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Research article

Inhibition of clinical multi-drug resistant *Klebsiella pneumoniae* biofilm by *Siphoviridae* bacteriophage Z

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Abstract
Klebsiella pneumoniae is an opportunistic pathogen responsible for a spectrum of nosocomial and community-acquired infections. It is also one of the important nosocomial enteric pathogen that causes biofilm-related infections. Bacterial biofilms are associated with a variety of human infections. Biofilms are extremely resistant to a variety of antimicrobial drugs; therefore alternative strategies such as bacteriophages have been suggested to control bacterial biofilms. Phage interaction with biofilm is still not clearly explored and further studies are required for deep understanding of phage behavior on biofilm. In this study, we have described a previously reported phage Z to control *K. pneumoniae* biofilm. The *K. pneumoniae* biofilms formed under static condition with renewal of media for 24 h, 72 h and 120 h showed biofilm biomass reduction of 2.5-log, 2.3-log and 3.3-log, respectively, after phage treatment for 4 hours. Similarly, biofilm formed under dynamic conditions with renewal of media for 24 h, 72 h and 120 h showed biofilm biomass reductions of 2.3-log, 2.4-log and 3.2-log, respectively, after phage treatment for 4 hours. We concluded from this study that bacteriophage Z was highly efficient in removing *K. pneumoniae* biofilm under both static and dynamic conditions.

Keywords: Bacteriophage, biofilm, *Klebsiella pneumoniae*, nosocomial.

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Introduction

Klebsiella spp. are one of the most frequently encountered nosocomial pathogens accounting for substantial increase in morbidity and mortality [1]. Multidrug resistant strains of *Klebsiella pneominae* are increasingly becoming difficult to be treated with existing therapeutic practices [2]. Moreover, pathogenecity and chronicity of *K. pneumonia* infections is increased by its biofilm forming ability [3]. *K. pneumoniae* typically infects patients with indwelling medical devices [4]. Many serous chronic infections are associated with biofilms formed on surfaces of different medical device such as intrauterine devices, uraniry catheter, surgical blades and pacemakers [5]. With the emerging threats of antibiotic resistance in biofilm forming bacteria, novel strategies for the prevention and eradication of biofilm are urgently needed [6, 7]. Donlan [8] states that bacterial biofilm is very infectious in nature and can result in severe nosocomal infections. Bacterial viruses (phages) have been used for treatment of human diseases, and researchers are interested to use of phages for bacterial biofilms control. Phage attacks on host bacterial cell by binding to specific receptor, inject its genetic material and causing lyses of the host cell [9]. Phage therapy is advantageous over chemotherapy due to safety to human, easy availability and replication in the host. Phages can cause lysis of multiple-drug resistant (MDR) pathogenic bacteria and mechanisms for bacterial lysis totally differ than those of antibiotics [10]. It has also been reported that the enzymes such as *depolymerases* produced by bacteriophages have the potential for biofilm degradation. Bacterial biofilm mainly consists of exopolysaccharide (EPS) matrix that acts as a barrier for antibiotic diffusion down the bacteria cells [11]. As the phage behavior on biofilms still requires further investigation and understanding, therefore, we design this study to investigate the effect of phage on biofilm. The objective of this study was to check the effects of previously isolated *Siphoviridae* phage Z on *K. pneumoniae* biofilm formed on steeliness steel plates developed under static and dynamic conditions at different time intervals.

Material and methods

Antibiotic sensitivity assay

The antimicrobial susceptibility profile of previously identified clinical strain *K. pneumoniae* (GenBank Accession No. KJ438818) [7] was determined along with a control strain of *Staphylococcus aureus* (ATCC 25923) according to Clinical and Laboratory Standards Institute (CLSI) protocol [12]. Fourteen different antibiotics were used to determine their effects on *K. pneumoniae* using conventional disc diffusion method (Table 1). The *K. pneumoniae* was uniformly swabbed on Luria Bertani agar plates and antibiotic discs were placed carefully at a specific distance (1.5 cm) from one another and incubated overnight at 37 ^{o}C.

Development of biofilm on stainless steel plates

In this study, stainless steel (SS) plates (1cm × 1cm) were used for the development of biofilms for different time periods in Microtiter 6-well plates. Proper sterilization of stainless steel (SS) plates was performed for the biofilm study as previously described by Sillankorva et al. [13]. SS plates were washed with 100 % acetone followed by immersing in alkaline detergent (1 % NaOH (w/v), pH 13.2) for 1 hour. Then, the SS plates were rinsed in sterilized distilled water and were dried cleaned with alcohol (70 % (v/v)). The SS plates were washed again with distilled water, dried for 2 h at 60 ºC and were sterilized by autoclaving at 121 ºC for 15 min. Two major conditions were considered for biofilm formation at 37°C i.e. static condition (with out shaking) and dynamic condition (constant shaking of 120 rpm) with the removal of media in both cases after each 12 hours.

Biofilm formation on SS plates was achieved using the method of Cerca et al. [14] with some modifications. Briefly, the SS plates were placed in six well plates and 50 µL bacterial cultures (OD = 1 at 600 nm) were deposited on SS plates. Later, 6 ml of trypic soy broth (TSB) was added to each well in such way not to disturb the bacterial culture on the plate surface. The six well plates having SS plates were incubated at different conditions (static and dynamic) at 37 °C for 24 h, 48 h and 72 h.

Colony forming units (CFU) of biofilm and planktonic cells

Bacterial cell counts in the biofilm after different time intervals on SS plates were determined by the method of Sillankorva et al. [13]. A negative control without phage was also established by putting biofilm samples in a solution of phage buffer and TSB for 24 hrs. The SS plates with biofilm were washed carefully by dipping in a freshly prepared phosphate buffer saline (PBS) (1×). These washed SS plates were transferred into a tube containing 3 ml of normal saline and vortexed vigorously with high speed. Afterwards, serial dilutions were prepared for biofilm cells and CFU was calculated by spreading various dilutions on TSA plates. Similarly, CFU for planktonic cells were counted from the 6-well Microtiter plates by the same serial dilution methods.

Application of bacteriophage

Bacterial bioflms formed under different conditions were treated with phage stock suspensions. Biofilms formed for 24 h, 48 h and 72 h were treated with phage suspensions for four hours. For phage treatment, the SS plates (biofilms) were immersed carefully in fresh PBS (1×) and then transferred to new fresh well containing 3 ml of TSB and 3 ml of phage suspension. The Microplates containing SS plates were incubated for 4 hours at 37°C. At the same time, negative control experiment was done. Effect of phage suspension on bacterial biofilms prior and post phage treatment was analyzed [13].

Plaque forming unit (PFU)

After phage treatment of SS plates, those were rained carefully in PBS (1×) and consequently transferred to tubes containing 3 ml of normal saline solution. Then the SS plates were vortexed vigiorously in such a way to remove all the phages adhering to SS plate surfaces. After this, serial dilutions were formed in SM buffer and the soft ager overlay assay was performed for PFU calculations as described by Cerca et al. [14].

Scanning electron microscopy of biofilm

Biofilms formed on SS slides were fixed by the method as described previously by Sillankorva et al. [13] with some modification. The SS plates were removed from the media with the help of forceps and carefully washed by dipping three times in PBS (1X) solution. After PBS washing the SS plates were placed in gluteraldehyde (2.5 %) at 4 °C and fixed for one hour. After fixation the SS plates were subjected to drying procedure by using ethanol. Biofilm samples were dehydrated with an ethanol series ranging from 30% to 100%. Each step of ethanol treatment was ranging from 5-10 min. After drying, the biofilm SS plates were further processed for SEM analysis. Biofilm formed on each SS plate was mounted on an adhesive carbon tabs on aluminium specimen. SS plates were rendered for conduction with Au/Pd (Denton Desk II sputter coater). For biofilm detection, both treated and control SS plates were examined under scanning electron microscope (Hitachi S-4000 FE-SEM, High Technologies America, USA) and scanning electron micrographs of high magnifications were obtained through software (PCI Quartz) at Electron Microscopy and Bio-Imaging lab (EMBL), Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida (UF), USA.

Results

Antibiotic sensitivity of *K. pneumoniae*

According to the CLSI guidelines antibiotic sensitivity assay displayed that the *K. pneumoniae* was resistant to amoxicillin (AM-25), sulphametoxazole/trimethoprim (SXT-25), trimethoprim (TMP-30), ciprofloxacin (CIP-5), cefazolin (CZ-30), tetracyclin (TE-30), streptomycin

(S-10), cefotaxime (TAZ-30) and amoxycillin/clavulanicacid (AMC-25). *K. pneumoniae* showed intermediate resistance to doxycyclin (D-30) and gentamycin (GM-30) while showed susceptibility to erethromycin (E-15), ceftazidime (CA-Z30) and levofloxacin (LEV-10) (Table 1).

Table 1 Sensitivity *of Klebsiella pneumoniae* to different group of antibiotics

Antibiotics	Conc. (µg/ml)	Resistance
Amoxycillin (AM-25)	25	R
Tetracyclin (TE-30)	30	R
Trimethoprim((TMP-30)	30	R
Ciprofloxacin(CIP- 5)	5	R
Sulphametoxazole/Trimethoprim (SXT- 25)	25	R
Erethromycin (E-15)	15	S
Streptomycin (S-10)	10	R
Doxycyclin (D-30)	30	I
Ceftazzidime (CAZ-30)	30	S
Gemtamycin (GM-30)	30	I
Cefotaxime (TAZ-30)	30	R
Cefazolin (CZ-30)	30	R
Amoxycillin/Clavulanicacid (AMC-25)	25	R
Levofloxacin (LEV-10)	10	S

R = resistant; S = sensitive; I = intermediate resistant

Biofilm formed under static conditions

The results showed that the biofilm formed by *K. pneumoniae* under static condition with renewal of media for 24 h, 72 h and 120 h with phage treatment for 4 h showed biomass reduction of 2.5-log, 2.3-log and 3.3-log, respectively (Fig. 1).

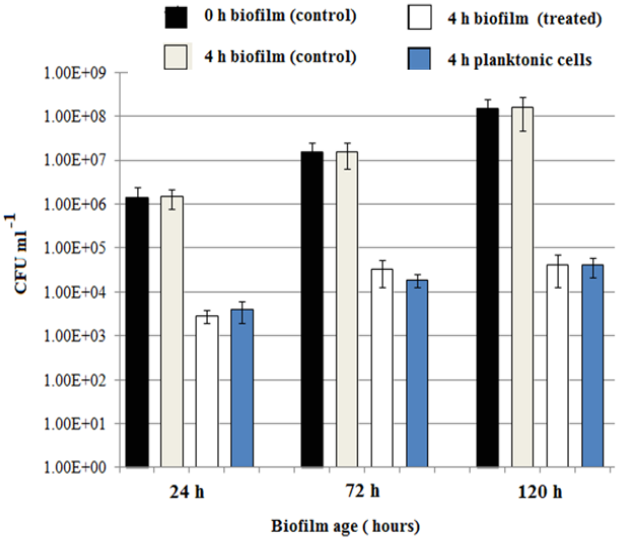

Fig 1 Number of biofilm and planktonic cells of *K. pneumoniae* before and after exposure to phage Z under static conditions. 0h biofilm (control) = Bacterial cells before phage Z treatment; 4h biofilm (control) = Bacterial cells after 4 h in phage buffer-TSB treatment. Values are the means of 4 determinations with ± SD

Biofilm formed under dynamic conditions

The results showed that the biofilm formed by *K. pneumoniae* under dynamic condition with the renewal of media for 24 h, 72 h and 120 h with phage treatment for 4 h showed biomass reduction of 2.3-log, 2.4-log and 3.2-log, respectively (Fig. 2).

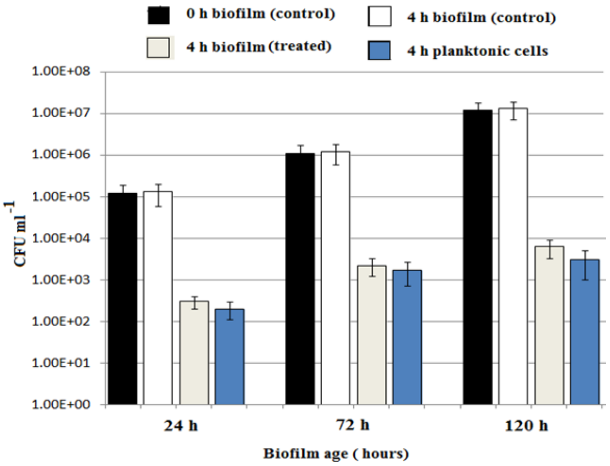

Fig 2 Number of *K. pneumoniae* biofilm and planktonic cells before and after exposure to phage Z under dynamic conditions. 0h biofilm (control) = Bacterial cells before phage Z treatment; 4h biofilm (control) = Bacterial cells after 4 h in phage buffer-TSB. The experiment was performed under static conditions with media renewal. Values are the means of 4 determinations with ± SD.

Biofilm scanning electron microscopy

We also performed scanning electron microscopy of *K. pneumoniae* biofilms to properly observe the physical appearance of biofilms and effect of phage Z on those developed biofilms. Fig. 3 shows the results of representative scanning electron micrographs of biofilms formed under static condition with renewal of media. The obvious differences between control and treated biofilms can be seen clearly.

Discussion

Biofilm formation occurs on both non-living and biological surfaces and this biofilm formation is the survival mechanism of bacterial communities. Biofilms in humans can give rise to a number of infections, in which most of them are acquired from medical devices [6]. It has been proved by microscopy that most of bacteria have the potential to produce biofilms on abiotic and biotic surfaces [15], and resistance to antibiotics and host defense system is a major problem posed by the biofilms [6].

Generally, biofilm forming micro-organisms show resistance to antibiotics, disinfectants and biocides. Prolong treatment with high concentrations of antibiotics are normally needed to reduce or remove biofilms [16]. We tested fourteen different

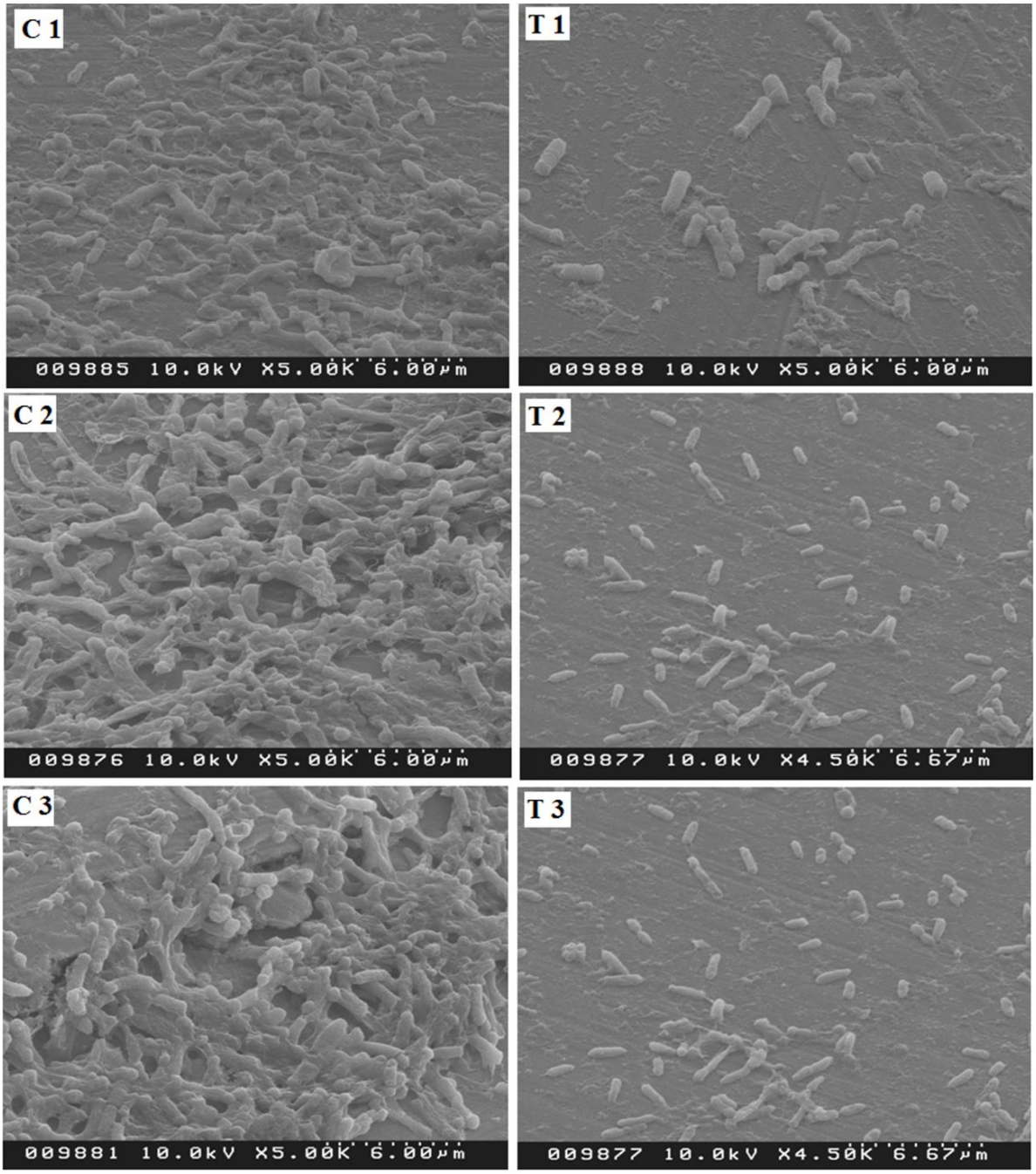

Fig 3 Scanning electron micrographs of *K. pneumonia* biofilms formed (37 °C) under static conditions at different time intervals. Biofilm micrograph after 24 h (C1), 72 h (C2) and 120 (C3) hours before phage treatment and biofilm micrograph after 24 h (T1), 72 h (T2) and 120 (T3) hours after four hours phage treatment.

commercially available antibiotics against the bacterial strain of *K. pneumoniae*, which was under consideration for biofilm formation. The selected *K. pneumoniae* strain was found resistant to 10 antibiotics out of 14 (Table 1). We developed *K. pneumoniae* biofilms on SS plates under both static and dynamic conditions at different time intervals. After the development of biofilms, the phage Z (2.8×10^7 PFU/ml) was applied as anti-biofilm agent. Phage-treated biofilms were incubated for 4 hours at 37°C. Under both static and dynamic conditions, a considerable reduction in biofilm was observed, which showed that phage Z was effective in reducing biofilm and probably has enzyme that can degrade extracellular polymeric substances (EPS) of biofilm matrix. Similarly, Sillankorva et al. [13] have

prevously reported *P. fluorescens* biofilm reduction using phage phiIBB-PF7 titer of 10^7 PFU/ml for biofilm treatment. They observed a biofilm biomass reduction ranging from 1 to 5-log. Biofilm of the host bacteria is infected initially by phage causing its EPS degradation, and finally lysis of the bacterial cells. There is an evidence that bacterial biofilm could be affected by phage-induced depolymerases [11]. Carson et al. [17] reported anti-biofilm potential of phages on the surface of medical devices. They observed about 90% eradication of *E. coli* biofilm by the direct application of phages on catheters as compared to untreated catheters.

We also used scanning electron microscopy (SEM) methods for detecting biofilms of clinical strain *K. pneumoniae* developed on stainless steel plates using time spans of 24 h, 48 h and 72 h under both static and dynamic conditions. Peters et al. [18] stated that scanning electron microscopy (SEM) is a well-established technique for the observation of the morphology of bacteria adhering to surface, bacterial biofilm and also the relationship between them. SEM also provides key structural information about biofilm and has long been used for this purpose [19, 20].

In conclusion, bacteriophage Z has a very good inhibitory activity against multidrug resistant *K. pneumoniae* biofilms under both static and dynamic conditions. However, for complete eradication of *K. pneumoniae* biofilm a cocktail of phages (a combination of different phages) may be more promising.

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