

Efficacy of Phage Cocktails Against Biofilms Formed by Antibiotic-Resistant Bacteria

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Aim: The rapid spread of multidrug-resistant strains of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, along with their ability to form biofilms on various medical devices, significantly complicate the treatment of infections caused by these microorganisms and render antibiotic therapy ineffective. In contrast, the use of bacteriophages is a promising alternative for combating antibiotic-resistant biofilm-forming strains of *K. pneumoniae* and *P. aeruginosa*.

Methods: Two cocktails of 14 bacteriophages (nine *Klebsiella* phages and five *Pseudomonas* phages) were used to control biofilms formed by XDR (Extensively Drug-Resistant) strains of *K. pneumoniae* and *P. aeruginosa* under in vitro conditions. The *K. pneumoniae* strain harbored genes associated with biofilm formation *fimH*, *mrkA*, *matBecp* and antibiotic resistance *bla_{NDM-1}*, *bla_{KPC}*, *bla_{OXA-48}*, *bla_{CTX-M-1}*, *bla_{TEM}*. The *P. aeruginosa* strain carried genes associated with biofilm formation *algD*, *PsID*, *PelF* and antibiotic resistance *bla_{NDM-1}*. Bacteriophages were isolated from the wastewater samples. Biofilms were formed on various substrates (glass slides, wells of polystyrene plates, and polyvinyl chloride vascular catheters) and analyzed using optical and scanning electron microscopy, as well as gentian violet staining assays.

Results: The results demonstrated that bacteriophage cocktails could effectively degrade biofilms of *K. pneumoniae* and *P. aeruginosa*. Biofilms formed on catheter segments, polystyrene plate wells, and glass slides were treated with lytic bacteriophages at concentrations of at least 10⁷ PFU/mL. After 24 h of treatment with phage cocktails, a 34.5% reduction in biofilm biomass was observed on the catheters for *K. pneumoniae* strain No. 361 and 34.1% for *P. aeruginosa* strain No. 7. In polystyrene plate wells, the reductions were 39.3% and 52.8%, respectively.

Conclusion: The experimental results indicate the effectiveness of phage cocktails in reducing biofilm biomass and bacterial viability. Given the ability of phages to degrade biofilms, phage therapy may become a promising adjunct to standard treatment methods for infections caused by multidrug-resistant pathogens.

Keywords: bacteriophages, biofilms, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, antimicrobial resistance

Introduction

The ability to form biofilms is a crucial virulence factor for opportunistic pathogens, such as *K. pneumoniae* and *P. aeruginosa*, which are gram-negative bacteria frequently associated with healthcare-associated infections. Over the past decade, there has been a significant increase in the incidence of infections caused by multidrug-resistant strains of *K. pneumoniae* and *P. aeruginosa*.¹ This highlights the importance of better understanding the pathogenesis of infections caused by these microorganisms.

To date, classic opportunistic strains of *K. pneumoniae* (cKp) are distinguished, which are primarily associated with nosocomial infections, and hypervirulent strains (hvKp), considered as community-acquired bacteria capable of causing community-acquired infections in individuals of all ages, including healthy individuals.² The emergence of hypervirulent and antibiotic-resistant strains of *K. pneumoniae* has prompted a series of clinical and epidemiological studies. According to data

from the European Centre for Disease Prevention and Control (ECDC), the epidemiological situation regarding the prevalence of carbapenem-resistant enterobacteria in the European Union/European Economic Area (EU/EEA) continues to worsen. An increase in the frequency of bloodstream infections caused by carbapenem-resistant *K. pneumoniae* and the spread of hypervirulent *K. pneumoniae* strains, particularly ST23, has been observed.³ The increase in cases of infection caused by carbapenem-resistant hvKp is of significant concern due to the severity of infections caused by hvKp in combination with their resistance to last-line antibiotics, which greatly complicates treatment. Since the last operational risk assessment by the ECDC in 2021, the number of EU/EEA countries reporting cases of hvKp has risen from four to ten, and the number of isolates sent by these countries for analysis has increased from 12 to 143.⁴

P. aeruginosa is a ubiquitously gram-negative bacterium that can survive under a wide range of environmental conditions.⁵ These bacteria, in their biofilm state, can survive in hypoxic or extremely harsh environments. Moreover, treating *P. aeruginosa* infections is exceptionally challenging because of rapid mutations in the pathogen and its adaptation to acquiring antibiotic resistance.⁶ *P. aeruginosa* has a high level of intrinsic resistance to most antibiotics owing to the limited permeability of its outer membrane, efflux systems that pump antibiotics out of the cell, and production of enzymes that inactivate antibiotics, such as β -lactamases.⁷ In 2023, the resistance rate of *P. aeruginosa* to carbapenems below 5% was reported in two (5%) of the 43 countries that provided data on this microorganism-antimicrobial combination, while seven (16%) countries reported resistance rates equal to or exceeding 50%. Comparing 2019 and 2023, there was an observed increase in the estimated incidence of invasive *P. aeruginosa* isolates by 11.7%.⁸ This highlights the significant role of *P. aeruginosa* in the pathology of human infection.

Biofilms are densely organized bacterial communities attached to a solid surface and surrounded by an exopolysaccharide matrix. The latter accounts for 50–90% of the total organic carbon in biofilms and is considered the primary structural component of the biofilm matrix. The chemical and physical properties of the exopolysaccharide matrix can vary; however, its main components are polysaccharides. Some polysaccharides are neutral or anionic. The anionic properties of the exopolysaccharide matrix are due to the presence of uronic acids (such as D-glucuronic, D-galacturonic, and mannuronic acids) or ketal-linked pyruvates.⁹ One of the key characteristics of bacteria within biofilms is their enhanced resistance to antimicrobial agents and the host immune system mechanisms. As a result, biofilm-related infections typically have a chronic course and are characterized by significant resistance to therapy. Bacteria living in biofilms can be up to 1000 times more resistant to antibacterial compounds than planktonic bacteria, indicating the presence of specific resistance mechanisms that may differ from those responsible for antimicrobial resistance in planktonic bacteria.¹⁰ Eradicating biofilms within the host is particularly challenging because methods that typically destroy unattached and unaggregated microbes are ineffective because of the physiological and physical barriers within the biofilm. Owing to the resistance of biofilms to antibiotics, the use of antibiotics alone is ineffective for treating biofilm-associated infections.¹¹

It has been shown that phages can penetrate the exopolysaccharide matrix of biofilms. The structural components of phages and their enzymes can degrade the components of the exopolysaccharide matrix.¹² It has been experimentally confirmed that the use of bacteriophages is highly effective against biofilm-forming forms of *K. pneumoniae* and *P. aeruginosa*.^{13,14} Combinations of bacteriophages and antibiotics for the treatment of bacterial infections have attracted increasing attention because of the synergistic effects often observed when they are used together. This synergy has also been found with bacteria in biofilms, as many phages are capable of breaking down the heterogeneous matrix, which typically hinders the penetration of antibiotics into bacteria embedded within the biofilm.¹⁵

One of the most promising directions in the development of phage therapy today is the combined use of bacteriophages with antibiotics of different classes to achieve the phenomenon of phage–antibiotic synergy (PAS). Under this phenomenon, antibiotics at sublethal concentrations can enhance phage particle production and increase their lytic activity. Numerous experimental studies described in the scientific literature have demonstrated that combinations of phages and antibiotics targeting various bacterial species exhibit consistent synergistic effects both in vitro and in vivo.^{16–18} The extent of PAS largely depends on the type of antibiotic, the characteristics of the phage, and the physiological state of the bacterial host. An essential step in the practical implementation of PAS is the selection of bacteriophages with high lytic activity against antibiotic-resistant bacterial strains. The most optimal phages are those that, in addition to potent lytic activity, possess the ability to effectively disrupt bacterial biofilms.

Our previous studies indicate a high prevalence of antibiotic-resistant microorganisms with enhanced biofilm-forming capacity – particularly *K. pneumoniae* and *P. aeruginosa* – among the causative agents of bloodstream infections in children in Ukraine.¹⁹ The next stage of this research involved evaluating the efficacy of bacteriophages isolated from environmental sources against biofilms formed by multidrug-resistant strains of *K. pneumoniae* and *P. aeruginosa* under in vitro conditions.

Materials and Methods

Bacterial Strains

This study used two clinical isolates, *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7, which were isolated from blood samples during routine microbiological screening for bloodstream infections. In preliminary experiments, these isolates showed a strong ability to form biofilms (OD 630 > 0.6 and 0.9, respectively).¹⁹ Microorganisms were identified using MALDI-TOF. The bacterial cultures were maintained under laboratory conditions on nutrient agar (HiMedia®).

Bacteriophages

To investigate the effects of phages on bacterial biofilms, 14 bacteriophages were isolated from municipal wastewater from Kyiv. Two phage cocktails (phage preparations) were formulated from the isolated phages: one was lytically active against *K. pneumoniae* strain No. 361 (comprising nine phages) and the other targeted *P. aeruginosa* strain No. 7 (comprising five phages). Phages were isolated from municipal wastewater by using an enrichment method. For this, 100 mL of pre-centrifuged and filtered wastewater (through membrane filters with a 0.45 µm pore size and low protein-binding capacity) was mixed with 1 mL of the corresponding bacterial culture and 3 g of dry nutrient agar (HiMedia®). The flasks containing the mixture were incubated for 24 h at 36±1°C. After the incubation period, 5% chloroform was added to the flasks, which were then vigorously mixed for 10 min and centrifuged at 3000 g for 10 min. The supernatant was collected and stored at 4°C. The specific activity of the isolated phages was determined using the spot test and the agar overlay method.

After selecting the target bacteriophages, stock suspensions were prepared at a concentration of no less than 10⁸ PFU/mL. For this purpose, 0.05 mL of the corresponding bacteriophage and 5 mL of the bacterial culture at equal initial concentrations (MOI = 0.01) were added to a flask containing 50 mL of nutrient broth (Nutrient Broth, HiMedia®). The resulting mixtures were incubated at 36 ± 1 °C for 20 hours. Following incubation, the clear bacteriophage lysates were combined in equal volumes to obtain two phage cocktails and subjected to preliminary filtration through 0.45 µm pore-size membrane filters (MF-Millipore™, Millex®GS, MCE membrane). To ensure sterility, an additional filtration step was performed using 0.22 µm pore-size filters (MF-Millipore™, Millex®GS, MCE membrane).

Antimicrobial Susceptibility Testing

The susceptibility of the isolated bacterial strains was determined according to the EUCAST and CLSI guidelines using disk diffusion and broth microdilution method.^{20,21}

In-vitro Biofilm Models

Biofilm Formation on Coverslips

For biofilm formation on glass coverslips, 24-hour cultures of *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7 were grown in nutrient broth (HiMedia®). After incubation, bacterial suspensions were adjusted to a concentration of 1×10⁵ CFU/mL. A total of 5 mL of the suspension was transferred to sterile Petri dishes, followed by the addition of 20 mL fresh nutrient broth (HiMedia®). Sterile and degreased glass coverslips were placed in dishes. The Petri dishes were incubated for 24 h at 36±1°C to allow biofilm formation on the coverslip surfaces.

Biofilm Formation in 96-Well Plates

A 24-hour broth culture of *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7 grown in nutrient broth (HiMedia®) was diluted 1:200. Next, 200 µL of the resulting bacterial suspension was added to each well of a sterile 96-well microtiter plate (Thermo Fisher Scientific). Plates were incubated at 36±1°C for 24 h to allow biofilm formation on the well walls.

Biofilm Formation on Catheters

Polyvinyl chloride (PVC) central venous catheters KV-3 (2.0×1.4 mm / F6) were used in the experiments. The catheters were cut into 0.5 cm segments and placed in sterile 96-well polystyrene plates. Each well containing a catheter segment was filled with 200 µL of bacterial suspension prepared as described above. The plates were incubated at 36±1°C for 24 h to facilitate biofilm formation on the catheter surfaces.

Infection of Biofilm with Bacteriophages

Coverslips

After 24 h of biofilm formation on the glass coverslips, the coverslips were washed with sterile phosphate-buffered saline (PBS). For control samples, 25 mL of fresh sterile nutrient broth (HiMedia®) was added. For experimental samples, 25 mL of fresh sterile nutrient broth (HiMedia®) and 0.5 mL of a bacteriophage cocktail targeting the corresponding bacterial strain (with a minimum concentration of 1×10^7 PFU/mL per phage) were added. Samples were then incubated for an additional 24 h at 36±1°C. A control containing only phages and sterile nutrient medium was not included in the experiments.

96-Well Plates

After 24 h of incubation, the contents of the wells were removed and the wells were washed twice with sterile PBS. Fresh sterile nutrient broth (200 µL; HiMedia®) was added to control wells. In the experimental wells, 200 µL of fresh sterile nutrient broth (HiMedia®) containing bacteriophage cocktail ($\geq 1 \times 10^7$ PFU/mL per phage) was added. The plates were incubated for another 24 h at 36±1°C. A control containing only phages and sterile nutrient medium was not included in the experiments.

Polyvinyl Chloride Catheters

After 24 h of incubation, the contents of the wells containing the catheter segments were removed and the wells were washed twice with sterile PBS. For the control wells with catheters, 200 µL of fresh sterile nutrient broth (HiMedia®) was added. To the experimental wells, 200 µL of fresh sterile nutrient broth (HiMedia®) containing the bacteriophage cocktail ($\geq 1 \times 10^7$ PFU/mL per phage) was added. Samples were incubated for an additional 24 h at 36±1°C. A control containing only phages and sterile nutrient medium was not included in the experiments.

Evaluation of Biofilm Formation and Phage Activity

Coverslips

To visualize the effect of bacteriophages on preformed biofilms under a light microscope, coverslips were carefully removed using forceps, washed twice with sterile PBS, and transferred to Petri dishes lined with paper filters without drying. To preserve the native biofilm structure, samples were fixed in 100% methanol for 2 min. Following fixation, the coverslips were stained with 1% crystal violet solution. The effect of the phage cocktail on biofilm formation and disruption was evaluated using a light microscope (AxioPlan; Carl Zeiss, Germany).

96-Well Plates

After 24 h of incubation at 36±1°C, the contents of each well were removed and the wells were washed three times with 300 µL of sterile PBS to eliminate residual planktonic cells. The Bacteria adhered to the well surfaces were fixed by exposure to a stream of hot air at 60°C for 60 min to preserve the biofilm structure. Each well was stained with 200 µL of 1% crystal violet solution for 15 min. After three washes with PBS, the stain was extracted with 200 µL 95% ethanol. The optical density (OD) was measured using a microplate reader (Humareader) at a wavelength of 630 nm. The biofilm biomass values from the control and experimental wells were compared.

Polyvinyl Chloride Catheters

After 24 h of incubation at 36±1°C, the contents of the wells containing the catheter segments were removed and the wells were washed three times with 300 µL of sterile PBS. The Biofilms on the catheter surfaces were fixed by exposure to a stream of hot air at 60°C for 60 min. Control and experimental catheter segments were stained with 200 µL 1%

crystal violet solution for 15 min. After three washes with PBS, catheter segments were transferred to a new 96-well plate. The stain was extracted using 200 μ L 95% ethanol. The optical density (OD) was measured using a microplate reader (Humareader) at a wavelength of 630 nm. Biofilm biomass values were compared between control and phage-treated catheter samples.

Transmission Electron Microscopy

Formvar was used as the substrate film on the grids. The samples were contrasted with 2% phosphotungstic acid (pH 6.8) for an exposure time of 1–2 minutes. The bacteriophage titer was at least 10^9 PFU/mL. Bacteriophage samples were applied dropwise to the substrate films. The prepared specimens were examined using a JEOL JEM 1230 transmission electron microscope.

Scanning Electron Microscopy

To fix the samples under study, a 4F:1G fixative (McDowell and Trump, 1976) was used.²² The Samples were placed in a fixative overnight at 4°C. After fixation, samples were rinsed with phosphate buffer. Sputtering was performed using platinum and palladium alloys (80:20). The prepared specimens were examined using a JEOL JSM-6060LA scanning electron microscope.

Gene detection of biofilm formation and antibiotic resistance

This was performed using the PCR method. In *P. aeruginosa*, biofilm formation genes *algD*, *PslD*, and *PelF* were detected. In *K. pneumoniae*, the biofilm genes *fimH*, *mrkA*, and *matBecp* were analyzed. In *P. aeruginosa*, resistance genes *bla_{NDM-1}*, *bla_{IMB}*, *bla_{VIM}*, *bla_{TEM}* were detected in *K. pneumoniae*, whereas the resistance genes *bla_{NDM-1}*, *bla_{KPC}*, *bla_{OXA-48}*, *bla_{CTX-M-1}*, *bla_{TEM}*, *qnrB*, and *gyrA* were examined.¹⁹

Bacterial DNA was extracted using an enzymatic method using an ExToPCR kit (A&A Biotechnology). Each reaction mixture, with a final volume of 25 μ L, contained 1 μ g DNA, 12.5 μ L PCR Mix Plus Green (A&A Biotechnology, Poland), 1 μ M of each forward and reverse primer (10 pmol/ μ L), and ultrapure water to a total volume of 25 μ L.

PCR conditions included: an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30s, primer annealing at 52–56 °C for 30–60 seconds, elongation at 72 °C for 45s, and a final elongation at 72 °C for 10 min.

The amplification products were analyzed under UV light after electrophoresis on a 1.5% agarose gel at 80 V for 30 min.

Statistical Analysis

Statistical significance of all results was assessed using one-way analysis of variance (ANOVA). Statistical significance was set at a p-value < 0.05. The normal distribution of samples within each group was verified using the Shapiro–Wilk test. All experiments were repeated three times and performed in triplicates. The mean values of the repetitions were calculated using standard deviation (SD).

Results

In the preliminary stage of the study, a collection of microorganisms was isolated from bloodstream infections that exhibited varying degrees of biofilm formation and antibiotic resistance. To further investigate the potential use of bacteriophages against biofilm-forming antibiotic-resistant strains, two isolates were selected: *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7. These microorganisms were resistant to all antibiotics used in the study. Antimicrobial susceptibility results are shown in Table 1.

Biofilm formation in 96-well plates demonstrated that both the strains were capable of strong biofilm formation. The PCR results revealed that *K. pneumoniae* contained the antibiotic resistance genes *bla_{NDM-1}*, *bla_{KPC}*, *bla_{OXA-48}*, *bla_{CTX-M-1}*, *bla_{TEM}*, as well as the biofilm formation genes *fimH*, *mrkA*, *matBecp*. *P. aeruginosa* No. 7 contained the resistance gene *bla_{NDM-1}* and the biofilm formation genes *algD*, *PslD*, and *PelF* (Figure 1).

Fourteen bacteriophage strains were selected from the bacteriophage collection of the Department of Microbiology and Parasitology with Basics of Immunology at Bogomolets National Medical University, based on their high lytic activity against clinical isolates *K. pneumoniae* strain No. 361 (nine bacteriophages) and *P. aeruginosa* strain No. 7 (five

Table 1 Antibiotic Susceptibility of Isolated Bacterial Cultures

Antibiotic	<i>K. pneumoniae</i> strain No. 361	<i>P. aeruginosa</i> strain No. 7
Amoxicillin/clavulanic acid	R	–
Cefepime	R	R
Meropenem	R	R
Amikacin	R	R
Trimethoprim/sulfamethoxazole	R	–
Ceftazidime	R	R
Ceftazidime/avibactam	R	–
Ceftriaxone	R	–
Imipenem	R	–
Ertapenem	R	–
Tobramycin	R	–
Colistin	R	–
Piperacillin/tazobactam	R	–
Piperacillin	–	R
Aztreonam	–	R
Ciprofloxacin	–	R
Levofloxacin	–	R
Norfloxacin	–	R
Gatifloxacin	–	R

Note: R – Resistant, – – not tested.

bacteriophages). The selected phages belong to three morphotypes: Myo-like, Sipho-like, and Podo-like (Figure 2). Two bacteriophage cocktails were formulated from the selected phages that were specifically active against *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7.

The experimental studies indicated pronounced antibiofilm activity of the applied phage cocktails after 24-hour treatment of preformed biofilms.

After 24 h of incubation on coverslips without the bacteriophage cocktail, microcolonies of bacterial cultures *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7 were observed covering the entire surface of the coverslips (Figure 3A). In samples with preformed biofilms treated with bacteriophage cocktails for 6 h, only individual bacterial cells and small cell aggregates were observed (Figure 3B).

After 24 h of incubation, mature biofilms formed by *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7 were observed on the control samples of the central venous catheters, which stained well with crystal violet. After 48 h, the biofilm formation intensified. In contrast, only isolated biofilm fragments were observed in the experimental catheters treated with bacteriophages after an additional 24-hour incubation. The antibiofilm activities of the formulated bacteriophage cocktails were similar for *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7. This pattern was confirmed using scanning electron microscopy (Figure 4).

To investigate whether phage cocktails can reduce the biomass and viability of 24-hour biofilms formed by *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7 on catheters and in the wells of polystyrene plates, quantitative crystal violet assays were performed after 24-hour treatment with the phage cocktails.

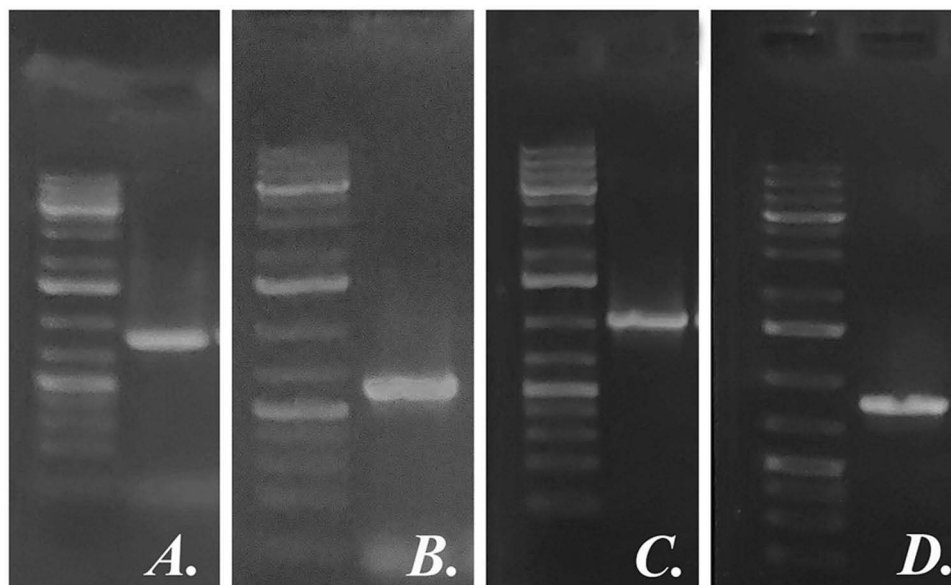


Figure 1 Investigation of the presence of antibiotic-resistance and biofilm-formation genes in *K. pneumoniae* strain No. 361: (A) *fimH*; (B) *bla*_{NDM-1}; (C) *bla*_{KPC}; (D) *bla*_{TEM}. DNA size marker – GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific™).

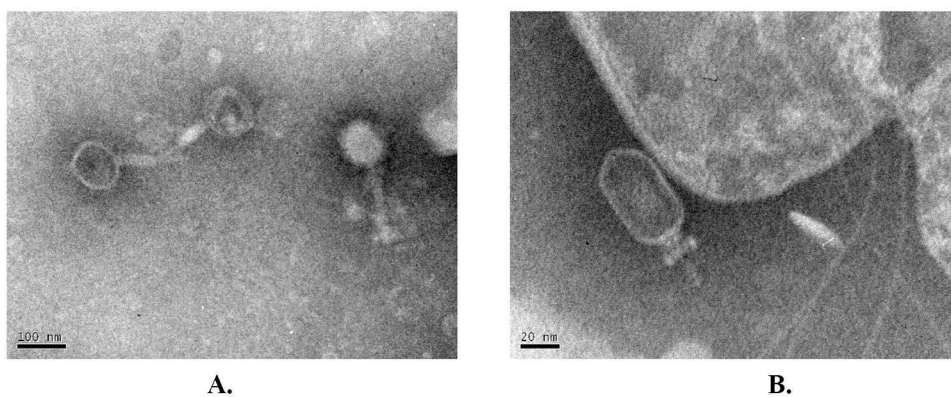


Figure 2 Morphology of the bacteriophages used in this study: (A) phages active against *P. aeruginosa* (Sipho-like morphotype); (B) Phages active against *K. pneumoniae* (Podo-like morphotype).

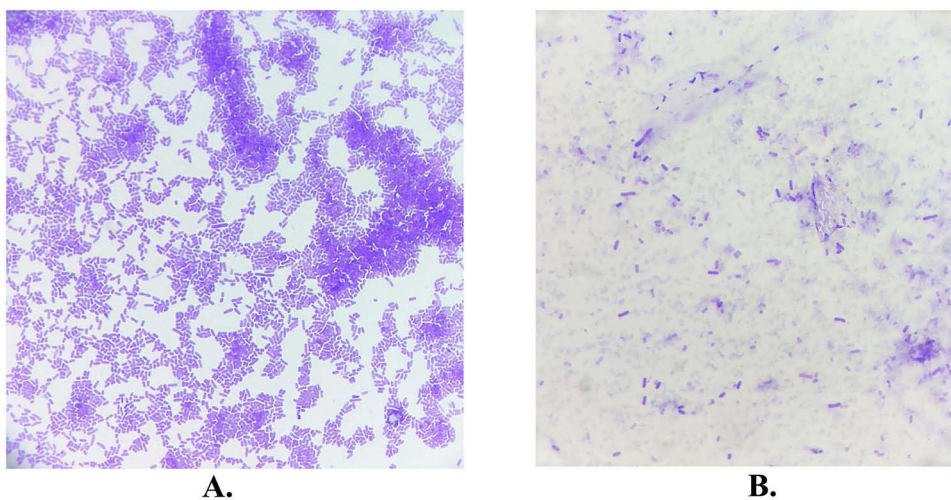


Figure 3 Biofilm formation by *K. pneumoniae* strain No. 361 on coverslips: (A) control, 24-h incubation; (B) preformed biofilm after 6-h exposure to the phage cocktail. Bright-field microscopy, 900× magnification.

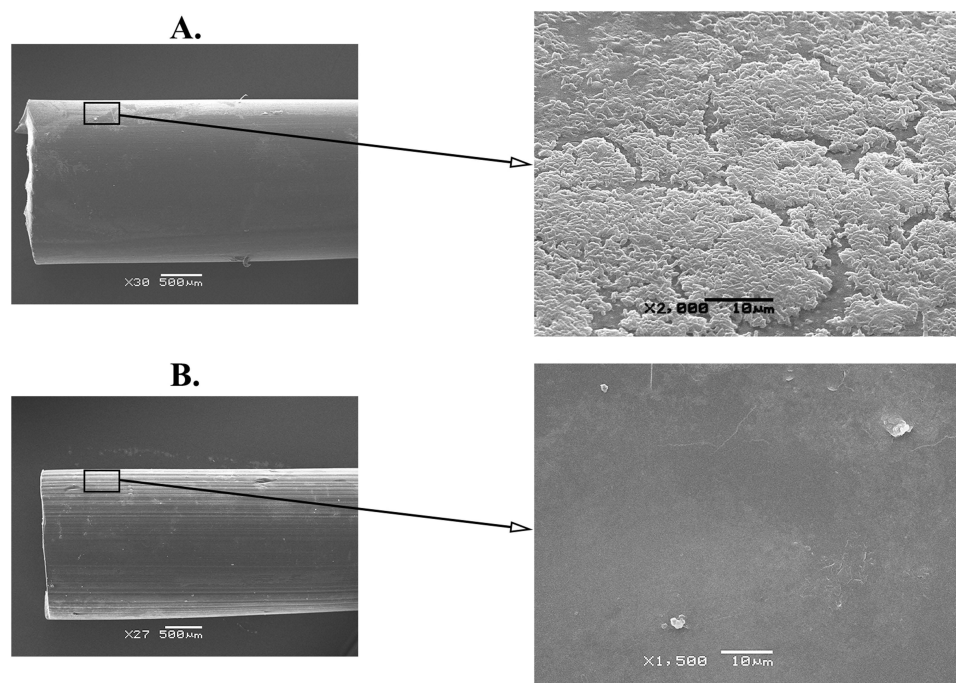


Figure 4 Effect of the phage cocktail on *P. aeruginosa* strain No. 7 biofilms. Scanning electron microscopy: (A) control of the formed biofilm; (B) treatment of the formed biofilm with the phage cocktail for 24 h.

Phage cocktails with a concentration of each phage no less than 10^7 PFU/mL significantly reduced the biomass of *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7 compared with the control sample without treatment. In polystyrene plates, the biofilm biomass decreased by 39.3% and 52.8%, respectively. Treatment of biofilms on catheters with phage cocktails showed that the phages led to a reduction in biofilms by 34.5% for *K. pneumoniae* strain No. 361 and 34.1% for *P. aeruginosa* strain No. 7 (Figure 5).

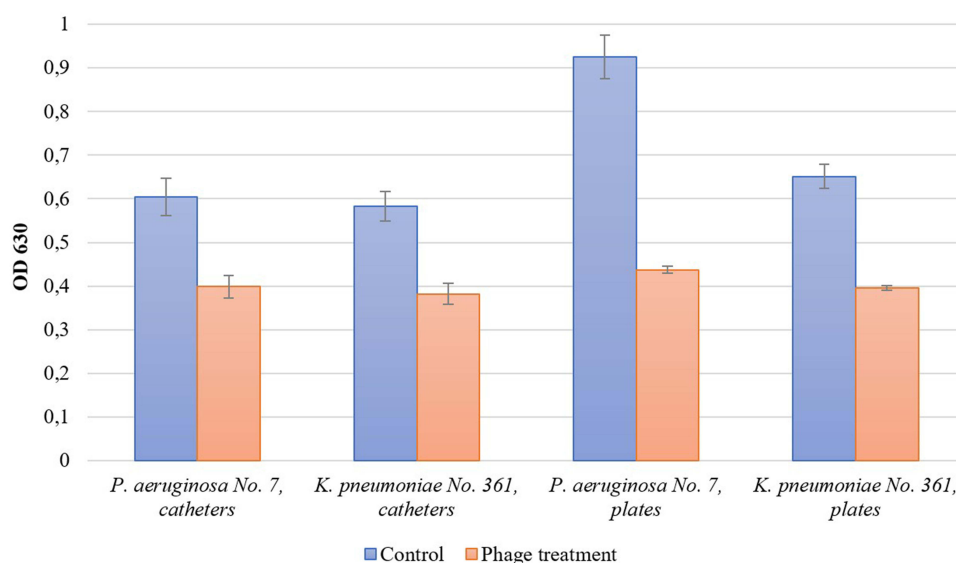


Figure 5 Effect of phage cocktails on established static biofilms on plates and catheters. Phage activity was assessed using biofilm assays and optical density (OD 630) measurements after 24 h of incubation with the phage cocktails. Statistically significant differences between treated and untreated biofilms were determined ($p < 0.05$).

Discussion

This study assessed the potential use of phage cocktails against Extensively Drug-Resistant (XDR) strains of *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7, which demonstrated a strong ability to form biofilms. Since biofilms are a crucial component in the development of many infections, including bloodstream infections, and significantly complicate antibiotic treatment, the results of this study highlight the need for the exploration of new therapeutic strategies. Phage therapy is an effective tool for combating biofilms formed by nosocomial strains of multidrug-resistant bacteria.²³ This finding is supported by numerous experimental studies.^{7,24} Currently, there is active research on the potential use of bacteriophages in combination with other antimicrobial agents including antibiotics. This strategy has also shown promising results.^{25,26}

We isolated 14 bacteriophages from Kyiv wastewater, which demonstrated high lytic activity against clinical isolates of *K. pneumoniae* (nine phages) and *P. aeruginosa* (five phages) with multidrug resistance. The use of monocomponent bacteriophage solutions can lead to the development of resistance to bacterial cultures. Additionally, it has been shown that bacterial strains, previously resistant to a specific phage, can regain sensitivity after developing resistance to an unrelated phage.²⁷ One approach to overcome this issue is to formulate mixtures of different bacteriophages known as phage cocktails. This approach was used in this study. Based on the isolated phages, two cocktails were formed that were active against *K. pneumoniae* strain No. 361 (nine phages) and *P. aeruginosa* strain No. 7 (five phages).

The results of this experimental study indicated the high efficacy of phages in combating biofilms formed by antibiotic-resistant microorganisms. This finding is supported by data from other studies. For example, in the study by Manohar et al, it was shown that the *Pseudomonas* *Motto* phage exhibited significant antibiofilm activity both in vitro and in vivo. The phage demonstrated pronounced degradation activity against 35 isolates with strong biofilm-forming abilities, resulting in at least a two-fold reduction in the biofilm mass within 24 hours.¹³ There are also reports that indicate a lack of biofilm-degrading activity. For example, Melo et al demonstrated that SEP1 phages, active against *Staphylococcus epidermidis*, are capable of lysing planktonic bacterial cells in various physiological states but proved ineffective against biofilms. To assess the impact of biofilm architecture and the role of phage enzymes in phage activity, SEP1 bacteriophages were tested on damaged biofilms. This resulted in a two-log reduction in the number of viable bacterial cells after 6 hours of infection.²⁸ Lysins and depolymerases are powerful enzymes that contribute to biofilm disruption and selective bacterial destruction. Depolymerases, found in phages, are capable of breaking down the extracellular matrix of bacteria, as these substances are abundantly present in the composition of biofilms.²³ Considering the mechanism of action of phage depolymerases, they can be divided into two classes—hydrolases and lyases. Both groups target the degradation of polysaccharides, including capsular polysaccharides, lipopolysaccharides, and exopolysaccharides produced as part of the biofilm matrix. It has also been noted that some of these enzymes are capable of cleaving polypeptides or lipids.²⁹ These bacteriophage-derived enzymes weaken the biofilm structure, allowing phages to penetrate and disperse it, while simultaneously leading to the lysis of bacterial cells and/or the release of individual bacteria, thereby increasing their susceptibility to the action of other antimicrobial agents.³⁰ This highlights the importance of assessing the antibiofilm activity of bacteriophages as a treatment for infections accompanied by intense biofilm formation.

In addition to studying the biofilm-degrading activity of phage cocktails on polystyrene plates, we also used polyvinyl chloride central venous catheters. The choice of this object was based on the fact that the development of catheter-associated bloodstream infections depends on various factors including biofilm formation on the catheter surface. These biofilms enable bacterial cells to survive in the presence of antimicrobial agents and protective mechanisms of the host's immune system.³¹ The obtained experimental data indicated the active degrading activity of the phage cocktails against the formed biofilms of *K. pneumoniae* strain No. 361 and *P. aeruginosa* strain No. 7 on polyvinyl chloride central venous catheters (KV-3). Our findings are consistent with the literature, confirming that bacteriophages can significantly weaken biofilms formed by antibiotic-resistant bacteria on catheters. Curtin et al demonstrated that treatment of silicone catheters with a hydrogel coating of *S. epidermidis* phage in an in vitro model system significantly reduced the formation of viable *S. epidermidis* biofilms during a 24-hour exposure.³² This pattern was also confirmed using a model of biofilms formed by MDR *Providencia stuartii* on urinary catheters.³³

Given the effectiveness of phage cocktails in reducing biomass and bacterial viability, further research should focus on identifying specific phages to treat infections caused by multidrug-resistant pathogens that form biofilms. One promising direction is the combination of phages with other therapeutic strategies including antibiotics and immunomodulatory agents. This could significantly improve the treatment effectiveness and reduce the risk of developing resistance to antibiotics and phages. A number of experimental studies have indicated the high effectiveness of this strategy.^{34,35}

Additionally, it is important to study in detail the impact of phage cocktails on dynamic biofilms. Literature suggests that dynamic biofilms have a substantial impact on the apparent effectiveness of treatment, as they may be more resistant to phage therapy than static biofilms.²⁶

Despite the encouraging results, this study has several limitations. Only two clinical isolates were tested, which restricts the generalizability of the findings, as microbial variability may significantly influence phage susceptibility and the efficiency of biofilm disruption. Furthermore, biofilms were formed exclusively under in vitro conditions, which, although standardized, do not fully replicate the complexity of the in vivo environment, including immune responses, blood flow dynamics, and serum components. In addition, the investigation focused solely on 24-hour biofilms representing early stages of development, while mature biofilms (3–5 days) with multilayered matrices and increased antimicrobial tolerance were not examined, limiting extrapolation to clinical scenarios involving long-term colonization of medical devices. Finally, potential interactions between bacteriophages and antibiotics or antiseptics were not assessed, precluding insights into possible synergistic effects of combined therapies.

Conclusion

Our results confirmed the high potential of bacteriophages to combat biofilms formed by multidrug-resistant bacteria, such as *K. pneumoniae* and *P. aeruginosa*. Given the effectiveness of phages in disrupting biofilms, phage therapy could become a promising addition to the traditional methods of treating infections, particularly those caused by multidrug-resistant pathogens.

Ethics Approval and Consent to Participate

This study was conducted as part of a doctoral dissertation with the approval of the Commission on Bioethical Expertise and Research Ethics at Bogomolets National Medical University in accordance with the principles of the Declaration of Helsinki.

Disclosure

The author(s) report no conflicts of interest in this work.

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