Exploiting Vpusm 8 Lytic Phage for The Bio-Control of V. Cholerae: A Novel Approach Against Cholera in The Era of Antibiotic Resistance

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Abstract— **Objective: The purpose of this study was to assess the effectiveness of Vibriophage Universiti Sains Malaysia 8 (VPUSM 8), a bacteriophage that destroys bacteria, in managing the proliferation of Vibrio cholerae, specifically the El Tor serotype, as an alternate therapeutic strategy.**

Methods: The study entailed subjecting water samples from Kelantan, Malaysia, to reproduce the natural circumstances that promote the growth of V. cholerae. Subsequently, the samples were contaminated with the V. cholerae O1 El Tor Inaba strain and treated using VPUSM 8. The study employed a controlled experimental design, wherein the samples were divided into three groups, each experiencing different treatment methods. Quantifying the number of colony-forming units (CFUs) and plaque-forming units (PFUs) using serial dilution methods and agar plate cultures was part of the evaluation of VPUSM 8.

Results: The study showed a notable decrease in V. cholerae populations in the groups treated with phages compared to the control group. The group that got an additional dose of VPUSM 8 had a more significant reduction in bacterial count.

Conclusion: This work emphasises the potential of phage therapy, specifically VPUSM 8, as a practical approach to controlling cholera, particularly the El Tor Inaba strain of V. cholerae.

Index Terms— **Antibiotic resistance, Bacteriophages, Cholera, Phage therapy, V. cholera.**

I. Introduction

Cholera, historically significant for sparking epidemics in developing nations since the 19th century, has been implicated in seven pandemics to date. The etiological agent, V. cholerae, a Gram-negative bacterium, precipitates a severe diarrheal disease with potentially life-threatening complications [1, 2]. It poses a considerable public health menace as a waterborne ailment, particularly in areas afflicted by substandard sanitation and limited access to potable water. V. cholerae demonstrates a range of strains, with those capable of producing cholera toxin exhibiting augmented virulence. Transmission predominantly occurs via contaminated water and food sources, especially in environments with appropriate sanitation infrastructure [3, 4]. V. cholerae is classified into over 200 serogroups based on the O antigen of their lipopolysaccharide. However, only two serogroups, O1 and O139, are responsible for epidemic and pandemic cholera. The O1 serogroup is further divided into the classical and El Tor biotypes. Since the seventh pandemic, the El Tor biotypes (Ogawa and Inaba serotypes) have been the dominant strain in most cholera outbreaks. El Tor, which stands out for having a distinct biotype, demonstrates greater environmental tenacity and a better ability to survive in aquatic environments, contributing to its widespread distribution. In contrast to the classical biotype, El Tor is recognised for its tendency to produce more asymptomatic carriers. These carriers operate as reservoirs for the bacteria, making disease surveillance and control methods more challenging. Infections induced by the El Tor strain are characterised by a milder severity but a longer duration of carriage, which contributes to their ongoing dissemination [5, 6, 7, 8, 9, 10].

Bacteriophages, often phages, have gained recognition as a new approach to bacterial management, specifically in addressing V. cholerae. These viruses have a unique affinity for and cause the destruction of bacterial cells, providing a focused method for eliminating bacteria. Their ability to specifically target and effectively combat particular bacterial strains, such as various biotypes like El Tor, renders them a promising instrument in the battle against diseases such as cholera. Bacteriophages adhere to the bacterial cell, introduce their genetic material, and exploit the bacterial apparatus to duplicate, generating more bacteriophages within the cell until it ruptures, liberating additional bacteriophages to invade other bacteria. Bacteriophages possess a distinctive mechanism of action wherein they specifically target and eradicate particular bacteria [11, 12, 13]. This characteristic makes them viable substitutes or additions to conventional antibiotic therapies. This method is crucial in escalating antibiotic resistance and needing more precise microbial control strategies. Hence, this investigation aimed to ascertain the potential of a VPUSM 8 lytic phage as a bio-control agent against V. cholerae that particularly enters an in vitro setting [14].

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II. MATERIAL AND METHODOLOGY

The assay was performed as described by Vinod et al. [15] with minor modifications. Water samples were obtained from Sungai Peng Datu, Kelantan, Malaysia, and subjected to low-speed centrifugation at 1500 ×g for 5 minutes to eliminate large particles. Subsequently, the samples underwent sterilisation through autoclaving at 121 °C for 15 minutes. The sterilised water was then filtered using a Sartolab use-and-throw vacuum filtration unit (Sartolab® RF | BT, Sartorius, Goettingen, Germany) and distributed among nine Sartolab units, each containing 500 ml.

For the bacterial culture, V. cholerae O1 El Tor Inaba was obtained from the Institute for Medical Research (IMR), Malaysia, and grew in 10 ml LB broth at 37 °C overnight in a shaking incubator. The bacterial cells were harvested by centrifugation at 6000 ×g for 15 minutes, washed twice with normal saline, and suspended in 5 ml of normal saline. After adjusting the cell concentration to an OD600 of 2.0, the V. cholerae culture was added to the sterilised and filtered water samples in the nine Sartolab units. It resulted in a final concentration of 106 CFU/ml.

The nine Sartolab units were grouped into three sets (Group A, Group B, and Group C), with each group containing three units. Initially, 100 µl of lytic bacteriophage VPUSM 8 suspensions were given to Groups A and B. These were known to target the V. cholerae O1 El Tor Inaba strain and had been previously isolated at a concentration of about 107 PFU/ml on day 0. Group C served as a control group without any virophage treatment. After 24 hours (day 1), Group B underwent additional treatment with a booster dose of 100 µl (107 PFU/ml) virophages. All nine Sartolab units were statically incubated at 37 ºC until the experiment's conclusion.

Colony-forming units (CFU) were used to measure viable cells in each treatment. After incubation, 1 ml aliquot was taken from each of the nine treatments at every 12-hour interval until 96 hours and serially diluted (100 –1012) using normal saline. 100 µl of each dilution was spread onto triplicates of LB agar plates. The plates were allowed to dry and then incubated at 37 ºC for 24 hours, and V. cholerae colony numbers were counted on dilution plates, yielding counts between 30 and 300 cells.

Quantitative plaque-forming unit (PFU) counts were monitored for each interval until 96 hours. Serial dilution (100 –1012) in SMG buffer was performed, and 100 µl was taken from both groups (A and B). The aforementioned soft agar overlay method in triplicate was used, and the plates were incubated at 37 ºC for 8 hours.

III. RESULTS AND DISCUSSION

This study aimed to assess the efficacy and dose-dependent effects of VPUSM 8 in controlling the growth of V. cholerae O1 El Tor Inaba within a laboratory microcosm. VPUSM 8 was found in a sample of sewage water from a community in Kelantan, Malaysia, during a cholera outbreak in 2010. It has unique properties, such as icosahedral capsids and double-stranded DNA with contractile tail sheaths. The preliminary classification categorises the bacteriophages within the family Myoviridae and order Caudovirales. Despite its relatively narrow host range, VPUSM 8 demonstrates specificity by infecting V. cholerae O1 El Tor Inaba selectively. The results of the impact on V. cholerae growth were determined by measuring the absorbance at 600 nm and bacterial titre (CFU/ml) for all treatments (in the presence of vibriophage) and control (without vibriophage). At the same time, a plaque assay was carried out to enumerate the vibriophage titre at every interval.

In all the groups, the initial concentration of V. cholerae at 0 hours was 106 CFU/ml, while the absorbance measurement at 600 nm was 0.025 nm. 100 µl of VPUSM 8 (at 107 PFU/ml) was added to groups A and B at 0 hours, while an additional 100 µl of VPUSM 8 was added after 24 hours to group B. Figure 1 shows all groups' viable V. cholerae (CFU/ml). In the control group, the V. cholerae concentration increased by two log units (from 106 CFU/ml to 108 CFU/ml) after 48 hours. It was then decreased slightly after 96 hours when the concentration of V. cholerae reached 106 CFU/ml. In contrast, the concentration of V. cholerae was remarkably reduced over time in both groups, A and B. The concentration of V. cholerae in group A decreased by four log units (102 CFU/ml) at 60 hours and remained constant from 1.92 to 1.46 log CFU/ml between hours 60 and 96. Similarly, in group B, V. cholerae concentration drastically decreased by four log units (102 CFU/ml) between 24 and 48 hours. It has then remained at a low concentration (101 CFU/ml) for 96 hours.

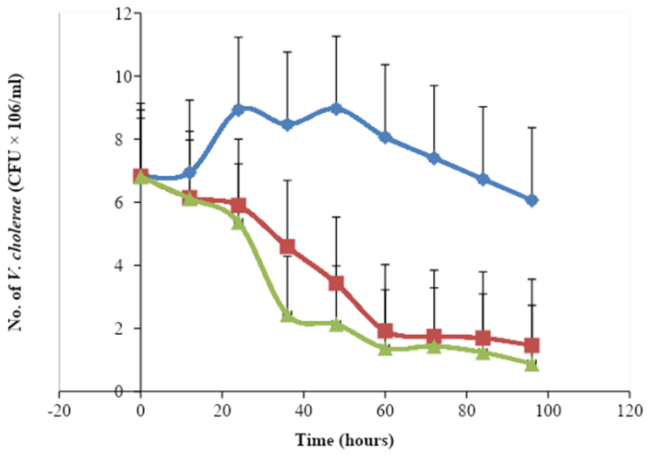

Fig. 1 The effect of vibriophage challenging on *V. cholerae* titre. (♦) Control group without vibriophage treatment, (■) Group A with vibriophage treatment at 0 hours and (▲) Group B with vibriophage treatment at 0 and 24 hours. The experiments were done in triplicates; the data shown are the mean titre.

Figure 2 compares the titre (CFU/ml) of V. cholerae and the titre (PFU/ml) of VPUSM 8 in group A. Group A shows a drop in the growth of V. cholerae titre after adding 100 µl of vibriophage. The VPUSM 8 titre, in contrast, increased over time due to the release of progeny vibriophages from the infected host cells. However, after the 60th hour, the VPUSM 8 titre decreased progressively due to insufficient vibriophage to infect new host cells.

A similar finding to that in group A was also observed in group B (Figure 2). However, interestingly, the second dose of vibriophage into the V. cholerae culture one day after the first

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dose slightly increased the VPUSM 8 titre and decreased the growth of V. cholerae compared to group A. The vibriophage titre increased over time and reached 2.7 log PFU/ml, compared with 2.4 log PFU/ml after 60 hours in groups A and B, respectively. Nevertheless, at 72 hours, in group B, the VPUSM 8 titre had reduced to 1.54 log PFU/ml, while in group A it had reduced to 1.12 log PFU/ml (Figure 3).

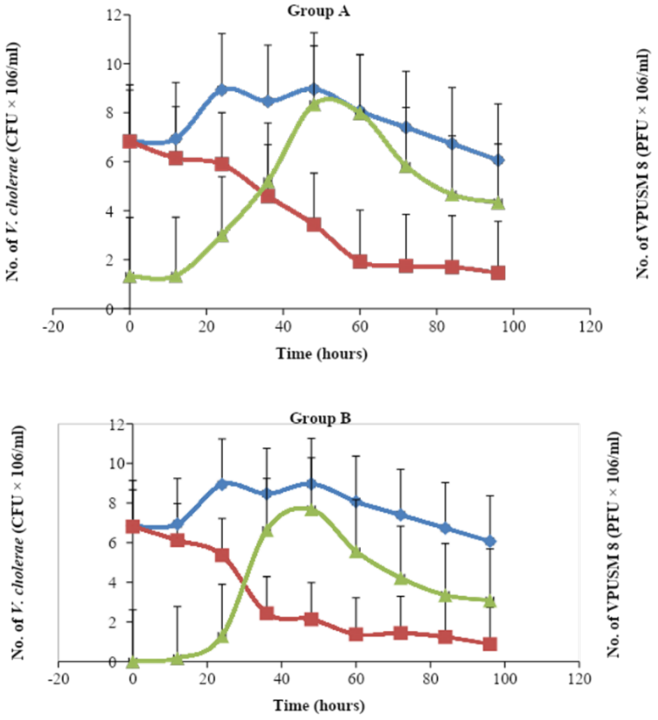

Fig. 2 The efficiency and the dose effect of VPUSM 8 on the population of viable *V. cholerae*. (♦) Control group without vibriophage treatment, (■) CFU/ml of *V. cholerae* and (▲) PFU/ml of VPUSM 8. The experiments were done in triplicates and the data shown are the mean titre.

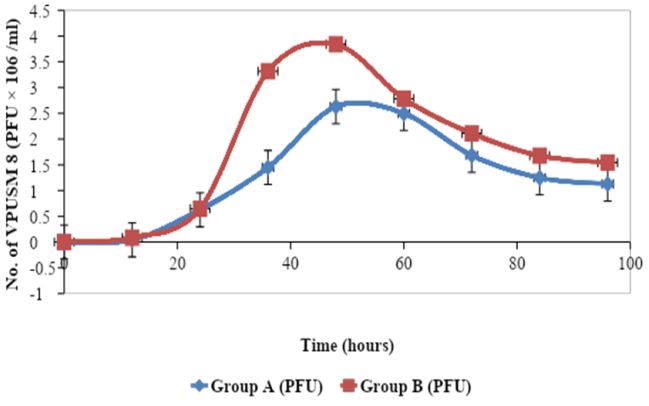

Fig. 3 Comparison of VPUSM 8 titre (PFU/ml). (♦) Group A treated with one dosage of 100 µl VPUSM 8. (■) Group B with two dosages of 100 µl VPUSM 8 at a 24-hour interval. The experiments were done in triplicates, and the data shown are the mean titre

Given the alarming increase in multidrug-resistant bacteria, there is a renewed emphasis on reevaluating the potential of phage therapy to address bacterial infections in both human and environmental settings [16, 17]. This trial explored the potential use of the isolated VPUSM 8 vibriophage to control the growth of V. cholerae O1 El Tor Inaba in a laboratory microcosm. In the laboratory microcosm, a reduction in V. cholerae count could be achieved through treatment with a VPUSM 8 vibriophage (see Figure 2). This study revealed that simultaneous exposure of V. cholerae VPUSM 8 (group A) to the treatment caused a four-log reduction in V. cholerae count. Interestingly, the concurrent addition of VPUSM 8 and V. cholerae, followed by a second exposure to the vibriophage 24 hours later, led to a remarkable five-log reduction in the V. cholerae count, demonstrating that the vibriophage VPUSM 8 acts against V. cholerae and inhibits its growth through cell lysis.

However, the results showed that the subsequent addition of vibriophage at 24 hours (second dose) could only prevent exponential growth but did not kill V. Cholerae. This observation was probably due to the reproduction of phage-resistant cells, which might appear during the treatment of bacteria with phages [18, 19].

It was confirmed that phage-resistant cells were present during vibriophage titration in a bio-control experiment by a spotting and overlay assay. In several lawns, small, non-spreading phage-resistant colonies were seen in clearing zones. Interestingly, diauxic growth was also observed in the control group, which decreased after 40 hours. This indicates that the cells died due to a lack of nutrients and the accumulation of toxic by-products [20, 21].

On the other hand, the comparison of bacteria titre (CFU/ml) and vibriophage titre (PFU/ml) in both groups (single dose and double dose of VPUSM 8) was evaluated. It was demonstrated that after VPUSM 8 was added, the growth of V. cholerae slowed and decreased. In contrast, the titre of VPUSM 8 continued to increase due to the release of progeny vibriophage from the infected host cells. However, after the 60th hour, the vibriophage titre started to decrease. The reduction of vibriophage titre was probably due to the lack of enough host cells to infect VPUSM 8; (i) the bacteria cell count was reduced due to infection of the vibriophage; and (ii) the host cells continued to grow before they reached the stationary phase and eventually a death phase. At this point, the vibriophage titre dropped dramatically [22, 23].

The study of VPUSM 8 demonstrates the potential of phage therapy as a therapeutic agent to combat public health risks. The observed decline in bacterial populations suggests that with further advancements, phage therapy could be an effective approach for controlling bacterial diseases in medical and environmental contexts, potentially reducing the impact of cholera outbreaks globally. However, challenges arise when transitioning from in-vitro models to in-vivo applications and environmental management. The complex nature of the human body and environmental systems can impact the effectiveness of phage therapy, necessitating thorough in-vivo research and field experiments. Additionally, the occurrence of resistant bacterial strains during the study highlights a significant constraint of phage therapy, potentially impairing its long-term efficacy. To address these constraints, future research should focus on creating phage mixtures engineered to hinder resistance strain generation, conducting longitudinal investigations and field trials, creating a favourable regulatory framework, and addressing public attitudes towards phage

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therapy. This comprehensive analysis places the study within the broader framework of antibiotic resistance and phage treatment research, offering a well-defined course of action to further the field [24, 25, 26].

CONCLUSION

In our conclusion, the study contributes substantially to our understanding of using bacteriophages to manage bacterial diseases, particularly cholera, in the context of increasing antibiotic resistance. The results are promising and provide a new strategy to address this issue.

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