ISOLATION AND CHARACTERIZATION OF POTENTIAL PHAGES AGAINST *Vibrio alginolyticus*

By

LEE RUI YING
(D18B0047)

A RESEARCH PAPER SUBMITTED TO THE
FACULTY OF VETERINARY MEDICINE
UNIVERSITI MALAYSIA KELANTAN
IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF VETERINARY MEDICINE

FEBUARY 2023

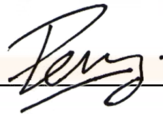
FACULTY OF VETERINARY MEDICINE
UNIVERSITI MALAYSIA KELANTAN

FYP FPV
UNIVERSITI
MALAYSIA
KELANTAN

CERTIFICATION

This is to certify that I have read this research paper entitled 'Isolation and Characterization of Potential Phages Against *Vibrio alginolyticus*' by Lee Rui Ying, and in my opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course DVT 55204 – Research Project.

Dr. Ruhil Hayati binti Hamdan
BSc (UMT), MSc (UMT), PhD (UPM)
Senior Lecturer
Faculty of Veterinary Medicine
University Malaysia Kelantan
(Supervisor)



Dr. Tan Li Peng
BSc, PhD (UPM)
Senior Lecturer
Faculty of Veterinary Medicine
University Malaysia Kelantan
(Co-Supervisor)

FYP FPV
UNIVERSITI MALAYSIA KELANTAN

ACKNOWLEDGEMENT

First of all, I would like to acknowledge as well as give my warmest thanks to my supervisor, Dr Ruhil Hayati binti Hamdan who guide me through the FYP journey. Her guidance and advice made me enjoyed all the me through all the project stages. Laboratory works cannot be done without the experienced guidance of the PHD student, Kak Ain, aquatic laboratory assistant, Kak Faten, molecular laboratory assistant, Kak Salma, virology laboratory assistant, Kak Nani, zoonotic laboratory assistant, Kak Ina, and histology laboratory assistant, Encik Fadli, much appreciated.

I would like to give special thanks to my Group 4 DVM class of 2018 rotamates, coursemates, and family as a whole for their understanding and support during all stages of my project preparation. Thanks to the course coordinator, Dr. Luqman Bin Abu Bakar for his supervision for all the students.

FYP FPV

UNIVERSITI
MALAYSIA
KELANTAN

DEDICATION

The thesis is dedicated to:

My lecturers (Dr. Ruhil Hayati binti Hamdan, Dr. Tan Li Peng)

My mom (Ooi Beng Hwa)

My dad (Lee Kit Chong)

My sis (Lee Pei Ying)

My best friend (Wong Mei Kei)

DVM class of 2018/ 2023

&

Faculty of Veterinary Medicine, Universiti Malaysia Kelantan

FYP FPV

UNIVERSITI
MALAYSIA
KELANTAN

Table of Contents

1.0 INTRODUCTION……………………………………………………………………1-2

1.1 Problem statement……………………………………………………………..2

1.2 Research questions ……………………………………………………………3

1.3 Research hypothesis…………………………………………………………...3

1.4 Research objectives……………………………………………………………3

2.0 LITERATURE REVIEW

2.1 Vibriosis in aquaculture………………………………………………………..4

2.2 Phage therapy for vibriosis……………………………………………………4-5

2.3 Classification / morphology of bacteriophage………………………………...5-6

3.0 MATERIALS AND METHODS

3.1 Bacterial strains………………………………………………………………..7

3.2 Sample preparation……………………………………………………………..8

3.3 Phage isolation………………………………………………………………...8-9

3.4 Electron microscopy…………………………………………………………..9

3.5 Host range analysis..……………..………………………………….................…9-10

4.0 RESULTS

4.1 *Vibrio alginolyticus* bacteriophage isolation…………………………………..11-13

4.2 Morphological analysis………………………………………………………...13-14

4.3 Host specificity………………………………………………………………….14

5.0 DISCUSSION…………………………………………………………...……15-17

6.0 CONCLUSION AND RECOMMENDATION……………………………………18

REFERENCES…………………………………………………………………………….19-21

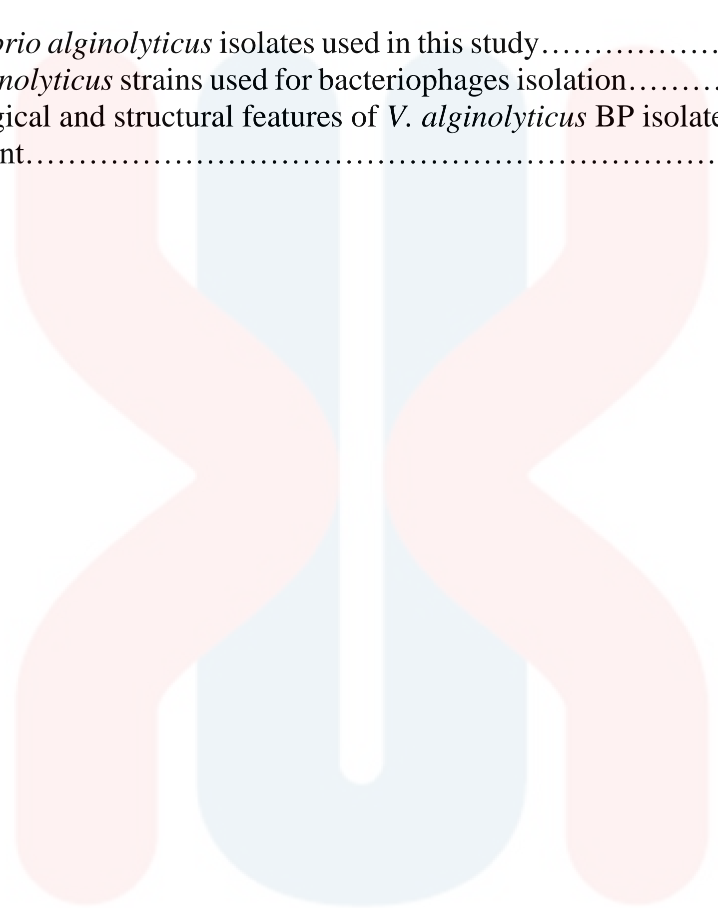
Appendix A……………………………………..………………………………...22-23

FYP FPV

UNIVERSITI MALAYSIA KELANTAN

LISTS OF TABLES

Table 1: Lists of *Vibrio alginolyticus* isolates used in this study…………………………………..17
Table 2*: Vibrio alginolyticus* strains used for bacteriophages isolation………………………...21
Table 3: Morphological and structural features of *V. alginolyticus* BP isolated by PHD student……………………………………………………………………………..24



FYP FPV

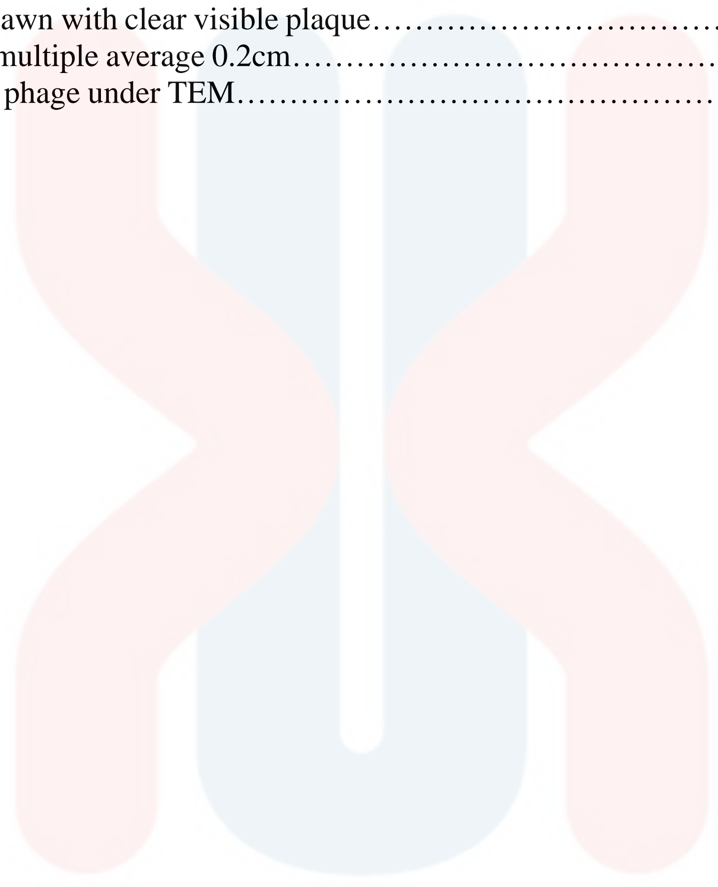
UNIVERSITI

MALAYSIA

KELANTAN

LIST OF FIGURES

Figure 1: Bacterial lawn with clear visible plaque……………………………………………...22
Figure 2: VA-CC1 multiple average 0.2cm……………………………………………………….23
Figure 3: VA-WO1 phage under TEM……………………………………………………………23



UNIVERSITI
MALAYSIA
KELANTAN
FYP FPV

LIST OF APPENDICES

Appendix A.1: Bean clam, *Donax cuneatus*……………………………………………………32
Appendix A.2: Carpet clam, *Paphia* textile……………………………………………………32
Appendix A.3: Wild oyster, *Crassostrea sp*..…………………………………………………32
Appendix A.4: Blending of clam sample………………………………………………………32
Appendix A.5: Centrifuged clam sample………………………………………………………32
Appendix A.6: Incubation of the bacterial strains………………………………………………32
Appendix A.7: Double layer agar………………………………………………………………33
Appendix A.8: TEM machine……………………………………………………………………33
Appendix A.9: Copper grid on filter paper………………………………………………………33
Appendix A.10: TEM phage observation in USM Penang with PHD student, Kak Ain and FYP SV, Dr Ruhil…………………………………………………………...33

FYP FPV

FYP FPV

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine in the partial requirement on the course DVT 55204 – Final Year Research Project.

Vibriosis is a disease that cause massive mortality in aquaculture farming in Asia which is commonly controlled by antibiotic. Since there is antimicrobial resistance (AMR) in vibrio bacterial species, bacteriophage can be an option for fish farmer to treat vibriosis. The objectives of this study were (i) to isolate and identify bacteriophage from different kind of bivalve mollusks (bean clam, carpet clam and wild oyster) in wet market against *Vibrio alginolyticus* strains and (ii) to determine the host specificity of the isolated phage. Spot test and plaque formation were done to isolate the phages from bivalve mollusks. Then, the isolated phages were identified morphologically using Transmission Electron Microscope (TEM). In this study, two isolates of phages were isolated from white oyster (VA-WO1) and carpet clam (VA-CC1). Unfortunately, only VA-WO1 phage was further studied due to technical error. Morphologically the bacteriophage identified using TEM belongs to the *Siphoviridae* family. This study showed VA-WOI only targeted specific strain of *V. alginolyticus* (K5). Further studies are needed to isolate other phages for effective vibriosis control caused by *V. alginolyticus*.

Keywords: *Vibrio alginolyticus*, bacteriophage, aquaculture

UNIVERSITI MALAYSIA KELANTAN

ABSTRAK

Ini adalah abstrak kertas penyelidikan dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan daripada bidang DVT 55204 – Projek Penyelidikan.

Vibriosis adalah penyakit yang menyebabkan kematian secara besar-besaran dalam penternakan akuakultur di Asia yang biasanye dikawalkan oleh antibiotik. Oleh disebabkan terdapat rintangan antimikrobial (AMR) dalam spesies bakteria Vibrio, bakteriofag boleh dijadikan pilihan oleh peternak ikan untuk merawat vibriosis. Objektif kajian ini adalah (i) untuk mengasingkan dan mengenal pasti bakteria daripada pelbagai jenis moluska bivalve (siput, lala bunga dan tiram) di pasaran terhadap strain *Vibrio alginolyticus* dan (ii) untuk menentukan perumah khusus bagi bakteriofag yang diasingkan. Ujian penitisan dan pembentukan plak dilakukan untuk mengasingkan fag daripada moluska bivalve. Kemudian, fag terpencil akan dikenal pasti secara morfologi melalui mikroskopi transmisi elektron (TEM). Dalam kajian ini, dua fag terpencil telah diasingkan daripada tiram (VA-WOI) dan lala bunga (VA-CC1). Namun, hanya VA-WO1 fag dapat dikaji dengan lebih lanjut disebabkan oleh kesilapan teknikal. Secara morfologi bakteriofaj yang telah dikenal pasti menggunakan TEM tergolong dalam keluarga *Siphoviridae*. Kajian ini menunjukkan VA-WOI hanya menyasarkan strain khusus *V. alginolyticus* (K5). Kajian lanjut diperlukan untuk mengasingkan fag lain bagi kawalan vibriosis yang berkesan yang disebabkan oleh *V. alginolyticus*.

Kata kunci: *Vibrio alginolyticus*, bakteriofag, akuakultur

FYP FPV

UNIVERSITI MALAYSIA KELANTAN

1.0 INTRODUCTION

The brackishwater and freshwater aquaculture sector in Malaysia has contributed a gross domestic product (GDP) of 5.7% in 2020 (*Department of Statistics Malaysia Official Portal*, 2022). Unfortunately, disease among other factors in farming has pressured aquaculture production (Mazuki, 2022). On top of that, heavily dependent use of antibiotic in fish farming to fight infectious disease further contribute to the spread of antimicrobial resistance (AMR) via acceleration of antibiotic resistance gene (ARG) in bacteria, which threatening public health (Reverter et al., 2020). The effect of AMR increases 1.3-2-fold of patient susceptibility to infection, higher cost of treatment for patient, higher morbidity as well as mortality (Eliopoulos et al., 2003).

Vibriosis, disease caused by pathogenic *Vibrio* species and strains poses a threat to economic, food demand and food security in human (Cecília & Wan, 2021). One of the Vibrio species, according to Sharma et al. (2013), *Vibrio alginolyticus* caused generalised patchy hemorrhagic body and ulcerated muscle with 5% mortality in fish. Vibrio strains also has been isolated from clinical sample that are resistant to few antibiotic like ampicillin, tetracycline, sulfonamide, gentamicin and so on (Letchumanan et al., 2016).

In aquaculture, phage therapy is one of the alternative control measures currently used that is cost effective, environmental friendly, and low risk for microbial resistance (Silva et al., 2014). Phages also treat infectious disease long time ago before broad spectrum antibiotic was discovered but limited knowledge lead to reduction in phage research (Letchumanan et al., 2016). Phages, or bacteriophages (BPs), are virus that cause negative effect to bacteria but not animal cells. Besides they are high host specificity of strains and species level which able to multiply themselves, causing mortality in wide host range. The phage against bacteria are mainly family *Siphoviridae*,

FYP FPV

UNIVERSITI MALAYSIA KELANTAN

Myoviridae, and *Podoviridae* from order *Caudovirales*. Each phage is specific to infect specific species or strains of bacteria (Ramos et al., 2021). They can be found widely in anywhere their host reside which include sewage, hatchery, thermal vents, natural bodies of water (oceans, seas, lakes, rivers), soil, as well as deep thermal vents (Letchumanan et al., 2016).

This study was carried out to isolate and characterise the potential phages against *Vibrio alginolyticus* and to investigate the ability of the specific isolated phage against *Vibrio alginolyticus* strains as an alternative to combat antimicrobial resistance.

1.1 Problem statement

One of the greatest challenges affecting public health and animal welfare is the antimicrobial resistance (AMR). Fish farming is heavily relied on the use of antibiotic which has adverse effects on long term application. In addition, *Vibrio* spp. is ubiquitous in water which makes aquatic organism easily exposed to the disease and threatening their health. Hence, phage therapy can be used as an alternative treatment to replace antibiotic.

Hence, this study is conducted to isolate and identify local phage as well as asses their ability against *V. alginolyticus* bacterial strains to control pathogen infestation in aquaculture and fisheries industries.

FYP FPV
UNIVERSITI
MALAYSIA
KELANTAN

1.2 Research questions

- Can bacteriophage against *Vibrio alginolyticus* strains be isolated and identified from different kinds of bivalve mollusks?
- Can the host specificity of the isolated phage be determined?

1.3 Research hypotheses

- Bacteriophage against *Vibrio alginolyticus* strains can be isolated and identified from different kinds of bivalve mollusks.
- The host specificity of the isolated phage can be determined.

1.5 Research objective

- To isolate and identify bacteriophage from different kind of bivalve mollusks (bean clam, carpet clam and white oyster) against *Vibrio alginolyticus* strains
- To determine the host specificity of the isolated phage

FYP FPV
UNIVERSITI
MALAYSIA
KELANTAN

2.0 LITERATURE REVIEW

2.1 Vibriosis in aquaculture

Vibrio is a bacteria that is ubiquitous in aquaculture. It is a Gram-negative, rod-shaped bacteria that can either be non-pathogenic, which commonly found in healthy aquatic animal, and pathogenic, which cause vibriosis disease in aquatic animal that aquatic farming are concerned with. According to Selvin & Lipton (2004) *V. alginolyticus* is among the common *Vibrio* species in aquaculture that had few outbreak incidence in aquaculture and it cause infection in human as well. Hence, preventive measures for instance health management plan, prophylaxis and nutritional enhancement were practised (Cecília & Wan, 2021).

2.2 Phage therapy for vibriosis

Phages or bacteriophages (BP) are host specific bacterial virus that can be found in ubiquitois environment and in aquatic animal. Phage has the ability to bind to specific receptors of lipopolysaccharide in *Vibrio*. Phage attack bacteria by inserting their nucleic acid into cytoplasm of bacteria, replicate themselves via lytic cycle in bacteria, causing destruction of the bacterial host. This phages are called virulent phage or lytic phage. Another process which is lysogenic cycle involve transfer and incorporate genetic information of phage into bacterium genome, and cell division of bacteria produce infected daughter cells which until a favourable condition was meet for new virus particles to be produced. This phages are called temperate phage or lysogenic phage. When phages introduced into fish body system, immune recognition mechanism started a systemic screening process which then stimulate host's innate immune response (Ramos et al., 2021).

FYP FPV
UNIVERSITI MALAYSIA KELANTAN

Research of phage therapy becoming active at 1980's after the emergence of AMR. It is proven that phage able to delay and reduce mortality in aquatic animal caused by *V. alginolyticus* as well as reduce total bacteria total load (Schulz et al., 2022). Culot et al. (2019) mentioned that the research of phage in China, USA, and Korea were the leading countries in phage research but only done by academic institution only, which there are still lack of large scale used.

FYP FPV

2.3 Classification / morphology of bacteriophage

Order *Caudovirales* or tailed bacteriophages consists of head (icosahedral or oblate), neck, and tail. It was divided further into three families that differentiated by different characteristic of tail. Bacteriophage from *Myoviridae* family has long contractile tail, bacteriophage from *Siphoviridae* family has long but noncontractile tail, and bacteriophage from *Podoviridae* family has short and noncontractile tail (Iwasaki et al., 2018).

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 phages dilution in agar matrix onto solid 1.5% agar medium. After 24-36 hours of incubation, visible plaque will be formed on medium, indication no bacterial growth in that zone, which shows the ability of bacteriophage to undergone replication-lysis-infection cycle (Daubie et al., 2022).

Bacteriophage is classified according to its morphology, type of nucleic acid replication strategy, targeted host as well as clinical sign they caused. For classification of bacteriophages in aquaculture, morphology and nucleic acid type in phages were opted which include Transmission Electron Microscopy (TEM) and DNA sequencing analysis (Ramos-Vivas et al., 2021).

UNIVERSITI MALAYSIA KELANTAN

3.0 MATERIALS AND METHODS

3.1 Bacterial strains

Ten bacteria *V. alginolyticus* strains were obtained from Aquatic Animal Health Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The bacteria were stored in the trypticase soy broth, TSB (Oxoid, England) supplemented with 1.5% NaCl and 50% glycerol stocks in -80˚C freezer. *V. alginolyticus* isolates were revived on trypticase soy agar, TSA + 1.5% NaCl (Oxoid, England) and incubated at 30°C for 24 hours (h). Ten *V. alginolyticus* were used in this present study (Table 1).

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

No.	Strain	Sampling location
1.	K1	Laguna Semerak, Kelantan
2.	K2	Laguna Semerak, Kelantan
3.	K3	Laguna Semerak, Kelantan
4.	K4	Laguna Semerak, Kelantan
5.	K5	Laguna Semerak, Kelantan
6.	T1	Kuala Ibai, Terengganu
7.	T2	Sungai Besut, Terengganu
8.	T3	Kuala Ibai, Terengganu
9.	T4	Sungai Besut, Terengganu
10.	T5	Sungai Besut, Terengganu

FYP FPV

3.2 Sample preparation

A total of 30 seafood samples consisting of 10 samples for each bean clam (*Donax cuneatus*), carpet clam (*Paphia textile*) and oyster (*Crassostrea sp.*) were purchased from different wet markets in Bachok, Kelantan. Ten grams of each sample was then weighed and transferred into 10 mL of salt of magnesium (SM) buffer (100 mM NaCl, 8 mM MgSO4·7H2O, 50 mM pH 7.5 Tris-HCl and 0.01% gelatin [Merck, Germany]) in a 50 mL sterile centrifuge tube. The mixture was shaken at 150 rpm for 15 min in an orbital shaker (Heidolph Unimax 1010). Large particles were pelleted in the tube via centrifugation at 10,000 × g for 5 min. The supernatant was filtered through a 0.2 µM pore size syringe filter (Pall, United States). A 200 µL of log-phase *V. alginolyticus* host culture (OD600nm = 0.4–0.6) was added into the 5 mL of filtrate and incubated at 30°C, 150 rpm in an orbital shaker overnight. After incubation, the mixture was centrifuged at 10,000 × g for 5 min to pelletize the bacterial cells and the supernatant was filtered with a 0.2 µM pore size syringe filter (Pall, United States).

3.3 Phage isolation

Isolation of bacteriophages was carried out according to the double agar overlay plaque assay (Kropinski et al., 2009). A 100 mL of each dilution filtrate and 100 mL of the log phase bacteria culture was aliquot into a 3 mL molten soft agar, which constituted of tryptic soy broth (TSB) (Oxoid, England) and 0.6% bacteriological agar (Oxoid, England), which was stored at 45˚C. The molten soft agar was mixed gently and poured evenly onto tryptic soy agar (TSA) (Oxoid, England) base plate. The plate was allowed to solidify for at least 15 min and incubated in an inverted position at 30˚C overnight. After incubation, a single colony plaque with clear lysis appearance from the highest dilution was picked using a sterile inoculation loop and transferred it into a 5 mL SM buffer with 200 mL log-phase bacteria culture. The mixture was incubated at 30˚C, 150 rpm

FYP FPV
UNIVERSITI MALAYSIA KELANTAN

in an orbital shaker overnight. After incubation, the sample was centrifuged at 10,000×g for 5 min, and the supernatant was filtered with a 0.2 mM pore size syringe filter.

The plates were check for the plaque by holding the plate up to a light source. The morphology and amount of plaque was recorded.

3.4 Electron microscopy

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

3.5 Host range analysis

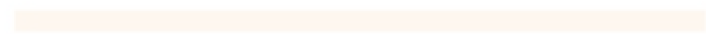
The host range of the obtained phage was determined using a spot assay and confirmed by the double-layer agar method. Ten microliters of the phage lysate (>10^7 PFU/mL) was dropped onto the overlaid top agar and mixed with each bacterial strain. The plates were then incubated overnight at 30°C and checked for the presence of a lysis zone. An efficiency of plating (EOP) assay was conducted to quantify the lytic activity of phage VA-WO1. The phage suspension (10^3 PFU/mL) was then assayed by the double-layer agar method. The total number of plaques was determined

FYP FPV
UNIVERSITI
MALAYSIA
KELANTAN

after 24 h of incubation and EOP values were calculated by comparing the ratios of PFUs of a susceptible strain to the indicator strain K5 in triplicate.

UNIVERSITI

MALAYSIA



KELANTAN

FYP FPV

4.0 RESULTS

4.1 *Vibrio alginolyticus* bacteriophage isolation

Ten *V. alginolyticus* strains tested on three types of bacteriophage obtained from bean clam (VA-BC1), carpet clam (VA-CC1) and wild oyster (VA-WO1) (Table 2). Two isolates of phages were isolated from wild oyster (VA-WO1) and carpet clam (VA-CC1). Unfortunately, only VA-WO1 phage was further studied due to technical error which include inappropriate pipetting technique or inappropriate volume of solution.

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

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

FYP FPV

	T4	-	-	-
	T5	-	-	-

\+ Bacteriophage isolated from the sample showed lysis spot.

\- No bacteriophage isolated from the sample.

Plaque formation was seen after 24 hours of incubation in VA-CC1 and VA-WO1 with K5. Figure 1 showed interaction of VA-CC1 and K5 with different plaque morphology and size. The presence of heterogenous mixture of plaques having different diameter. The plaques formed were pin-point size that were unable to be measure and 0.2cm size both with visible clear zone (Figure 2).

Figure 1: Bacterial lawn with clear visible plaque.

FYP FPV
UNIVERSITI MALAYSIA KELANTAN

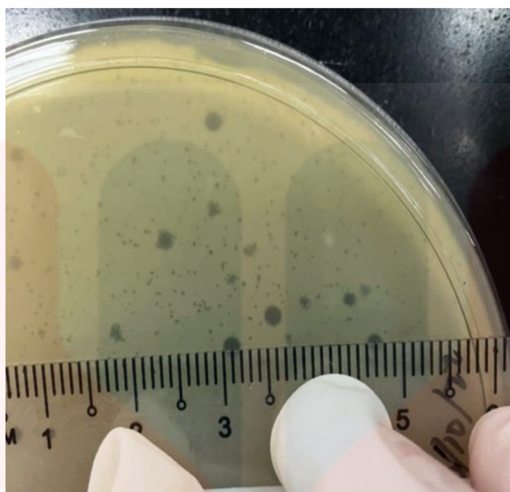


Figure 2: VA-CC1 multiple average 0.2cm.

4.2 Morphological analysis

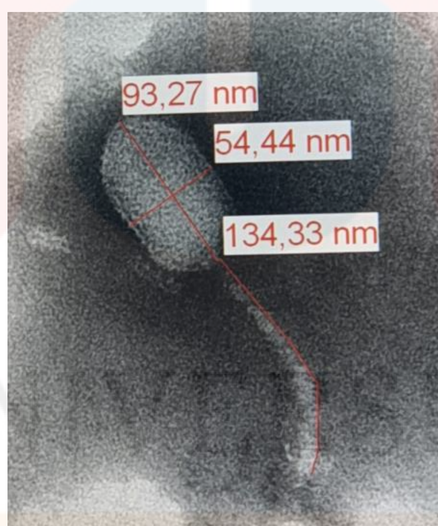


Figure 3: VA-WO1 phage under TEM.

To classify the *Vibrio alginolyticus* BP into morphotype specific group, the BP particles were examined by transmission electron microscopy (Figure 3). Figure 3 shows VA-WO1 has icosahedral-isometric head with diameter of 54.44 nm and thin, long flexible tail length is 134.33 nm. The total phage length is about 227.6 nm, the base plate and tail pipe can be differentiated.

FYP FPV

Hence, it can be concluded that this BP is belong to the family of *Siphoviridae* in *Caudovirales* based on the International Committee on Taxonomy of Viruses (ICTV).

Table 2: Morphological and structural features of VAKϕ.

Bacteriophage	Morphology		Family	Plaque size
	Caspid diameter, nm	Tail length, nm		
VA-WO1	54.44	134.33	*Siphoviridae*	small

4.3 Host specificity

The phages were tested for its host range specificity and lysis zone were observed on double-layer agar plate seeded with different *V. alginolyticus* strains (K1-K5 and T1-T5). None of the *V. alginolyticus* isolates showed plaque formation using VA-WO1.

FYP FPV

UNIVERSITI
MALAYSIA
KELANTAN

5.0 DISCUSSION

According to the SEA-PHAGES Resource Guide (Poxleitner et al., 2022), the flow of phage discovery started with collecting samples, followed by phage isolation either direct isolation or enriched isolation technique were carried out. For direct isolation, it can detect different phages with various plaque morphology on double layer agar plaque combine with plaque assay which detect BP in both solid and liquid environmental sample. For enriched isolation, it amplify the BP concentration before identify plaque on solid media with spot test which to screen multiple samples for phages on same plate. After confirmed 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, characterization of phages can be done via the morphology of phage under TEM and further genome analysis of the phage DNA.

According to Glonti & Pirnay (2022), spot test is suitable as quantitative activity detection before double agar overlay plaque assay for confirmation. This was supported by Silva et al. (2014) which used spot test as initial approach to detect bacterial infection before testing the efficiency on double-layer agar method.

According to Dan (2017), lytic and temperate phage express different appearance on plaque which clear plaque indicating lytic phage since all bacterial cell had been infected and killed while for temperate phage, there are mixture of killed bacterial cell and lysogens resulting in cloudy plaque. In Figure 1 and Figure 2, the plaque formed are clear visible lytic spot indicating the killing of the phage. Besides, plaque assay also indicates the ability of the phage to adsorb the bacterium, generate and release more phage progeny in order to kill more bacterium (Daubie et al., 2022).

FYP FPV

As mentioned by Daubie et al. (2022), plaque size can be affected by intrinsic factor of phage such as the size, latency period as well as burst size. 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. (2016), the common causes of error happened in laboratory including the procedural error. Since identification of phages took long yet complicated procedure, improper adhere to certain procedure might happened especially lack of familiarize of new experiment. Nevertheless, incidents of careless which include incorrect measurement especially during pipetting technique and spilling of material can also be expected.

Since there is an antimicrobial resistance, there is bacterial phage resistance as well. As mentioned by Fang et al. (2022), the bacteria demonstrated rapid phage resistance in just only 4 hours by low production of capsule and minimize the phage virulance. This bacterial phage resistance might happened at different infection stages for instance during adsorption stage, there are no receptor or presence of physical barrier result in failure of phage adherence to bacterium, or blockage of phage-genome uptake results in inefficient distribution of phage particles by infected bacterium (Daubie et al., 2022).

Labrie et al. (2010) stated that majority of the bacterial phage resistance targeted double-stranded DNA phages which include *Siphoviridae* that observed under TEM. Skliros et al. (2021) also reported the drawback of phage therapy on *V. alginolyticus* via transcriptome and metabolome

FYP FPV
UNIVERSITI MALAYSIA KELANTAN

analysis that reduce expression of phage receptor leading to phage resistance. Mutation detected in *V. alginolyticus* when they are under phage infection stress as well (Zhou et al., 2021).

According to Ramos et al. (2021), mostly isolated bacteriophage against *V. alginolyticus* are from family *Myoviridae* which they are obtained from marine sediment and marine water. This was supported by Kim et al. (2019) stating bacteriophage from family *Myoviridae* is a good candidate as biocontrol agent against *V. alginolyticus.* However in Li et al. (2021) research, they isolated phage from family *Siphoviridae* that was obtained from seafood market sewage. Among 14 types of phages infecting *V. alginolyticus,* 7 phages belongs to family *Myoviridae*, 3 are from family *Siphoviridae*, 2 from family *Podoviridae*, 1 from *Demerecviridae* and *Schitoviridae* (Droubogiannis et al., 2022).

FYP FPV

UNIVERSITI
MALAYSIA
KELANTAN

6.0 CONCLUSION AND RECOMMENDATION

In conclusion, two phages were isolated from white oyster (VA-WO1) and carpet clam (VA-CC1), respectively. Unfortunately, only VA-WO1 phage was further studied due to technical error. Morphologically identification of the bacteriophage using TEM showed that it belongs to the *Siphoviridae* family. This study showed that VA-WO1 only targeted specific strain of *V. alginolyticus* (K5).

Recommendation includes 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 *V. alginolyticus*. Next, other studies should include on the use of cocktail phage or mixing of several types of phages against bacterial strains as recommended by Fang et al. (2022).

FYP FPV

UNIVERSITI
MALAYSIA
KELANTAN

REFERRENCES:

Culot, A., Grosset, N., & Gautier, M. (2019). Overcoming the challenges of phage therapy for industrial aquaculture: A review. *Aquaculture*, 513, 734423.

Dan. (2017). *SEA-PHAGES | Home*. Seaphages.org. https://seaphages.org/

Daubie, V., Chalhoub, H., Blasdel, B., Dahma, H., Merabishvili, M., Glonti, T., De Vos, N., Quintens, J., Pirnay, J.-P., Hallin, M., & Vandenberg, O. (2022). Determination of phage susceptibility as a clinical diagnostic tool: A routine perspective. *Frontiers in Cellular and Infection Microbiology*, *12*. https://doi.org/10.3389/fcimb.2022.1000721

Department of Statistics Malaysia Official Portal. (2022). Dosm.gov.my. https://www.dosm.gov.my/v1/index.php?r=column/ctwoByCat&parent_id=45&menu_id=Z0VTZGU1UHBUT1VJMFlpaXRRR0xpdz09

de Souza V. Cecilia., & Wan, A. H. L. (2021). Vibrio and major commercially important vibriosis diseases in decapod crustaceans. *Journal of Invertebrate Pathology*, *181*, 107527. https://doi.org/10.1016/j.jip.2020.107527

Droubogiannis, S.; Pavlidi, L.; Tsertou, M.I.; Kokkari, C.; Skliros, D.; Flemetakis, E.; Katharios, P. (2022). Vibrio Phage Artemius, a Novel Phage Infecting Vibrio alginolyticus. *Pathogens 2022, 11*, 848. https:// doi.org/10.3390/pathogens11080848

Eliopoulos, G. M., Cosgrove, S. E., & Carmeli, Y. (2003). The Impact of Antimicrobial Resistance on Health and Economic Outcomes. *Clinical Infectious Diseases*, *36*(11), 1433–1437. https://doi.org/10.1086/375081

Fang, Q., Feng, Y., McNally, A., & Zong, Z. (2022). Characterization of phage resistance and phages capable of intestinal decolonization of carbapenem-resistant Klebsiella pneumoniae in mice. *Communications Biology*, *5*(1). https://doi.org/10.1038/s42003-022-03001-y

Glonti T., Pirnay J.-P. (2022). *In vitro* techniques and measurements of phage characteristics that are important for phage therapy success. *Viruses* 14 (7), 1490–1513. doi: 10.3390/v14071490

Ina-Salwany, M. Y., Al-saari Nurhidayu, Mohamad, A., Fathin-Amirah, M., Mohd, A., Amal, M. N. A., Hisae K., Sayaka, M., Tomoo, S., & Zamri-Saad, M. (2018). Vibriosis in Fish: A Review on Disease Development and Prevention. *Journal of Aquatic Animal Health.* doi:10.1002/aah.10045

Iwasaki, T., Yamashita, E., Nakagawa, A., Enomoto, A., Tomihara, M., & Takeda, S. (2018). *Three-dimensional structures of bacteriophage neck subunits are shared in Podoviridae, Siphoviridae and Myoviridae . Genes to Cells, 23(7), 528– 536.* doi:10.1111/gtc.12594

FYP FPV

FYP FPV

Kim, S. G., Jun, J. W., Giri, S. S., Yun, S., Kim, H. J., Kim, S. W., Kang, J. W., Han, S. J., Jeong, D., & Park, S. C. (2019). Isolation and characterisation of pVa-21, a giant bacteriophage with anti-biofilm potential against Vibrio alginolyticus. Scientific Reports, 9(1). https://doi.org/10.1038/s41598-019-42681-1

Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., and Johnson, R. P.(2009). "“Enumeration of bacteriophages by double agar overlay plaque assay,”,”in Bacteriophages: Methods in Molecular Biology, eds M. R. Clokie and A. M. Kropinski (Totowa, NJ: Humana Press), 69–76.

Labrie, S., Samson, J. & Moineau, S. Bacteriophage resistance mechanisms. *Nat Rev Microbiol* 8, 317–327 (2010). https://doi.org/10.1038/nrmicro2315

Letchumanan, V., Chan, K. G., Pusparajah, P., Saokaew, S., Duangjai, A., Goh, B.H., Syakima M,, Lee, L.H. (2016). *Insights into Bacteriophage Application in Controlling Vibrio Species. Frontiers in Microbiology, 7. doi:10.3389/fmicb.2016.01114* .

Letchumanan, V., Chan, K.-G., Pusparajah, P., Saokaew, S., Duangjai, A., Goh, B.-H., Ab Mutalib, N.-S., & Lee, L.-H. (2016). Insights into Bacteriophage Application in Controlling Vibrio Species. *Frontiers in Microbiology*, 7. https://doi.org/10.3389/fmicb.2016.01114

Li, J., Tian, F., Hu, Y., Lin, W., Liu, Y., Zhao, F., Ren, H., Pan, Q., Shi, T., & Tong, Y. (2021). Characterization and Genomic Analysis of BUCT549, a Novel Bacteriophage Infecting Vibrio alginolyticus With Flagella as Receptor. Frontiers in Microbiology, 12. https://doi.org/10.3389/fmicb.2021.668319

Mazuki Hashim (2022). National Aquaculture Sector Overview: FAO Fisheries & Aquaculture. *Food and Agriculture Organization of the United Nations.* https://www.fao.org/fishery/en/countrysector/my/en

Penziner, S., Schooley, R. T., & Pride, D. T. (2021). Animal Models of Phage Therapy. *Frontiers in Microbiology*, 12. https://doi.org/10.3389/fmicb.2021.631794

Poxleitner, M., Pope, W., Deborah, J.S., Sivanathan, V., & Hatfull, G. (2022). Phage Discovery Guide. *The Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science*. https://seaphagesphagediscoveryguide.helpdocsonline.com/3-0-overview

Ramos-Vivas, J., Superio, J., Galindo-Villegas, J., & Acosta, F. (2021). Phage Therapy as a Focused Management Strategy in Aquaculture. *International journal of molecular sciences*, *22*(19), 10436. https://doi.org/10.3390/ijms221910436

Reverter, M., Sarter, S., Caruso, D., Avarre, J.-C., Combe, M., Pepey, E., Pouyaud, L., Vega-Heredía, S., de Verdal, H., & Gozlan, R. E. (2020). Aquaculture at the crossroads of global warming and antimicrobial resistance. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-15735-6

FYP FPV

Schulz, P.; Pajdak-Czaus, J.; Siwicki, A.K. (2022). In Vivo Bacteriophages' Application for the Prevention and Therapy of Aquaculture Animals– Chosen Aspects. Animals, 12, 1233. https://doi.org/10.3390/ani12101233

Selvin, J., & Lipton, A. (2004). *Vibrio alginolyticus associated with white spot disease of Penaeus monodon*. ResearchGate; Inter Research. https://www.researchgate.net/publication/8909120_Vibrio_alginolyticus_associated_with_white_spot_disease_of_Penaeus_monodon

Sharma, K., Rathore, G., Dev Kumar Verma, & Philipose, K. K. (2013). *Vibrio alginolyticus infection in Asian seabass (Lates calcarifer, Bloch) reared in open sea...* ResearchGate;Wiley. https://www.researchgate.net/publication/235788743_Vibrio_alginolyticus_infection_in_Asian_seabassLates_calcarifer_Bloch_reared_in_open_sea_floatingcages_in_India

Silva, Y. J., Costa, L., Pereira, C., Mateus, C., Cunha, Â., Calado, R., Gomes, N. C. M., Pardo, M. A., Hernandez, I., & Almeida, A. (2014). Phage Therapy as an Approach to Prevent Vibrio anguillarum Infections in Fish Larvae Production. *PLoS ONE*, *9*(12), e114197. https://doi.org/10.1371/journal.pone.0114197

Skliros, D., Kalatzis, P. G., Kalloniati, C., Komaitis, F., Papathanasiou, S., Kouri, E. D., Udvardi, M. K., Kokkari, C., Katharios, P., & Flemetakis, E. (2021). The Development of Bacteriophage Resistance in Vibrio alginolyticus Depends on a Complex Metabolic Adaptation Strategy. *Viruses*, *13*(4), 656. https://doi.org/10.3390/v13040656

Staub, N. L., Poxleitner, M., Braley, A., Smith-Flores, H., Pribbenow, C. M., Jaworski, L., Lopatto, D., & Anders, K. R. (2016). Scaling Up: Adapting a Phage-Hunting Course to Increase Participation of First-Year Students in Research. *CBE—Life Sciences Education*, *15*(2), ar13. https://doi.org/10.1187/cbe.15-10-0211

Zhou, W., Li, Y., Li, Z., Ma, B., Jiang, X., Hu, C., Ai, Y., & Luo, P. (2021). Genomic Changes and Genetic Divergence of Vibrio alginolyticus Under Phage Infection Stress Revealed by Whole-Genome Sequencing and Resequencing. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.710262

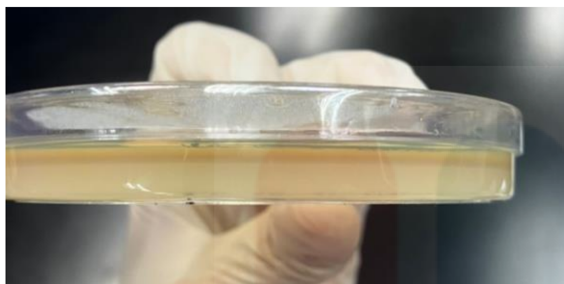
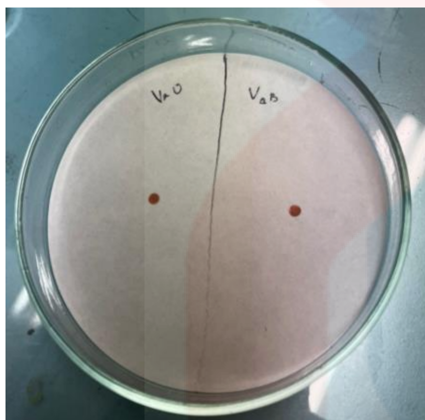
UNIVERSITI
MALAYSIA
KELANTAN

FYP FPV

APPENDIX A

 Appendix A.1: Bean clam, *Donax cuneatus*	 Appendix A.2: Carpet clam, *Paphia textile*
 Appendix A.3: Wild oyster, *Crassostrea sp*	 Appendix A.4: Blending of clam sample
 Appendix A.5: Centrifuged clam sample	 Appendix A.6: Incubation of the bacterial strains

FYP FPV

 Appendix A.7: Double layer agar	Appendix A.8: TEM machine
 Appendix A.9: Copper grid on filter paper	Appendix A.10: TEM phage observation in USM Penang with PHD student, Kak Ain and FYP SV, Dr Ruhil

UNIVERSITI
MALAYSIA
KELANTAN