HS-04

Activity of Novel Bacteriophages from Hospital Wastewater on Antibiotic-Resistant Acinetobacter *Baumannii* Biofilm

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Abstract

Antimicrobial resistance in nosocomial infections caused by *Acinetobacter baumannii* is a significant complication for hospitalized patients. Biofilm formation of *A. baumannii* contributes to its survival in adverse environments such as medical devices and hospital settings. Bacteriophages have emerged as a potential alternative treatment for antimicrobial-resistant *A. baumannii* infections. In this study, we evaluated the activity of novel bacteriophages isolated and purified from hospital wastewater on the biofilm of *A. baumannii*. Five phages, namely PAB1928, PAB436, PAB391, PAB026, and PAB018, were isolated and purified using an enriched method and a Double-Layer Plaque Assay, respectively. The host range assay showed that all phage isolates could invade bacterial host cells, except PAB018. PAB1928 was selected for further evaluation in a Multiplicity of Infection (MOI) assay, which demonstrated its ability to efficiently eliminate bacterial host cells within 1 hour at various MOIs. However, bacteria regrowth was observed after 8 hours of phage treatment, indicating the development of phage-resistant bacteria. For biofilm prevention and elimination experiments, PAB1928 was shown to efficiently eliminate *A. baumannii* biofilm in a 96 well plate, as measured by the crystal violet assay. The findings of this study suggest that phage therapy could be a potential alternative for antibiotic-resistant *A. baumannii* infections and provide basic knowledge to optimize and develop phage-based therapy. This study may benefit patients with antibiotic-resistant bacterial infections in the future.

Keywords: Bacteriophage, Biofilm, Phage, Antibiotic-resistant

Introduction

Nosocomial infections were a significant complication in hospitalized patients worldwide. In the United States alone, approximately 1.7 million people were infected each year. Among multidrug-resistant microorganisms, *A. baumannii* is a major cause of healthcare-associated infections. According to the CDC in

2017, the United States had approximately 85,000 cases of drug-resistant *A. baumannii*, resulting in over 700 deaths and an estimated cost of $281 million for treatment. Antibiotic treatments were expected to lose their effectiveness in the future due to the high potential of bacteria for developing antibiotic resistance. It was projected that the death toll from these infections will rise from 700,000 to 1,000,000 by 2050 (Di Lallo et al., 2021).

A. baumannii is a Gram-negative, non-motile, strictly aerobic, non-fermentative, non-sporing coccobacillus that was notorious for its ability to survive in various environmental conditions. Its ability to form biofilms contributes to its survival in adverse environmental conditions, including hospital environments and medical devices (Amankwah et al., 2021). The most important virulence factors identified by genomic and phenotypic investigations were outer membrane porins, phospholipases, capsular polysaccharides, lipopolysaccharides (LPS), proteases, iron-chelating systems, and protein secretion systems.

Biofilms are communities of microorganisms that adhere to biotic and/or abiotic surfaces (Francesca Longo, 2014)] encased by an extracellular polymeric substance (EPS) matrix, physiologically different from planktonic (free-floating) bacteria. Biofilm-encased cells have limited metabolic activity and are shielded by the extracellular matrix, making them more resistant to antibiotics and innate immune components of the host (Yang et al., 2019).

A. baumannii frequently causes biofilm-related infections, especially ventilator-associated pneumonia and catheter-related infections. These infections can be unresponsive to antibiotic therapy, creating a significant challenge to the clinical management of *A. baumannii*-related biofilm infections. The rapid spread of medical device-associated infections and its antibiotic resistance have made *A. baumannii* biofilms one of the most critical global. Therefore, understanding the extent of *A. baumannii* biofilm formation, its role in pathogenesis, and antimicrobial resistance are essential to limit medical device-associated infections (Lee et al., 2017).

Bacteriophages are viruses that have been studied for the treatment of bacterial infections for approximately 100 years (Liu et al., 2021). They invade and killed targeted bacteria by lysis and do not attack mammalian cells. Phages are specific to bacterial strains. They bind to receptors on bacterial cell walls to inject deoxyribonucleic acid into the cell and ultimately lyse the cell in the lytic phase (Jamal et al., 2019). During the lysogenic cycle, phages integrate into their host genome or exist in the cell as plasmids, evolving to coexist with bacteria. Bacteriophages were abundant in the environment, such as marine, soil, human gut, and wastewater. Wastewater is an extremely diverse environment for microorganisms, allowing for a variety of interactions between bacteria. Therefore, hospital and sewage waste are potential sources of bacteriophage. In this study, bacteriophages are isolated and purified from wastewater sludge from King Chulalongkorn Memorial Hospital.

Purposes

1) To evaluate the activity of novel bacteriophages from hospital wastewater on the biofilm of *A. baumannii*

Research Methodology

1. **Bacterial host isolates.**

50 isolates of *A. baumannii* were collected from patients in the microbiology laboratory of King Chulalongkorn Memorial Hospital. All cultures were grown at 37°C with aeration (200 revolutions per minute) in Luria–Bertani (LB) broth overnight for 18-24 hours. The stocks were prepared in LB with a 20% (vol/vol) glycerol supplement and stored at -80°C.

2. **Bacteriophage selection**

2.1. Phage isolation

Bacteriophage candidates were isolated from wastewater sludge obtained from King Chulalongkorn Memorial Hospital. The wastewater samples were incubated with host strain culture in Luria–Bertani (LB) broth at 37°C overnight. After incubation, the samples were centrifuged at 5000 g for 30 minutes, filtered through a 0.45 µm syringe filter, and then centrifuged for 2 hours at 40,000 g at 4°C (using a Beckman Coulter model Optima XL-100K, Type 45 Ti Rotor). The resulting supernatant was discarded, and the pellet was resuspended with SM buffer followed by filtration through a 0.2 µm syringe filter. For subsequent experiments, phage stocks were prepared in SM buffer and maintained at 4°C (Bonilla et al., 2016). Bacteriophages were isolated using a modified method of the Phage On Tap (PoT) protocol for the quick and efficient preparation of homogeneous bacteriophage (phage) stocks. Briefly, the following steps were performed: (i) Bacterial hosts of *A. baumannii* were produced in LB broth overnight at 37°C and 200 rpm; (ii) Phage stock was diluted in LB broth; (iii) LB top agar was completely molten by heating it in a water bath at approximately 70-90°C, and then cooled in a water bath at 56°C or until it was warm to the touch; (iv) In a microcentrifuge tube, the overnight bacterial host and the phage dilution were combined in a 1:1 ratio and incubated for 10 minutes at 37°C; (v) The liquid mixture was poured quickly onto an LB agar plate, tilting the plate to spread the agar uniformly, and allowed to sit until the agar has gelled; (vi) After 5 minutes, the plate was inverted and incubated at 37°C overnight.

2.2. Phage purification

To isolate a single bacteriophage, these steps were followed: i) Using a sterile Pasteur pipette, pick up a single plaque from the plate and resuspend it in a microcentrifuge tube containing 1 mL of filter-sterilized phage diluent (SM buffer). Vortex the tube for 5 minutes. ii) Centrifuge the tube at 4,000g for 5 minutes to remove any remaining debris. iii) Filter the supernatant through a 0.2 µm filter into a 1.5 mL microcentrifuge

tube. iv) In another microcentrifuge tube, combine the overnight bacterial host and the phage dilution in a 1:1 ratio and incubate for 10 minutes at 37°C. v) Pour the liquid mixture quickly onto an LB agar plate, tilting the plate to spread the agar uniformly. Allow it to sit until the agar has gelled. vi) Invert the plate after 5 minutes and incubate it at 37°C overnight. Repeat this step for three rounds.

2.3. Phage host range assay

50 isolates of *A. baumannii* overnight culture were mixed with the top agar and then poured onto the bottom agar plate for each isolate. Next, the top agar mixture was allowed to gel before spotting 10 µl of phage isolates onto it. The plate was then inverted and incubated at 37°C overnight.

2.4. Multiplicity of Infection (MOI) assay

In the MOI assay, the host bacterium *A. baumannii* AB1928 was cultured overnight in LB medium at 37 °C until it reached an OD_{600} of approximately 0.3-0.6. The overnight cultures were then adjusted to 0.5 McFarland. Next, 230 µl of 0.5 McFarland overnight culture was added to each well of an ELISA 96-well plate and treated with phage PAB1928 at different multiplicities of infection (MOIs) of 100, 10, 1, 0.1, and 0.01. The well plate was incubated at 37 °C with 200 rpm for 24 hours. Bacterial turbidity was measured at 30-minute intervals for 24 hours by spectrophotometry at OD_{600}, and these tests were performed in triplicate.

3. Biofilm elimination

The bacterial cultures were incubated for 18-24 hours at 37°C in LB broth, shaking at 200 rpm. Subsequently, the *A. baumannii* AB1928 culture turbidity was adjusted to 0.5 McFarland and added to 96-well plates at a volume of approximately 300 µl per well, followed by the addition of 1% glucose. The plates were then incubated for 24 hours at 37°C with shaking at 200 rpm. After removing the planktonic cells, the attached cells were gently washed three times with phosphate-buffered saline (PBS) solution and 270 µl of LB broth was added to each well. Next, 30 µl of phage was added to each well, followed by incubation for 24 hours at 37°C with shaking at 200 rpm. The wells were then washed three times with PBS solution, air-dried, and fixed with 300 µl of absolute methanol for 15 minutes. After air-drying again, the wells were stained with 300 µl of 0.1% crystal violet (CV) for 5 minutes. Following three more washes with PBS, the surface-associated dye was dissolved in 300 µl of 33% acetic acid for 20 minutes. The OD_{600} of the CV eluate was measured using a microplate reader (Thermo Scientific, Multiskan GO) and calculated.

Results

4.1. Bacteriophage isolation and host spectrum.

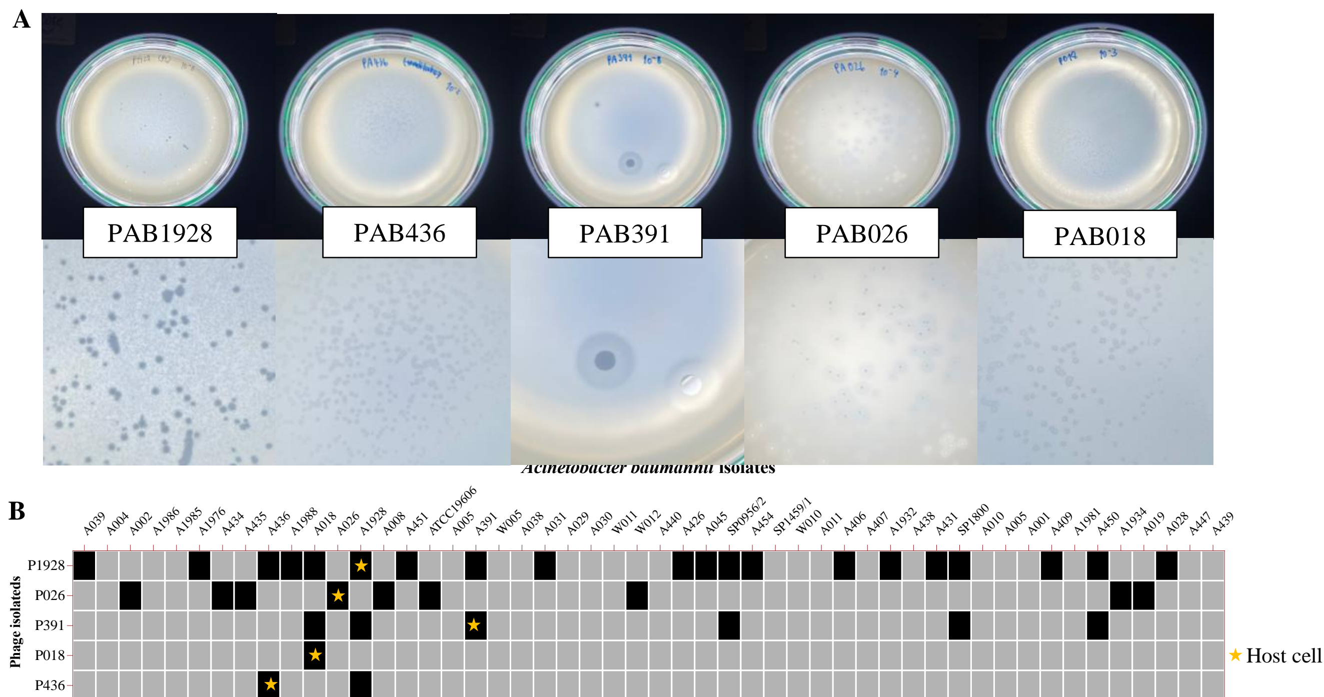

Figure 1. Demonstrates the plaque morphology of PAB198, PAB391, PAB026, PAB018, and PAB436, respectively, in (A) and the host range of phages with 50 isolates of antibiotic-resistant *A. baumannii* in (B).

Figure 1A presents the morphology of the phages on a double-layer agar, where the phage PAB1928 formed small transparent round plaques of approximately 0.4 mm in diameter on the lawn of the host *A. baumannii* AB1928. Similarly, phage PAB436 formed turbid, small, round plaques of approximately 0.2-0.4 mm in diameter on the lawn of *A. baumannii* AB436 host. On the other hand, phage PAB391 formed large, round, transparent plaques of approximately 1 cm in diameter with an observable halo on the lawn of *A. baumannii* AB391 host. Phage PA026 formed small, round, transparent plaques of approximately 0.2 mm in diameter with an observable halo outside on the lawn of *A. baumannii* AB026 host. Finally, phage PAB018 formed turbid, small, round plaques of approximately 0.2-0.4 mm in diameter on the lawn of *A. baumannii* AB018 host. Figure 1B illustrates the host range of 5 phages based on the bacterial genera, species, and strains they can lyse. Phage PAB1928 can lyse various *A. baumannii* isolates, including A039, A1976, A436, A1988, A018, A1928, A451, A391, A031, A426, A045, SP0956/2, A454, A406, A1932, A431, SP1800, A409, A450, and A028. Similarly, phage PAB026 can lyse several *A. baumannii* isolates, such as A002, A434, A435, A026, A008,

ATCC19696, W012, A1934, and A019. Phage PAB391 can lyse *A. baumannii* isolates including A018, A1928, A391, SP0956/2, SP1800, and A450. Phage PAB018 can only lyse *A. baumannii* host cell isolates, while phage PAB436 can lyse *A. baumannii* isolates A436 and A008. The yellow stars indicate the bacterial host cells for each phage. PAB1928 was selected for the multiplicity of infections (MOIs) assay due to its demonstrated potential to efficiently lyse multiple bacterial strains. This characteristic makes it an ideal candidate for assessing the effectiveness of treatments targeting various bacterial infections in vitro.

4.2. Bactericidal Kinetics

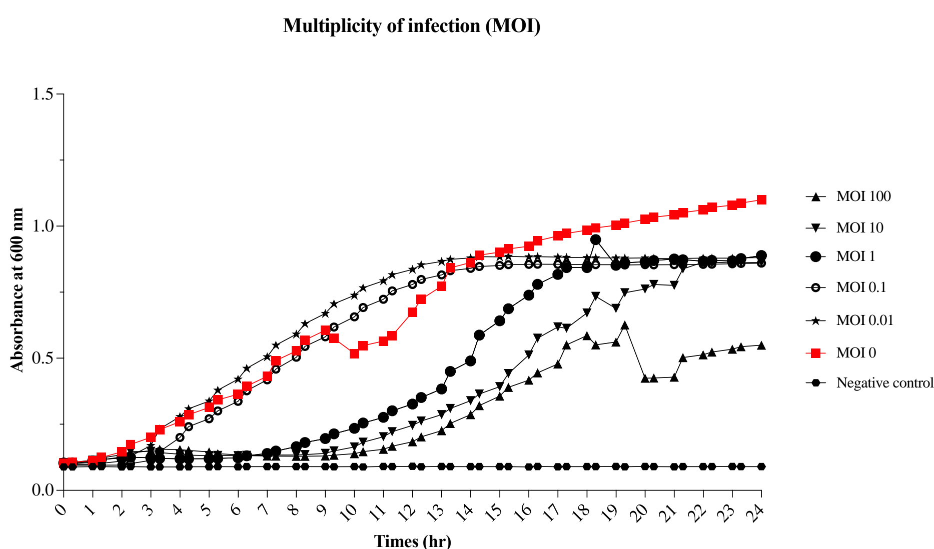

Figure 2. illustrates the impact of phage PAB1928 on controlling the growth of pathogenic *A. baumannii* isolate A1928. The experiment involved co-cultivating the isolate with phage PAB1928 at a different multiplicity of infections (MOIs), while a separate isolate that was not treated with phage was used as a control.

Phage therapy efficiency can be hindered by the emergence of phage-resistant bacteria due to the co-evolution of phage PAB1928 and host cell bacterium. In this study, we analyzed the generation of phage-resistant bacteria by co-incubating the phage with the bacterial strain. Our findings showed that the phage PAB1928 significantly inhibited bacterial growth during the initial 7-hour period.

4.3. Biofilm formation

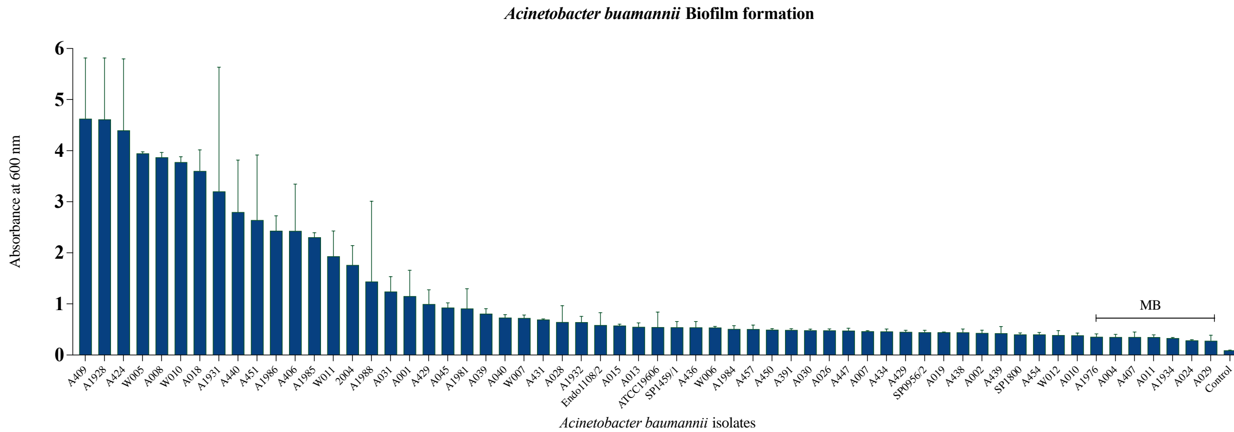

Figure 3. Demonstrates the biofilm-forming capacity of 60 *A. baumannii* isolates at King Chulalongkorn Memorial Hospital.

In this study, we evaluated the biofilm-forming capacity of *A. baumannii* isolates for the subsequent experiment. As shown in Figure 3, biofilm mass formation was measured using the CV assay (absorbance at 600 nm) after 24 hours of formation. The biofilm formation capacity was classified as N/A for non-adherent ($OD < OD_{control}$), WB for weak biofilm producer ($OD_{control} < OD <= 2xOD_{control}$), MB for moderate biofilm producer ($2xOD_{control} < OD <= 4xOD_{control}$), and SB for strong biofilm producer ($4xOD_{control} < OD$). Our findings indicated that all *A. baumannii* isolates exhibited a strong biofilm-forming capacity, except A1976, A004, A407, A1934, A024, and A029.

4.4. Anti-biofilm activity

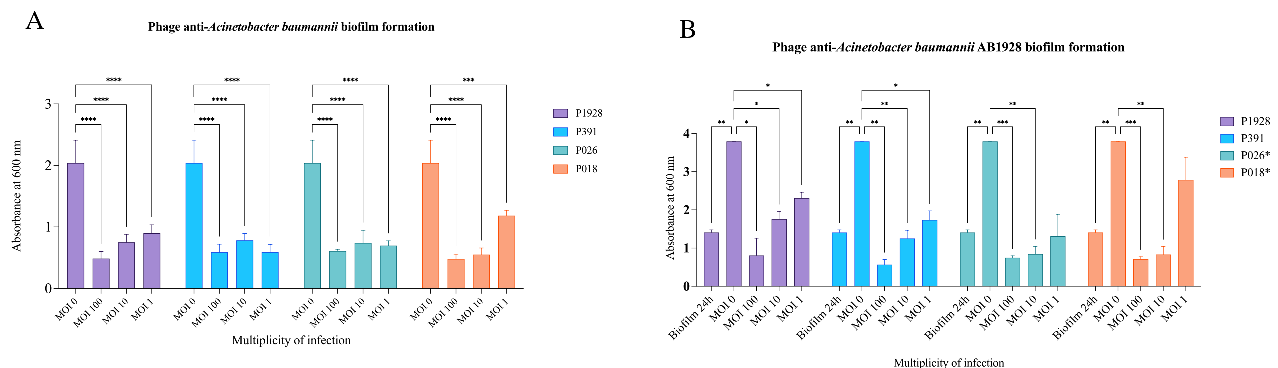

Figure 4. Illustrates the biofilm inhibition effect of phages on *A. baumannii* isolate A1928 by co-incubating and treating it with phages at different MOIs after 24 hours of biofilm formation. Panel A shows

the results of adhered cell biomass co-culture quantified by CV assay (absorbance at 600 nm) at 0, 1, 10, and 100 MOIs. Panel B shows the results of adhered cell biomass after 48 hours of treatment with phages at 0, 1, 10, and 100 MOIs. The data represent the means ± SD of three independent experiments, and bars with an asterisk indicate statistically significant difference from the untreated control. The statistical significance was determined using the two-way ANOVA followed by Bonferroni's multiple comparison tests for different variances. $P < 0.1$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) were considered statistically significant (n=3 per group).

In this study, we investigated the efficacy of bacteriophages in eradicating biofilms formed by *A. baumannii* isolate 1928. Figure A depicts the biofilm formation and co-culture of the isolate with bacteriophages (P1928, P391, P026, and P018) at various MOIs (MOI 0, MOI 1, MOI 10, MOI 100). Our results indicate that all bacteriophages were capable of reducing biofilm formation when co-cultured at each MOI. P1928 showed statistically significant reductions in biofilm formation at MOI 1, 10, and 100 ($P < 0.0001$). P391, P026, and P018 showed statistically significant reductions in biofilm formation at all MOIs tested. We also observed that although some phages were unable to lyse the host cells of the *A. baumannii* isolate 1928, they were still able to decrease biofilm formation. To assess the potential of these phages for the eradication of biofilms, we performed a biofilm eradication test. Figure B shows the biofilm formation after infecting with bacteriophages P1928, P391, P026, and P018 at various MOIs (0, 1, 10, 100). We found that all phages were able to significantly reduce biofilm formation compared to the biofilm-negative control at MOI 0, except for P026 and P018 at MOI 1 where there were no statistically significant differences. These findings demonstrate the potential of bacteriophages for eradicating biofilms formed by *A. baumannii* isolate 1928.

Discussion

Over the past ten years, there has been a concerning worldwide increase in the occurrence and prevalence of Gram-negative bacteria that are resistant to carbapenem antibiotics. Among these, *A. baumannii* is particularly threatening, as it leads to severe infections that are associated with a high level of illness and death. In addition, *A. baumannii* tends to quickly develop resistance to various types of antibiotics, making it a challenging infection to treat. In 2017, the World Health Organization designated carbapenem-resistant *A. baumannii* a crucial target, emphasizing the need for research and development of new antibiotics. The lack of effective antibiotics or antibiotics in progress has prompted the US Centers for Disease Control to increase the level of threat from "serious" to "urgent" in 2019 (Centers for Disease Control and Prevention, 2019). This situation clearly demonstrates the necessity for alternative solutions. In recent years, bacteriophages have emerged as a potential solution to control bacterial infections as they are natural enemies of bacteria. They

offer promising possibilities for the treatment of infectious diseases, even serving as a last resort for treating diseases caused by multidrug-resistant pathogens. This is supported by an increasing amount of research and publications on both natural and engineered phages(Lood et al., 2015; Menon et al., 2021; Song et al., 2021). However, there is still a great need to gain a deeper understanding of phage biology and to conduct further research on their potential applications.

1. Bacteriophage selection

The study identified five phages, namely PAB1928, PAB436, PAB391, PAB026, and PAB018, which were obtained from a clinical host strain of *A. baumannii* that was both biofilm-producing and multidrug-resistant. These five phages had different morphologies. Figure 1A showed that PAB436 and PAB018 formed small plaques and turbid zones, suggesting low efficacy against all bacterial isolates in their host ranges. In our study, it was observed that PAB018 exhibited a narrow host range, as it selectively eradicated only host cells. These findings are consistent with previous research that indicated the phage had only targeted host cells, which could opportunity to become a lysogenic phage (Daubie et al., 2022). However, in a previous study conducted by Romain Gallet et al., the impact of various phage characteristics on different plaque properties was investigated. The findings of their study indicated that the adsorption rate had a negative impact on plaque size, productivity, and phage concentration in plaques. Conversely, the maximum plaque size was observed when the lysis time was moderate, although differences in lysis time did not significantly affect plaque productivity. Additionally, the phage with a larger expected virion size displayed a smaller plaque size. In the case of both PAB391 and PAB026, phages capable of producing a halo spread beyond the lysis zone were observed. This particular feature is a hallmark of depolymerase-containing phages (Wintachai et al., 2022), and there are two hypotheses to explain it (Knecht et al., 2019). According to our study, two hypotheses have been proposed to explain the halo spread observed outside the lysis zone of both PAB391 and PAB026 phages. The first hypothesis suggests that it is due to the diffusion of offspring virions, while the second hypothesis states that soluble enzymes are responsible for this phenomenon. A previous study reported that the phage plaque was analyzed for three days as the halo continued to expand. Free phages were only detected in the first halo zone, which was close to the spot, while no free phages were found in the second and third halo zones.(Vukotic et al., 2020).

2. Host spectrum

The host spectrum or host range of a bacteriophage is determined by the bacterial species, genera, and strains that it is capable of killing. Our results demonstrate the broad spectrum of phage P1928. It was selected for further assay in Figure 1B due to its broad-spectrum activity. As reported by Alexa Ros et al., broad host range phages are considered more useful in certain bacteriophage applications. In the case of phage therapy, a broad host range phage that can eliminate multiple bacterial species is equivalent to broad-spectrum

antibiotics. Thus, a smaller number of broad host range phages may be more beneficial than a larger number of narrow host range phages (Ross et al., 2016).

3. Bactericidal Kinetics

MOI studies (Figure 2) have shown that the optimal MOI needed for phage amplification varies among *Acinetobacter* phages. Our result demonstrates the absorbance increase after 8 hours of phage treatment. Previous studies also found that the results indicated a rise in bacterial growth between 6 and 24 hours after phage treatment (Wintachai et al., 2022). However, the turbidity of the cultures increased rapidly thereafter, possibly due to the emergence of cell debris or the growth of phage-resistant strains. Phage-resistant bacteria can emerge due to various factors such as spontaneous mutations, adsorption resistance, blocking of penetration systems, receptor blocking, and the activation of adaptive immunity associated with CRISPR/Cas systems. Our results indicated that at MOI 0.01 and MOI 0.1, the phage was not effective in eliminating the bacteria. The efficiency of viral infection is generally dependent on the MOI and the susceptibility of the host cell towards the infection. When the MOI is too low, there might not be enough viral particles present to initiate a productive infection in the host cell.

4. Biofilm formation

The ability of the isolates to form biofilms was assessed using crystal violet staining. The ability to form biofilms and resistance to antibiotics can vary across different sites, and the factors responsible for this resistance may also differ. Current evidence suggests that the high resistance of biofilms to antibacterial agents cannot be fully explained by conventional mechanisms. Multiple mechanisms have been investigated as key factors in the high resistance of biofilms, including limited diffusion, enzyme-mediated neutralization, heterogeneous function, slow growth rate, persistent non-dividing cells, and adaptive mechanisms of the biofilm phenotype (Yang et al., 2019). All *A. baumannii* isolates included in our experiment, which were obtained from the medical microbiology lab, were found to have the virulence genes *ompA* that are associated with the biofilm formation of multidrug-resistant *A. baumannii* clinical isolates. (Dolma et al., 2022; Yang et al., 2019). In our local database, 91.6% of the *A. baumannii* strains were found to harbor the *ompA* gene. It was observed that some of the strains that did not form biofilm also had the *ompA* gene. However, there is no sufficient evidence to confirm that *OmpA* is responsible for inducing biofilm formation (Wintachai et al., 2022).

5. Anti-biofilm activity

We evaluated the impact of phage on biofilm formation, including the prevention of biofilm formation through co-culturing bacteria with phage, and the elimination of pre-existing biofilm by treating it with various MOIs of phage. The biofilm was formed within 24 hours before phage treatment. The study showed that Phage PAB1928 had depolymerase activity that could eliminate bacterial capsule polysaccharides and

exopolysaccharides were similar to other studies (Shahed-Al-Mahmud et al., 2021; Wintachai et al., 2022). During our study, we observed a significant reduction in biofilm formation when treated with phage, particularly at a high MOI, as previously reported in similar studies. Although PAB026 and PAB018 did not demonstrate bactericidal activity against the *A. baumannii* isolate 1928, they were observed to be effective against biofilm formation. This indicates that the phages could act against biofilms, even if they are not capable of killing bacterial host cells. However, further experimentation is necessary to confirm these results.

Conclusions

The objective of this study was to evaluate the efficacy of bacteriophage treatment in vitro for eradicating persisting cells and biofilms. Our findings demonstrated that the bacteriophages we examined successfully prevented *A. baumannii* biofilm formation and eradicated biofilm formation compared to the untreated control group. Bacteriophages have been found to be a promising alternative therapy for bacterial infections, particularly against multidrug-resistant bacteria. This study provides valuable insights into optimizing and developing phage-based therapies. Additionally, the results of this study have the potential to benefit patients with antibiotic-resistant bacterial infections in the future.

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