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Phage Cocktail against *Acinetobacter baumannii* biofilm on Endotracheal Tube: An in vitro study

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ABSTRACT

Although biofilms on endotracheal tube (ET) surfaces represent a major clinical challenge, studies addressing the effect of lytic bacteriophages on these biofilms are relatively scarce. This study focused on examining the anti-biofilm capability of three specific phage against an XDR isolate of *Acinetobacter baumannii* in a 48-hour pre-formed biofilm on an ET surface. For this purpose, crystal violet staining, colony counting, and scanning electron microscopy (SEM) were employed. The results demonstrated a significant decrease in biofilm mass and bacterial count after 24 hours of exposure to the phage cocktail. SEM images confirmed a dramatic reduction in the biofilm. Based on these findings, phage therapy has the potential to reduce and disrupt biofilms on ET surfaces.

Keywords: Bacteriophage, Biofilm, *Acinetobacter baumannii*, Endotracheal Tube

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Introduction

The intensive care unit (ICU) uses mechanical ventilation to support critically ill patients, but this intervention predisposes them to serious complications. Ventilator-associated pneumonia (VAP), the most common infection in ventilated patients, typically develops within 48 hours after intubation. Its incidence ranges from 1–2.5 cases per 1000 ventilator-days in North America and Europe, with reports exceeding 18 per 1000 in some regions. Mortality attributable to VAP is estimated to be nearly 50%, making it a major clinical challenge that prolongs hospital stays and increases healthcare costs (1-4).

Microorganisms associated with VAP vary according to factors such as the duration of mechanical ventilation and length of hospitalization. The most common bacterial agents involved in VAP include *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. Generally, VAP is categorized into two types: early- and late-onset. The early-onset form occurs within the first four days of hospitalization and is often associated with colonization by normal oropharyngeal flora. The late-onset form arises after at least five days of hospitalization and is typically caused by multidrug-resistant (MDR) organisms (5).

Acinetobacter baumannii is responsible for a variety of infections, including bacteremia, pneumonia, meningitis, wound infections, and urinary tract infections (UTIs) (6). Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is a significant pathogen associated with high mortality rates. Due to its extensive resistance to multiple classes of antibiotics, physicians are often compelled to use polymyxins (colistin or polymyxin B) as a last-line treatment (7, 8). *Acinetobacter baumannii* is capable of colonizing and forming biofilms on both abiotic and biotic surfaces, such as tracheal tubes and epithelial cells (9). Biofilm formation on both living and non-living surfaces, such as endotracheal tubes (ETs), plays a key role in hospital-acquired infections. Shortly after intubation, biofilms form, serving as a reservoir for microorganisms. These biofilms act as physical barriers that reduce antibiotic penetration, increase antibiotic resistance, and protect microorganisms from the host's immune system, making biofilm eradication challenging (10,11). Infections associated with biofilms can be thousands of times more resistant to antibiotics than planktonic cells. This increased resistance occurs because bacteria enter a dormant and persistent phase within the biofilm (12, 13). Phages are viruses capable of lysing their bacterial hosts. Their activity is typically restricted to strains of a single species (14). The antibacterial properties of phages were recognized even before the discovery of antibiotics. In recent years, phage therapy has re-emerged as a promising alternative treatment for antibiotic-resistant pathogens (15, 16).

Given that using a single phage for treatment can lead to phage resistance, employing a mixture of phages, or a cocktail, can mitigate this limitation. Phage cocktails are often more effective than a single phage at preventing and eradicating biofilms (15). Despite the prevalence of VAP and biofilm-associated antibiotic resistance, few studies have evaluated the use of bacteriophages to disrupt biofilms on ET surfaces. The present study addresses this clinically relevant gap by focusing specifically on biofilms formed on ETs, which are major reservoirs for pathogens in mechanically ventilated patients. This approach highlights the novelty of our work in applying phage cocktails to a setting directly linked to ventilator-associated pneumonia. This study aimed to address this gap by in vitro assessment of phage cocktail efficacy against *Acinetobacter baumannii* biofilms formed on ETs. The goal of this research was to identify specific lytic phages of *Acinetobacter baumannii* from wastewater sources and evaluate the in vitro activity of a phage cocktail against biofilms formed on ETs.

Methods

Isolation and identification of bacterial strains

A XDR strain of *Acinetobacter baumannii* was isolated from patients admitted to ICU ward of Velayat Hospital (Qazvin, Iran). This strain was identified through culturing on differential media (CondaLab, Spain) and by employing phenotypic and genotypic methods. Qazvin University of Medical Sciences approved this study through its ethics committee (Approval no. IR.QUMS.REC.1401.208). Written informed consent was obtained from all of the participants in the study.

Biofilm Formation Assay

To study biofilm formation in bacterial isolates, the crystal violet staining technique was employed.

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Briefly, bacterial suspensions were prepared from fresh cultures grown in Tryptic Soy Broth (TSB) medium (CondaLab, Spain) and adjusted to an optical density (OD) of 0.03 at 570 nm. Two hundred microliters of these suspensions were added to all the wells of a 96-well microtiter plate and incubated at 37°C for 48 hours. The contents of the wells were then aspirated and washed with normal saline.

Subsequently, 200 µL of a 1% crystal violet solution was added to each well and left for 20 minutes, after which the wells were washed again. Next, 200 microliters of 95% ethanol were added to dissolve the crystal violet, and the optical density of the solutions was measured at 570 nm. The OD cut-off value (ODc) was calculated as the mean OD of the negative control plus three standard deviations. Biofilm formation was defined as follows: ($OD \leq ODc$) non-producer, ($ODc < OD \leq 2 \times ODc$) weak producer, ($2 \times ODc < OD \leq 4 \times ODc$) moderate producer, and ($OD > 4 \times ODc$) strong producer (17,18).

Antimicrobial susceptibility testing

The disk diffusion method was used to assess antibiotic susceptibility to the following antibiotics: doxycycline (30 µg), cefepime (30 µg), trimethoprim/sulfamethoxazole (23.75/1.25 µg), ciprofloxacin (5 µg), ampicillin/sulbactam (10/10 µg), ceftazidime (30 µg), meropenem (10 µg), gentamicin (10 µg), cefotaxime (30 µg), and piperacillin/tazobactam (100/10 µg) (Mast, UK). The procedure followed the Clinical and Laboratory Standards Institute (CLSI) 2024 guidelines (17). The Clinical and Laboratory Standards Institute (CLSI) 2024 guidelines were followed for determining the minimum inhibitory concentration (MIC) of colistin using the broth microdilution method. Colistin sulfate salt (Sigma-Aldrich) was used to prepare a two-fold dilution series ranging from 0.125 to 64 µg/mL. The test was conducted in a 96-well polystyrene plate containing Mueller-Hinton broth (QUELAB) (19).

Phage isolation

Hospital wastewater samples from Qazvin, Iran, were mixed with Luria-Bertani (LB) broth (1X, CondaLab, Spain) to enrich for potential phages. Host bacteria were then added, and the mixture was agitated in a shaker incubator for 24 hours. Following incubation, the broth was subjected to centrifugation at 8,000 × g for 10 minutes, and the supernatant was passed through a 0.22 µm syringe filter. The filtered supernatant was subsequently added to a bacterial suspension in the logarithmic growth phase and incubated at 37°C for 24 hours. If the LB medium remained clear, indicating bacterial lysis, the lysate was filtered and stored at 4°C (20).

Spot test and plaque assay

Spot tests and plaque assays were carried out using double-layer agar (DLA) with Luria-Bertani (LB, 1X) agar (CondaLab, Spain). Spot and plaque assays were performed using the standard double-layer agar method as previously described. The presence of a lytic phage was indicated by the formation of a clear plaque on the bacterial lawn (21).

For the plaque assay, serial dilutions of the filtered lysate were prepared. A 100 µL aliquot of each dilution was combined with 100 µL of a bacterial suspension and incubated for 10 minutes at 37°C. This mixture was then added to 5 mL of molten LB soft agar (0.7% agar) and poured onto an LB base agar plate (1.5% agar). After incubation for 24 hours, the formation of clear plaques indicated the presence of lytic phages. This process was repeated through several rounds of plating to purify individual phage isolates. Purified phage stocks were stored in SM buffer at 4°C (22).

Transmission Electron Microscopy (TEM)

A drop of the phage suspension was placed on a carbon-coated copper grid for 4 minutes. The sample was then negatively stained with 2% uranyl acetate. The grid was examined using a transmission electron microscope (TEM; Zeiss LEO 906) operating at an accelerating voltage of 90 kV (23).

Host range determination

Determination of the host of the studied phages was performed using standard strains of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Shigella flexneri* (ATCC 12022), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028), and *Pseudomonas aeruginosa* (ATCC 27853) as well as 15 *Acinetobacter baumannii* clinical isolates using single phage and phage cocktail by the spot test method (20).

Phage titration

The phage titer was determined using the double-layer agar (DLA) method. After 24 hours of incubation,

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the number of plaques was counted, and the phage titer was calculated using the following formula: (Number of plaques) × (dilution factor) = PFU/mL (24).

Determination of the multiplicity of infection (MOI)

With slight modifications of previous studies (25, 26), serial dilutions of the host bacteria (from 0 to 10) were prepared, and the optical density at 600 nm (OD600) of the culture was measured using a spectrophotometer. Then, 100 microliters (µL) of each bacterial dilution were spread onto the surface of an agar plate and incubated at 37°C. After incubation, bacterial colonies were counted, and the bacterial titer was determined in colony-forming units per milliliter (CFU/mL). To determine the optimal multiplicity of infection (MOI), the lytic activity at five MOIs (MOI-100, MOI-10, MOI-1, MOI-0.1, MOI-0.01) was tested for each individual phage (Ab.ph1, Ab.ph2, Ab.ph3).

A bacterial suspension equivalent to 1.5×10^5 CFU/ml from a two-hour culture was mixed with the appropriate phage dilution (10^3–10^7 PFU/ml). A bacteria-only control without phage was included. Optical density (OD600) was measured every 30 minutes for 6 hours.

Phage Heat and pH Stability Test

To determine thermal stability, 500 µL of a phage suspension (10^7 PFU/mL) was incubated at temperatures of -80, -20, 4, 25, 37, 50, 60, 70, and 80 °C for one hour. The phage titer was subsequently determined using the double-layer agar (DLA) method. To assess stability at different pH levels, aliquots of LB broth (pH 7.0) were adjusted to various pH values (ranging from 1 to 14) using NaOH and HCl. Then, 100 µL of the phage suspension (10^7 PFU/mL) was added to 1 mL of the adjusted medium at each pH and incubated for one hour at 37 °C.

The phage titer was determined immediately after incubation using the DLA method. The samples incubated at 37 °C and pH 7.0 were used as controls. All experiments were performed in triplicate. (20).

Bacteriophage activity against established biofilms
Phage cocktail activity against established biofilm in 96-well microtiter plate

The activity of bacteriophages against pre-formed biofilms was investigated using a modified protocol (27, 28). The bacterial strain was cultured in LB broth. A fresh culture was adjusted to a concentration of 10^5 CFU/mL, and 200 µL of this suspension was added to each well of a 96-well polystyrene microtiter plate (n = 7 wells per multiplicity of infection (MOI) group). The plates were incubated for 48 hours at 37°C to allow for biofilm formation. Wells containing sterile LB broth served as the negative control. Following incubation, the biofilms were washed with phosphate-buffered saline (PBS) and subsequently treated for 24 hours with the phage cocktail at three multiplicities of infection (MOI 100, MOI 10, and MOI 1), all incubation steps were performed at 37 °C unless otherwise stated.

Biofilm-only wells (n=7) served as positive controls (no phage added). Following incubation, the contents of all wells were aspirated and washed twice with sterile phosphate-buffered saline (PBS, pH 7.4). The attached biofilm biomass was quantified using the crystal violet staining method, as previously described. The optical density (OD) of the dissolved crystal violet was then measured at 570 nm using a microplate reader (BioTek, USA).

Biofilm formation on ET

Polyvinyl chloride ET segments were cut into 1 cm piece and sterilized by autoclaving. Subsequently, each piece was placed in a tube containing 10 mL of LB broth inoculated with approximately 10^5 CFU/mL of bacteria and incubated for 24 hours at 37°C to facilitate initial biofilm formation. To promote thicker biofilm development, half of the spent culture medium was aseptically removed and replaced with an equal volume of fresh, sterile LB broth, followed by a further 24 hours of incubation. Following incubation, the ET fragments were gently washed three times with sterile phosphate-buffered saline (PBS, pH 7.4) to remove non-adherent cells and transferred to new tubes (arranged in 7 groups, with n=7 tubes per group), then treated with the phage cocktail at an MOI of 1. The seventh group (control) received no phage. All tubes were then incubated again under the same conditions (29,30).

Biofilm dislodgement by EDTA

At 1, 2, 4, 6, 8, and 24 hours after phage cocktail addition, one group (n=7) of ET pieces was washed three times with sterile PBS (pH 7.4). Each piece was then treated with 1 ml of 25 mM EDTA. The segments were vortexed for 60 seconds, held for 15 minutes at room temperature, and vortexed again for 60 seconds. 100 µl of the solution was spread on the surface of an

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LB agar plate and incubated overnight. The number of colonies was counted and multiplied by the dilution factor. Untreated control samples, which did not receive phage cocktail treatment, were processed in the same way with EDTA exposure and vertexing before colony counting (31).

Characterization of biofilms using SEM

To compare and evaluate the effect of the phage cocktail on the biofilm formed on the endotracheal tube (ET), biofilm mass was visualized using scanning electron microscopy (SEM; TESCAN MIRA3, Czech Republic). Samples consisted of an ET fragment exposed to the phage cocktail for 24 hours and a control sample not exposed to phage.

Statistical analysis

The Shapiro-Wilk test was used to assess the normality of the data. Continuous variables are presented as mean ± standard deviation (SD) for parametric data or median and interquartile range (IQR) for non-parametric data. Categorical and ordinal variables are expressed as proportions (n, %). An independent sample t-test was used to compare means between two groups for parametric data. The Mann-Whitney U test was employed to compare medians for non-parametric data. Proportions were compared using the Pearson chi-square test or Fisher's exact test, as appropriate. Results are presented as the median with 95% confidence intervals (CIs). A p-value of less than 0.05 was considered statistically significant for all tests. All statistical analyses were performed using SPSS software Version 16 (SPSS Inc., Chicago, IL, USA).

Results

Characterization of the bacterial isolate

XDR was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) (32). The studied *Acinetobacter baumannii* isolate demonstrated resistance to all antibiotics tested by disk diffusion, including doxycycline, cefepime, trimethoprim/ sulfamethoxazole, ciprofloxacin, ampicillin/ sulbactam, ceftazidime, meropenem, gentamicin, cefotaxime, and piperacillin/ tazobactam. The minimum inhibitory concentration (MIC) for colistin, determined by broth microdilution, was ≤2 µg/mL, indicating intermediate resistance according to CLSI guidelines. Note that only resistant and intermediate interpretive categories are defined for colistin. Using a 96-well microtiter plate assay with crystal violet staining, the *Acinetobacter baumannii* isolate was confirmed to be a strong biofilm producer (OD570 = 0.831± 0.01, n= 5).

Phage morphology and Lytic Activity

A total of three *Acinetobacter baumannii*-specific phages were isolated from municipal and hospital wastewater samples. These phages were designated Ab.ph1, Ab.ph2, and Ab.ph3. All three phages produced transparent plaques approximately 1–2 mm in diameter, surrounded by a clear halo zone measuring 2–3 mm. Transmission electron microscopy (TEM) analysis revealed that all three phages belong to the class *Caudoviricetes*, characterized by a double-stranded DNA (dsDNA) genome, an icosahedral head measuring 95 ± 5 nm in diameter, and a contractile tail approximately 150 nm in length (Figure 1).

Host range results

Determining the host range by spot test showed that none of the phages exhibited lytic activity against the non-*Acinetobacter* bacterial strains used in the assay, including Escherichia coli (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), Enterococcus faecalis (ATCC 29212), Shigella flexneri (ATCC 12022), and *Salmonella enterica subsp. enterica serovar Typhimurium* (ATCC 14028). This indicates that the isolated phages are specific to their *Acinetobacter baumannii* host. Of the 15 *Acinetobacter baumannii* clinical isolates tested, 4 (26.6%), 2 (13.3%), 7 (46.6%), and 8 (53.3%) were sensitive to Ab.ph1, Ab.ph2, Ab.ph3, and the phage cocktail, respectively. Based on these results, Ab.ph1 showed a narrow host range, while Ab.ph3 showed a broader host range. The phage cocktail retained lytic activity against all isolates for which at least one constituent phage was active.

Bacteriophage titer determination

Based on the double-layer agar (DLA) method, plaques were counted after 24 hours of incubation. Using the standard formula, the titers of phages Ab.ph1, Ab.ph2, and Ab.ph3 were calculated as 5.1 ×

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10^8 PFU/mL, 4.3 × 10^8 PFU/mL, and 5.2 × 10^8 PFU/mL, respectively.

MOI determination

The examination of phage lytic activity against planktonic bacteria across a range of multiplicities of infection (MOIs: 100, 10, 1, 0.1, and 0.01) demonstrated that all tested phage concentrations resulted in a significant reduction in optical density at 600 nm and inhibition of host bacterial growth compared to the untreated positive control. MOI-100 was the most effective phage concentration, exhibiting the lowest optical density (OD600). This result was consistent for all three phages (Figure 2).

Phage thermal and pH Stability

In this study, the effect of different temperatures and pH range on stability of three phages (Ab.ph1, Ab.ph2, and Ab.ph3) were tested and results showed the titers of phages were stable at temperatures of -80°C, -20°C, 4°C, 26°C, 37°C and 50°C, for 1 h.

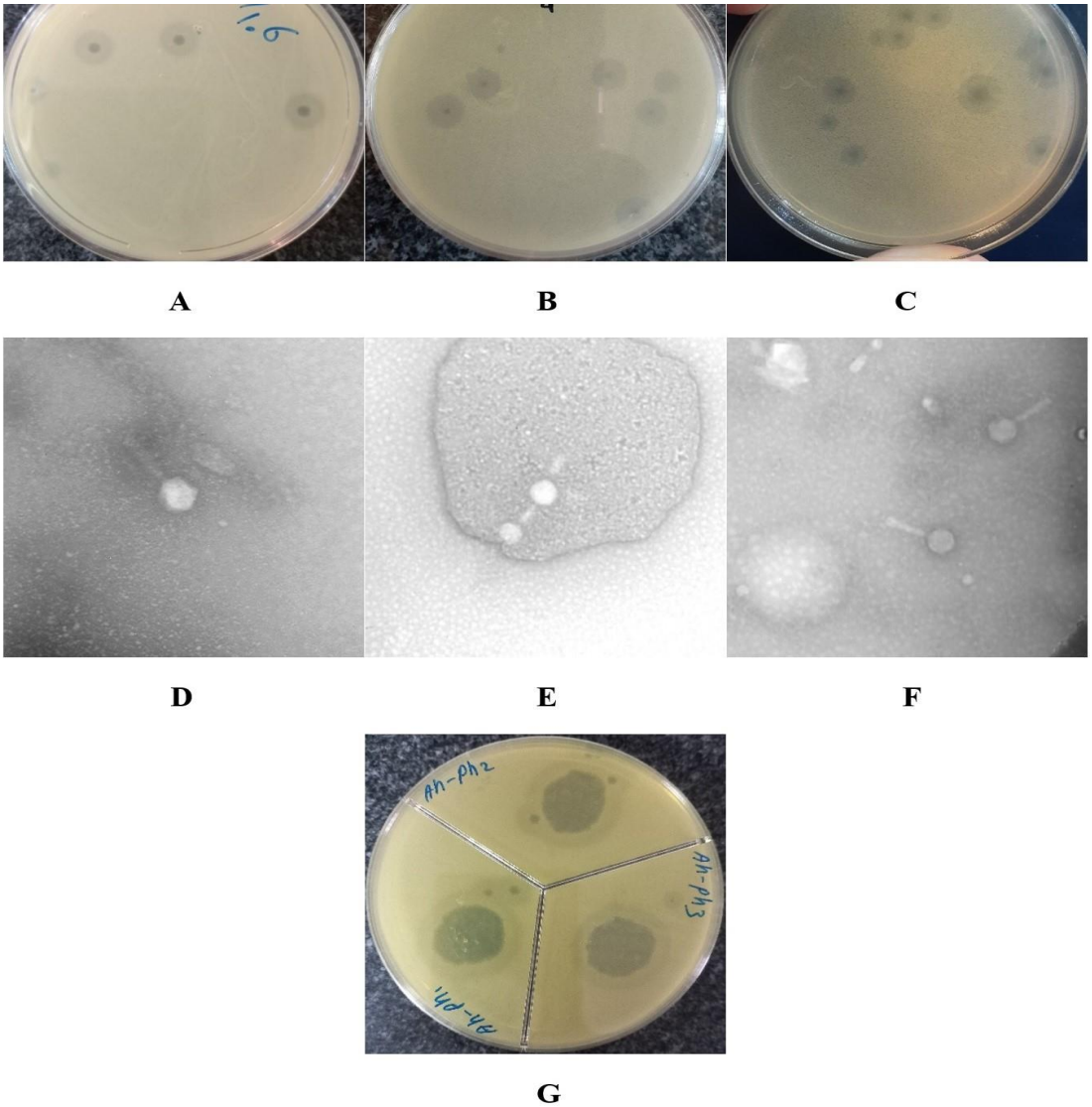


Figure 1. Morphologies and spot test of anti- *Acinetobacter* phages. Plaques of Ab.ph1 (A), Ab.ph2 (B), Ab.ph3 (C). TEM microscopy of Ab.ph1 (D), Ab.ph2 (E), Ab.ph3 (F), represent 30 nm, 100 nm and 50 nm respectively. Spot test of three phages (G).

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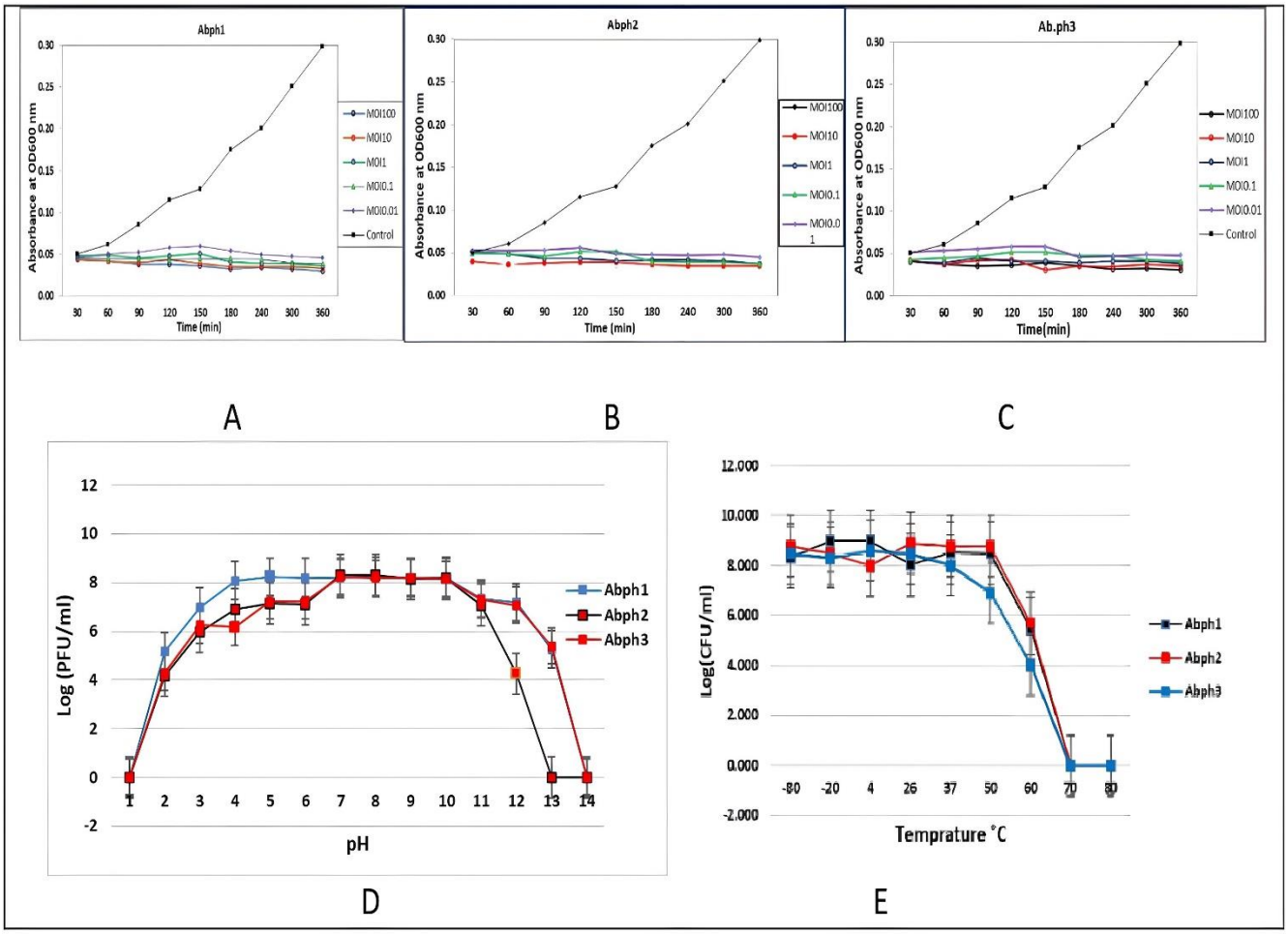


Figure 2. General characterization of Ab.ph1, Ab.ph2 and Ab.ph3. (A), (B) and (c) Multiplicity of infection. (D) PH stability of phages in pH range of 1 to 14. (E) Thermal stability of the phages in different temperatures. pH and thermal data represent the mean ± SD of three independent experiments.

The number of all three phages decreased significantly at 60°C the titer of three phages dropped by approximately 99%, whereas to 70°C led to complete inactivation, with no PFU/ ml detected. Therefore, the behaviour of all three phages in response to temperature changes was the same (Figure 2). The pH stability results showed that the titers of all three phages remained constant within the pH range of 4 to 11. Under alkaline conditions, the titers of Ab.ph1 and Ab.ph3 decreased at pH 13, and no phage activity was detected for these phages at pH 14. The titer of Ab.ph2 reduce substantially at pH 13 and this phage became no detectable phage activity at pH 14. Under acidic condition all three phages exhibited a strong decrease in titer at pH 3 and 2 and were activity fell below detection limit at pH 1. Ab.ph2 was slightly more sensitive to both alkaline and acidic pH than the other two phages (Figure 2).

Bacteriophage activity against established biofilms

The biofilm degradation activity was assessed by biomass staining, viable bacterial counting, and SEM analysis.

Phage cocktail activity against established biofilm as determined by Biomass Staining

The results were calculated as an average for each MOI and for the controls. Based on the data analysis, Phage cocktail treatment significantly reduced biofilm biomass at all tested MOIs after 24 h. The $\log_{10}$ reductions relative to untreated controls were 0.758 ± 0.064, ($p < 0.001$) for MOI 100, 0.711 ± 0.036, ($p < 0.001$) for MOI 10 and 0.807 ± 0.053, ($p < 0.001$) for MOI 1 (mean ± SD, n=7).These results indicate that, contrary to the determination of the most effective multiplicity of infection (MOI) for individual phages (MOI = 100), an MOI of 1 was the most effective

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concentration for the phage cocktail in reducing pre-formed biofilm biomass.

Viable cell count in established biofilm on ET dislodgement by EDTA

Bacterial biofilm biomass on ET dislodged after applying 25 mmol EDTA to both phage-treated and untreated biofilms for 15 minutes, followed by plating and overnight incubation, the number of grown colonies was counted. This number was then multiplied by the dilution factor, and the average was calculated for each parameter.

According to the results, at 1h, 2h and 4h after phage cocktail treatment, the number of viable cells present in the biofilm biomass was gradually reduced by Log_{10} 0.43 ± 0.06, ($p < 0.001$), Log_{10} 1.21 ± 0,03, ($p < 0.001$), Log_{10} 1.84 ± 0.19, ($p < 0.001$) respectively, which was more heightened after 6h and 8h, achieving Log_{10} 2.36 ± 0.14, ($p < 0.001$) and Log_{10} 2.91 ± 0.12, ($p < 0.001$) log respectively, compared to the control. After 24h, the viable cell number reached zero ($p < 0.001$) (mean± SD, n=7). (Figure 3). After 24h, the viable cell number reached zero ($p < 0.001$) (mean ± SD, n=7) (Figure 3). The corresponding log reductions with 95% confidence intervals are summarized in Table 1.

Scanning Electron Microscopy (SEM) Analysis

To more evaluate phage cocktail activity against *Acinetobacter baumannii* biofilm on ET, SEM was used to visualize the effect of phage on a 48h-old biofilm. Biofilms were pictured by scanning electron microscopy. The images obtained from SEM demonstrated that the phage cocktail (MOI=1) effectively eradicated the biofilm formed on the ET. As clearly shown in Figure 4, biofilm structure and the cells within the matrix were visible before phage cocktail treatment, while after 24 hours of phage cocktail treatment, both the biofilm matrix and the embedded cells were dramatically redacted.

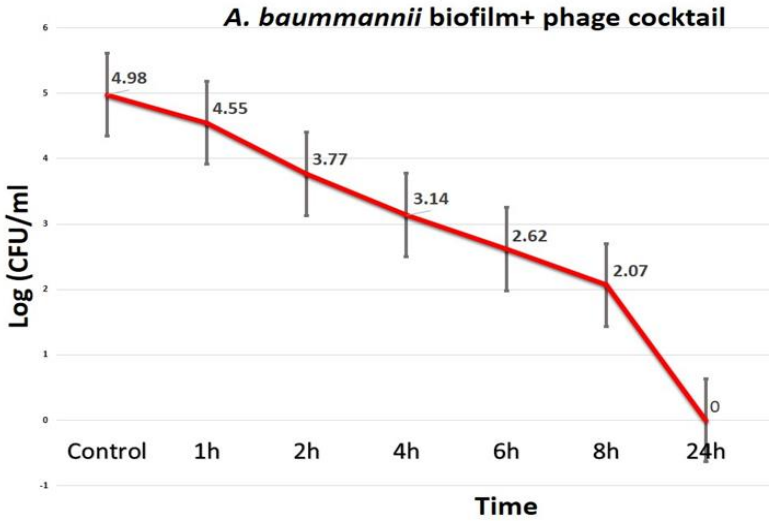


Figure 3. Phage disruption of established *Acinetobacter baumannii* biofilm on ET. Biofilms grown on ET surfaces for 48 h, were initially inoculated with 10^5 PFU/ml of phage cocktail. After 1, 2, 4, 6, 8 and 24 h of phage treatment at 37 °C, average biofilm viable cell count, were recorded comparatively to untreated control samples. Values are expressed as the mean ± SD from seven independent replicates.

Table 1. Log reduction of viable cells on pre-formed ET biofilms treated with phage cocktail. Values are mean ± 95% CI (n=7)

Time (h)	Log reduction (mean ± 95% CI)
1	0.43 (0.37 -49)
2	1.21 (1.18- 1.24)
4	1.86 (1.68- 2.04)
6	2.37 (2.24- 2.51)

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Time (h)	Log reduction (mean ± 95% CI)
8	2.92 (2.81- 3.03)
24	No detectable CFU/ ml

Data present mean log eradication relative to untreated control (n=7). 95% confidence intervals were calculated based on standard error of the mean. Complete eradication at 24 h indicate no detectable CFU/ml

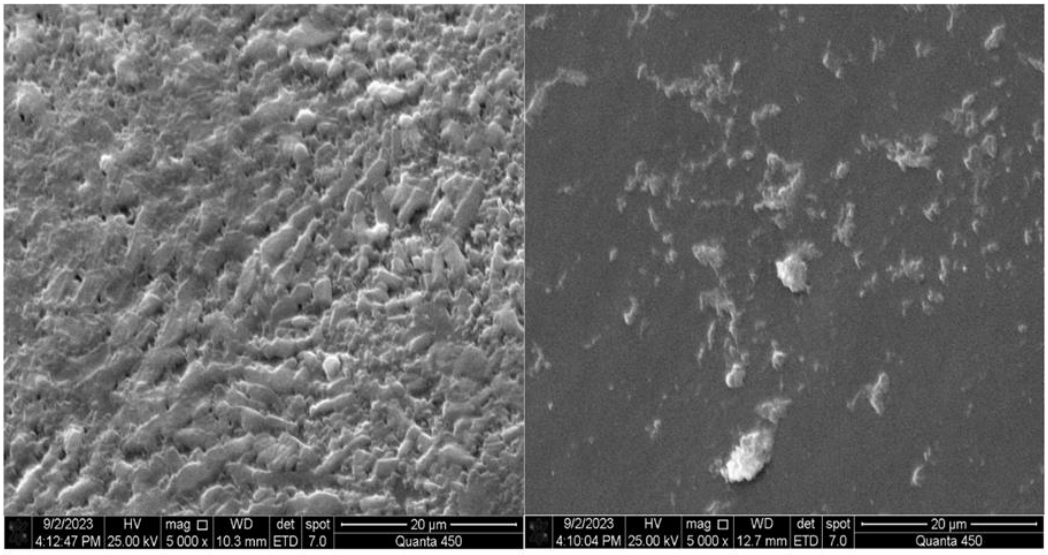


Figure 4. SEM images of *Acinetobacter baumannii* biofilm on ET(A) and treatment of *Acinetobacter baumannii* biofilm on ET with phage cocktail resulted in a dramatic reduction of biofilm matrix (B).

Discussion

This study investigated the in vitro efficacy of a bacteriophage cocktail against biofilms formed by an XDR Acinetobacter baumannii isolate on endotracheal tubes (ETs), a key contributor to VAP. Bacteriophages can lyse and kill bacteria within biofilms and enzymatically degrade the biofilm matrix (33, 34). Investigation of biofilm production by the XDR *Acinetobacter baumannii* isolate under study revealed it as a strong biofilm producer, a characteristic often associated with increased drug resistance. Consistent with previous studies (38, 39), we isolated phages targeting Acinetobacter baumannii from wastewater. This confirms that sewage is a reliable source of lytic phages and supports its use for isolating phages active against clinical isolates. Consistent with previous studies (38, 39), we isolated phages targeting *Acinetobacter baumannii* from wastewater. This confirms that sewage is a reliable source of lytic phages and supports its use for isolating phages active against clinical isolates.

Observation of plaques produced by the studied phages using the DLA method showed that all three phages formed haloed plaques, indicating the presence of depolymerase enzymes capable of degrading biofilm matrix and bacterial cells. This enzymatic activity has been reported to facilitate biofilm disruption, supporting the reductions observed in SEM images and viable cell counts (30, 35-37).

Based on analysis of TEM electron microscope images, all three specific phages belonged to the class Caudoviricetes. These stability characteristics support their potential for therapeutic applications. The host range of a bacteriophages is a notably property to be noted in the choice of phages for potential therapeutic usage. In our study, the phage cocktail demonstrated a broader activity than single phages, with more than half of the isolates being sensitive. This highlights the benefit of combining multiple phages to expand host range and reduce resistance development (30, 38). The application of broad host-range phages decreases the reliance on numerous phage types for the management of infections due to pathogenic bacteria (30).

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Previous studies have demonstrated that phage cocktails can effectively reduce biofilms in medical device models through co-cultivation of bacteria, phages, and biofilms (39, 40). However, few studies have directly assessed their lytic activity on biofilms formed specifically on ETs. In this study, the phage cocktail significantly reduced biofilm mass at different MOIs and eliminated viable cells after 24h exposure.

These results underscore the potential of phage cocktails as a promising strategy to eradicate biofilms on ETs, which are critical reservoirs for pathogens in VAP. Interestingly, biofilm assays revealed optimal biomass reduction at MOI-1, suggesting that biofilm–phage interactions differ from planktonic systems. One possible explanation is that very high phage concentrations may limit effective interactions with bacterial cells within the biofilm, potentially due to receptor saturation or steric hindrance. Similar observations have been reported in other systems, where phage predation efficiency was influenced by environmental factors or phage density (39, 40). However, direct evidence for this effect in *Acinetobacter baumannii* biofilms is still lacking, and further studies are needed to clarify this phenomenon.

Researchers investigated the antibiofilm effect of single phages against Uropathogenic *E. coli* strains using the crystal violet staining method in synthetic urine at 2, 4, 8, and 24 hours. The results indicated that the greatest reduction in biofilm mass occurred after 8 hours (28). Contrary to our findings, the study reported an increase in biofilm mass at 24 hours, suggesting potential biofilm reformation due to bacterial resistance to single phage. This problem might be addressed by using a phage cocktail. Also, lower phage concentrations have been shown to yield better results, as discussed previously. In another study (41), pre-treatment and post-treatment tests of *P. aeruginosa* biofilm with single and phage cocktail were performed on ETs.

Phage concentration, exposure time, and biofilm age differed from our study. Pre-treatment with both phages resulted in a substantial reduction in microbial load inhibition compared with the phage-untreated control group. In post-treatment, low phage concentrations led to a slight decrease in microbial load, while higher concentrations yielded better results. The optimal concentration was determined to be 10^6 PFU/ml, indicating a dose-dependent efficacy of the phage. The results of this study showed that the applied phage cocktail effectively reduces biofilm biomass on the surface of the ET. Our findings indicate that phage therapy can be utilized as an adjunctive therapy for the treatment of infections affected by resistant bacteria, potentially reducing the incidence of VAP. By focusing on ET-associated biofilms, this work provides new insights into phage application in a clinically relevant context, reinforcing the novelty and potential translational impact of the study.

In this study, the use of lytic phages Ab.ph1, Ab.ph2, and Ab.ph3 as a cocktail demonstrated significant in vitro reduction of biofilm biomass formed by an XDR *Acinetobacter baumannii* isolate on the ET. These findings suggest that lytic phages may represent a promising approach for future therapeutic strategies against drug-resistant *Acinetobacter baumannii* causing nosocomial infections, including VAP. Further studies, including in vivo experiments, are needed to confirm their clinical potential.

Limitations

This study has several limitations, including use of a single *Acinetobacter baumannii* isolate, in vitro experiments only, short duration, and lack of in vivo validation. Additionally, the phages were isolated from wastewater and tested against a limited number of isolates, which may restrict generalizability. Although phage stability was evaluated across different temperatures and pH, long-term stability and storage conditions were not assessed. The study focused solely on biofilms formed on endotracheal tube surfaces, whereas biofilms on other medical devices or tissues may behave differently.

Finally, genomic characterization of the phages was not performed to confirm the absence of undesired genes such as toxins or antibiotic resistance. Future studies should address these limitations by including in vivo models, testing a broader panel of bacterial isolates, evaluating long-term phage stability, performing whole-genome sequencing, and investigating phage delivery systems to optimize therapeutic application.

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