THE EFFECT OF BACTERIOPHAGE T4 AND PB-1 INFECTION WITH TOBRAMYCIN ON THE SURVIVAL OF *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA* BIOFILMS

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ABSTRACT

THE EFFECT OF BACTERIOPHAGE T4 AND PB-1 INFECTION WITH TOBRAMYCIN ON THE SURVIVAL OF *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA* BIOFILMS

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Populations of bacterial cells growing as biofilms demonstrate greater resistance to antibiotics compared to planktonic cells. Consequently, there is renewed interest in bacteriophage therapy as an alternative to antimicrobial chemotherapy. Although phages may be more effective than antibiotics alone in reducing biofilm mass, they are often not able to eradicate the biofilm. The aim of this study was to determine if bacteriophage in combination with an antimicrobial would be more effective in decreasing biofilm mass compared to the use of either antibiotic or phage alone. It was also of interest to determine if the combination of phage and antibiotic could reduce the emergence of either antibiotic or phage resistant mutants. *Escherichia coli* or *Pseudomonas aeruginosa* 48 h biofilms were challenged with phage T4 or PB-1, respectively, in combination with tobramycin. At 6 h and 24 h post challenge, total cells, tobramycin resistant cells, and

phage resistant cells were determined. The use of phage in combination with antibiotic resulted in an enhanced reduction of *E. coli* biofilms compared to either phage or antibiotic alone. The combination of phage and antibiotic resulted in a reduction in *P. aeruginosa* biofilms compared to phage alone. The combination of phage with antibiotic resulted in a reduced emergence of phage resistant (39% to 99%) and antibiotic resistant (26% to >99%) cells compared to treatment with either phage or antibiotic alone. The study suggests the combination of phage and antibiotic is more effective in reducing both biofilm mass and the emergence of resistance than the use of either phage or antibiotic alone. The study also suggests biofilm survival is dependent on the phage-host system.

INTRODUCTION

Bacterial biofilms are populations of cells adhered to an abiotic or biotic surface that can grow to be several millimeters thick (1, 17, 65). Biofilms have been found to form on medical implants such as catheters and artificial hips (17, 53) as well as in the lungs of cystic fibrosis patients (13, 22, 54, 64). The community of cells are coated in a sticky matrix made of extracellular DNA, secreted proteins, and polysaccharides (39, 55) called extracellular polymeric substances (EPS) that allow for adherence to surfaces as well as protection from antimicrobial agents (22). The EPS is diverse between species, for example the EPS *Pseudomonas aeruginosa* produces is polyanionic (24) while *Staphylococcus epidermidis* produces a polycationic EPS (44, 59). Confocal images haves shown water channels are formed in the EPS which allow for nutrient and oxygen delivery to the cells (13, 17, 40). Biofilms are considered to be highly tolerant to antimicrobials (7, 17, 46, 52, 66) due to the ability of the EPS to hinder diffusion of antimicrobials (17, 26, 30) also biofilms contain slow-growing cells due to the lack of nutrients and oxygen throughout the structure which can decrease the efficacy of antimicrobials that rely on actively growing cells (7, 17, 32, 33, 46). There is also evidence of EPS trapping phage particles (24, 36). Hanlon and colleagues passed bacteriophage through 4% commercial alginate (comparable to purified *P. aeruginosa* EPS) and found a five log decrease in the number of phage that were able to pass through alginate compared to buffer (24). The ability of EPS to hinder diffusion has been greatly

debated and seems to depend on the antimicrobial (7, 57) or phage particle (31, 60, 63). A study found mature *E. coli* biofilm cells (72 h) more resistant to ampicillin than young biofilms (2 h and 24 h) (33) . Another study found *P. aeruginosa* 2 day biofilms were reduced by 97% when treated with 5 μg ml^{-1} of tobramycin and 7 day biofilms were reduced 50% when treated with up to 50 μg ml^{-1} of tobramycin (3). These studies indicate the older the biofilm, the greater the resistance to antimicrobials.

E. coli and *P. aeruginosa* form biofilms and are commonly present in infection. *E. coli* is a Gram-negative bacterium often found in the mammalian gut. It is one of the major contributors to urinary tract infections and can be found growing as biofilms on urinary catheters (34). *P. aeruginosa* is a Gram-negative soil bacterium. It is commonly seen growing as biofilms in patients with cystic fibrosis (6, 13, 62) and can also be found on urinary catheters (34) as well as other medical devices.

Due to the increase in antibiotic resistance, alternatives such as bacteriophage therapy are being pursued to treat infection (38). Bacteriophages are viruses that are specific for the infection of bacterial cells. Phages can be highly specific or have a broader range for its host. Lytic phages, phage that replicate in the bacterial cell until lysis to release progeny virus, have been most commonly used in phage therapy. Frederick Twort and Felix d'Herelle are credited with the discovery of phage in the early 1900's, though there is evidence other scientists observed the effects of phage on bacteria before Twort and d'Herelle (58). It was d'Herelle, and his wife, that coined the name *bacteriophage*. The first studies on phage therapy were performed by d'Herelle in Paris in 1919 and later he produced several phage preparations under the company name L'Oréal (58). Phage therapy was a common method of treating infections before replaced

by the discovery of antibiotics (10, 25, 38). In parts of the former Soviet Union phage therapy is still being used for the treatment of a variety of afflictions ranging from acne and urinary tract infections to methicillin resistant *Staphylococcus aureus* (25, 58). A major concern of phage therapy is the development of phage resistance (20).

It has been proposed that phage therapy is effective on biofilm infections due to the ability of some phage to degrade EPS (20). There have been several studies on the effect of phage on biofilms. A recent study used phage SAP-26 on *S. aureus* biofilms and found the phage to kill 28% of the biofilm after 24 h (52). Another study using λW60 on *E. coli* biofilms and PB-1 on *P. aeruginosa* biofilms found the phages were not effective at maintaining low cell densities and therefore concluded the efficacy of phage treatment on biofilms may be dependent upon phage type (36). There is some evidence that shows phage polysaccharide depolymerase can degrade bacterial capsules (31). Verma and colleagues observed non-depolymerase bacteriophage (ΦNDP) on *Klebsiella pneumoniae* biofilms was unable to significantly decrease biofilm mass, however, the depolymerase bacteriophage (KPO1K2) was able to significantly decrease biofilm mass (63). When phage ΦNDP and KPO1K2 were applied to planktonic cultures of *K. pneumoniae*, both phages had similar efficacies (63). Hughes and colleagues further showed the importance of depolymerase and lytic bacteriophage in the treatment of *Enterobacter agglomerans* biofilms, where treatment with depolymerase phage SF153b was more effective at decreasing the biofilm than depolymerase alone (31). The reoccurring theme with phage treatment on biofilms seems to be that phage is often able to decrease biofilm mass, however, it is not able to eradicate the biofilm (60).

Tobramycin is a broad-spectrum aminoglycoside that targets the 30S subunit of prokaryotic ribosomes and inhibits protein synthesis (37, 45, 48, 49). Tobramycin is derived from the bacterium *Streptomyces tenebrarius* (28). The antibiotic is stable at varying pHs and range of temperatures (49) and is effective against Gram-negative bacilli, Gram-positive cocci, and *Mycobacterium* species (45). Tobramycin is the drug of choice to treat *P. aeruginosa* infection in cystic fibrosis patients (27, 28, 48, 62). For tobramycin to be effective the cells must be metabolically active (24, 56, 62), a problem when treating the slow-growing biofilm cells.

Biofilms have been found to be highly tolerant to antimicrobial agents, often requiring much higher concentrations of the antibiotic than what is effective on planktonic cells. A study on the effect of antibiotics on the survival of *S. aureus* biofilms resulted in 60%, 25%, and 17% kill due to rifampicin, azithromycin, and vancomycin, respectively, after 24 h of treatment with concentrations reportedly 10-fold higher than the MIC (52). Seven day old *S. aureus* biofilms were exposed to ten times the minimal bactericidal concentration of tetracycline, vancomycin, and benzylpenicillin for 24 h and exhibited no reduction in viability after treatment with tetracycline and vancomycin, and 40% reduction after treatment with benzylpenicillin (66). Another study using ciprofloxacin on *K. pneumoniae* biofilms found an approximate two log decrease in biofilm mass after 6 h with a concentration of antibiotic much higher than the planktonic MIC (63). Cerca and colleagues treated *Staphylococcus epidermidis* planktonic cells and biofilm cells with cefazolin, vancomycin, dicloxacillin, tetracycline, and rifampicin and found cefazolin, vancomycin, and dicloxacillin to produce a three log decrease in the planktonic cells after 6 h (7). However, the treatment of the antibiotics on biofilms

produced less than 0.5 log decrease after 6 h (7). Recent evidence suggests that the application of aminoglycosides, tobramycin in particular, can lead to an increase in biofilm formation in *P. aeruginosa* and *E. coli* cells (28, 41).

There have been few studies on the effect of the combination of phage and antibiotic on biofilms. Rahman and colleagues used a combination of phage SAP-26 and rifampicin, azithromycin, or vancomycin on *Staphylococcus aureus* biofilms (52). The combination was more effective in decreasing biofilm mass after 24 h compared to either phage or antibiotic alone, and the combination of rifampicin with phage SAP-26 was most effective (52). A study by Verma and colleagues found the combination of lytic phage KPO1K2 in combination with ciprofloxacin was more effective at reducing older *K. pneumoniae* biofilms than either phage or antibiotic alone (63). Another study on *K. pneumoniae* treated with amoxicillin and bacteriophage B5055 found the combination of the phage and antibiotic to be more effective than either alone (4). The combination of phage and antibiotic has been tested in a limited number of phage-host systems, but has been shown effective.

The purpose of this study was to determine if *E. coli* bacteriophage T4 or *P. aeruginosa* bacteriophage PB-1 in combination with tobramycin could decrease *E. coli* and *P. aeruginosa* 48 h biofilms more effectively than either phage or tobramycin alone. The results showed the combination of T4 and tobramycin was more effective than either the use of phage or tobramycin alone. The combination of PB-1 and tobramycin was just as effective as tobramycin indicating the efficacy is dependent on the phage-host system. In addition the study showed combinational phage and antibiotic therapy reduced the

emergence of both phage and antibiotic resistant cells compared to the use of either phage or antibiotic alone.

MATERIALS AND METHODS

Bacteria and bacteriophage. *Escherichia coli* B (ATCC 11303) and *Pseudomonas aeruginosa* PAO1 (obtained from V. Deretic, University of New Mexico) were used in the experiments. Bacteriophage T4 (ATCC 11303-B4) was used to infect *E. coli* and PB-1 (ATCC 15692-B3) was used to infect *P. aeruginosa*.

Antibiotic preparation. Tobramycin (T4014, Sigma-Aldrich Co., St. Louis, MO) stock concentrations of 40 μg ml^{-1} and 50 μg ml^{-1} were prepared by diluting tobramycin in deionzed water and filter sterilizing (0.22 μm; Fisher 25-mm syringe filter; Fisher Scientific Inc., Dublin, Ireland).

Growth of bacteria. *E. coli* and *P. aeruginosa* were grown in Luria Bertani (LB) broth (Accumedia Manufacturers, Inc., Lansing, Michigan) at 37°C in an orbital rotating shaker water bath (Lab-Line Instruments, Inc. model 3540 Orbital Shaker Bath, Melrose Park, IL).

Bacteriophage propagation. Bacteriophage T4 and PB-1 stocks were prepared by infecting early log phase of *E. coli* or *P. aeruginosa*, respectively, at a multiplicity of infection (MOI) of approximately 1000. Infected cultures were placed into a 37°C reciprocal shaking water bath (Blue M Electric Company Magni Whirl MSB-1122A-1 Shaker Bath, Blue Island, Illinois) until the cultures cleared, or 2 h. Infected cultures were then placed into a glass centrifuge tube with 0.5 ml of chloroform at 4°C. After 5 min at

4°C the infected cultures were shaken for 1 min and placed at 4°C for 5 min. The cultures were centrifuged (Eppendorf Centrifuge model 5810 R, Hamburg, Germany) at 4,000 rpm for 20 min at 4°C. The supernatant was filtered (0.45 µm) and phage titers were determined by soft-agar overlay plaque assay (2).

Biofilm growth. Silicone rubber disks, 7 x 1 mm (Dapro Rubber Inc., Tulsa, Oklahoma), were placed into 125 ml flasks containing 50 ml of LB broth and inoculated with overnight cultures of *E. coli* or *P. aeruginosa* to a cell density of approximately $1x10^6$ CFU ml^{-1} (OD_{600nm}=0.1). Monocultures were incubated in an orbital shaking water bath at 100 rpm for 48 h at 37°C. Biofilm growth was measured using sonication followed by determination of CFU's, as described by Whiteley et al. (65).

Determination of antibiotic concentration and MOI on cell survival. *E. coli* and *P. aeruginosa* biofilms were grown as described previously. After 48 h disks containing biofilms were rinsed in phosphate buffered saline (PBS) (Sigma-Aldrich, Co., St Louis, MO) and placed in individual 20 ml scintillation vials containing LB broth and phage at various MOI (0.0001-10) or tobramycin (0.25-4 µg ml^{-1}) (8). Treated biofilms were incubated in a reciprocal shaking water bath at 100 rpm for either 6 h or 24 h at 37°C. Biofilms were rinsed in PBS to remove planktonic cells and placed in 5 ml PBS, sonicated (Branson Ultrasonic Cleaner 1510, Danbury, CT) for 10 mins, and vortexed for 1 min. Cell density was determined by dilution plating on LB agar plates (65).

Bacteriophage challenge. Following 48 h growth, biofilm-coated disks were gently rinsed with 5 ml of PBS in sterile test tubes to remove planktonic cells. The disks were then placed into individual 20 ml scintillation vials containing 10 ml of LB broth

with either T4 or PB-1 at an MOI of 0.01. The vials were incubated at 37°C at 70 cycles per minute in a reciprocal shaking water bath. The biofilm-coated disks were selected at 6 h and 24 h, sonicated for 5 min, vortexed for 2 min and assayed for colony forming units (CFU) on LB to determine the total number of surviving cells. CFU's were converted to biofilm density (CFU mm^{-2}). Tobramycin resistant cells were determined by plating on LB agar containing tobramycin (2 µg ml^{-1} for *E.* coli and 0.5 µg ml^{-1} for *P. aeruginosa*). Phage resistant cells were determined by agar overlay containing 0.1 ml of 10^8 PFU ml^{-1} and 0.1 ml of the diluted biofilm cells. CFU's were used to determine the number of resistant cells.

Antibiotic challenge. 48 h biofilms were gently rinsed with 5 ml of PBS in sterile test tubes to remove planktonic cells. Biofilms were placed in individual 20 ml scintillation vials containing 10 ml of LB broth with 2 µg ml^{-1} of tobramycin for *E.* coli and 0.5 µg ml^{-1} of tobramycin for *P. aeruginosa*. Biofilms were incubated at 37°C at 70 cycles per minute in a reciprocal shaking water bath. The biofilm-coated disks were selected at either 6 h or 24 h, sonicated for 5 min, vortexed for 2 min and assayed for CFU's on LB agar plates to determine the total number of surviving cells. CFU's were converted to biofilm density (CFU mm^{-2}). Tobramycin resistant and phage resistant cells were determined as described previously.

Antibiotic and bacteriophage challenge. 48 h biofilms were gently rinsed with 5 ml of PBS in sterile test tubes to remove planktonic cells. Biofilms were placed in individual 20 ml scintillation vials containing 10 ml of LB broth with tobramycin (2 µg ml^{-1}) and T4 at an MOI of 0.01 for *E.* coli and tobramycin (0.5 µg ml^{-1}) and PB-1 at an MOI of 0.01 for *P. aeruginosa*. Biofilms were incubated 70 cycles per minute in a

reciprocal shaking water bath at 37°C. Biofilms were selected at random after 6 h or 24 h, sonicated for 5 min, vortexed for 2 min and assayed for CFU to determine the total number of surviving cells. CFU's were converted to biofilm density (CFU mm^{-2}). Tobramycin resistant and phage resistant cells were determined as described previously.

RESULTS

Determination of antibiotic concentration on cell survival. *E. coli* and *P. aeruginosa* 48 h biofilms were exposed to varying concentrations of tobramycin (0.25-4 μg ml^{-1}) for 6 h and 24 h then assayed to determine which concentrations produced 90% kill, the results for *E. coli* and *P. aeruginosa* (Table 1 and Table 2). After *E. coli* biofilms were exposed to tobramycin for 6 h, the percent kill for tobramycin concentrations 1-4 μg ml^{-1} were greater than 90%. After *E. coli* biofilms were exposed to tobramycin for 24 h, 2 μg ml^{-1} yielded approximately 90% kill and was used for further experiments. After *P. aeruginosa* was exposed to tobramycin for 6 h, the percent kill for tobramycin concentrations 0.5-1.5 μg ml^{-1} yielded greater than 90% kill and 0.25 μg ml^{-1} yielded approximately 90% kill. After *P. aeruginosa* was exposed to tobramycin for 24 h, 0.5 μg ml^{-1} gave 90% kill and was used for further experiments.

Determination of MOI on cell survival. To determine the MOI of bacteriophage T4 and PB-1 that would yield approximately 90% kill, *E. coli* and *P. aeruginosa* 48 h biofilms were treated with MOI's ranging from 0.0001 to 10 for 6 h and 24 h, the results for *E. coli* and *P. aeruginosa* (Table 3 and Table 4). After 6 h and 24 h, T4 MOI's of 0.001, 0.01, and 0.1 resulted in approximately the same percent kill of greater than 90%. An MOI of 0.01 was used for future experiments on *E. coli*. After 6 h, PB-1 MOI's of 0.0001, 0.01, and 1 resulted in greater than 90% kill. After 24 h, PB-1 MOI's of 0.001,

0.01, 0.1, and 10 resulted in percent kills of less than 90%. An MOI of 0.01 was used for PB-1 infection for future experiments on *P. aeruginosa*.

Effect of tobramycin and T4 on *E. coli* biofilm cell survival. *E. coli* biofilms were exposed to tobramycin, T4, or a combination of the two for 6 h and 24 h then assayed to determine the number of surviving cells (Table 5 and Fig. 1). After 6 h and 24 h, there was a greater decrease in the number of surviving cells treated with a combination of T4 and tobramycin than T4 alone or tobramycin alone. After the 6 h treatment with tobramycin, T4, and a combination of tobramycin and T4 -0.27 ± 0.55, -0.10 ± 0.21, and -1.6± 0.32 CFU mm^{-2} remained, respectively. After the 24 h treatment with tobramycin, T4, and a combination of tobramycin and T4 2.1 ± 0.66, -0.79 ± 0.20, and -1.8 ± 0.43 CFU mm^{-2} remained, respectively.

Determination of *E. coli* biofilm cell resistance. After 6 h and 24 h treatments with tobramycin, T4, and a combination of tobramycin and T4, biofilm cells were assayed to determine T4 resistant cells and tobramycin resistant cells (Table 6 and Fig. 1). The number of T4 resistant cells in the combination challenge were compared to the number of T4 resistant cells in the T4 challenge and the number of tobramycin resistant cells in the combination challenge were compared to the number of tobramycin resistant cells in the tobramycin challenge. After 6 h, there was a 99% decrease in the number of T4 resistant cells and an 80% decrease in the number of tobramycin resistant cells in the combination challenge. After 24 h, there was a 39% decrease in the number of T4 resistant cells and a >99.99% decrease in the number of tobramycin resistant cells in the combination challenge.

Effect of tobramycin and PB-1 on *P. aeruginosa* biofilm cell survival. *P. aeruginosa* biofilms were exposed to tobramycin, PB-1, or a combination of tobramycin and PB-1 for 6 h and 24 h and the number of surviving cells were determined (Table 5 and Fig. 2). After the 6 h treatment with tobramycin, PB-1, and a combination of tobramycin and PB-1 2.7 ± 0.30, 2.4 ± 0.21, and 2.0 ± 0.35 CFU mm^{-2} remained, respectively. After the 24 h treatment with tobramycin, PB-1, and a combination of tobramycin and PB-1 1.8 ± 0.36, 3.2 ± 0.17, and 1.6 ± 0.33 CFU mm^{-2} remained, respectively.

Determination of *P. aeruginosa* biofilm cell resistance. After 6 h and 24 h treatments with tobramycin, PB-1, and a combination of tobramycin and PB-1, the cells were assayed to determine PB-1 resistant cells and tobramycin resistant cells (Table 6 and Fig. 2). The number of PB-1 resistant cells in the combination challenge were compared to the number of PB-1 resistant cells in the PB-1 challenge and the number of tobramycin resistant cells in the combination challenge were compared to the number of tobramycin resistant cells in the tobramycin challenge. After 6 h, there was an 81% decrease in the number of PB-1 resistant cells and a 26% decrease in the number of tobramycin resistant cells in the combination challenge. After 24 h, there was a 99% decrease in the number of PB-1 resistant cells and a 60% decrease in the number of tobramycin resistant cells in the combination challenge.

Table 1. Effect of tobramycin concentration on the survival of *E. coli* biofilms

Time (h)	Tobramycin (µg/ml)			
	1	2	3	4
t_6	93.61[a]	>99.99[a]	99.99[a]	>99.99[a]
t_{24}	64.23[a]	81.95[a]	96.52[a]	99.79[a]

[a] Percent kill

Table 2. Effect of tobramycin concentration on the survival of *P. aeruginosa* biofilms

Time (h)	Tobramycin (µg/ml)			
	0.25	0.5	1	1.5
t_6	92.52[a]	99.98[a]	>99.99[a]	99.99[a]
t_{24}	73.50[a]	89.89[a]	99.96[a]	99.97[a]

[a] Percent kill

Table 3. Effect of T4 MOI on the survival of *E. coli* biofilms

Time (h)	Multiplicity of Infection				
	0.0001	0.001	0.01	0.1	1.0
t_6	-	98.67[a]	99.46[a]	98.69[a]	-
t_{24}	99.61[a]	99.67[a]	99.82[a]	99.78[a]	99.82[a]

[a] Percent kill

Table 4. Effect of PB-1 MOI on the survival of *P. aeruginosa* biofilms

Time (h)	Multiplicity of Infection					
	0.0001	0.001	0.01	0.1	1	10
t_6	98.53[a]	-	98.10[a]	-	98.63[a]	-
t_{24}	-	77.76[a]	68.77[a]	79.18[a]	-	85.80[a]

[a] Percent kill

Table 5. Survival of *E. coli* and *P. aeruginosa* biofilm cells treated with bacteriophage and tobramycin

Time (h)	*E. coli*			*P. aeruginosa*		
	CFU/mm^2					
	Tobramycin[a]	T4[a]	Tob+T4[a]	Tobramycin[b]	PB-1[b]	Tob+PB-1[b]
t_6	-0.27 ± 0.55	-0.10 ± 0.21	-1.6 ± 0.32	2.7 ± 0.30	2.4 ± 0.21	2.0 ± 0.35
t_{24}	2.1 ± 0.66	-0.79 ± 0.20	-1.8 ± 0.43	1.8 ± 0.36	3.2 ± 0.17	1.6 ± 0.33

[a] log Mean ± SE (n=12)
[b] log Mean ± SE (n=15)

Table 6. Bacteriophage and tobramycin resistance in *E. coli* and *P. aeruginosa* biofilms

	E. coli		*P. aeruginosa*	
Time (h)	T4 Resistance[a]	Tobramycin Resistance[b]	PB-1 Resistance[c]	Tobramycin Resistance[d]
t_6	99%	80%	81%	26%
t_{24}	39%	>99.99%	99%	60%

[a] % decrease in resistance following T4+Tob challenge compared to T4 alone
[b] % decrease in resistance following T4+Tob challenge compared to tobramycin alone
[c] % decrease in resistance following PB-1+Tob challenge compared to phage alone
[d] % decrease in resistance following PB-1+Tob challenge compare to tobramycin alone

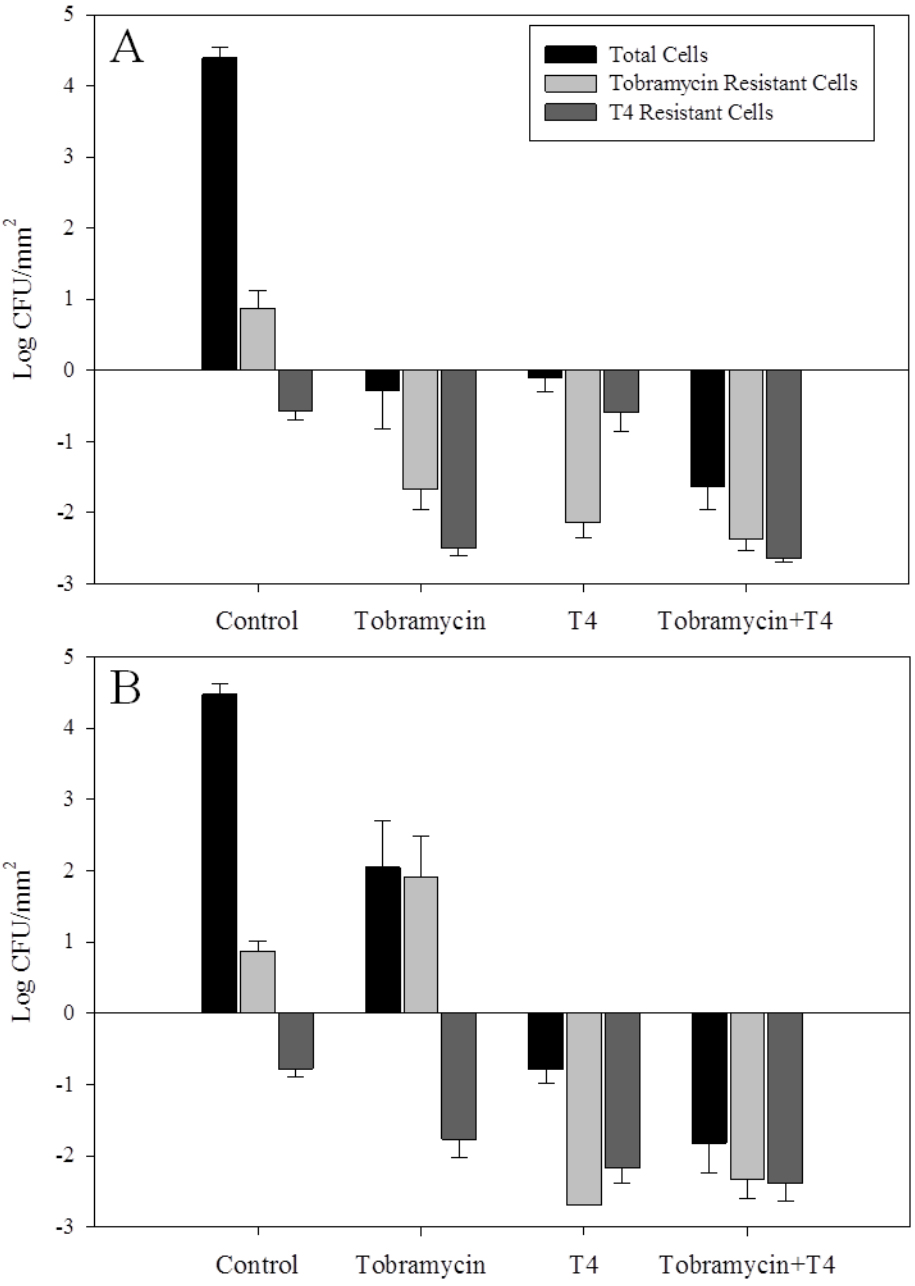

Fig. 1. *E. coli* biofilms challenged with tobramycin, T4, and a combination of tobramycin and T4. *E. coli* biofilm survival, tobramycin resistance, and T4 resistance was determined after 6h (A) and 24h (B).

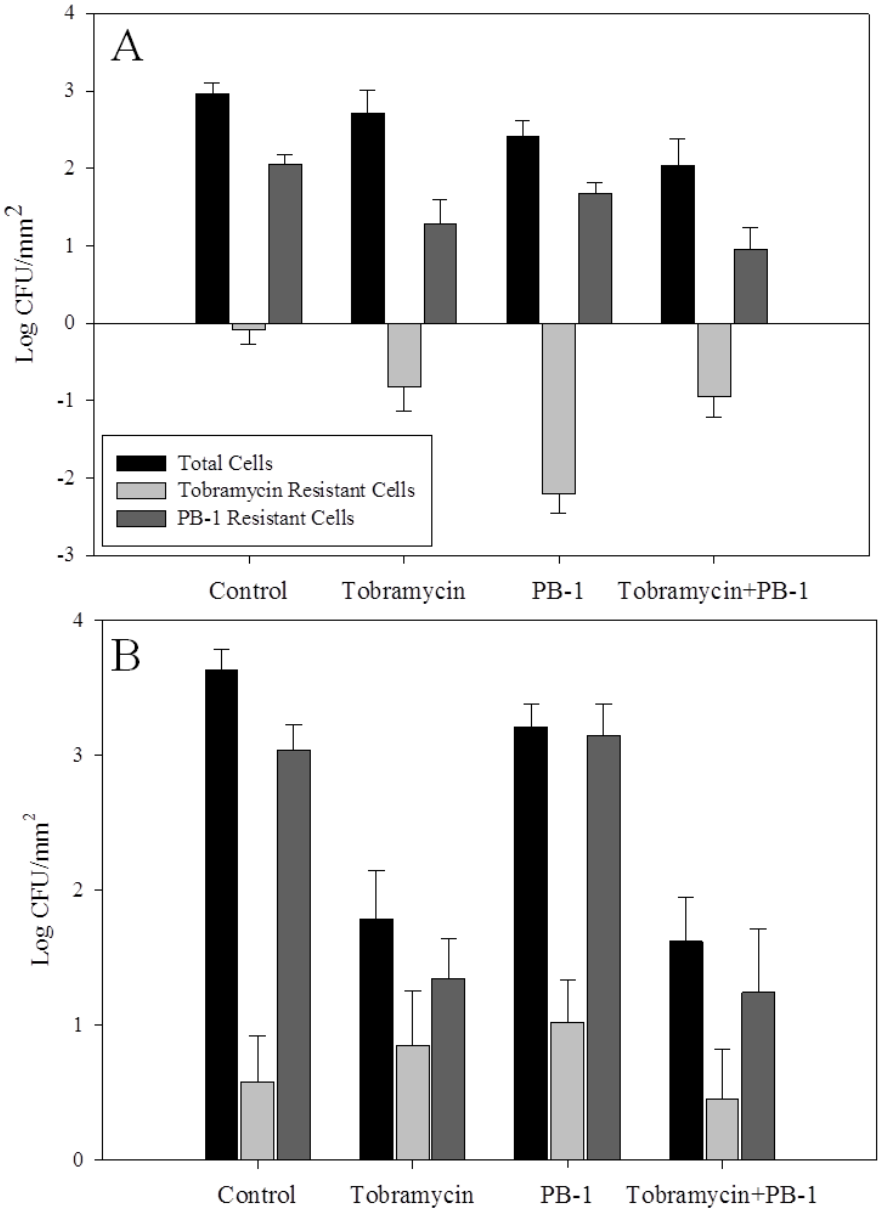

Fig 2. *P. aeruginosa* biofilms challenged with tobramycin, PB-1, and a combination of tobramycin and PB-1. *P. aeruginosa* biofilm survival, tobramycin resistance, and PB-1 resistance was determined after 6h (A) and 24h (B).

DISCUSSION

Infection due to biofilms are difficult to treat because of their high tolerance to antimicrobials (52, 66). Bacteriophage therapy has been suggested for the treatment of biofilms (38), and although effective in decreasing biofilm cell density, phage therapy alone not been shown to eradicate biofilms (60). In addition, the use of phage therapy may also lead to phage resistance (20). The purpose of this study was to determine if the combination of phage infection with an antibiotic is more effective at reducing *E. coli* and *P. aeruginosa* biofilm mass compared to the use of either phage or antibiotic alone. This study also examined the effect of combined therapy on the emergence of *E. coli* and *P. aeruginosa* phage resistant cells and antibiotic resistant cells.

This study found bacteriophage T4 infection combined with tobramycin on *E. coli* biofilms to be more effective at decreasing biofilm mass than the use of either phage or antibiotic alone. Similar results have been found on studies with *K. pneumoniae* and *S. aureus* biofilms treated with combinations of phage and antibiotics (4, 52, 63). The present study also found that although the combination of phage PB-1 and tobramycin was more effective at decreasing *P. aeruginosa* biofilm mass than phage alone after 24 h, the combination was just as effective as the use of tobramycin alone. Phage type has been found to be an important factor in biofilm survival (63). A study of *P. aeruginosa* and *E. coli* mixed biofilms found PB-1 was not significantly effective at decreasing biofilm mass of *P. aeruginosa* mixed and monoculture biofilms (36). PB-1 receptors are

lipopolysaccharides found in the outer membrane of *P. aeruginosa* cells (9, 35). *P. aeruginosa* biofilms form a thick EPS which could restrict the ability of PB-1 to attach to cells (24). In the present study, PB-1 was used at an MOI of 0.01 which resulted in 69% kill of *P. aeruginosa* biofilm after 24 h. Possibly, an increase in MOI could increase the efficacy of PB-1 infection (23).

Combinational drug therapy is commonly used to prevent the emergence of antibiotic resistance in infections. A mathematical model to determine if a single antibiotic or multiple antibiotics could more effectively reduce the occurrence of antibiotic resistance found the combination of antibiotics to be more effective on bacterial infections where recovery and termination of transmission coincide (5). Vancomycin-resistant *Staphylococcus aureus* treated with vancomycin and naficillin resulted in a reduction in bacterial load by 1.5 logs or greater after 3 days compared to the use of either antibiotic alone (19). Tré-Hardy and colleagues showed some combinations of antibiotics are more effective than others in treating infection (62). *P. aeruginosa* biofilms resistant to tobramycin and clarithromycin were completely eradicated with the combination of the two after 28-day exposure, showing a synergistic relationship (62). *P. aeruginosa* biofilms sensitive to tobramycin and azithromycin became resistant to tobramycin when treated with the combination of the two antibiotics, showing an antagonistic relationship (62).

Due to the high specificity of the phage-host relationship, combinations of different phages have been used to treat infection (38, 47). These combinations of phage called *phage cocktails* are currently offered for the treatment of human infections through the ELIAVA Institute in the Republic of Georgia (18). It has been suggested that phage

cocktails containing phage with different bacterial receptors could increase kill and therefore reduce the emergence of phage resistance (43). A study using a phage cocktail to prevent *P. aeruginosa* biofilm formation on catheters found treatment with a single phage (M4) resulted in a 2.8 log decrease after 24 h with all recovered variant types resistant to the phage (20). However, a combination of five phages resulted in a three log decrease after 48 h with only one variant resistant to all phages (20).

Studies have shown combination therapy, whether it be antibiotics or phage, are more effective at reducing bacterial resistance than the use of a single treatment. In this study, the combination of phage T4 or PB-1 and tobramycin on *E. coli* or *P. aeruginosa* biofilms, respectively, was more effective at reducing the emergence of phage resistant cells and tobramycin resistant cells. In the combination challenge, *E. coli* biofilms had a 39% and >99.99% decrease, in T4 resistance and tobramycin resistance, respectively, after 24 h, compared to either T4 or tobramycin alone. In the combination challenge, *P. aeruginosa* biofilms had a 99% and 60% decrease in PB-1 resistance and tobramycin resistance, respectively, after 24 h, compared to either PB-1 or tobramycin alone.

Bacteria have been found to create resistance to phage in the formation of clustered, regularly interspaced, short palindromic repeats known as CRISPRs (29, 61). *Streptococcus therophilus* CRISPRs provide protection against *pac*-type and *cos*-type phages (15). *E. coli* B2 strains do not have the CRISPR2/CAS-E system (16, 61) which has been suggested to be the result of a lack of phage diversity in their environments, notably the meninges and urogential tract (16). A sensitivity analysis also found several strains of *E. coli* compared to 59 coliphages did not have a correlation in *E. coli* CRISPR sequence (16). This could indicate *E. coli* does not have a strong reliance on CRISPRs as

a mechanism of phage resistance (61). Palmer and colleagues found a significant relationship between antibiotic resistance and the presence or absence of CRISPRs in enterococci (50). *Enterococcus faecalis* and *Enterococcus faecium* strains with multiple drug resistance (MDR) were found to lack CRISPRs, whereas *E. faecalis* strains lacking MDR contained CRISPR1-*cas* or CRISPR3-*cas* and all contained CRISPR2 (50). The lack of functional CRISPR-*cas*, and therefore phage resistance, could aid phage infection in MDR strains of enterococcus. *P. aeruginosa* (PA14) infected with temperate phage DMS3 or containing CRISPR-*cas* genes coding phage genes DSM3-24 and DSM3-13 inhibited swarming motility and biofilm formation (67). It has been suggested that behavioral inhibition of *P. aeruginosa* could be the bacterium's way of isolating itself to prevent further infection to other cells or it could provide an unknown advantage to the phage (67). In either event, phage could be used to reduce biofilm formation in *P. aeruginosa* infections and provide a better opportunity for antibiotic treatments.

Engineered phages to enhance antibiotic efficacy by reducing the occurrence of antibiotic resistance could be used in the treatment of biofilm infection (43). A study modified *E. coli* K-12 M13 phage to over-express *lexA3* which suppresses the SOS system (42) which lead to the inhibition of antibiotic resistance (11). The engineered phage improved the bactericidal effect of the antibiotic (loxacin) by 2.7 and 4.5 orders of magnitude compared to unmodified phage and no phage, respectively (43). Lu and colleagues also found the engineered phage was able to reduce antibiotic resistant cells to a median of 2.5 resistant CFU's compared to the unmodified phage with a median of 43.5 resistant CFU's (43).

Several studies have found the use of a subinhibitory level of antibiotic can increase biofilm mass. Another benefit to combinational phage and antibiotic therapy could be to decrease this effect. *S. epidermidis* biofilms were exposed to tetracycline, quinupristin, dalfopristin, and quinupristin-dalfopristin at levels below the MIC (51). The antibiotics were found to increase the formation of biofilm due to the induction of the *ica* operon which mediates the production of the polysaccharide intercellular adhesion, important for biofilm formation (51). Subinhibitory levels of tobramycin have been found to induce biofilm formation, as an increase in cell numbers, in *P. aeruginosa* (28, 41) and *E. coli* (28). Tobramycin was found to induce swimming and swarming of *P. aeruginosa* (41). Ciprofloxacin and tetracycline were also tested on *P. aeruginosa* in subinhibitory levels and were found to increase biofilm formation (41). Interestingly, Comeau and colleagues found what they called "Phage Antibiotic Synergy" (PAS) whereby sub-lethal doses of β-lactam antibiotics on *E. coli* MFP increased phage (ϕMFP, RB32, and RB33) growth by increasing cell lysis and inducing the filamentation phenotype allowing for increased phage replication (12). The PAS response was also seen in *E. coli* AS19 with T4 phage resulting in a titer ~11-fold greater in the presence of cefotaxime and ~9-fold greater in the presence of mitomycin C, when compared to no antibiotic (12). In the present study, tobramycin was found to decrease *E. coli* and *P. aeruginosa* biofilms by approximately two logs after 24 h, suggesting tobramycin was not at subinhibitory levels that would increase biofilm formation.

Combinational phage and antibiotic therapy could benefit cystic fibrosis patients with *P. aeruginosa* infections. Current treatment includes the use of nebulized antibiotics, in combination, often only effective on new, as opposed to chronic, infection (62). A

study using nebulized phage (21) in mice with *P. aeruginosa* lung infections found similar results of phage alone effectively decreasing strains in primary infection but not strains from chronic infections (14). Phage in combination with antibiotic may be effective in treating chronic infections.

This study agrees with others that the combination of phage and antibiotic is more effective at reducing biofilm mass compared to the use of either phage or antibiotic alone (4, 52, 63). The combination of T4 and tobramycin on *Escherichia coli* biofilms was more effective at decreasing biofilm mass compared to either phage or antibiotic alone. The combination of PB-1 and tobramycin on *Pseudomonas aeruginosa* biofilms was more effective than phage alone and just as effective as antibiotic alone in decreasing biofilm mass. This study also agrees with others that the efficacy is dependent of the phage-host system (36, 63). In all instances the combination of phage and antibiotic was more effective at decreasing the emergence of phage resistant cells and antibiotic resistant cells.

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