International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2024.1135.1142

Research Article
Phage Isolation, Characterization and Antibiofilm Activity Against Multidrug-Resistant *Pseudomonas aeruginosa*

[1]Büşra Merve Sarıtaş, [2]Ayşe Karacalı Tunç, [3]Medine Çotak, [1]Gamze Alçi, [4]Nejdet Çeri and [4]Deniz Ğezal

[1]Department of Medical Microbiology, Faculty of Medicine, Kocaeli University, Kocaeli 41380, Turkey
[2]Department of Basic Sciences, Faculty of Dentistry, Iğdır University, Iğdır 76000, Turkey
[3]Mikroliz Biyoteknoloji, Ankara 06000, Turkey
[4]Department of Medical Services and Techniques, Health Services Vocational School, Iğdır University, Iğdır 76000, Turkey

Abstract

Background and Objective: Biofilms are structured microbial communities that occur as surface-attached communities aggregates. By penetrating the biofilm matrix and infecting bacterial cells, bacteriophages can disrupt the structure of the biofilm. Therefore, bacteriophages can be used as an alternative strategy in the control and pathogenic bacteria that form antibiotic-resistant biofilms. Factors such as the specificity of bacteriophages, infection persistence, mechanism of action and their ability to penetrate bacterial cells within the biofilm need better understanding. This study may contribute to developing new therapeutic options and reveal the potential of bacteriophages in the treatment of multidrug-resistant *Pseudomonas aeruginosa* infections. **Materials and Methods:** The double layer agar technique was used to isolate bacteriophage. The phage was characterized using a transmission electron microscope. The host range of the phage as well as its antimicrobial and antibiofilm properties were determined. The antibiofilm effect of the bacteriophage was evaluated using crystal violet method a 96-well flat-bottom microplate setup. **Results:** Clear results were obtained for 5 isolates on 10 bacterial plates with strong biofilm ability. It was observed that the isolated bacteriophage has a wide thermal range and does not lose its infectivity at different pH levels. Bacteriophages showed an average antibiofilm effect of 82 and 59% at varying bacterial dilutions of 1/100 and 1/1000, respectively. **Conclusion:** It was observed that it showed a stronger antibiofilm effect against the isolate with strong biofilm formation ability than the control. However, further research is needed to assess the anti-biofilm effects of bacteriophages. In general, the results suggest that the bacteriophage isolated may be a strong antibiofilm candidate.

Key words: Bacteriophage therapy, bacterial biofilm, multidrug resistant (MDR), *P. aeruginosa*, antibiofilm, phage isolation

Citation: Sarıtaş, B.M., A.K. Tunç, M. Çotak, G. Alçi, N. Çeri and D. Ğezal, 2024. Phage isolation, characterization and antibiofilm activity against multidrug-resistant *Pseudomonas aeruginosa*. Int. J. Pharmacol., 20: 1135-1142.

Corresponding Author: Büşra Merve Sarıtaş, Department of Medical Microbiology, Faculty of Medicine, Kocaeli University, 41380, Kocaeli, Turkey Tel.: +905073005115

Funding: The project numbered 2209-A TÜBITAK supported 2023 1st term 1919B012309443.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pseudomonas aeruginosa causes high morbidity and mortality in patients with underlying conditions such as cystic fibrosis, chronic obstructive pulmonary disease, burn wounds, Acquired Immunodeficiency Syndrome (AIDS) and bronchiectasis. The resistance mechanisms it develops complicate effective treatment strategies. In recent years, there has been a significant increase in infections caused by this bacterium, particularly infections caused by extensively drug-resistant (XDR) and Multidrug-Resistant (MDR) strains[1].

Antibiotic resistance emerges as a result of the capacity to develop biofilms, which consist of bacterial communities entrenched in an exopolysaccharide matrix (EPS). The National Institutes of Health (NIH) involved that biofilm formation is linked to all microbial and chronic infections[2].

Pseudomonas aeruginosa can develop biofilms on diverse animate and inanimate surfaces, including mucus plugs within Cystic Fibrosis (CF) lungs, catheters and contact lenses. The bacterium is known for forming biofilms on medical equipment and is commonly associated with nosocomial infections. The *P. aeruginosa* proliferates on these surfaces, creating biofilms characterized by clusters of cells enveloped in a protective alginate polysaccharide[3].

Antibiotic therapy diminished the effectiveness against bacterial infections owing to the escalating resistance levels. Pervasive antibiotic resistance poses a substantial global health hazard. Antimicrobial resistance (AMR) is a recognized and formidable health challenge acknowledged by prominent international professional organizations, including the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO). Although, the current annual global mortality attributed to AMR is estimated at 700,000, this figure is anticipated to increase significantly, reaching a 10 million deaths annually by 2050. Hence, there is an urgent imperative need to develop novel strategies, such as monoclonal antibodies, immunomodulators, bacteriophages and phage-encoded enzymes[4].

Phages are the most abundant biological entities. Their population size is estimated to be around 10. Over the last two decades there has been a resurgence of interest in phage therapy within Western medicine[4]. This therapeutic approach serves as an alternative method, typically employed as a last resort, to address infections induced by multidrug-resistant bacteria when conventional treatments have proven ineffective[1].

The objective of this study was to isolate and characterize a lytic phage against multiple drug-resistant *P. aeruginosa* strains and to figure out the efficacy of the phage efficacy against planktonic cells and biofilms. Specifically, this study focused on utilizing the bacteriophages to eradicate biofilms formed on both living and nonliving surfaces and to mitigate the dissemination of resistant strains. This study positproposed that understanding the antibiofilm efficacy of bacteriophages can serve as a preventive measure against nosocomial infections.

MATERIALS AND METHODS

Study area: The study was carried out at Iğdır University, Turkey and antibiofilm trials conducted at Kocaeli University, Turkey, in the period from November, 2023 to February, 2024.

Bacterial isolation: In this study, P130582-D, which has multidrug resistance properties, was purchased from Mikroliz Biotechnology. Passages were revived under the required incubation conditions and stored at -80°C.

Isolation of bacteriophages: Lytic phages were isolated using the double-layer agar method against the multidrug-resistant *P. aeruginosa* strain. Samples from sewage were centrifuged at 12,000×g for 10 min. It was passed through a 0.22 µm filter to remove bacteria and residues. *Pseudomonas aeruginosa* culture and LB broth were added in logarithmic phase to filtered sewage supernatants in a 50 mL tube. The coculture was incubated at 37°C for 4 hrs and then centrifuged at 12,000×g for 2 min at 4°C. Supernatants were passed through a 0.22 µm filter. It was then diluted with Tris-HCl-$MgSO_4$ buffers in 10-fold concentration gradients. The 10 µL of the supernatant of different dilutions was mixed with 100 µL of *P. aeruginosa* culture in logarithmic growth phase, 5 mL of 0.7% semisolid LB agar. It was then poured onto a 1.5% LB agar plate. Plaque formation was observed after overnight incubation at 37°C. Isolated plaques were collected by micropipette aspiration using filter tips and immersed in Tris HCl-$MgSO_4$ buffer. The MTBB P13fang0582-D bacteriophage was isolated from sewage waste and can effectively infect *P. aeruginosa* strains[5].

Bacteriophage host range: One hundred microliters of the isolates, which we adjusted to 0.5 McFarland, were planted on the tryptone soy agar (TSA) we prepared and after waiting for a while for the agar to freeze, 10 µL of this phage was inoculated on it as a spot. After waiting for 1 hr to prevent the phages from flowing, the plates were incubated upside down at 37°C for 24 hrs. Plaque formation was examined the next day.

Plaque morphology: The morphological features of the plaques were characterized by determining the diameter, aperture and presence of a halo zone around the plaques.

Multiplicity of infection assay: The ratio of phages transferred to host bacteria was determined as the multiplicity of infection (MOI). The MTBB P130582-D stock phage (3×10^{11} PFU/mL) was serially diluted tenfold. *Pseudomonas aeruginosa* bacteria that had grown overnight were adjusted to a concentration of 3×10^{8} CFU/mL, corresponding to a McFarland density of 1. Subsequently, the phage was introduced into the bacteria at different ratios (0.001, 0.01, 0.1, 1, 10 and 100). The combination of bacteria and phage was cultured at 37°C. Bacterial growth was measured by turbidity every 120 min for 24 hrs using a UV-Vis spectrophotometer (Agilent Cary 60) at 600 nm. A culture that solely contained bacteria was used as a control.

Influence of temperature on bacteriophage stability: The thermal stability of the phages was estimated by subjecting the P130582-D phage to four distinct temperatures: 40, 50, 70 and 90°C for durations of both 1 and 2 hrs. Subsequently, the double plaque method was employed to evaluate the survival rate of the bacteriophage[2].

Influence of pH on bacteriophage stability: The bacteriophage was subjected to varying pH levels ranging from 3-12 using solutions of 0.1 M HCl and 0.1 M NaCl at 37°C for 1 hr. Subsequently, a double plaque assay was conducted to assess the survival of the bacteriophage[2].

Transmission Electron Microscope (TEM) scanning: A freshly prepared phage lysate (10^{9} PFU/mL) was used for TEM image analysis. Then, 10 µL of phage solution was added to the carbon-coated copper grid and air-dried, followed by negative staining with phosphotungstic acid. The phages were viewed at the Osmangazi University Central Research Laboratory Application and Research Centre (ARUM) using a Transmission Electron Microscope (TEM-HT7800) set at 100 kV and a magnification of 10,000×15. The classification of the phage was determined by the standardized classification guidelines provided by the International Committee on Taxonomy of Viruses (ICTV)[6].

Antibiofilm effect of the bacteriophage: The antibiofilm effect of the bacteriophage was evaluated using a 96-well flat-bottom microplate setup. Tryptic soy broth (TSB) medium was dispensed into the wells. Subsequently, bacteria suspended in a 0.5 McFarland standard were inoculated into the wells. Following the designated incubation period, the medium was decanted and the wells were washed twice with phosphate-buffered saline (PBS). The bacteriophages were added to the wells and allowed to incubate for 24 hrs. Afterward, the bacteriophage solution was removed and the biofilm was fixed with methanol for 15 min. A 0.1% solution of crystal violet was applied to the wells. Following staining, the wells were rinsed twice with physiological saline, air-dried and prepared for spectrophotometric analysis using ethanol. Optical density (OD) values at 630 nm were determined. Control wells containing TSB and the investigated bacterial isolate were included for comparison[2].

RESULTS

Isolation of bacteriophage: The MTBB P130582-D bacteriophage was isolated from slaughterhouse waste and can effectively infect *P. aeruginosa* strains. A phage was isolated that developed clear plaques against host bacteria (Fig. 1).

Host range: Mikroliz Biotechnology determined the biofilm properties of all multidrug-resistant *P. aeruginosa* isolates using the crystal violet method. The bacteriophage with the highest OD for biofilm properties against the isolate was isolated. The host range of the obtained bacteriophages was determined. Clear results were obtained for 5 isolates on 10 bacterial plates with strong biofilm ability. The host range of the obtained bacteriophages was determined. Clear results were obtained for 5 isolates in 10 bacterial plates with strong biofilm ability. Four of the five isolates were measured as clear with a diameter of 4 mm and 1 isolate was measured as clear with a diameter of 3 mm (Table 1). The presence of bacteriophage did not affect five of the isolates. The presence of the bacteriophage did not affect five of the isolates (Fig. 2a-d).

Fig. 1: Phage isolation

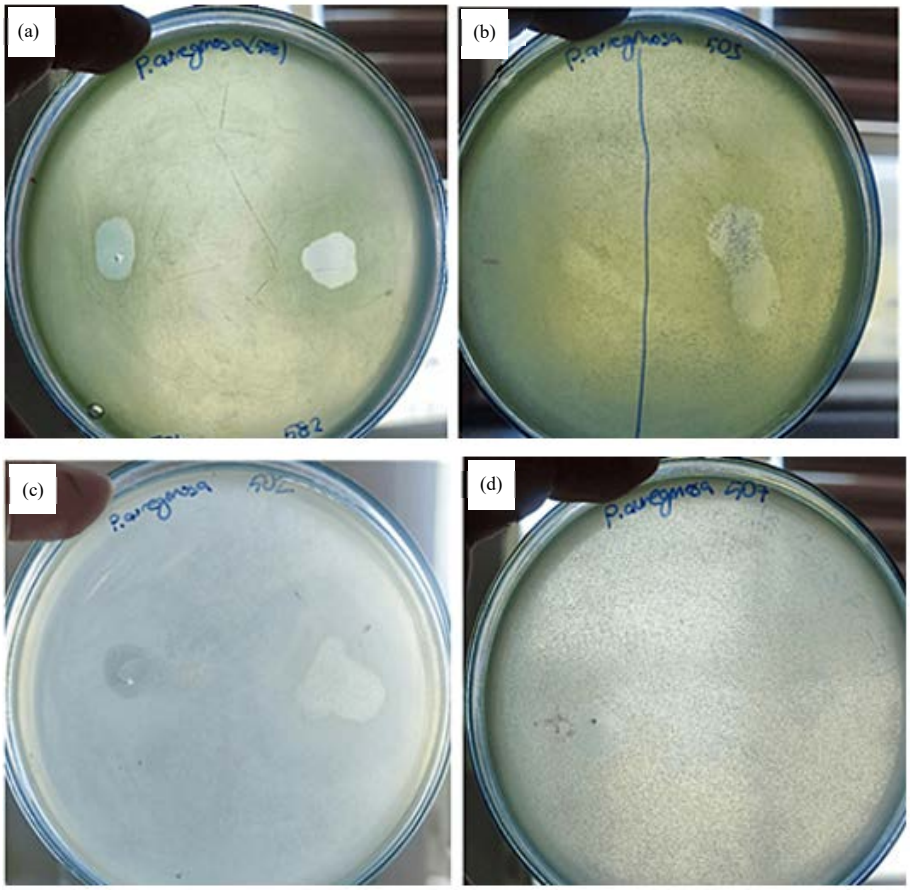

Fig. 2(a-d): Plaque morphology of the phage was evaluated using the double-layer agar method, (a-c) Large plaque with hollow areas (diameter: up to 4 mm) and (d) No plaques

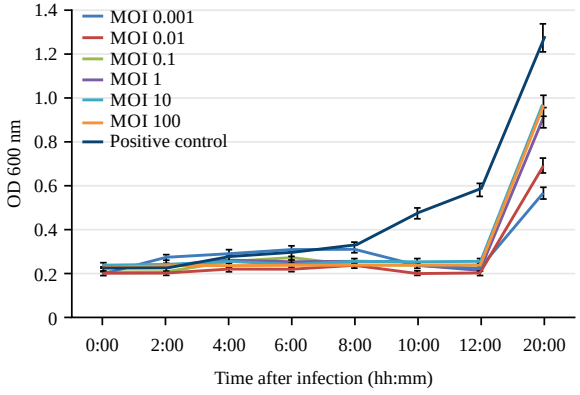

Fig. 3: Effect of phage MTBB 130582-D on growth at different MOIs at 37°C (n = 3)
Positive control no phage was added line navy blue, MOI 0.001: Line blue, MOI 0.01: Line red, MOI 0.1: Line green, MOI 1: Line purple, MOI 10: Line blue and MOI 100: Line orange

Multiplicity of infection (MOI) assay: The reduction in bacterial growth caused by phage was compared with that of the control. The decrease continued in phage-supplemented cultures compared to the control (Fig. 3).

Effect of temperature on bacteriophage stability: The P130582-D phage demonstrated lytic activity at different temperatures after 1 and 2 hrs. It showed lytic activity at 70 and 90°C (Fig. 4).

Effect of pH on bacteriophage stability: The bacteriophage did not lose its infectivity at the different tested pH values (3-12).

Transmission electron microscope scanning: According to Table 2, according to data from TEM and the International Committee on Taxonomy of Viruses, tailed phages belonging to the family Myoviridae had heads (dimension ranges: 55.95 nm) of 61.72×11.05 (length×width nm) (Fig. 5a-d).

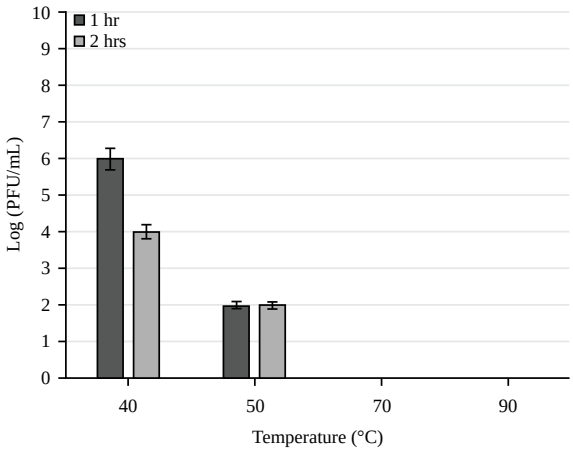

Fig. 4: Stability of phage MTBB P130582-D at different temperatures

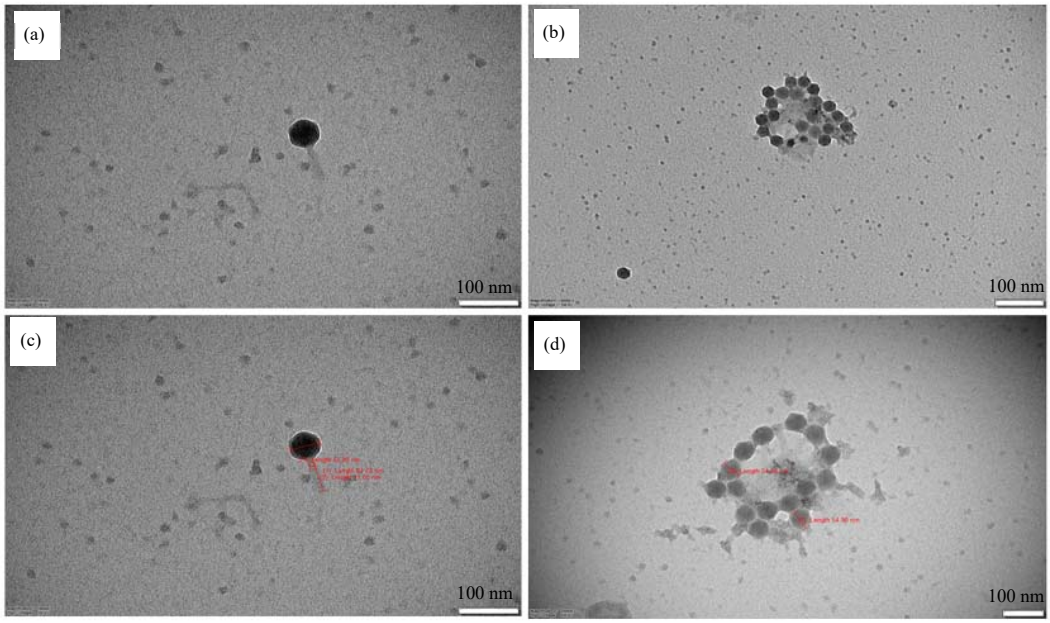

Fig. 5(a-d): TEM images of the bacteriophage MTBB P130582-D belonging to the family Siphoviridae, (a-c) Solitary phage itself and its dimensions and (b-d) Aggregated phages and their sizes

Table 1: Bacterial strains used for the host range spectrum of the bacteriophage

Bacterial strain	Sources	Diameter (mm)
Pseudomonas aeruginosa	130501	-
Pseudomonas aeruginosa	130502	4
Pseudomonas aeruginosa	130503	4
Pseudomonas aeruginosa	130504	3
Pseudomonas aeruginosa	130505	4
Pseudomonas aeruginosa	130506	-
Pseudomonas aeruginosa	130507	-
Pseudomonas aeruginosa	130508	4
Pseudomonas aeruginosa	130509	-
Pseudomonas aeruginosa	130510	-

Table 2: Dimensions of the phage by using electron microscopy

Phage	Head diameter	Tail length	Tail width
P130582-D	55.95 nm	61.72	11.05

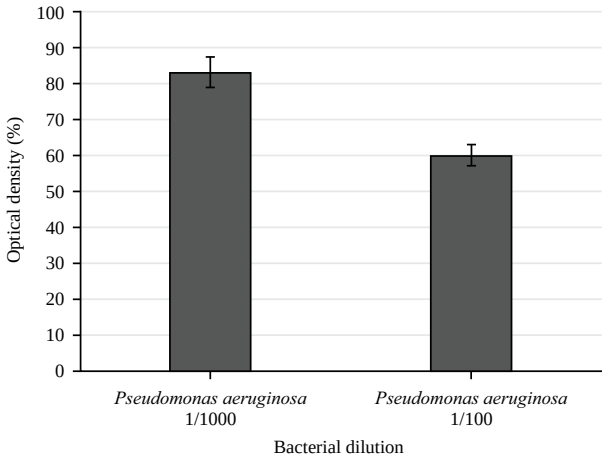

Fig. 6: Percentage of biofilm formation was assessed in different dilutions (1/1000, 1/100) of bacterial suspension adjusted to 0.5 MacFarland, both before and after treatment with phage, compared to the control

Each bar on the graph represents the average of three independent measurements

Bacteriophage activity on biofilms: The outcomes were computed as the mean value for each bacterial dilution and subsequently as an overall average for the bacteriophage. The findings indicated that the examined bacteriophages led to a reduction in biofilm formation across varying bacterial dilutions of 1/100 and 1/1000, resulting in average reductions of 82 and 59%, respectively, compared to those in the control group (Fig. 6).

DISCUSSION

Pseudomonas aeruginosa is an opportunistic nosocomial infection agent that can be found everywhere[7]. High susceptibility to biofilm formation and antibiotic resistance are among the problems we encounter in the eradication of bacteria. This represents an important challenge to current treatment modalities. Currently, researchers were examining new antibiofilm factors, e.g., phages or phage-derived endolysins[8]. The phage identified in this study is promising for use in biofilms and planktonic cells. However, it also brings problems such as the narrow host range[9].

This study examined the biofilm-forming abilities of *P. aeruginosa* isolates and the isolation and characterization of bacteriophages against these isolates. In our research, we showed that bacteriophages isolated via Mikroliz Biotechnology were effective against only some isolates. These results suggested that bacteriophages may be a potential strategy to combat *P. aeruginosa* biofilms but their effectiveness may vary among isolates.

In a study by Adnan *et al.*[10] a bacteriophage with antibiofilm activity against *P. aeruginosa* was isolated and characterized. The bacteriophage MA-1, which has a moderate host range, was obtained from samples collected from wastewater. The phage exhibited significant stability to heat and pH. They determined by electron microscopy that phage MA-1 belongs to the Myoviridae family. It was found that the phage exerted a strong effect on biofilm formation at 6, 24, 48 and 74 hrs.

Similar to current study, in 2017, a phage for multidrug-resistant *P. aeruginosa*-2995 was isolated by Jamal *et al.*[11] and its effect on biofilms was investigated. In this study, the preformed biofilm mass decreased 3-fold. These results were compatible with current study. The JHP phage reduced biofilm biomass by 60-90% and reduced the bacterial load, which highlights the potential to prevent biofilm formation from permanent medical devices[12].

Studies have reported biofilms in approximately 40-80% of *P. aeruginosa* strains isolated from patients with chronic rhinosinusitis (CRS). In phage applications to prevent and reduce biofilms in bacterial infections, it has been reported that bacterial isolates taken from the sinuses of CRS patients reduce the biofilm biomass by an average of 70%[13]. In a similar study, the biofilm biomass of *P. aeruginosa* isolates from CRS patients was reduced by 76% using a single dose of phage cocktail[14].

In a study conducted by Guo *et al.*[15], two phages were isolated. These two bacteriophages inhibited the growth of the bacteria and produced good results even at low infection levels. It has also been found to be successful in preventing biofilm formation and eliminating previously formed biofilms.

Bacteriophages are a viable therapeutic option for treating infections connected to biofilms because of their fast bactericidal activity, ability to self-amplify and ability to degrade biofilms. *Pseudomonas aeruginosa* and *Staphylococcus aureus* can form mixed biofilms together. These dual-species biofilms can lead to treatment failure. In Tkhilaishvili *et al.*[16] study, the *in vitro* effect of commercially available phages on dual-species biofilms was investigated and the bacteria were found to be sensitive. In support of current study, phages showed good and moderate activity in biofilms of both bacterial species.

Bacteriophages are present in all environments that support microbial life, which makes them an essential part of current ecosystem. These include hot springs, whose temperatures can range from 40-98°C and whose pH can range from 1 to 9[17]. Two lytic bacteriophages (Pa1 and Pa2) isolated from sewage water samples specific to *P. aeruginosa* were stated to be stable at 90°C in the temperature test and at low and high levels in the pH analysis. Results determined that it showed lytic activity at 70 and 90°C [18].

The temperature and phage stability were studied at 25, 37, 40, 50, 60 and 70°C. In this study, they showed that the member of the phage family *Myoviridae* was largely stable at temperatures of 25 and 37°C and moderately stable at temperatures of 40 and 50°C. However, 72% of the phages survived at temperatures of 60°C and above and 14% survived at 70°C. Their pH analysis showed that it was most stable at pH 6.0, 7.0 and 8.0 and no significant loss in titer was detected. However, at pH values less than 3.0 and greater than 10.0, very small fractions of viable phages have been reported. Our phage was not affected by different pH values[19].

No significant loss in phage activity was observed in the temperature range of 37-50°C. The lowest activity was observed at 70°C, while it was inactivated at 75 and 80°C. In current study, no activity was observed at 70°C or above[20].

In this study, biofilm formation was carried out on polystyrene microplates. While the experiment was performed on polystyrene microplates, it can also be tested on medical devices such as catheters. However, a limitation of this study was that the experiment was not conducted directly on medical devices, limiting the direct applicability of current findings to clinical settings. The genomic characterization of the phages was not conducted. Instead, morphological characterization was performed using electron microscopy.

CONCLUSION

Antibiotic resistance of *P. aeruginosa*, an important cause of nosocomial infections, is being prevented all over the world. Its ability to form biofilm, especially on living and non-living surfaces, also increases its spread in hospitals. Bacteriophages, which were used for therapeutic purposes in the past, can now be a therapeutic treatment option against *P. aeruginosa*. In this study, the antibiofilm and antimicrobial effects of bacteriophage against multidrug-resistant *P. aeruginosa* were observed. It also appeared to have a stronger antibiofilm effect on preformed biofilms. For this reason, it will provide an alternative in reducing their ability to adhere to hospital surfaces as a result of the antibiofilm effect. Further studies have shown that nosocomial infections can be prevented by reducing *P. aeruginosa* colonization of medical devices.

SIGNIFICANCE STATEMENT

Multidrug resistant bacteria have become alarming all over the world. *Pseudomonas aeruginosa*, which is a serious problem in hospitals, can easily spread everywhere with its biofilm feature. This study aimed to find out how to deal with the antibiotic resistance with therapeutic methods. In this study, bacteriophages belonging to multidrug-resistant *Pseudomonas aeruginosa* were isolated. Results are effective on both multidrug-resistant *Pseudomonas aeruginosa* and biofilm. Results showed that bacteriophages isolated from the natural environment, which do not have any side effects, should be the primary method in the treatment of multidrug-resistant *Pseudomonas aeruginosa*.

REFERENCES

1. Santamaría-Corral, G., A. Senhaji-Kacha, A. Broncano-Lavado, J. Esteban and M. García-Quintanilla, 2023. Bacteriophage-antibiotic combination therapy against *Pseudomonas aeruginosa*. Antibiotics, Vol. 12. 10.3390/antibiotics12071089.
2. El-Atrees, D.M., R.F. El-Kased, A.M. Abbas and M.A. Yassien, 2022. Characterization and anti-biofilm activity of bacteriophages against urinary tract *Enterococcus faecalis* isolates. Sci. Rep., Vol. 12. 10.1038/s41598-022-17275-z.
3. Sharma, G., S. Rao, A. Bansal, S. Dang, S. Gupta and R. Gabrani, 2014. *Pseudomonas aeruginosa* biofilm: Potential therapeutic targets. Biologicals, 42: 1-7.
4. Chang, R.Y.K., S.C. Nang, H.K. Chan and J. Li, 2022. Novel antimicrobial agents for combating antibiotic-resistant bacteria. Adv. Drug Delivery Rev., Vol. 187. 10.1016/j.addr.2022.114378.
5. Fang, Q., Y. Feng, A. McNally and Z. Zong, 2022. Characterization of phage resistance and phages capable of intestinal decolonization of carbapenem-resistant *Klebsiella pneumoniae* in mice. Commun. Biol., Vol. 5. 10.1038/s42003-022-03001-y.
6. Bozdeveci, A., M. Karali, R. Akpinar and Ş.A. Karaoğlu, 2022. Isolation, characterization, and comparative genomic analysis of vB_PlaM_Pd22F, a new bacteriophage of the family Myoviridae. Arch. Virol., 167: 1269-1284.
7. Rolston, K.V.I. and G.P. Bodey, 1992. *Pseudomonas aeruginosa* infection in cancer patients: Infectious complications of cancer. Cancer Invest., 10: 43-59.
8. Wang, X., J. Tang, W. Dang, Z. Xie and F. Zhang *et al*., 2023. Isolation and characterization of three *Pseudomonas aeruginosa* viruses with therapeutic potential. Microbiol. Spectr., Vol. 11. 10.1128/spectrum.04636-22.
9. Mirzahosseini, H.K., M. Hadadi-Fishani, K. Morshedi and A. Khaledi, 2020. Meta-analysis of biofilm formation, antibiotic resistance pattern, and biofilm-related genes in *Pseudomonas aeruginosa* isolated from clinical samples. Microb. Drug Resist., 26: 815-824.
10. Adnan, M., M.R.A. Shah, M. Jamal, F. Jalil and S. Andleeb *et al*., 2020. Isolation and characterization of bacteriophage to control multidrug-resistant *Pseudomonas aeruginosa* planktonic cells and biofilm. Biologicals, 63: 89-96.

11. Jamal, M., S. Andleeb, F. Jalil, M. Imran and M.A. Nawaz *et al*., 2017. Isolation and characterization of a bacteriophage and its utilization against multi-drug resistant *Pseudomonas aeruginosa*-2995. Life Sci., 190: 21-28.
12. Shafique, M., I.A. Alvi, Z. Abbas and Shafiq ur Rehman, 2017. Assessment of biofilm removal capacity of a broad host range bacteriophage JHP against *Pseudomonas aeruginosa*. APMIS, 125: 579-584.
13. Łusiak-Szelachowska, M., B. Weber-Dąbrowska, M. Żaczek and A. Górski, 2021. Anti-biofilm activity of bacteriophages and lysins in chronic rhinosinusitis. Acta Virol., 65: 127-140.
14. Fong, S.A., A. Drilling, S. Morales, M.E. Cornet and B.A. Woodworth *et al*., 2017. Activity of bacteriophages in removing biofilms of *Pseudomonas aeruginosa* isolates from chronic rhinosinusitis patients. Front. Cell. Infect. Microbiol., Vol. 7. 10.3389/fcimb.2017.00418.
15. Guo, Y., P. Chen, Z. Lin and T. Wang, 2019. Characterization of two *Pseudomonas aeruginosa* viruses vB_PaeM_SCUT-S1 and vB_PaeM_SCUT-S2. Viruses, Vol. 11. 10.3390/v11040318.
16. Tkhilaishvili, T., L. Wang, C. Perka, A. Trampuz and M.G. Moreno, 2020. Using bacteriophages as a trojan horse to the killing of dual-species biofilm formed by *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus*. Front. Microbiol., Vol. 11. 10.3389/fmicb.2020.00695.
17. Marks, T.J. and I.R. Rowland, 2024. The Diversity of Bacteriophages in Hot Springs. In: Bacteriophages: Methods and Protocols, Tumban, E. (Ed.), Humana, New York, ISBN: 978-1-0716-3549-0, pp: 73-88.
18. Marei, E.M., 2020. Isolation and characterization of *Pseudomonas aeruginosa* and its virulent bacteriophages. Pak. J. Biol. Sci., 23: 491-500.
19. Sharma, S., S. Datta, S. Chatterjee, M. Dutta and J. Samanta *et al*., 2021. Isolation and characterization of a lytic bacteriophage against *Pseudomonas aeruginosa*. Sci. Rep., Vol. 11. 10.1038/s41598-021-98457-z
20. Mohamed, W.F., A.A. Askora, M.M.H Mahdy, E.A. EL-Hussieny and H.M. Abu-Shady, 2022. Isolation and characterization of bacteriophages active against *Pseudomonas aeruginosa* strains isolated from diabetic foot infections. Arch. Razi Inst., 77: 2187-2200.