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APPLICATION OF BACTERIOPHAGES AGAINST MULTIDRUG-RESISTANT STRAINS OF KLEBSIELLA PNEUMONIAE

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Background. The emergence of antimicrobial-resistant microorganisms poses a serious global public health challenge. *Klebsiella pneumoniae* is among the most common pathogens responsible for healthcare-associated infections, particularly in critically ill patients. The prevalence of multidrug-resistant (MDR) *K. pneumoniae* has increased dramatically worldwide over recent decades, presenting an urgent threat to public health. In the absence of effective treatments for severe bacterial infections caused by antibiotic-resistant strains, bacteriophages represent a targeted and promising adjunct – and in some cases, an alternative – to conventional therapy.

Aim: to explore the possibility of isolating bacteriophages against PDR (pan-drug resistant)/XDR (extensively drug-resistant) strains of *K. pneumoniae* using urban wastewater samples, and to investigate the spectrum of their activity under *in vitro* conditions.

Materials and Methods. Bacteriophages were isolated using the enrichment method, and their specificity was evaluated using a modified Gratia method and the spot test. Morphological characterization of the isolated phages was performed via electron microscopy. Antimicrobial susceptibility testing was conducted using disk diffusion and broth microdilution methods. Detection of antibiotic resistance genes (*blaNDM-1, blaKPC, blaCTX-M-1*, and *gyrA*) was performed by PCR.

Results. A total of 212 bacteriophages were isolated from municipal wastewater samples, demonstrating lytic activity against a broad spectrum of clinical isolates and reference strains of *K. pneumoniae*, including antibiotic-resistant variants. Specifically, 56.1% of the phage isolates showed specific activity against a pre-characterized panel of 15 PDR/XDR K. pneumoniae strains. Notably, each clinical isolate with acquired extensive drug resistance was susceptible to multiple phages, and 14 phages lysed more than 50% of the tested bacterial cultures. The phages exhibited both narrow and broad host ranges, supporting the formulation of effective phage cocktails for potential therapeutic use.

Conclusions. The findings demonstrate the high potential of bacteriophages as an alternative or adjunctive strategy in combating multidrug-resistant *K. pneumoniae*. These experimental results highlight the importance of further development of phage therapy approaches, especially in the context of escalating multidrug-resistant infections.

Keywords: Klebsiella pneumoniae, antibiotic resistance, phage therapy, phage cocktail, isolation.

Background. *Klebsiella pneumoniae* is a Gram-negative, capsulated opportunistic bacteria belonging to the large *Enterobacteriaceae* family. Various species of the *Klebsiella* genus are capable of causing infections at different anatomical sites, including the lungs, urinary tract, bloodstream, surgical wounds, soft tissues, and the central nervous system. These infections primarily occur in patients with underlying health conditions or immunosuppression. The prevalence of *K. pneumoniae* in healthcare-associated infections has been reported to reach up to 10% [1]. Currently, *K. pneumoniae* is considered one of the major pathogens of global concern due to the emergence of hypervirulent and carbapenem-resistant strains [2].

In recent years, the extensive use of antimicrobial agents has exacerbated the issue of resistance among *Klebsiella* spp., with antimicrobial resistance progressing from multidrug resistance (MDR) to extensively drug-resistant (XDR) and even pan-drug-resistant (PDR) phenotypes [3]. A recent meta-analysis indicated that carbapenem-resistant strains accounted for 28.69% of *Klebsiella*-associated infections [4]. According to the CAESAR (Central Asian and Eastern European Surveillance of Antimicrobial Resistance) network, the proportion of carbapenem-resistant *K.pneumoniae* isolates in Ukraine increased from 27.6% in 2017 to 64.4% in 2021. Resistance to third-generation cephalosporins rose from 56.7% to 89.9% over the same period [5]. In addition to the growing presence of antibiotic-resistant *K. pneumoniae* in clinical settings, there has been an increasing number of reports of MDR K.pneumoniae being isolated from rivers, coastal areas, municipal wastewater, and other environmental sources [6, 7].

The lack of effective treatment options for infections caused by multidrug-resistant organisms necessitates the exploration of alternative and adjunctive therapeutic strategies. Among these, bacteriophage therapy stands out as a promising option for combating antibiotic-resistant bacteria. Bacteriophages (phages) are viruses that specifically target and infect bacteria at the species or even strain level. They have been employed in the treatment of bacterial infections since their discovery in the early 20th century [8].

Environmental isolation of bacteriophages plays a crucial role in current research across medicine, biotechnology, and ecology, as naturally occurring phages are often used in the development of therapeutic and diagnostic applications. Municipal wastewater and soil samples are among the most common sources of phage isolation [9].

In this study, we investigated the possibility of isolating bacteriophages from urban wastewater samples that are active against multidrug-resistant clinical isolates of *K. pneumoniae* and their potential use for combating PDR/XDR *K. pneumoniae* strains under in vitro conditions.

MATERIALS AND METHODS

Bacterial Cultures. To assess the potential use of bacteriophages against antibiotic-resistant microorganisms, 15 clinical isolates of *K.pneumoniae* with varying degrees of antibiotic resistance were selected. These isolates originated from various clinical specimens including blood, urine, wound exudates, and lavage fluids. In the laboratory, cultures were maintained on tryptic soy agar (Merck), while long-term storage was performed using tryptic soy broth supplemented with 10% glycerol at – 80 °C.

Bacteriophages. A bacteriophage collection was isolated from municipal wastewater samples collected in Kyiv, Ukraine. Reference and clinical strains of *K.pneumoniae* were used as host bacteria. The collection of microorganisms used for phage isolation was gathered over three calendar years (2021–2023) from various healthcare facilities in Kyiv. From the urban wastewater samples, we isolated and described 212 bacteriophages that demonstrated lytic activity against 207 reference strains and clinical isolates of *K. pneumoniae* with varying degrees of antibiotic resistance. Each of the 207 bacterial strains tested was susceptible to at least one phage from the created collection, indicating the feasibility of isolating phages from the environment that are capable of infecting a wide range of *K. pneumoniae* isolates with different capsular types and cell wall receptors.

The isolation of bacteriophages was carried out as follows: wastewater samples were centrifuged at 3000 rpm for 10 minutes and filtered through 0.22 μm pore size membranes (MF-Millipore™, Millex® GS MCE Membrane) to remove large particles and bacterial cells.

Next, 1 mL of a *K. pneumoniae* culture in ear-

ly logarithmic growth phase and 3 g of tryptic soy agar (Merck) were added to 100 mL of the prepared wastewater sample to support bacterial growth. The mixture was incubated at 37 °C for 24 hours. After incubation, the culture was centrifuged at 4000 × g for 20 minutes at 4 °C. The resulting supernatant was re-filtered through a 0.22 μm membrane to eliminate any remaining bacterial cells.

The presence of bacteriophages in the supernatant was assessed using the single-layer agar method. The appearance of clear plaques indicated lytic phage activity. Individual plaques were isolated for subsequent amplification. Purification steps were repeated at least three times to obtain pure phage lines. Phage suspensions were stored at 4 °C.

Antimicrobial Susceptibility Testing. Antimicrobial susceptibility of the isolated bacterial strains were determined using the disk diffusion method and broth microdilution, performed on the VITEK® 2 Compact system in accordance with EUCAST guidelines [10].

Detection of Resistance Genes. To identify antibiotic resistance genes in *K.pneumoniae* (*blaNDM-1, blaKPC, blaCTX-M-1*, and *gyrA*), specific primers (Table 1) were used. Bacterial DNA was extracted enzymatically using the ExToPCR commercial kit (A&A Biotechnology, Poland). Each 25 μL PCR reaction mixture contained: 1 μg of DNA template, 12.5 μL of PCR Mix Plus Green (A&A Biotechnology, Poland), 1 μM of each forward and reverse primer (final concentration: 10 pmol/μL), and ultrapure water to a total volume of 25 μL.

PCR cycling conditions included an initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of: denaturation at 95 °C for 30 seconds, primer annealing at 52–56 °C for 30–60 seconds, and extension at 72 °C for 45 seconds. A final extension was performed at 72 °C for 10 minutes.

Amplification products were analyzed under UV illumination using a transilluminator following electrophoresis on 1.5% agarose gel at 80 V for 30 minutes.

Electron Microscopy. The size and morphology of the phages were examined using transmission electron microscopy (TEM). All analyses were conducted at the Electron Microscopy Laboratory of the Department of Microbiology and Parasitology with Basics of Immunology, Bogomolets National Medical University, Ministry of Health of Ukraine, as well as at the Shared Research Facilities Center for Electron Microscopy of the National Academy of Sciences of Ukraine.

Phage suspensions were prepared in SM buffer at a concentration of no less than 10^9 PFU/mL. Enriched phage particles were purified using PEG/NaCl precipitation [15]. Support films on grids were prepared using formvar-coated copper grids. The phage samples were applied onto the film by dropwise deposition. Negative staining was performed using a 2% phosphotungstic acid solution (pH 6.8). The exposure time in the staining solution was 1–2 minutes. Morphological characteristics of phages active against *K.pneumoniae* were examined using a JEOL JEM-1230 transmission electron microscope.

RESULTS

Our preliminary studies indicate the growing role of K.pneumoniae in the etiology of infectious diseases among hospitalized patients in Ukraine. Strains of *K.pneumoniae* exhibiting multidrug resistance are being isolated with increasing frequency, prompting the need for alternative and adjunctive approaches to combat antibiotic-resistant microorganisms.

In the initial phase of the experimental study, 15 bacterial isolates of *K.pneumoniae* with PDR/XDR phenotypes were selected. The results of antimicrobial susceptibility testing are presented in Table 2.

Table 1

Primers used for PCR detection of antibiotic resistance genes in clinical K.pneumoniae isolates

Gene	Primer (5´ to 3´)	Size of amplicon	Reference
blaNDM-1	GGTTTGGCGATCTGGTTTTC	621 bp	[11, 12]
	CGGAATGGCTCATCACGATC		
blaKPC	TGTCACTGTATCGCCGTC	1100 bp	[11]
	GTCAGTGCTCTACAGAAAACC		
blaCTX-M-1	AAAAATCACTGCGCCAGTTC	415 bp	[13]
	AGCTTATTCATCGCCACGTT		
gyrA	AATGAACAAGGTATGACACC	368 bp	[14]
	GCGATACCTGATGCACCATT		

Table 2

Antimicrobial susceptibility of clinical K.pneumoniae isolates

Clinical isolate / Antibiotic	1538/22	1299/22	1395/22	234/23	1409/22	1266/22	828	314a	1403/22	16a	1422/22	135/22	1279/22	1545/22	1513/22
Ampicillin-sulbactam	R	R	R	–	R	R	R	R	R	R	R	R	R	R	R
Amoxicillin/ clavulanate	R	R	–	R	R	R	R	R	R	R	R	R	R	R	R
Piperacillin/ tazobactam	R	R	–	R	R	R	–	R	R	R	R	R	R	–	R
Cefepime	R	R	R	–	R	R	R	R	R	R	R	R	R	R	R
Ceftazidime/ avibactam	R	S	–	R	R	R	R	R	R	R	S	S	S	R	R
Ceftazidime	R	R	R	R	R	R	–	R	R	R	R	R	R	R	R
Cefuroxime	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefoperazone/ sulbactam	–	R	–	R	R	R	R	R	R	R	R	–	R	R	–
Cephalexin	R	R	–	R	R	R	R	R	R	–	R	R	R	–	R
Cefazolin	R	R	R	–	R	R	–	R	R	R	R	R	R	R	R
Ceftriaxone	R	R	–	–	R	R	R	R	R	–	R	R	R	–	R
Ertapenem	R	R	R	R	R	R	–	R	R	–	R	R	R	R	R
Meropenem	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I
Imipenem	R	–	–	R	–	–	–	R	–	R	–	R	–	–	R
Aztreonam	R	R	R	R	S	R	–	R	R	R	R	R	R	R	R
Ciprofloxacin	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
Levofloxacin	R	R	R	–	R	R	R	R	R	R	R	R	R	R	R
Moxifloxacin	R	R	–	–	R	R	R	–	R	R	R	R	R	–	R
Norfloxacin	R	R	R	–	R	R	–	R	R	R	R	R	R	–	R
Ofloxacin	R	R	–	–	R	R	–	–	R	R	R	R	R	R	R
Amikacin	R	S	R	R	R	R	S	–	R	–	R	S	S	R	R
Gentamicin	R	I	–	R	R	R	S	–	R	–	R	R	I	R	R
Netilmicin	–	S	–	–	R	R	–	R	R	R	R	–	S	–	–
Tobramycin	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
Tigecycline	R	S	S	–	S	S	S	R	S	R	S	R	R	S	R
Chloramphenicol	R	R	R	–	S	S	R	R	R	S	R	R	R	R	S
Co-trimoxazole	R	R	–	R	R	R	R	R	R	R	R	I	S	R	R

Note:
S – Susceptible, standard dosing regimen.
R – Resistant.
I – Susceptible, increased exposure.
«-» – Not tested

Additionally, specific genes associated with certain resistance mechanisms were identified in the selected strains using polymerase chain reaction (PCR). This allowed us to establish that 93.3% of the strains carried the blaCTX-M gene, which encodes the CTX-M extended-spectrum beta-lactamase (ESBL). Furthermore, the blaNDM gene, which encodes one of the most clinically significant enzymes, metallo-beta-lactamase (MBL), was detected in 73.3% of the isolates, conferring high resistance to carbapenems. Additionally, the blaKPC gene, responsible for carbapenem resistance, was found in 33.3% of the K. pneumoniae isolates. Resistance-associated genes to fluoroquinolones were detected in 40% of the strains (Fig. 1, Table 3).

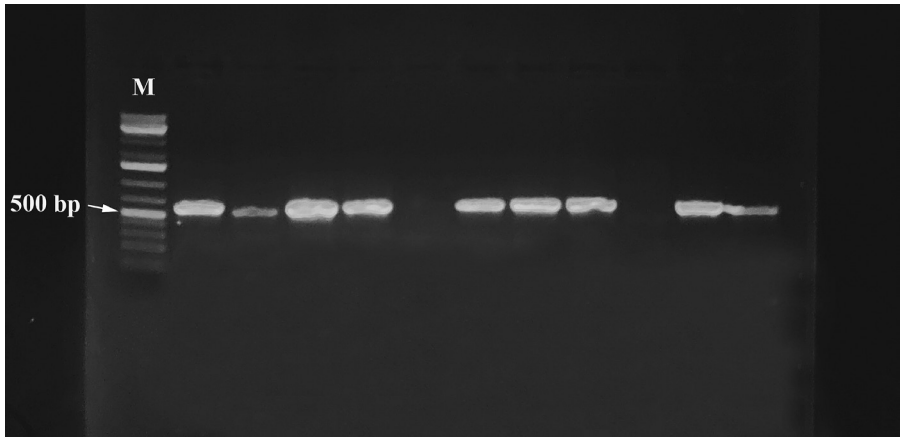

Fig. 1. Detection of blaNDM genes in PDR/XDR isolates of Klebsiella pneumoniae using PCR

Table 3

Antibiotic resistance genes in clinical isolates of K.pneumoniae

Gene / Strains	blaNDM	blaKPC	blaCTX-M	gyrA
1538/22	+	+	+	–
1299/22	+	+	+	+
1395/22	+	–	+	–
234/23	+	–	+	+
1409/22	+	–	–	–
1266/22	+	+	+	–
828	–	–	+	+
314a	+	–	+	–
1403/22	+	+	+	+
16a	+	–	+	+
1422/22	–	–	+	+
135/22	–	–	+	–
1279/22	–	+	+	–
1545/22	+	–	+	–
1513/22	+	–	+	–
Total	**73,3 %**	**33,3 %**	**93,3%**	**40 %**

The created phage collection was tested against 15 specifically selected and characterized PDR/XDR isolates of *K.pneumoniae*. Using a modified spot test, it was determined that 56.1% (119 out of 212) of the phage isolates from the collection exhibited specific activity against the tested PDR/XDR *K.pneumoniae* isolates. The lytic activity of the phages ranged from 6.7% to 73.3% (Fig. 2).

Phages with a broad host range are generally considered more beneficial for phage therapy than those with a narrow host range, as they enable simultaneous targeting of multiple bacterial strains. Among the 119 bacteriophages from the collection that were lytically active against PDR/XDR isolates of *K. pneumoniae*, 46 demonstrated specific activity only against individual strains. Fourteen bacteriophages were able to lyse more than 50% of the PDR/XDR K. pneumoniae strains used in the study. Conversely, each of the PDR/XDR isolates of K. pneumoniae was susceptible to multiple bacteriophages (ranging from 4 to 42 phages).

Currently, so-called "phage cocktails" are most commonly used in medicine. These are mixtures of several bacteriophages intended for the treatment or prevention of bacterial infections [16]. The pattern of K. pneumoniae susceptibility to bacteriophages that we established underscores the necessity of including multiple phage isolates with lytic activity against the same bacterial strain when formulating phage cocktails. Furthermore, phages within cocktails may exhibit a synergistic effect, further enhancing their efficacy.

During the assessment of the specific activity of various bacteriophages against PDR/XDR K. pneumoniae isolates, no correlation was found between bacterial susceptibility to phages and the presence of specific antibiotic resistance genes. This finding supports the feasibility of using a single phage cocktail to combat different strains of K. pneumoniae with diverse antibiotic resistance profiles.

The use of electron microscopy allowed us to determine that the bacteriophages active against PDR/XDR strains of Klebsiella pneumoniae exhibited different morphological types (Fig. 3). The most common variant was the Myo-type phage morphotype.

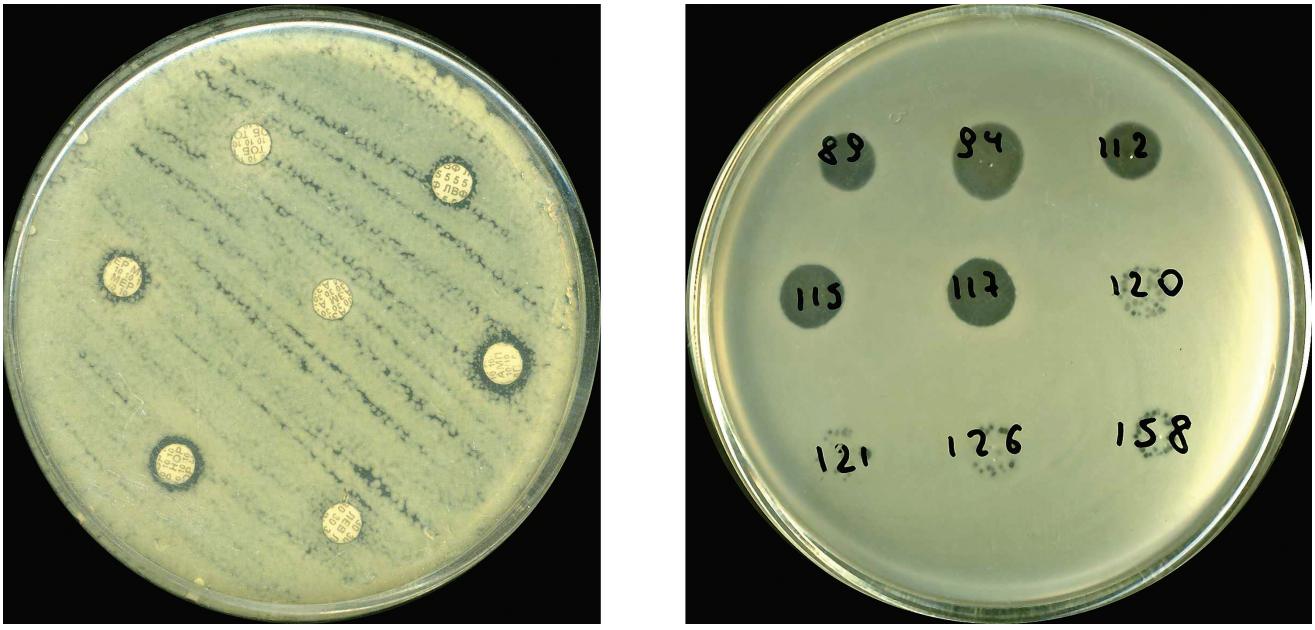

Fig. 2. Sensitivity of strain 1395/22 to antimicrobial agents and bacteriophages:
a – Resistance to antimicrobial agents; b – Sensitivity to bacteriophages as determined by the small drop method

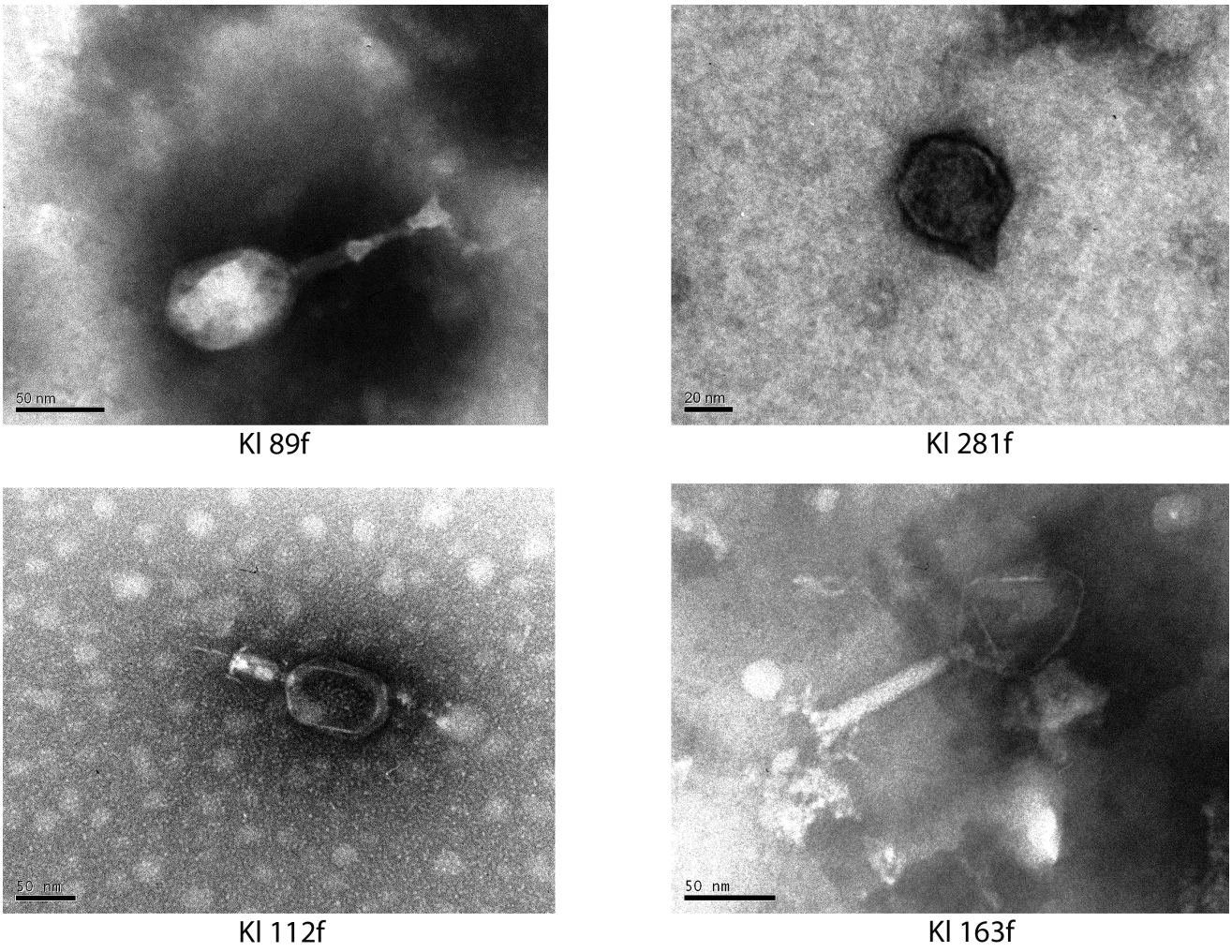

Fig. 3. Morphological forms of bacteriophages active against PDR/XDR strains of *K.pneumoniae*

DISCUSSION

Recently, due to the growing number of infections caused by bacteria such as *Klebsiella pneumoniae*, bacteriophages have reemerged as a potential supplement to modern antimicrobial agents. In this study, Klebsiella-specific phages were isolated from samples of municipal wastewater in Kyiv (Ukraine). The activity of these phages was subsequently tested against PDR/XDR strains of *K. pneumoniae*.

The results of our experimental studies indicate that Klebsiella-specific phages are quite prevalent in municipal wastewater. We successfully isolated phages that were active against all 15 PDR/XDR strains of *K. pneumoniae* used in the experiment. The possibility of isolating bacteriophages from wastewater samples that are specific to MDR/XDR/PDR strains of *K. pneumoniae* has been confirmed by several other researchers [17, 18]. For instance, Martins WMBS and colleagues, using wastewater from various parts of the world, obtained a set of highly effective phages against XDR *K. pneumoniae* ST16 [19]. Thus, municipal wastewater can serve as a source for isolating phages active against clinical isolates of *K. pneumoniae* with varying levels of antibiotic resistance.

Some phages have a polyvalent host range, allowing them to infect many microbial strains, while others exhibit more narrow specificity, infecting only certain strains. This is primarily due to the receptor specificity of the phages. Phage adsorption

to the host cell can occur through various external structures, depending on the type of phage and host; in Gram-negative bacteria such as *K.pneumoniae,* receptors may include capsules, pili, outer membrane proteins, sugar fragments, or lipopolysaccharides (LPS) [20]. This process determines the range of hosts, i.e., the spectrum of bacteria that a particular phage can infect. In this study, 38.7% of the isolated phages showed activity against only specific bacterial strains, which confirms the mosaic antigen-receptor structure of Klebsiella spp. The narrow host range of a significant portion of the phages isolated in our study that were active against K.pneumoniae aligns with the findings of other researchers. For example, Celia Ferriol-González and colleagues, using cross-infection matrices, established that most Klebsiella phages from a collection of 86 isolates were highly specific and capable of infecting a limited number of hosts (on average, 2.67). Specifically, 84.88% of bacteriophages could infect one to three strains [21].

The creation of a collection of phages with a narrow host range can be considered advantageous for personalized phage therapy, as it allows phages to specifically target individual bacterial strains without affecting the normal microbiota, thereby reducing the risk of side effects. At the same time, phages with a broad activity spectrum also offer certain benefits, as they can lyse a wide range of bacterial strains. This makes them particularly useful in cases where an infection is caused by multiple pathogens or for prophylactic use [21].

Klebsiella spp. are ubiquitous Gram-negative bacteria that are commonly found in the natural environment, including the human microbiome. *Klebsiella* species are involved in the development of many infectious diseases. In recent years, their resistance to multiple drugs, especially carbapenems and β-lactam antibiotics, has posed significant challenges for treatment. This is reflected in the growing number of antimicrobial agents to which these bacteria demonstrate resistance. Klebsiella species possess a variety of antibiotic resistance mechanisms. For example, the presence of the *blaKPC* and *blaNDM* genes confers resistance to β-lactams, including carbapenems; the mcr-1 gene causes resistance to colistin; *blaCTX-M* confers resistance to many β-lactams; *aac(3)-II* confers resistance to aminoglycosides; and tet genes provide resistance to tetracyclines [22]. Our findings indicate that the PDR/XDR strains of *K.pneumoniae* isolated from patients in inpatient wards in Ukraine have a range of different antibiotic resistance genes. In almost all cases, combinations of several resistance mechanisms were detected. The most frequently identified gene was blaCTX-M (93.3%). The bacteriophages isolated during the study lysed all PDR/XDR K. pneumoniae strains, regardless of the presence of resistance genes or their combinations.

CONCLUSIONS

The results obtained show that municipal wastewater can be a valuable source of phages active against multidrug-resistant *K. pneumoniae*, regardless of their genetic resistance profile. The use of bacteriophages with a broad or combined activity spectrum could become a promising direction in the fight against infections caused by PDR/XDR strains of *K. pneumoniae*, especially in the context of limited effectiveness of traditional antimicrobial agents.

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ВИКОРИСТАННЯ БАКТЕРІОФАГІВ ПРОТИ ШТАМІВ KLEBSIELLA PNEUMONIAE З МНОЖИННОЮ ЛІКАРСЬКОЮ СТІЙКІСТЮ

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Актуальність. Поява резистентних до протимікробних препаратів мікроорганізмів є серйозною глобальною проблемою охорони здоров'я. Однією з найпоширеніших бактерій, що спричиняють внутрішньолікарняні інфекції, є *Klebsiella pneumoniae*, особливо у важкохворих пацієнтів. Частота поширеності *K. pneumoniae* з множинною лікарською стійкістю різко зросла в усьому світі за останні десятиліття, створюючи негайну загрозу для громадського здоров'я. За відсутності ефективних методів лікування важких бактеріальних інфекцій, спричинених антибіотикорезистентними штамами бактерій, бактеріофаги є індивідуальним та ефективним терапевтичним доповненням, а в деяких випадках – альтернативою традиційним антибактеріальним стратегіям.

Мета: вивчити можливість виділення бактеріофагів проти штамів *K.pneumoniae* з повною (PDR) та розширеною (XDR) лікарською стійкістю, використовуючи зразки міських стічних вод, а також дослідити спектр їхньої активності *in vitro*.

Матеріали та методи. Бактеріофаги ізолювали за допомогою методу збагачення, а їх специфічну активність визначали модифікованим методом Граціа та методом малих крапель. Морфологічні особливості ізольованих бактеріофагів досліджували за допомогою електронної мікроскопії. Визначення чутливості до протимікробних препаратів здійснювали методом диско-дифузійного тесту (ДДМ) та методом мікророзведень. Гени антибіотикорезистентності (*blaNDM-1, blaKPC, blaCTX-M-1* та *gyrA*) виявляли методом полімеразної ланцюгової реакції (ПЛР).

Результати. У рамках дослідження було ізольовано 212 бактеріофагів із міських стічних вод, які проявили активність щодо широкого спектра клінічних ізолятів та референтних штамів *K.pneumoniae*, включаючи антибіотикорезистентні варіанти. Зокрема, 56,1 % фагових ізолятів продемонстрували специфічну активність до попередньо відібраних і охарактеризованих 15 PDR/XDR-штамів *K.pneumoniae*. Важливо, що кожен клінічний ізолят із набутою широкою антибіотикорезистентністю був чутливий до кількох фагів, а 14 бактеріофагів лізували понад 50 % бактеріальних культур. При цьому фаги демонстрували як вузький, так і широкий спектр літичної активності, що дозволяє сформувати ефективні фагові коктейлі для потенційної фаготерапії..

Висновки. Отримані результати свідчать про високу потенційну ефективність бактеріофагів як альтернативного або допоміжного засобу у боротьбі з *K. pneumoniae* з множинною лікарською стійкістю. Результати експериментального дослідження підкреслюють доцільність подальшого розвитку фаготерапевтичних підходів, особливо в умовах поширення мультирезистентних інфекцій.