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Anti-biofilm potential of purified environmental bacteriophage preparations against early stage *Pseudomonas aeruginosa* biofilms

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ABSTRACT

Aims This paper presents the potential of environmentally-sourced bacteriophages to affect the growth of clinical isolates of *Pseudomonas aeruginosa* biofilms, and assesses the respective plaque morphotypes presented by each bacteriophage, *in vitro*.

Methods and Results Bacterial host strains were typed for their ability to produce the quorum sensing-controlled virulence factor pyocyanin, and then tested for bacteriophage susceptibility using the spot test method. The bacteriophages were co-administered with ciprofloxacin in order to determine whether the bacteriophages would demonstrate synergistic or antagonistic behaviour to the antibiotic *in vitro*. Results suggest a potential relationship between the bacteriophage plaque size and biofilm inhibition, where those producing smaller plaques appear to be more effective at reducing bacterial biofilm formation.

Conclusions This phenomenon may be explained by a high adsorption rate leading to the rapid formation of smaller plaques, and greater biofilm reduction associated with the loss of viable bacterial cells before the cells can adhere to the surface and form a biofilm. Results from the coadministration of bacteriophage & ciprofloxacin suggest that the two work synergistically to affect *P. aeruginosa* biofilms.

Significance and Impact of Study The data indicate enhanced efficacy of ciprofloxacin by ≥50%. This could offer an alternative strategy for targeting antibiotic-resistant infections.

Key words: bacteriophage, biofilms, environmental, environmental/recreational water, antibiotics

1 INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium, commonly isolated from soil and environmental waters. However, it is most often noted for its multi-drug resistance (MDR) to a high number of antibiotics commonly prescribed to treat infectious disease in humans (Lambert *et al.*, 2002; Hirsch & Tam, 2010). The bacterium is frequently isolated from incidents of wound and burn infection, as well as cystic fibrosis, and is noted as a leading cause of morbidity and mortality by healthcare providers (Kosorok *et al.*, 2001). The persistence of *P. aeruginosa* in up to 80% of all

infections is attributed to its ability to form complex biofilms. In one study, Milivojevic *et al.* (2018) showed that the infection ability of forty *P. aeruginosa* can be predicted based on different virulence factors, where a positive correlation between cytotoxicity (an indicator of infection) and biofilm formation was found. Bacteria in a biofilm display many different features from their planktonic counterparts. These structured and aggregated bacterial communities are known to be notorious for their resistance to a variety of antibiotics and the action of host defences (Singh *et al.,* 2017; Skariyachan *et al.*, 2018). Thus, a biofilm provides *P. aeruginosa* a protective mode of growth, where individual bacteria can reproduce whilst circumventing external stresses.

Ciprofloxacin is a fourth-generation quinolone antibiotic, routinely prescribed to treat *P. aeruginosa* infection. The rate of ciprofloxacin resistance by *Pseudomonas sp.* associated with bacteraemia was reported to be static at 11% between 2007 to 2011 in the UK (Anon., 2012). In 2017, the English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR) reported that the rate of *Pseudomonas sp.* resistant to ciprofloxacin varied across to the UK between 9.4 to 10.9% of isolates (Anon., 2017). Evidence suggests that a delay in prescribing antibiotics to treat *P. aeruginosa* infections is a significant factor leading to increased morbidity of patients, further leading to increased mortality (Kang, *et al.,* 2003; Nathwani *et al.*, 2014). Research by Zelenitsky *et al.* (2005) recommended the use of 400 mg i.v. q8h to improve pharmacodynamic target attainment and to effectively treat *P. aeruginosa* infections. Such a treatment regime might prove to be effective to prevent long-term morbidity. However, it has been reported that the rate of consumption of antibiotics, including ciprofloxacin, is linked to the development of resistance *in vitro* (Miliani *et al.*, 2011). Indeed, Zelenitsky *et al.* (2005) also suggested that breakpoints should be lowered from 1 to 0.5 mg/mL, and then prescription of a maximal dosage might be the most appropriate course of antibiotic treatment for such infections. This approach, although appropriate for individual cases of treatment, might lead to complications and the spread of antibiotic resistance between bacterial species. For these reasons, alternatives to antibiotic prescription must be evaluated alongside potential alternative therapies.

There are many published reports of alternative therapies being trialled as antibacterial and antibiofilm agents, such as microbial exoproducts, plant products, and bacteriophage preparations. For example, Papa *et al.* (2015) reported the antibiofilm but non-biocidal effects of supernatants from Arctic and Antarctic bacteria against *Staphylococcus aureus, Staphylococcus epidermidis* and *P. aeruginosa.* Artini *et al*. (2018) reported that essential oil extracts from Mediterranean plants were able to destabilise *P. aeruginosa* biofilms without affecting bacterial viability.

Bacteriophages are obligate intracellular parasites of bacteria. They are specific to only one species, sometimes even to one strain of a single bacterial species (Hanlon 2007). For these reasons, bacteriophages have been promoted as an alternative therapeutic agent for bacterial infections. For *P. aeruginosa*, research has demonstrated that administration of a single bacteriophage to bacterial biofilm cultures achieved a 3-$\log_{10}$ reduction in cell number (Pires *et al.*, 2011). Other than their bactericidal properties, bacteriophages have several attractive properties such as low production costs, low inherent toxicity and minimal disruption of normal flora (Gorski *et al.,* 2018; Gosh *et al.,* 2019). Although their application in Eastern Europe is well documented (Merabishvili *et al.,* 2009), the western world remains sceptical about the efficacy and safety of phage therapy.

This paper reports the efficacy of environmentally-sourced bacteriophages against clinical strains of *P. aeruginosa.* Bacteriophages from three different environmental surface waters, were used in this study, and characterised according to the morphological characteristics of the plaques developed through plating. The potential efficacy of the bacteriophages against bacterial biofilms was evaluated in a microtitre plate in single application, or in combination with ciprofloxacin, in order to determine whether co-administration of the bacteriophage and antibiotic enhanced the disruption of *P. aeruginosa* biofilms, *in vitro*.

2 MATERIALS AND METHODS

The following materials and reagents were used: L.B. Agar (Liofilchemsrl ReF 610245), L.B. Medium (Liofilchemsrl ReF 610084) were used for bacterial growth, glucose (Sigma-Aldrich), casamino acids (Sigma-Aldrich), ethanol (Sigma-Aldrich), crystal violet (BioMerieux®SA), acrodisc syringe filters with supor membrane, 32 mm, 0.45 µm (Pall corporation), ciprofloxacin (Laboratorio Farmaceutico C.T). Plastic and disposable lab consumables were purchased from (VWR), and the centrifuge used in this work was manufactured by IEC MultiRF (ThermoIEC).

2.1 Characterisation of bacteriophages according to their lytic activity against host bacteria

Eight clinical strains of *P. aeruginosa,* provided by the culture collection at The Eliava Institute, Republic of Georgia, were used in this study (Table 1). These isolates were screened against a total of 40 bacteriophages isolated from the Mtkvari (Kura) River, Lisi Lake and Turtle Lake in Tbilisi (Republic of Georgia), using the spot test technique (Merabishvili *et al.,* 2009). Briefly, overnight cultures of *P. aeruginosa* were diluted to a suspension containing 10^8 CFU ml^{-1}. An aliquot of 200µL was added to 4mL molten 0.7% (w/v) LB agar, and deposited on the surface of 1.5% (w/v) LB agar plates. Plates were air-dried, spotted with 5µL (10^7 PFU ml^{-1}) of each of the bacteriophage suspensions, and incubated at 37°C for 18h. Lytic zones were evaluated according to the following
criteria.

(i) Confluent lysis (Cl), a zone of clearing.

(ii) Opaque lysis (Ol), a film of fine growth on area of dropped phage.

(iii) Semi-confluent lysis (Scl), high amount of growth on area of dropped phage.

(iv) Several plaques (Sp), multiple zones of clearing.

(v) Resistant (R), no zone of clearing.

2.2 Assessment of plaque morphotype

Using the overlay agar method (Merabishvili *et al.,* 2009), suspensions of bacteriophages that demonstrated Cl or Ol in the screening assay were used to determine the titre, and assess the morphology and clarity of the plaques produced. Briefly, serial dilutions of each bacteriophage were prepared in LB broth. An aliquot of 100µl of each dilution was mixed with 4ml molten 0.7% (w/v) LB agar, containing a suspension of *P. aeruginosa* PS 573 at 10^8 CFU ml^{-1}. This mixture was deposited on the surface of 1.5% (w/v) LB agar plates, and plates were incubated at 37°C for 24h. Plates with 1 to 200 distinguishable homogenous plaques were enumerated, and the number of plaque forming units (PFU ml^{-1}) was determined using equation (a).

(a) *PFU* ml^{-1} *= number of plaques* ***x*** *(1* ***/*** *volume of phage plated [ml])* ***x*** *dilution factor*

To ensure the purity of the selected bacteriophage suspensions (*i.e.* that they did not contain a mixture of bacteriophage clones), all plaques with different morphology were removed with a sterile pipette tip, and placed in 1ml LB broth. These were incubated at 37°C for 1h to allow lysis of adherent bacterial cells. The tubes were then placed at 4°C for 1h, before the lysate suspension was filtered using a 0.45µm membrane filter. Serial dilutions of each suspension were prepared in LB broth, and each dilution was titrated using the overlay agar method. Plates showing 5-20 plaques were analysed in detail. This complete cycle was repeated until homogenous plaques were obtained.

Pure suspensions, containing one clear plaque morphotype, were obtained after several cycles at a PFU ml^{-1} of 10^7. Following several cycles of purification, pure stock suspensions of selected PTbacteriophages were prepared using the double-agar overlay method previously described. Briefly, 1ml LB was added to the surface of plates with homogenous plaques, and incubated at 4°C for 1h. The top (soft) agar layer was then removed using a sterile L-shaped rod, and transferred to a 50ml centrifuge tube. The mixture was centrifuged at 6000*g* for 20 min, at 4°C. The supernatant was

aspirated using a sterile 10ml syringe with a 30G needle, and filtered through a 0.45µm membrane filter. The titre of the stock suspension was determined as described using the agar overlay method. Stock suspensions of purified PT-bacteriophages were obtained at 10^9-10^{10} PFU mL^{-1}.

2.3 Pyocyanin assessment

Overnight cultures of *P. aeruginosa* strains (detailed in Table 1) were grown in nutrient broth at 37°C with agitation of 120rpm for 24h. The supernatants were recovered by centrifugation at 4500 *g* for 20 min at 4°C, and filtered using a 0.2µm syringe filter. Pyocyanin production was visually assessed and scored: 0, no pigmentation, 1+ weak pigmentation, 2+ moderate pigmentation, and 3+ strong pigmentation (*El-Shouny et al.*, 2011).

2.4 Biofilm studies

The anti-biofilm potential of purified bacteriophages, ΦPT-18[b], ΦPT-20[a], ΦPT-1S[a], ΦPT-5[a] and ΦPT-2[b] (Glonti, 2004; Glonti *et al.,* 2010; Ceyssens *et al.,* 2011), was assessed on *P. aeruginosa* PS 573 alone, and in combination with ciprofloxacin, using the crystal violet biofilm assay (O'Toole, 2011). This static batch-growth biofilm assay was therefore used for examining the formation of biofilms in the early stages of biofilm maturation, termed microcolony development (≤24h). This method is preferable for this work, as opposed to continuous-flow methods, as it can be used to monitor microbial attachment to an abiotic surface and detect the transition from planktonic to biofilm mode of growth (O'Toole & Kolter, 1998; Caiazza & O'Toole, 2004). In brief, the assay involved the growth of cells in a 96-well polystyrene microtiter plate or individual polystyrene plates at pre-determined nutrient conditions. After incubation, any non-adherent cells were removed by washing and adherent cells were stained with a dye to allow visualisation of the biofilm. The dye was then solubilised to provide semi-quantitative data. Biofilm media was prepared using 1x LB broth, supplemented with 0.4% (w/v) glucose and 0.5% (w/v) casamino acids. These nutrient conditions were

designed through in-house optimisation to provide maximal biofilm growth and result in the formation of a robust biofilm, which can be used to test the effectiveness of potential biofilm inhibitors.

Fresh cultures of *P. aeruginosa* PS 573 were prepared in 5mL LB broth and incubated at 200rpm for 3h. Cultures were then diluted 1:10 in biofilm growth media to achieve a final concentration of 1 x 10 CFU ml^{-1}. Purified bacteriophages were prepared at low and high titres in biofilm media to achieve a final titre of 1 x 10^3 and 1 x 10^8 PFU ml^{-1}, respectively. For bacteriophage and antibiotic combination studies, purified bacteriophages were prepared in biofilm media at low titres (10^3 PFU ml^{-1}) and supplemented with ciprofloxacin at the minimal inhibitory concentration of 0.5µg ml^{-1}. All components were added to a sterile tube to a total volume of 5mL, mixed for 30s, and poured into 50mm polystyrene UV-sterilised plates. These plates were sealed with parafilm and incubated at 37°C for 22 ± 2h, without agitation. Growth control wells containing untreated *P. aeruginosa* PS 573 cultures and sterile biofilm media were also included.

After incubation, the contents of each plate were decanted, and plates were washed with distilled water to remove any non-adherent cells. Plates were air-dried for 5-10 minutes, and adherent cells were stained with 6mL 10% (v/v) crystal violet for 15 minutes. The dye was removed, and each plate was washed 6 times with distilled water to remove excess stain. To quantify the biofilm, stain was solubilised in 6mL 95% (v/v) ethanol for 25 minutes. Two-mL aliquots were transferred into sterile cuvettes, and absorbance was measured at 550nm, using 95% (v/v) ethanol as the blank. Preliminary studies confirmed that PS 537 produces a biofilm biomass similar to that of PAO1, a biofilm-forming lab strain (data not shown). Absorbance values lower than the untreated control were indicative of less crystal violet staining and therefore low biofilm growth.

2.5 Data analysis

All experiments were performed at least three independent times unless stated otherwise (n). Data are expressed as the mean of independent experiments ± the standard deviation (SD). Data obtained from each experiment were processed using Microsoft Office Excel 2011 (USA) and/ or GraphPad Prism 6 (GraphPad Software Inc., USA). Statistical analysis was performed using the statistical software package of GraphPad Prism 6. Significant differences between mean were assessed using one-way analysis of variance (ANOVA) and Tukey's Post-Hoc test, with a 95% confidence interval. Two-sided Student's t-test was used where appropriate. P-values of < 0.05 were considered statistically significant.

3 RESULTS

3.1 Lytic activity of environmental bacteriophages against host *P. aeruginosa*

The screening of the bacteriophages against clinical strains of *P. aeruginosa* identified varying degrees of lytic activity, which was assessed by the relative clarity of the plaques (Table 2, and Figure 1). The bacteriophages showed 10-98% lytic activity against the clinical isolates, however, it must be noted that this was only observed against pyocyanin-deficient strains.

Results indicate that bacteriophage PT-1(B/M/S/X), belonging to the *Myoviridae* family of bacteriophages, was capable of forming clear plaques on all pyocyanin-deficient *P. aeruginosa* strains (63% of all isolates); specifically, PS 112, PS 114, PS 317, M 317 and PS 573, and displayed a broader host range against the *P. aeruginosa* strains than the remaining bacteriophages. None of the bacteriophages were able to produce clear plaques or demonstrate any signs of lytic activity against pyocyanin-proficient strains (PS 118, PS 119 and PS 1123). Pyocyanin production is a terminal signal of quorum sensing, and a potent virulence factor of *P. aeruginosa* (Dietrich *et al.,* 2006; Milivojevic *et*

al., 2018), and the results may therefore suggest that a reduction in pathogenicity enhances the lytic activity of the aforementioned bacteriophages. This observation is, however, limited to three bacterial strains, and the hypothesis needs to be further assessed using several wild-type and quorum sensing-deficient strains and/or by combining these bacteriophages with a compound that reduces pathogenicity such as a quorum sensing inhibitor. The bacteriophages produced the clearest plaques on PS 573 (98% lytic activity, Figure 2), and this strain was therefore selected as host for subsequent purification and propagation of the bacteriophages.

3.2 Assessment of plaque morphotype

The bacteriophages were selected based upon their plaque morphotype, which was determined by using *P. aeruginosa* PS 573 as the host strain. Results are shown in Figure 4 and Table 3, and the data are defined as follows.

(i) ΦPT-18[b]: very small, clear plaques with a diameter of ≤ 0.5mm.

(ii) ΦPT-20[a]: very small, clear plaques with a diameter of ≤ 0.5mm.

(iii) ΦPT-1S[a]: small, clear plaques with a diameter of 1-2mm.

(iv) ΦPT-5[a]: small, clear plaques with a diameter of 2mm.

(v) ΦPT-2[b]: large, clear plaques with a diameter of ≥ 3mm.

3.3 Biofilm studies

The crystal violet biofilm assay stains both live and dead bacterial cells, as well as extracellular matrix polymers. Results indicate that *P. aeruginosa* PS 573 is a strong biofilm-producing strain. The biofilm formed by this strain was moderately resistant to disruption during the mechanical washing steps. The addition of bacteriophages, ΦPT-18[b], ΦPT-20[a], ΦPT-1S[a], ΦPT-5[a] and ΦPT-2[b] in to the test media indicated that all of the bacteriophages tested were all able to reduce the rate at which planktonic cells of *P. aeruginosa* formed a biofilm (Figure 4[a-c] and 5[a-c]). A higher titre (10^8

PFU/mL) was more effective at preventing biofilm formation than a lower titre of 10^3 PFU/mL (Figure 3[a-c] and 4[a-c]), respectively. Of the five bacteriophages tested, ΦPT-18[b] and ΦPT-20[a] (which form plaques with a diameter of ≤ 0.5mm) appeared to be most effective and reduced biofilm biomass by approximately 87.6 and 84.6% when applied at 10^8 PFU/mL, respectively (Figure 5[a-c]).

3.4 Co-administration of ciprofloxacin and PT bacteriophages to biofilms

Exposure to bacteriophages ΦPT-18[b], ΦPT-20[a], ΦPT-1S[a], ΦPT-5[a] and ΦPT-2[b], rendered the biofilm-associated bacterial cells more susceptible to ciprofloxacin (Figure 6[a-c]). Administration of ciprofloxacin alone was able to reduce biofilm biomass production by approximately 68.5%, when compared to the untreated *P. aeruginosa* PS 573 control. However, the combination of phages with ciprofloxacin appeared to provide a synergistic effect against the establishment of a biofilm, increasing the inhibitory effects to 61.8%, when compared to the growth control ($P < 0.05$, ANOVA). Additionally, phage-antibiotic-treated biofilms retained a limited amount of stain, which is likely to be due to the presence of a low number of cells. Therefore, these biofilms resembled negative control plates, suggesting that the bacteriophages appeared to enhance the efficacy of ciprofloxacin,
in vitro.

4 DISCUSSION

From the total number of forty bacteriophages derived from environmental surface waters in this study, the majority were only effective against pyocyanin-deficient strains of *P. aeruginosa*. Five of these bacteriophages produced unique plaque morphologies of ≤ 0.5 to ≥ 3mm in diameter, which is are notably smaller than the plaques produced by the other isolated bacteriophages. Analysis of the data suggests a potential relationship exists between the plaque size that each bacteriophage produced using the agar overlay method and biofilm inhibition, *in vitro*. Different bacteriophages for

the same host bacterial species have been reported to produce different plaque sizes on agar plates, *in vitro* (Bremner *et al*., 2016). The literature further indicates that the adsorption rate contributes to the phenotype of plaques, where high-adsorption phages tend to produce smaller plaques (Gallet *et al.,* 2011). It must be noted that high rates of adsorption may not necessarily lead to all bacteriophages completing their infection cycle. Mitarai *et al*. (2016) reported that plaque morphology can be affected by the nutritional state of the host bacterial cell, and the number of bacteriophages that can infect them. This is important, as the nutritional and metabolic state of bacterial cells within biofilms will be different to those in planktonic culture, as well as differences existing between early-stage and mature biofilm-dwelling cells. A high adsorption rate may explain the greater reduction in biofilm formation observed in this study. If bacteriophages ΦPT-18[b] and ΦPT-20[a] are indeed able to adhere to cells more efficiently, then this may cause lysis before *P. aeruginosa* is able to attach to the abiotic surface. Extracellular polymers that constitute the biofilm are likely to exert a protective effect for bacterial cells (Ghafoor *et al.,* 2011; Colvin *et al.,* 2012; Irie *et al.,* 2012; Zhao *et al.,* 2013). Therefore, the potential for bacteriophages to reduce bacterial cell counts would be assumed to be maximal against planktonic cells, followed by early-stage, and then mature biofilms. The anti-biofilm potential of purified PT-bacteriophage preparations suggested that when added in combination with ciprofloxacin, ΦPT-18[b], ΦPT-20[a], ΦPT-1S[a], ΦPT-5[a] and ΦPT2[b] were found to act synergistically and to enhance the reduction of bacterial load by ≥50%. The findings presented in this paper suggest the potential synergy of bacteriophage-antibiotic preparations to effectively reduce biofilm formation by *P. aeruginosa.* This could offer an alternative strategy for targeting antibiotic-resistant infections.

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CONFLICT OF INTEREST

The authors state no conflict of interest is declared in the submission of this manuscript for publication.

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Table 1. Strains of *P. aeruginosa* used in this study.

Strain number	**Description ***
PS 112	*rhl* and/or *PQS*-deficient; PYO negative (0+)

PS 114	*rhl* and/or *PQS*-deficient; PYO negative (0+)
PS 118	*rhl* and/or *PQS*-proficient; PYO positive (2+)
PS 119	*rhl* and/or *PQS*-proficient; PYO positive (3+)
PS 317	*rhl* and/or *PQS*-deficient; PYO negative (0+)
M 317	*rhl* and/or *PQS*-deficient; PYO negative (0+)
PS 573	*rhl* and/or *PQS*-deficient; PYO negative (0+)
PS 1123	*rhl* and/or *PQS*-proficient; PYO positive (3+)

* Bacterial strains were grouped based on their ability to produce the quorum sensing-controlled virulence factor pyocyanin (PYO): (i) *rhl* and/or *PQS*-deficient strains that produced no detectable pyocyanin levels, and (ii) *rhl* and/or *PQS*-proficient strains that produced detectable pyocyanin levels. Pyocyanin levels were qualitatively determined; 0+ no pigmentation, 1+ weak pigmentation, 2+ moderate pigmentation, and 3+ strong pigmentation.

Table 2. Susceptibility of *P. aeruginosa* strains to a series of PT-bacteriophages *.

	PS 112	**PS 114**	**PS 118**	**PS 119**	**PS 317**	**M 317**	**PS 573**	**PS 1123**
PT-1	Cl	Cl	R	R	Cl	Cl	Cl	R
PT-2	R	R	R	R	R	R	Ol	R
PT-3	R	R	R	R	R	R	Ol	R
PT-4	R	R	R	R	R	R	Ol	R
PT-5	R	R	R	R	R	R	Ol	R
PT-6	R	R	Sp	R	R	R	Ol	R
PT-7	Scl	R	R	R	Cl	Cl	Ol	R
PT-7x	Scl	R	R	R	Cl	Cl	Ol	R
PT-8	R	R	R	R	Sp	Sp	Sp	R
PT-10	R	R	Sp	R	R	R	Ol	R
PT-12	R	Scl	R	R	Sp	R	Scl	R
PT-14	R	R	R	R	Sp	R	Cl	R

PT-14x	R	R	R	R	Sp	R	Cl	R
PT-15	R	R	R	R	R	R	R	R

***Pseudomonas aeruginosa* test strains**

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PT-2.K8	R	R	R	R	R	R	Ol	R
PT-4.K2	R	R	R	R	R	R	Ol	R

PT-17	R	R	R	R	Sp	Sp	Ol	R
PT-18	R	R	R	R	R	R	Cl	R
PT-19	R	R	R	R	R	R	Cl	R
PT-20	R	R	R	R	R	R	Cl	R
PT-35	R	R	R	R	R	Sp	Scl	R
PT-40	R	R	R	R	R	Sp	Cl	R
PT-1B	Cl	Cl	R	R	Cl	Cl	Cl	R
PT-1M	Ol	Cl	R	R	Cl	Cl	Cl	R
PT-1S	Cl	Cl	R	R	Cl	Cl	Cl	R
PT-1X	Cl	Cl	R	R	Cl	Cl	Cl	R
PT-2.K2	R	R	R	R	R	R	Ol	R
PT-2.K4	R	R	Sp	R	R	Sp	Ol	R
PT-2.K5	R	R	R	R	R	R	Ol	R
PT-2.K7	R	R	R	R	R	R	Ol	R

PT-4.K3	R	R	R	R	R	R	Ol	R

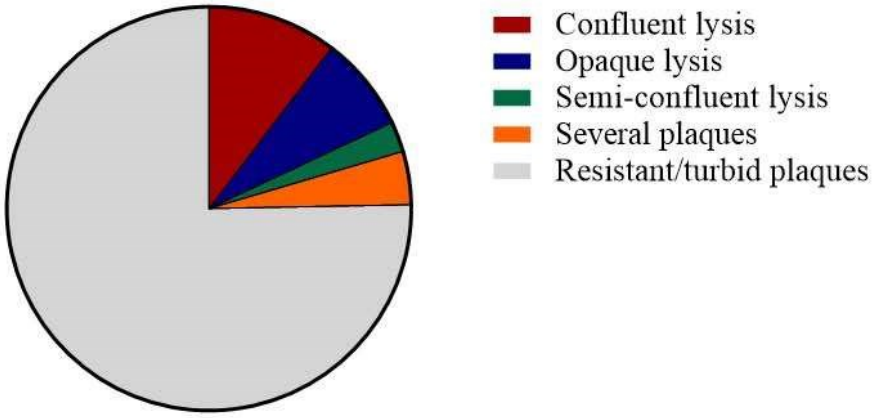
PT-4.K4	R	R	Scl	R	R	R	Ol	R
PT-4.K5	R	R	R	R	R	R	Scl	R
PT-4.K7	R	R	R	R	R	R	Ol	R
PT-4.K8	R	R	R	R	R	R	Ol	R
PT-5.K2	R	R	R	R	R	R	Ol	R
PT-5.K3	R	R	R	R	R	R	Ol	R
PT-5.K5	R	R	R	R	R	R	Ol	R
PT-5.K7	R	R	R	R	R	R	Ol	R
PT-5.K8	R	R	R	R	R	R	Ol	R
PT-7.K3	R	R	R	R	R	R	Scl	R

* The extent of lysis is indicated by letters, as follows: Cl, confluent lysis; Ol, opaque lysis; Scl, semi-confluent lysis; Sp, several plaques; R, resistant/turbid plaques.

Table 3. Purification cycles and stock suspension titres of PT-bacteriophages with *P. aeruginosa* PS 573.

	Purification cycle *					Pure stock suspension
Φ	**I**	**II**	**III**	**IV**	**V**	
PT-18[b]	Plaques: 0.5 - 1mm	Plaques: 0.5 - 1mm	Plaques: 0.5mm	--	--	**PFU/mL: 3.9 x 10^9**
	PFU/mL: 2.1 x 10^7	PFU/mL: 1.7 x 10^7	PFU/mL: 1.5 x 10^7			
PT-20[a]	Plaques: 0.5 - 2mm	Plaques: 0.5 - 1mm	Plaques: 0.5mm	--	--	**PFU/mL: 9 x 10^9**
	PFU/mL: 1.8 x 10^7	PFU/mL: 1.4 x 10^7	PFU/mL: 7 x 10^7			
PT-1S[a]	Plaques: 0.5 - 4mm	Plaques: 1 - 3mm	Plaques: 1 - 2mm	Plaques: 1 - 2mm	--	**PFU/mL: 9.5 x 10^{10}**
	PFU/mL: 1.9 x 10^7	PFU/mL: 6.8 x 10^7	PFU/mL: 8 x 10^7	PFU/mL: 8.3 x 10^7		
PT-5[a]	Plaques: 0.5 - 4mm	Plaques: 0.5 - 4mm	Plaques: 1 - 3mm	Plaques: 1-2mm	Plaques: 2 mm	**PFU/mL: 1.6 x 10^{10}**
	PFU/mL: 1.1 x 10^6	PFU/mL: 6.5 x 10^6	PFU/mL: 2.1 x 10^7	PFU/mL: 6.9 x 10^7	PFU/mL: 9.9 x 10^7	
PT-2[b]	Plaques: 3 - 4mm	Plaques: 3 - 4mm	Plaques: 3 - 4mm	Plaques: 3 - 4mm	--	**PFU/mL: 3.7 x 10^{10}**
	PFU/mL: 8.7 x 10^7	PFU/mL: 8.9 x 10^7	PFU/mL: 7.2 x 10^7	PFU/mL: 8.6 x 10^7		

* Plaques: x mm, indicates the size range of plaques observed on plates at each purification cycle.



Total=328

(A)

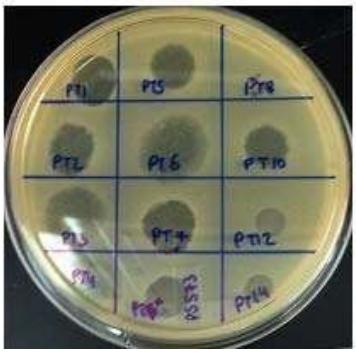
ΦPT-1	ΦPT-5	ΦPT-8
ΦPT-2	ΦPT-6	ΦPT-10
ΦPT-3	ΦPT-7	ΦPT-12
ΦPT-4	ΦPT-7x	ΦPT-14

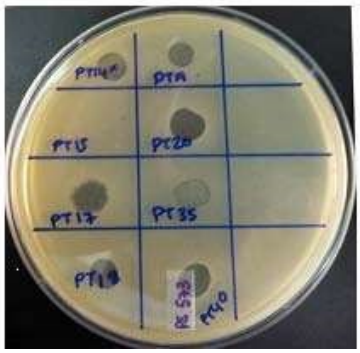
(B)

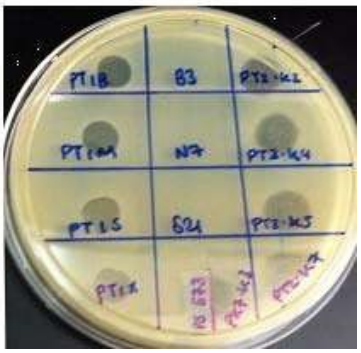
ΦPT-14x	ΦPT-19	–
ΦPT-15	ΦPT-20	–
ΦPT-17	ΦPT-35	–
ΦPT-18	ΦPT-40	–

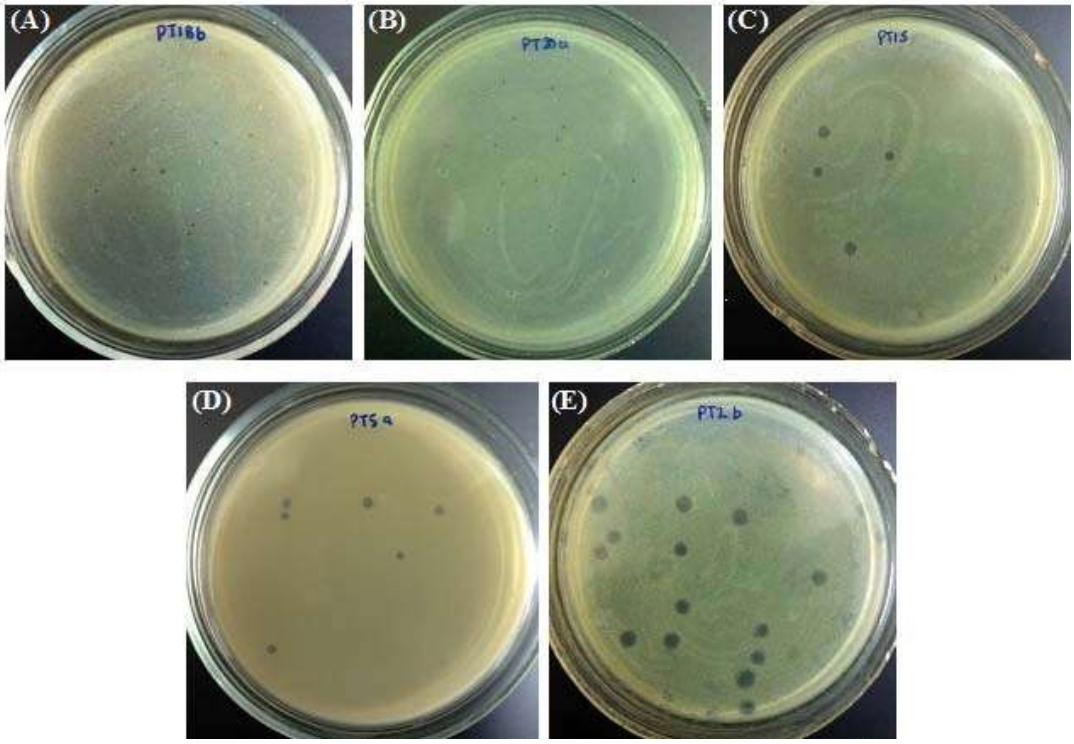
(C)

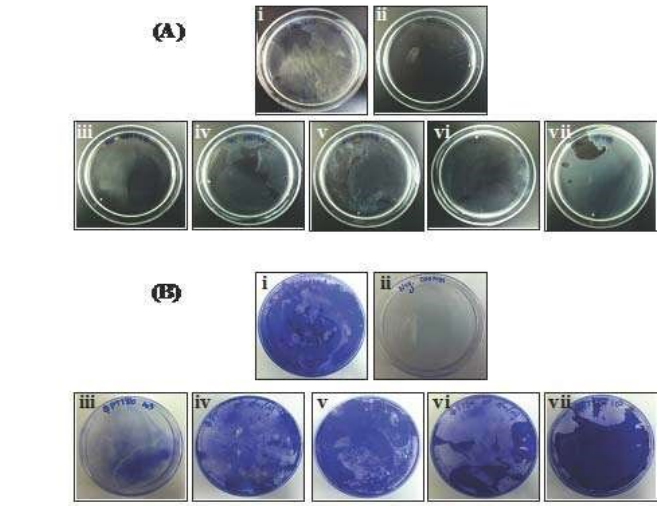
ΦPT-1B	ΦB3	ΦPT-2K2
ΦPT-1M	ΦN7	ΦPT-2K4
ΦPT-1S	ΦS21	ΦPT-2K5
ΦPT-1X	ΦPT-7K3	ΦPT-2K7

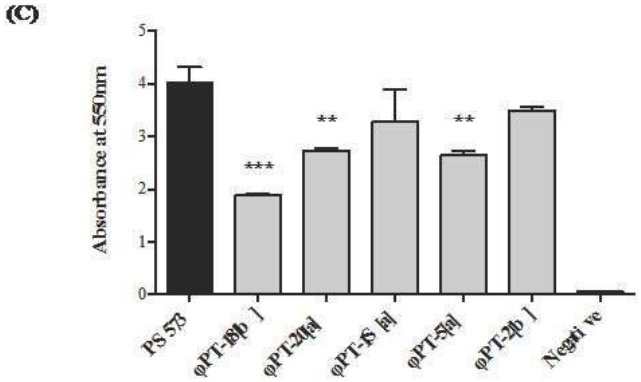


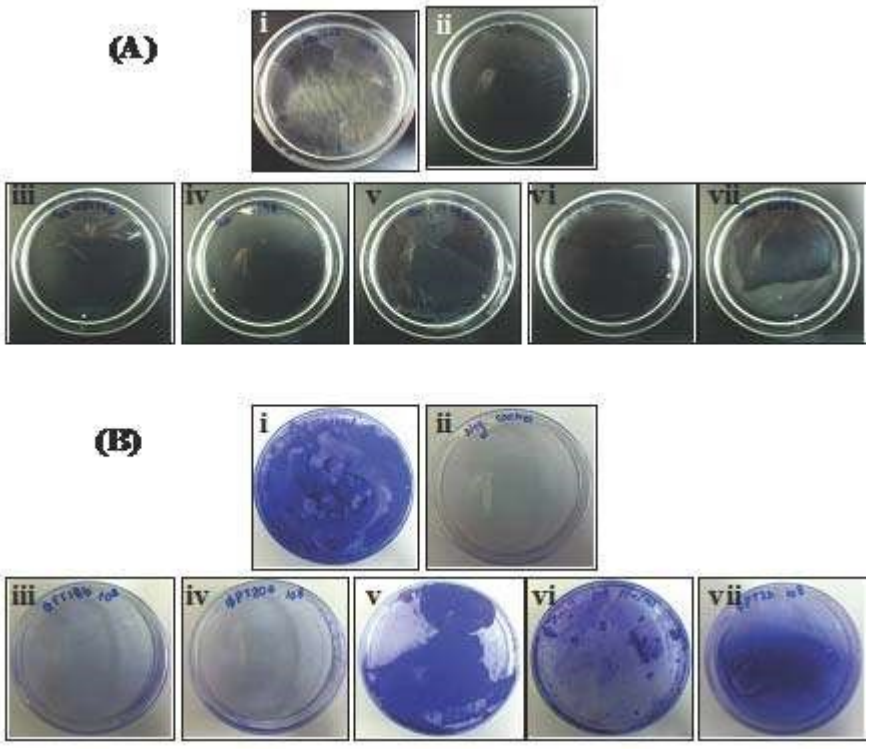


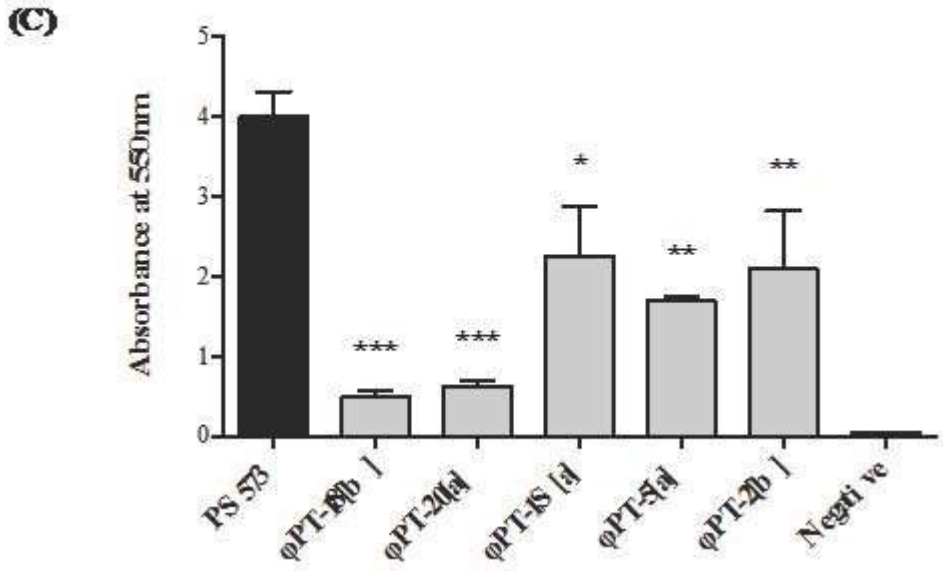


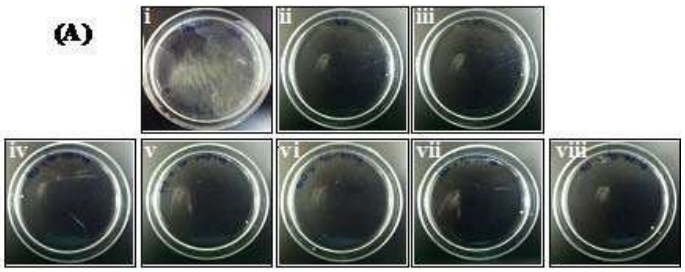


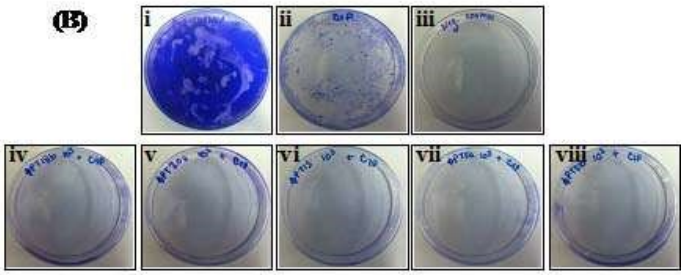












(C)