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# Research Article
# Identification of Clinical *Pseudomonas* Pf Phages Inducible by Mitomycin and Investigation of the Antibiotic Relevance of Pf1 Phage

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## Abstract

**Background and Objective:** *Pseudomonas aeruginosa* boasts a significantly expansive genome relative to its microbial counterparts. This genome size endows *Pseudomonas aeruginosa* with the capacity to thrive across diverse environments, generate an array of virulence factors and manifest resistance against a wide spectrum of antibiotics. Notably, *Pseudomonas aeruginosa* is responsible for both community-acquired and hospital-acquired infections. In this study, *Pseudomonas aeruginosa* isolates were subjected to mitomycin C treatment and bacterial isolates with all induced filamentous Pf's, including Pfu-a, Pfu-b, Pf1, Pf4 and Pf5, were selected and it was investigated how the presence of Pf1 differed in these isolates. **Materials and Methods:** The study included 125 clinical isolates of *Pseudomonas aeruginosa* that were identified using automated systems from patient samples that were requested from different clinics of Gaziantep University Şahinbey Research and Application Hospital. The ethical permission for the collection of *Pseudomonas aeruginosa* bacterial isolates was acquired on September 18, 2019, with decision number 2019/275 from the Gaziantep University Clinical Research Ethics Committee. Antibiotic susceptibilities were also determined for each isolate. For each sample, ethical guidelines were followed to record and proceed analysis. **Results:** The presence of Pfu-a, Pfu-b, pf4 and Pf5 filamentous phages were detected by PCR. The presence of Pf1 filamentous phage was investigated in 33 clinical *Pseudomonas aeruginosa* strains that were detected as filamentous phage positive. The correlation between the presence of Pf1 filamentous phage and antibiotic sensitivities was investigated. **Conclusion:** The presence of pf1 phages in *Pseudomonas aeruginosa* genome is associated with antibiotic effects.

Key words: *Pseudomonas aeruginosa*, Pf1 phage, antibiotic relevance, mitomycin, Pf4 phage, Pf5 phage

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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* is a prominent pathogen that has a deleterious influence on human health as a nosocomial infectious agent. The proliferation of these pathogens in our surroundings is rapid[1-3]. As an opportunistic pathogen, *Pseudomonas aeruginosa* genetic variants produce a wide spectrum of infections in humans, from minor to life-threatening diseases[3]. The pathogen has been found to colonize tissues such as the lung epithelium and the urinary system, causing infections and increasing mortality in patients with burns, AIDS and cystic fibrosis (CF)[4-6]. The World Health Organization (WHO) has classified *Pseudomonas aeruginosa* as a critical priority pathogen causing loss of billions of dollars in healthcare[7-9].

The most preferred antibiotics such as ceftazidime, amikacin, colistin and meropenem are employed that can be applied with monotherapy and multi-therapy for treating bacterial infections. Antibiotic susceptibility and genomic structure of pathogens are major factors in designing treatment regimes. However, these factors resulting from the resistance mechanisms of bacteria cause ineffective antibiotic regimes to treat bacterial infections[5]. Recent studies showed that evolved resistance mechanisms have emerged novel variants multidrug-resistant (MDR) and extensively drug-resistant (XDR) clones[10,11].

Phages are categorized as siphoviridae, podoviridae, myoviridae and filamentous phages based on their morphological differences and genomic compositions. (International Committee on Taxonomy of Viruses, ICTV)[12]. Tail phages of the order Caudovirales are the best described phages for phage therapeutic applications due to their lytic activity. As Pf phages are lysogenic, they can make *P. aeruginosa* with Pf phages more virulent[13,14]. This phage group is quite different from other bacteriophages in terms of morphology and life cycle[15,16]. In addition to this, Pf phages, one of the filamentous bacteriophages (Pf phage) play roles in the production of biofilm[14,17,18]. The crystalline structure of Pf phages increases the adhesion and viscosity of *Pseudomonas aeruginosa* biofilms, making it difficult for antibiotics to diffuse and reducing their effectiveness[14].

The Pf phage is found in more than half of the *P. aeruginosa* strains cataloged in the genome database[19]. Burgener *et al.*[19] analysed approximately 2226 genome sequences, showed that Pf phage is common in *P. aeruginosa* bacteria, but not in all isolates. Some of them do not integrate into the genome but replicate only outside the chromosome. Others integrate into the genome and persist as prophages[20,21]. The Pf1 and Pf3 are filamentous phages that re not integrated into the *P. aeruginosa* genome. Other *P. aeruginosa* filamentous phages are known to be integrated into the bacterial genome. These are the Pf4, Pf5 and Pf7 phages, which persist as prophages[13,18,22,23].

Applications of prophages have been promising an effective solution to treat life-threatening infections[15]. Induction of prophage extraction from pathogen genomes has become popular tool in bacterial research, as documented in several clinical trials and supported by numerous successful phage studies[24-30]. Burgener *et al.*[19] have shown that *P. aeruginosa* prophages contribute to its pathogenicity and resistance. Existence of diverse prophages profiles in *P. aeruginosa* genomes increase the chances of survival among strains by producing toxins or triggering biofilm formation[15].

Examining the phage profiles and capacity for biofilm formation as well as the antibiotic susceptibility of clinical *P. aeruginosa* is crucial. Improved knowledge of the effects of prophage will help create more potent defenses against *P. aeruginosa* infections. Therefore, clinical *P. aeruginosa* isolates were evaluated for their filamentous phage profile. While 125 clinical *P. aeruginosa* isolates were subjected to mitomycin C treatment and bacterial isolates with all induced filamentous Pf's, including Pfu-a, Pfu-b, Pf1, Pf4 and Pf5, were selected and it was investigated how the presence of Pf1 differed in these isolates.

## MATERIALS AND METHODS

**Study area:** The study was carried out from December, 2019 to January, 2023. The study included 125 clinical isolates of *Pseudomonas aeruginosa* that were identified using automated systems from patient samples that were requested from different clinics of Gaziantep University Şahinbey Research and Application Hospital. Antibiotic susceptibilities were also determined for each isolate.

For each sample, ethical guidelines were followed to record and proceed analysis. The ethical permission for the collection of *Pseudomonas aeruginosa* bacterial isolates was acquired on September 18, 2019, with decision number 2019/275 from the Gaziantep University Clinical Research Ethics Committee.

**Bacteriophage induction:** Bacterial cultures of clinical *Pseudomonas aeruginosa* samples were incubated on Luria-Bertani (LB) agar for 48 hrs and their purity was determined by Gram staining, morphological characteristics

and specific biochemical analyses. The 125 bacterial suspensions from bacterial cultures of *Pseudomonas aeruginosa* isolates with an optical density of 0.2, measured by OD600, were treated with mitomycin C (0.5 µg/mL) and incubated at 37°C for 3 hrs on a shaker. Bacterial suspensions were centrifuged at 16,100 g for 5 min and the supernatants were filtered through a 0.22 µm pore diameter filter (EMD Millipore Co.). For phage detection, plaque formation was determined using the double agar technique. Briefly, 100 µL of diluted phage and 100 µL of host bacteria ($10^8$ CFU/mL) were mixed with 5.0 mL of soft agar (0.75% agar, w/v) and rapidly poured onto nutrient agar. The Petri dishes were incubated overnight at 37°C. After incubation, plaque formation was analyzed.

**Storage of phages:** After observation of plaque formation, plaques were counted according to their appearance and differences and a single plaque was removed and added to a mid-log phase *P. aeruginosa* culture supplemented with 0.1 M $CaCl_2$ using a sterile capillary tube to obtain pure phage separately. The 10 µL of the culture and phage mixture were incubated at 37°C overnight. The lysate was filtered using a disposable 0.22 µm pore injection filter (EMD Millipore Co.). The purified phages were transferred to 50% saline magnesium phage buffer (SM) (20 mg/L Tris HCl, 10 mg/L $MgSO_4$, 10 mg/L $CaCl_2$, 100 mg/L NaCl, pH 7.5) containing 50% glycerol and different stocks were obtained at -80°C and +4°C[31-34]. The 125 *Pseudomonas aeruginosa* isolates were phage-induced according to the above protocol. Phage induction of all samples was completed and the phages obtained were stored according to the method described above.

**DNA extraction:** The DNA was isolated from 55 *P. aeruginosa* samples induced with mitomycin-C according to the NORGEN DNA Isolation Kit protocol as recommended by the manufacturer.

**Detection of (pro)phage genetic elements:** The presence of Pf phages in the *P. aeruginosa* genome was determined by PCR. Two pairs of universal Pf primers (Pfu-a and Pfu-b) and three pairs of specific Pf primers (Pf1, Pf4 and Pf5) were used for this purpose (Table 1). Primers were synthesized with study of Mooij *et al.*[22] and Knezevic *et al.*[35]. The PCR was performed using genomic DNA as template and primers were added to 25 µL of standard reaction medium. Primers were amplified by multiplex PCR. Standardized PCR conditions for all primers were set as follows: Initial cycle at 94°C for 5 min, 35 cycles of 94°C for 30 sec, annealing at 53°C for 20 sec, extension at 72°C for 60 sec and final extension at 72°C for 7 min. The PCR products were run on 1-2% agarose gel and visualised by ethidium bromide staining.

**Antibiotic susceptibility testing:** The VITEK® 2 COMPACT automated system (VITEK® 2 Biomérieux, France) was used to determine antibiotic susceptibilities. Antibiotic susceptibilities were evaluated according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) the VITEK® 2 COMPACT system detects bacterial growth and metabolic changes in microwells on thin plastic cards using fluorescence-based technology. The fluorescence detection region where the lowest antibiotic concentration is detected, at which bacterial growth is completely inhibited, is evaluated as the MIC and the results are reported. In accordance with EUCAST recommendations, the defined MIC values of anti-pseudomonal antibiotics are used as a reference. In the VITEK® 2 COMPACT system, results were obtained after 8-12 hrs of incubation.

***Pseudomonas aeruginosa* colistin minimum inhibitory concentration (MIC) values:** In accordance with EUCAST recommendations, it was determined by the liquid microdilution method according to International Organisation for Standardisation (ISO) 20776-1. Colistin MIC testing was performed using the diagnostic MIC COL kit

Table 1: Primers used in this study

| | Primer | Sequences (5'-3') | Amplicon (bp) | References |
|---|---|---|---|---|
| **General** | | | | |
| pf1-like | PfUa-F | GTGTCGATCAAGATCCACCA | 872 | Mooij *et al.*[22] |
| | PfUa-R | GGAGGAAGAAAGCTATTCGCA | | |
| | PfUb-F | TTGTGTACGACAGCGGGAA | 569, 570, 593, 1242 | Knezevic *et al.*[35] |
| | PfUb-R | TCAATTCGCCTTTTTCGGC | | |
| **Specific** | | | | |
| Pf1 | Pf1-F | GTGCTTGTTGCCTTCGTTATTC | 519 | Knezevic *et al.*[35] |
| | Pf1-R | GTGGAGCCTTTGACCAGATAG | | |
| Pf4 | Pf4-F | TCGAATTCCGCTTCCATCAC | 1001 | Mooij *et al.*[22] |
| | Pf4-R | CCTGATGCTTGGTCAGGTACG | | |
| Pf5 | Pf5-F | CGGGAATCGTATTGAGCCGA | 440 | Knezevic *et al.*[35] |
| | Pf5-R | GAGGACAGCCAGGGTCATTC | | |

(European Committee on Antimicrobial Susceptibility Testing, 2016). Commercial diagnostic MIC-COL (Diagnostics I.n.c., Galanta, Slovakia) with lyophilised colistin sulphate concentrations of 0.25-16 mg/L was used as recommended by the manufacturer. Results were interpreted according to the EUCAST colistin cut-off values (≤2 mg/L sensitive; >2 mg/L resistant).

**Phenotypic tests:** Isolates containing Pf phage were inoculated onto eosin-methylene blue agar (Merck) and cetrimide agar (Merck). After inoculation at 37°C for 24 hrs, colony colours and whether the colonies were mucoid or not were determined.

## RESULTS AND DISCUSSION

**Mitomycin induction and screening phage profiles:** A total of 125 clinical *Pseudomonas aeruginosa* isolates from blood, urine, tracheal aspirate, tracheal aspirate wound, sputum, broncho-alveolar lavage and throat cultures aged 2-87 years from different clinics of Gaziantep University Şahinbey Research Hospital were included in the study. All isolates were treated with mitomycin-C and total genome isolations of isolates with phage plaques were performed using the overload plate method.

The 125 lysates were applied to detect their lytic activity on all *Pseudomonas aeruginosa* isolates. The results revealed that only 26.4% (33/125) of *Pseudomonas aeruginosa* isolates were able to be induced with mitomycin-C application and plaques were observed with different sizes (Fig. 1a). Induciblity of mitomycin-C in diverse pathogens was investigated in previous studies[36-38]. The present study results were consistent with these studies.

The DNA from induced each lysate was isolated. As Pfu-a and Pfu-b are universal phages, Pf4 and Pf5 are integrated into the bacterial genome and Pf1 is not integrated into the genome. The presence of phages of Pfu-a, Pfu-b, Pf1, Pf4 and Pf5 were screened using appropriate primers (Table 1). Anydifference for the presence of Pfu-a, Pfu-b, Pf4 and Pf5 phages did not found (Fig. 1b-e), but phage profile for Pf1 was diverse for 33 lysates (Fig. 1f).

**Phenotype and resistance patterns of clinical *Pseudomonas aeruginosa* Pf1 positive isolates:** The 33 isolates with Pfu-a, Pfu-b, Pf4 and Pf5 bands were cultured and the mucoid of the colonies was recorded. Antibiotic susceptibility was determined. In this study used amikacin, aztreonam, gentamicin, imipenem, colistin, levofloxacin, meropenem, netilmicin, piperacillin, piperacillin/tazobactam, cefepime, ceftazidime, ciprofloxacin and tobramycin antibiotics to investigate the effects of antibiotic efficiency.

This study performed ANOVA test to investigate relationship between present/absent Pf1 and diverse antibiotics and mucoidity. Our results showed that the presence of pf1 phages in *P. aeruginosa* genome is associated with antibiotic effects (Table 2). Colistin, levofloxacin, netilmicin, piperacillin and ciprofloxacin antibiotics were not statistically associated with the presence of the pf1 phages. Gentamicin and cefepime antibiotics are most significantly associated with the absence of pf1 phages with $p = 0.004$, but also amikacin, imipenem, meropenem, piperacillin/tazobactam and ceftazidime antibiotics showed significant association with cut off value 0.05. These results suggested that the presence of pf1 phages profiles might be considered to treat *P. aeruginosa* infection.

*Pseudomonas aeruginosa* is an opportunistic pathogen that is constantly improving its ability to cause infections that are resistant to antibiotic therapy. As an opportunistic pathogen, *Pseudomonas aeruginosa* is the leading cause of nosocomial infections and in some cases the leading cause of death. Therefore, identification of the phage profiles in *P. aeruginosa* might be extremely important for fighting against resistant strains.

The lack of scientific data on the consequences of the interaction of therapeutic phages with bacteria under different conditions and environments is the main limiting factor. In addition, it is very important to determine the prophage profiles of resistant strains in order to reveal possible interactions between antibiotic sensitivity in clinical strains. *Pseudomonas aeruginosa* carrying filamentous (Pf) phage showed enhanced resistance to amikacin, aztreonam and meropenem, but not to ciprofloxacin, tobramycin, cefepime, ceftazidime and piperacillin-tazobactam[19]. *Pseudomonas aeruginosa* LESB58 inserted filamentous (Pf4) phage showed enhanced susceptibility to ciprofloxacin and gentamicin[15].

Throughout the lytic cycle, *Pseudomonas aeruginosa* filamentous phages are less prolific than tailed phages. To obtain enough filamentous phages from each lysate, bacterial growth was measured and used in this study. Then, a novel double-layer approach was used to induce phage profiles using mitomycin C[15]. This method allowed us to calculate and validate the abundance of filamentous in each lysate. This study is able to induce only 26.4% *P. aeruginosa*

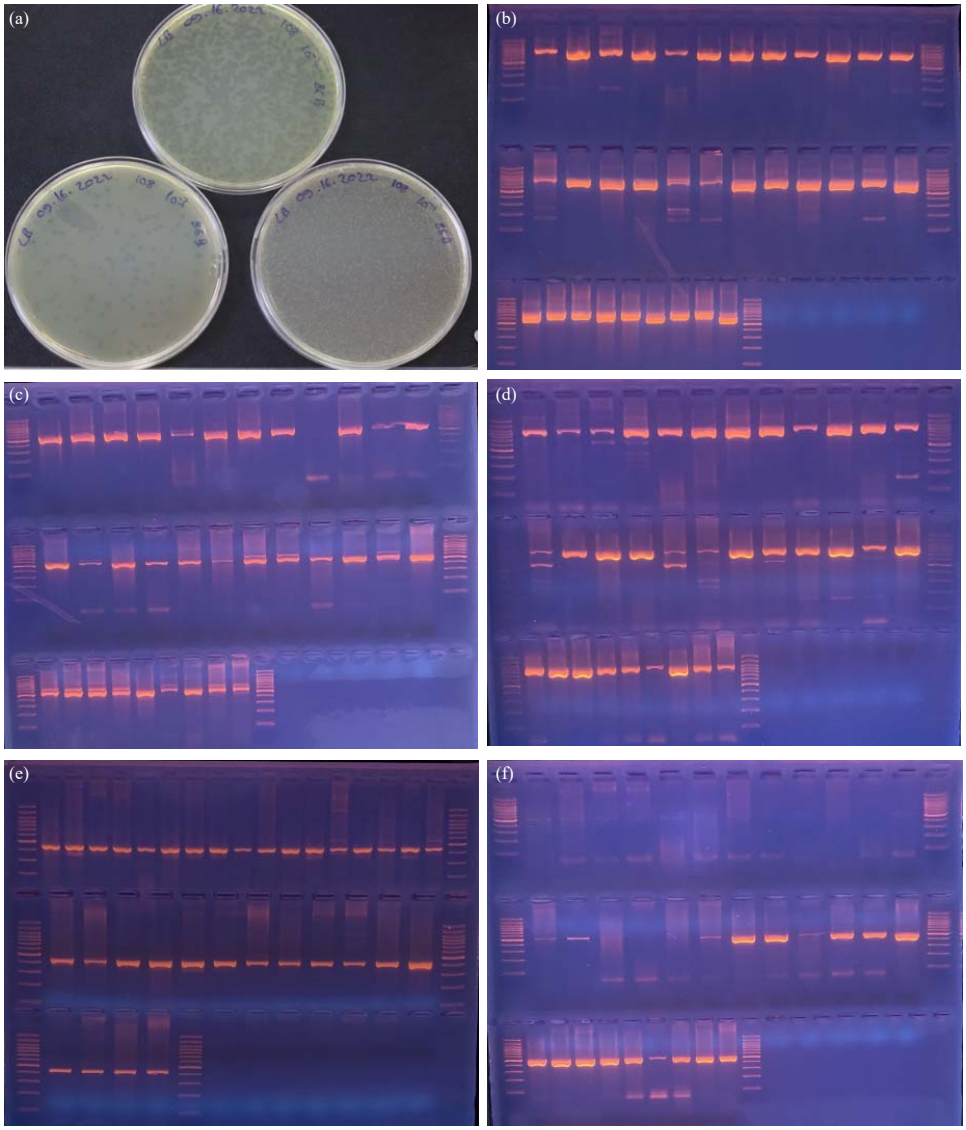


Fig. 1(a-f): Selection of *Pseudomonas aeruginosa* isolates, (a) Phage plaque assay after mitomycin-C induction for *Pseudomonas aeruginosa*, (b) A gel image of PfU-a amplicons, (c) A gel image of PfU-b amplicons, (d) A gel image of Pf4 amplicons, (e) A gel image of Pf5 amplicons and (f) A gel image of Pf1 amplicons

(33/125 clinical samples) phages using mitomycins C, suggesting that mitomycin-C might be limited to induce whole phage profiles in *P. aeruginosa* genome, or their genome is not abundant with phages. Burgener *et al.*[19] proposed Pf phage is common in *P. aeruginosa* bacteria, but not in all isolates. In our studied population, observed some different results about abundance of phages in *P. aeruginosa*. But it would be worth mentioning that our whole isolates are clinical samples compared to their study. These findings might suggest that sources of *P. aeruginosa* isolates should be considered and investigated for future study in detail.

The clinical *P. aeruginosa* genomes have common Pfu-a, Pfu-b, Pf4 and Pf5 phages, but not Pf1 phages. The presence of Pf1 was investigated in 33 isolates in which Pfu-a, Pfu-b, Pf4 and Pf5 bands were detected in PCR results using appropriate primers in 55 samples in which phage plaque was detected by the overload plaque method as a result of induction. The presence of Pf1 was detected in 15 of them (45.4%). It was concluded that Pf profiles of *P. aeruginosa* affect both antibiotic susceptibility and biofilm production potential, pyoverdin and pyocyanin production.

Table 2: Antibiotics effects and mucoid characterizations

| | Resistance | Susceptible | Total | p-value |
|---|---|---|---|---|
| **Amikacin** | | | | |
| Absent | 9 | 8 | 18 | 0.027 |
| Present | 1 | 10 | 14 | |
| Total | 10 | 18 | 32 | |
| **Aztreonam** | | | | |
| Absent | 12 | 4 | 16 | 0.074 |
| Absent | 5 | 7 | 12 | |
| Total | 17 | 11 | 28 | |
| **Gentamicin** | | | | |
| Absent | 10 | 8 | 18 | 0.004** |
| Present | 1 | 13 | 14 | |
| Total | 11 | 21 | 32 | |
| **Imipenem** | | | | |
| Absent | 12 | 4 | 16 | 0.047 |
| Present | 5 | 8 | 13 | |
| Total | 17 | 12 | 29 | |
| **Colistin** | | | | |
| Absent | 3 | 15 | 18 | 0.419 |
| Present | 1 | 13 | 14 | |
| Total | 4 | 28 | 32 | |
| **Levofloxacin** | | | | |
| Absent | 11 | 5 | 16 | 0.153 |
| Present | 6 | 8 | 14 | |
| Total | 17 | 13 | 30 | |
| **Meropenem** | | | | |
| Absent | 11 | 4 | 17 | 0.034 |
| Present | 4 | 9 | 13 | |
| Total | 15 | 13 | 30 | |
| **Netilmicin** | | | | |
| Absent | 11 | 5 | 16 | 0.796 |
| Present | 9 | 5 | 14 | |
| Total | 20 | 10 | 30 | |
| **Piperacillin** | | | | |
| Absent | 11 | 5 | 16 | 0.219 |
| Present | 6 | 7 | 13 | |
| Total | 17 | 12 | 29 | |
| **Piperacillin/tazobactam** | | | | |
| Absent | 12 | 6 | 18 | 0.048 |
| Present | 4 | 9 | 13 | |
| Total | 16 | 15 | 31 | |
| **Cefepime** | | | | |
| Absent | 13 | 4 | 17 | 0.004** |
| Present | 3 | 10 | 13 | |
| Total | 16 | 14 | 30 | |
| **Ceftazidime** | | | | |
| Absent | 12 | 5 | 17 | 0.010 |
| Present | 3 | 10 | 13 | |
| Total | 15 | 15 | 30 | |
| **Ciprofloxacin** | | | | |
| Absent | 10 | 6 | 16 | 0.282 |
| Present | 6 | 8 | 14 | |
| Total | 16 | 14 | 30 | |
| **Tobramycin** | | | | |
| Absent | 7 | 9 | 16 | 0.024 |
| Present | 1 | 13 | 14 | |
| Total | 8 | 22 | 30 | |
| **Phenotype (mucoid)** | | | | |
| Absent | 9 | 9 | 18 | 0.074 |
| Present | 12 | 3 | 15 | |
| Total | 21 | 12 | 33 | |

**Levene's test is significant ($p<0.05$), suggesting a violation of the equal variance assumption

The antibiotic susceptibilities of Pf1-positive *P. aeruginosa* isolates to amikacin, aztreonam, gentamicin, imipenem, meropenem, piperacillin-tazobactam, cefepime, tobramycin and ceftazidime were significantly higher. No significant difference was found in susceptibility to ciprofloxacin, piperacillin, netilmicin, levofloxacin and colistin. The current results were slightly consistent with of studies Burgener *et al*.[19] and Gavric and Knezevic[15]. They also reported that Pf phage infection may allow the transformation of *P. aeruginosa* bacteria from intermediate resistance to susceptible to ceftazidime and tetracycline[15].

## CONCLUSION

Increasing antibiotic resistance is critical for determining new alternative treatments. First of all, it is known that MDR and later XDR isolates of *P. aeruginosa*, which is the subject of the research, are gradually increasing. It is even reported that PDR isolates have been seen recently. In this case, to determine effective control strategies, it is important to know the genome characteristics of *P. aeruginosa* as well as the Pf phage profiles they have to investigate the ways of control. This study was conducted in order to draw attention to the importance of determining the antibiotic susceptibilities of *P. aeruginosa* bacteria isolated from clinics together with their Pf phage profiles. The results obtained showed the necessity of evaluating the presence of Pf1 phage as a factor in determining the ways of control and revealing their role in antibiotic susceptibility.

## SIGNIFICANCE STATEMENT

*Pseudomonas aeruginosa* isolated from clinics seem to be developing resistance to more and more antibiotics. The WHO has emphasized the importance of this situation by including *Pseudomonas aeruginosa* among the critical agents. It should not be neglected that Pf phages are among the resistance factors of *Pseudomonas aeruginosa*. This study contains important results in terms of determining the Pf phage profiles of clinic *Pseudomonas aeruginosa* and revealing whether there is a relationship between Pf1 and antibiotic susceptibility. The results of the study provide important data showing that there is a relationship between the antibiotic resistance status of this agent and the filamentous phage profiles they have.

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