Research Article

Broad Immunity of Acinetobacter baumannii lysogens: Implications for Phage Therapy Strategies

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Phage therapy is increasingly considered an alternative treatment for multi-drug-resistant bacteria, including *Acinetobacter baumannii* (AB), a major nosocomial pathogen. A challenge in phage therapy is the induction of bacterial lysogens, which can become immune to the phage that infects them. In this study, an AB isolate, designated ABU-3, was found to be susceptible to three different AB-phages. Typically, lysogens exhibit immunity to the phage that infects them. However, two of the ABU-3 lysogens also developed resistance to additional AB-phages. This finding highlights a potential complication in phage therapy and underscores the need for strategies to prevent such resistance to ensure its success. The varying immunity of ABU-3 lysogens to AB-phages presents an intriguing topic for further research, particularly in developing strategies to prevent lysogen induction and enhance phage therapy approaches.

Introduction

Acinetobacter baumannii (AB) is a bacterium commonly found in hospitals, where it acts as a nosocomial pathogen, causing infections in various systems of the body, particularly the respiratory tract, bloodstream, urinary tract, and skin[1]. AB contamination in hospitals often leads to opportunistic infections in long-term patients. Multi-drug-resistant AB (MDR-AB) strains are a major cause of mortality among patients with weakened immune systems[2].

AB is an aerobic, non-sugar-fermenting bacterium. It does not form filaments but can move in a twitching manner using pili[3]. The World Health Organization (WHO) recognizes AB as a highly

important antibiotic-resistant bacterium, along with five others: *Enterobacter spp.*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Due to the limited scope for developing new antibiotics, alternative treatments for MDR bacteria, including AB, have been proposed, with phage therapy emerging as a promising option.

Bacteriophages, or phages, are viruses that naturally infect and kill specific susceptible bacteria. Phages can follow two pathways to infect a bacterial host: the lytic and lysogenic pathways. In the lytic pathway, phage infection leads to bacterial lysis and the release of new phage progeny, which can then infect other bacterial cells. In contrast, the lysogenic pathway involves the integration of the phage genome into the bacterial chromosome, forming a lysogen. The lysogen does not produce phage progeny unless induced by agents such as mitomycin-C[4], nalidixic acid, and ciprofloxacin[5]. Ultraviolet light has also been shown to induce lysogens to enter the lytic cycle through the SOS regulatory system[6]. The presence of lysogens limits the success of phage therapy, as lysogenic bacteria are resistant to the phage that induced them, making the same phage ineffective for subsequent treatments.

There are two main categories of phages: virulent and temperate. Virulent phages are ideal for phage therapy because they exclusively follow the lytic pathway and do not form lysogens[7]. However, virulent phages are rarely found in natural resources, and most reported phages are temperate. Using temperate phages for therapy requires careful consideration, as lysogens usually become resistant to the same phage, complicating treatment.

This study explored the use of more than one type of AB-phage to infect a specific isolated AB strain. Ultimately, three types of AB-phages were isolated to treat a particular AB strain, referred to as ABU-3. The lysogens of ABU-3, induced by all three AB-phages, were isolated to study the possibility of resistance to each AB-phage infection. This research aims to understand the potential for phage resistance in AB lysogens induced by each phage. Also, the proper way of phage therapy to prevent induction of the bacterial lysogen is proposed. Hopefully, the information will lead to establishing a way to access phage therapy in optimal practice.

Materials and Methods

Acinetobacter baumannii (AB)

Various AB isolates were collected from the microbiology laboratory of Srinakarin Hospital, Khon Kaen, Thailand. These isolates were obtained from the tracheal suction fluid of bronchitis patients and identified through biochemical testing. The isolates were stored in 50% glycerol at -80°C until further use. Prior to experimentation, the isolates were sub-cultured, and their biochemical profiles and antibiotic susceptibility to doxycycline, amikacin, imipenem, and levofloxacin were reconfirmed.

Isolation and Preparation of AB Phage

Phage samples were collected from wastewater sources in the vicinity of Srinakarin Hospital, Khon Kaen University, Khon Kaen Province. The wastewater samples, approximately 40-45 ml in volume, were filtered through a 0.22 µm membrane filter. For enrichment, 9 ml of the filtered sample was mixed with 1 ml of a mixture of 4-5 AB isolates at a concentration of 10^6-10^7 CFU/ml. The mixture was supplemented with an equal volume of 2X brain heart infusion (BHI) broth and incubated at 37°C with shaking for 4 hours.

After incubation, the mixture was centrifuged at 5,000 g for 15 minutes, and 2 ml of the supernatant was incubated with a single isolate at 37°C with shaking for 2-4 hours. The presence of phage was indicated by a clearer appearance of the test tube compared to the control. The sample was then centrifuged at 10,000 g for 15 minutes, and the supernatant was filtered through a 0.22 µm membrane and stored at 4°C for further study.

AB-phages were amplified by infecting AB cultures at different multiplicities of infection (MOI), ranging from 0.1 to 10. Phage titres were determined using 10-fold dilution with SM buffer (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$) and quantified by spotting and double-layer agar methods[8].

Screening for AB-Phage Susceptibility

Forty AB isolates were screened for susceptibility to the isolated phages using the spot test method. AB lawns were prepared on MacConkey or nutrient agar plates, mixing bacterial cultures with 0.5% nutrient agar. Ten microlitres of phage suspensions were spotted onto the lawns, and plates were incubated at 37°C for 8-12 hours. Clear plaques indicated phage susceptibility. Only AB isolates

susceptible to at least two different phages were selected for further analysis.

If a particular AB strain showed different plaque appearances, individual plaques were isolated, purified using a pipette tip, amplified with the specific AB strain, and incubated overnight at 37°C. The supernatant from 10,000 g centrifugation was then processed again with the spot test and double-layer agar methods.

Physical Characterization of AB-Phages

The stability of AB-phages was evaluated under varying pH, temperature, and freeze-thaw conditions. Phages at a concentration of 10^7 PFU/ml were diluted 100-fold in SM buffer prepared at different pH values (ranging from pH 2 to 13). The mixtures were incubated at room temperature for 4 hours. For thermal stability, each of the AB-phages was kept at temperatures ranging from 45°C to 75°C by a Bio-Rad C1000Dx Thermal Cycler for 1 hour. Freeze-thaw stability was assessed by freezing phage suspensions at -20°C for 8 hours to overnight, followed by repeated freezing and thawing for 6 cycles. Phage survival was confirmed by spot test and double-layer agar methods.

Phage Morphology Examination

AB-phage morphology was examined using transmission electron microscopy (TEM). Phage suspensions, at least 10^9 PFU/ml, were applied to a copper grid and allowed to settle for 3 minutes. The grid was dried with filter paper and stained with 1% uranyl acetate. The grid was stored in a desiccator overnight before TEM imaging.

Bioassay of AB Phage Therapy

AB-phage concentrations were prepared at 10^9-10^{10} PFU/ml. Each phage was diluted in serial 10-fold dilutions and delivered to each well of a microtiter plate in a volume of 90 µl. For tests involving multiple AB-phages (e.g., three phages), 30 µl of each phage was added to make a total volume of 90 µl. Ten microlitres of AB suspension were then added to achieve different MOIs (0.1, 1, 10, 100, etc.). The plate was incubated at room temperature and 37°C with shaking at 80 rpm overnight. Ten microlitres of the AB and AB-phage mixture were spotted on MacConkey medium and incubated at 37°C overnight to observe AB survival. AB suspensions were re-counted to confirm by the pour plate technique and adjust MOI calculations in each experiment.

Isolation of AB-Phage Lysogens and Phage Immunity of AB-Lysogens

AB lysogens were isolated by exposing AB cultures to phages at sub-lethal MOI levels and incubating for 18 hours at 37°C. The mixtures were centrifuged, and the precipitated bacteria were cultured and tested for lysogeny using spot tests. Alternatively, bacterial colonies that survived within phage plaques of ABU-3 were isolated[9]. The candidate AB lysogens were tested for their immunity to all three AB-phages by spotting phage suspensions on AB-lysogen lawns and comparing the results to the parent ABU-3 isolate. Phage solutions ranging from 10^4 to 10^8 PFU/ml were spotted on bacterial lawns, incubated overnight at 37°C, and observed for plaque formation on AB-phage lysogens and ABU-3 lawns.

Results

Characterization of the AB-Phages

To study the association between AB lysogens and other susceptible AB-phages, we selected the ABU-3 isolate, which was susceptible to three types of AB-phages: AB-phage-22, AB-phage-27, and AB-phage-32, for further study in this project. The plaque formations of the three phages are shown in Figure 1. AB-phage-22 formed clear plaques around 1.3-2.1 mm in diameter, with a thick, turbid surrounding zone. AB-phage-27 produced plaques less than 1 mm in diameter, and AB-phage-32 formed clear plaques approximately 1.2-1.7 mm in diameter, with a thinner surrounding zone compared to AB-phage-22. Notably, AB-phage-27 appears distinct from AB-phage-22 and AB-phage-32, with a plaque diameter less than 1 mm. TEM morphologies of the three AB-phages are distinguished by the shape and pattern of their tails. AB-phage-22 is more like a Siphovirdae, while AB-phage-27 is Podoviridae-like, and AB-phage-32 has a long, contractile tail, considered a Myoviridae-like phage.

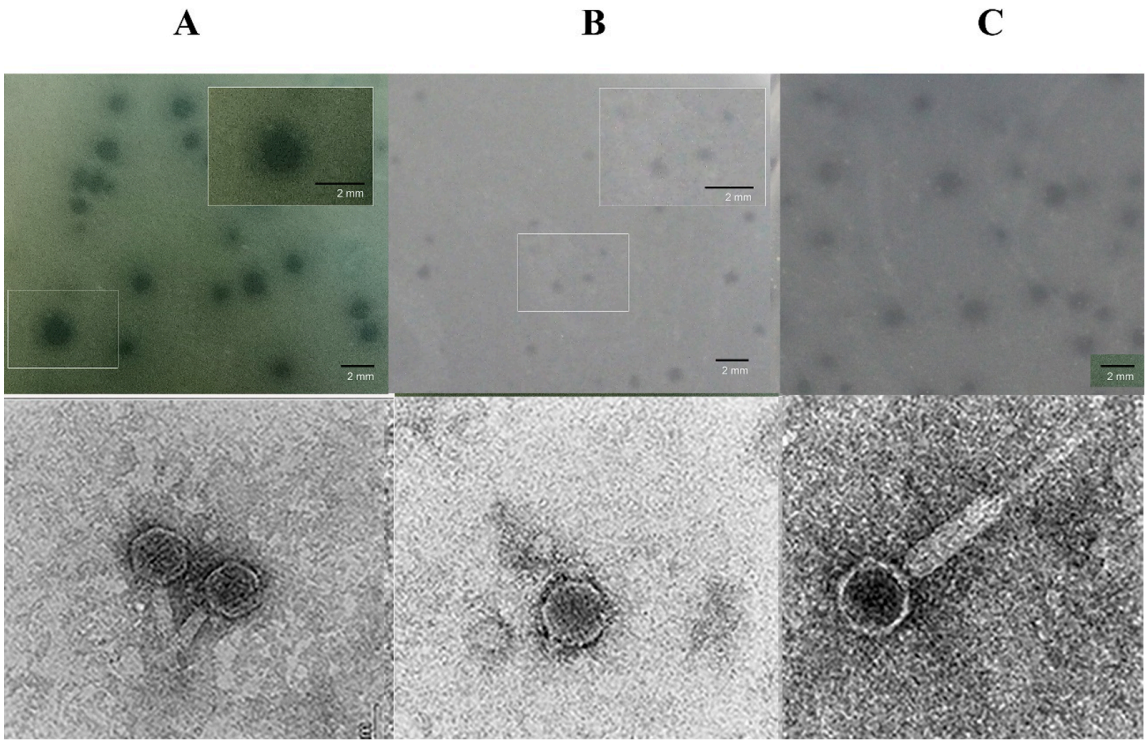

Figure 1. Plaque formation and morphologies of AB-phage-22 (panel A), AB-phage-27 (panel B), and AB-phage-32 (panel C).

We then tested the physical characteristics of these phages, particularly their tolerance to pH and temperature. AB-phage-27 became inactive after 1 hour at 56°C, while AB-phage-22 and AB-phage-32 tolerated temperatures of 68-69°C and 66-67 °C, respectively, for the same duration. All three AB-phages remained stable under freeze-thaw conditions for at least six cycles, as evaluated by both spot tests and plaque formation methods. Regarding pH tolerance, AB-phage-22 remained partially active after 2 hours at pH 2, whereas AB-phage-32 was completely inactivated at the same pH. On the other hand, AB-phage-27 became inactive at pH 3. All three AB-phages tolerated alkaline conditions at pH 12 and became inactive at pH 13.

In Vitro Bioassay of Phage Therapy

We conducted in vitro bioassays to determine the multiplicity of infection (MOI) of each AB-phage that could clear ABU-3, referred to herein as the "MOI clearance of ABU-3." After five tests, the MOI clearance results for single AB-phage-22, AB-phage-27, and AB-phage-32 are shown in Table 1. AB-phage-27 showed lower efficiency in clearing ABU-3, with an MOI clearance of 100 in all five

experiments. AB-phage-22 cleared ABU-3 once at MOI 1 and four times at MOI 10, while AB-phage-32 consistently cleared ABU-3 at MOI 10 in all five experiments. Although AB-phage-22 achieved MOI clearance at 1, MOI 10 provided consistent results. For safety considerations and to prevent the induction of AB-lysogens, MOI 10 should be chosen for clearing ABU-3 with AB-phage-22, not MOI 1. This model serves as an example for clinical trials aimed at determining the optimal ratio of AB and AB-phage for AB clearance, with the goal of preventing the induction of AB-lysogens. The combination of AB-phages in this study, whether using two AB-phages or all three, did not prove to be more effective than single-phage treatments in terms of MOI clearance of ABU-3.

AB-phage	pH resistance	Temperature toleration (°C)	MOI clearance of ABU-3			
			0.1	1	10	100
AB-phage 22	2-12	68-69	None	1 exp.	4 exp.	5 exp.
AB-phage 27	3-12	56	None	None	None	5 exp.
AB-phage 32	2-12	66-67	None	None	5 exp.	5 exp.

Table 1. Physical characteristics of each AB phage in terms of pH and temperature tolerance. The MOI clearance of the AB phage against ABU-3 is shown based on 5 experiments (exp.)

Phage Resistance of AB-Lysogens

To gain more insight into the phage resistance of each AB-lysogen, we isolated lysogens of each phage, referred to as 22-AB-lysogen, 27-AB-lysogen, and 32-AB-lysogen, respectively. These lysogens were tested as target cells against single AB-phages alongside ABU-3 using the spot test. As shown in Figure 2, the 22-AB-lysogen completely resisted AB-phage-22 and displayed partial resistance to AB-phage-27 and AB-phage-32, with a reduction of approximately 2 logs compared to the parental ABU-3 isolate. In the case of the 32-AB-lysogen, it was more adapted and resistant to all three AB-phages. In contrast, the 27-AB-lysogen did not exhibit increased resistance to other AB-phages except its own phage. These AB-lysogens were re-identified as AB by biochemical tests in a microbiology routine laboratory.

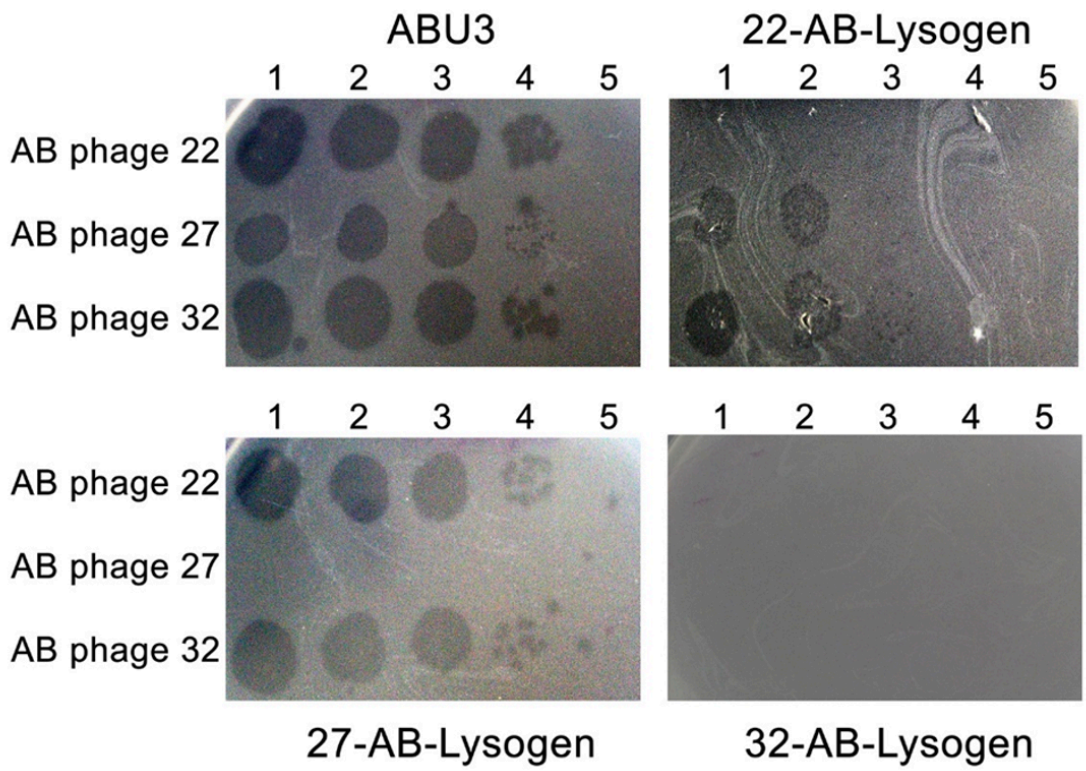

Figure 2. Super-infection immunity of each AB lysogen tested with a spot method. Ten-fold serial dilutions of three AB-phages (AB-phage-22, AB-phage-27, and AB-phage 32) were spotted on lawns of ABU-3 (panel A), 22-AB-lysogen (panel B), 27-AB-lysogen (panel C), and 32-AB-lysogen (panel D).

Discussion

This study isolated three AB-phages (AB-phage-22, AB-phage-27, and AB-phage-32) that infect the same AB host, ABU-3, as evidenced by differences in plaque formation and TEM morphology. Although the TEM morphology of AB-phage-22, AB-phage-27, and AB-phage-32 seems to classify them as *Siphovirdae, Podoviridae*, and *Myoviridae*, respectively, more information is required to confirm these classifications. This article primarily demonstrates that these three AB-phages are likely different types of AB-phages based on their distinct plaque formation and TEM morphology. The physical properties of these phages show that they are quite resistant to a wide range of pH levels and temperatures, as shown in Table 1, which is not surprising given that they were isolated from wastewater—a challenging environment. AB-phage-27 is less tolerant to temperature than the other two AB-phages, becoming inactive at temperatures over 57°C for 1 hour. It is also notable that these

phages can withstand freeze-thaw cycles, simplifying storage, as they can be frozen without a preservative agent while maintaining their activity. Additionally, AB-phage-22 has been tested for lyophilization and retained its activity. However, long-term preservation requires further observation[10][11].

Given the increasing incidence of bacterial resistance to antibiotics, phage therapy presents an attractive option for preventing and treating bacterial infections, including those caused by AB. However, phage therapy has limitations, particularly due to the highly specific interaction between each phage and its bacterial host. No single phage can infect all strains of a bacterial species, although some can infect multiple strains. Addressing this limitation requires a collection of phages ready for therapy, which demands time, cost, and labour. Another significant limitation is the formation of lysogens, which can make bacteria resistant to the same phage. This could occur if an insufficient phage dose is used during the initial treatment. With multi-phage infections of ABU-3, there is an opportunity to study phage immunity more deeply.

As shown in Figure 2, this study found varying levels of super-infection immunity in three different lysogens: definite immunity in the 27-AB-lysogen, which was resistant to its original AB-phage-27 but not the other two AB-phages. On the other hand, the 32-AB-lysogen was not only resistant to its initial AB-phage but also showed broad immunity to the other two AB-phages. Meanwhile, the 22-AB-lysogen was completely resistant to its initial phage, as expected, but exhibited partial resistance to AB-phage-27 and AB-phage-32, with almost two log reductions compared to the original ABU-3 host.

Different theories explain super-infection immunity[12]. One early proposal is the expression of a repressor gene, such as the *CI* repressor gene, which represses the lytic cycle and prevents super-infection by the same phage. This hypothesis extends to explain that super-infection immunity can occur with other related phages, causing a wider range of immunity—a phenomenon known as super-infection exclusion[13]. However, the definition of "related phages" remains unclear in this context and does not fully explain the mechanism behind super-infection with related phages. Another proposal, the inducible receptor concept, suggests that phages or viruses using the same or related cellular molecules as receptors/co-receptors for entry may be blocked by the down-regulation of these molecules during phage penetration, similar to sperm-egg fertilization[14]. However, this concept may not fully explain the partial broad immunity of the 22-AB-lysogen, which remains sensitive to higher concentrations of AB-phage-27 and AB-phage-32. A more recent proposal, based

on the CRISPR-Cas system—an adaptive immunity-like mechanism in prokaryotes—explains super-infection immunity more clearly[15]. However, this proposal also does not explain the mechanism of partial super-infection exclusion, indicating a need for further investigation. Understanding the mechanisms behind super-infection immunity could be fundamental in developing strategies, such as modifying the phage genome, to prevent lysogen induction and ensure the success of phage therapy. While progress has been made, much remains to be explored.

To overcome lysogen induction during phage therapy, we emphasize the conventional strategy of using sufficient phage amounts to clear all ABU-3 cells and prevent lysogen induction. We propose determining the MOI (multiplicity of infection) clearance value as a procedure for phage therapy. In this study, as shown in Table 1, the MOI clearance values for AB-phage-22, AB-phage-27, and AB-phage-32 were 10, 100, and 10, respectively. These MOI clearance values represent the optimal concentrations that can effectively eliminate all ABU-3 cells. We propose testing the MOI clearance to prevent lysogen induction. Previous studies have shown that phage cocktails are more effective in phage therapy[16][17]. However, this study did not support the notion that combined or cocktail phages are more effective than single-phage treatments. It is important to note that this study evaluated the efficiency of phage therapy by clearing all bacterial cells to prevent lysogen induction, rather than statistically reducing bacterial numbers, which might still lead to lysogen formation. The AB lysogen could overgrow and become resistant not only to the initial AB-phage but potentially to others as well. While phage cocktails could be advantageous for targeting a broader spectrum of pathogenic bacteria, they may induce lysogeny over a wider range. The benefits and drawbacks of cocktail phage therapy should be carefully assessed. We propose determining the MOI clearance of bacterial pathogens when it is not an emergency. The priority is to ensure that patients are cured without any later complications. Based on our pilot testing, which involved a single experiment for the MOI clearance of 12 other AB isolates that are susceptible to these three AB-phages, an MOI of 100 was consistently effective in clearing all the AB isolates. Although an MOI of 10 showed a significant reduction with a few surviving colonies, MOI 100 was consistently effective. This suggests that an MOI of 100 might be the clearance value for AB by all the AB-phages. Further study with a larger number of AB and AB-phage samples is required. If so, this could be advantageous for practical phage therapy with cocktail phages in emergencies, even if phage therapy is processed without testing the MOI clearance value.

In conclusion, this study demonstrated that induced lysogens not only exhibit immunity to their primary AB-phage but can also develop immunity to other AB-phages. Careful consideration of the

MOI clearance value of the infected bacteria is essential for effective phage therapy, as simply reducing the target bacterial population may not eliminate lysogens, leading to potential complications in treatment. Understanding the causes of super-infection immunity in lysogens could be beneficial for developing strategies to modify phage genomes to prevent lysogen induction. At present, preventing lysogen induction by determining the MOI clearance value before phage therapy is proposed.

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Declarations

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