

Dynamics of Bacteriophages as a Promising Antibiofilm Agents

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Pseudomonas aeruginosa is an ubiquitous organism which has emerged as a major threat in the hospital environment. Overuse of antibiotics has also significantly increased the emergence of antimicrobial multiresistant bacteria. *P. aeruginosa* has an innate ability to adhere to surfaces and form virulent biofilms. Bacteriophage might represent one attractive solution to this problem. In this study, *Paeruginosa* phage were utilized to Biofilm inhibition and remove. Sample collected from University sewage. Isolation was done according to Martha.R.J.Clokie protocol. Serial dilution prepared, then equally incubated with bacteria to investigate Biofilm inhibition potential. Biofilm formed base on Microplate Biofilm Assay. The effect of isolated phage investigated on biofilm remove of *Pseudomonas putida*, *E.coli* and *Acinetobacter baumannii*. *Paeruginosa* biofilm had OD: 1.688 in 492n.m. Pure phage, 10-2 and 10-3 diluted phage decreased OD to 1.587, 1.341 and 1.461, respectively. Isolated phage dramatically decline OD of Biofilm of all strains. Phages have various affinity to attach to hosts, thereby it is supposed to phages compete for their receptors. Therefore it is supposed phages have most efficiency in optimum concentration to remove biofilm or growth inhibition.

Key word: Bacteriophage, Biofilm, Planktonic, Biofilm remove and Biofilm inhibition.

Pseudomonas aeruginosa is an ubiquitous organism which has emerged as a major threat in the hospital environment. This bacterium is the most frequently isolated Gram-negative organism in blood stream and wound infections, pneumonia and intra-abdominal and urogenital sepsis, and is a serious problem, infecting immunocompromised patients with cystic fibrosis

(CF), severe burns, cancer, AIDS, etc¹. One of the most worrying characteristics of this bacterium is its low antibiotic susceptibility, which can be attributed to a concerted action of multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes and the low permeability of the bacterial cellular envelopes². Overuse of antibiotics has also significantly increased the emergence of antimicrobial multiresistant bacteria; consequently, treatment of most chronic *P. aeruginosa* infections with antibiotics is notoriously difficult^{3,2}. Additionally, *P. aeruginosa* has an innate ability to adhere to surface and form virulent biofilms

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particularly difficult to eradicate^{4,5,6}. Biofilm formation is an important bacterial survival strategy and, in humans, biofilms are responsible for numerous pathologies usually associated with use of medical devices^{7,8,9}. Thus, new alternative strategies to antibiotic therapy are highly in demand by the worldwide medical and scientific community. Bacteriophages (phages) are the natural enemies of bacteria and might represent one attractive solution to this problem¹⁰. 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 are very specific and efficient for their target bacteria, which reduces destruction of the host's natural flora; they are not pathogenic for man; and they persist only as long as the targeted bacteria are present^{7,11}. Lytic bacteriophages of *Paeruginosa* belong to main family of phages; *Myoviridae* and *Podoviridae* and they are dsDNA by 20-200 nm length^{12,13}. Here, we describe the isolation of phages for *Paeruginosa* and their application to planktonic cultures and biofilms. The main goal is to determine phage potential to control these two types of cells. In this study, *Paeruginosa* phage were utilized to biofilm inhibition and remove.

METHODS

Samples were collected from University sewage. Isolation was done according to Martha R.J.Clokic protocol¹⁴. 40cc sewage centrifuged by 4000rpm in 4c for 15min. Supernatant filtered by 0.45µm filter and 2-3 drops chloroform added. Treated sample added equally into LB broth with 1-2cc Mg₂SO₄ and 5 cc of bacteria. After one night, centrifuge and filtering repeated and finally plaque assay was done by overlay culture. *Paeruginosa* ATCC PAO recruited as standard controls and 2 clinical isolates used to isolation (Fig. 2).

Biofilm Inhibition

The serial dilution prepared from phage in series 10⁻¹-10⁻³, then incubated at 37°C for 24h with bacteria based on biofilm formation method in ELISA plate. Triple test done to evaluate unexpected default and decrease unwanted errors. After overnight incubation, liquid content with planktonic cells was removed from the wells and each well was washed twice with 250 µl of Normal

Saline and left to dry. Attached bacterial cells were fixed with 250 µl of absolute methanol for 15 min. The fixative was removed and the plates were air-dried. Into each well, 200 µl of 0.4% crystal violet was added and after 15 min stain was removed. The plates were washed by stream of tap water in order to remove excessive amount of the stain and left to dry. Into each well of dried plates, 250 µl of 33% acetic acid was added and left for 20 min to allow stain to dissolve. The absorbance was measured at 492 nm using the microtiter plate reader (Table 1)¹⁵.

Biofilm removal

The effect of isolated phage investigated on biofilm of *P. putida*(ATCC;49128), *E.coli* and *Acinetobacter baumannii*. Biofilm formed by microtitre plate¹⁵, Triple test done to evaluate unexpected default and decrease unwanted errors. After one night incubation, liquid content was removed and each well was washed twice with 250 µl of Normal Saline and left to dry. Then 190 µl LB broth and 10 µl of pure, 10⁻¹, 10⁻² and 10⁻³ phages added to each well. Next day washing done like above and then attached bacterial cells were fixed with 250 µl of absolute methanol for 15 min. The fixative was removed and the plates were air-dried. Into each well, 200 µl of 0.4% crystal violet was added and after 15 min stain was removed. The plates were washed by stream of tap water in order to remove excessive amount of the stain and left to dry. Into each well of dried plates, 250 µl of 33% acetic acid was added and left for 20 min to allow stain to dissolve. The absorbance was measured at 492 nm using the microtiter plate reader (Table 2).

RESULTS

Our funding indicates phages shown strain specification manner because there is phage against one strains among four strains (Fig. 1,2).

Biofilm inhibition

The recruited *Paeruginosa* strains had strong biofilm by OD: 1.688 in 492nm. Pure phage reduced OD to 1.587 but, unexpectedly there is an increase by 10⁻¹ phage. The 10⁻² and 10⁻³ phage decreased OD to 1.341 and 1.461, respectively. The most prevention belongs to 10⁻² phage.

Biofilm removal

P.putida formed strong biofilm by OD:0.221 in 492nm but dramatically declines after

of phage recruitment. Manipulated phages in serial dilution 10^{-1} , 10^{-2} , 10^{-3} decrease OD to 0.078, 0.067, 0.062, 0.065, respectively. Biofilm removal graph

for *E.coli* and *Acinetobacter baumannii* shown similar results (Table 1,2).

Table 1. Biofilm inhibition method

Blank	Biofilm assay	Pure phage	1/10 phage	1/100 phage	1/1000 phage
200µl LB broth	190 µl LB borth	180 µl LB borth	190 µl LB borth	190 µl LB borth	190 µl LB borth
None	10µl Bacteria	10µl Bacteria	10µl Bacteria	10µl Bacteria	10µl Bacteria
None	None	10µl pure phage	10µl 1/10 phage	10µl 1/100 phage	10µl 1/1000 phage

Table 2. Biofilm remove method

Blank	<i>P.putida</i>	<i>E.coli</i>	<i>A. baumannii</i>
200µl LB broth	190 µl LB borth	190 µl LB borth	190 µl LB borth
None	10µl Bacteria	10µl Bacteria	10µl Bacteria
24h-37	24h-37	24h-37	24h-37
Washing	Washing	Washing	Washing
190 µl LB borth	190 µl LB borth	190 µl LB borth	190 µl LB borth
10 µl phage	10 µl phage	10 µl phage	10 µl phage
24h-37	24h-37	24h-37	24h-37
Washing,	Washing,	Washing,	Washing,
Fixation	Fixation	Fixation	Fixation
and Staining	and Staining	and Staining	and Staining

Table 3. OD of Biofilm inhibition in 492 n.m

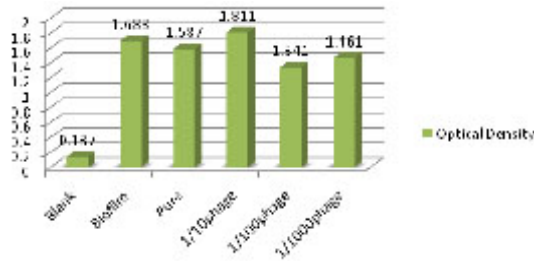
P.aer Biofilm	P.aer Biofilm	Blank	Blank	Pure phage	Pure phage	10^{-1} phage	10^{-1} phage	10^{-2} phage	10^{-2} phage	10^{-3} phage	10^{-3} phage
1.610	1.767	0.143	0.132	1.487	1.688	1.830	1.793	1.222	1.222	1.557	1.365
0.895	1.128	0.148	0.131	1.721	1.393	1.198	0.999	1.166	1.166	1.097	1.118
0.975	0.867	0.167	0.190	1.543	1.615	0.937	1.012	1.103	1.103	0.770	1.255



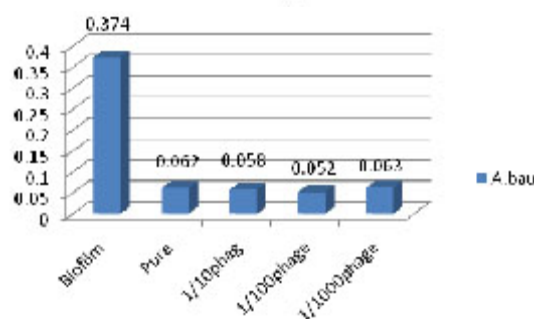
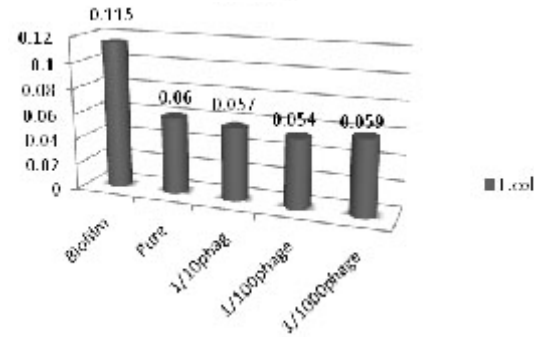
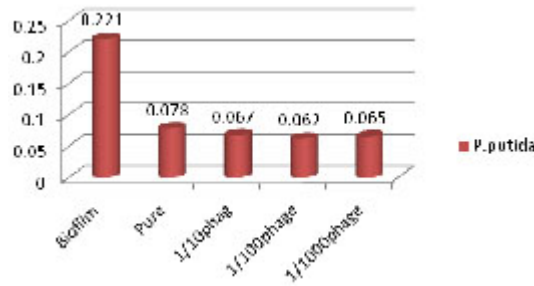
Fig. 1. (Phage plaques): phage isolated against clinical strain



Fig. 2. (phage against PAO ATCC): Left-down is phage plaques against an PAO strain but there is not any phages against others



Graph 1. Biofilm Inhibition of *P. aeruginosa*



Graph 2. Biofilm Removal (*P.putida*, *E.coli* and *Acinetobacter baumannii* Biofilm)

DISCUSSION

Phage obviously eliminates bacteria in planktonic and biofilm form therefore they would be useful to bacterial growth inhibition and biofilm remove. These funding illustrated *P.aeruginosa*

phages have effect on other bacteria such as *E.coli*, *P.putida* and *Acinetobacter baumannii*. This phenomenon could be explained by proteolytic enzymes of phages which have affect on Exopolysaccharide (EPS) of Biofilm. Strikingly, our study shown phages have best efficiency in 10^{-2} concentration in comparison by to pure phage, 10^{-1} and 10^{-3} concentrations. *Pseudomonas* phage exploits LPS and pilus as receptor on the bacteria surface so LPS diversity and a pilus count might be a reason of this event. Indeed, phages have various affinity to attach to host cells, thereby it is supposed to phages compete for their receptors^{16,17} Consequently, phages have most efficiency in optimum concentration to remove biofilm or growth inhibition. Recent studies in Eastern Europe indicates cocktails of various of phages could be more efficient, although researchers in West believe that recruiting of one phage is better^{18,19}. Based on our experiments and registered patents in United States Patents such as chewing gum which contains proteolytic enzymes of phage, it is supposed to recruit cocktail of proteolytic enzymes of these phages could be more efficient to treat biofilm²⁰⁻²³.

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