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Research Article

Isolation of H2S-generating Bacterium (*Desulfovibrio* sp.) and *Vibrio parahaemolyticus* from Aquatic Farming and *In Vitro* Evaluation of the Ability of Bacteriophages as Biocontrol

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Abstract

Shrimp aquaculture faces environmental challenges from H_2S production and bacterial pathogens like *Vibrio* spp. and *Desulfovibrio* spp.. This can create harmful compounds like H_2S and provide a breeding ground for *Vibrio* bacteria, causing diseases in shrimp. Antibiotics are commonly used to treat these diseases, but they can lead to resistance and pollution. Therefore, using bacteriophages as a treatment option is a more sustainable approach. This study isolated and evaluated bacteriophages capable of selectively inhibiting these bacteria, demonstrating their potential as sustainable biocontrol agents for improving water quality and reducing reliance on antibiotics. Bacteria were identified through biochemical and molecular tests, and bacteriophages were isolated using plaque and spread methods. This study isolated the *Desulfovibrio vulgaris* strain (12D) and identified bacteriophages capable of inhibiting *Vibrio parahaemolyticus* in shrimp aquaculture systems. Three potential bacteriophage strains were identified that can inhibit *Desulfovibrio vulgaris* and *V. parahaemolyticus* bacteria by altering the size, shape, and number of colonies in treatments supplemented with phages. Although they do not change the genetic sequence of these bacteria, they effectively control their population. Among the three bacteriophage lineages, ϕTT2H was the most effective in inhibiting *Desulfovibrio vulgaris.* This research demonstrates the potential of using bacteriophages in shrimp culture to control bacterial populations.

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1. Introduction

Aquaculture represents a cornerstone of Vietnam's economy, particularly in the Mekong Delta, which contributes significantly to shrimp farming. However, this industry faces severe challenges, including disease outbreaks such as Acute Hepatopancreatic Necrosis Disease (AHPND) driven by *Vibrio* spp., and the accumulation of toxic H_2S from decomposing residual feed in shrimp ponds. Current water treatment methods heavily rely on antibiotics and chemicals due to their rapid effectiveness. However, excessive use of these substances has resulted in severe consequences, such as antibiotic residues in seafood, environmental pollution, and the emergence of antibiotic-resistant bacteria. For instance, antibiotic-resistant pathogens are implicated in over 30,000 deaths annually in Europe, with projections indicating up to 10 million deaths worldwide by 2050, posing a substantial economic and health burden (Himanshu *et al.,* 2022). *Vibrio* strains isolated from clinical and environmental samples show high resistance to many antibiotics such as amoxicillin, ampicillin, carbenicillin, cefazolin, tetracycline, chloramphenicol, oxytetracycline, ceftazidime, cephalothin and colistin (Dutta *et al.,* 2021; Mancini *et al.,* 2023; Nguyen *et al.,* 2024; Tan *et al.,* 2020). The most popular antibiotic residues of *Vibrio* spp. are polymyxin B (100%), azithromycin (100%), and ciprofloxacin (16.1%) (Gxalo *et al.,* 2021). *Desulfovibrio* spp. are sensitive to imipenem, metronidazole, clindamycin, and chloramphenicol (Fujihara *et al.,* 2023).

Finding the primary cause of antibiotic resistance in bacteria found in aquatic products is incredibly difficult, and controlling its detrimental effects on shrimp and the environment is much more difficult (Pepi and Focardi, 2021). Thus, using bacteriophages (ϕ) is one of the most powerful techniques to replace antibiotics for controlling pathogens. The phages specifically attack certain host bacteria and do not have any effect on other beneficial ones. A report by (Van and Thu, 2023) showed that bacteriophages L12 and T0 isolated from shrimp pond water were capable of infecting *Desulfovibrio* spp. and preventing the growth of 11D bacteria as well as preventing the generation of H_2S gas. Bacteriophage BP14 reduces the expression of the virulence protein AHPND in *V. parahaemolyticus* (Hsu *et al.*, 2024). Phage vB_VpaP_SJSY21 is an effective tool to prevent AHPND in shrimp farming (Xu *et al*., 2023), and there are reports of effective inhibition of *V. parahaemolyticus* in vitro by bacteriophages (Lee *et al.,* 2023; Wahjuningrum *et al.,* 2024).

This study was conducted to find bacteriophage strains that can suppress *Desulfovibrio* spp. and *Vibrio* spp. In addition, it may pave the way for expanding shrimp farming and enhancing clean and safe fishery production.

2. Materials and Methods

2.1 Materials

Desulfovibrio spp. were isolated from shrimp pond water samples in Kien Giang, Ca Mau, Bac Lieu, and Soc Trang provinces. The *Vibrio parahemolytius* ATCC 17802 bacterial strain and 18 bacteriophages (Table 2), were supplied by The Biomolecular Laboratory, Institute of Food and Biotechnology, Can Tho University. The strains were cultivated in Thiosulfate citrate bile salts sucrose (TCBS) (Himeida, India), Postgate B medium and Tryptic soy agar (TSA) (Himeida, India) at 37°C with shaking at 120 rpm for 24 hours.

Table 2. Interaction of bacteriophages with *Vibrio parahaemolyticus* 17802 and *Desulfovibrio vulgaris* 12D.

***V. parahaemolyticus* 17802**		***D. vulgaris 12D***	
Position	**Phage**	**Position**	**Phage**
1	ϕMix	1	ϕMA
2	ϕA2223	6	ϕA2223
4	ϕCM	8	ϕLAX1
5	ϕKM2	9	ϕTT1H
7	ϕKM14	10	ϕTT2H
8	ϕKM16		
10	ϕTT1H		
14	ϕTT2H		

2.1.1 Ethical approval

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

2.2 Methods

2.2.1 Isolation and identification of Desulfovibrio vulgaris and Vibrio parahaemolyticus

A total of 7 mL of shrimp farming water and 13 mL of Postgate B liquid medium were mixed and placed at room temperature for 24 hours. Then, the mixture was spread in the TCBS (thiosulphate citrate bile salt) medium, incubated at room temperature, and selected for black-forming colonies.

The morphological characteristics (shape, color, and size) of *Vibrio* spp. and *Desulfoyibrio* spp. were observed under the stereoscope (A.KRÜSS Optronic GmbH, Germany) as described previously (Ab-

delaziz *et al.,* 2017; Huynh *et al.,* 2021). The catalase reaction (H_2O_2 degradation) was described by (Aryal, 2015a). The isolated bacteria were placed in the lamen, into which a 3% H_2O_2 solution was dropped. The foamy appearance indicates a positive reaction. If not, the reaction is negative. The oxidase reaction (Aryal, 2015b) was carried out by spreading bacterial colonies cultured on TSA medium onto an oxidase test pad. The blue-violet or blue color or no color appearance revealed a positive or negative reaction.

The ability of *Desulfovibrio vulgaris* and V*ibrio parahaemolyticus* bacteria to produce H_2S was determined using the method of (Warren *et al.,* 2005). The colonies grown on TSA medium were deeply inoculated into SIM (sulfide indole motilily) medium containing 3 g/L of beef extract, 30 g/L of peptone, 0.2 g/L of ferrous ammonium sulfate, 0.025 g/L of sodium thiosulfate, and 3 g/L of agar. The medium, or the infusive lines reversing to black (black precipitate), demonstrated the H_2S-generating abilities.

2.2.2 In vitro evaluation of host spectrum of bacteriophages by spot test

The host range of the phages was determined using the agar spot test on TCBS agar plates supplemented with 0.5% agar (Van and Thu, 2023).The bacterial solution was triple spread on the surface of the medium and incubated at room temperature for 30 to 45 minutes. Amount of 2 µL of bacteriophages were dropped into the dishes and incubated at room temperature for 24 h.

2.2.3 Identification of bacteria by biomolecular

DNA was amplified using ToxR primers of *Vibrio* spp.. The PCR contained 25 µl H_2O, 3 µl DNA, 20 µl My TaqMix 2X (Bioline, England), 1 µl F-primer 5'–GTCTTCTGACGCAATCGTTG–3', and 1 µl R-primer 5'–ATACGAGTGGTTGCTGTCATG–3'. The amplifier was carried out at 94°C for 10 min with 20 cycles: 94°C (one minute), 63°C (90 seconds), 72°C (90 seconds), and 72°C (10 minutes) (Abdelaziz *et al.,* 2017). Then, products were examined by 2% agarose gel electrophoresis with 10 mM Tris, 5 mM borate, and 0.1 mM EDTA in 1X TBE to find the bank at 368 bp. Then, the nucleotides were sequenced and compared to the Genbank using the BLAST tool.

For identifying *Desulfovibrio* spp., the experiment was carried out similarly to that of *Vibrio* spp. except for the addition of 16S rRNA with 1 µl F-primer 5'–CAGGCCTAACACATGCAAGTC–3', 1 µl R-primer 5'– GCATCTGAGTGTCAGTATCTGTCC–3'. The PCR was carried out at 95°C for 10 min with 30 cycles: 95°C (one minute), 55°C (90 seconds), 72°C (60 seconds), and 72°C (5 minutes) (Montieri *et al.,* 2010). Then, products were examined as described above to find the bank at the position of 1500 bp. Then, the nucleotides were sequenced and compared to the Genbank using the BLAST tool.

2.3 Analysis Data

MS Excel (Microsoft Corp., Redmond, WA, US) and Minitab 16 software (Sydney, NSW, Australia) were used for ANOVA statistical analysis by Tukey's and Fisher's tests. Data were shown ± standard deviations (SDs) as triplicate experiments' means.

3. Results and Discussion

3.1 Results

From eight isolated bacterial strains, bacterial strain 12D was initially identified as *Desulfovibrio* spp. based on morphological characteristics such as black color and smooth surface of the colonies measuring 2-3 mm. Figure 2 showed the biochemical test results and colony characteristics of two bacterial strains, *V. parahaemolyticus* 17802 and 12D. On TCBS medium, bacteria 17802 appeared as green, smooth, round colonies measuring 1-2 mm (Figure 2A), similar to the round-shaped, smooth, black colonies of bacteria 12D which produce H_2S gas (Figure 2B).

Bacteria 12D are comma-shaped, gram-negative (Figure 2D), motile (Figure 2F), negative for catalase (Figure 2H), oxidase (Figure 2K), and capable of producing H_2S gas (Figure 2M). Similarly, bacterium 17802 is comma-shaped, gram-negative (Figure 2C), motile (Figure 2E), and positive for catalase (Figure 2G) and oxidase (Figure 2J). Results Table 1 summarizes the biochemical characteristic of two bacterial strains, 12D and 17802, initially identifying bacterial strain 12D as *Desulfovibrio* spp..

Table 1. Physiological and biochemical characteristics of *Vibrio* sp. 17802 and *Desulfovibrio* sp. 12D.

Characteristics	Bacterium	
	17802	12Đ
Shape	Curved-rod	Curved-rod
Colony	Blue	Black
Gram	–	–
Spore	–	–
Mobility	+	+
Catalase activity	+	–
Oxidase activity	+	–
H_2S generation	–	+

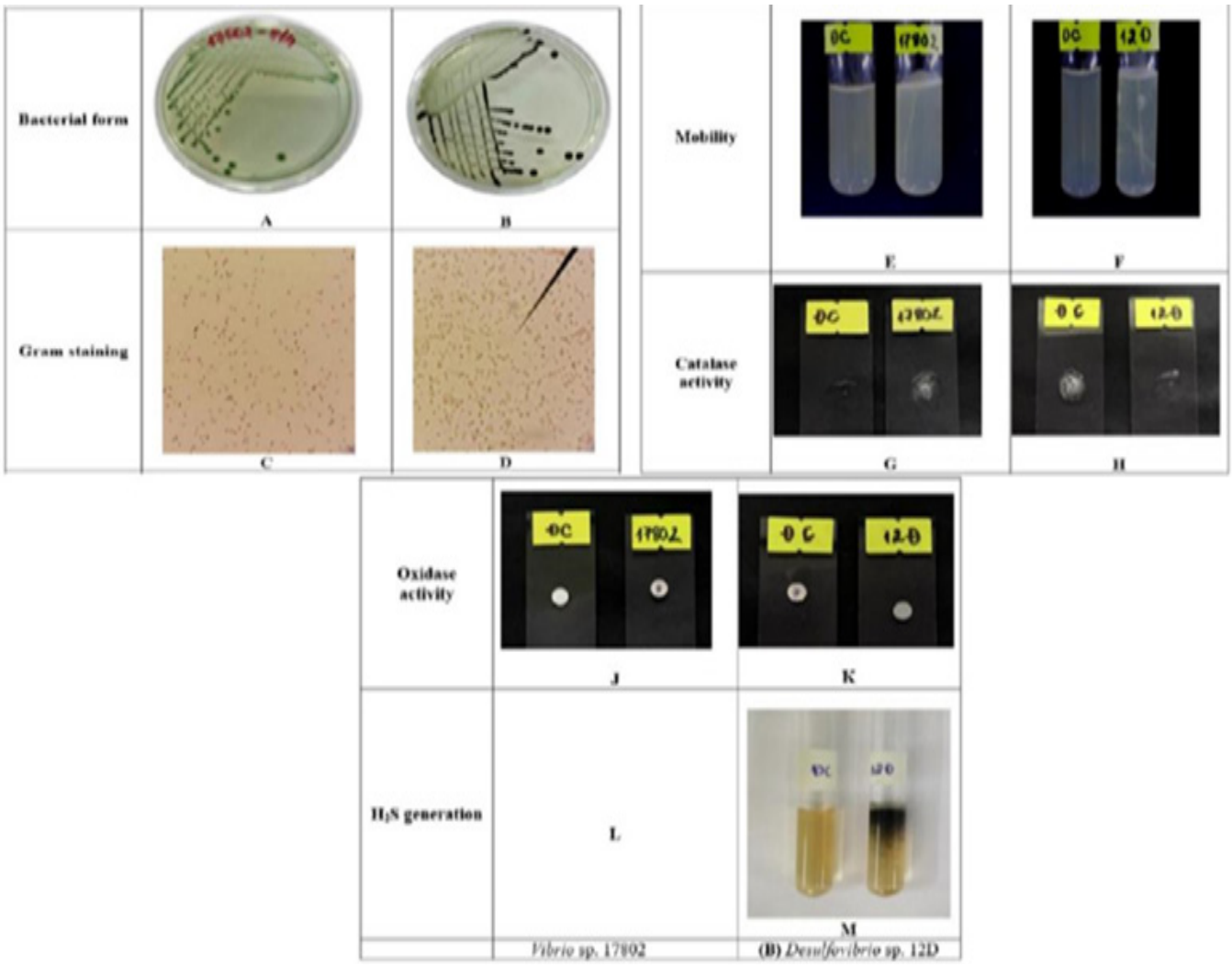

Figure 2. Characteristics of bacteria in TCBS medium. (A) 17802 and (B) 12D colonies.

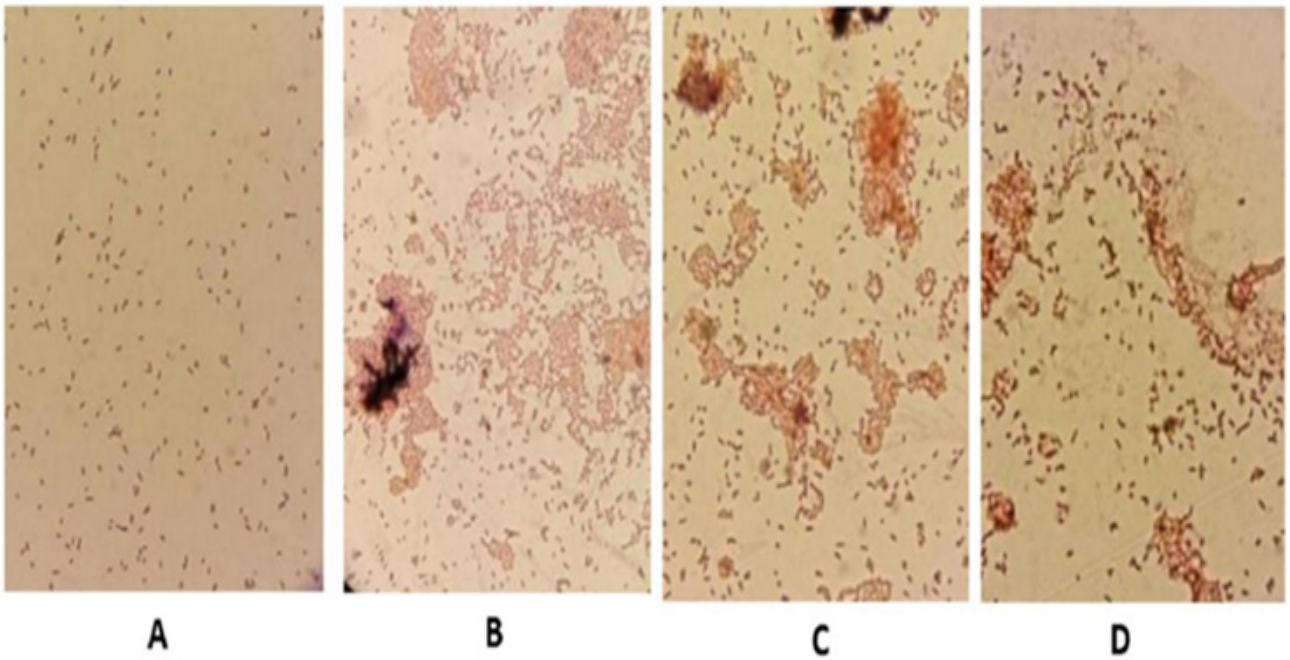

Figure 3. Gram dyeing of 17802 bacterium. The bacterium The bacterium infected by non-bacteriophages (A) Control, ϕTT1H (B), ϕTT2H (C), and ϕA2223 (D).

In order to study the relationship between bacteria and bacteriophages, a microscopic examination was conducted as part of an experiment. In the control group (Figure 3A), bacteria 17802 exhibited a curved rod shape, pink color, and were evenly distributed in the medium. Following a 30-minute incubation with phages ϕTT1H, ϕTT2H, and ϕA2223, the bacteria appeared to clump together and form stacks (Figure 3B, C, and D). This clustering phenomenon was also observed in the case of 12D bacteria in both the control group (Figure 4A) and the phage-treated groups (Figure 4B, C, and D).

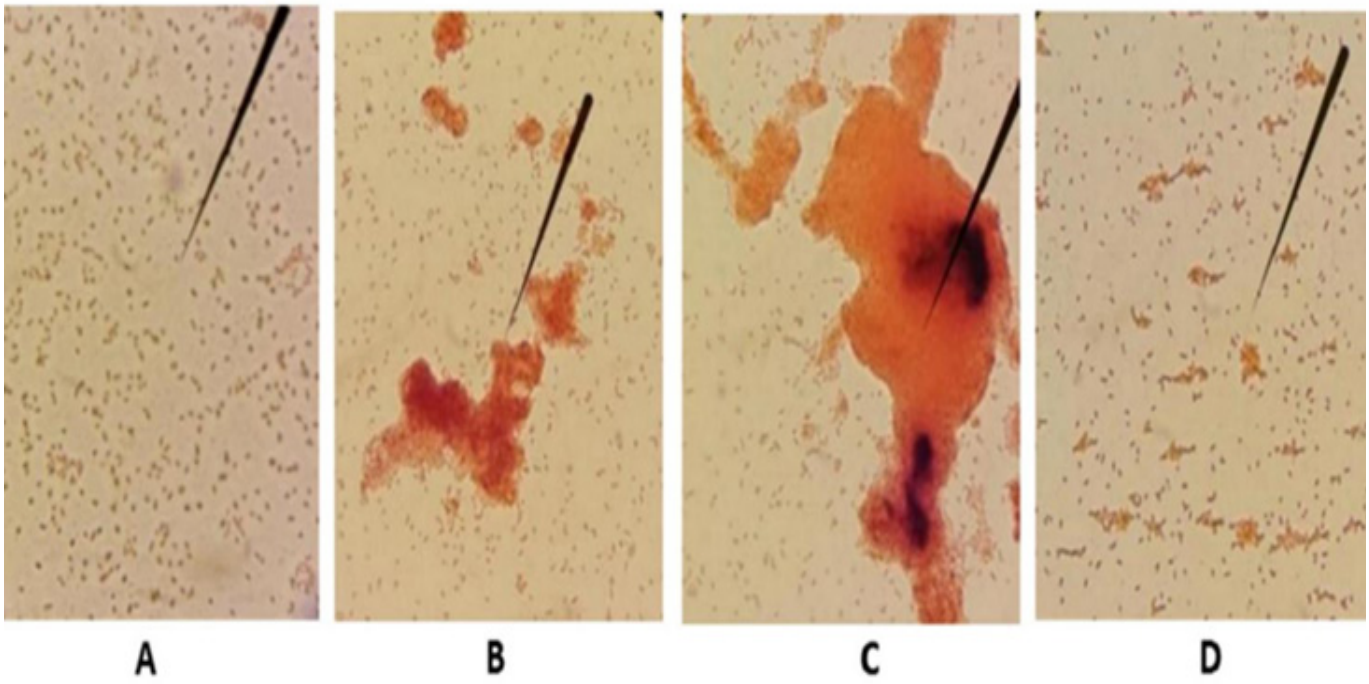

Figure 4. Gram dyeing of 12D bacterium. The bacterium infected by non-bacteriophages (A), (B), (C), and (D) are described in the legends of Figure 3.

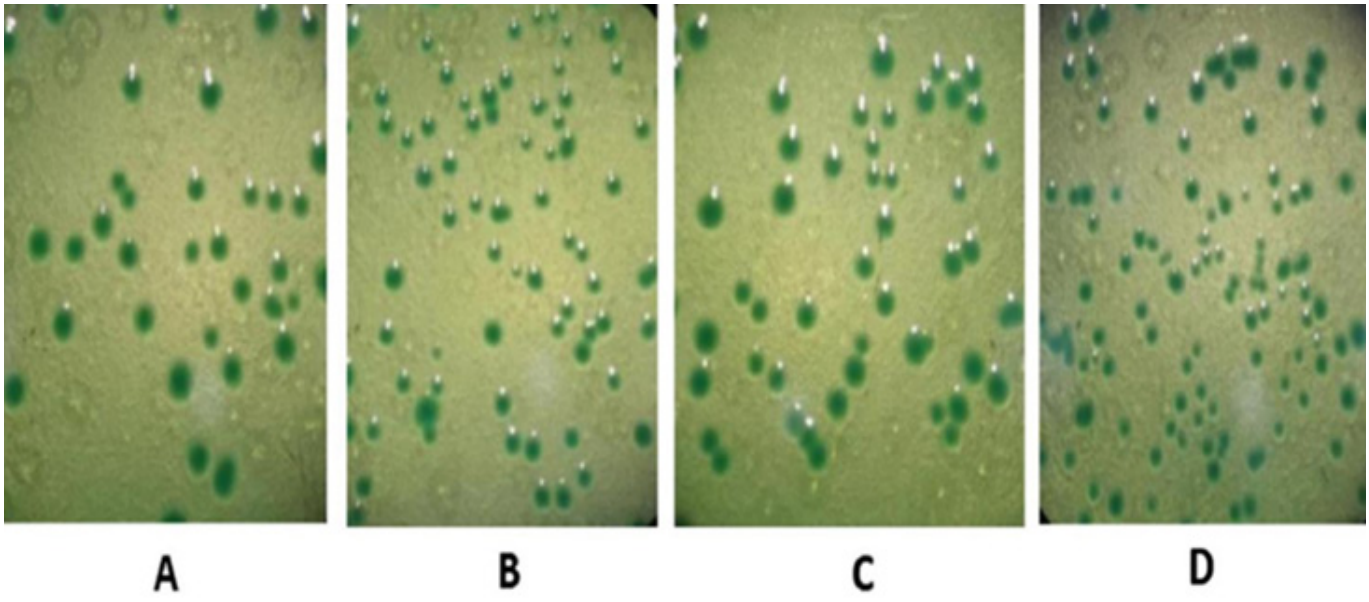

Figure 5. 17802 colony shapes observed by microscope. (A), (B), (C), and (D) are described in the legends of Figure 3.

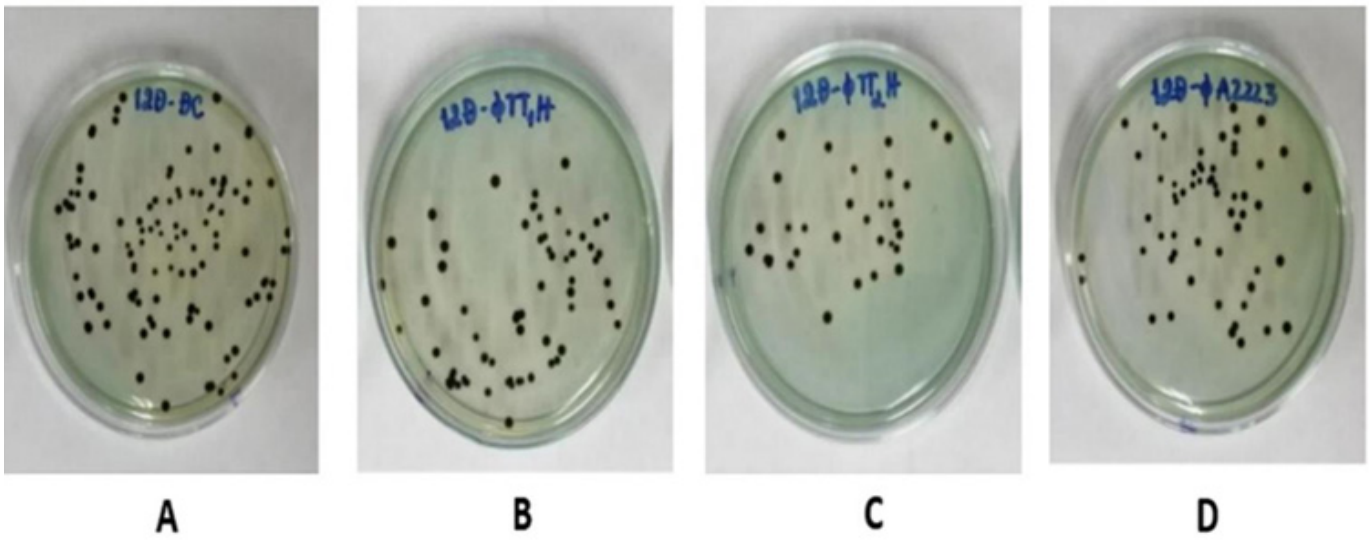

Figure 6. 12D colony shapes. (A), (B), (C), and (D) are described in the legends of Figure 3.

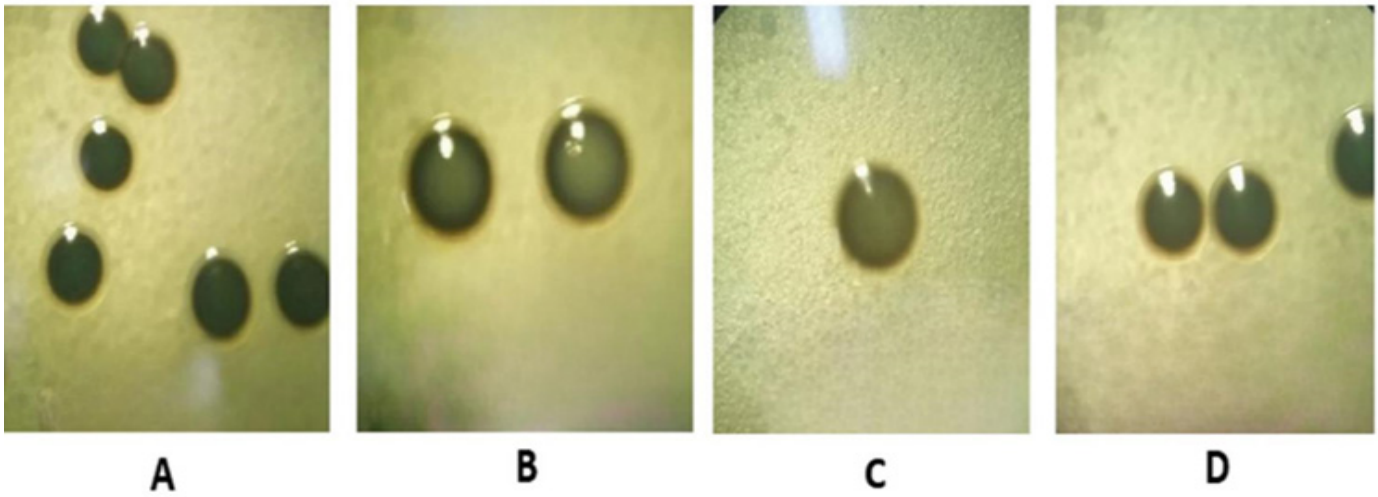

Figure 7. 12D colony shapes. (A), (B), (C), and (D) are described in the legends of Figure 3.

Table 3. Sizes of bacteriophages infecting bacterial colonies (mm).

Strain	Bacteria			
	Control	+ ϕTT1H	+ ϕTT2H	+ ϕA2223
17802	$2.2^{a} \pm 0.42$	$1.3^{b} \pm 0.48$	$1.4^{b} \pm 0.52$	$1.5^{b} \pm 0.53$
12Đ	$3.1^{a} \pm 0.32$	$3^{a} \pm 0.67$	$2.5^{b} \pm 0.52$	$1.7^{c} \pm 0.48$

Table 4. Quantity of bacteriophages infecting bacterial colonies (CFU/ml).

Strain	Bacteria			
	Control	+ ϕTT1H	+ ϕTT2H	+ ϕA2223
17802	$8.32^{a} \pm 0.03$	$8.55^{b} \pm 0.01$	$8.48^{c} \pm 0.06$	$8.71^{d} \pm 0.04$
12Đ	$8.95^{ab} \pm 0.04$	$9.12^{a} \pm 0.06$	$8.69^{c} \pm 0.17$	$8.81^{bc} \pm 0.08$

After exposure to bacteriophages, changes in colony size and shape were observed in bacteria 17802 (Figure 5) and 12D (Figures 6 and 7). The bacteriophages ϕTT1H, ϕTT2H, and ϕA2223 reduced the size of 17802 colonies by 40.9%, 36.4%, and 31.8% respectively. For 12D bacteria, ϕTT2H and ϕA2223 reduced colony size by 19.4% and 45.2% respectively, while ϕTT1H did not have a significant impact on colony size (Table 3). Results Table 4 demonstrates that ϕTT1H, ever, there was a difference in band length between the control wells and the wells supplemented with phage, with the most significant difference observed in the well supplemented with ϕA2223. Similar, 12D bacteria (Figure 8B), which amplified the target sequence of the 16S rRNA gene segment, the results indicated the presence of DNA bands in both the control wells and the wells supplemented with bacteriophages.

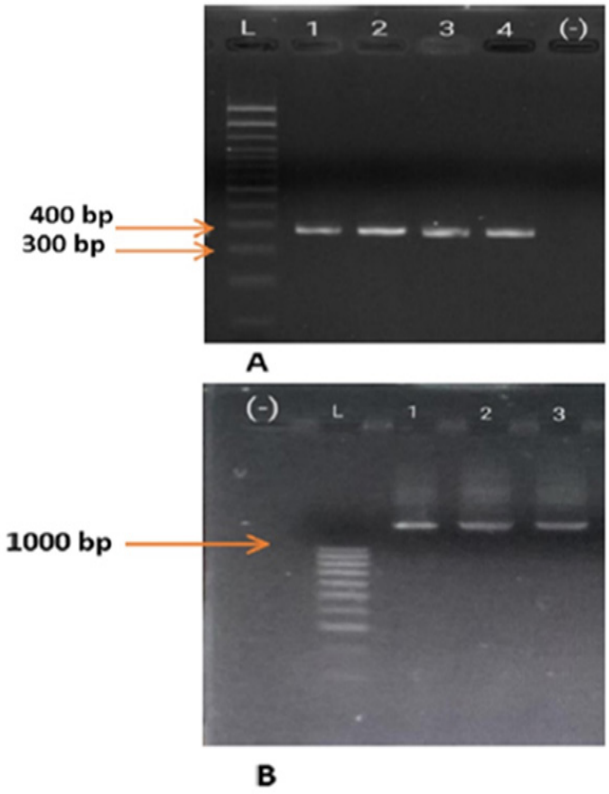

Figure 8. PCR product of *Vibrio* sp. 17802 and *Desulfovibrio* sp. 12D strains. (A) Identify 17802 toxic gene ToxR. L well – 1200 bp ladder. (B) 12D gene sequenced with 16S rRNA primers. The bacteria infected by non-bacteriophages (Well 1) – Control, ϕTT1H (Well 2), ϕTT2H (Well 3), and ϕA2223 (Well 4). Well -, negative control.

ϕTT2H, and ϕA2223 increased the number of colonies of bacteria 17802 by 2.76%, 1.92%, and 4.69%, respectively, compared to the control. Additionally, ϕTT2H significantly reduced the number of 12D bacteria by 2.9% compared to the control. Strain ϕA2223 did not show a significant reduction in the number of colonies, while ϕTT1H increased the number of 12D bacteria colonies by 1.89%.

Figure 8A illustrates the results of amplifying the 368 bp target sequence of the ToxR gene in bacteria 17802 with and without phage addition. All four samples exhibited a target DNA band of approximately 300-400 bp in size. This is consistent with the results of toxin gene identification (Quang *et al.*, 2020). How

The PCR products of the DNA sample with ToxR primers of ϕTT1H, ϕTT2H, and ϕA2223 infected *Vibrio* sp. 17802 and the control one were chosen to be sequenced and compared nucleotide sequences by NCBI. Figure 9A indicated that the 17802 ToxR sequence was 100% homologous to the *Vibrio parahaemolyticus* strain with 98% query coverage. From this sequence and the mentioned results, 17802 is the *Vibrio parahaemolyticus* strain (Figure 9B). The 12D 16S rRNA gene was compared in GenBank by Blast to identify a strain, indicating that the nucleotide sequence of 12D was 96.69% homogenous to that of the *Desulfovibrio vulgaris* strain with 71% query coverage and the E-value = 0.0 (Figure 10A). It means that 12D is the *Desulfovibrio vulgaris* strain (Figure 10B).

Figure 9. Nucleotide sequences of *Vibrio* sp. 17802 Species (A) and ToxR genes (B) in NCBI using BLAST tool. Query: 17802 strain, Sbjct: *Vibrio parahaemolyticus* (ID: CP014046.2) strain.

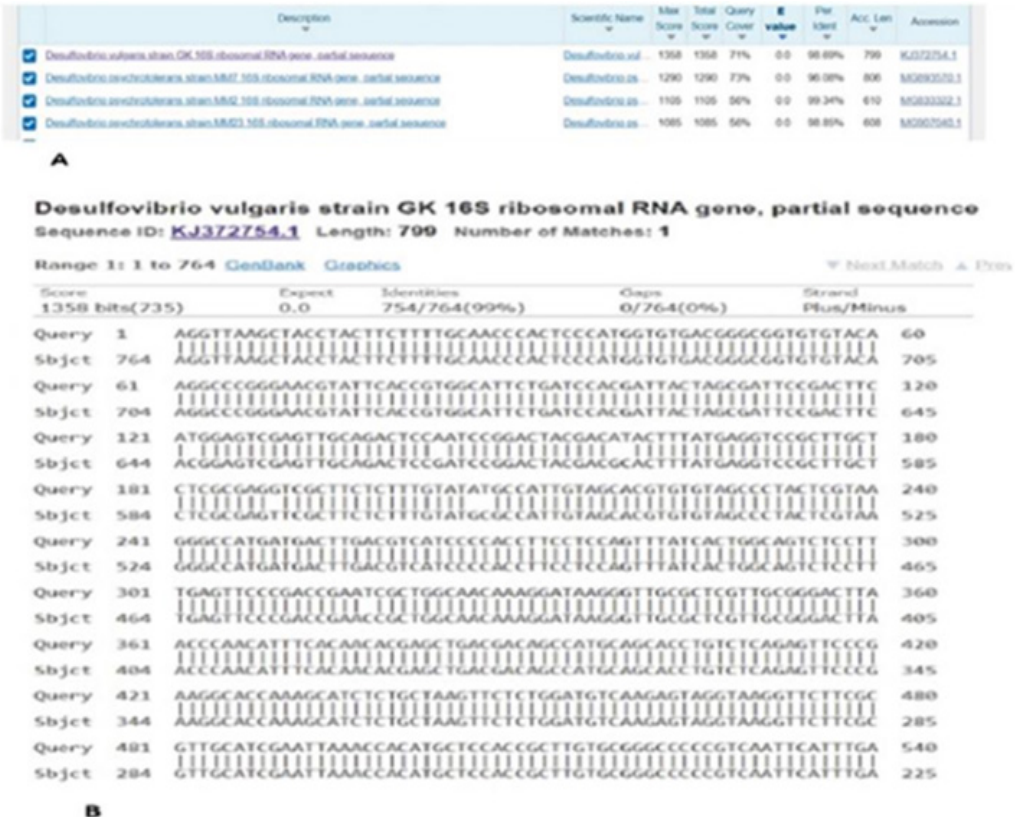

Figure 10. Nucleotide sequences of 12D Species (A) and 16S genes (B) in NCBI using BLAST tool. Query: 12D strain, Sbjct: *Desulfovibrio vulgaris* (ID: KJ372754.1).

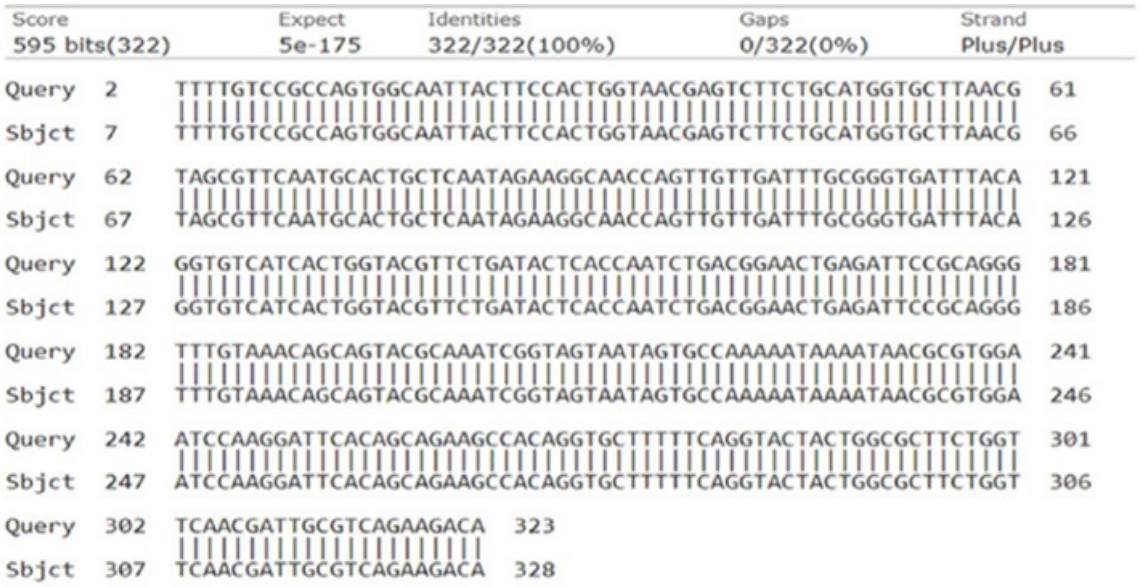

Figure 11. Nucleotide sequences of *Vibrio parahaemolyticus* and its interaction with ϕTT1H. Query: 17802 strain, Sbjct: *Vibrio parahaemolyticus* (ID: CP014046.2) strain. Sbjct: *Vibrio* sp. 17802 + ϕTT1H.

The 17802 ToxR nucleotides and those of ϕTT1H, ϕTT2H, and ϕA2223 infected bacterium are 100% homologous to those of *V. parahaemolyticus* (ID: CP014046.2). It can be explained that the 17802 receptors are not comparable to those of ϕTT1H, ϕTT2H, and ϕA2223, or they can be hidden by a physical wall (Dy *et al.,* 2014; Labrie *et al.,* 2010). Figure 11 revealed that ϕTT1H and ϕTT2H could not affect the toxic gene of the surveyed bacteria.

3.2 Discussion

These results are consistent with the description of *Vibrio* spp. and *Desulfovibrio* spp. by (Abdelaziz *et al.,* 2017; Alcaide *et al.,* 1999; Huynh *et al.,* 2021; Hao *et al.,* 1996; Warren *et al.,* 2005; Al-Tayyar *et al.,* 2018). Besides, these results suggest the development of bacterial antiphage mechanisms, leading to the formation of bacterial communities. In the context of bacteriophage-bacteria interactions, competitive co-evolution between bacteriophages and bacteria, known as the "evolutionary arms race," has resulted in the emergence of defense mechanisms. Bacteria can inhibit various stages of the phage life cycle (Rostøl and Marraffini, 2019). Bacteria primarily reside in biofilms, which are extracellular matrices composed of polymers where bacteria live in close proximity, aiding in their protection against bacteriophages (Simmons *et al.,* 2018). This could explain the tendency of the two bacterial strains 12D and 17802 to cluster together when phages were introduced. Additionally, gram-negative bacterial strains can release outer membrane vesicles (OMVs) containing exposed outer membrane proteins that serve as phage receptors. OMVs can act as decoys, trapping extracellular bacteriophages and preventing them from attacking the bacteria (Schwechheimer and Kuehn, 2015).

The decrease in bacterial colony size when exposed to bacteriophages is due to the bacteria's ability to resist phage attachment and infection, resulting in a noticeable reduction in colony size after phage treatment (Koonjan *et al.,* 2022). The impact of bacteriophages on bacterial colony numbers depends on the type of phage involved. Virulent phages decrease colony numbers by lysing bacteria, while temperate phages can increase colony numbers by integrating their genome into the bacterial chromosome (Gummalla *et al.,* 2023; Pelzek *et al.,* 2013; Spriewald *et al.,* 2020).

4. Conclusion

The study successfully isolated the bacterial strain Desulfovibrio vulgaris (12D) from shrimp ponds and identified three potential bacteriophage strains: ϕTT1H, ϕTT2H, and ϕA2223. These bacteriophages can inhibit the growth of Desulfovibrio vulgaris and V. parahaemolyticus bacteria by affecting the size, shape, and number of bacterial colonies in treatments with bacteriophages. While they do not change the genetic makeup of these bacterial strains, they effectively control the bacterial population. This research suggests that these bacteriophages could be valuable biological control agents in shrimp ponds.

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Authors' Contributions

All authors have contributed to the final manuscript. The contribution of each author as follow: Nguyen Thi Loan Anh: Collected materials and the data; performed the experiments; wrote first draft of methods; drew figures. Nguyen Huu Tri: Collected materials and the data; Performed the experiments. Chau Thanh Tuan: Analyzed and interpreted the data; wrote and formed the paper. Truong Thi Bich Van: Gave ideals; supervised the research; paid and contributed reagents, materials, and analysis tools and performed the experiments and manuscript preparation and funding.

Conflict of Interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential 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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