ISOLATION OF POTENTIAL PHAGES AGAINST *Aeromonas veronii*

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DOCTOR OF VETERINARY MEDICINE

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Isolation of Potential Phages against Aeromonas veronii

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Faculty of Veterinary Medicine
UNIVERSITI MALAYSIA KELANTAN

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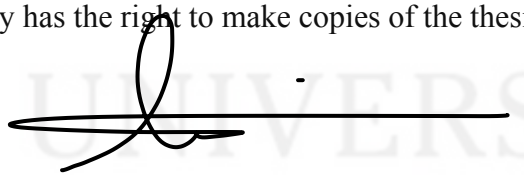

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ISOLATION OF POTENTIAL PHAGES AGAINST

Aeromonas veronii

ABSTRACT

An abstract of the research paper was presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement for the course DVT 55204 – Research Project.

In today's era of emerging antimicrobial resistance, there has been an increased emphasis on bacteriophages, though it is surprising that bacteriophages against Aeromonas veronii is not much studied. Therefore, this study focuses on the isolation of potential bacteriophages against *Aeromonas veronii* from sewage water, river and soil in Kota Bharu, Kelantan.. The research involved the collection of environmental samples such as water and soil, followed by the isolation of potential phages using *A. veronii* strains as hosts. Enrichment technique using pond water along with bacterial culture was employed for the isolation of phage followed by double layered agar plaque assay for titre enumeration. In this study, two bacteriophages were isolated from Universiti Malaysia Kelantan (UMK) and Sungai Pengkalan Chepa (SPC) unfortunately only one bacteriophage sample were further studies which is sample from Universiti Malaysia Kelantan (UMK) due to time constraint. The isolated phages underwent host range determination and plaque forming units were conducted to enumerate the titer of the isolated bacteriophage and the highest countable dilution is 10^{-5} and the titer is 1.25×10^{-3} PFU/mL. These findings contribute valuable insights into the potential use of bacteriophages as targeted agents for controlling *Aeromonas veronii* infections. Further exploration of these isolated phages could pave the way for the development of tailored phage therapies, offering a precise and effective approach to combating *A. veronii*-related illnesses.

Keywords: ***Aeromonas veronii*****, Bacteriophage, antimicrobial resistance**

PENGASINGAN BAKTERIOFAJ BERPOTENSI MENENTANG *Aeromonas veronii*

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.

Dalam era ketahanan antimikrobial yang semakin mencuat pada hari ini, terdapat penekanan yang meningkat terhadap bakteriofaj, walaupun agak mengejutkan bahawa bakteriofaj terhadap Aeromonas veronii kurang dikaji. Oleh itu, kajian ini memberi tumpuan kepada pengasingan bakteriofaj yang berpotensi terhadap Aeromonas veronii daripada air sisa, sungai, dan tanah di Kota Bharu, Kelantan. Kajian ini melibatkan pengumpulan sampel alam sekitar seperti air dan tanah, diikuti dengan pengasingan bakteriofaj yang berpotensi menggunakan strain A. veronii sebagai hos. Teknik pengayaan menggunakan air kolam bersama kultur bakteria digunakan untuk pengasingan faj, diikuti dengan ujian plat agar lapisan berganda untuk pengukuran titer.
Dalam kajian ini, dua bakteriofaj diasingkan dari Universiti Malaysia Kelantan (UMK) dan Sungai Pengkalan Chepa (SPC), tetapi malangnya hanya satu sampel bakteriofaj yang dikaji lanjut iaitu sampel dari Universiti Malaysia Kelantan (UMK) kerana kekangan masa. Bakteriofaj yang diasingkan menjalani penentuan julat hos dan unit pembentukan plat dilakukan untuk mengukur titer bakteriofaj yang diasingkan dan Dilusi yang dapat dihitung tertinggi adalah pada 10^{-5}, dan titernya adalah 1.25 x 10^{-3} PFU/mL.. Penemuan ini memberikan pandangan berharga tentang penggunaan bakteriofaj sebagai agen bergerak sasaran untuk mengawal jangkitan Aeromonas veronii. Penjelajahan lanjut terhadap bakteriofaj yang diasingkan ini boleh membuka jalan untuk pembangunan terapi bakteriofaj yang disesuaikan, menawarkan pendekatan yang tepat dan berkesan dalam memerangi penyakit yang berkaitan dengan A. veronii.

Kata kunci: ***Aeromonas veronii*****, Bakteriofaj, Ketahanan antimikrobial**

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I would like to express my sincere gratitude to all those who have contributed to the completion of this research project. First and foremost, I extend my deepest gratitude to God Almighty for his blessing throughout the entire journey of completing my Final Year Project (FYP). My gratitude towards my supervisor, Dr. Ruhil Hayati Binti Hamdan for thorough supervision, patience and guidance throughout the entire process of completing the project, and my appreciation towards my co-supervisors Prof. Dr. Maizan Binti Mohamed and Dr. Tan Li Peng. Not to forget Cik Ain Auzureen Mat Zin for sharing her knowledge and time helping us throughout the process and to all FPV laboratory staff for their patience and guidance. I want to acknowledge the support from my family members and friends for their endless support and encouragement until the end of my project

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LIST OF ABBREVIATION

1M $CaCl_2$	1 Molar of Calcium Chloride
AMR	Antimicrobial resistance
ARG	Antibiotic resistance gene
MAS	Motile Aeromonas septicemia
spp.	Species
SPC	Sungai Pengkalan Chepa
SPG	Sungai Pulau Gajah
SPGS	Sungai Pulau Gajah Soil
UMK	Universiti Malaysia Kelantan
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth

LIST OF SYMBOLS

%	Percentage
µm	Micrometer
µL	Micro liters
kV	Kilovolts
℃	Degree celsius
µl	Microliter
µm	Micrometer
PFU/mL	Plaque forming units per milliliter
rpm	Round per minute

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Freshwater and brackish water aquacultures plays a significant role in Malaysia's economy, providing a sustainable source of protein, generating employment opportunities, and contributing to the country's food security (Ismail, Mohamad.2021). Despite its contribution, freshwater and brackishwater aquaculture faces various challenges and one of the challenges is disease outbreak, where in a recent investigation of a disease outbreak in Northern province Vietnam shows that out of 506 diseases fish 46.4% were positive for *Aeromonas spp.* Infection. (Nhinh,, 2021). Infection by *Aeromonas spp* causes Motile Aeromonas Septicemia and it's a challenge for farmers to combat this disease in their farm. In addition, fish farmers are heavily dependent on using antibiotics in fish farming to treat disease further contributing to the spread of antimicrobial resistance (AMR) via acceleration of antibiotic resistance gene (ARG) in bacteria which threatens public health (Reverter et al., 2020). According to Lulijwa et al. (2019), 73% of the main aquaculture producing countries use florfenicol, sulphadiazine and oxytetracycline and 55% applied amoxicillin, erythromycin, enrofloxacin and sulfadimethoxine where most of this antibiotics are frequently used in human medicine. This poses a threat to public health where there would be an increased number of foodborne antimicrobial resistance due to high amounts of residual drugs. Furthermore, *Aeromonas spp* is commonly regarded as pathogens of aquatic creatures and several other animals, have been gaining fame on medical trial due to its ability to colonize and infect human beings, symptoms range from acute self-limiting diarrhea to lethal sepsis however, wounds, skin, bones, heart, lungs, eyes, and other organs can be potentially

affected (Pessoa, R. B. G, et al, 2022). Due to these challenges, phage therapy is being implemented in aquaculture as a potential antimicrobial to combat the disease as it is cost-effective, eco-friendly, safe for aquaculture species and end-users such as humans and animals (Pereira, C.2022).

1.2 Problem Statement

One of the greatest challenges affecting public health and animal welfare is the antimicrobial resistance (AMR). Fish farming heavily relies on use of antibiotics which has adverse effects in long term application and improper dosing. *Aeromonas* spp. is abundant in fresh and brackishwater which makes aquatic organisms easily exposed to the disease and threatening their health. Recent studies also revealed that *Aeromonas veronii* is resistant to multiple antibiotics such as Ampicillin, Cefoxitin, Cephalothin, Gentamicin, Erythromycin, Penicillin, Sulfonamide and Tetracycline (Dubey et al., 2022). Hence phage therapy can be used as a substitute to replace antibiotics, phage therapy has specific host range which has the ability to only infect specific pathogens which prevents from disrupting the normal flora. Other than that, the mechanism of bacteriophages enables it to infect and kill bacteria that is resistant towards multiple antibiotics (Loc-Carrillo, 2011).

1.3 Research Question

Can bacteriophage against *Aeromonas veronii* be isolated from sewage water?

1.4 Research Hypothesis

Bacteriophage against *Aeromonas veronii* can be isolated from sewage water.

1.5 Research Objective

To isolate bacteriophage against *Aeromonas veronii* from sewage water, river and soil samples in Kota Bharu, Kelantan.

CHAPTER 2

LITERATURE REVIEW

2.1 Aeromonas in Aquaculture

Aeromonas is widely distributed in nature with good environmental adaptability(Parte,2003). It is a Gram-negative, rod-shaped and facultative anaerobic bacteria. *Aeromonas hydrophila, A. dhakensis, A. jandaei, A. sobria* and *A. veronii* are the *Aeromonas* species most frequently associated with fish diseases (Rahman et al., 2002; Sreedharan et al., 2012; Yi et al., 2013; Musa & Laith, 2014). Infection by *A. veronii*, *A. hydrophila* and *A. caviae* on fresh and brackish water animals causes Motile Aeromonas Septicemia (MAS) (Lee et al., 2018). This results in animals showing clinical signs such as reddening of fins, diffuse hemorrhage on skin and inflammation of the anus.

2.2 Phage Therapy on *Aeromonas spp.*

Phages are bacteria-infecting viruses that can be found abundant in the environment and essential in controlling bacterial populations in the natural system (Almeida et al., 2009; Pereira et al., 2021). Based on a research conducted by Liu et al. (2020), three phages were able to be isolated that showed infectivity towards *A. veronii* which are N21, W3 and G65. A bacteriophage can pursue either lytic or lysogenic replication once it has attached to a susceptible host. A phage attaches to a receptive host bacterium during a lytic replication cycle, inserts its genome into the cytoplasm of the host cell, and uses the host's ribosomes to produce its proteins. Resources from

the host cell are quickly transformed into viral genomes and capsid proteins, which come together to form numerous copies of the original phage. It is either actively or passively lysed when the host cell decomposes, releasing the new bacteriophage to infect another host cell. The phage additionally binds to a receptive host bacterium during the lysogenic replication cycle and inserts its genome into the cytoplasm of the host cell. Instead, the phage genome is either maintained as an episomal element or incorporated into the chromosome of the bacterial cell, where it is duplicated and transmitted to progeny bacterial cells without killing them in either situation. (Kasman & Porter, 2022)

2.3 Isolation and Identification of *Aeromonas spp* Phages

Bacteriophages can be found abundant in the environment where they can be isolated from various samples, A study conducted at Dong Thap province Vietnam, samples that was taken are water, sludge, intestine, liver and kidney shows that all sample was positive with bacteriophage where sludge has the highest percentage of 100% (Nguyen et al., 2021). Research conducted by Liu et al., (2020), which isolates and identifies bacteriophages against *Aeromonas hydrophila* shows broad spectrum activity that shows infectivity towards *Aeromonas veronii*. The characteristics of each individual phages show icosahedral head with the diameter of (62.6 ± 1.9) nm, (64.9 ± 3.2) nm and (58.8 ± 4.1) nm, respectively, a contractile tail with the length of (153.1 ± 6.2) nm, (154.1 ± 1.4) nm and (152.3 ± 9.8) nm with six long fibers, respectively, and collar and base plate structures (Liu et al., 2020).

In general, bacteriophage samples should be taken from areas where we suspect the host be (Hyman ,2019). This is because bacteriophage targets a specific host for it to replicate, so when collecting samples we can yield a larger number of bacteriophages in the sample. Phages for farmed fish pathogens provide high yield when samples are taken from coastal waters and fish farm water (Stenlhom et al,2008). Phages for mammalian intestinal bacteria can readily be isolated from fecal materials, sewage water and farm run off(Jakhetia et al. ,2013). To isolate phages from samples, a study conducted by Czajkowski et al. (2016) stated that zinc chloride could be used to concentrate phages from water or plant soil extracts sufficiently to detect phages by direct plating without enrichment culture. For soil samples, Trubl et al. (2016) stated that it requires extensive washing to release phages that might adhere to soil particles. Based on a study conducted by Hyman (2015), they get better results by omitting the filtration process for fecal and soil samples although for water sample in general filtration is needed after centrifugation to remove debris,(Nikkhahi et al., 2017)

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Bacterial collection

Table 1 shows *Aeromonas veronii* and *Aeromonas hydrophila* were used for this study. The bacterial stocks were obtained from Aquatic Animal Health, Faculty of Veterinary Medicine. The bacterial stocks were revived on TSA at 30℃ for 24 h.

Table 1: Bacterial stocks used in this study.

Sample ID	Bacterial Isolates	Location
K3K22	*Aeromonas veronii*	Kg. Tujuh Tumpat, Kelantan
KIP5	*Aeromonas veronii*	Nilam Puri, Kota Bharu, Kelantan
K2P6(b)	*Aeromonas veronii*	Kg. Pantai Melawi, Bachok, Kelantan
P1T2	*Aeromonas veronii*	Kg. Baru Pulau Keladi, Pekan, Pahang
P3T7(b)	*Aeromonas veronii*	Kolam Zia, Kg. Pandan Dua, Kuantan Pahang
P3T10	*Aeromonas veronii*	Kolam Zia, Kg. Pandan Dua, Kuantan Pahang
PIK5(a)	*Aeromonas veronii*	Kg. Baru Pulau Keladi, Pekan Pahang
K3T8	*Aeromonas hydrophila*	Kg. Tujuh, Tumpat, Kelantan

3.2 Sample Collection and Processing

Fifty milliliter of water sample was collected from three different area in Kota Bharu Kelantan which are from University Malaysia Kelantan(UMK), Sungai Pengkalan Chepa (SPC) and Sungai Pulau Gajah(SPG) and one dirt sample was collected from Sungai Pulau Gajah (SSPG). The samples were then centrifuged at 4000 rpm for 10 minutes to remove debris. For the soil sample, approximately 5 g of dirt was inserted into another sample container and was added with SM buffer until it reached fifty milliliters then centrifuged. The supernatant of each sample was filtered through a 0.45 µm filter and was partitioned into two containers where for portion A we used it for direct isolation for spot test and portion B will be used for enrichment isolation using double strength tryptic soy broth.

3.3 Enrichment Isolation

Ten milliliters of filtered sample from portion B was added into another container containing 10 ml of double strength TSB and 40 microliter of 1M $CaCl_2$. The sample was then incubated in a shaker incubator at 30℃ for 24 h at 200 rpm. Then the sample was filtered using 0.45 µm syringe filter.

3.4 Spot test

Four milliliters of soft agar was prepared and 100 µL of bacterial sample was added with 10 µL of 1M CaCl2 and the solution was poured into TSA plates. Seven microlitres of the phage

sample was applied onto the marked section of the TSA plates. The plates were incubated for 24 h at 30℃.

3.5 Picking Plaques

Each plaque spot on the plate was observed y and individual plaques were selected. One hundred microliters of SM buffer was added into the microcentrifuge tube. Next pipette and pipette tip was poked to the centre of the plaque. Then, the pipette tip was added into the microcentrifuge tube and stored at 4℃ to allow bacteriophage to diffuse from the overlay agar for 30 min up to an hour. The sample was then centrifuged at 14,000 rpm for 20 min and the supernatant was filtered using 0.45 μm to remove any bacteria residue.

3.6 Serial Dilution

Eight microcentrifuge was labeled accordingly from 10^{-1} until 10^{-8} and was added with 900 μL of SM buffer. One hundred microliters of stock phage was added into the microcentrifuge labeled 10-1 and mixed up and down several times and the pipette tip was discarded. 100 μL from the microcentrifuge is transferred into the next microcentrifuge and is mixed up and down. The same procedure was applied until the microcentrifuge labeled 10^{-8} and 100 μL from it was discarded.

3.7 Plaque Assay

The TSA plates were labeled according to serial dilution (10^{-1} until 10^{-8}).Hundred microlitres from serial dilution, 10^{-1},100 µL of bacteria and 10 µL of 1M $CaCl_2$ was added into the 4 ml overlay agar The mixture was poured onto TSA plates. The plates were then incubated at 30℃ for 24 h. The same procedure applies to other serial dilution (10^{-2} until 10^{-8}).

The number of plaques were calculated within the range of 20-200. The plaque forming unit per milliliter (PFU/mL) was calculated using this formula.

$$\frac{Number\ of\ plaques\ counted\ (PFU)}{Volume\ plated\ (in\ mL)} \times dilution\ factor\ = titre\ (\frac{PFU}{mL})$$

Picking plaques were conducted on the selected plates and centrifuged at 14,000 rpm for 20 min.The supernatants were filtered using a 0.2 µm filter syringe and stored at -80℃, the collected supernatants were used to purify the phages.

3.8 Purification

The plaque assay was repeated 3 times to purify the bacteriophage.

3.9 Host Range

Bacterial stock of *Aeromonas veronii and Aeromonas hydrophila* were incubated overnight at 30℃ at 200 rpm using tryptic soy broth. Serial dilution was prepared using the purified phage stock labeled from 10^{-1} until $10^{=8}$. Confluent bacterial lawn was prepared by mixing one hundred micro milliliter of stock bacteria into a 4mL overlay agar with 10 µL 1M $CaCl_2$ and poured onto TSA plates. Spot tests were then performed on each plate and left for a few hours to let it dry. The plates were then incubated at 30 ℃ overnight.

CHAPTER 4

RESULTS

Isolation of *Aeromonas veronii* Phages

Initially, there was no inhibition zone observed on all samples when conducting spot tests before enrichment isolation. Table 1 shows there was presence of a clear inhibition zone on sample Universiti Malaysia Kelantan and Sungai Pengkalan Chepa. No inhibition zone on water and soil samples from Sungai Pulau Gajah after enrichment isolation.

Table 1 : Spot Test After Enrichment

Location	Sample type	Result
Universiti Malaysia Kelantan (Taman Bendahara),(UMK)	Water	Clear zones were observed with spots of bacterial colonies.
Sungai Pengkalan Chepa(SPC)	Water	Clear zone was observed with distinct spots of bacterial colony
Sungai Pulau Gajah	Water	No clear zone observed
	Soil	No clear zone observed

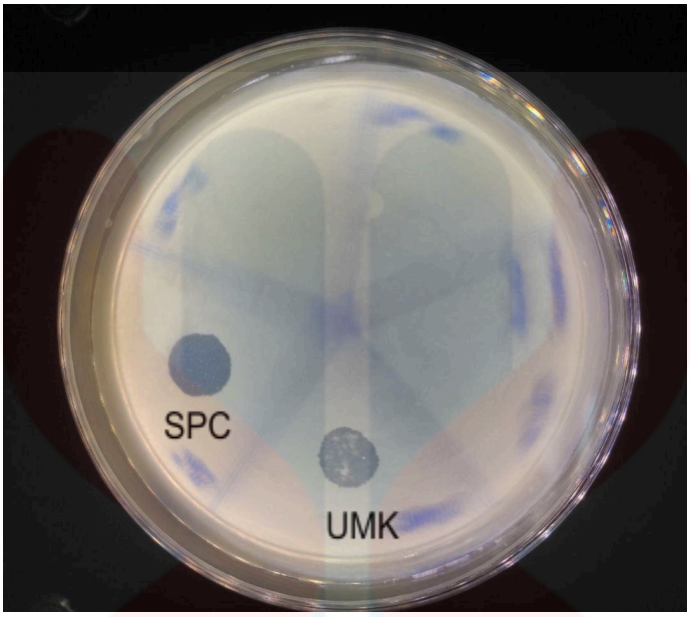

Figure 1 : Zone of inhibition from spot test after samples enrichment taken at UMK and SPC.

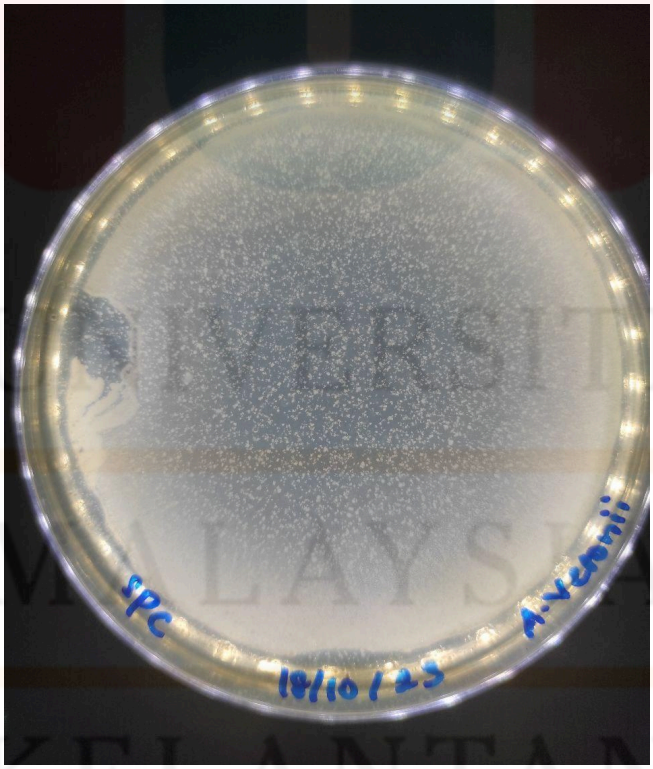

Figure 2 : Overlay result of bacteriophage from SPC

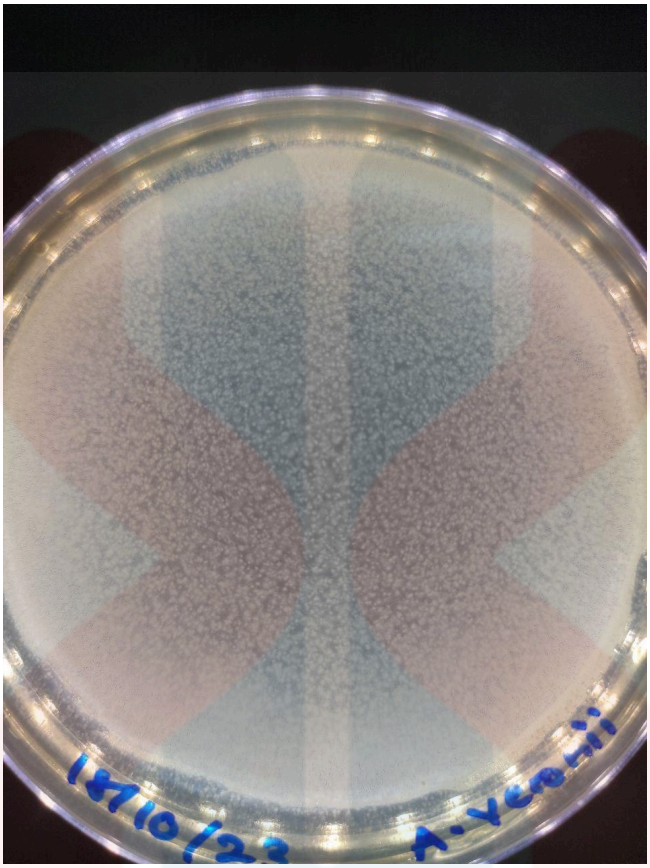

Figure 3 : Overlay result of bacteriophage from UMK

Table 2 : Plaque forming units per milliliter (PFU/ml)

Table 2 shows the titer (PFU/mL) gradually decreasing as we increase the dilution factor. Dilution 10^{-3} was collected for further purification.

Stock Phage (UMK) Dilution	Sample volume (ml)	Number of PFU	Titer (PFU/mL)
-1	0.2	Too many to count	-
-2		Too many to count	-
-3		85	0.425
-4		11	$5.5x10^{-3}$
-5		3	$1.5x10^{-4}$
-6		1	$5x10^{-6}$
-7		0	0
-8		0	0

Table 3 shows the titer sample from phage stock dilution 10^{-3} from table 2 gradually decreasing as the dilution factor increases. Dilution 10^{-2} was collected and stored at -80℃.

Table 3 : Plaque forming units per milliliter (PFU/mL) from Phage stock dilution 10^{-3}

Stock Phage (UMK) from Dilution 10^{-3}	Sample volume (µL)	Number of PFU	Titer (PFU/mL)
-1	0.2	Too many to count	-
-2		141	7.05
-3		76	0.38
-4		42	0.021
-5		25	$1.25x10^{-3}$

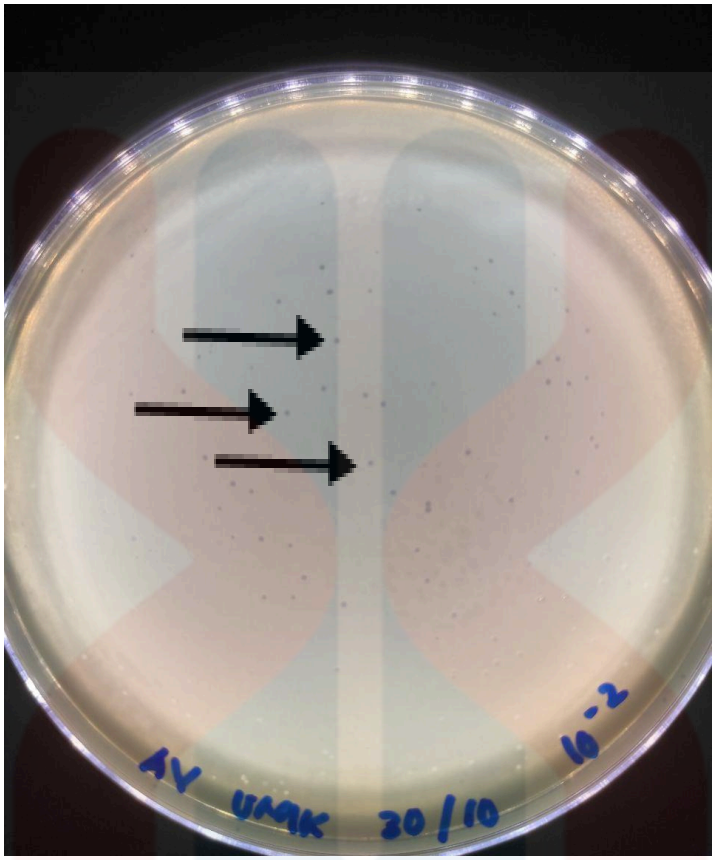

Figure 4 : Third purification from sample UMK at dilution 10^{-2} .

Host Range

All *Aeromonas veronii* samples from various locations tested negative for the inhibition zone as well as *Aeromonas hydrophila*.

CHAPTER 5

DISCUSSION

This study was aimed to identify and characterize bacteriophage against *Aeromonas veronii* for further research in Malaysia to expand the use of bacteriophage in commercializing it for daily uses, especially for aquatic farmers as an alternative medication against *Aeromonas spp.* infection. Bacteriophage are viruses that infect bacteria causing either lytic or lysogenic replication towards the susceptible bacteria to produce more phages against the specific bacteria (Kasman & Porter, 2022). To commercially use bacteriophage in aquaculture, we aimed to isolate lytic bacteriophages.

In this study, bacteria stock samples for *Aeromonas veronii* were collected from African catfish (*C. gariepinus*) at Kg. Pulau Keladi Pekan, Pahang. Two water samples from Universiti Malaysia Kelantan (UMK) and Sungai Pengkalan Chepa (SPC) were positive for lytic bacteriophage against *Aeromonas veronii* after enrichment isolation. For samples from UMK we were able to observe a clear zone of inhibition with small spots of bacterial colonies whereas samples from SPC, a clear inhibition zone can be observed but there were spots of bacterial colonies in which might indicate that there is a mix of lysogenic and lytic phages in the sample. For the two other samples from Kampung Pulau Gajah, Kelantan there were no inhibition zones observed after enrichment isolation; it could be because the bacteriophage present are not specific towards the strain of bacteria being used.

In the host range, the result shows that all stock bacteria for *Aeromonas veronii* from different locations, tested negative for the spot test using the purified stock phage sample from UMK. Like all viruses, bacteriophages are very species-specific with regard to their hosts and usually only infect a single bacterial species or even specific strains within a species (Kasman and Porter, 2022). This is due to a combination of factors including specificity of phages' host binding proteins, biochemical interactions during infection, and bacteriophage-resistance mechanisms (Hayman & Abedon, 2010).

The diameter of inhibition represents the size of bacteriophage, in this study we could observe that the inhibition zone is small where it was approximately about 0.1mm in diameter. The size of the plaque is negatively correlated with the size of the virion, where larger virions produce smaller plaques, as seen in this study (Gallet et al., 2011). This is presumably because larger virions would diffuse more slowly through the double-layer agar than smaller virions, resulting in smaller plaques (Jurczak-Kurek et al., 2016). Although there were other factors affecting the size of inhibition zone as stated by Gallet et al.(2011), mentioned that the lysis time, which is the time taken for the progeny to burst out of the host cell, also affects the size of the plaque, where long lysis time is positively correlated to plaque size.

The result for plaque forming units using a bacteriophage sample from UMK shows that the highest countable dilution of 10^{-5} yields a titer of 1.25×10^{-3} PFU/mL. Testing for plaque forming units (PFU) is a common method for determining the number of bacteriophages in a sample (Acs et al., 2020). To effectively assess the outcome of clinical treatments and to unravel the complex

dynamics of microbial communities, detection and enumeration of phage particles in complex samples is of fundamental importance (Dąbrowska, 2019). Furthermore, phage enumeration is needed for production and development of phage-based products, as well as for using phages for detection of bacterial infections and biocontrol of food products (Monk et al., 2010).

Even though the morphology characterization of the bacteriophage using electron microscope could not be done in time, the expected family of the bacteriophage are *Myoviridae* and *Podoviridae*. According to Liu et al. (2020), most bacteriophages against *Aeromonas* spp. have been from the family *Myoviridae* with the characteristic of tailed bacteriophage. The study also stated that bacteriophages against *Aeromonas* spp. were from the *Podoviridae* family with characteristics of icosahedral heads with short non-contractile tails. In addition, Anand et al. (2018) also reported about isolation and characterization of Podoviridae family phage against *Aeromonas veronii*, where the PCR result confirms its classification T7 like genus. Furthermore, a study conducted by Li Z et al. (2022) isolated bacteriophages against *Aeromonas veronii* that belong to the family *Myoviridae*.

Chapter 5 : Conclusion

In conclusion, two lytic phages were isolated by taking water samples from Universiti Malaysia Kelantan(UMK) and Sungai Pengkalan Chepa (SPC). Only one bacteriophage sample was further studied which is samples from UMK due to time constraints. After purification, the bacteriophage sample from UMK only targeted specific strains of *Aeromonas veronii*. However, limitations to this research relies on characterizing the bacteriophage morphology. Unfortunately, we were unable to characterize the bacteriophage using transmission electron microscopy due to time constraints. Therefore, I recommend that the time frame for Final Year Project (FYP) should be extended to have enough time to complete the project.

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APPENDIX

Appendix 1: Water sample from Sungai Pengkalan Chepa

Appendix 2: Water sample from Universiti Malaysia Kelantan

Appendix 3: Water and Soil sample from Sungai Pulau Gajah

Appendix 4: Collecting water sample from Universiti Malaysia Kelantan

Appendix 5: Collecting water sample from Sungai Pengkalan Chepa

Appendix 6: Collecting water and soil sample from Sungai Pulau Gajah