Characterization of Siphoviridae phage Z and studying its efficacy against multidrug-resistant Klebsiella pneumoniae planktonic cells and biofilm

Muhsin Jamal,1,2 Tahir Hussain,1 Chythanya Rajanna Das2 and Saadia Andleeb1

1Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan
2Emerging Pathogens Institute, University of Florida, FL, USA

Biofilm has many serious consequences for public health and is a major virulence factor contributing to the chronicity of infections. The aim of the current study was to isolate and characterize a bacteriophage that inhibits multidrug-resistant Klebsiella pneumoniae (M) in planktonic form as well as biofilm. This phage, designated bacteriophage Z, was isolated from wastewater. Its adsorption rate to its host bacterium was significantly enhanced by MgCl2 and CaCl2. It has a wide range of pH and heat stability. From its one-step growth, latent time and burst size were determined to be 24 min and about 320 virions per cell, respectively. As analysed by transmission electron microscopy, phage Z had an icosahedral head of width 76 ± 10 nm, length 92 ± 14 nm and icosahedron side 38 nm, and a non-contractile tail 200 ± 15 nm long and 14–29 nm wide. It belongs to the family Siphoviridae in the order Caudovirales. Six structural proteins ranging from 18 to 65 kDa in size were revealed by SDS-PAGE. The genome was found to comprise double-stranded DNA with an approximate size of 36 kb. Bacteria were grown in suspension and as biofilms to compare the susceptibility of both phenotypes to the phage lytic action. Phage Z was effective in reducing biofilm biomass after 24 and 48 h, showing more than twofold and threefold reduction, respectively. Biofilm cells and stationary-phase planktonic bacteria were killed at a lower rate than exponential-phase planktonic bacteria.

INTRODUCTION

Klebsiella pneumoniae accounts for a significant proportion of hospital-acquired infections. The most important reservoirs for transmission of Klebsiella spp. are the gastrointestinal tract and the hands of hospital personnel; hence they are crucially involved in causing outbreaks of nosocomial infections (Podschun & Ullmann, 1998).

Among the nosocomial pathogens, K. pneumoniae is one of the most important biofilm-forming bacteria, causing a large number of infections (Podschun & Ullmann, 1998). Biofilms have been involved in many nosocomial infections associated with medical devices, equipment used in hospitals and other hard surfaces, which can act as reservoirs for biofilm-acquired infections (Rao et al., 2005). It has been estimated that biofilm is involved in more than 60% of nosocomial infections (Donlan & Costerton, 2002; Vinh & Embel, 2005). The National Institutes of Health claim that about 80% of all chronic infections are caused by biofilms (Monroe, 2007), and in all about 65% of microbial infections are related to biofilms (Potera, 1999). These infections are very difficult to treat owing to the resistance of bacteria to numerous antimicrobial agents (Costerton et al., 1999). As the threat of emergence of antibiotic resistance and the inability to eradicate the biofilm structures have increased, novel strategies for preventing the biofilm mode of growth are urgently needed (Mah & O'Toole, 2001).

Being obligate parasites of bacteria, bacteriophages bind to microbial surfaces, injecting their genetic material into and replicating within the bacterial host, causing lysis of the host cell (Sulakvelidze et al., 2001). Phage therapy has gained increasing attention because it has many advantages over antibiotic therapy. Phage are effective against multidrug-resistant pathogenic bacteria because the mechanisms by which they induce bacteriolyis differ from those of antibiotics (Nakai & Park, 2002). Phage have also been reported to produce depolymerases that are able to degrade biofilm exopolysaccharide matrix, which acts as a barrier for antimicrobials, and can cause extensive disruption of biofilm (Hughes et al., 1998).
It has been postulated that approximately $10^{30}$ bacteriophages are present in the biosphere (Ashelford et al., 2000). Despite this rich reservoir of phages present in the environment, very few (about 300) have been characterized (Casjens, 2008). Hence, it is very important to isolate and characterize new phages, especially in light of the observation that most disease-causing organisms live in matrix-enclosed environments called biofilms (Watnick & Kolter, 2000), which inherently show increased resistance towards all antibiotics (Gilbert et al., 1997). The objective of this study was to isolate and characterize a new lytic phage from wastewater that infects K. pneumoniae (M), and also to investigate the phage lytic activity against the bacterial planktonic cells as well as biofilms under controlled conditions in the laboratory.

**METHODS**

**Bacterial identification.** A clinical strain of K. pneumoniae obtained from the Railway General Hospital, Pakistan, was identified by ribotyping. Sequencing of the 16S rRNA gene was performed. For molecular identification of K. pneumoniae, bacterial genomic DNA was isolated by using the ZR Fungal/Bacterial DNA kit as instructed by the manufacturer (Zymo Research). The 16S rRNA gene sequence was amplified by using the PCR universal 16S rRNA primers (RS1, 5'-AAAACTCAATGATTTGACGG-3', and RS3, 5'-ACGAGGCGGTG-TGTGAC-3') as described by Ul Haq et al. (2012). The PCR-amplified product was electrophoresed in 1% agarose gel. The PCR product was eluted from the gel using a gel extraction kit (Invitrogen). Purified PCR-amplified product was sequenced at the Cancer Genetics Department, University of Florida, USA. The 16S rRNA sequence was identified by alignment using BLAST (National Center for Biotechnology Information).

**Bacteriophage isolation and purification.** Bacteriophage Z was isolated from a wastewater sample collected from Rawalpindi, Pakistan. Phage Z was isolated using methods previously described by Jamalludeen et al. (2007) with some modifications. For determination of phage titre, plaque assay was performed by adding 100 µl diluted suspension and 100 µl K. pneumoniae strain (M) (OD$_{600}$ 1.0) to a tube containing 3 ml soft Luria–Bertani (LB) agar (50°C). The mixture was poured onto the surface of LB agar plates and allowed to solidify for 20 min. The plates were incubated overnight at 37°C and were examined for the presence of plaques; a single clear plaque was isolated for purification of the phage.

**Host range determination.** The host range of phage Z was assessed on a range of Gram-positive and Gram-negative clinically isolated bacteria that were obtained from the Microbiology Laboratory, Railway General Hospital, Pakistan (Table 1). The clinical bacterial strains used for host range determination included strains of Klebsiella spp., Escherichia coli, Pseudomonas spp. and Staphylococcus spp., and Achromobacter xylosoxidans, as shown in Table 1. To test the susceptibility of bacterial isolates, a spot test was used (Zimmer et al., 2002). After overnight incubation at 37°C, plates were checked for plaque formation against an uninfected negative control.

**Thermal stability of phage Z.** Thermal stability tests for the phage were conducted according to the methodology described by Capra et al. (2006), with some modifications. Phage suspensions (9.0 × 10^5 c.f.u. ml$^{-1}$) were poured into Eppendorf tubes and treated at 37°C (control), 45, 50, 55, 60, 65, 70 and 80°C for 1 h. After incubation at the respective temperatures, we used the soft agar overlay method to determine the rate of survival of each treated phage as described above.

**pH stability.** Experiments for testing pH stability were carried out as described by Capra et al. (2006), with some modifications. We established a pH gradient ranging from 1 to 11 (pH 1, 3, 5, 7, 9, 11). We added 1 ml phage suspension to 9 ml trypic soy broth (TSB) medium having a specific pH, and incubated it overnight at 37°C. Each sample was tested against the host bacterium by the soft agar overlay method after incubation.

**Effect of calcium and magnesium ions on the adsorption rate of the phage.** A 50 ml K. pneumoniae (M) culture was divided equally into two flasks. One flask was inoculated with 250 µl (2.8 × 10^5 p.f.u.) phage, and the other flask with 250 µl phage and 250 µl CaCl$_2$ or MgCl$_2$ (each at a concentration of 10 mM). Samples were taken from both flasks at time intervals of 0, 10, 20 and 30 min to measure the number of free phage in control and calcium- or magnesium-containing suspensions. The effect of the calcium or magnesium ions was evaluated by adsorption according to the percentage of free phage, N/N$_0$ × 100, where N$_0$ is p.f.u. ml$^{-1}$ at

<table>
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<th>Sample no.</th>
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<th>Activity*</th>
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<tbody>
<tr>
<td>1</td>
<td>Klebsiella strain (M)</td>
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<tr>
<td>2</td>
<td>K. pneumoniae 3206</td>
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*+, Lysis; –, no lysis.
was determined. Each sample was also diluted 10-fold and plated in triplicate on tryptic soy agar. The plates were then incubated overnight at 37 °C. This experiment was repeated three times. To determine the concentration of phage in suspension during bacterial growth, a sample was collected every hour and phage were quantified by the serial dilution method described earlier.

Susceptibility of biofilm to phage Z. For studies of the susceptibility of the clinical bacterial strain *K. pneumoniae* (M) to phage Z, biofilm was formed as described previously, with some modifications (Cerca et al., 2007). Briefly, 10 μl bacterial culture (2.5 × 10^8 cells) grown overnight was diluted 100 times in TSB, and 100 μl cell suspensions at 2.0 × 10^7 cells ml^{-1}, prepared in 0.9% NaCl solution, were added to 96-well microtitre plates containing TSB plus 1% glucose (TSBG). Biofilm was allowed to form during 24 and 48 h at 37 °C with rotation at 130 r.p.m. Each biofilm was washed twice in 0.9% NaCl to remove planktonic cells. Then the phage (titre 5.2 × 10^9 p.f.u. ml^{-1}) was diluted in 0.9% NaCl and added to half of the wells, while normal saline was added to the other wells as a negative control. The plates were incubated for 24 or 48 h at 37 °C with constant shaking at 130 r.p.m. The biofilms were washed twice in 0.9% NaCl, and the total biomass of the biofilm was determined by crystal violet staining as described previously, with some modifications. Briefly, biofilms were washed twice with 0.9% NaCl solution, dried in an inverted position, and stained with 1% crystal violet for 20 min. The plates were washed with distilled water and air-dried. An aliquot of 200 μl 0.9% NaCl solution was added to each well, and the OD_{570} was measured in an ELISA plate reader (BioTek). For each condition studied, three separate experiments were performed.

Susceptibility of biofilm-grown cells versus planktonic cultures to phage Z. Susceptibility of biofilm and planktonic cells was determined using the methods previously described by Cerca et al. (2007). *K. pneumoniae* (M) biofilm was allowed to form for 24 h in TSBG as described above. The biofilm was then scraped from the surface and resuspended in 0.9% NaCl. Resuspended biofilms were then vortexed for 20 s and sonicated for 5 s at 10 W, to disaggregate the bacteria in such a way as to minimize cell disruption. Planktonic bacteria were grown for 24 h in TSB, in order to obtain cells in the stationary growth phase. The suspension was centrifuged at 10 000 g for 5 min and resuspended in 0.9% NaCl by vortexing for 20 s and sonication for 5 s at 10 W. Then both suspensions were diluted in a nutrient-poor medium (10% TSB diluted with 0.9% NaCl) to OD_{600} ~0.4. Bacterial cell count was determined by the serial dilution method. Phage at an appropriate m.o.i. was added to each suspension and reduction in OD_{600} was monitored with a spectrophotometer (Biomate3; Thermoscientific) for 5 h, compared with a control containing no phage. This experiment was repeated three times.

Statistical analysis. Data are expressed as means ± SD. Statistical analysis was performed with Excel 2007, using Student’s *t*-test for biofilm experiments. Difference at *P* ≤ 0.05 was considered statistically significant.

RESULTS

Bacterial identification by ribotyping

The bacterial strain *K. pneumoniae* (M) was identified by ribotyping and sequence information was derived from its 16S rRNA gene. A 470 bp amplicon was amplified and subjected to DNA sequencing from both directions. The resulting sequence was deposited in GenBank (accession no. KJ438818) and aligned to search for the most similar
sequences. BLAST analysis showed a high (99%) nucleotide sequence identity to *K. pneumoniae*.

**Isolation of phage Z**

A phage against multidrug-resistant *K. pneumoniae* (M) was isolated from a wastewater sample. The antibiotic-resistance profile of *K. pneumoniae* (M) is shown in Table S1 (available in the online Supplementary Material). The phage produced clear plaques on the lawn of the host, indicating that it was a virulent phage. The phage has a plaque size ranging from 1.0 to 3.0 mm in diameter and well-defined boundaries. The isolated phage was designated phage Z (Fig. 1).

**Host range determination**

Thirty-four strains of bacteria were used to determine the host range of phage Z by using the spot test method. Phage Z infected *K. pneumoniae* (M), *K. pneumoniae* 3206, *K. pneumoniae* 3, *A. xylosoxidans* and *Pseudomonas aeruginosa* 2995, as shown in Table 1. The other bacterial strains used in this study were insensitive to the phage. These results suggested that the phage had a narrow host range among different bacterial strains.

**Thermal stability**

A thermal stability test was carried out to analyse the heat-resistance capability of the phage at pH 7.0. It retained almost 100% infection activity (8.4 x 10^3 c.f.u. ml^-1) after incubation at 37 °C. The results suggested that the phage was stable at temperatures ranging between 37 and 70 °C. At 80 °C, there were no plaques (Fig. 2).

**pH stability**

Optimal pH for the phage was determined by testing its stability at different pH values at 37 °C for 16 h. Phage Z showed maximum stability at pH 7, while also showing good stability at pH 5, 9 and 11; at pH 1.0 and 3.0 no actively infectious phage was detected. The results showed that the phage might be unstable at low pH. At pH 1.0 and 3.0, no plaques were observed, while the number of plaques was found to increase with increasing pH, reaching the highest number at pH 7.0. A small decrease was observed in the number of plaques when the phage was incubated at pH values above 7.0 (Fig. 3).

**The effect of Ca^{2+} and Mg^{2+} ions on the adsorption rate of phage Z**

The effect of Ca^{2+} and Mg^{2+} ions on the adsorption of phage was analysed by adding 10 mM calcium chloride or 10 mM magnesium chloride to a mixture of the phage with *K. pneumoniae* (M). The number of free phage left in the solution (i.e. those not bound to the bacteria) was detected at time intervals of 0, 10, 20 and 30 min using the plaque assay. Data analysis showed a significant difference between the control and phage Z treated with Ca^{2+} or Mg^{2+} ions. The results showed that Ca^{2+} and Mg^{2+} ions stabilize the process of adsorption. The number of free phage was decreased, as shown by the lower line, compared with the upper line representing the control, in Fig. 4.

**Fig. 1.** Soft agar overlay plates, showing spot test assay (a) and a higher dilution (10^-7) of phage titre showing clear plaques 1.0–3.0 mm in diameter (b).

**Fig. 2.** Stability of phage Z held at different temperatures for 60 min. All values represent means of three determinations ± SD.

**Fig. 3.** Stability of phage Z treated with different pH overnight at 37 °C. All values represent means of three determinations ± SD.
Latent time period and burst size

The single-step growth experiment was performed for determining the latent time period and burst size of the phage. A triphasic curve was obtained showing the latent phase, log or rise phase, and stationary or plateau phase. From the data, the latent time period was calculated to be 24 min. The burst size was 320 phage per cell. Determination of burst size was based on the ratio of the mean yield of phage that infected the bacterial cells to the mean number of phage particles liberated (Fig. 5).

Morphology of phage Z

As analysed by transmission electron microscopy, phage Z had an icosahedral head of width $76 \pm 10$ nm, length $92 \pm 14$ nm and icosahedron side 38 nm, and a non-contractile tail 200 $\pm 15$ nm long and 14–29 nm wide; it therefore falls into the family *Siphoviridae* of the order *Caudovirales*. All values were determined as means $\pm$ SD from three measurements (Fig. 6).

Phage Z structural proteins

Ultracentrifuge-purified phage particles were subjected to 12 % SDS-PAGE, and protein bands were obtained after Coomassie Blue G-250 staining and destaining. A total of six proteins composing phage Z were detected by SDS-PAGE. Their molecular mass ranged approximately from 18 to 65 kDa (Fig. 7).

Genome isolation of the bacteriophage

The phage genome was characterized by agarose gel electrophoresis. The genome was found to be approximately 36 kb (Fig. 8a), and upon EcoRI restriction it produced two bands of different sizes (Fig. 8b). Thus, the genome consisted of double-stranded DNA, phage nucleic acid being digested by EcoRI.

Susceptibility of planktonic cultures to phage Z

The highly susceptible *K. pneumoniae* (M) strain was treated with different m.o.i. values of phage Z. The lytic activity of phage Z against planktonic cultures of *K. pneumoniae* (M) in the exponential phase of growth is illustrated in Fig. 9. The bacterium showed high susceptibility to phage Z at all m.o.i. values. An abrupt reduction in c.f.u. was observed in the first hour with m.o.i. 1.0 and 5.0, from $6.0 \times 10^8$ to $7.0 \times 10^4$ and $1.0 \times 10^5$ c.f.u. ml$^{-1}$, respectively, while m.o.i. 0.1 and 0.5 gave a small, gradual reduction; but after 2 h all m.o.i. values showed approximately similar efficacy, i.e. $4.0 \times 10^5$ to $1.0 \times 10^5$ c.f.u. ml$^{-1}$.

Susceptibility of biofilms to phage Z

To determine the action of phage Z in *K. pneumoniae* (M) biofilms, the biofilm-forming susceptible strain was grown in TSBG in microtitre plates (Costar) for 24 and 48 h, after which biofilms were challenged with phage Z. The reduction in biomass of the biofilms compared with the controls was evident (Fig. 10). Phage Z showed interesting results on both 24 and 48 h biofilms. Biofilm formed in 24 h showed 2.5-fold reduction while 48 h biofilm also showed about threefold reduction, but total removal was not observed in either case as determined statistically by paired samples t-test ($P<0.05$) when compared with the control.

Susceptibility of biofilm-grown cells versus planktonic cultures to the phage

To compare planktonic and biofilm-grown cell susceptibilities to phage Z, the lytic assay was performed using strain *K. pneumoniae* (M) planktonic cells at the stationary growth phase (after 24 h of growth) and 24 h biofilm-grown cells, in...
a low-nutrient medium to slow down the growth rate of cells and to preserve the biofilm cells for the longest possible time. Fig. 11 presents the effect of phage Z at m.o.i. 1 on biofilm-grown cells and also on planktonic stationary-phase cells. Both cell types demonstrated little susceptibility to phage Z. The biofilm cells were a little more resistant than the stationary planktonic cells. For biofilm cells the drop in $\text{OD}_{600}$ occurred relatively slowly compared with planktonic stationary cells, while in the control the stationary planktonic cells showed slightly higher growth than did biofilm cells (Fig. 11).

**DISCUSSION**

Biofilms can be found everywhere and have been implicated in a variety of nosocomial infections associated with medical devices, hospital equipment and other hard and moist surfaces (Rao et al., 2005). Microscopic observations have shown that most bacteria (99.9%) are capable of producing biofilms on a wide variety of biotic and abiotic surfaces (Costerton et al., 1978). There is renewed interest in phage therapy owing to the very high level of emergence of antimicrobial resistance in healthcare institutions worldwide due to frequent use of antimicrobials (Archibald et al., 1997; Tenover, 2001). Bacteriophages are often considered alternative agents for controlling bacterial infection and contamination, and increased antibiotic resistance in bacteria. In the last few years, researchers have looked forward to controlling the emergence of antibiotic-resistant bacteria through phage therapy (Nakai & Park, 2002).

Most of the known phages interact only with a specific set of bacteria that express specific binding sites. This narrow host range is a challenge for phage therapy. Consequently, there is no known phage that is lytic for all strains of *K. pneumoniae*. This high specificity of phage–host relationship leads to a need for phages to inhibit newly isolated strains of *K. pneumoniae*.

Phages are generally isolated from environments that are habitats for their respective host bacteria (Nakai & Park, 2002). Phage Z was isolated from sewage water. It is known that sewage generally contains a large diversity of microorganisms owing to contamination from faecal material.

**Fig. 6.** Transmission electron micrographs of the purified phage Z. Bars, 200 nm. Three representative images (a), (b) and (c) are shown.

**Fig. 7.** SDS-PAGE analysis of phage Z structural proteins. Lane 1, broad-range protein molecular mass markers (Precision Plus Protein; BioRad); lane 2, phage Z proteins.

**Fig. 8.** (a) Genome of phage Z: 0.6% (w/v) agarose gel. Lane 1 shows DNA ladder (GeneRuler High Range) and lane 2 shows band of phage DNA with size approximately 36 kb. (b) Lane 1 shows 1 kb DNA Ladder (New England Biolabs) and lane 2 shows restriction analysis of phage Z DNA with EcoRI.
and hospital drainage water (Piracha et al., 2014). Phage Z is highly lytic and capable of producing clear plaques ranging from 1.0 to 3.0 mm in diameter. It has a narrow host range, infecting only *K. pneumoniae* (M), *K. pneumoniae* 3206, *K. pneumoniae* 3, *A. xylosoxidans* and *P. aeruginosa* 2995. Many phages have been reported that are highly specific for receptors present on the host cell surface. They only interact with their own specific receptors and not with receptors having different structures (Piracha et al., 2014).

Several studies have documented that different phage strains vary in thermal and pH stability. Phage Z was tolerant to relatively high temperatures, ranging from 37 to 65 °C, but was killed at 70 °C. It also showed good pH stability over a broad range of pH values, pH 5–11, and maximum stability at pH 7.0. The results are consistent with the previous observations by Ackermann & Dubow (1987) and Jamalludeen et al. (2007) that most phages are able to survive well over a wide range of pH (5–9) under physiological conditions which maintain the native virion structure and stability. The inactivity of the phage at lower pH values of 1 and 3 in our study can be attributed to protein denaturation in acidic environments (Hazem, 2002). These characteristics may be useful for the application of the phage in different environments.

The infectivity of phage Z was shown to be increased at 10 mM calcium chloride or magnesium chloride solution concentration. According to Guttman et al. (2005), cofactors such as Ca$^{2+}$, Mg$^{2+}$, other divalent cations or sugars may be required for successful binding to occur. Ca$^{2+}$/Mg$^{2+}$ ions stabilize the weak interaction of virion with receptors during adsorption. Diverse concentrations of calcium ions give maximum infectivity for various phages (Donlan, 2005; Reese et al., 1974). It is also postulated that Ca$^{2+}$ ions may increase the concentration of phage particles at the host surface or alter the structure of a cell surface receptor so as to increase accessibility to the receptor molecules or transfer of phage nucleic acids (Russel et al., 1988; Watanabe & Takesue, 1972). Yang et al. (2010) have described a burst size and duration of the latent phase that nearly correlate with our results (latent period 24 min; burst size 320 virions per cell), while Sillankorva et al. (2004) found a smaller burst size and shorter duration of the latent
phase. Thus, variations have been reported in the literature regarding latent time and burst size of bacteriophages.

The International Committee on Taxonomy of Viruses recognizes one order, 13 families and 31 genera of bacteriophages. As a non-contractile-tailed virus, our phage falls into the order Caudovirales, which contains three families of tailed viruses that infect bacteria and archaea (Van Regenmortel et al., 2000). Possession of an icosahedral head and a long non-contractile tail would place it in the family Siphoviridae (Van Regenmortel et al., 2000).

Phage specific for a bacterium can infect biofilm cells by first degrading the extracellular polysaccharide and then ultimately lysing the bacterial cells. There is evidence that phage-induced depolymerases could affect biofilms and have potential for biofilm control (Hughes et al., 1998; Roy et al., 1993).

Carson et al. (2010) have reported the potential utility of bacteriophages to reduce bacterial biofilms on medical device surfaces and in prevention of biofilm via direct incorporation of phages, with an approximately 90% reduction in E. coli biofilm formation on bacteriophage-treated catheters compared with untreated controls.

Out of the ordinary was the finding that planktonic cells of K. pneumoniae (M) were much more sensitive to phage Z lysis in exponential growth phase than in stationary growth phase. This effect was previously demonstrated in Pseudo- monas fluorescens planktonic cultures (Sillankorva et al., 2004) and Staphylococcus epidermidis planktonic cells (Cerca et al., 2007). It seems that biofilms are killed slowly by phage Z, not due to specific biofilm cells, but probably due to the low metabolic activity of biofilm cells (Brindt et al., 2008; Corbin et al., 2001; Costerton, 1995). Corbin et al. (2001) studied the impact of phage T4 on E. coli biofilm and observed a 1.5-log reduction, while Moons et al. (2006) studied the effect of phage T7 on E. coli biofilms. They gave phage T7 treatment for 1 h at a concentration of $1 \times 10^{10}$ c.f.u. ml$^{-1}$ and observed approximately twofold reduction. Similarly, we observed a slightly more than twofold reduction for both 24 and 48 h biofilms when treated with a phage titre of $4.5 \times 10^{9}$ p.f.u. ml$^{-1}$.

In conclusion, this study suggests that phage Z is a tailed, DNA-lytic phage having good heat tolerance and a wide range of pH stability. It also has activity against multidrug-resistant K. pneumoniae (M) in both plankonic cells and biofilms but does not result in total eradication of K. pneumoniae (M) biofilms. Thus, for efficient and complete eradication of biofilms, a combination of phage types (phage cocktail) may be needed.

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