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NEW BACTERIOPHAGE OF MULTIDRUG RESISTANT STRAIN OF *PSEUDOMONAS AERUGINOSA*

Aim: *Antimicrobial resistance in bacteria is one of the most discussed and important theme in healthcare field now. Bacteriophages are known as the most promising alternative to antibiotics and a method of fighting the multi-resistant pathogens. In this study, our main aim was to isolate lytic specific phages of multidrug resistant Pseudomonas aeruginosa, one of the most dangerous bacterial human pathogens.* ***Methods:*** *Disk-diffusion method, spot-test, agar overlay method, electron microscopy.* ***Results:*** *Antimicrobial resistance range of P. aeruginosa 458 strain was tested and it appeared to be resistant to all available antibiotics. Phages were isolated from waste waters in Kyiv. They appeared to have a typical morphology of Myoviridae family. Isolated viruses successfully lysed all the bacteria in vitro.* ***Conclusions:*** *Considering high effectiveness of isolated phage in vitro, we propose it as a candidate for phage therapy, though further studies on physical and genetic characteristics are required.*

Keywords: *bacteriophages, Pseudomonas aeruginosa, antimicrobial resistance, phage therapy.*

Introduction. *Pseudomonas aeruginosa* is a heterotrophic, motile, Gram-negative rod-shaped bacterium. It is a facultative anaerobe that preferably uses aerobic respiration [1], but it is also able to grow under anaerobic conditions in the presence of nitrates [2]. *P. aeruginosa* optimal temperature is 37 °C, though bacteria can persist in temperatures from 4 to 42°C which allows it to attach and survive on medical equipment, hospital surfaces [3]. It often occurs as opportunistic infection in patients with compromised immune systems including those with cystic fibrosis, cancer, AIDS, burn and eye injuries [4]. It was also reported multiple times that *P. aeruginosa* is often responsible for urinary tract infections, especially those associated with catheter injections [5], [6]. *P. aeruginosa* is really difficult to cure and eradicate because of its ability to acquire resistance to different antibiotics [7]. This bacterium uses different pathways to resist the antimicrobials; for instance, it produces broad-spectrum β-lactamases and metallo-β-lactamases. *P. aeruginosa* carbapenem-resistance associated with the production of metalloenzymes [8], [9]. Resistance to other various antibiotics and antimicrobial substances is associated with biofilm formation [10]. It is known that type IV pili and exopolysaccharide are involved in this process [11]. That is why the search of alternative antimicrobials is of a high importance as it can improve the treatment of *P. aeruginosa*-induced infections and decrease the mortality level.

Bacteriophages are widely present in the environment and are easy to isolate and propagate. They are highly specific to their hosts (bacteria) and do not lyse other bacterial species in particular human microbiome which makes them more reliable than antibiotics. Number of phages of this bacterium were already isolated, characterized and tested under various conditions. Some of them showed to be able to lyse the bacterial biofilm [12], [13]. There were also successful animal tests showing the safety of phage therapy against *P. aeruginosa* pneumonia [14]. However, even though phages were able to lyse the biofilm, complete eradication may require a combination of different phages [15]. Taking these facts into account, it is highly important to search for new virulent phages, characterize them and use in medical treatment of such difficult infections as provoked by *P. aeruginosa.* That is why our main aim in this work was to find new lytic phages, specific to this pathogen.

Materials and methods

Bacteria cultivation. *P. aeruginosa* isolate 458 was taken from the collection of NeoProBioCare Ltd, previously isolated from the urine of the patient with urinary tract infection and was cultured at 37°C in tryptic soy medium (casein peptone 17 g, soymeal peptone 3 g, D(+) glucose monohydrate 2.5 g, Sodium chloride 5 g, di-Potassium hydrogen phosphate 2.5 g per litre, Millipore, Merck Germany) supplemented with 1.4 % agar on Petri plates or in glass tubes. While working with phages we used an overnight culture of bacteria, in which bacteria was in exponential phase of growth. The concentration of bacteria cell culture was 10^8-10^9 CFU/mL.

Disk-diffusion method. To investigate the range of antimicrobial resistance of *P. aeruginosa* the modified disk-diffusion method was used according to the existing protocols [16]. Briefly, the Mueller-Hinton agar plates were prepared. Overnight culture of bacteria was washed with 0.9 % NaCl and then plates were inoculated with it by sterile swab. Antibiotics discs were put on agar using sterile forceps. Plates were incubated as described previously.

Bacteriophage isolation. Phages in this study were isolated from waste waters taken from Bortnychi Sewage treatment plant in Kyiv. To isolate phage a modified protocol of an enrichment procedure involving a double-layer agar method was used [17]. Briefly, a molten1.4 % agar was poured into Petri plates and incubated at room temperature for 5 min. Then 500 mL of filtered water sample (centrifuged at 4000 rpm for 15 min and filtered through 0.22-μm pores) were mixed with 100 mL of *P. aeruginosa* cells and added to 2 mL of molten 0.7 % medium and poured into plates with 1.4 % agar medium. Plates were incubated overnight in 37°C. Single plaques were picked with a pipette tip and transferred into NaCl solution (0.9 %), followed by centrifugation and mixing to release phages and then was stored at 5 °C.

Bacteriophage host range. The phage host range was assessed by spotting 10 μl of phage suspension on plates

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with bacterial lawn, prepared similar to spot-test assay protocol. After 24 h incubation at 37°C, phage efficiency was evaluated by the presence or absence of the lysis zones on the bacterial lawn. For this assay, we took a list of strains of *P. aeruginosa* from the collection of NeoProBioCare Ltd. and also DSM strains of Gram-negative bacteria *Escherichia coli, Klebsiella pneumoniae, Shigella flexneri, S. sonnei.*

Spot-test assay. Serial dilutions of phage samples were added on bacterial lawn (10 µL of each dilution) and kept at room temperature for 20 minutes to let phages diffuse into upper layer of medium. Then plates were incubated overnight at 37°C. Titer of the phages was approximately calculated by the last dilution where phage plaques were detected.

Agar overlay method. To calculate the exact titer of phages the agar overlay method was used. Phages were serially diluted in NaCl 0.9 % in 2 mL microfuge tubes. Then 0.1 ml of overnight bacterial culture (10^8 CFU/mL) together with 500 µL of phages solution (one dilution per plate) was added to 2.5 mL of 0.7 % agar. Plates were incubated overnight at 37°C. After incubation single plaques were counted.

Electron microscopy. Virions morphology was investigated using the electron microscope JEM 1230 (M.G. Kholodny Institute of Botany of the National Academy of Sciences of Ukraine, Kyiv, Ukraine). Formvar films placed on 400-mesh copper grids were dipped into sample for 2 min and contrasted in 2 % uranyl acetate. The preparations were dried and viewed under the electron microscope at an instrumental magnification of 20,000.

Results and discussion

Antimicrobial resistance test

Disc-diffusion tests were done prior to bacteriophage search in order to investigate the range of antimicrobial resistance of *P. aeruginosa.* Resulted inhibition zones were measured in mm and are listed in Table 1.

***Table* 1. Antibiotic susceptibility of *P. aeruginosa* 458. Results interpretation was performed according to Clinicaland Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 31st ed. CLSI supplement M100 (ISBN 978-1-68440-104-8 [Print]; ISBN 978-1-68440-105-5 [Electronic]). Clinicaland Laboratory Standards Institute, USA, 2021**

№	Antibiotic	Antibiotic group	Reference values			Results
			S	I	R	Ps 458
1.	Piperacillin	ureidopenicillin	≥ 21	15–20^	≤14	12 **R**
2.	Cefepime	cephalosporin	≥ 18	15–17^	≤ 14	0 **R**
3.	Ceftazidime	cephalosporin	≥ 18	15–17^	≤ 14	0 **R**
4.	Imipenem	carbapenems	≥ 19	16–18^	≤ 15	0 **R**
5.	Meropenem	carbapenems	≥ 19	16–18^	≤ 15	0 **R**
6.	Ciprofloxacin	fluoroquinolone	≥ 25	19–24^	≤ 18	0 **R**
7.	Ofloxacin	fluoroquinolone	≥ 16	13–15^	≤ 12	0 **R**
8.	Norfloxacin	fluoroquinolone	≥ 17	13–16	≤ 12	0 **R**
9.	Gatifloxacin	fluoroquinolone	≥ 18	15–17^	≤ 14	0 **R**
10.	Levofloxacin	fluoroquinolone	≥ 22	15–21^	≤ 14	0 **R**
11.	Amikacin	aminoglycoside	≥ 17	15–16^	≤ 14	0 **R**
12.	Gentamicin	aminoglycoside	≥ 15	13–14^	≤ 12	0 **R**

This isolate appeared to be resistant to all available antibiotics, even though it had a small inhibition zone around Piperacillin disc. Such multiresistant strains could be very dangerous if appear in hospital conditions. Considering this, bacteriophage cocktail of specific lytic phages is the only available option now to cure the infection that can be provoked by this resistant *P. aeruginosa*.

Bacteriophage tests

After waste waters purification and inoculation on *P. aeruginosa* using agar overlay method, single plaques of phages were picked and propagated. There were transparent plaques, less than 2 mm in diameter, without halo or any turbidity (fig.1).

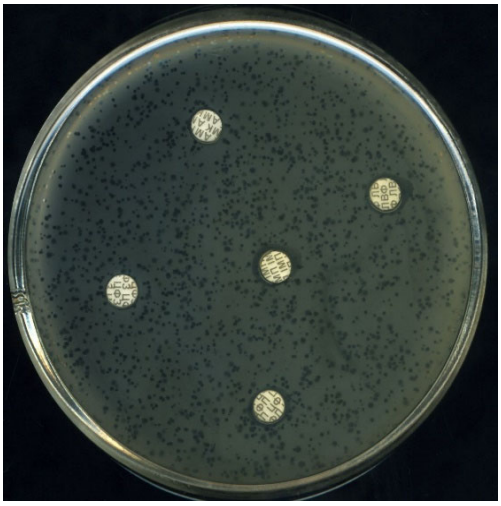

Figure 1. Phage plaques on *P. aeruginosa* lawn with antibiotics discs

Phage concentration reached 10^{10} PFU/mL. Following this, we performed an electron microscopy of isolated phages in order to check its morphological features.

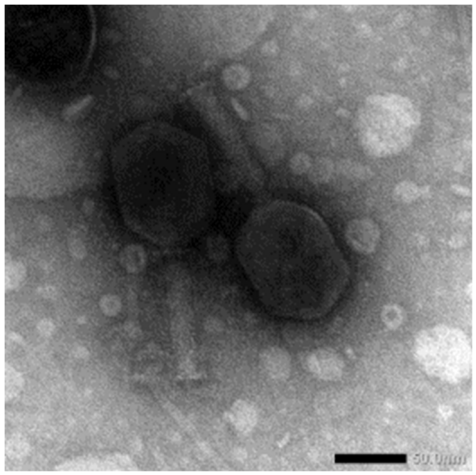

Figure 2. Electronic microscopy of *P. aeruginosa* 458 phage

Phage appeared to have a typical morphology of *Myoviridae* family, with elongated icosahedral capsid and contractile tail (55x72x88 nm) (fig.2). Many myophages of *P. aeruginosa* were reported to this time. Phages of PB1-like virus genus have been used as therapeutic phages already [18]; phage 14-1, which is also the member of *Myoviridae*, was used in experiments to optimize spray-dried phage powders [19], so that type of morphology seems to be usual generally among *P. aeruginosa* phages.

Bacteriophage host range

To estimate the potential of isolated phage in therapy, we tested its host specificity on different strains of *P. aeruginosa*, isolated from patients in SI "The Institute of Traumatology and Orthopedics" by NAMS of Ukraine, and also on strains from DSM collection of NeoProBioCare Ltd. The results are listed in table 2.

***Table* 2. Host range of isolated bacteriophage.**
Positive result or lysis was indicated as "+", negative or absence of lysis – as "-".

Bacteria	Result
Pseudomonas aeruginosa DSM	+
P. aeruginosa 458	+
P. aeruginosa 677	+
P. aeruginosa 688	+
P. aeruginosa 519	-
P. aeruginosa 500	+
P. aeruginosa 738	-
P. aeruginosa 770	-
P. aeruginosa 858	+
E. coli DSM 1103	-
K. pneumoniae DSM 30104	-
S. flexneri DSM 4782	-
S. sonnei DSM 5570	-

Phage appeared to be specific not only to mentioned bacterial isolate 458, but also to some other *P. aeruginosa* strains. All used bacterial strains were susceptible only to colistin, while resistant to other antibiotics. Specificity of our phage to multiresistant microbial strains is crucial as we propose it as potential therapeutical agent.

Conclusion

In this work, we isolated phage, specific to multiresistant strain of *Pseudomonas aeruginosa*. Mentioned bacterial strain appeared to be resistant to piperacillin, cefepime, ceftazidime, imipenem, meropenem, ciprofloxacin, ofloxacin, norfloxacin, gatifloxacin, levofloxacin, amikacin, gentamicin. Isolated phage successfully lysed bacteria *in vitro*. Morphology of the virion was typical for *Myoviridae* members. Phage was able to lyse not only the investigated bacteria but also some other multiresistant strains. This phage can be used for therapeutical purposes if the further characteristics will be provided.

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НОВИЙ БАКТЕРІОФАГ МУЛЬТИРЕЗИСТЕНТНОГО ШТАМУ *PSEUDOMONAS AERUGINOSA*

Наразі антибіотикорезистентність у бактерій є однією з найбільш обговорюваних і важливих тем у сфері охорони здоров'я. Бактеріофаги відомі як найбільш перспективна альтернатива антибіотикам і метод боротьби з мультирезистентними збудниками. У цьому дослідженні нашою головною метою було виділити літичні специфічні фаги, активні проти мультирезистентної Pseudomonas aeruginosa – одного з найнебезпечніших бактеріальних збудників людини. Для цього використовували диско-дифузійний метод, спот-тест, метод подвійних агарових шарів, електронну мікроскопію. У результаті досліджено діапазон антибіотикорезистентності клінічного ізоляту P. aeruginosa 458 і виявлено, що зазначена бактерія є стійкою до всіх відомих антибіотиків. Фаги були ізольовані зі стічних вод Києва. Вони мали типову морфологію представників родини Myoviridae. Ізольовані віруси успішно лізували бактерію in vitro. Отже, із врахуванням високої ефективності ізольованого фага in vitro авторами запропоновано його як кандидата до складу фаготерапевтичного препарату, проте необхідні подальші дослідження фізичних і генетичних характеристик вірусу.

Ключові слова: бактеріофаги, Pseudomonas aeruginosa, антибіотикорезистентність, фаготерапія.