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Original Article

Isolation and Characterization of Bacteriophages Active against *Pseudomonas aeruginosa* Strains Isolated from Diabetic Foot Infections

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Abstract

Diabetic foot infection has become one of the most important public health concerns and is a growing problem**.** *Pseudomonas aeruginosa* is an important opportunistic multidrug-resistant bacterium in diabetic foot infections. In the absence of antibiotics active against MDR strains of *P. aeruginosa*, phage therapy becomes a key way to deal with *P. aeruginosa* infections. Out of 185 samples collected from diabetic foot ulcers, 50 (27.02%) isolates were identified as *P. aeruginosa*. The incidence increases with older ages, and males (n=34, 68%) predominated in all age groups. The tested isolates showed maximum susceptibility towards colistin (80%), imipenem (72%), amikacin (66%), and piperacillin/tazobactam (62%), while these isolates showed moderate susceptibility towards ceftazidime (58%), cefepime (52%) and gentamicin (46%). However, it showed complete resistance (100%) to ampicillin, cefaclor, and sulphamethoxazole/trimethoprim and highly resistance to clindamycin (90%) and amoxicillin/clavulanic acid (84%). Two bacteriophages (ϕPAE1 and ϕPAE2) isolated from sewage samples showed a broad host range against *P. aeruginosaa* clinical strains. ϕPAE2 infected 74% (37/50) and ϕPAE2 58% (29/50). Furthermore, both phages were host-specific, infecting only *P. aeruginosa* strains and could not infect other bacterial species in the cross-infectivity studies. Both phages were found to be relatively heat stable as over a period of 1 h, after exposure to a temperature range of 37–50°C, no significant loss in phage activity was observed. On the other hand, the lowest activity was observed at 70°C (39.15%) for ϕPAE1 whereas it was inactivated at 75°C while the lowest activity was observed at 75°C (38.01%) for ϕPAE2 whereas it was inactivated at 80°C. Isolated phages were able to survive and lyse host bacteria over a wide pH range. The optimum pH range for infection was from 6 to 8. Furthermore, ϕPAE1 lost its ability to lyses at pH 2, 3, 11 and 12, whereas; ϕPAE2 lost its infectivity at pH 2, 3 and 12. Chloroform was the most effective solvent that reduced the infectivity of ϕPAE1 and ϕPAE2 to 63.27% and 77.88%, respectively. On the other hand, petroleum ether showed the lowest effect on the infectivity of ϕPAE1 and ϕPAE2; it was reduced to 96.4% and 97.48%, respectively, followed by acetone and ethyl alcohol. The ability of *P. aeruginosa* phages to form plaques after different storage temperatures (4°C, 30°C, 37°C and 44°C) for a month was slightly affected. The storage of ϕPAE1 and ϕPAE2 at 4ºC showed the least effect on its infectivity, and the storage at 44ºC showed the highest reduction in its infectivity. Moreover, Phage counts were slightly decreased by increasing storage period and temperature.

Keywords: Bacteriophages, Phage treatment, *Pseudomonas aeruginosa*, Diabetic foot, Antibiotic resistance

1. Introduction

Diabetic foot disease is one of the most difficult-to-treat complications of diabetes and has become an important cause of non-traumatic amputation. The probability of diabetic patients suffering from diabetic foot ulcers (DFUs) during their lifetime can reach 25% (1). The risk of a DFU complicated by a diabetic foot infection (DFI) is high. DFIs not only extend the average length of the hospital stay, resulting in a huge economic burden but also increase the risk of amputation (2), which seriously affects the quality of life and life expectancy of patients with diabetes. Control of DFIs promptly and effectively has become an urgent problem for clinicians.

Pseudomonas aeruginosa is a Gram-negative aerobic bacillus, and the natural skin and intestinal tract flora found in water and soil. It is an opportunistic pathogen, and one of the leading causes of nosocomial infections causes severe diseases like cystic fibrosis, urinary tract infections, acute purulent meningitis, otitis media, otitis external, eye infections, wound and burn infections, septicaemia and infantile diarrhea (3). The multiple drugs resistant to the most commonly used antibiotics are quite common in *P. aeruginosa* due to the possession of a high number of virulence factors, which is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes and the low permeability of bacterial cellular envelopes as well as biofilm formation (4).

Around the world, *P. aeruginosa* plays a vital role in diabetic foot infections. As a Gram-negative opportunistic pathogen, *P. aeruginosa* causes recurrent and refractory infections characterized by biofilm formation and antibiotic resistance (3). So, there is considerable interest in developing low-cost, large-scale alternative remedies to prevent or reduce the multidrug resistance of *P. aeruginosa.* In this regard, bacteriophage may close the therapeutic gap.

Bacteriophage therapy is designed to treat bacterial infections using bacteriophages that infect bacteria and has become a promising alternative to conventional antibiotics. Bacteriophages, in many ways, are advantageous over antibiotics, as they are self-replicating in the presence of host cells, have low inherent toxicity, are highly specific with minimal disruption to normal flora, and disappear with the absence of a host (5). Some bacteriophage therapies have been evaluated experimentally using animal models and clinical trials in recent years to determine their suitability to control *P. aeruginosa* infections. These studies have demonstrated a potential therapeutic effect of specific bacteriophages in reducing mortality and morbidity (6, 7). Thus, intensive research efforts have focused on the prevalence of bacteriophages from different sources to accelerate or expand their applications in various fields.

2. Materials and Methods

2.1. Isolation, purification and identification of *P. aeruginosa*

Pus samples for bacterial culture were collected from 185 patients with diabetic foot ulcers admitted to Zagazig University Hospitals, Zagazig, Egypt. Each sample was inoculated onto selective media (cetrimide agar). Suspected colonies were picked up and re-streaked onto a new plate of the same media till obtaining pure separate colonies. The purified cultures were identified and confirmed after investigating morphological, cultural characters and biochemical tests according to standard clinical laboratory methods (8).

2.2. Antibiotic Sensitivity of Clinical Strains of *P. aeruginosa*

The antibiotic resistance patterns of *P. aeruginosa* isolates were assessed using the Kirby-Bauer disk diffusion method, following the recommendations of the clinical and standard laboratory institute (9). Fourteen antibiotics (Oxoid Ltd. UK) were selected (Table 1).

Table 1. Comparative susceptibility of *P. aeruginosa* isolates against different antibiotics

Antibiotic	Symbol	µg/disc	Susceptible %	Intermediate %	Resistant %
Colistin	CT	10	80	2	18
Imipenem	IPM	10	72	6	22
Amikacin	AK	30	66	8	26
Piperacillin/Tazobactam	TZP	100/10	62	8	30
Ceftazidime	CAZ	30	58	12	30
Cefepime	FEB	30	52	6	42
Gentamicin	CN	10	46	12	42
Levofloxacin	LEV	5	36	10	54
Ciprofloxacin	CLIP	5	30	10	60
Amoxicillin/Clavulanic acid	AMC	20/10	6	10	84
Clindamycin	DA	2	4	6	90
Ampicillin	AMP	10	0	0	100
Cefaclor	CEC	30	0	0	100
Sulphamethoxazole/Trimethoprim	NEXT	23.75/1.25	0	0	100

2.3. Molecular Identification of the Most Resistant Isolates

Identifying the most resistant isolates (DF8, DF15, DF24 and DF39) that were resistant to all tested antibiotics was confirmed by investigating 16S *rRNA* gene sequences according to (10).

The obtained sequences of each isolate were compared to published sequences in GenBank using the Basic Local Alignment Search Tool (BLAST)

2.4. Isolation of *P. aeruginosa* Lytic Phages

Anti-*P. aeruginosa* virulent phages were detected in four crude sewage wastewater samples collected from the 10th of Ramadan wastewater treatment plant, Zagazig university hospital, sewage treatment plant in Alqnayat and sewage treatment plant in Abu Khalil at El-Sharkia Governorate, Egypt.

Sewage samples were clarified by centrifugation at 6000 rpm for 20 min. After particle removal by paper filters, the supernatant was filtered through a 0.45µm membrane filter (Steradisc, Kurabo Co., Ltd. Osaka, Japan). From these filtrates, 100 ml were added to an equal volume of double-strength nutrient broth supplemented with 1 mM MgSO4 and 1 mM CaCl2 in a 250 ml Erlenmeyer flask. The flask was inoculated with 1ml of the fresh culture of different pathogenic *P.aeruginosa* (2×10^9 CFU/ml) isolated from diabetic foot ulcers and incubated on a shaker at 120 rpm at 37 ºC/24hr. Ten ml chloroform was added to this culture and centrifuged at 6000 rpm for 30 minutes. The supernatant was filtered through a 0.45 µm membrane filter and was used as a possible source of phages (11).

2.5. Detection of Phages

2.5.1. Spot Test

The presence of phages in the supernatant was detected by spot test as an initial test measuring lytic activity. 100µl of an overnight culture of *P. aeruginosa* ($3.2\ x10^9$ CFU/ml) was added to 4ml semi-solid nutrient agar (0.7% agar), mixed gently, and poured over solid nutrient agar plates. After solidification, 10µl droplets of the supernatant previously prepared were spotted on the lawns of the bacterial isolates and left to dry. The plates were incubated overnight at 37 ºC and checked for the presence of lysed zones. A clear zone in the plate, resulting from the lysis of host cells, indicated the presence of phage. Positive tests were confirmed by plaque assay (12).

2.5.2. Plaque Assay (Double Agar Overlay) Method

This method was used for both the detection and titration of existing phage, according to Pires *(13)*. Titration of phages occurred by serial dilution of phage filtrate, and the diluent was sterile saline (0.9% NaCl). To a 3 ml melted semi-solid agar kept at 45 ºC, 100µl of bacterial overnight culture of the tested bacterial isolate was mixed with 100µl of phage suspension, shaken and poured quickly onto solid nutrient agar plates. After solidification of the double agar overlay, the plates were incubated overnight at 37 ºC. After

incubation, the types of plaques and counting were detected. The titer of the phage expressed as plaque-forming units per ml (PFU/ml) was calculated as follows:

$$PFU/ml = \frac{\text{Number of plaques} \times \text{Dilution Factor}}{\text{Phage volume plated (ml)}}$$

2.6. Purification of Phages

Phages were purified from single-plaque isolates, according to Sekhar (14). The isolated phages were purified by three successive single-plaque isolation with a sterile pasture pipette until homogenous plaques were obtained. Briefly, a single plaque (different plaque phenotypes, if present, from each plate) was picked and put into 5.0 ml nutrient broth, in which 100 µl of the bacterial host (overnight culture of *P. aeruginosa*) was added and incubated at 37 ºC under shaking condition with 1200 rpm. After incubation, the phage-host mixture was centrifuged at 6000 rpm for 10 min, and supernatants were filtered through a sterilized Millipore filter (0.45µm pore size) to remove any bacterial contamination. Dilutions were then prepared, and plating was performed to allow the purification and isolation of a single phage.

Repeated subcultures on the respective host strain performed consecutive rounds of plaque purification. The plaque purification process has repeated a minimum of three times for each plaque or until a purified plate of uniform plaques was obtained, reflected by single plaque morphology. Purified phages were stored in 50 % glycerol (v/v) in nutrient broth at -80°C for long-term use. Short-term stock preparations were maintained at 4°C.

2.7. Host Range and Cross-Infectivity of the Isolated Phages

The isolated phages (ϕPAE1 and ϕPAE2) were investigated for host range specificity by spot test as described before. Bacterial lawns of the 50 different *P. aeruginosa* strains isolated from diabetic foot ulcers, and other 4 different bacterial species (*Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus* and *Enterococcus faecalis;* kindly provided by Microbiology Department, Faculty of Science, Ain Shams University, Egypt) were propagated on nutrient plates, and 10µl droplets of phages were put on the lawns. The plates were incubated overnight at 37 ºC and checked for the presence of plaque. Phages were named according to the name of the host and the country isolated from (6).

2.8. Thermal Stability Determination

To determine the thermal stability of phages, two ml of each phage suspension were incubated in a water bath for 60 min at the following temperatures: 37ºC, 45ºC, 50ºC, 55ºC, 60ºC, 65ºC, 70ºC, 75ºC, 80ºC, 85ºC and 90ºC. Samples were obtained at 10-minute intervals and immediately plated for phage titration. Three independent experiments were performed. Phage infectivity was determined by plaque assay technique, and the results were presented as the percentage of PFUs in the treated group compared with the initial PFUs (6).

2.9. pH Stability Assay

In the pH stability assay, phage stocks were incubated in media ranging from 2.0-12.0 (with 1.0 intervals) using 0.1 N HCl and/or 0.1 N NaOH for 60 min. After incubation of the mixtures at 37ºC overnight, the residual phage activity was determined by plaque assay technique as previously reported. Three independent experiments were performed. Phage infectivity was determined by plaque assay technique, and the results were presented as the percentage of PFUs in the treated group compared with the initial PFUs at pH = 7 (6).

2.10. Phage Suspension with Different Organic Solvents

2 ml of different organic solvents (petroleum ether (60-80), acetone (99 %), ethyl alcohol (95 %) and chloroform (99 %)) was added to 2 ml of phage suspension. Two ml of nutrient broth was added to 2 ml of phage suspension and used as a control. After 30 minutes in a shaking incubator, the phage suspension was centrifuged at 4500 rpm for 10 minutes. Twenty µl were diluted to 5 ml to stop the effect of chloroform and assayed for the infectivity of phages by plaque assay (14).

2.11. Storage of Phages at Different Temperatures

The effect of storage of phages at different temperatures was studied with a known titer of $5.9x10^7$ PFU/ml for ØPAE1 and $4.3x10^8$ PFU/ml for ØPAE2 which was dispensed in sterile screw-capped tubes and then stored at different temperatures: 4°C, room temperature (30°C), 37°C and 44°C. Samples were withdrawn and assayed for infectivity weekly for a month (15).

3. Results

Of the 185 samples collected from different patients, 50 (27.02%) isolates were identified as *Pseudomonas aeruginosa* (positive on cetrimide agar), which were further confirmed through a series of different biochemical tests. The results showed this bacterium was positive for motility, catalase, oxidase, citrate, and gelatin liquefaction, while negative for methyl red, Voges-Proskauer, urease, and indole tests.

The majority of the patients were elderly (mean age 62.4). The patient ages ranged between 42 to 85 years. The incidence increases with older ages, and the majority of strains (n=17, 34%) were derived from patients aged 61-70 years. More organisms were isolated from males (n=34, 68%) than from females (n=16, 32%).

The identification of the most resistant isolates (DF8, DF15, DF24 and DF39) was confirmed by using 16S *rRNA* gene sequencing as *Pseudomonas aeruginosa DF8*, *Pseudomonas aeruginosa DF15*, *Pseudomonas aeruginosa DF24*, and *Pseudomonas aeruginosa DF39*. The sequences were submitted to GenBank at the NCBI website (www.ncbi.nlm.nih.gov) in accession numbers: MT158652, MT158653, MT158654, and MT158655, respectively.

The tested *P. aeruginosa* isolates showed maximum susceptibility towards colistin (80%), imipenem (72%), amikacin (66%), and piperacillin/tazobactam (62%), while these isolates showed moderate susceptibility towards ceftazidime (58%), cefepime (52%) and gentamicin (46%). On the other hand, the tested isolates showed complete resistance (100%) to ampicillin, cefaclor, and sulphamethoxazole/trimethoprim and highly resistance to clindamycin (90%) and amoxicillin/clavulanic acid (84%). Finally, it was noted that *P. aeruginosa* isolates showed increased resistance to quinolones (ciprofloxacin (60%) and levofloxacin (54%)).

Two sewage samples out of four (Zagazig university hospital and 10th of Ramadan wastewater treatment plant) gave lytic activity against *P. aeruginosa* clinical isolates using the spot test technique, as shown in figure 1. The plaque assay test confirmed the presence of bacteriophages specific for *P. aeruginosa* isolates, as shown in figure 2. Two plaques with different diameters (2 mm and 4 mm) and clear appearance were selected, purified and characterized (Figure 2). These two plaques types were designated as ØPAE1 and ØPAE2 (Ø= phage, P= *Pseudomonas*, A= *aeruginosa* and E= Egypt) and were used for further studies.

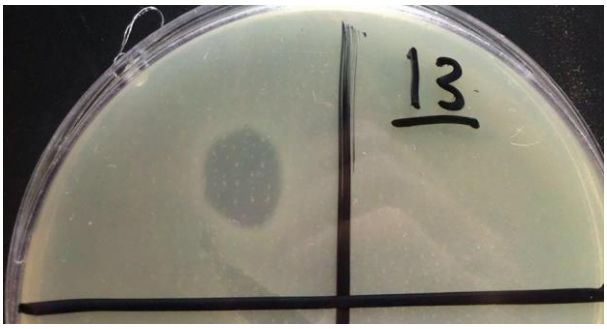

Figure 1. Spot test on a lawn of *P. aeruginosa*

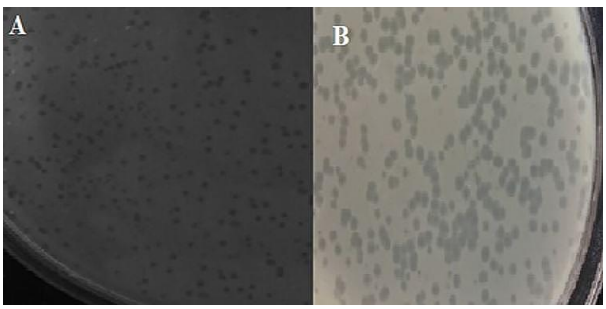

Figure 2. Plaque morphology of *P. aeruginosa* phages. (**A**) Bacteriophage ϕPAE1 produced distinct, circular, transparent plaques with defined margins with a plaque diameter of about 2mm. (**B**) Bacteriophage ϕPAE2 produced distinct, circular, transparent plaques with defined margins with a plaque diameter of about 4mm

The results presented in table 2 showed that ϕPAE1 and ϕPAE2 bacteriophages showed a broad host range against *P. aeruginosa* clinical strains isolated from diabetic foot ulcers. ϕPAE2 bacteriophage infected 74% (37/50) and ϕPAE2 58% (29/50) of *P. aeruginosa* clinical strains, of which multidrug-resistant isolates used as a host in this study which showed a quite broad host range of these phages. Furthermore, both phages were host-specific, infecting only *P. aeruginosa* strains and could not infect other bacterial species.

The four (100% Multidrug resistant) *pseudomonas aeruginosa* strains were found to be all infected by bacteriophage ϕPAE2

The data obtained in this study showed that phages ϕPAE1 and ϕPAE2 differed in lytic activity against *P. aeruginosa* strains but complemented each other in the lysis of at least 86% (43/50) of the used *P. aeruginosa* strains. This can be considered an important positive result of the design of therapeutic bacteriophage-based preparations.

Plaque assay results showed that the highest titer of phage was observed with ϕPAE2 (4.3×10^{12}) followed by ϕPAE1 (3.1×10^{9}). Furthermore, bacterial strain *P. aeruginosaDF25* was the most sensitive strain to the tested phages under investigation. So it was used as a phage host for the following studies.

The results in figure 3 indicated that both phages were found to relatively heat stable as over a period of 1 h, after exposure to a temperature range of 37–50°C, no significant loss in phage activity was observed. However, at 60°C after 1h, a 36.58% and 21.29% reduction in phage activity for ϕPAE1 and ϕPAE2, respectively, was observed that showed heat stability of bacteriophage ϕPAE2. On the other hand, the lowest activity was observed at 70°C (39.15%) for ϕPAE1 bacteriophage, whereas it was inactivated at 75°C and above while the lowest activity was observed at 75°C (38.01%) for ϕPAE2 bacteriophage whereas it was inactivated at 80°C and above. It was noticed that ϕPAE1 was more sensitive to temperature than ϕPAE2.

The results in figure 4 revealed that phages could survive and lyse host bacteria over a wide pH range. The optimum pH range for infection was from 6 to 8, with almost 100% infectivity with maximum activity observed at pH 7. Moreover, bacteriophages tolerate the alkaline medium more than the acidity medium.

Furthermore, the tested ϕPAE1 phage lost its ability to lyse (no plaque formation was observed) at pH 2, 3, 11 and 12, whereas ϕPAE2 phage lost its infectivity at pH 2, 3 and 12. Moreover, plaque formation of ϕPAE1 was more sensitive to pH exchange than ϕPAE2.

Data illustrated by figure 5 show that incubating phage suspensions with chloroform, acetone, petroleum ether and ethyl alcohol in a ratio of 1:1 (v/v) for 30 min at 37º C reduced its infectivity to different percentages. Chloroform was the most effective solvent that reduced the infectivity of ϕPAE1 and ϕPAE2 to 63.27% and 77.88%, respectively. On the other hand, petroleum ether showed the lowest inhibiting effect on the infectivity of ϕPAE1 and ϕPAE2; it was reduced to 96.4% and 97.48%, respectively. ϕPAE1 was the most sensitive one for solvents. In contrast, the infectivity of ϕPAE2 was slightly affected by solvents. Moreover, data showed that the infectivity of both phages was still above 63 % after incubation for 30 min with each of the tested organic solvents.

The results of storage of *P. aeruginosa* phages at different temperatures (4°C, 30°C, 37°C and 44°C) for the month were shown in tables 3 and 4. The ability of phages to form plaques after different storage temperatures were slightly affected. The storage of ϕPAE1 and ϕPAE2 at 4ºC showed the least effect on its infectivity after extended incubation for one to four weeks, while the storage of ϕPAE1 and ϕPAE2 at 44ºC showed the highest reduction in its infectivity after extended incubation for one to four weeks. Phage counts were slightly decreased by increasing storage period and temperature.

Table 2. Host range of isolated *P. aeruginosa* phages

	Formation of lytic area (±) and phage titre (PFU/ml)	
Bacterial species	ϕPAE1	ϕPAE2
Pseudomonas aeruginosa DF1	-	+(3.3×10^{9})
Pseudomonas aeruginosa DF2	-	-
Pseudomonas aeruginosa DF3	-	-
Pseudomonas aeruginosa DF4	+(3.1×10^{7})	+(2.1×10^{8})
Pseudomonas aeruginosa DF5	+(2.1×10^{9})	+(3.5×10^{6})
Pseudomonas aeruginosa DF6	-	-
Pseudomonas aeruginosa DF7	+(2.3×10^{9})	+(3.1×10^{9})
Pseudomonas aeruginosa DF8	-	+(3×10^{7})
Pseudomonas aeruginosa DF9	-	+(2×10^{9})
Pseudomonas aeruginosa DF10	+(1.1×10^{9})	+(3.1×10^{10})
Pseudomonas aeruginosa DF11	-	-
Pseudomonas aeruginosa DF12	+(1.8×10^{5})	+(2.5×10^{11})
Pseudomonas aeruginosa DF13	+(3.2×10^{8})	+(3×10^{9})
Pseudomonas aeruginosa DF14	+(2×10^{7})	+(1.9×10^{11})
Pseudomonas aeruginosa DF15	+(3×10^{6})	+(3.1×10^{7})
Pseudomonas aeruginosa DF16	+(2.6×10^{8})	+(2.5×10^{9})
Pseudomonas aeruginosa DF17	+(2.4×10^{9})	+(1.6×10^{9})
Pseudomonas aeruginosa DF18	+(3.5×10^{7})	+(2.8×10^{11})
Pseudomonas aeruginosa DF19	+(1.9×10^{9})	+(3×10^{6})
Pseudomonas aeruginosa DF20	-	+(1.6×10^{7})
Pseudomonas aeruginosa DF21	-	+(2×10^{9})
Pseudomonas aeruginosa DF22	+(2.3×10^{7})	-
Pseudomonas aeruginosa DF23	+(2.6×10^{8})	-
Pseudomonas aeruginosa DF24	-	+(3.4×10^{10})
Pseudomonas aeruginosa DF25	+(3.1×10^{9})	+(4.3×10^{12})
Pseudomonas aeruginosa DF26	+(1.4×10^{5})	+(3×10^{9})
Pseudomonas aeruginosa DF27	-	+(4×10^{4})
Pseudomonas aeruginosa DF28	-	+(1.8×10^{10})
Pseudomonas aeruginosa DF29	+(2×10^{4})	+(3.2×10^{6})
Pseudomonas aeruginosa DF30	-	-
Pseudomonas aeruginosa DF31	-	+(2.8×10^{9})
Pseudomonas aeruginosa DF32	+(2×10^{7})	+(3.1×10^{11})
Pseudomonas aeruginosa DF33	+(2.1×10^{4})	+(3.8×10^{9})
Pseudomonas aeruginosa DF34	-	+(2.1×10^{7})
Pseudomonas aeruginosa DF35	-	+(1.6×10^{10})
Pseudomonas aeruginosa DF36	+(3.1×10^{4})	-
Pseudomonas aeruginosa DF37	-	-
Pseudomonas aeruginosa DF38	+(1.5×10^{7})	+(2×10^{9})
Pseudomonas aeruginosa DF39	+(3×10^{8})	+(1.9×10^{9})
Pseudomonas aeruginosa DF40	+(2.6×10^{5})	+(3.1×10^{11})
Pseudomonas aeruginosa DF41	+(3.1×10^{9})	+(3×10^{5})
Pseudomonas aeruginosa DF42	-	-
Pseudomonas aeruginosa DF43	+(2.4×10^{5})	-
Pseudomonas aeruginosa DF44	+(2.7×10^{8})	+(2.7×10^{10})
Pseudomonas aeruginosa DF45	-	+(3.6×10^{9})
Pseudomonas aeruginosa DF46	-	+(2.8×10^{11})
Pseudomonas aeruginosa DF47	+(3×10^{7})	-
Pseudomonas aeruginosa DF48	+(3.4×10^{6})	+(1.8×10^{10})
Pseudomonas aeruginosa DF49	+(1.8×10^{9})	-
Pseudomonas aeruginosa DF50	-	+(3.8×10^{8})
Escherichia coli	-	-
Klebsiella pneumonia	-	-
Staphylococcus aureus	-	-
Enterococcus faecalis	-	-
Total	29/50 (58%)	37/50 (74%)

+ = phage produced lytic area in spot test.− = no lytic area in the spot test.
Numbers between brackets are phage titre (PFU/ml) using plaque assay technique

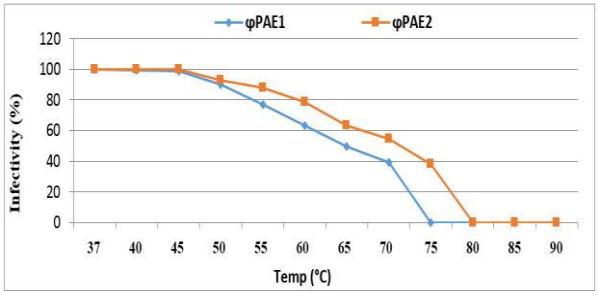

Figure 3. Thermal stability of *P. aeruginosa* phages

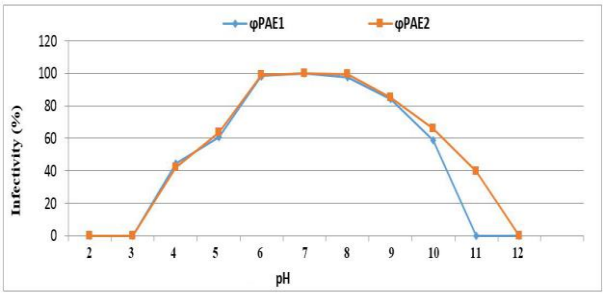

Figure 4. Stability of *P. aeruginosa* phages at different pH values

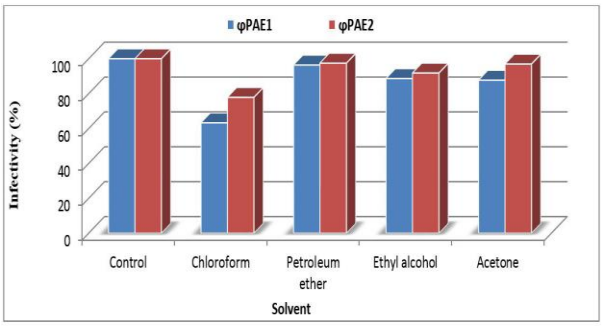

Figure 5. Effect of organic solvents on the infectivity of *P. aeruginosa* phages

Table 3. Storage of ϕPAE1 phage at different temperatures

	1st week			2nd week		
Temp (°C)	**PFU/ml**	**Log PFU/ml**	**Infectivity (%)**	**PFU/ml**	**Log PFU/ml**	**Infectivity (%)**
Initial	$5.9x10^7$	7.77	100			
4	$5.7×10^7$	7.75	99.74	$4.6×10^7$	7.66	98.58
30	$4.6×10^7$	7.66	98.58	$4×10^7$	7.6	97.81
37	$4.6×10^7$	7.66	98.58	$3.7×10^7$	7.56	97.3
44	$3.1×10^7$	7.49	96.4	$1.5×10^7$	7.18	92.4
	3rd week			**4th week**		
Temp (°C)	**PFU/ml**	**Log PFU/ml**	**Infectivity (%)**	**PFU/ml**	**Log PFU/ml**	**Infectivity (%)**
4	$4.1×10^7$	7.61	97.94	$3×10^7$	7.48	96.26
30	$3.4×10^7$	7.53	96.91	$2×10^7$	7.3	94
37	$3×10^7$	7.48	96.26	$1.5×10^7$	7.18	92.4
44	$5.8×10^6$	6.76	87	$2.4×10^6$	6.38	82.11

Table 4. Storage of ϕPAE2 phage at different temperatures

	1st week			2nd week		
Temp (°C)	**PFU/ml**	**Log PFU/ml**	**Infectivity (%)**	**PFU/ml**	**Log PFU/ml**	**Infectivity (%)**
Initial	$4.3x10^8$	8.63	100			
4	$4.2×10^8$	8.62	99.88	$4×10^8$	8.6	99.65
30	$4×10^8$	8.6	99.65	$3.8×10^8$	8.58	99.42
37	$3.9×10^8$	8.59	99.53	$3.8×10^8$	8.58	99.42
44	$3.5×10^8$	8.54	98.95	$3.2×10^8$	8.5	98.49
	3rd week			**4th week**		
Temp (°C)	**PFU/ml**	**Log PFU/ml**	**Infectivity (%)**	**PFU/ml**	**Log PFU/ml**	**Infectivity (%)**
4	$3.5×10^8$	8.54	98.95	$2.9×10^8$	8.46	98.03
30	$2.6×10^8$	8.41	97.45	$1.8×10^8$	8.26	95.71
37	$2×10^8$	8.3	96.17	$1.8×10^8$	8.26	95.71
44	$1.2×10^8$	8.08	93.62	$6.2×10^7$	7.79	90.26

4. Discussion

Diabetic foot ulcers (DFU) are responsible for more than 20% of diabetes-related hospital admissions, and it also causes complications in lower limbs or life-threatening diabetic foot infections (DFI) among admitted patients. One of the major reasons for amputation among diabetic patients is usually triggered by the development of a chronic wound, clinically defined as a wound that fails to heal within 30 days. Chronic wound leads to significant tissue destruction and is highly susceptible to the rapid spread of infections leading to subsequent amputation of the foot (16).

In the current study, 50/185 (27.02%) samples produced positive growth on a selective medium. The majority of the patients were elderly (mean age 62.4). The incidence increases with older ages, and the majority of strains (n=17, 34%) were derived from patients aged 61-70 years. More organisms were isolated from males (n=34, 68%) than females (n=16, 32%) and males predominated in all age groups. These results were in agreement with recent data (16, 17).

In this study, we have noticed significant variations in certain variables, such as gender and age. Males outnumbered females by a factor of 2.12:1. A similar gender ratio was reported by Bansal (18) in Gujarat, India (2.1:1), Al Benwan (19) in a study conducted in Kuwait (2.8:1), Saltoglu (20) in Turkey (2.03:1) and Machado (21) in Portuguese (3:1). Male predominance in DFU could be linked to factors such as gender-related differences in lifestyles and professional roles that require the feet to tolerate more pressure. Increased levels of outdoor work and poor compliance with foot care practices were more dominant among males than females (14).

Most of the patients were elderly (mean age 62.44), similar to a study reported by Chavan (22). Previous studies also reported that people in the elder group are at a greater risk of developing foot ulcers and are more susceptible to abscesses and osteomyelitis (17, 21).

Adequate knowledge about the microbes that cause infection is very important and helps determine appropriate antibiotic therapy and proper management of these infections. *Pseudomonas aeruginosa* is an important human opportunistic bacterium in the diabetic foot. It is a gram-negative aerobic, rod-shaped non-fermenting bacterium with unipolar motility. It can be responsible for various presentations, from superficial colonization of ulcers to extensive tissue damage, including osteomyelitis, septic arthritis and bacteremia (23, 24).

In the current study, out of the 185 samples collected from different diabetic foot ulcers, 50 (27.02%) isolates were identified as *Pseudomonas aeruginosa.* Similarly, previous studies reported that *P. aeruginosa* is the most common gram-negative rod-shaped bacterium isolated from the soft tissue or ulcerated skin of diabetic foot infections (DFIs) (14, 17, 19).

Different studies have revealed that samples from diabetic foot infections have grown a variety of bacteria in a culture medium. According to a study conducted in Malaysia on diabetic foot, *S. aureus* was isolated in 44% of cases, *Proteus* in 28%, *P. aeruginosa* in 25% and *Klebsiella* in 15% of cases (25). In a study by Kurup & Ansari (17), Gram-negative bacteria (63.0%) were more prevalent than gram-positive bacteria (37.0%). Among DF patients, *Pseudomonas aeruginosa* (18.8%) was the most common isolate, followed by *Escherichia coli* (13.9%) among the gram-negative group. 12.1% MRSA, followed by MSSA (7.9%), dominated among the gram-positive group in diabetic foot patients.

In a multicenter (19 centres) observational study in Turkey, in total, 791 patients with DFI were included, 531(67%) were male, and the median age was 62 (19–90). Five hundred thirty-six microorganisms were isolated; the most common microorganisms were *S. aureus* (20%), *P. aeruginosa* (19%) and *E. coli* (12%). The Methicillin resistance (MR) rate among *Staphylococcus aureus* isolates was 31%. Multidrug-resistant bacteria were detected in 21% of *P.*

aeruginosa isolates. Among Gram-negative bacteria, *Pseudomonas sp*. was predominant and accounted for 33% of the isolated bacteria (20). On the other hand, *P. aeruginosa* infection has been documented from DFU with lower frequency (6–13%) by various authors (21, 26). These findings could be explained based on the duration of DFIs, ulcer grade and empirical antimicrobial agents.

The rapid emergence of multidrug-resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives. Many decades after the first patients were treated with antibiotics; bacterial infections have become a threat. The antibiotic resistance crisis has been attributed to the overuse and misuse of these medications and a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements (27). The selection of an appropriate antibiotic regimen has become the need of the hour to manage diabetic foot ulcers (3, 4) correctly.

The results in the current study revealed that the tested *P. aeruginosa* isolates showed maximum susceptibility towards colistin (80%), imipenem (72%), amikacin (66%), and piperacillin/tazobactam (62%), while these isolates showed moderate susceptibility towards ceftazidime (58%), cefepime (52%) and gentamicin (46%). On the other hand, the tested isolates showed complete resistance (100%) to ampicillin, cefaclor, and sulphamethoxazole/trimethoprim and highly resistance to clindamycin (90%) and amoxicillin/clavulanic acid (84%). Finally, it was noted that *P. aeruginosa* isolates showed increased resistance to quinolones (ciprofloxacin (60%) and levofloxacin (54%)). Similarly, this study's sensitivity pattern of *P. aeruginosa* to imipenem, piperacillin/tazobactam and amikacin was inconsistent with Amjad's findings *(25)*. Muthu (24) reported that antibiotic sensitivity of *P. aeruginosa* from diabetic foot ulcers showed high resistance to gentamicin (50%), cefoperazone (54.5%), cefotaxime (63.6%); intermediate resistance to ceftazidime (36.4%), ciprofloxacin (45.5%); and least resistance to piperacillin (9%), amikacin (9%), and imipenem (13.6%). Furthermore, Fu (28) investigated antibiotic resistance patterns in clinical isolates of *P. aeruginosa* and showed that 96.5% of the isolates were resistant to trimethoprim-sulphamethoxazole.

Imipenem, amikacin and tobramycin were the most effective antimicrobial agents for Gram-negative bacterial species isolated from diabetic foot infections, in which *P. aeruginosa* (18.8%) was the most common isolate according to Kurup and Ansari (17). Moreover, El-Shibiny (5) found that all strains of *P. aeruginosa* showed 100% resistance to ampicillin, ampicillin/sulbactam, cefazolin, tigecycline, nitrofurantoin and trimethoprim/sulphamethoxazole while imipenem, meropenem, amikacin, gentamicin and tobramycin showed 100% sensitivity followed by ciprofloxacin 69.23%.

Pseudomonas aeruginosa isolates collected in the Asia-Pacific region largely showed acquired resistance (<80% susceptibility) to carbapenems (imipenem, doripenem, meropenem) and fluoroquinolones (levofloxacin, ciprofloxacin), while, colistin, ceftolozane/tazobactam and amikacin demonstrated higher susceptibility rates among *P. aeruginosa* (29). Also, Al Benwan (19) observed that imipenem, amikacin and piperacillin/tazobactam were the most effective antibiotic against aerobic Gram-negative bacteria in Kuwait. Nevertheless, imipenem-resistant *Pseudomonas* is a new challenging problem, especially in hospitalized patients (30). Imipenem resistance was observed in (61.2%) of the isolates in Brazil (31).

Of the therapies found to be effective against *P. aeruginosa*, colistin is considered an antimicrobial of last resort owing to potential kidney toxicity. Amikacin is teratogenic and has concerning side effects such as hearing loss and kidney damage toxicity. Both are old drugs that have come back into favour due to acquired resistance to modern antibiotics (29).

P. aeruginosa is a major nosocomial pathogen, intrinsically resistant to many drugs and able to become resistant to virtually any antimicrobial agent. The

resistance mechanisms include the production of β-lactamases, efflux pumps, and target-site or outer membrane modifications. Resistance to multiple drugs usually results from the combination of different mechanisms in a single isolate or the action of a single potent resistance mechanism (4, 32).

The rapid development and spread of drug resistance create an urgent need to identify new antibacterial agents. As phages can be highly specific in identifying and lysing pathogenic bacteria, phage therapy has attracted increasing attention over the past few years (12, 28). As one of the most abundant biological entities, bacteriophages are widely distributed in various ecosystems, including the oceans, soil, wastewater, hot water springs, and animal gut, which provide unlimited resources for phage applications (33). The essential characteristics of phages include their specificity and proliferation within the host and at the site of infection, with no side effects (34).

In the present study, two sewage samples gave lytic activity against *P. aeruginosa* clinical isolates. Sewage water has proved to be a good source of bacteriophages ((35). Sewage water contains a huge genetic diversity of microbes, probably due to contamination from different sources, including fecal and hospital wastes (1).

Bacteriophages generally are very specific in their target and can inhibit particular species or strains of bacteria. To date, no potential phage is known to clear/kill all strains of *P. aeruginosa*. This high specificity of the phage-host relationship leads to the need for the isolation of novel phages, which can be lytic for MDR strains of *P. aeruginosa* (36, 37).

In this study, ϕPAE1 and ϕPAE2 phages showed a broad host range against *P. aeruginosa* clinical strains. ϕPAE2 infected 74% (37/50) and ϕPAE2 58% (29/50) of *P. aeruginosa* clinical strains, of which multidrug-resistant isolates. Phages with a broad host range have been reported previously, including those that lyse 50% of *P. aeruginosa* clinical isolates (38). Similarly, in the study of Balarjishvili (39), the range of antibacterial action of three phages (isolated from wastewater) and their mixtures on 427 multi-drug-resistant *P. aeruginosa* clinical isolates was assessed. PAT-5 bacteriophage lysed 78% *of P. aeruginosa* strains, PAT-13 lysed 57%, and PAT-14 lysed 58%. The lytic activity range of the multi-component preparation obtained by combining three phages was significantly higher and comprised 91.7%. The lytic rates presented here are higher than in other phages, which can be related to the origin and the number of tested strains. Host broad range and species specificity are desirable for bacteriophage application in phage therapy.

Furthermore, our results reveal that both phages (ϕPAE1 and ϕPAE2) were host-specific, infecting only *P. aeruginosa* strains and could not infect other bacterial species in the cross-infectivity studies. These results are comparable to that in the literature (3, 40) but contrary to Bielke's (41). Some phages have an expansive host range tendency. These pages are called broad host range, which was first introduced by Jensen (42), and also they are known as gene diversity carriers in nature. These phages could infect *Pseudomonas*, *Enterobacteriaceae*, Gram-positive bacteria and *Bacillus*. In the study of Oliveira (3), the lytic activity of bacteriophages was tested against 37 strains (33 *P. aeruginosa* and four different bacterial species). Their results reveal that all bacteriophages could efficiently lyse 69.7% of the *P. aeruginosa* strains, of which seven were multidrug resistant but did not lyse other bacterial strains in the cross-infectivity studies.

The data obtained in this study showed that phages ϕPAE1 and ϕPAE2 differed in lytic activity against *P. aeruginosa* strains but complemented each other in the lysis of at least 86% (43/50) of the used *P. aeruginosa* strains. This can be considered an actual positive result of the design of therapeutic bacteriophage-based preparations. Similarly, Kwiatek (12) investigated the effects of two bacteriophages, MAG1 and MAG4, and their capability to control carbapenem-resistant *P. aeruginosa* in planktonic and biofilm models. It was found that each phage individually affected

approximately 50% of *P. aeruginosa* isolates, but when they were used as a cocktail, the anti-biofilm property was increased to 72.9%.

In the current study, the ability of *P. aeruginosa* phages to form plaques after different storage temperatures (4°C, 30°C, 37°C and 44°C) for a month was slightly affected. The storage of ϕPAE1 and ϕPAE2 at 4ºC showed the least effect on its infectivity after extended incubation for one to four weeks, while the storage of ϕPAE1 and ϕPAE2 at 44ºC showed the highest reduction in its infectivity. Phage counts were slightly decreased by increasing storage period and temperature. Similarly, Olson (43) recommends 4°C as the optimum temperature for short (no longer than 40 days) phage storage in wastewater. As shown by Ackermann (15), tailed phages were the most resistant to storage and showed the longest survivability; some retained viability even after 10–12 years at 4°C. Some phages, such as lipid-containing ones, were not stable after storage at 4°C but could be stored at −80°C or in liquid nitrogen.

In the absence of antibiotics active against MDR strains of *P. aeruginosa*, phage therapy becomes the main way to deal with *P. aeruginosa* infections. Thus, it is currently important for phage therapy to become a routine medical procedure in opposition to what it is now: occasional demonstrations of success in relatively rare applications. Such a transition will require an accumulation of large collections of phages specific for *P. aeruginosa* and deep studies of these phages.

It was concluded from this study that wastewater is a good source for the isolation of phages against MDR bacteria. The isolated phages ϕPAE1 and ϕPAE2 were lytic phages showing good heat and pH stability and differ but complement each other in their lytic activity against *Pseudomonas aeruginosa*, which is a very promising parameter for using it as a potential candidate for phage therapy single or in combination.

Authors' Contribution

Study concept and design: W. F. M.
Acquisition of data: A. A. A.
Analysis and interpretation of data: M. M. H. M.
Drafting of the manuscript: E. A. E.
Critical revision of the manuscript for important intellectual content: A. A. A.
Statistical analysis: H. M. A.
Administrative, technical, and material support: A. A. A.

Ethics

Ethical approval for this Study was obtained from the ethics committee at the University Specialized Hospitals, Cairo, Egypt.

Conflict of Interest

The authors declare that they have no conflict of interest.

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