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ORIGINAL ARTICLE

Elimination of multidrug-resistant *Proteus mirabilis* biofilms using bacteriophages

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Abstract

Proteus mirabilis is responsible for a wide range of infections that affect the urinary tract, the respiratory tract, burns, wounds and the feet of individuals with diabetes. They are highly resistant to antimicrobial agents, and new therapeutic options are therefore needed to combat this pathogen. The use of bacteriophages is one option that may be useful in treating multidrug-resistant (MDR) *Proteus mirabilis* infections, especially biofilm-based infections. The aim of this study was to control biofilms formed by MDR *Proteus mirabilis* using bacteriophages. *Proteus mirabilis* isolates were identified based on biochemical tests, and their resistance profiles were determined by the disk diffusion method. The biofilm-forming capacity of the isolates was assessed by the spectrophotometric method. Bacteriophages attacking *Proteus mirabilis* were isolated from sewage. The effect of phage on biofilm formation was investigated by the viable count method. A high rate of drug resistance was found (87.2%). Strong biofilm formation was observed in 80.5% of isolates, while moderate production was found in 19.5%. Five bacteriophages were isolated from sewage and were tested for their ability to eliminate biofilms. Significant disruption of pre-formed biofilms was observed that reached up to 99.9% decrease in the number of viable cells. The use of bacteriophages is considered a promising strategy against the biofilm infections caused by MDR *Proteus mirabilis* isolates.

Introduction

Proteus mirabilis is a Gram-negative bacillus that belongs to the family *Morganellaceae* [3]. It is an opportunistic pathogen that can cause many types of infections, with urinary tract infections being the most prevalent [31]. The risk of infection is high in patients who are catheterized for long periods of time or have structural or physiological abnormalities of the urinary tract [63]. These bacteria can also cause diabetic foot ulcers in diabetic patients [58], respiratory tract infections, and infections of burns and wounds [55].

The routine use of antibiotics has led to the development of antibiotic resistance, particularly in Gram-negative bacteria [67]. Multidrug resistance (MDR) also varies depending on the isolation source, as reported by Abbas *et al.* [2].

Biofilms are communities of cells that adhere to a surface and are enclosed within a matrix of polysaccharides, proteins and nucleic acids [30]. Bacteria within a biofilm exhibit high levels of resistance to biocides and antimicrobial agents. These agents are needed at high concentrations, which can be 1000-fold higher than that required in the case of planktonic cells to produce an antibacterial effect [10]. The most widely investigated *P. mirabilis* biofilms are those that occur in the urinary tract, especially in catheterized patients. The formation of crystalline biofilms on catheters can lead to catheter encrustation and obstruction [31]. Moreover, a study performed by Abbas and Gad [1] investigated the biofilm-forming capacity of different bacteria isolated from diabetic foot infections, and it was found that the most prevalent isolates were *P. mirabilis* and that these all showed strong biofilm formation capacity.

Biofilm resistance to antimicrobial agents may be due to several mechanisms, including slow growth rates and low metabolic activities. Moreover, antimicrobial agents can be inactivated by the negatively charged extracellular matrix, which limits the penetration of positively charged molecules such as aminoglycosides. As a result, the penetration of these antibiotics is significantly retarded. In addition, a low oxygen level can prevent the action of some antibiotics [26, 42, 46, 62]. Another reason for resistance is the presence of

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dormant "persister cells" within the biofilm matrix that are resistant to antibiotic therapy [43].

To treat biofilm-related infections, new approaches are required. One of these is the use of bacteriophages [25], which can infect and lyse bacterial cells present in biofilms [24, 41]. Various studies have investigated the effect of phage on biofilm removal. Lehman and Donlan [41] and Melo *et al.* [47] studied the effect of pre-treatment of silicone hydrogel catheters with phage and observed a reduction in biofilm formation caused by *P. mirabilis* in artificial urine medium. Moreover, Nzakizwanayo *et al.* [51] utilized a phage tablet to effectively remove *P. mirabilis* from catheters in an *in vitro* bladder model system. Furthermore, the use of lytic bacteriophages in the prevention and eradication of biofilms of *Proteus mirabilis* and *Escherichia coli* was reported by Carson *et al.* [9].

The use of bacteriophages provides several advantages over traditional antibiotic therapy. They are host specific and do not affect natural microflora, they are not harmful to humans or animals, and there are many possible routes of administration. Moreover, phage preparation production is rapid, simple and inexpensive [14, 52, 60]. In this study, we investigated the ability of phages to eliminate *Proteus mirabilis* biofilms.

Materials and methods

Media and chemicals

Mueller Hinton broth and agar, tryptone soya broth (TSB) and agar (TSA), and MacConkey agar were purchased from Oxoid (St. Louis, USA). Cystine lactose electrolyte-deficient (CLED) medium, triple sugar iron (TSI) agar, and Simmons' citrate agar were obtained from LAB-M (UK). Other chemicals were of pharmaceutical grade, including alpha naphthol, sodium chloride, potassium hydroxide, hydrogen peroxide, dipotassium hydrogen phosphate, barium chloride, sulphuric acid, methyl red, glycerol, 95% ethanol, isoamyl alcohol, methanol, glacial acetic acid and hydrochloric acid, which were obtained from the El Nasr Pharmaceutical Chemical Company, Cairo, Egypt. Crystal violet and *p*-dimethyl amino benzaldehyde (DMAB) were obtained from Avondale Laboratories, Oxon, England. The antibiotic disks that were used in this study were obtained from Oxoid (Hampshire, England). These disks contained ampicillin (AM; 10 µg), piperacillin (PRL; 100 µg), ampicillin-sulbactam (SAM; 20 µg), cefotaxime (CTX; 30 µg), cefoperazone (CEP; 75 µg), ceftazidime (CAZ; 30 µg), cefepime (FEB; 30 µg), imipenem (IPM; 10 µg), meropenem (MEM; 10 µg), aztreonam (ATM; 10 µg), gentamicin (CN; 10 µg), amikacin (AK; 30 µg), tetracycline (TE; 30 µg), ciprofloxacin (CIP; 5 µg), levofloxacin (LEV; 5 µg), sulphamethoxazole-trimethoprim (SXT; 5 µg) or chloramphenicol (C; 30 µg).

Table 1 Isolation of *P. mirabilis* from different clinical specimens

Specimen type	No. of samples	No. (%) of isolates
Urine sample	125	19 (15.2%)
Endotracheal aspirate	50	6 (12%)
Burn swab	34	4 (11.8%)
Diabetic foot swab	79	9 (11.4%)
Surgical wound swab	112	9 (8%)
Total	400	47 (11.75%)

Table 2 Bacterial stains used in the host range test and their sources

Host bacterium	Sample type
Escherichia coli	Urine sample
Klebsiella pneumoniae	Nosocomial pneumonia specimen
Serratia marcescens	Nosocomial pneumonia specimen
Proteus penneri	Diabetic foot swab
Proteus vulgaris	Nosocomial pneumonia specimen
Pseudomonas aeruginosa	Burn swab
Salmonella Typhimurium	Reference strain (ATCC 14028)

Bacterial strains and growth conditions

A total of 41 *P. mirabilis* isolates were used in this study. They were recovered from 400 specimens (Table 1). Specimens were obtained from patients with urinary tract infections, diabetic foot ulcers, burn infections, wound infections, and respiratory tract infections hospitalized in Zagazig University Hospital and Al Ahrar Hospital in Zagazig Sharkia Governorate, Egypt. The strains were identified based on morphology and biochemical activity according to Koneman *et al.* [35]. Clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae, Serratia marcescens, Proteus penneri, Proteus vulgaris* and *Pseudomonas aeruginosa* were obtained from the stock culture of the Department of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University. *Salmonella* Typhimurium ATCC 14028 was kindly provided by Egyptian Pharmaceutical Industries Company (EPICO), (Table 2). All isolates were maintained in Mueller Hinton broth with 10-15% glycerol and kept at -80 °C.

Antibiotic susceptibility testing

Antibiotic susceptibility was tested using the Kirby-Bauer disc diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines [15]. The bacterial suspensions were prepared from overnight cultures on Muller-Hinton agar (Oxoid, Hampshire, England). Suspension

densities were adjusted to the approximate turbidity of 0.5 McFarland standard (1.5×10^8 colony-forming units [CFU]/ml). The surface of the Muller-Hinton agar plate was evenly inoculated with the suspensions using a sterile swab. The plates were dried before applying antibiotic disks. The disks were placed on the surface no less than 24 mm apart from center to center. The plates were incubated overnight at 37 °C, after which the diameters of the inhibition zones around the disks were measured. The results were interpreted according to Clinical Laboratory Standards Institute guidelines [15].

Quantitative assessment of biofilm formation by spectrophotometry

The ability of *Proteus mirabilis* isolates to form biofilms was tested according to Stepanovic *et al.* [61]. *P. mirabilis* suspensions with turbidity matching that of 0.5 McFarland standard were diluted 1:100 in tryptone soya broth (TSB). Aliquots of 200 µL of the prepared suspensions were added to individual wells of 96-well U-shaped microtiter plates, which were then incubated for 24 h at 37 °C. Negative-control wells containing 200 µL of TSB alone were included in each plate. After incubation, the contents of wells were decanted and the plates were washed three times with water and left to dry in air.

To fix the biofilm, 150 µL of 99% methanol was added to each well and left for 20 min. The methanol was then removed, and the wells were left to dry in air. The fixed biofilms in the wells were stained for 15 min with 150 µL of crystal violet (1%), and the excess dye was removed by washing with water. The plates were air dried, and the bound dye was dissolved by adding 150 µL of 33% glacial acetic acid to each well. Optical density was measured spectrophotometrically at 570 nm using a Bio Tek synergy HT microplate reader (USA). Measurements were performed in triplicate, and the experiment was repeated three times. The cutoff optical density (ODc) was calculated as three standard deviations above the mean OD of the negative control.

The tested strains were classified according to the criteria of Stepanovic *et al.* [61] into non-biofilm producer (OD ≤ ODc), weak biofilm producer (OD > ODc, but ≤ 2× ODc), moderate biofilm producer (OD > 2× ODc, but ≤ 4× ODc) and strong biofilm producer (OD > 4× ODc).

Isolation, purification and propagation of bacteriophages

Bacteriophages were isolated from raw sewage water samples collected from different areas in Sharkia Governorate, Egypt, and identified by transmission electron microscopy (TEM). The phages were isolated by the enrichment technique [11]. Briefly, 4.5 mL of the sewage sample was mixed with 0.5 mL of *Proteus mirabilis* culture in early exponential phase (4-h cultures) in TSB and 5 mL of TSB in 15-mL Falcon tubes. The tubes were incubated at 37 °C for 24 h with shaking. After centrifugation at 4000 rpm for 20 min at 4 °C using a Hermle cooling centrifuge (Germany), the supernatants were filtered using 0.22-µm membrane filters (Millipore Corporation, Bedford, MA, USA) and were screened for presence of phages using the double-layer agar (DLA) technique [7]. Aliquots of 100 µL of the filtrates were mixed with 100 µL of an early-log-phase culture *Proteus mirabilis* and added to 3 mL of prewarmed molten soft TSA and poured into TSA plates. The plates were incubated at 37 °C for 24 h, and the presence of phages was indicated by the appearance of clear zones (plaques) on bacterial lawns.

Phages were purified by multiple rounds of single-plaque selection, resuspended in 1 mL of TSB, serially diluted, and plated until a single plaque morphology was observed [34]. The purified phages were propagated to obtain stocks with a high titer according to Carey-Smith *et al.* [7]. Purified phages were serially diluted in saline to give a concentration that would provide confluent lysis of the host in a soft agar overlay plate. For each dilution, three to five plates were overlaid, and after incubation, plates with almost confluent lysis were chosen. To recover the phages, 5 mL of saline was added to the plates with confluent lysis, and after 1 h at room temperature, the liquid was poured into centrifuge tubes and centrifuged. The supernatants containing phages were then filtered and stored at 4 °C [7].

Transmission electron microscopy (TEM)

Bacteriophages were visualized using transmission electron microscopy (TEM) as described by Fard *et al.* [20]. A single drop of purified phage was deposited on a carbon-coated copper grid (200 mesh) for 60 s, and the excess liquid was removed. The suspension was then negatively stained with 1% phosphotungstate, air dried, and observed using a JEM-2100 transmission electron microscope (JEOL Ltd.).

Host-range test

The phage host specificity against different bacterial species was tested according to Jun *et al.* [32] with some modifications. Aliquots of 100 µL of early-log-phase cultures of the tested bacterial suspensions were mixed with 3 mL of soft TSA (0.7%) and plated on TSA plates (1.5%). After solidification of the upper agar layer, 10 µL of a phage stock of 10^{10} plaque-forming units per mL (PFU/mL) was applied to the surface. The plates were then dried, incubated at 37 °C for 24 h and checked for the presence of clear zones.

Lytic spectra of isolated bacteriophages

Phage activity was tested against different clinical isolates of *Proteus mirabilis* according to Jun *et al.* [32] with some modifications. Ten microliters of diluted phage stock (10^{10} PFU/mL) was spotted on a bacterial lawn. After incubation, the spots were described as showing strong bacterial lysis, weak bacterial lysis, very weak bacterial lysis, or no bacterial lysis.

Anti-biofilm activity of bacteriophages

Phage isolates showing strong lytic activity were tested for their ability to remove established biofilms as described by Harrison *et al.* [28] with some modifications. Biofilms were formed on acid-washed 4-mm sterile glass beads [66]. Equal quantities of glass beads were added to 15-mL Falcon tubes containing 5 mL of TSB and sterilized by autoclaving. Then, 50 microliters of *P. mirabilis* suspensions with turbidity matching that of 0.5 McFarland standard was added to the tubes containing the beads and incubated for 24 h at 37 °C to allow biofilm formation.

The beads were washed three times with 0.9% saline to remove unattached bacteria and transferred to fresh Falcon tubes, and 2 mL of phage (10^9 PFU/bead) was added. Untreated beads were used as a control. The control and treated beads were incubated for 6 h at 37 °C without shaking, and the beads were washed again. For counting viable bacteria, glass beads with biofilms were transferred to fresh Falcon tubes containing 5 mL saline and disrupted by vortexing for 5 min. A serial dilution was made in saline, and 100 µL of each dilution was spread on CLED agar plates. Visible colonies were counted after 24 h of incubation at 37 °C. The number of total viable cells was calculated from the plate count. The results were expressed as $\log_{10}$ CFU/cm^2.

Statistical analysis

Statistical analysis was performed using OneWay ANOVA test followed by Dunnett's Multiple Comparison Test, GraphPad Prism® Software version 5.0.1 (San Diego, CA, USA). A probability value (*P*-value) <0.05 was considered significant.

Results

Isolation and identification of *Proteus mirabilis*

From a total of 400 specimens, 47 isolates of *P. mirabilis* were recovered with an overall frequency of 11.75% (Table 1). The frequency of *P. mirabilis* isolation from different sources was as follows: 15.2% from urinary tract infections, 12% from endotracheal aspirates of mechanically ventilated patients, 11.8% from burn infections, 11.4% from diabetic foot infections, and 8% from surgical wound infections. *P. mirabilis* isolates were identified as Gram-negative rods that did not ferment lactose on MacConkey agar, showed swarming motility on nutrient agar, and had a characteristic foul odor. Complete identification of the isolates was performed based on their biochemical characteristics (Table 3).

Table 3 Biochemical identification of *P. mirabilis*

Biochemical test	Results
IMViC test	-+-+
Urease test	+
Phenylalanine deaminase test	+
Maltose utilization test	-

Table 4 Percentage of *P. mirabilis* isolates resistant to different antibiotics

Antibiotic disk	No. of resistant isolates (%)
Ampicillin	43 (91.5)
Piperacillin	22 (46.8)
Ampicillin-sulbactam	39 (83)
Cefotaxime	25 (53.2)
Cefoperazone	27 (57.4)
Ceftazidime	21 (44.7)
Cefepime	34 (72.3)
Imipenem	5 (10.6)
Meropenem	4 (8.5)
Aztreonam	13 (27.7)
Gentamicin	25 (53.2)
Amikacin	15 (31.9)
Tetracycline	47 (100)
Ciprofloxacin	25 (53.2)
Levofloxacin	15 (31.9)
Sulphamethoxazole-trimethoprim	38 (80.9)
Chloramphenicol	35 (74.5)

Antimicrobial susceptibility

It was found that 41 (87.2%) *P. mirabilis* isolates were resistant to at least one antibiotic of three or more different classes and were therefore considered multi-drug resistant (MDR). An antibiotic susceptibility test showed a variable degree of resistance to different antibiotics (Table 4).

Quantitative assessment of biofilm formation by spectrophotometry

Biofilm quantification was performed for all 41 MDR *P. mirabilis* isolates. The cutoff optical density (ODc) was calculated to be 0.08. According to the OD of the bacterial biofilm, isolates were divided into four categories: not biofilm-forming (OD ≤ 0.08), weakly biofilm-forming (OD > 0.08, but ≤ 0.16), moderately biofilm-forming (OD > 0.16, but ≤ 0.32), and strongly biofilm-forming (OD > 0.32). Among the 41 *P. mirabilis* isolates, strong biofilm formation was observed in 80.5%, while moderate production was found in 19.5%.

Isolation, purification and propagation of *Proteus mirabilis* phages

Five different phages specific for *Proteus mirabilis* were isolated from raw sewage water. They were named PMP1 (*Proteus mirabilis* phage 1), PMP2, PMP3, PMP4 and PMP5. PMP1, PMP2 and PMP5 produced large, circular, clear plaques surrounded by a halo, PMP3 produced small, circular, clear plaques surrounded by a halo, and PMP4 produced small, circular, clear plaques with no halo (Fig. 1).

Transmission electron microscopy (TEM) imaging of *Proteus mirabilis* phages

TEM (Fig. 2) showed that PMP1 had an icosahedral head of 45.10 × 44.41 nm and a short tail of 21.38 nm in length and 7.75 nm in width, suggesting that it probably belongs to the family *Podoviridae*.

No tail was observed with the phages PMP2-5. PMP2 appeared to be pleomorphic, with a head size of 45.31 × 43.76 nm diameter and thus probably belong to *Plasmaviridae* family. PMP3, 4 and 5 had icosahedral heads with sizes of 45.59 × 45.23 nm, 43.10 × 41.60 nm, and 49.75 × 49.16 nm, respectively. These phages could belong to any of the families *Microviridae*, *Corticoviridae, Tectiviridae, Leviviridae* or *Cystoviridae*.

Host range test

The five isolated phages were tested against six bacterial isolates belonging to different bacterial species in the order *Enterobacteriales* (*Salmonella* Typhimurium, *Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Proteus penneri* and *Proteus vulgaris*) and *Pseudomonas*

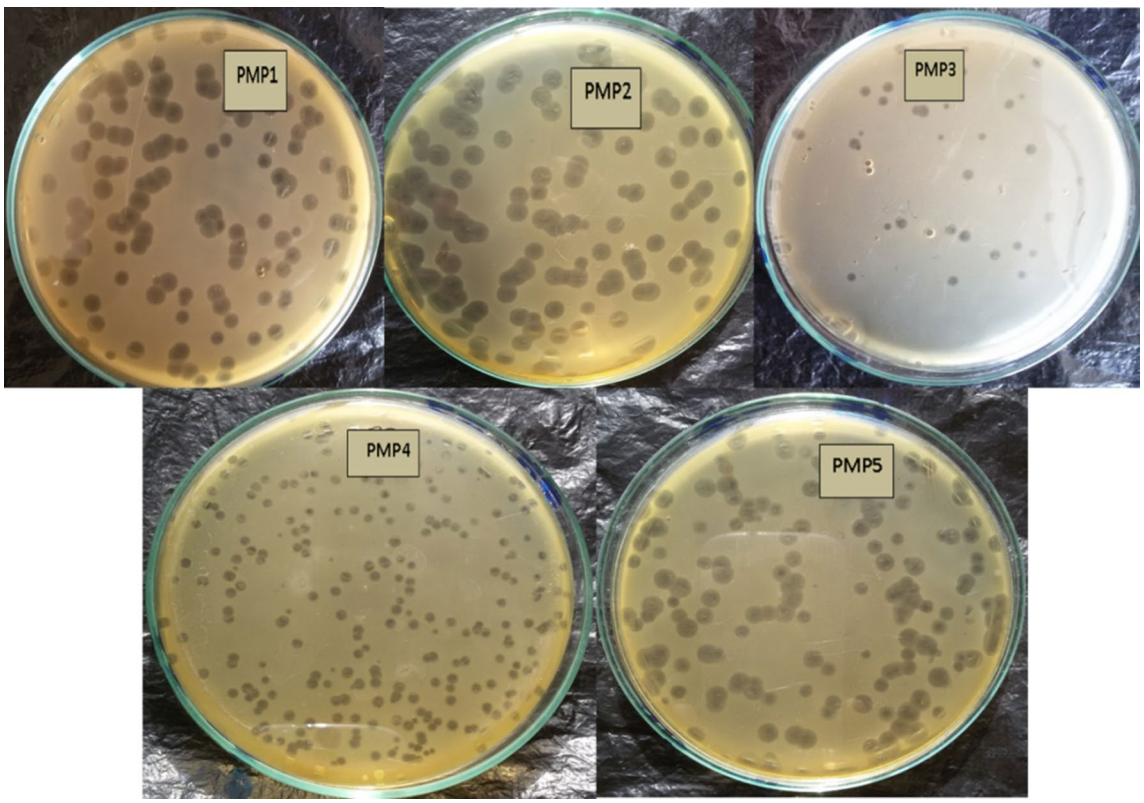

Fig. 1 Plaque morphology of the isolated phages after 24 h of incubation. PMP1, PMP2 and PMP5 formed large, circular, clear plaques surrounded by halos. PMP3 formed small, circular, clear plaques surrounded by halox. PMP4 formed small, circular, clear plaques with no halo

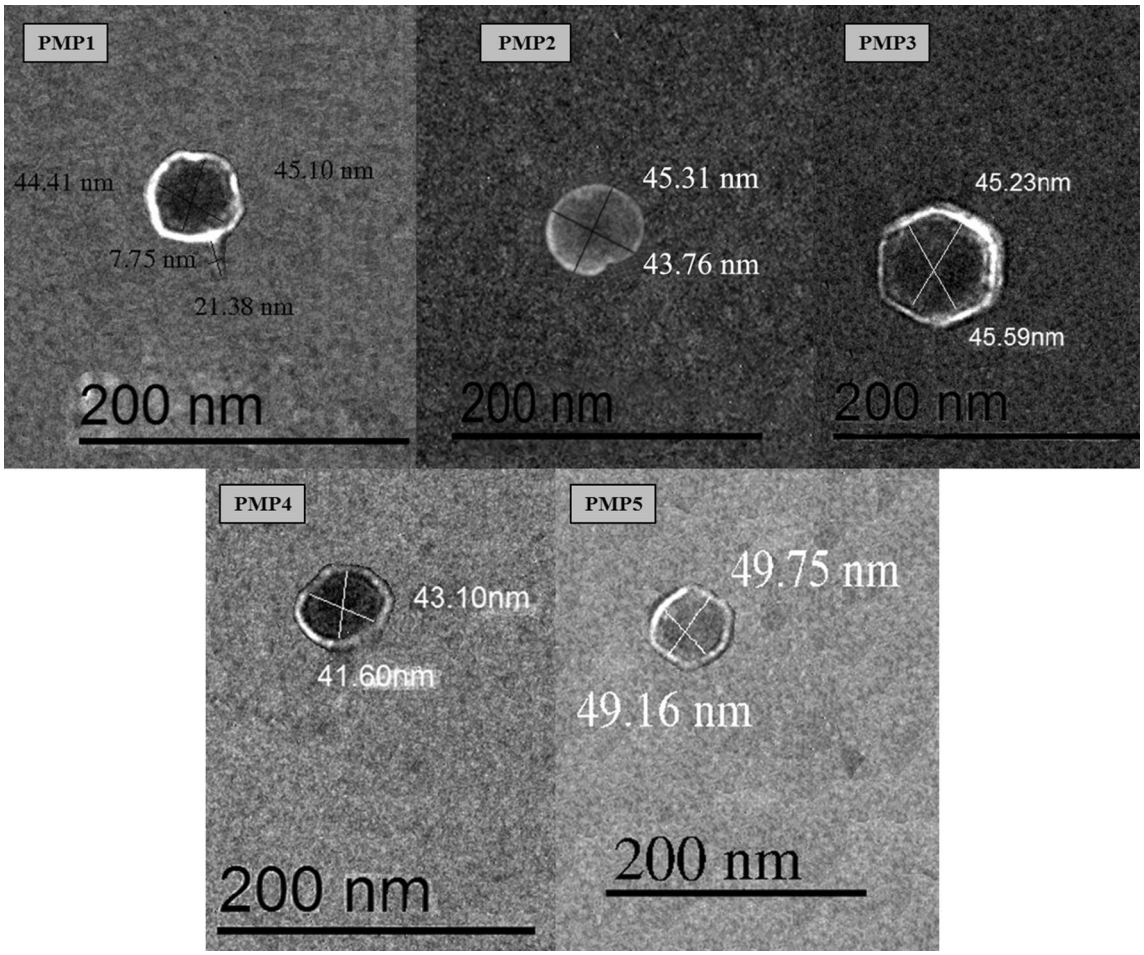

Fig. 2 Transmission electron micrographs of *P. mirabilis* phages (PMP1, PMP2, PMP3, PMP4 and PMP5) using a JEM-2100 transmission electron microscope (JEOL). Bars = 200 nm. Phage PMP1 has a head of 45.10 × 44.41 nm and short tail of 21.38 nm length and 7.75 nm width. PMP2 has a head of 45.31 × 43.76 nm, PMP3 has a head of 45.59 × 45.23 nm, PMP4 has a head of 43.10 × 41.60 nm PMP5 has a head of 49.75 × 49.16 nm

aeruginosa. No plaque formation was observed with the tested bacteria.

Phage lytic spectra of isolated bacteriophages

The susceptibility of the 41 multidrug-resistant *Proteus mirabilis* isolates to bacteriophages was tested using a modified double-layer agar technique. Only 10 clinical isolates showed lysis by one or more of the tested phages. PMP1, PMP3 and PMP4 exhibited a narrow spectrum of activity. Each lysed only 10% of the tested isolates, producing a strong lysis zone. PMP5 had a broader spectrum and lysed 20% of the bacterial isolates. A strong lytic zone was seen in 12% of the tested isolates, and a very weak lysis zone was seen in 8% of the tested isolates. PMP2 had the broadest spectrum of lysis activity. It lysed 22% of the bacterial isolates with a strong lysis area in 20% of the tested isolates and a weak lysis area in 2% of the tested isolates (Table 5).

Biofilm disruption by bacteriophages

The viability of the cells in the biofilms was measured by counting. The tested bacteriophages had different abilities to reduce the number of viable cells in the biofilms (Table 6). Statistically significant reduction in biofilm in the presence and absence of phages was found at <0.05 (Fig. 3). PMP1, PMP2 and PMP3 were able to cause more than a 90% reduction in the count of bacterial cells embedded in the biofilm matrix. PMP4 caused a 3.8% to 40.2% reduction in viable counts for all of the isolates tested except for PM36, in which a 92% reduction in viability was achieved. PMP5 also had a limited effect on the viable count, with reduction ranging between 2.4% and 35.1%.

Table 5 Lytic spectra of the isolated phages against *P. mirabilis* strains

Isolate no.	Specimen type	Lysis area formed by the isolated phages				
		PMP1	PMP2	PMP3	PMP4	PMP5
PM2	Urine sample	S	-	S	S	VW
PM7	Diabetic foot swab	-	S	-	-	S
PM9	Surgical wound swab	S	S	S	S	VW
PM11	Urine sample	-	W	-	-	-
PM14	Urine sample	-	S	-	-	S
PM16	Surgical wound swab	-	S	-	-	S
PM17	Urine sample	-	S	-	-	S
PM18	Surgical wound swabs	-	S	-	-	-
PM36	Urine sample	S	S	S	S	S
PM43	Urine sample	S	S	S	S	VW

S, strong; W, weak; VW, very weak; -, no bacterial lysis zone

Table 6 Effect of phages on biofilm formed by MDR *P. mirabilis*

Isolate no.		Log_{10} CFU/mL				
		PMP1	PMP2	PMP3	PMP4	PMP5
PM2	C	7.52	7.52	7.52	7.52	7.52
	T	5.76		5.94	7.2	
PM7	C	7.31	7.31	7.31	7.31	7.31
	T		6.06			7.3
PM9	C	7.79	7.79	7.79	7.79	7.79
	T	6.31	6.45	5.51	7.77	
PM14	C	7.38	7.38	7.38	7.38	7.38
	T		5.88			7.2
PM16	C	7.11	7.11	7.11	7.11	7.11
	T		6.07			7.01
PM17	C	7.53	7.53	7.53	7.53	7.53
	T		4.29			7.4
PM18	C	7.68	7.68	7.68	7.68	7.68
	T		7.41			
PM36	C	7.42	7.42	7.42	7.42	7.42
	T	5.36	4.43	6.13	6.32	
PM43	C	7.52	7.52	7.52	7.52	7.52
	T	6.09	5.66	6.25	7.4	

C, control; T, test

Discussion

Proteus mirabilis is the etiological agent of many human infections [13, 16, 19, 53, 57]. *P. mirabilis* shows high antimicrobial resistance, and its resistance to multiple drugs complicates therapy [5].

Bacteriophage therapy is a potential alternative to control biofilm infections [6, 27]. Some phages produce polysaccharide depolymerases. These enzymes degrade exopolysaccharides (EPS), allowing phages to enter bacteria embedded in the biofilm matrix [56, 59, 64]. Bacteriophages have high specificity for their hosts [36], thus minimizing overall damage to the microbial population [48]. However, this high specificity also means that phage therapy using natural lytic phages requires isolation and identification of appropriate phages, which may delay treatment. This can be partially overcome by using phage cocktails to broaden the phage host range [12, 22]. There is still a lack of properly documented clinical research on phage therapy, and there are no established protocols for the route of administration, dose, frequency or duration of treatment. There is also limited knowledge regarding phage behavior *in vivo* [21]. Bacteria can also become

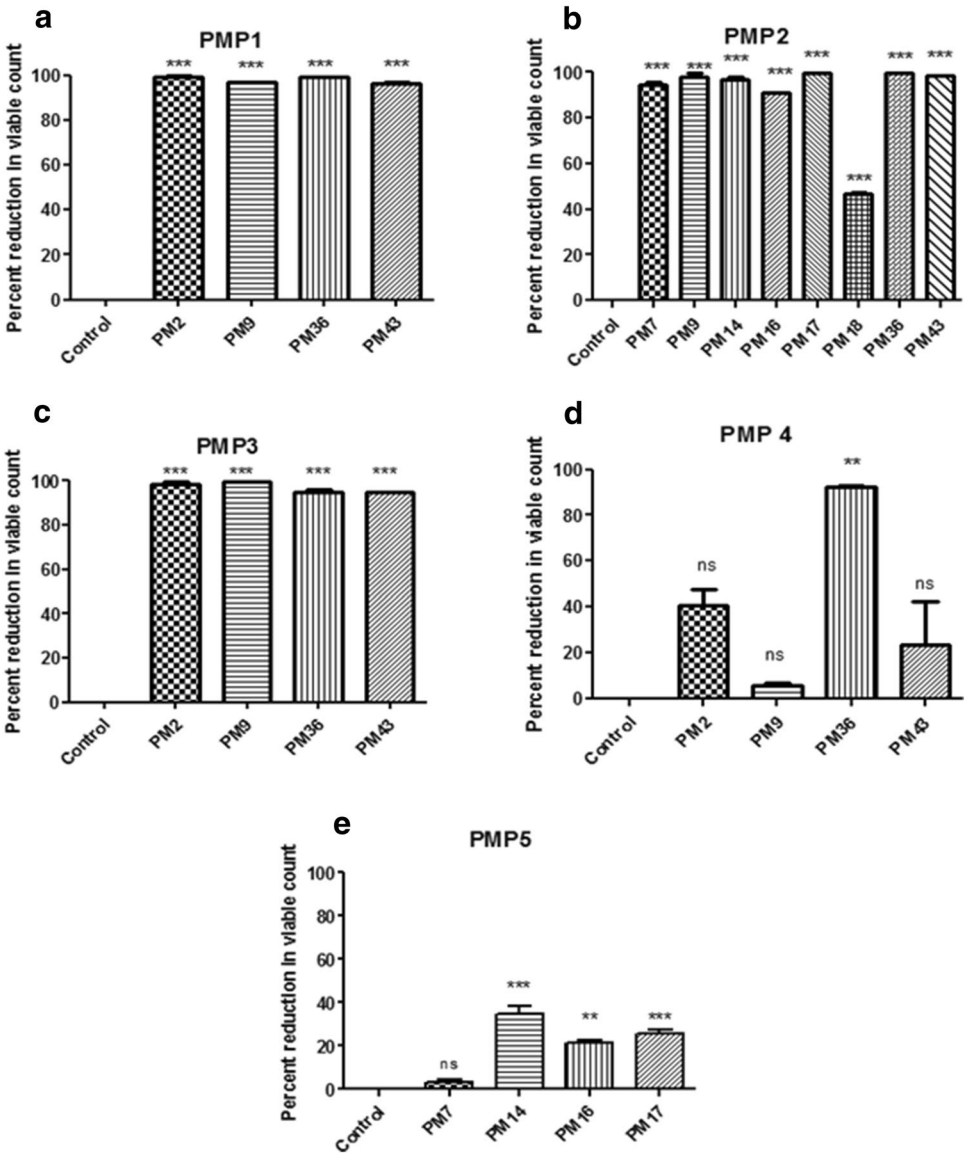

Fig. 3 Effect of phages on biofilm formed by MDR *P. mirabilis*. (*) significant difference ($P < 0.05$)

phage resistant through the lack or loss of a receptor [44] and through structural modifications and/or masking of the receptor [40]. Phages can be used in cocktails or in combination with antibiotics to overcome phage resistance [29, 38].

In this study, five different phages specific for *Proteus mirabilis* isolates were isolated from raw sewage samples. On culture plates, these phages produced plaques that varied in size and morphology (turbid/clear appearance, presence/absence of a halo). Some produced large plaques, while others produced small ones. All of the phages produced round, clear plaques, most of which were surrounded by a halo. These data were similar to those reported by Morozova *et al.* [50], who isolated four lytic *P. mirabilis* phages that produced large, clear plaques surrounded by a halo. Comparable results were reported by Morozova *et al.* [49], who isolated a phage against a *Proteus mirabilis* isolate that formed large clear plaques surrounded by a halo on a culture plate. Nzakizwanayo *et al.* [51] also isolated three phages with halo-producing plaques. Yazdi *et al.* [68] isolated a phage that produced clear plaques. However, Melo *et al.* [47] isolated two different phages infecting *Proteus mirabilis*. One formed clear plaques and the other formed less-clear ones. Numerous phages that induce enzymes capable of degrading the EPS of many Gram-negative bacteria, including bacteria capable of biofilm formation, have been isolated. On culture plates, such phages are characterized by halos of different sizes, surrounding the plaques obtained after the infection of a single bacterial cell. The halos are formed by bacteria from which the EPS has been removed by excess phage enzyme released during the lysis of infected cells [64].

For further identification, TEM imaging was performed on the isolated phages. It was found that the phages PMP1 and PMP2 probably belong to the families *Podoviridae* and *Plasmaviridae*, respectively, while the other three remaining phages could belong to any of the families *Microviridae*, *Corticoviridae, Tectiviridae, Leviviridae* or *Cystoviridae*. Further studies using techniques such as DNA sequencing are needed to determine to which families these phages belong. The present results agree with those of Nzakizwanayo *et al.* [51], Morozova *et al.* [49], and Morozova *et al.* [50], who showed that their phages belonged to the family *Podoviridae*. Moreover, Melo *et al.* [47] isolated two phages infecting *Proteus mirabilis* that belonged to the families *Podoviridae* and *Myoviridae*. However, TEM studies by Yazdi *et al.* [68] revealed that their phages belonged to the family *Siphoviridae*, and three phages reported by Thompson [65] were found to belong to the families *Siphoviridae, Myoviridae* and *Podoviridae*.

The tested phages could infect *P. mirabilis* isolates, but could not infect any of the other bacterial species tested. This specificity might be due to the presence of specific phages receptors present on the *P. mirabilis* cell surface. Receptors used by bacteriophages include flagella, polysaccharides, lipopolysaccharides, capsules, cell wall proteins, pili, and fimbria [23]. Variations in these receptors among various bacterial species have a strong effect on phage specificity [54].

In many previous reports, only a limited number of phages and hosts were used [47, 49, 50, 65, 68]. The results of this study are similar to those reported by Afriani *et al.* [4], who used *P. mirabilis,* enteropathogenic *E. coli, Salmonella* spp., *Bacillus pumilus*, and *Photobacterium damselae* to determine *P. mirabilis* phage specificity. They showed that the isolated phage exhibited high specificity against *P. mirabilis.* A phage isolated by Morozova *et al.* [49] against *P. mirabilis* was strain specific, lysed only one clinical strain, and did not infect the other 12 *P. mirabilis* isolates used in the test. However, Melo *et al.* [47] isolated two different phages specific for *P. mirabilis* isolates and tested their specificity against 43 stains of the family *Enterobacteriaceae* and found that both phages exhibited a much broader spectrum than in the current study; one of them lysed 16 out of 26 of *Proteus* strains tested, including (18 *P. mirabilis* strains, six *P. vulgaris* strains, one *P. hauseri* strain and one *P. penneri* strain), while the other lysed 26 out of 26 isolates. Both had no activity against any other tested strains in family *Enterobacteriaceae.*

Biofilm infections pose a therapeutic problem due to their extreme resistance to antimicrobial agents. In order to treat such infections, very high concentrations of antimicrobials are required. These concentrations may be toxic for the patient. Thus, alternative therapeutic approaches are necessary. One such approach is the use of bacteriophages [6], which have several advantages over antibiotics. First, they cause only minimal disruption of the natural microflora. Second, new phages can be discovered quickly, and their production cost is low. Third, phages can be administered by various routes and are also available in many formulations, such as creams and liquids [8, 37–39]. Fourth, they can be used at low dosage. Finally, no side effects have been reported with phage application, in contrast to antibiotics [45].

In this study, three bacteriophages were able to eliminate more than 60% of a pre-formed biofilm, while the other two phages had low (1%-30%) and intermediate (31%-60%) activity. The biofilm eradication activity of the bacteriophages used in the current study was higher than that reported by Carson *et al.* [9], who found that there was nearly complete eradication of pre-formed biofilms caused by *Escherichia coli* and only 10% reduction in case of *Proteus mirabilis* biofilm. Furthermore, the results of the current study were similar to those reported by Danis-Wlodarczyk *et al.* [18], who applied *Pseudomonas aeruginosa* PAO1 phage treatment for 4 h at 37 °C. Colony counts revealed that both phages used in the study were able to decrease a 24- to 72-h-old *Pseudomonas aeruginosa* PAO1 biofilm by 70%-90%. This was also reported by Cornelissen *et al.* [17], who observed maximum biofilm degradation 8 h after addition of high doses (10^4 and 10^6 PFU) of T7-related *Pseudomonas putida* phage φ15 (96% for 10^6 PFU) and development of resistance after 24 h. This might indicate that there is a negative correlation between biofilm degradation and time of exposure to phage. Some authors have studied the relationship between biofilm eradication and exposure time, such as Karaca *et al.* [33], who applied P22 phage at different titers (10^4-10^8 PFU/mL) to 24-h-old *Salmonella* Typhimurium DMC4 biofilms for 24h at 37 °C. They found that the highest eradication activity was 57.1% and 69% for a 24-h-old biofilm at phage titers of 10^7 and 10^8 PFU/mL, respectively. This was also reported by Danis-Wlodarczyk *et al.* [18]. Moreover, the resistance of biofilm to phage therapy has been studied as a time-dependent process. Yazdi *et al.* [68] studied the capability of a *P. mirabilis* phage to degrade a 24-h-old biofilm formed by *P. mirabilis* and found that at a multiplicity of infection (MOI) of 100, this phage removed 89% of the biofilm's biomass after 8 h of phage treatment. However, this proportion decreased to 70% after 24 h. The development of resistance was also reported by Cornelissen *et al.* [17].

Conclusion

Proteus mirabilis is an opportunistic Gram-negative bacterium that can cause many types of infections in humans, such as infections of the urinary tract, respiratory tract, burns

and wounds, in addition to infections of diabetic foot ulcers. High rates of drug resistance were found in this study. *P. mirabilis* showed high capability of biofilm formation. Biofilm formation is considered a virulence factor and contributes to antibiotic resistance. Bacteriophages have been used as an alternative to treat biofilm-based *P. mirabilis* infections and have been effective in elimination of biofilms. We recommend the use of bacteriophages in controlling biofilm-based infections caused by MDR *P. mirabilis* strains.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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