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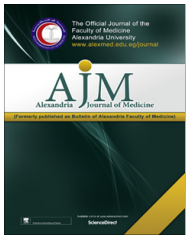

Bacteriophage-antibiotic synergism to control planktonic and biofilm producing clinical isolates of *Pseudomonas aeruginosa*

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KEYWORDS

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Abstract *Introduction:* *Pseudomonas aeruginosa* (*P. aeruginosa*) is a highly resistant opportunistic pathogen and is capable of forming biofilms on medical devices. Bacterial biofilms, which are micro-colonies encased in extracellular polysaccharide material are so difficult to be treated by conventional antibiotics. During the last decade, *P. aeruginosa* phages have been extensively examined as an alternative to antimicrobial agents. The aim of the study was to assess bacteriophage-antibiotic combination on planktonic and biofilm states of *P. aeruginosa* isolates.

Materials: In this study, we isolated 6 lytic phages, from hospital effluents, they were tested against 50 *P. aeruginosa* strains, isolated from different clinical specimens delivered to the Diagnostic Microbiology Laboratories, Faculty of Medicine, Alexandria University.

Results: Out of the 50 isolates, 15 were susceptible to these phages. So the biofilm forming capacity of these 15 isolates was investigated. The results showed that 14 isolates (93.33%) produced detectable biofilm. The minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) assays were used to evaluate the antibiotic sensitivity patterns of these *P. aeruginosa* isolates in their planktonic and biofilm phases to amikacin and meropenem. Also, the effects of phage on the planktonic and biofilm states of isolates at different multiplicities of infections (MOI) were tested. On the planktonic state, the amikacin-phage combination showed synergistic effect ($P = 0.001$), and the meropenem-phage combination showed synergistic effect ($P = 0.003$). On the biofilm state, the amikacin-phage combination showed biofilm eradication

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in 50% of the isolates ($P = 0.003$). On the other hand, the meropenem-phage combination showed biofilm eradication in 14.3% of the strains.

Conclusion: The combination of phage and antibiotics could have potentially more benefits on *P. aeruginosa* planktonic and biofilm states than just using phages or antibiotics alone.

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the leading causes of nosocomial infections, being responsible for high rates of mortality and morbidity.[1] This bacterium is the most frequently isolated Gram-negative organism in bloodstream, wound infections, pneumonia, intra-abdominal and urogenital sepsis. This is a serious problem, also infecting immunocompromised patients with cystic fibrosis, severe burns, cancer, AIDS, etc.[2]

One of the most worrying characteristics of this bacterium is its low antibiotic susceptibility. Overuse of antibiotics has also significantly increased the emergence of antimicrobial multidrug-resistant bacteria; consequently, treatment of most chronic *P. aeruginosa* infections with antibiotics is difficult. Additionally, *P. aeruginosa* has an innate ability to adhere to surfaces and form virulent biofilms particularly difficult to eradicate.[3]

It is well recognized that biofilm mode of growth can promote resistance to antimicrobial agents, and its occurrence during the infectious process has been considered a limiting factor for therapeutic success.[1]

Biofilm is a structured community of bacterial cells adherent to an inert or living surface and/or embedded in a self-produced extracellular polymeric substances' (EPS) matrix. The bacteria within the biofilms are protected from physical, chemical and biological stresses, including antimicrobial agents, antibodies and the antimicrobial products of phagocytic cells.[4] The mechanism of biofilm resistance is multifactorial. Treatment with antibiotics may kill planktonic bacteria (free living bacteria) shed from the biofilm surface; however, they fail to eradicate those embedded within the biofilm, which can then subsequently act as a nidus for recurrent infection.[5]

So, new alternative strategies to antibiotherapy are highly needed by the worldwide medical and scientific community. Bacteriophages (phages) are considered the natural enemies of bacteria and may represent an attractive solution to this problem.[6]

Phage therapy is based on the use of lytic phages to combat bacterial infections, including multidrug-resistant bacteria and has many advantages compared to antibiotics: they persist as long as the targeted bacteria are present, they are very specific and efficient for their target bacteria, which reduce destruction of the host's natural flora; and they are not pathogenic for human.[7]

The aim of this study was to assess bacteriophage-antibiotic (amikacin/meropenem) synergism to control planktonic and biofilm states of clinical isolates of *P. aeruginosa* by determining their MICs (minimum inhibitory concentrations) and MBECs (minimum biofilm eradicating concentrations) values.

2. Material and methods

2.1. Bacterial strains

The material of this study consisted of 50 strains of *P. aeruginosa* isolated from various clinical specimens processed in the Microbiology Laboratory, Faculty of Medicine, Alexandria Main University Hospital, over a period of time from June through September 2013.

2.2. Identification of strains

Colonies of *P. aeruginosa* on blood agar were identified according to the standard microbiological techniques.[8]

2.3. Isolation of Bacteriophages:[2,9]

Phages were isolated from Alexandria University Hospital effluents during the same period of collection of the study strains. These effluents were enriched with different isolated *P. aeruginosa* strains in double strength Tryptone Soya Broth (TSB) medium (Oxoid, UK). This solution was incubated for 48 h, then centrifuged for 10 min at 2500 rpm to collect the supernatant. The supernatant was filtered through a 0.45-μm membrane filter. This supernatant was then checked for the presence of lytic phages by spotting 10 μl onto lawns of different *P. aeruginosa* isolates on Tryptone Soya Agar (TSA) plates. After overnight incubation, the formation of clear zones (plaques) suggested the presence of lytic phage specific for each strain.[9]

2.3.1. Titration of bacteriophages by Double Agar Overlay (Plaque Assay)[10]

TSA plates were prepared. Three ml of Soft agar (TSB with 0.7% agar) was prepared in sterile tubes. TSB was prepared and used as a diluent for the bacteriophages (in 1 ml quantities). One hundred microliters of phage lysate were added to the first tube and serial 10-fold dilutions were made. One hundred microliters of phage lysate from each dilution were added to 100 μl of overnight grown *P. aeruginosa* strain in TSB as 0.5 Mcfarland[11], specific to that phage, and mixed with three ml sterile molten soft agar. This mixture was poured over sterile TSA plate for each phage dilution. The overlays were left to harden for 30 min, and then the plates were incubated inverted at 37 °C for 24 h. Plates were checked for plaque formation and plaque forming units were calculated according to the following equation.[9,10]

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

2.3.2. Study of biofilm production[4]

P. aeruginosa isolates were cultured overnight in TSB. A volume of 200 µl was transferred to each of three wells of a sterile 96 wells polystyrene microtiter plate flat bottom (Becton Dickinson, Franklin Lakes, NJ, USA), so that each isolate was tested in triplicate. After incubation for 24 h at 37 °C, the contents of the wells were discarded and the wells were gently washed three times with 200 µl sterile phosphate-buffered-saline (PBS) (pH 7.2). 200 µl of 2% Sodium acetate solution was added to each well and kept for 5 min (for biofilm fixation). The plate was then washed using PBS, followed by addition of 200 µl crystal violet (0.5%) for 30 min at room temperature for biofilm staining. The plate was then washed with tape water. In each run a negative control was included (non-biofilm forming *P. aeruginosa*). The absorbance (optical density, OD) of the remaining surface-adsorbed cells of the individual wells was read using a spectrophotometer (ELX 800 Universal Microplate Reader Bio-TEC Instruments, INC.) at a wave length of 545 nm. The optical density cut-off value (ODc) = OD of the negative control.

OD the isolate $\leqslant$ ODc was considered absent biofilm, and OD the isolate between ODc and 2X ODc is considered weak biofilm. OD the isolate between 2X ODc and 4X ODc is considered moderate biofilm and OD the isolate > 4X ODc is considered strong biofilm producing strain.[1]

2.3.3. MIC determination

Based on studies in Egypt amikacin was the most active drug against *P. aeruginosa* followed by meropenem, hence the choice of theses antibiotics for further testing.

MICs of amikacin (range 0.25–512 mg/L) and meropenem (range 0.25–512 mg/L) for the *P. aeruginosa* strains selected for biofilm studies were determined using cation adjusted Mueller Hinton broth (CAMHB) (Oxoid, Basingstoke, UK) according to the CLSI broth microdilution method. Results were interpreted as follows: for amikacin (Sensitive: $\leqslant$16 µg/ml, Intermediate: 32 µg/ml, Resistant: $\geqslant$64 µg/ml), for meropenem (Sensitive: $\leqslant$2 µg/mL, Intermediate: 4 µg/ml, Resistant: $\geqslant$8 µg/mL).[12]

2.3.4. MBEC determination

A single colony from the selected biofilm producing *P. aeruginosa* isolate was added to a test tube containing 2 ml TSB. After overnight incubation, the turbidity was adjusted[11] and 200 µl of the bacterial suspension was added to wells of the microtiter plate. These were incubated for 24 h at 37 °C aerobically. Planktonic cells were removed by washing with PBS. The remaining attached bacteria were resuspended in 100 µL of CAMHB and challenged with 100 µl of amikacin at different 2 fold dilutions (2–512 µg/ml) or meropenem at different 2 fold dilutions (2–512 µg/ml). Each dilution was tested in triplicate and the plates were incubated for another 24 h at 37 °C. The drug was removed and the wells were rinsed three times with PBS. The subsequent steps (i.e., fixation and staining) were performed as in the biofilm formation assay. A positive control (the biofilm producing strain without adding amikacin or meropenem) and a negative control (a biofilm negative strain without adding amikacin or meropenem) were also included in the experiment, each in its respective well. The MBEC was defined as the minimum concentration of amikacin (or meropenem) required to eradicate the biofilm. Eradication of biofilm gave a reading $\leqslant$ ODc negative control.[4,13]

Testing the effect of phage on the planktonic cells: Specific phages were tested against the specific *Pseudomonas* isolates at different multiplicities of infection(MOI), where MOI is the average number of virus particles infecting each cell (MOI = Plaque forming units (PFU) of virus used for infection/number of cells).[14] A 0.5 Mcfarland (1×10^8 cfu/ml) suspension of *P. aeruginosa* isolate was prepared in TSB. One hundred microliters of the suspension were added to 100 µl of phage lysate of concentration 10^8 PFU/ml (to give MOI = 1) in a well of the microtiter plate. Plates were incubated for 24 h at 37 °C, then examined for clearance of the wells.[13,15]

Testing phage on bacterial Biofilm at different MOI (1, 2, 4): Each dilution was tested in triplicates and the plates were incubated for 24 h at 37 °C. The phage was removed and the wells were rinsed three times with PBS. The subsequent steps (i.e., fixation and staining) were performed as in the biofilm formation assay. Eradication of the biofilm gave an OD reading similar to that of the negative control.[16]

Antibiotic and phage synergism against the planktonic cells: MIC method was done as mentioned before, where 100 µl of different concentrations of the Antibiotic (Amikacin) (ranging from 0.25 to 512 µg/ml) were put in wells of microtiter plate. One hundred microliters of phage lysate 10^8 PFU/ml were added to these wells and finally 100 ml of bacterial suspension 10^8 cfu/ml were added to the wells. The same steps were repeated for the meropenem.[17]

Antibiotic and phage synergism against the biofilm: After doing the same biofilm assay, planktonic cells were removed by washing with PBS leaving only the biofilm bacteria attached to the microtiter plate. One hundred microliters of antibiotic at different 2 fold dilutions (2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml) and 100 ml Phage 10^8 PFU/ml (to give MOI = one), were added to each well, this was applied for both amikacin and meropenem. Each dilution was tested in triplicates and the plates were incubated for 24 h at 37 °C. The Antibiotic and the phage were removed and the wells were rinsed three times with PBS.[13,16]

2.3.5. Statistical analysis

The data were analyzed using SPSS package version 18. The following statistical measures were used:

Descriptive statistics including frequency, distribution, median, and inter quartile range were used to describe different characteristics. Univariate analyses including the following: Mann Whitney test and Wilcoxon Signed ranks test were used to test the significance of results of quantitative variables. Fisher's Exact test and McNemar test were used to test the significance of results of qualitative variables. The significance of the results was at the 5% level of significance.

3. Results

Fifty *P. aeruginosa* strains were isolated. The isolates were mostly from urine (50%), followed by pus (40%) and (10%) were from broncho–alveolar lavage.

Six phages were isolated from Alexandria University Hospital effluents, they were tested against the 50 strains. Out of the 50 isolates, 15 were susceptible to these phages.

All 15 *P. aeruginosa* strains that had a specific lytic phage isolated were tested for their ability to form biofilm by microtiter plate method (MTP). Fourteen isolates (93.33%) produced detectable biofilm by MTP. Among these 14 isolates, eight isolates produced strong biofilm and six isolates produced moderate/weak biofilm (Table 1).

Determination of amikacin/meropenem MBEC: The 14 biofilm producing isolates (100%) had MBEC for greater than the defined planktonic MIC breakpoint for resistance ($\geqslant 64$) for amikacin and ($\geqslant 8$) for meropenem.

Table 1 shows that amikacin reduced the biofilm in (50%) of the strong *P. aeruginosa* biofilm producing isolates and in (66.7%) of the moderate biofilm producing isolates, yet this was statistically insignificant ($p = 0.627$). Similarly, meropenem produced reduction of the biofilm in (25%) of the strong biofilm producers and in (50%) of the moderate biofilm producers, this was also statistically insignificant ($p = 0.58$).

By testing the effect of the corresponding phage on planktonic state of *P. aeruginosa* strains at different MOI, clearance of the wells was produced in 5 out of 15 isolates (33.3%). These 5 isolates were susceptible at MOI 4. The remaining 10 isolates (66.6%) needed MOI > 4. As for phage effect on biofilm, three out of the 14 *biofilm producing P. aeruginosa* isolates (21.42%) showed biofilm eradication at MOI = 4, two of these isolates showed this effect by using phage cocktail, whereas 11 of the isolates (78.57%) needed MOI > 4 to eradicate the biofilm.

Testing the effect of combination of the sub MIC concentration of amikacin/meropenem and the corresponding phage at a MOI = one on the planktonic *P. aeruginosa* strains revealed that the amikacin-phage combination produced synergistic effect on 13 out of 15 strains (86.66%), but had no effect on the other two strains (13.33%). The meropenem-phage combination showed synergistic effect on 11 out of the 15 strains (73.33%) and had no effect on the other four strains (26.67%). Statistically, it was found that the median MIC value of amikacin alone on *P. aeruginosa* strains was 64 and with phage combination it was reduced to 16. Also, the median MIC value of meropenem alone was 64 and with phage combination it was reduced to 32 (Table 2).

Statistically, Table 2 shows that the antibiotic-phage combination caused considerable great reduction of the MIC for *P. aeruginosa* strains in the planktonic state (amikacin $P = 0.001$)/(meropenem $P = 0.003$).

The effect of combination of amikacin (at MIC of $\leqslant 512$) and the corresponding bacteriophage at MOI = (one) on the 14 biofilm producing *P.aeruginosa* strains was attempted. Eradication of the biofilm occurred in 7 out of the 14 strains (50%). As for meropenem (at MIC $\leqslant$ 512) with phage combination, eradication of the biofilm occurred in two out of the 14 strains (14.28%) (Table 3).

Statistically, Table 3 shows that the amikacin-phage combination caused significant eradication of *P. aeruginosa* biofilm ($p = 0.003$), whereas the meropenem-phage combination caused no significant eradication of *P. aeruginosa* biofilm ($p = 0.481$).

4. Discussion

P. aeruginosa is a ubiquitous organism which has emerged as a major threat in hospital environment. It is one of the most resistant bacterial pathogens. Many medical implants such as catheters, artificial hips and contact lenses can easily get colonized by *P. aeruginosa*.[18]

Table 1 Relation between strength of biofilm production in *P. aeruginosa* strains and effect of Amikacin and Meropenem on the biofilm.

Antibiotics	Strong ($n = 8$)		Moderate/weak ($n = 6$)		Significance
	No.	%	No.	%	
Amikacin					
Detected effect on biofilm					$^{FE}P = 0.627$
No reduction	4	50	2	33.3	
Reduced to weak	4	50	4	66.7	
Meropenem					
Detected effect on biofilm					$^{FE}P = 0.58$
No reduction	6	75	3	50	
Reduced to weak	2	25	3	50	

FEP: Fisher's Exact test, Z: Mann Whitney test.

Table 2 The effect of combination of the sub MIC concentration of Amikacin or Meropenem with the corresponding bacteriophage on Planktonic state of *P. aeruginosa* strains.

The effect	Amikacin-phage ($n = 15$)		Meropenem-phage ($n = 15$)	
	No.	%	No.	%
Synergism	13	86.7	11	73.3
No effect	2	13.3	4	26.7
Total	15	100	15	100
Significance	$Z = 3.185$ $P = 0.001^*$		$Z = 2.944$ $P = 0.003^*$	

Z: Wilcoxon Signed ranks test.
* Significant at $P \leqslant 0.05$.

Table 3 Effect of using Amikacin or Meropenem at different concentrations with and without combination with the corresponding bacteriophage on eradication of *P. aeruginosa* biofilm.

Antibiotic	Without adding the phage		With adding the phage		Significance
	No	%	No	%	
Amikacin					$^{MN}P = 0.003^*$
Eradication	0	0	7	50	
No eradication	14	100	7	50	
Meropenem					$^{MN}P = 0.481$
Eradication	0	0	2	14.3	
No eradication	14	100	12	85.7	

MNP: McNemar test.
* Significant at $P \leqslant 0.05$.

In the present study 25 out of 50 *P. aeruginosa* strains (50%) were isolated from urine, (40%) from pus and (10%) from BAL.

Our study revealed that, only 15 of our isolates had specific lytic phages. Fourteen out of these 15 isolates (93.33%) had the ability to produce biofilm by the MTP. Out of these 14 isolates 8 produced strong biofilm (57.1%) and six produced moderate/weak biofilm (42.8%).

These results were close to those obtained by Deligiann et al.[19] who studied *P. aeruginosa* biofilm producing isolates from sputum samples taken from children with cystic fibrosis. They found that (47.9%) produced strong biofilm, (19.7%) produced moderate biofilm and (32.2%) produced weak biofilm. However, those isolated from cystic fibrosis were expected to have strong biofilm.

A group of Egyptian-Saudi investigators[20] conducted a study on *P. aeruginosa* MDR strains in 2013. They found that 18 out of 40 strains (45%) produced biofilm. Sixteen (89%) of these strains were strong biofilm-producers, one strain was moderate and another one was weak biofilm-producer. These results might reflect the different sources from which the strains were isolated.

In this study, (42.8%) of the biofilm producing *P. aeruginosa* isolates were isolated from urine, (42.8%) from pus and (14.2%) from BAL.

In our study 4 out of 15 (27%) planktonic isolates were susceptible to amikacin (MIC $\leqslant$ 32 μg/ml) whereas 11 isolates (73%) were resistant. On the other hand, all the 14 (100%) biofilm producing isolates showed high amikacin MBEC ($\geqslant$64 μg/ml) and cannot be considered susceptible to amikacin according to the 2014 CLSI breakpoints.[12] This reflects the antibiotics abuse in the community, leading to such high rates of resistance.

Similar results were obtained when strains were challenged with meropenem where 3 out of 15 (20%) planktonic isolates were susceptible to meropenem (MIC $\leqslant$ 2 μg/ml) whereas 12 isolates (80%) were resistant. Similarly, all the 14 (100%) biofilm producing isolates showed high meropenem MBEC ($\geqslant$8 μg/ml) and cannot be considered susceptible according to the 2014 CLSI breakpoints.[12]

However in cases of biomaterial associated infections, in which biofilm formation is the main characteristic, conventional MIC only predicts the ability of an antimicrobial agent to inhibit the growth of the bacteria released from the biofilm, not the bacterial growth within biofilm matrix.[21]

However, Liu stated that Brown et al.[22] found that 20% of *P. aeruginosa* were resistant to amikacin in their planktonic state and 55% of *P. aeruginosa* isolates were resistant in their biofilm state. In another study, Saxena et al.[23], in India, found that 31.8% of the biofilm producing *P. aeruginosa* strains isolated from patients with lower respiratory tract infections were resistant to amikacin.

The higher resistance of amikacin and meropenem in the biofilm state more than planktonic state of *P. aeruginosa* can be explained by the decreased diffusion of antimicrobial agents through the extensive biofilm matrix where there is increase in alginate synthesis in biofilm. Aminoglycoside antibiotics are clearly less effective against the same micro-organism in anaerobic conditions which are provided by the biofilm than in aerobic conditions.[24] Alternatively, the depletion of a substrate or accumulation of an inhibitive waste product might cause some bacteria to enter a non-growing state, in which they are protected from killing.[5] β-lactam antibiotics, which target cell–wall synthesis, kill only growing bacteria and cannot act on the metabolically inactive bacteria in the biofilms.[25]

The development of new alternatives to antibiotherapy for eradication and control of virulent biofilms from surfaces, mainly of medical devices, has become a great challenge in the scientific community. Several studies have shown the potential of use of phages to treat infectious diseases in animals[26] and humans[27], even those caused by multidrug-resistant bacteria.[28]

The 15 strains of the present study were susceptible to 6 lytic phages isolated from effluents isolated from the same hospital over several months.

It is expected that phage action on planktonic cells is more efficient than in biofilms due to biofilm architecture, which prevents easy access of phages to the bacteria.[29] Sillankorva et al.[30] studied the effect of phage on *P. fluorescens* planktonic and biofilm states. They found that phage infection of planktonic culture of *P. fluorescens* resulted in a sharp increase of cell lysis, whereas the rate of cell lysis in biofilms was significantly lower.

Contrary to that expectation, our study showed low activity of phage against planktonic cells where 5 out of 15 strains (33.3%) were susceptible at MOI = 4.

On the other hand, 3 out of the 14 biofilm producing isolates (21.4%) were eradicated by their specific phages. It is noteworthy that two of these isolates showed biofilm eradication by using phage cocktail. Similarly, Pires et al.[2] studied the use of newly isolated phages to control *P. aeruginosa* PAO1 and ATCC 10145 and found that both planktonic and biofilm states were resistant to the reference phage PhiIBB-PAP21. They explained this by that after 9–10 h of infection, planktonic *P. aeruginosa* PAO1 cell cultures began to grow again.

This finding can be explained that the interaction of phages and bacteria during long periods of time can result in the emergence of phage-resistant bacteria and increase bacterial density.[31] However, other authors found that bacterial density was not observed to increase in vivo, since the combination of phage and host defences is sufficient to keep the bacterial density below the lethal threshold after phage therapy.[32] Thus, maybe short periods of treatment seem to be a better solution for avoiding the emergence of phage-resistant hosts. The use of phage cocktails also, presented advantages over the use of single phage, since phages can be selected in such a way to overwhelm host resistance mutations.[2]

Nevertheless, despite the potential of phages as antimicrobial agents, it is well known that bacteria can quickly adapt and create new survival strategies and the emergence of phage-resistant phenotypes is inevitable. Thus, the combination of phage and antibiotic therapies could have potentially more benefits than just using phages or antibiotics alone.[33]

The results of the present study demonstrated that the association of phage at MOI = 1 with the sub-MIC values of amikacin on *P. aeruginosa* strains, in their planktonic state, was statistically significant, where 13 out of 15 (86.66%) strains showed synergistic effect, whereas two strains (13.33%) showed no effect by this combination. Moreover, the combination of Sub MIC of meropenem and the corresponding phage at MOI = 1 on the 15 *P. aeruginosa* strains in planktonic state demonstrated that 11 (73.33%) strains showed synergistic effect, whereas four (26.67%) showed no effect by this combination. So the Median dose for amikacin and meropenem has

decreased by the combination of each with phage on the *P. aeruginosa* in the planktonic state.

Similar to our findings, Hagens et al.[34] studied the combination of phage and low doses of antibiotics on *P. aeruginosa* strains and found that this combination was able to inhibit the growth or kill *P. aeruginosa* and that the resistance to tetracycline was drastically reduced upon infection with phage.

These findings can be explained by two postulations: The first is the fact that the principal barrier to antibiotics in Gram-negative bacteria is the outer membrane, which in *P. aeruginosa* is augmented by the active efflux system MexAB-OprM. However, during filamentous phage progeny extrusion, this barrier for antibiotic penetration may be less effective in the bacterial cell.[38] The second is the cell filamentation in the presence of subinhibitory concentrations of β-lactams. This was explained by the fact that these antibiotics, although exhibiting different mechanisms of action, finally block bacterial cell division.[35,36] According to Comeau et al.[37] phage antibiotic synergism is a result of a change in morphology that permits faster assembly of phages through altered or larger pools of precursors important to phage maturation and accelerates the timing of cell lysis.

The results of this study demonstrated that the combination of amikacin and the corresponding phage at MOI = 1 on the *P. aeruginosa* biofilm state was synergistic on 7 (50%) strains with complete biofilm eradication whereas 7 (50%) showed no eradication. On the other hand, the combination of meropenem and the corresponding phage at MOI = one on the *P. aeruginosa* biofilm state showed synergistic effect in two (14.28%) strains with complete biofilm eradication, whereas 13 (85.7%) strains showed no biofilm eradication.

Statistical analysis of the current results demonstrated that the amikacin-phage combination caused statistically significant eradication ($p = 0.003$) of *P. aeruginosa* biofilm, whereas the meropenem-phage combination caused no significant eradication ($p = 0.481$) of *P. aeruginosa* biofilm.

This effectiveness of combined treatment of phages and amikacin on *P. aeruginosa* biofilm can be explained by several factors, namely altered surface charges of phage resistant phenotypes and disruption of the biofilm matrix induced by some of the phages which can enhance the antibiotic penetration.[37]

The results our study were similar to those of Fothergill et al.[38] They found that ciprofloxacin and norfloxacin caused a level of phage induction higher than that observed with other antibiotics (including meropenem) against the Liverpool epidemic strain of *P. aeruginosa* in the United Kingdom. Their data suggest that the choice of antibiotic could dramatically affect the levels of free *Pseudomonas* phages. *P. aeruginosa* phage mobilization has been associated with increased diversification and the transduction of antibiotic resistance.

Bowler et al.[39] reported that meropenem exposure stimulated significantly more β-lactamase production in mature biofilm and there was decreased β-lactamase production by all planktonic bacteria exposed to meropenem after 24 h.

However, Sillankorva et al.[33] observed antagonism with the amikacin-phage combination to control *P. aeruginosa* biofilms.[33] This antagonism could be explained as Kaplan[40] stated in his study that *P. aeruginosa* biofilm induction in response to tobramycin is a specific response to aminoglycoside antibiotics rather than a non-specific response to translation inhibition or other cell stressors. It was found that mutants carried transposon insertions in a gene designated arr, stands for aminoglycoside response regulator. The arr gene encodes a c-di-GMP phosphodiesterase that degrades c-di-GMP and reduces intracellular c-di-GMP concentrations. In which reduced c-di-GMP levels induce *P. aeruginosa* biofilm formation.

In Conclusion, the amikacin-phage combination could have potentially more benefits on *P. aeruginosa* biofilms than using phages or antibiotics alone. Meropenem is not recommended for non-growing bacteria in biofilm. Combination of phage and sub inhibitory concentrations of amikacin/meropenem results in avoidance of antibiotic side effects occurring after administration of high doses. It may be possible to commercialize phage-based products containing a combination of bacteriophages, further studies are needed to use these phage cocktails as phage therapy.

Conflict of interest

The authors declare that there is no conflict of interest in performing this study.

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