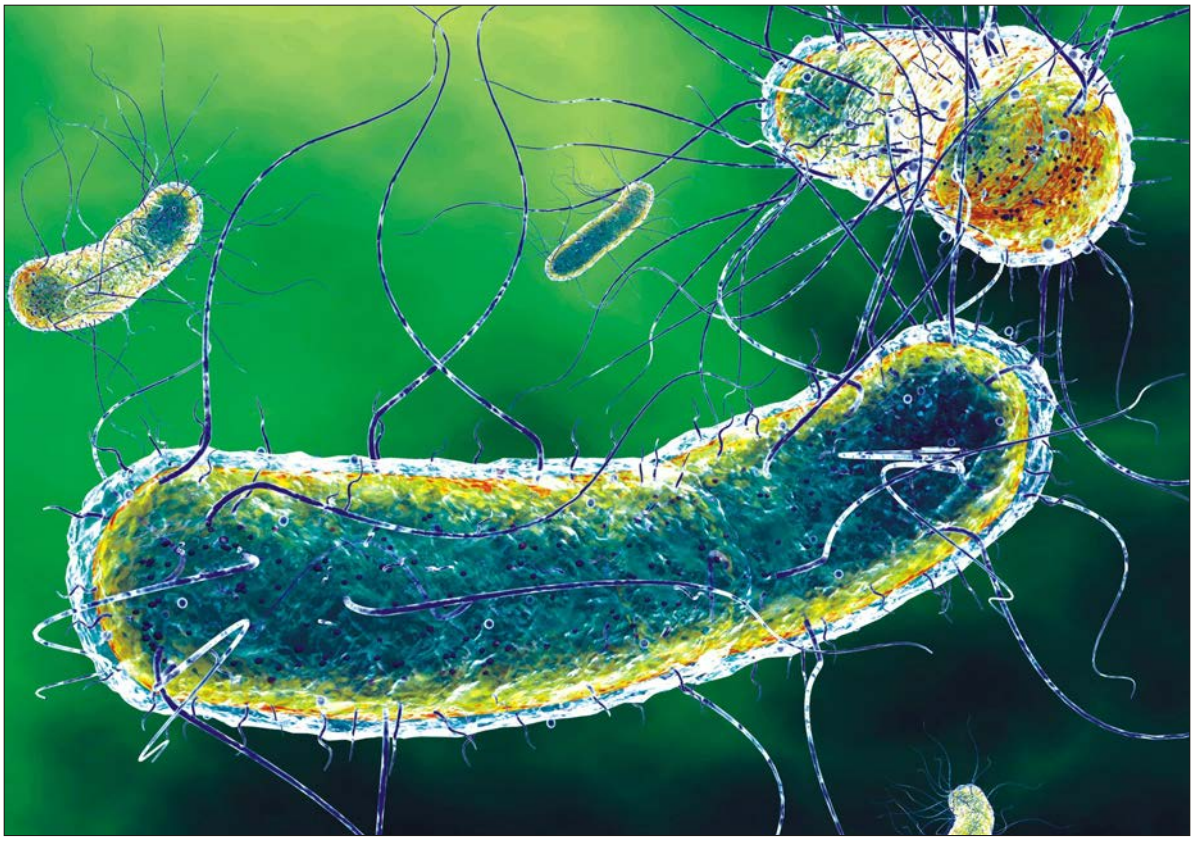

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# Research Article
# Potential Phages Against *Vibrio alginolyticus* from Oyster and Clams

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## Abstract

**Background and Objective:** Vibriosis is a disease that causes massive mortality in aquaculture farming in Asia which is commonly controlled by antibiotics. Increasing Antimicrobial Resistance (AMR) in *Vibrio* species may lead to bacteriophage therapy as an option for fish farmers to treat vibriosis. The objective of this study was to isolate and identify bacteriophage from bean clam, carpet clam and wild oysters against *Vibrio alginolyticus* strains. **Materials and Methods:** The 30 bivalve samples including 10 each for bean clams, carpet clams and wild oysters were purchased from local and street wet markets around Pengkalan Chepa and Bachok, Kelantan. Spot tests and plaque formation were done to isolate the phages from bivalve molluscs. Then, the isolated phages were identified morphologically using a Transmission Electron Microscope (TEM). **Results:** The two isolates of phages were isolated from white oysters (VA-WO1) and carpet clams (VA-CC1). Unfortunately, only the VA-WO1 phage was further studied due to technical errors. Morphologically the bacteriophage identified using TEM belongs to the Siphoviridae family. The VA-WOI phage only targeted specific strains of *Vibrio alginolyticus* (K5). **Conclusion:** Further studies are needed to isolate other phages for effective vibriosis treatment. Thus, the obtained phages will further characterize the molecular and genome sequences examination. Phages have a huge potential to become supportive tools development of antibiotic treatment.

Key words: *Vibrio alginolyticus*, bacteriophage, oyster, clam, Transmission Electron Microscope (TEM), spot test, plaque formation test

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Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The freshwater aquaculture sector in Malaysia contributed a Gross Domestic Product (GDP) of 3.1%, however, the production of brackish water aquaculture has decreased by 10.3% in 2018[1]. Unfortunately, disease among other factors in farming has pressured aquaculture production[2]. On top of that, Reverter *et al.*[3] mentioned the heavily dependent use of antibiotic in fish farming to fight infectious disease further contribute to the spread of Antimicrobial Resistance (AMR) via the acceleration of antibiotic resistance gene (ARG) in bacteria, which threatens public health[3]. The effect of AMR increases 1.3-2 fold of patient susceptibility to infection, higher cost of treatment for patients, higher morbidity as well as mortality[4].

*Vibrio* is a bacterium that is ubiquitous in aquaculture. It is a Gram-negative, rod-shaped bacterium that can either be non-pathogenic, which is commonly found in healthy aquatic animals or pathogenic, which causes vibriosis disease in an aquatic animal that aquatic farming is concerned with. According to Selvin and Lipton[5], *Vibrio alginolyticus* is among the common *Vibrio* species in aquaculture that had few outbreak incidences in aquaculture and it causes infection in human as well. Hence, preventive measures, for instance, health management plans, prophylaxis and nutritional enhancement were practiced[6]. Vibriosis, the disease caused by pathogenic *Vibrio* species and strains poses a threat to the economy, food demand and food security in humans[6]. One of the *Vibrio* species, according to Sharma *et al.*[7], *Vibrio alginolyticus* caused the generalised patchy haemorrhagic body and ulcerated muscle with 5% mortality in fish. *Vibrio* strains also have been isolated from the clinical sample that are resistant to a few antibiotics like ampicillin, tetracycline, sulphonamide, gentamicin and so on[8].

In aquaculture, phage therapy is one of the alternative control measures currently used that are cost-effective, environmental friendly and low risk for microbial resistance[9]. Phages also treat infectious diseases a long time ago before broad spectrum antibiotic was discovered but limited knowledge led to a reduction in phage research[8]. Bacteriophages also called phages, are viruses that cause negative effects on bacteria but not animal cells. Besides they are high host specificity of strains and species levels which are able to multiply themselves, causing mortality in a wide host range. The phage against bacteria is mainly family Siphoviridae, Myoviridae and Podoviridae from the order *Caudovirales*. Each phage is specific to infect specific species or strains of bacteria[10]. They can be found widely in anywhere their host residues which include sewage, hatchery, thermal vents, natural bodies of water (oceans, seas, lakes, rivers), soil, as well as deep thermal vents[8].

Research on phage therapy became active in the 1980s after the emergence of Antimicrobial Resistance (AMR). It is proven that phage can delay and reduce mortality in aquatic animals caused by *Vibrio alginolyticus* as well as reduce total bacteria total load[11]. Culot *et al.*[12] mentioned that the research of phage in China, United States of America and Korea were the leading countries in phage research done by academic institutions and there is still a lack of large-scale use. Order *Caudovirales* or tailed bacteriophages consist of a head (icosahedral or oblate), neck and tail. It was divided further into three families that were differentiated by the different characteristics of a tail. Bacteriophage from Myoviridae family has a long contractile tail, bacteriophage from Siphoviridae family has a long but noncontractile tail and bacteriophage from Podoviridae family has a short and noncontractile tail[13]. Double agar overlay plaque assay is used to visualize and detect bacteriophage activity before phage therapy in aquaculture. This method is growing both freshly cultured bacteria and phage dilution in an agar matrix onto a solid 1.5% agar medium. After 24-36 hrs of incubation, visible plaque will be formed on the medium, indicating no bacterial growth in that zone, which shows the ability of the bacteriophage to undergo a replication-lysis-infection cycle[14]. Bacteriophage is classified according to their morphology, type of nucleic acid replication strategy, targeted host as well as clinical signs they caused. For the classification of bacteriophages in aquaculture, morphology and nucleic acid type in phages have opted which include Transmission Electron Microscopy (TEM) and DNA sequencing analysis[10].

The present study aimed to isolate the potential bacteriophage against the pathogenic *Vibrio alginolyticus* from oysters and clams. In addition, the characterization of obtained phages was determined morphologically.

## MATERIALS AND METHODS

**Study area:** From June until December, 2022, 30 bivalve samples (10 bean clams, 10 carpet clams and 10 wild oysters) were randomly purchased from wet markets in Pengkalan Chepa and Bachok, Kelantan. The samples were packed in an insulated box containing ice packs and transferred immediately to the Aquatic Animal Health Laboratory at the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan.

Table 1: Lists of *Vibrio alginolyticus* isolates were used in this study

| Isolates | Sampling location |
|---|---|
| K1 | Laguna Semerak, Kelantan |
| K2 | Laguna Semerak, Kelantan |
| K3 | Laguna Semerak, Kelantan |
| K4 | Laguna Semerak, Kelantan |
| K5 | Laguna Semerak, Kelantan |
| T1 | Kuala Ibai, Terengganu |
| T2 | Sungai Besut, Terengganu |
| T3 | Kuala Ibai, Terengganu |
| T4 | Sungai Besut, Terengganu |
| T5 | Sungai Besut, Terengganu |

K: Kelantan (*Vibrio alginolyticus* isolates were collected from farms in Kelantan) and T: Terengganu (*Vibrio alginolyticus* isolates were collected from farms in Terengganu)

**Bacterial samples:** Ten isolates of *V. alginolyticus* were obtained from the Aquatic Animal Health Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The 10 bacteria samples were originally isolated from liver and kidney organ samples in farmed Asian seabass (*Lates calcarifer*) from Laguna Semerak in Kelantan, Kuala Ibai and Sungai Besut in Terengganu (Table 1). The bacteria were stored in the trypticase soy broth, TSB (Oxoid, England) supplemented with 1.5% $NaCl_2$ and 50% of glycerol solution in a -80°C freezer. *Vibrio alginolyticus* isolates were revived on trypticase soy agar, TSA with 1.5% $NaCl_2$ (Oxoid, England) and incubated at 35°C for 24 hrs. Ten *V. alginolyticus* were used in this present study (Table 1).

**Sample preparation:** A total of 30 seafood samples consisting of 10 samples for each bean clam (*Donax cuneatus*), carpet clam (*Paphia textile*) and wild oyster (*Crassostrea* sp.) were purchased from different wet markets in Kelantan. A 100 g of each sample were weighed and transferred into 100 mL of SM (buffer containing 100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM pH 7.5 Tris-HCl and 0.01% gelatin (Merck, Germany)[15]. The mixtures were left for 15 min before dissipating using a commercial blender machine. Large particles were pelleted in a 1.5 mL microcentrifuge tube at 14,000×g for 10 min. The supernatant was filtered through a 0.45 and 0.22 µm pore size syringe filter (Pall, United States). The filtrate of phages was maintained in a 4°C chiller for storage of phage.

**Phage isolation:** Isolation of bacteriophages was carried out using a double agar overlay technique of plaque assay according to method done by Tan *et al.*[15]. Ten microlitres of phage filtrate and 250 µL of log-phase *V. alginolyticus* host culture (OD = 600 nm) were aliquoted into a 3 mL of 0.5% molten soft agar (Trypticase soy broth and bacteriological agar, Oxoid, England), the temperature of soft agar was maintained at 55°C. The mixture of molten soft agar was swirled gently and poured onto a solidified TSA plate. The plate was allowed to solidify for at least 20 min and incubated in an inverted position at 35°C overnight. A single colony plaque with a clear lysis appearance was picked using a 200 µL sterile pipette tip and transferred into a 1.5 mL microcentrifuge tube containing 1 mL of SM buffer. The phage lysates were purified a minimum of three times by using the same procedure to obtain a single phage lysate colony.

**Electron microscopy:** The VA-WO1 phage was visualized via Transmission Electron Microscopy (TEM) (Zeiss TEM Libra 120) at Universiti Sains Malaysia, Penang, Malaysia. A droplet of phage sample was placed on a carbon film coated 400 mesh copper grids using fine forceps and rested for 3 min. After that, the suspension was removed by absorption onto filter paper and the sample was stained with 2% uranyl acetate for 1 min. The grid was air-dried for 10 min and then viewed with a JEM-1010 (Jeol, Tokyo, Japan) operated at 80 kV. Phage dimensions were calculated by measuring the dimensions of five independent phages.

## RESULTS

Ten *V. alginolyticus* strains were tested on three types of bacteriophage obtained from bean clams (VA-BC1), carpet clams (VA-CC1) and wild oysters (VA-WO1) (Table 2). Two isolates of phages were isolated from wild oysters (VA-WO1) and carpet clams (VA-CC1). Only VA-WO1 phage was further studied due to limited sources.

Plaque formation was seen after 24 hrs of incubation in VA-CC1 and VA-WO1 with K5. Figure 1 showed the interaction of VA-CC1 and K5 with different plaque morphology and size. The presence of a heterogenous mixture of plaques having different diameters. The plaques formed were pin-point size that was unable to be measured and 0.2 cm in size both with the visible clear zone (Fig. 1a).

To classify the *Vibrio alginolyticus* BP into morphotype-specific groups, the BP particles were examined by transmission electron microscopy. Figure 1b showed that VA-WO1 has an icosahedral-isometric head with a diameter of 54.44 nm and a thin, long flexible tail length is 134.33 nm. The total phage length is about 227.6 nm, the base plate and tailpipe can be differentiated. Hence, it can be concluded that this BP belongs to the family of Siphoviridae in *Caudovirales* based on the International Committee on Taxonomy of Viruses (ICTV).

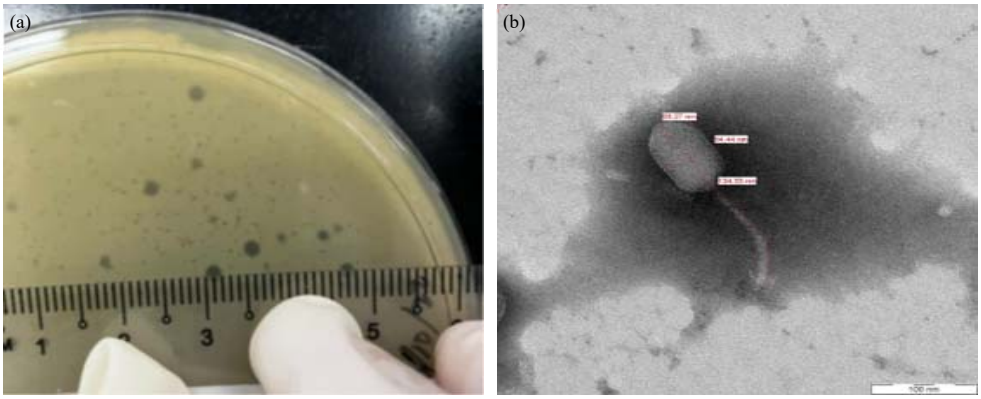


Fig. 1(a-b): Morphology of phage on agar and TEM, (a) Bacterial lawn with clear visible plaque and (b) VA-WO1 phage under TEM

Table 2: *Vibrio alginolyticus* strains used for bacteriophages isolation

| | | Bacteriophage | | |
|---|---|---|---|---|
| Bacterial strain | Isolate ID | VV-CC1 | VA-BC1 | VA-WO1 |
| *Vibrio alginolyticus* | K1 | - | - | - |
| | K2 | - | - | - |
| | K3 | - | - | - |
| | K4 | - | - | - |
| | K5 | + | - | + |
| | T1 | - | - | - |
| | T2 | - | - | - |
| | T3 | - | - | - |
| | T4 | - | - | - |
| | T5 | - | - | - |

+: Bacteriophage isolated from the sample showed lysis spot and -: No bacteriophage isolated from the sample

## DISCUSSION

In aquaculture, phage therapy has become a selective application that can provide alternative solutions to bacterial disease infection[16]. *Vibrio alginolyticus* is one of the most pathogenic bacterial species in freshwater environments and definite reports are indicating this bacterium in disease outbreaks[7,17,18]. In the present study, we represented the isolation and characterization of a lytic phage specific for a *V. alginolyticus* isolated from Asian seabass in a cage farm, which is a repetitive problem on the East Coast of Malaysia. The results showed an attempt to isolate the bacteriophage from shellfish samples using *V. alginolyticus* bacterial hosts. In this study, the phage host range was determined by overlay plague assays and checked using different bacterial strains mentioned earlier. According to the phage assay on agar, VA-CC1 and VA-WO1 showed the lyse clear zone against K5 of *V. alginolyticus*.

Bacteriophage approaches have been revitalized because of the growth of antibiotic resistance considering their high-efficiency features, specificity and environmental friendliness and promising of control of pathogenic bacteria, especially in aquaculture[19]. The bacteriophage application idea was neglected after the invention of antibiotics about 40 years ago. Lately, bacteriophage has been seen as an effective way to prevent and control of bacterial pathogens and as a biocontrol agent towards aquaculture diseases such as *Vibrio, Pseudomonas, Aeromonas* and *Flavobacterium* to reduce fish mortality[11,20]

In this study, we were using a spot test for direct isolation of *Vibrio* phages from fresh clam samples. According to Štrancar *et al.*[21], the flow of phage discovery started with collecting samples, followed by phage isolation either direct isolation or enriched isolation technique was carried out. For direct isolation, it can detect different phages with various plaque morphology on double-layer agar plaque combined with plaque assay which detects BP in both solid and liquid environmental samples. For enriched isolation, it amplifies the BP concentration before identifying plaque on solid media with a spot test which screens multiple samples for phages on the same plate. After confirming the presence of phages, phage purification is continued to get pure phage or homogenous population and phage amplification can be done to increase phage stock. At this stage, the characterization of phages can be done via the morphology of phage under TEM and further genome analysis of the phage DNA.

Besides that, the isolation continued with the purification of isolated phages with a double agar overlay technique. According to Glonti and Pirnay[22], a spot test is suitable as quantitative activity detection before double agar overlay plaque assay for confirmation. This was supported by Silva *et al.*[9], who used spot test as the initial approach to

detect bacterial infection before testing the efficiency of the double-layer agar method. According to Hyman[23], lytic and temperate phage express different appearances on the plaque. Clear plaque indicates lytic phage since all bacterial cells had been infected and killed while for temperate phage, there are mixture of killed bacterial cells and lysogens resulting in cloudy plaque. In Fig. 1-2, the plaque formed are clear visible lytic spots indicating the killing of the phage. Besides, plaque assay also indicates the ability of the phage to adsorb the bacterium and generate and release more phage progeny to kill more bacterium[13]. As mentioned by Daubie *et al.*[14], plaque size can be affected by the intrinsic factors of phage such as the size, latency period as well as burst size. The latent period means the time of phage to infect, attach and translocate its nucleic acid into the bacterial cell causing bacterial lysis. While burst size is defined as the average amount of phage particles generated per lysed infected bacterium.

In this study, only one phage was further studied, which might be due to different factors including laboratory error and bacterial phage resistance. According to Staub *et al.*[24], the common causes of error happened in the laboratory including the procedural error. Since the identification of phages took a long yet complicated procedure, improper adherence to certain procedures might happen especially a lack of familiarity with new experiments. Nevertheless, incidents of carelessness which include incorrect measurement, especially during pipetting technique and spilling of material can also be expected. Since there is antimicrobial resistance, there is bacterial phage resistance as well. As mentioned by Fang *et al.*[25], the bacteria demonstrated rapid phage resistance in just only 4 hrs by low production of the capsule and minimized phage virulence. This bacterial phage resistance might happen at different infection stages for instance during the adsorption stage, there are no receptors or presence of a physical barrier failing phage adherence to the bacterium, or blockage of phage-genome uptake results in inefficient distribution of phage particles by the infected bacterium[14].

In this study, the BP belonged to the family of Siphoviridae in *Caudovirales*, isolated from clams samples. Labrie *et al.*[26] stated that most of the bacterial phage resistance targeted double-stranded DNA phages which include Siphoviridae that were observed under TEM. Skliros *et al.*[27] also reported the drawback of phage therapy on *V. alginolyticus* via transcriptome and metabolome analysis that reduces the expression of phage receptors leading to phage resistance. Mutation is detected in *V. alginolyticus* when they are under phage infection stresses as well[28]. According to Ramos-Vivas *et al.*[10], mostly isolated bacteriophages against *V. alginolyticus* are from the family Myoviridae which are obtained from marine sediment and marine water. This was supported by Kim *et al.*[29] stating bacteriophage from the family Myoviridae is a good candidate as a biocontrol agent against *V. alginolyticus*. However, in Li *et al.*[30] research, isolated phage from the family Siphoviridae was obtained from seafood market sewage. Among 14 types of phages infecting *V. alginolyticus*, 7 phages belong to the family Myoviridae, 3 are from the family Siphoviridae, 2 are from the family Podoviridae, 1 is from Demerecviridae and Schitoviridae[31].

The results of this study suggested that in response to antimicrobial resistance towards aquatic disease, there is an increased demand for bacteriophage innovation. Bacteriophage therapy offered a promising novel alternative for the treatment and prevention of vibriosis in aquaculture. There are many industries using bacteriophage applications such as aquaculture, novel approaches against antibiotic-resistant bacteria (*Escherichia coli* and *Salmonella*), skin care, medicinal administration and vaccine development, etc. Pires *et al.*[32] found that phage treatment revealed no negative side effects and there were no notable differences in the frequency and rate of healing between the treated and control groups. However, bacteriophage scientific research has many challenges and still needs further examination to be presentable and has commercial potential for the aquaculture industry sector. Various bacteria were developed as antimicrobial resistance to pathogenic bacteria in aquatic disease, so the researchers have to consider the broad bacteriophage application. The limitations of the study were time restriction for phage samplings collection and optimization of laboratory procedure, lack of laboratory equipment and financial support for TEM characterization.

The phages have the potential to be commercialized as an alternative therapy to combat antimicrobial resistance in *Vibrio* species. Phage selection, MOI, environmental factors that affect lytic phage viability (e.g., temperature, salinity, pH, UV radiation), administration routes and bacterial resistance to phages are several factors that affect the ability of phages to control *Vibrio* species in aquaculture systems. Furthermore, the data obtained in *in vitro* assays cannot be directly applied to *in vivo* assays, nor can *in vivo* data for one phage be deduced from another phage. Efficacy testing to demonstrate the effectiveness and safety of the phages is crucial before

applying this approach commercially. The MOI that produces the best bacterial inactivation, stability of phage preparations, administration method and cost-effectiveness are the factors that should be considered and standardized. Several studies of the potential impact on the natural bacterial community and fish health, function of the type of bacteria and different environmental conditions are required to explore its integration as a new antimicrobial processing technology in aquaculture.

## CONCLUSION

The 2 phages were isolated from white oysters (VA-WO1) and carpet clams (VA-CC1), respectively. Only the VA-WO1 phage was further studied due to limited access and time. Morphologically identification of the bacteriophage using TEM showed that it belongs to the Siphoviridae family. This study showed that VA-WO1 only targeted a specific strain of *Vibrio alginolyticus* (K5). The recommendation includes a longer research period for better isolation and characterization of the phage. Further studies are needed to isolate other phages for effective vibriosis control caused by *Vibrio alginolyticus*. The other studies should include the use of cocktail phage or mixing of several types of phages against bacterial strains for future research studies.

## SIGNIFICANCE STATEMENT

The purpose of this study was to identify the bacteriophage isolates from bivalve molluscs such as bean clam, carpet clam and wild oysters in the wet market against *Vibrio alginolyticus*. The advantages of phage therapy are specific to their host. From this present study, it is notable that this bacteriophage can be used to combat antimicrobial-resistant antibiotics. Thus, the bacteriophage alternative can be a great prevention to reduce the risks of mortality and disease outbreaks in aquaculture. As a result, two phages were isolated and characterized which are VV-CC1 and VA-WO1 against K5 *Vibrio alginolyticus*.

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