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ISOLATION AND IDENTIFICATION OF COLIPHAGE FROM UNTREATED WASTEWATER IN KUALA PILAH NEGERI SEMBILAN

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Abstract

Bacteriophages are viruses that can only infect bacterial cells and are perceptible in almost all places where living bacteria are found, as they are highly host specific. Wastewater comprises microorganisms that have become contaminated by the environment. These microorganisms, particularly bacteria, may develop antibiotic resistance and constitute a serious threat to human health. Therefore, the goal of this research is to isolate coliphage from untreated wastewater and to identify the susceptible coliform host. The method used for bacteriophage isolation is plaque formation assay and the selected plaque was further enriched and purified using the double-layer plaque technique to classify bacteriophages. Identification of susceptible coliform host using gram stain and biochemical analysis results were compared with other coliform bacteria (Klebsiella sp, *Escherichia coli*, and Shigella sp.) to identify the susceptible coliphage host. The isolated coliphages appear late from 2 out of 3 samples collected around Kuala Pilah town. The plaque shows were small and turbid after 7 days of incubation in 37°C. It was suspected to be a temperate phage as the plaque developed was turbid. The susceptible host shows similar biochemical characteristics as Klebsiella sp. In conclusion, the bacteriophage obtained from this study is a temperate bacteriophage from environmental wastewater that could infect a coliphage like Klebsiella sp. Further identification of susceptible host was suggested including determination of its medical usage.

Keywords: bacteriophage, wastewater, *E. coli*, plaque.

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Introduction

Bacteriophages are spread through the environment, especially wastewater. It is a type of viruses that can only infect prokaryotes and kill them, especially bacteria. Leptospirosis is a deadly disease that is possible to cause sporadic epidemics disease commonly in the urban area (Abdul Mutalip et al., 2019). Following the integration of their genomes into those of the bacteria, they provide a massive gene pool from which bacteria can rapidly evolve and were utilized as biocontrol agents. In 1915 and 1917, when phages were discovered by Frederick Twort and Felix d'Herelle, the phage has the potential ability as a laboratory research subject (Pelzek et al., 2008). The interactions between the bacteriophage and the bacteria in the sewage can give impacts towards the dynamics and functions of bacteria that are abundant in the sewage (Runa et al., 2021). Most of the research done proven that benefits from bacteriophage infection has lysing and destroying multiple antibiotic resistance bacteria from becoming pathogenic (Elahi et al.,2021). There are two types of bacteriophages, virulent and temperate phage. A virulent phage results in the bacterium's death, while for temperate phage, the phage may become a prophage by integrating its nucleic acid with the host DNA or by being extrachromosomal. In fact, for

the last century, the antibacterial application of phage or 'phage therapy' has been used to treat diseases among people and had shown great success in managing pathogenic bacterial infections. The phage genetic material represents a substantial number of unique unknown genetic sequences and could be the world's largest repository of undiscovered genes (Chen et al., 2020).

Methods

Isolation of Bacteriophage

The 50 mL of water samples were collected in conical tubes from the stagnant drain in Kuala Pilah town, and 2 bottles of samples collected from each reservoir in Taman Senimas (A), near an eatery place (B) and a marketplace (C). The wastewater samples were filtered using Whitman filter paper and the sediment was added into a 50 mL falcon tubes and were centrifuged for 15 min. The supernatant was decanted into a new sterile conical tube and mixed with Tryptic soy broth. The cultures were then incubated at 37°C for 24 h in static condition to allow specific phage enrichment (Fortier and Moineau, 2009). 2

Plaque Formation Assay

The quantification of phage particles in the lysates was determined using the soft agar overlay method (Double layer agar method) (Dubey et al., 2015). The samples were diluted through a series of 10-fold dilutions in a total of 100 µL Tryptic soy broth, from 10−1 until 10−4. Each of the tube samples was mixed with 2 mL of molten soft agar. The mixture was then poured onto petri dishes containing the TS bottom agar and incubated at 37°C for 24 hours. The plaques formation in the plates were observed everyday up to 10 days. The test has been done on all 3 samples. Bacteriophage quantification was carried out by counting the amount of plaque formed in NA media plates as plaque forming units/mL (PFU/mL) (Sjahriani et al., 2021). The statistical analysis has not been done in this study due to lack of samples collected and the result obtained.

Purification of bacteriophage

The phage was concentrated and purified using polyethylene glycol (PEG) 3000 in order to get precipitation (Mclellan et al., 2021). The suspected host bacteria was previously grown in 50 mL of TS broth at 37° C. The isolated phage was mixed with suspected host and agitated overnight at 37°C for 200 revolutions per minute. Phage pellet was transferred to a sterile microcentrifuge tube. The supernatant was filtered through a 0.22 µm millipore filter membrane and placed in a sterile microcentrifuge tube (Sjahriani et al., 2021). Both were stored at low temperature (4°C) as bacteriophage stock. Identification of host Biochemical processes can provide crucial information for correctly identifying the genera of bacteria present in a sample (Moore, 2021). The host bacteria were identified using gram staining method as well as Mac Conkey agar analysis and biochemical test including TSI test, MRVP and Citrate test. Triple Sugar Iron test is mainly used to distinguish between Enterobacteriaceae based on their sugar fermentation patterns. The Vogues-Proskauer test was used to determine the ability of the test organism to produce non-acidic or neutral end products from glucose fermentation (Dubey et al., 2015).

Identification of host

Biochemical processes can provide crucial information for correctly identifying the genera of bacteria present in a sample (Moore, 2021). The host bacteria were identified using gram staining method as well as Mac Conkey agar analysis to confirm the lactose fermenter ability and biochemical test including TSI test, MRVP and Citrate test. Triple Sugar Iron test is mainly used to distinguish between Enterobacteriaceae based on their sugar fermentation patterns. The Vogues-Proskauer test was used to determine the ability of the test organism to produce non-acidic or neutral end products from glucose fermentation (Dubey et al., 2015).

Result and Discussion

All samples were collected in Kuala Pilah, with each site having its own unique characteristic on the surface of the wastewater. Sample A was taken from a reservoir in the Taman Senimas residential area, which had been polluted by both organic and inorganic waste disposal. The wastewater sample is brown in colour and contains some sediments. The wastewater for sample B was collected from a drain near an eatery having brown to greenish colour and soiled with some sediments. Sample C was collected from a drain near the marketplace with greenish colour, and an abundance of algae and some waste disposal floating on top. The wastewater in the drain was stagnant and not flowing.

Table 1: Morphology and size of the isolated plaques

Sampling site	Plaque size/ diameter (mm)	Plaque Morphology
A	0.06	Turbid
B	1.10	Turbid
C	1.00	Turbid

Referring to Table 1, sediments containing both particle-adsorbed virus and virus in pore water were obtained to successfully extract bacteriophage. Based on the results, bacteriophages were successfully isolated from all three plates. Table 2 showing the number of plaques from each sample according to the titration done. The plaque appears to be medium in diameter, circular, and turbid in all plates. After one week of incubation, all the plaques manifested. According to Rees (2014) plaque growth is dependent on several factors; it may be due to the delay in the expression of the phage receptor on the cell surface that would only be expressed by the host cells in the stationary phase thus requiring several days for the plaque to appear. The quantity of plaque formation was regulated by environmental factors (temperature, pH, and aeration) as well as the bacteriophage's accessibility to the target bacteria (Sjahriani et al., 2021). The viral degradation and dilution experiments indicated that viruses may be responsible for a considerable portion of the microbial activity in sediments (Borrel et al., 2012).

Table 2: Determination of Phage Titre

Plate no.	**Volume of Phage plated (mL)**	**Dilution factor (DF)**	**Plaque per Plate**	**PFU/mL= Plaque count/ (volume lysate infected with) (dilution)**	**Titer PFU**
A	0.01	10^{-2}	14	14/ (0.01) ($1x10^{-2}$)	$1.4x10^{5}$
B	0.01	10^{-2}	2	2/ (0.01) ($1x10^{-2}$)	$2.0x10^{4}$
C	0.01	10^{-2}	4	4/ (0.01) ($1x10^{-2}$)	$4.0x10^{4}$

The plaque size is proportional to the adsorption efficiency, the length of the latent period, and the phage burst size. It could be worth noting that samples B and C had a low number of bacteriophages because of the mixture of rainwater and flash floods just a few days before the samples were taken. The simulated rainwater pulse produced a front of low ionic strength water, resulting in significant phage remobilization (Wang et al., 2022). On the contrary, sample A reported the highest number of plaques because the sample was taken from the residential area, which directly contained human disposal. The 3 area of wastewater also remained stagnant, contributing to a higher concentration of phages. Table 1 shows the morphology and size of the isolated plaques. The plaque may be uniformly turbid if the host is partially resistant to the phage. The lysogeny stage is preferred when cells develop slowly, and when the multiplicity of infection (MOI) is high (Smaloy, 2002). Table 3 shows the biochemical characterization of the host bacteria. In general, wastewater contains a large diversity of coliforms due to fecal contamination and a reservoir of enteric pathogens.

Table 3: Biochemical Characterization of the Host Bacteria

Biochemical tests	**Sample A**	***E. coli***	***Klebsiella.*sp**	***Shigella.*sp**
Gram's stain	Gram Negative	Gram Negative	Gram Negative	Gram Negative
Cellular Morphology	Bacilli	Bacilli	Bacilli	Bacilli
Triple Sugar Iron Agar test	Yellow Slant Yellow Butt + Gas + H2S	Yellow Slant Yellow Butt + Gas + H2S	Yellow Slant Yellow Butt + Gas + H2S	Red Slant Yellow Butt + Gas + H2S
Methyl Red test	Negative	Negative	Negative	Positive
Voger Proskauer's test	Negative	Negative	Negative	Positive
Citrate test	Negative	Negative	Negative	Negative

Conclusion

In conclusion, bacteriophage isolates collected from Kuala Pilah area were able to infect coliform bacteria. All the 3 isolated phages A, B and C are temperate phages which produced round, turbid and medium sized plaques. Sample collection from sites A, have higher plaque count compared to B and C possibly due to the environmental factor. The result showed that the samples' host bacteria have a similar biochemical characterization with *Klebsiella* sp. Bacteriophages from environmental wastewater could lyse coliform bacteria and their specificity towards host can prevent any harm to other organisms. Therefore, it could be an alternative biocontrol agent that infects and kills waterborne pathogenic bacteria. For further recommendation, it is recommended that specific primer for Pseudomonas aeruginosa should be used when doing the PCR and strict aseptic technique must be followed when handling the PCR samples. More studies also should be done on the effects of bacteriophage against the virulence gene of the host.

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Author Contribution

The authors confirm their contribution to the paper as follows: study conception and design: Rashidah Iberahim, Nurhamimah Zainal Abidin; data collection: Muhammad Hafizhullah Anuar, Nur Surya Zulkifly; analysis and interpretation of results: Sharifah Aminah Syed Mohamad, Nur Intan Hasbullah, Rashidah Iberahim; draft manuscript preparation: Nur Surya Zulkifly, Muhammad Hafizhullah Anuar, Rashidah Iberahim. All authors reviewed the results and approved the final version of the manuscript.

Conflict of Interest

This research do not have conflict of interest.

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