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Original Article

Phage resistance profiles of AB-phages treated *Acinetobacter baumannii*: implications for phage therapy strategies

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Abstract

Introduction: Phage therapy is a promising alternative for combating multidrug-resistant bacteria, including *Acinetobacter baumannii* (AB). However, the development of phage-resistant variants after treatment, particularly when using phage cocktails, poses a significant challenge. This resistance can hinder the effectiveness of future phage-based treatments against pathogenic AB.

Methodology: Three AB-specific phages—AB-phage 22, AB-phage 27, and AB-phage 32—susceptible to an AB isolate, designated ABU-3, were used as a model to study phage resistance development in AB following phage treatment. This study proposes a strategy to effectively eliminate pathogenic AB using an optimal multiplicity of infection (MOI), referred to as the MOI clearance value.

Results: The MOI clearance values required for complete elimination of ABU-3 were determined to be 10 for AB-phages 22 and 32 and 100 for AB-phage 27. Surviving ABU-3 colonies from lower MOI treatments were analyzed for phage resistance. ABU-3 treated with AB-phage 27 developed resistance to AB-phage 27 but remained susceptible to AB-phages 22 and 32. ABU-3 treated with AB-phage 22 developed resistance to AB-phage 22 but retained partial susceptibility to the other phages at reduced MOI. In contrast, ABU-3 treated with AB-phage 32 displayed complete resistance to all three phages.

Conclusions: These findings highlight a key challenge in phage therapy: insufficient MOI ratio can promote phage resistance. The distinct resistance profiles observed emphasize the importance of optimizing phage combinations and dosages to prevent resistance development during treatment.

Key words: *Acinetobacter baumannii*; phage therapy; multiplicity of infection; phage-resistance variants; cocktail phage therapy.

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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 *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 bacteria. Phages can infect bacterial hosts through two main pathways: the lytic and lysogenic cycles. In the lytic pathway, phage infection results in bacterial lysis, releasing new phage progeny that can then infect additional bacterial cells. In contrast, the lysogenic pathway involves the integration of the phage genome into the bacterial chromosome, forming what is known as a lysogen. A lysogen does not produce phage progeny unless induced by agents such as mitomycin C [4]. Ultraviolet light has also been shown to induce lysogens to enter the lytic cycle through activation of the SOS regulatory system [5]. Phages are broadly categorized into two types: virulent and temperate. Virulent phages are preferable for phage therapy because they exclusively follow the lytic pathway and do not form lysogens [6].

Virulent phages can be identified by their formation of clear plaques in plaque assays, in contrast to temperate phages, which produce turbid plaques [7,8]. Virulent phages are theoretically more suitable for phage therapy due to these properties. Although virulent phages do not form lysogens, bacterial resistance to these phages can still develop, posing a challenge to successful phage therapy [9-11]. Addressing this resistance is essential for improving therapeutic outcomes.

This study established an *in vitro* phage treatment model to explore the use of multiple virulent AB-phages to infect an AB isolate, designated ABU-3. Ultimately, three distinct AB-phages, based on the plaque formation and electron microscopy, were isolated to target the ABU-3 isolate. This research aims to assess the likelihood of phage resistance ABU-3 variants induced by individual phages of the three distinct AB-phages. Additionally, strategies to optimize phage therapy and prevent induction of the phage resistance variants are proposed. The findings aim to provide insights into achieving the most effective practices in phage therapy.

Methodology

Acinetobacter baumannii [*AB*]

Forty AB isolates, including ABU-3 isolate, were collected and stored in the microbiology laboratory of Srinakarin Hospital, Khon Kaen, Thailand, between October 2020 and June 2023. The isolates originated from tracheal suction fluid specimens submitted for routine diagnostic testing and were identified using standard biochemical and antibiotic susceptibility assays. The samples were preserved in 50% glycerol at -80 °C without any link to patient-identifiable information. Prior to further use, the isolates were sub-cultured, and their biochemical characteristics and susceptibility to doxycycline, amikacin, imipenem, and levofloxacin were reconfirmed. Notably, there are no new data associated with this article.

Enrichment and Preparation of AB Phage

Phage samples were collected from waste water sources in the vicinity of Srinakarin Hospital, Khon Kaen University, Khon Kaen Province. The waste water 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. The final concentration of each AB-phage was determined using a serial 10-fold dilution in SM buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$) followed by a spot test. The lowest dilution of the AB-phage that produced plaques was subsequently evaluated by the double-layer agar method to determine the exact phage titer [12]. If the phage concentration was too low, the preparation was repeated with the specific AB- until the phage titer reached at least 10^9 PFU/mL.

Screening and isolation 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 nutrient agar plates by mixing bacterial cultures with 0.5% nutrient agar. Ten microliters 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 exhibited different plaque appearances using the double-layer agar method, individual plaques were isolated [13]. Phage purification was performed by isolating plaques with a pipette tip, amplifying them with the susceptible AB isolate, and incubating overnight at 37 °C to evaluate the unique clear plaque. This process was repeated to ensure the purity of the isolated phage.

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 Bio-Rad C1000Dx Thermal cycle 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.

Spot Test and Double Layer Agar Methods

For the spotting assay, a 1:100 dilution of an overnight-incubated bacterial suspension was inoculated into 10 mL of lactose broth and incubated at 37 °C for 2 hours. The bacterial density was checked using a spectrophotometer at OD_{600} (Biosan, Latvia, MacFarland densitometer adaptor) aiming for a range of 0.3–0.4. Two hundred microliters of the bacterial suspension were mixed with 0.6% agar at approximately 45–50 °C and poured onto a nutrient agar plate. Ten microliters of the diluted phage sample were then spotted onto the bacterial lawn as desired.

For the double-layer agar method, after mixing the bacterial suspension with 0.6% agar as described above, the desired amount and dilution of the phage sample were added, vortexed for a few seconds, and poured onto the nutrient agar plate. The plate was incubated at 37 °C overnight, and results were evaluated. Each sample was typically tested in triplicate. However, if any variations were observed, the test was conducted up to five times, with the coefficient of variation (CV%) kept below 5%.

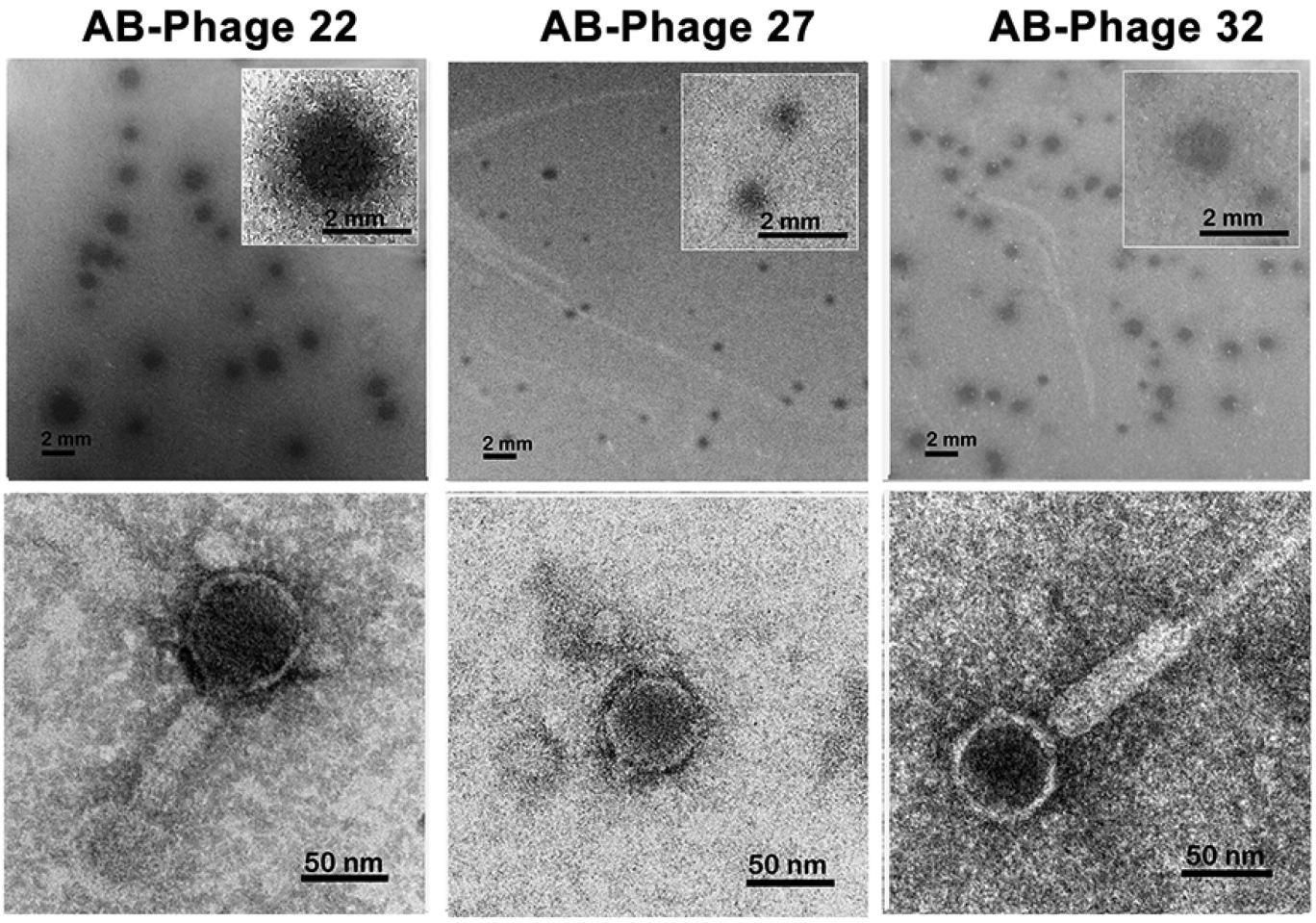
Phage Morphology Examination

AB-phage morphology was examined using transmission electron microscopy (TEM). Phage suspension, at least 10^9 PFU/mL, were applied to copper grids and allowed to settle for 3 minutes. Dried with filter paper, and stained with 1% uranyl acetate. The grid was stored in a desiccator overnight before TEM imaging [14].

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

Figure 1. Plaque formation and TEM images of AB-phage-22 (Left)), AB-phage-27 (Middle), and AB-phage 32 (Right). The plaques of AB-phage-22 exhibit unique morphology, characterized by larger clear plaques, more than 2 mm. in diameter with expanding turbid zone resembling an eye. AB-phage-27 forms the smallest clear plaque, less than 1 mm in diameter, while AB-phage-32 forms clear plaques with a peripheral turbid zone of 1.2-1.8 mm. in diameter. The TEM images of AB-phage-22, AB-phage -27 and AB-phage-32 resemble Siphoviridae, Podoviridae and Myoviridae, respectively.



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 microliters 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 microliters 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 pour plate technique and adjust MOI calculations in each experiment.

Isolation of phage-resistance variants

The phage-resistance variants, which survived exposure to each of the three AB-phages, was isolated by exposing ABU-3 cultures to AB-phages at 1 log lower than the MOI clearance level, followed by culturing on nutrient agar. The phage- treated ABU-3 isolates were tested for resistance to each corresponding AB-phage and confirmed to be resistant to the same phages that induced resistance. The phage resistant variants of each AB-phage were sub-cultured and tested for their resistance in the next day by spot test assay. In addition, the phage resistant variants to each AB-phage were also re-evaluated after storage in nutrient medium at 4 °C for approximately one week. Lawns of each phage-resistant ABU-3 variants were then tested for immunity to all three AB-phages by spotting phage suspensions and comparing the results to the parent ABU-3 isolate. Phage solutions ranging from 10^4 to 10^8 PFU/mL were spotted onto each bacterial lawn, incubated overnight at 37 °C, and observed for plaque formation on both the phage-resistant ABU-3 and the parent ABU-3 lawns.

Results

Characterization of the AB-Phages

The objective of this study was to investigate multiple phage infection of *Acinetobacter baumannii* (AB), 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. Plaque formations of the three phages are shown in Figure 1. AB-phage-22 formed clear plaques 1.3-2.1 mm in diameter, surrounded by a thick, turbid zone. AB-phage-27 produced plaques smaller than 1 mm, and AB- phage-32 formed clear plaques approximately 1.2-1.8 mm in diameter with a thinner surrounding zone compared to AB-phage-22. Notably, AB-phage-27 appeared distinct from AB-phage-22 and AB-phage-32 due to its smaller plaque diameter. TEM images revealed unique tail morphologies for each phage: AB-phage-22 resembled *Siphoviridae*, AB-phage-27 was *Podoviridae*-like, and AB-phage-32 displayed a long, contractile tail characteristic of *Myoviridae*.

We next examined the physical characteristics of these phages, focusing on 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 after at least six freeze-thaw cycles, as assessed by 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 under the same conditions. AB-phage-27 became inactive at pH 3. All three AB-phages tolerated alkaline conditions at pH 12 but were inactivated at pH 13. AB-phage-22 was tested for lyophilization and retained its activity; however, long-term preservation requires further observation [15,16]. The details were summarized in Table 1

In Vitro Bioassay of Phage Therapy

We conducted *in vitro* bioassays to determine the MOI of each AB-phage that could clear ABU-3, referred to here as the MOI clearance of ABU-3. After five tests, MOI clearance results for single AB-phage-22, AB-phage-27, and AB-phage-32 are presented in Table 1. AB-phage-27 showed lower efficiency, with an MOI clearance of 100 in all five experiments. AB-phage-22 cleared ABU-3 once at an MOI of 1 and four times at MOI 10, while AB-phage-32 consistently cleared ABU-3 at an MOI of 10 in all five experiments. While AB-phage-22 could achieve MOI clearance at 1, MOI 10 provided more consistent results, and for safety considerations and to avoid inducing AB-phage resistance AB, MOI 10 was recommended for clearing ABU-3 with AB-phage-22. This model could serve as a basis for clinical trials to determine the optimal AB-phage-to-bacteria ratio for effective AB clearance while avoiding phage resistant induction. Phage combinations in this study, whether with two or all three phages, did not prove more effective than single-phage treatments in achieving MOI clearance of ABU-3.

Phage Immunity of Each Phage-Resistant ABU-3 Variants

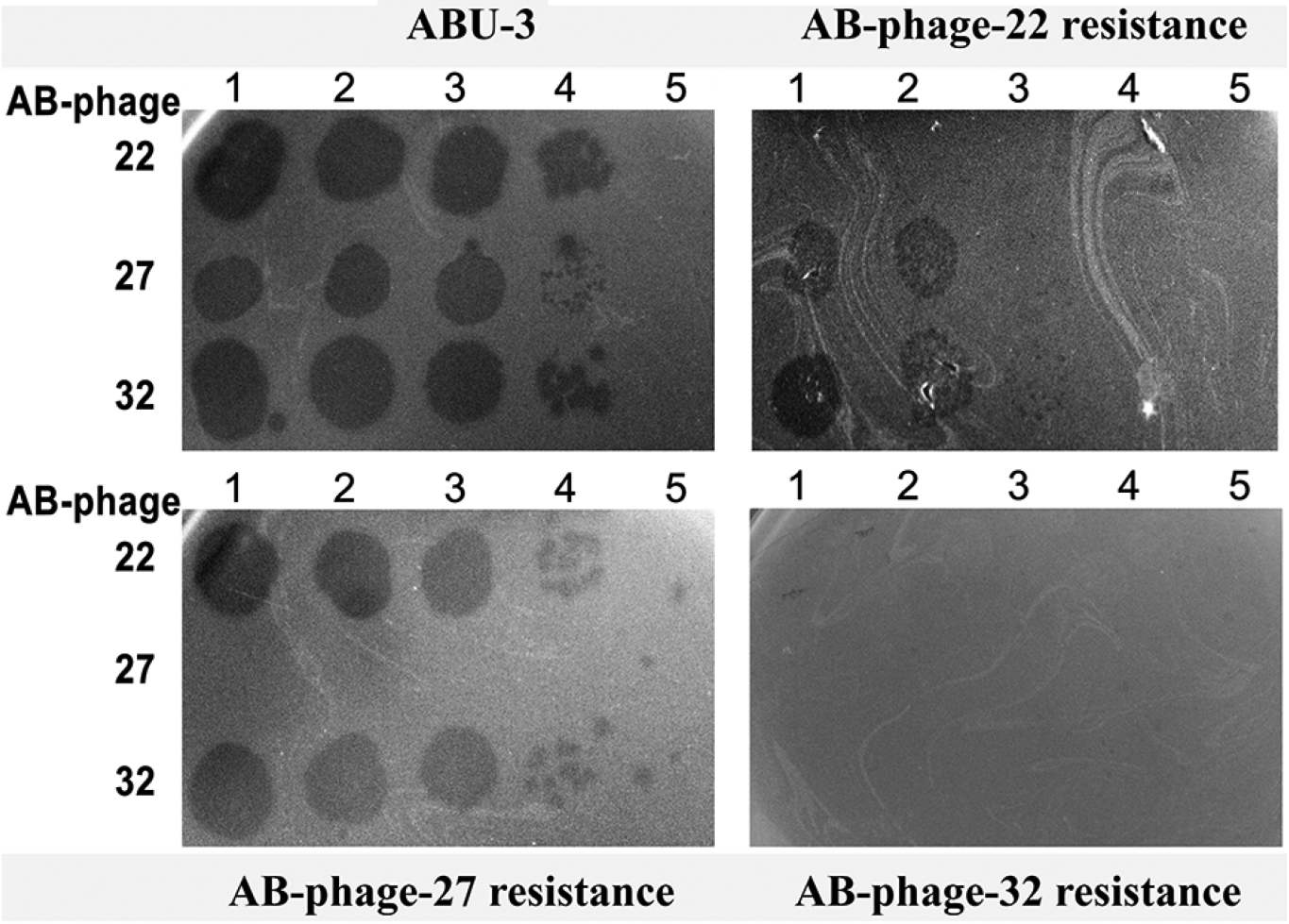
To gain further insight into the phage resistance of each AB-phage-treated ABU-3, we isolated phage-resistant ABU-3 variants: 22-AB-resistant ABU-3, 27-AB-resistant ABU-3, and 32-AB-resistant ABU-3

variants. These phage-resistant ABU-3 variants were tested as target cells against single AB-phages, alongside the parental ABU-3, using the spot test. As shown in Figure 2, 22-AB-resistant ABU-3 variant completely resisted AB-phage-22 and displayed partial resistance to AB-phage-27 and AB-phage-32, showing approximately a 2-log reduction compared to the parental ABU-3 isolate. In contrast, 32-AB-resistant ABU-3 variant was resistant to all three AB-phages. On the other hand, 27-AB-resistant ABU-3 variant did not exhibit increased resistance to the other AB-phages, except for its own. These AB-phage-resistant ABU-3 variants were re-identified as AB by routine biochemical tests and confirmed by their 16S RNA sequence.

Discussion

This study isolated three AB-phages—AB-phage-22, AB-phage-27, and AB-phage-32—capable of infecting the ABU-3 host, with distinctions in plaque formation and TEM morphology. While TEM morphology suggests that AB-phage-22, AB-phage-27, and AB-phage-32 belong to *Siphoviridae*, *Podoviridae*, and *Myoviridae*, respectively, further confirmation is necessary. The results demonstrate that these three AB-phages are likely distinct types, based on their plaque formation and TEM morphology. Their physical properties reveal tolerance to a broad range of pH levels and temperatures, likely reflecting their origin in wastewater. Notably, AB-phage-27 is less heat-tolerant than the other two, becoming inactive after 1 hour at 57°C, whereas all three tolerate freeze-thaw cycles, simplifying storage. Furthermore, AB-phage-22 was tested for lyophilization and retained its activity; however, long-term preservation requires further

Figure 2. Super-infection immunity of each AB-phage resistant ABU-3 tested using the 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 (Top/Left), AB-phage- 22 resistance ABU-3 (Top/Right), AB-phage-27 resistant ABU-3 (Bottom/Left), and AB-phage-32-resistant ABU-3 (Bottom/Right). The dilutions are arranged with position 1 representing the highest concentration of phages and position 5 representing the lowest concentration.



observation [15,16].

Given the increasing incidence of bacterial resistance to antibiotics, phage therapy presents an attractive alternative 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 labor [15,17].

To counteract the induction of phage-resistant AB during phage therapy, this study emphasizes the importance of using sufficient phage quantities to completely eliminate ABU-3 cells and prevent resistance development. Determining the MOI clearance value as a standard procedure for phage therapy is proposed. In this study, to completely clear ABU-3 isolate, the MOI clearance values for AB-phage-22, AB-phage-27, and AB-phage-32 were 10, 100, and 10, respectively (Table 1). These MOI values represent the optimal concentrations needed to clear all ABU-3 perfectly. Previous studies suggest that phage cocktails are generally more effective in phage therapy [18,19]. However, this study does not support the hypothesis that phage cocktails are more effective than single-phage treatments. It is important to note that the study focused on complete bacterial clearance to prevent the induction of phage-resistant strains, rather than merely reducing bacterial numbers based on statistical analysis, which could still lead to resistance if a single phage resistance variant was developed.

Phage-resistant AB variants could proliferate and develop resistance not only to the initial phage but also to others. While phage cocktails may target a broader range of pathogenic bacteria, they could also induce resistance across a wider range. The pros and cons of cocktail therapy must be carefully evaluated with the sufficient MOI. Based on pilot tests involving the MOI clearance of 12 other AB isolates susceptible to these three phages, an MOI of 100 consistently cleared all AB isolates, while an MOI of 10 resulted in significant reduction but with some surviving colonies. This suggests that an MOI of 100 might serve as a clearance threshold for AB by all three phages. Further research with larger sample sizes is needed to validate this approach. If successful, it could serve as a practical solution for emergency use of phage cocktails without prior MOI clearance testing.

To understand how AB develops resistance during insufficient phage doses, this study examined the immune phenotype of phage-treated AB strains. As shown in Figure 2, three phage-resistant ABU-3 variants exhibited varying levels of superinfection immunity to the three phages. The AB-phage-27-resistant strain was resistant to its original phage but remained susceptible to the other two. In contrast, the AB-phage-32-resistant variant displayed broad immunity to all three phages. Meanwhile, the AB-phage-22-resistant strain was fully resistant to its original phage and partially resistant to the other two, showing a nearly two-log reduction compared to the original ABU-3 host [20]. Interestingly, over 50% of surviving AB bacteria from suboptimal phage ratios remained susceptible to the same phage, potentially due to transient resistance mechanisms during infection. [21]

The limitation of this study is that we focus for the outcome of the ABU-3 that has been treated with different three AB-phages and tested for the phage resistance after surviving from the low MOI. At this moment we have not studied for the mechanism of different phage resistant phenotype. Different theories have been proposed to explain superinfection immunity. One hypothesis involves the expression of a repressor gene, such as the *CI* repressor, which suppresses the lytic cycle and prevents superinfection by the same phage [22]. Another hypothesis, the inducible receptor concept, suggests that phages or viruses using the same or related cellular molecules as receptors are blocked by the down-regulation of these molecules during penetration, similar to mechanisms in sperm-egg fertilization [23] A more recent proposal, the CRISPR-Cas system, describes an adaptive immunity-like mechanism in prokaryotes [24,25]. However, none

Table 1. Physical characteristics of each AB phage in terms of pH (n = 3) and temperature tolerance (n = 5). The MOI clearance of the AB phage against ABU-3 were shown based on 5 experiments (exp.). For the pH tolerance, the AB-phages maintained consistent infection activity against ABU-3 across all three experiments. For temperature tolerance, each of the AB-phages maintained its infection activity as indicated by the mean and SD. For MOI clearance, the study was conducted 5 times and the results are presented based on the MOI (0.1- 100) ratio in each experiment that could clear ABU-3.

AB-phage	pH tolerance	Temperature toleration (mean ± SD) °C	MOI clearance of ABU-3			
			0.1	1	10	100
AB-phage 22	2-12	68-69 (66.4 ± 0.55)	None	1 exp.	4 exp.	5 exp.
AB-phage 27	3-12	56 (56 ± 0.00)	None	None	None	5 exp.
AB-phage 32	2-12	66-67 (66.4 ± 0.55)	None	None	5 exp.	5 exp.

of these models fully explains partial superinfection exclusion, indicating a need for further investigation using molecular biology, biochemistry, and related approaches. Understanding the mechanisms behind superinfection immunity is essential for designing strategies to prevent resistance and enhance phage therapy success.

Conclusions

This study demonstrates that phage-resistant AB strains can develop immunity not only to their primary phage but also to other phages. Determining the MOI clearance value of infected bacteria is critical for effective phage therapy to prevent the emergence of resistant strains. Establishing an appropriate MOI clearance protocol prior to treatment can help reduce this risk. The findings highlight diverse immunity phenotypes in phage-resistant AB strains, especially in cases where AB isolates remain susceptible to multiple phages. Improper MOI values in phage cocktail therapy could promote the emergence of broader resistance, complicating treatment and potentially rendering phage therapy ineffective.

Further research is needed to explore similar immunity patterns in other pathogenic bacteria susceptible to multiple phages. Addressing this gap will deepen our understanding of virus-host interactions and improve the design and application of phage therapies.

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Conflict of interest

No conflict of interest is declared.

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