL of the supernatant resulting from centrifugation was mixed with 10 mL of Tryptic Soy Broth (TSB) media and 5 mL of 10^4 CFU/mL of *Vibrio* sp. bacteria, followed by incubation for 24 hours at 37°C. The phage suspension produced was centrifuged at 10,000 rpm at 4°C for 10 minutes. The resulting supernatant was then filtered using a 0.22 µm membrane filter. The phage suspension produced was detected using plaque assay method and double-layer technique.

Density of bacteriophage

Bacteriophage density was determined from the initial stock using the dilution method. Three plaques on 0.1 mL aliquot of bacteriophage stock were serially diluted in SM Buffer up to 10^{-7}. Simultaneously, *V. parahaemolyticus* was cultured in TSB at 37°C for 2 hours until the early log phase. For each dilution, 100 µL of bacteriophage was mixed with 100 µL of host culture in Tryptic Soy Broth (TSA) 0.6%, vortexed, and poured onto TSA 1.5% plates. The plates were then incubated at 37°C for 24 hours. Plaques formed within the 25-250 range were counted and expressed in PFU/mL. If the bacteriophage density was too high, the stock was further diluted using SM Buffer (Lukman et al. 2020).

Multi-host activity assay

The host range test on bacteria other than *Vibrio* sp. followed the protocol outlined by Moller et al. (2021) using TSB and TSA media containing 0.5% NaCl. The foodborne pathogenic bacteria tested included *Salmonella* Typhi, *Pseudomonas aeruginosa, Aeromonas hydrophila, Vibrio alginolyticus, Acinetobacter baumannii, Vibrio cholerae,* and *Vibrio harveyi*. All of the bacteria are obtained from our laboratory culture collection, which were isolated from local sources. Each bacterial strain was inoculated onto a separate TSA plate. A 0.1 mL aliquot of each strain was swabbed onto the TSA surface and left at room temperature for 30 minutes. Then, 5 µL of bacteriophage stock was spotted onto the TSA plates. The plates were divided into three sections: quadrants 1 and 2 contained 37 different phage samples, while quadrant 3 served as the SM Buffer control. All samples were incubated at 37°C for 24 hours, and plaque formation was observed at the end of the incubation period.

Morphological characterization of the bacteriophage

Morphological characterization of the isolated bacteriophage was performed using Transmission Electron Microscopy (TEM), which showed its typical morphology. The phage filtrate was negatively stained with uranyl acetate on a carbon-coated grid and examined under a Zeiss EM10CR microscope. The size and morphology were determined from three identical phage particles, confirming the specific structural features associated with this type of phage. Further molecular characterization is required for a comprehensive analysis (Alilesh et al. 2024).

RESULTS AND DISCUSSION

Isolation of *Vibrio* sp.

The isolation of *Vibrio* bacteria was conducted on dead shrimp in triplicate using marine TCBS media. Among 9 Petri dishes with dilutions up to 10^{-3}, 3 Petri dishes (at the 10^{-1} dilution) yielded colonies suspected to be *Vibrio*. These were identified in 6 colonies: 3, 2, and 1 colonies from plates 1, 2, and 3, respectively. The suspected *Vibrio* colonies exhibited green and yellow coloration, a circular shape, smooth edges, and convex elevation. These morphological features are consistent with the description of *Vibrio* colonies reported by Sugiharta et al. (2024), particularly the distinct green to yellow coloration and convex, circular form. Morphological identification was then performed on the suspected colonies. Among six colonies, only two colonies met the morphological criteria, likely indicating *V. parahaemolyticus* (Figure 1). These findings demonstrate that the isolation process effectively identified *Vibrio* species based on their characteristic colony morphology and provide a foundation for further investigation of *Vibrio* in seafood, particularly in relation to foodborne illness.

Molecular identification was performed to confirm the presence of *Vibrio* in the selected isolates. The electrophoresis results revealed that the selected isolate had a sequence length of approximately 1500 bp. The nucleotide sequences revealed that the selected colony was most closely related to the *V. parahaemolyticus* strain GRSPKGRMB-L1. The phylogenetic relationship between the selected isolate and this strain confirms the identity of the selected isolate as *V. parahaemolyticus*, reinforcing its significance in the study and potential applications for controlling this pathogen in foodborne disease research. Judging from the phylogenetic tree, it can be concluded that the A1 isolate is *V. parahaemolyticus* (Figure 2).

The isolation and molecular identification results confirm the presence of *V. parahaemolyticus* in shrimp samples from shrimp ponds in Probolinggo. This finding aligns with Kusmarwati et al. (2020), who reported a high prevalence and distribution of *V. parahaemolyticus* in marine environments, ponds, and beaches. The bacterium is commonly found in these locations, which contribute to its widespread occurrence in aquatic environments. Furthermore, *V. parahaemolyticus* is detected throughout all stages of the shrimp processing chain, including ponds, raw materials, intermediates, and finished products. This highlights the potential for contamination at various stages, making it a significant concern for food safety in the shrimp industry (Elshopakey et al. 2024; Eissa et al. 2025). The widespread presence of *V. parahaemolyticus* underscores the need for rigorous monitoring and control measures to prevent foodborne outbreaks and ensure the safety of seafood products.

Figure 1. Isolated bacteria presumed to be *Vibrio* sp.

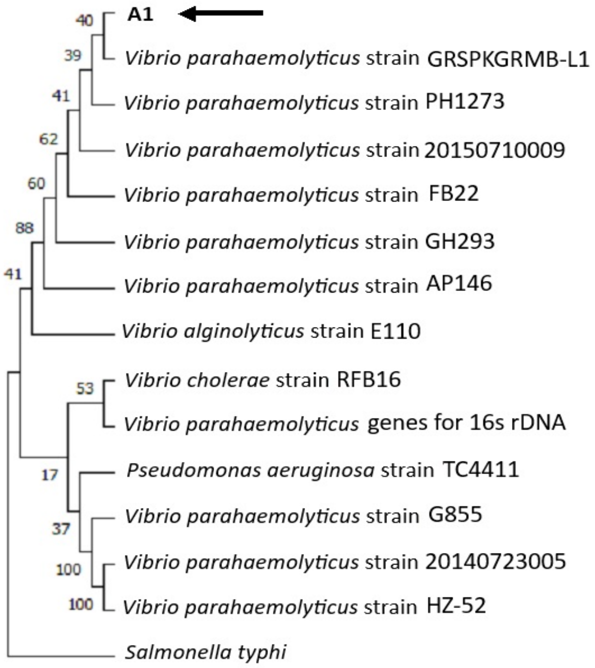

Figure 2. Phylogenetic tree for *Vibrio parahaemolyticus*

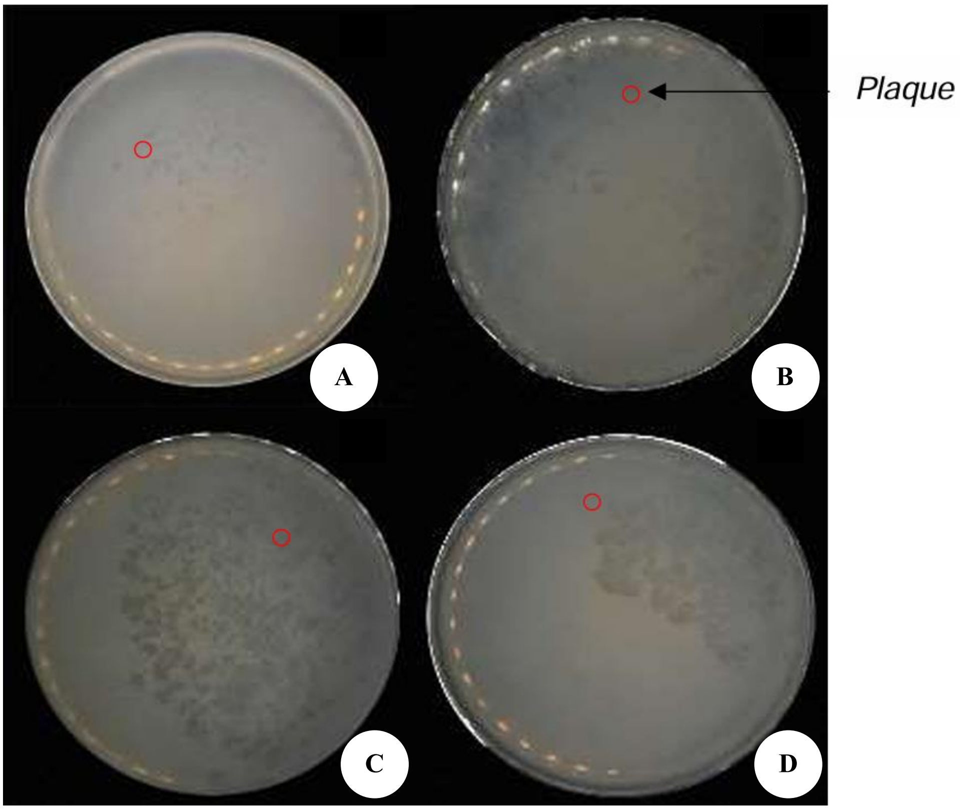

Figure 3. Plaque forming-isolated bacteriophage. Red circles are the plaque

Isolation of the bacteriophage

The isolation of *V. parahaemolyticus* bacteriophage from dead shrimp shells and shrimp pond wastewater samples was conducted using the plaque assay method with a double-layer technique. The results revealed the formation of plaques on *Vibrio* colony agar plates for both shrimp shell (KU1 and KU2) and pond wastewater samples (ALT1 and ALT2). The appearance of these plaques indicates the presence of *V. parahaemolyticus* bacteriophages in both shrimp shell and pond wastewater samples. In the plaque assay, plaques represent bacterial cells that have been lysed by the bacteriophage virus. Each plaque corresponds to one lysed bacterial cell, signifying successful infection and viral replication (Choliq et al. 2020; Navok et al. 2024).

The size of the plaques formed during the plaque assay can provide insight into the level of bacteriophage activity. Larger plaques typically indicate a higher production of new viruses, as the increased viral replication leads to the lysis of a larger number of bacterial cells. Conversely, smaller plaques suggest that fewer new viruses are produced, which could imply lower bacteriophage activity or a less effective lysis process. The ability to observe and measure plaque size is important for evaluating the efficacy of bacteriophages in lysing target bacteria (Abedon 2021; Tajima et al. 2024). The plaque morphology observed in the experiments can be seen in Figure 3.

Density of the bacteriophage

The measurement of *V. parahaemolyticus* bacteriophage density revealed a value of 9.27×10^4 PFU/mL in shrimp carapace samples (Figure 4) and 5.34×10^7 PFU/mL in shrimp pond wastewater samples (Figure 5). The significantly higher density in the wastewater sample compared to the carapace sample can be attributed to the greater abundance of *V. parahaemolyticus* host bacteria present in the wastewater. Wastewater, due to its higher bacterial load, creates a more conducive environment for bacteriophage replication and proliferation, resulting in a higher bacteriophage density.

Bacteriophage density is influenced by various factors such as the host's growth phase, pH, temperature, and host bacterial count. These factors can significantly impact the efficiency of phage propagation in different environments. Additionally, variations in bacteriophage density across the samples may also arise from differences in the viability of each sample. Viability can be affected by environmental conditions or sample preservation methods, which may lead to discrepancies in the observed phage concentrations (Lukman et al. 2020; Geng et al. 2024).

Two recent studies from Indonesia highlighted the significant knowledge gap regarding bacteriophage diversity and multi-host activity. The research carried out by Wahjuningrum et al. (2025) successfully isolated phages from shrimp pond water in Tasikmalaya and North Jakarta. Their findings suggest a narrow strain specificity, as evidenced by the phages' protective effects on Artemia. Similarly, the study by Takwin et al. (2024) demonstrated that their isolated phages had limited cross-genus activity, only capable of lysing one of six tested bacterial strains. These findings collectively underscore the need for further exploration to isolate and identify new and more effective potential phages from diverse ecological niches within Indonesian aquaculture systems.

Bacteriophages' host range

The host range test revealed that after adding 5 µL of bacteriophage lysate to the bacterial cultures, plaques (clear zones) formed in the cultures of *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. cholerae*, *V. harveyi*, and *Aeromonas hydrophila* (Table 1). These clear zones indicated that the bacteriophage was successful in lysing the bacterial cells, demonstrating the efficacy of the bacteriophages in targeting and killing specific pathogens. These results suggested that bacteriophages isolated from shrimp shells and pond wastewater possess substantial potential for use as biocontrol agents in the food industry. Further research into the application of bacteriophages as biocontrol agents against foodborne pathogens has shown promising results, especially when isolating bacteriophages from natural sources like shrimp shells and pond wastewater. These bacteriophages were analyzed for their host range, which refers to the range of bacterial species that they can infect and lyse. In a recent study, bacteriophages isolated from these natural environments were tested for their ability to lyse various foodborne pathogens (Paranos et al. 2024; Kinanti et al. 2025).

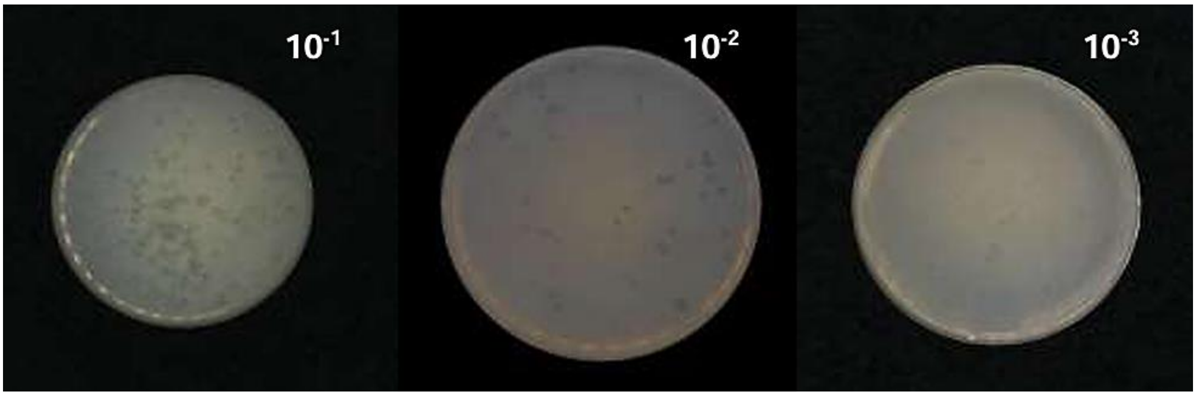

Figure 4. Density of the bacteriophages in shrimp carapace samples (10^{-1}, 10^{-2}, 10^{-3})

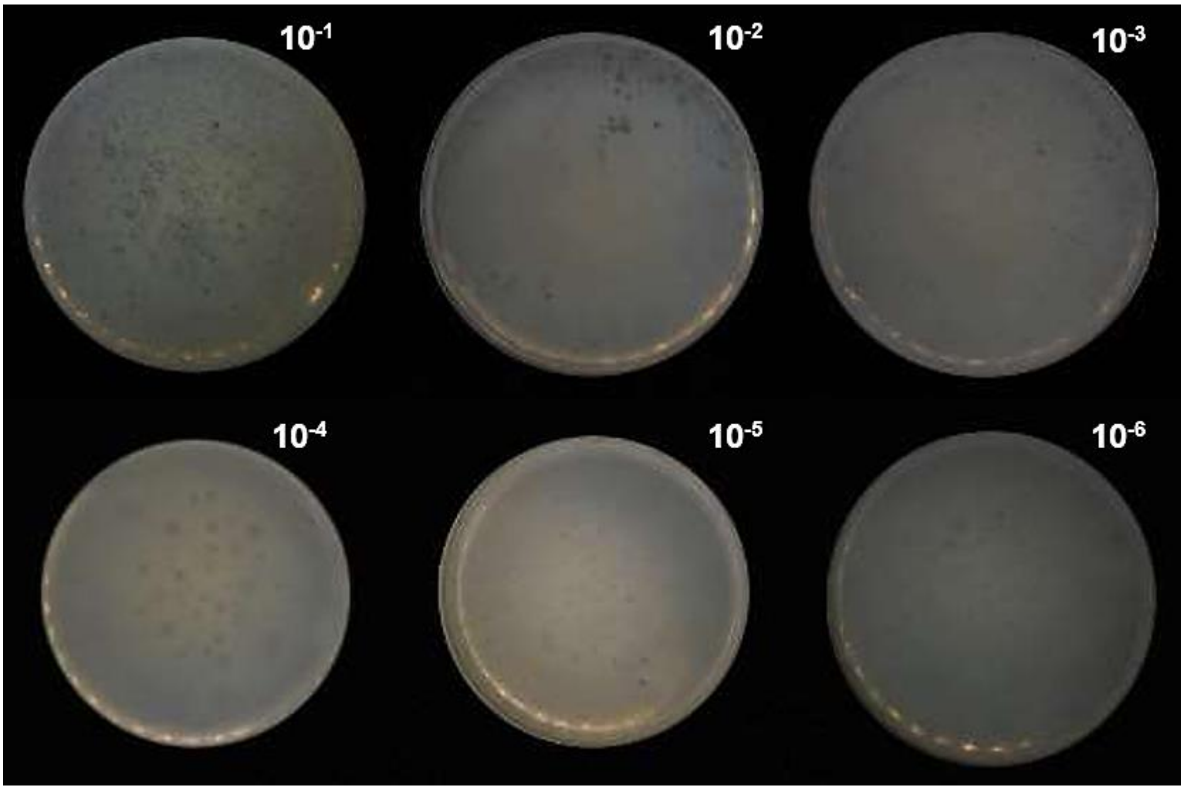

Figure 5. Density of the bacteriophages in pond wastewater samples (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6})

Table 1. Host range of the bacteriophage over several foodborne pathogens

Bacterial strain	Plaque formation
Vibrio parahaemolyticus	+
Vibrio cholerae	+
Vibrio alginolyticus	+
Vibrio harveyi	+
Salmonella Typhi	-
Pseudomonas aeruginosa	-
Aeromonas hydrophyla	+
Acinetobacter baumannii	-

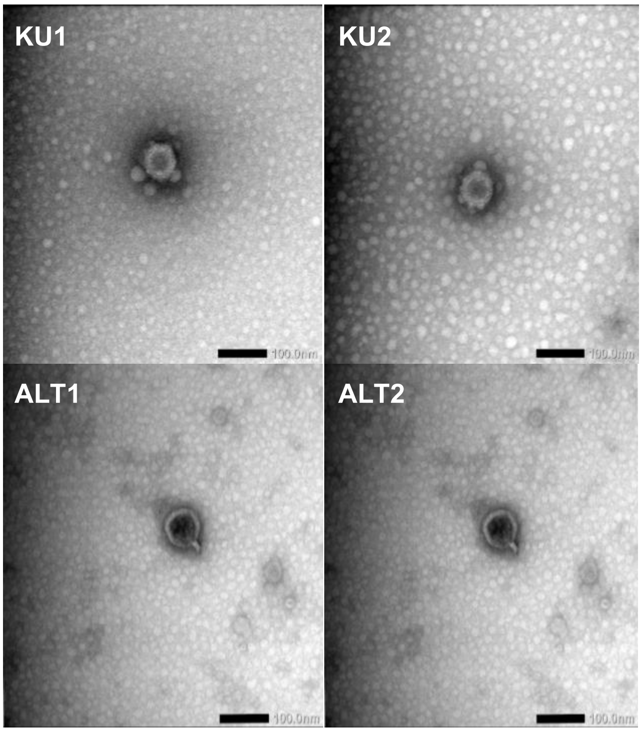

Figure 6. The morphology of the bacteriophages. (ΦKU1 and ΦKU2: shrimp shell); (ΦALT1 and ΦALT2: pond wastewater)

On the other hand, the bacteriophages did not produce plaques when tested against *Salmonella* Typhi, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, indicating that these bacterial species were not susceptible to the isolated bacteriophage. Although most bacteriophages are highly selective, several reports have demonstrated their ability to infect multiple strains, indicating a relatively broad host range and underscoring their potential as effective biocontrol agents (Göller et al. 2021). A broad host range means that the bacteriophage can target a wide variety of bacterial species, making it more versatile and effective in controlling multiple pathogens in the food supply (Kassa et al. 2021; Peng et al. 2024). The ability of the isolated bacteriophages to lyse both *Vibrio* and *Aeromonas* species may be due to the recognition of conserved surface epitopes or receptor structures shared between these genera, suggesting a possible molecular basis for their broad host range. The ability to lyse a variety of foodborne pathogens enhances the applicability of bacteriophages in improving food safety, especially in environments prone to contamination by *Vibrio* species, which is commonly found in seafood. The host range results of the bacteriophages isolated in this study are listed in Table 1, providing a clear representation of their potential applications in foodborne pathogen control.

Morphological identification of the isolated bacteriophage

The morphological characterization of the bacteriophages was performed on all isolated samples, namely ΦKU1, ΦKU2, ΦALT1, and ΦALT2, using Transmission Electron Microscopy (TEM). The primary objective of using TEM was to determine the classification or grouping of bacteriophages based on their morphological features. TEM analysis revealed that the KU and ALT isolates exhibited similar morphological characteristics, representing a single isolate, as shown in Figure 6. The two bacteriophages isolated from the KU samples, ΦKU1 and ΦKU2, demonstrated nearly uniform morphology and size. Specifically, the morphology of these two isolates was characterized by a distinct head structure and the absence of a tail (Figure 6).

Meanwhile, the morphological characterization of the bacteriophage isolates ΦALT1 and ΦALT2 showed results distinct from the ΦKU isolates. Based on the electron microscopy observations, both bacteriophages exhibited a short tail structure. This morphology is a defining characteristic that differentiates ΦALT1 and ΦALT2 from the other isolates and leads to their classification into a different group. These results from the present study highlight the unique and taxonomically significant characteristics of the ΦALT bacteriophages, which may contribute to broadening our understanding of phage diversity and classification. Notably, the findings are consistent with the observations of Li et al. (2024), whose morphological characterization of phage A1432 also emphasized distinctive structural traits with important taxonomic implications.

In conclusion, this study successfully isolated *Vibrio parahaemolyticus* bacteriophages from shrimp shells and pond wastewater, demonstrating their potential as effective natural antimicrobials. The isolates exhibited a broad host range, lysing not only *V. parahaemolyticus* but also *V. alginolyticus, V. cholerae, V. harveyi*, and *Aeromonas hydrophila*. Such multi-host lytic activity is noteworthy, as most phages are typically host-specific, suggesting that these isolates may serve as versatile tools for controlling diverse foodborne pathogens in aquaculture and seafood systems. Morphological characterization revealed distinct differences between the isolates: ΦKU1 and ΦKU2 (from shrimp shells) displayed an icosahedral, tailless structure, whereas ΦALT1 and ΦALT2 (from pond wastewater) possessed a hexagonal head and short tail. These contrasting morphologies highlight the natural diversity of phages in aquaculture environments and suggest ecological adaptations that may contribute to their host range variation.

Overall, these findings reinforce the potential of aquaculture systems as valuable sources of bacteriophages with promising food safety applications. By specifically targeting pathogenic bacteria without affecting beneficial microbiota, phages offer sustainable alternative to antibiotics, supporting safer seafood production and reducing the risk of antimicrobial resistance. Future research should focus on the purification, molecular characterization, and genomic profiling of these bacteriophages, along with stability and efficacy testing under real aquaculture and food-processing conditions. Such studies are critical to advance their development as safe and targeted biocontrol agents for improving seafood safety and aquaculture sustainability.

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