

RESEARCH ARTICLE

Phage Therapy Against Biofilm of Multidrug-Resistant *Klebsiella Pneumoniae* Isolated from Zakho Hospital Samples

Yousif Abdullah AlBany^{1*}, Mohammad Ismail Al-Berfkani², Mahde Saleh Assafi³

¹Department of Nursing, Bardarash Technical Institute, Duhok Polytechnic University, Duhok, Kurdistan Region - F.R. Iraq, ²Department of Medical Laboratory Technology, Zakho Technical Institute, Duhok Polytechnic University, Duhok, Kurdistan Region - F.R. Iraq, ³Department of Biology, College of Science, Duhok University, Duhok, Kurdistan Region - F.R. Iraq

***Corresponding author:**

Yousif Abdullah AlBany,
Department of Nursing,
Bardarash Technical
Institute, Duhok Polytechnic
University, Duhok, Kurdistan
Region - F.R. Iraq.
E-mail: yousif.albany@dpu.
edu.krd

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ABSTRACT

Klebsiella pneumoniae causes infection in human, especially in immunocompromised patients. About 80% of nosocomial infection caused by *K. pneumoniae* is due to multidrug-resistant strain. The emergence of antibiotic-resistant bacterial strains necessitates the exploration of alternative antibacterial therapies, which led to studying the ability of viruses that infect the bacteria (known as bacteriophage) to treat infection with *K. pneumoniae*. Bacterial biofilm which are crucial in the pathogenesis of much clinically important infection and are difficult to eradicate because they exist resistant to many antimicrobial treatment. Biofilm formation by *K. pneumoniae* is responsible for the catheter associated infection such as urinary tract infection and respiratory tract infection due to the colonization of the polymeric surface by forming multilayered cell cluster embedded in extracellular materials. In this study *K. pneumoniae* isolated from the hospital environment and characterized it and form the biofilm of that organism by microplate quantitative assay. Similarly bacteriophage specific for *K. pneumoniae* isolated from river water. The aim of work is the use of bacteriophage as a possible alternative for the treatment of bacterial infection of *K. pneumoniae*. We showed that biofilm is reduced by isolated phages by the comparative account of colony-forming unit versus plaque-forming unit. The result of this study, therefore, suggests that the timing of starting the phage therapy after initiation of infection significantly contributes toward the success of the treatment.

Keywords: Bacteriophage; Biofilm; *Klebsiella pneumoniae*; Multidrug resistant

INTRODUCTION

Biofilms have been found to be involved in a wide variety of microbial infections in the body, by one estimate 80% of all infections (Costeron et al., 1999; Matthew et al., 2005). Infectious processes in which biofilms have been implicated include common problems such as urinary tract infections, catheter infections, middle ear infections, formation of dental plaque, gingivitis, coating contact lenses, and less common but more lethal processes such as endocarditic, infections in cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses and heart valves (Lewis et al., 2001; Parsek and Singh, 2003). *Klebsiella pneumoniae* is the common pathogen of the urinary tract in both community and hospital-acquired infections (Al-Berfkani et al., 2016). It is resident in the intestine tract of 40% man and animals, and it can cause an opportunistic infection that means it may cause infection under certain condition. For example, nosocomial infection is those which hospitalized patients

picked up because they are in a weakened state (Otman et al., 2007). The ability to adhere materials and to form biofilm is an important feature in the pathogenesis of *K. pneumoniae* which associated with catheter-associated infection due to the colonization of polymer surface by forming multilayered cell clustered embedded in an extracellular matrix. The wide spectrum of infection caused by *K. pneumoniae* has been reported, which includes septicemia, meningitis, pneumonia, chronic renal failure, and adrenal insufficiency (Jagnow and Clegg, 2003). Bacteriophages have been found to be controlled biofilm formation (Lu and Collins, 2007; Bedi et al., 2009). The bacteriophage is the virus that can infect and destroy the bacteria, and it can refer to as parasite to the bacteria act as host with each phage type depending upon the strain of bacteria. At present, phage therapy is widely used for control disease in plants (Fox, 2000), animals (Biswas et al., 2002), and infectious disease in humans (Sillankorva et al., 2004), especially against infections caused by multidrug-resistant bacteria (Sillankorva et al., 2004).

The aim of this study is to isolate the *K. pneumoniae* from hospital samples and to check the efficiency of phage onto biofilm degradation in a comparative account of colony-forming unit (CFU) with plaque-forming unit (PFU).

MATERIALS AND METHODS

Microorganisms and Culture Media

Eight samples of *K. pneumoniae* were isolated from urine samples obtained from the general Zakho hospital. The eosin methylene blue agar medium was used as a selective medium. The isolate colonies were identified according to the identification scheme as gram staining and motility (Godkar, 2003). The isolates were confirmed using a set of biochemical tests which include indole test, methyl red test, Voges–Proskauer test, citrate + and H₂S test, catalase test, capsule staining test, and flagella staining test. In all, eight isolates were obtained, of which three isolates were confirmed as *K. pneumoniae* on the basis of cultural and biochemical characteristics.

Determination of Resistant to Antimicrobial Agent

Isolates of three *K. pneumoniae* were screened for their sensitivity to various antimicrobial agents that included antibiotics and commercial chemical disinfectant as chloroxylenol (Dettol) and ethyl alcohol (70%). Incubation for 24 h at 37°C and grown culture of respective isolates were used for sensitivity testing. The Kirby–Bauer modified disc-diffusion technique was used to determine the sensitivity to the antibiotics. The poly disc was evenly distributed on sterile Mueller Hinton agar medium. In a way that they was 10 mm from the edge of the plate, each disc was lightly pressed down to ensure its sufficient contact with the agar medium. The plates were then incubated at 37°C for 24 h; the result shows that *K. pneumoniae* is resistant to all antibiotics.

Effect of Dettol and Ethyl Alcohol on Growth of *K. pneumoniae*

To test the effect of Dettol and ethyl alcohol to growth of *K. pneumoniae*, different concentrations of Dettol and

alcohol were prepared starting with dilution with sterile distilled water 1:10–1:50 (v/v) and were inoculated with 0.5 ml of culture and incubated at 37°C for 24 h. After that, tubes were observed for turbidity and samples from tubes were also analyzed for viable bacteria by spread plate technique (Russell et al., 1988).

Isolation of Phage Specific for *K. pneumoniae*

K. pneumoniae was isolated from surface water of the Khabur River which flows through the Zakho city. Water sample was collected in a sterile capped bottles having 250 ml capacity. Water sample was kept in a refrigerator until used. Then, it was filtered through a sterile membrane filter (0.20 µm). The filtrate was used as a source of phage. Phages were isolated from the filtrate by double agar layer plaque assay method (Hancockm, 1959), in which 0.2 ml filtrate and 0.5 ml culture of *K. pneumoniae* are added in sterile soft agar (0.6%w/v) and then poured onto sterile nutrient agar medium. Plates were incubated at 37°C for 24 h, and after that, plates were checked for plaques (Hancockm, 1959).

Purification and Enrichment of Phages

A single plaque was picked from the plate with a sterile cork borer and then transferred into the flask containing 250 ml phage broth which contains (peptone – 10 g, sodium chloride – 5 g, meat extract – 3 g, CaCl₂ – 0.2 g, and MgSO₄·7H₂O – 0.5 g, per 1000 ml and pH – 7.4), with 1 ml overnight grown host bacterium. Flask was incubated at 37°C for 24 h. The content of the flask was centrifuged at 20000 rpm for 10 min. The supernatant was collected in a sterile 250 ml flask and filtered through a membrane filter of 0.20 µm. The filtrate was used as a source of phage. Phage activity was checked against all isolates of *K. pneumoniae*. Phages were stored at 4°C (Hancockm, 1959).

Bacteriophages Titration

Bacteriophage titer in the lysate was analyzed by 0.1 ml diluted phage lysate and 0.5 ml overnight grown bacterial culture was mixed in 3 ml sterile molten or soft agar and poured onto a sterile nutrient agar plate (Hancockm, 1959). Plates were incubated overnight at 37°C, after which PFUs were counted.

Biofilm Growth

K. pneumoniae was selected for biofilm formation on polyethylene glycol (PEG) of 96 well microtiter plates equipped with PEGs of plastic grain material on lids of a microtiter plate. Cells were grown in sterile nutrient broth

Table 1: Identification of hospital isolates by biochemical test

HS	Indol	MR	VP	Citrate	Capsule staining	Catalase test	Urease test
HS1	+	+	+	+	+	+	+
HS2	+	+	+	+	+	+	+
HS3	+	+	+	+	+	+	+

HS: Hospital strain, MR: Methyl Red, VP: Voges–Proskauer, + positive result/- negative result

Table 2: Antibiotic resistant pattern for hospital strain

HS	AK	BA	CF	CET	CH	CB	CL	TE	TOB	TMP	AK	GM
HS1	R	R	R	R	R	R	R	R	R	R	R	R
HS2	R	R	R	R	R	R	R	R	R	R	R	R
HS3	R	R	R	R	R	R	R	R	R	R	R	R

for 24 h, at 37 °C to get an absorbance range (0.01–0.50) at 560 nm. 200 ml of culture suspension of different optical densities (0.01–0.50) was added to wells of a microtiter plate. Control wells ($n = 6$) with only media but no bacteria were included. PEG lids were placed in the microtiter plate and inserted into plastic bags to prevent evaporation of the medium. Microtiter plate was incubated at 37°C under static condition for 24 h. PEGs were then analyzed for biofilm. PEGs with pre-grown biofilm were removed from the wells and then rinsed 3 times with sterile 200 ml of sterile phosphate buffer saline. PEGs were removed from the lids and dipped into the wells containing 200 ml of phosphate-buffered saline (PBS). PEGs were scrapped aseptically with the help of sterile forceps into the well. The content of the well was serially diluted in sterile PBS. Total viable count of bacteria was determined on plates with sterile nutrient agar medium; CFUs were counted after overnight incubation at 37°C.

Prevention of Biofilm Formation

The culture of *K. pneumoniae* grown in nutrient broth (optical density [OD] at 560 nm) having cell count 5.6×10^5 CFU/ml was used and 0.15 ml culture was dispensed in wells of a sterile microtiter plate. Then, 0.05 ml phage lysate with 2.5×10^2 PFU was added to the above wells. Well with 0.2 ml culture and no phage lysate was kept as a control. Plates were incubated at 37°C for 24 h and analyzed for phages in the well in terms of PFU. The content of the wells was also analyzed for host cells in terms of CFU. After 24 h, PEGs were rinsed with sterile PBS 3 times and then immersed in sterile PBS. PEGs were scrapped aseptically with the help of forceps to dislodge bacteria from PEGs into well. The content of well was analyzed for a total viable count of bacteria (Curtin and Donlan, 2006).

Biofilm Dispersion

Biofilm of *K. pneumoniae* of hospital isolates was allowed to form on PEG lid of microtiter plate under static condition for 24 h at 37°C. Then, the PEG with pre-grown biofilm was immersed twice in fresh PBS aseptically and placed in new microtiter plate with 0.2 ml phage broth containing 2.5×10^2 PFU. Microtiter plate with the PEG lid was incubated at 37°C for 24 h. PEG lids were removed from the wells, rinsed with sterile PBS, 3 times, and inserted into new wells containing 0.2 ml sterile PBS. PEGs were separated from the lid and dipped into the wells, scrapped with sterile forceps to dislodge bacteria into the wells, and kept at room temperature for 5 h. The contents of the well were serially diluted in PBS, and total viable count in terms of CFU/PEG was determined. Phage counts in the liquid phase (well) as well as on PEGs were determined.

RESULTS

Isolation of Resistant Strain of *K. pneumoniae*

K. pneumoniae was confirmed according to the cultural and biochemical tests (Godkar, 2003). Totally, three different hospital isolates were isolated and confirmed [Table 1]. The aim of the isolates was to determine whether hospital isolates of *K. pneumoniae* show resistant to antimicrobial agent, namely antibiotics and chemical disinfectant.

The result of antibiotic sensitivity test showed that all isolates of *K. pneumoniae* are resistant to all antibiotics [Table 2]. Survival studies in the presence of varying concentration of Dettol and alcohol showed that all isolates of *K. pneumoniae* could survive in all concentration of Dettol and alcohol, thereby showing resistant to Dettol and alcohol as shown in [Table 3].

Isolation of Lytic Bacteriophage

The main aim of this work was to isolate, select, and enrich an effective phage specific for *K. pneumoniae* hospital isolates. Majority of plaque showed clear plaques, whereas some showed turbid with centered colony [Figure 1]. Clear plaques are considered for further studies. Phage isolated from river water shows activity against all hospital strains of *K. pneumoniae* [Table 4].

Biofilm Growth

In this work, *K. pneumoniae* biofilm was allowed to form onto the PEGs of microtiter plate under static condition for 2 h at 37°C using the bacterial culture of different cell

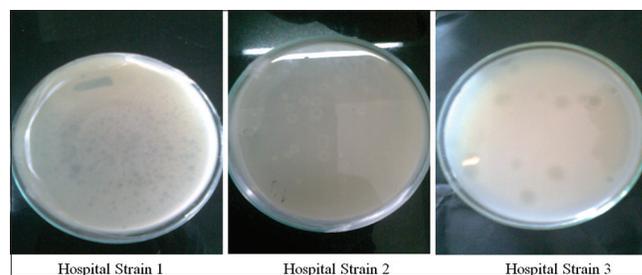


Figure 1: *In vitro* lytic activity of phage, showing with plaque formation in the bacterial lawn of *Klebsiella pneumoniae*

Table 3: Effect of chemical disinfectants on the growth of *K. pneumoniae* isolates

Dettol (Con.)	Growth	Alcohol (Con.)	Growth
1:10	+	1:10	+
1:15	+	1:15	+
1:20	+	1:20	+
1:25	+	1:25	+
1:30	+	1:30	+
1:35	+	1:35	+
1:40	+	1:40	+

K. pneumoniae: *Klebsiella pneumoniae*

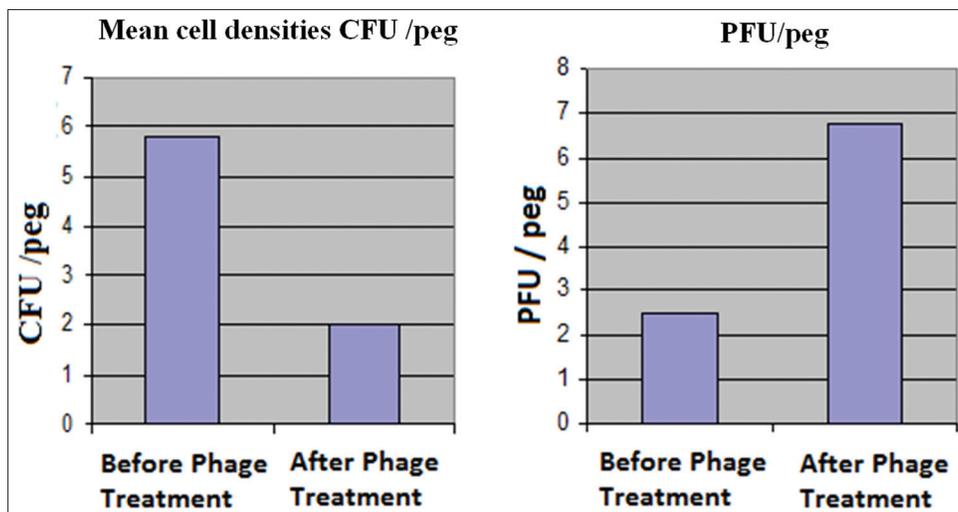


Figure 2: Dispersion of *Klebsiella pneumoniae* biofilm

Table 4: Phage activity against isolated strains

	Hospital strain 1	Hospital strain 2	Hospital strain 3
Phage activity	+	+	+
Phage morphology	Clear circular plaque	Turbid and some clear circular plaques	Clear circular plaque

Table 5: Mean cell density required for biofilm formation

OD at 560 nm	CFU/PEG	Biofilm formation
0.01	1.0×10 ¹	+
0.02	1.0×10 ²	+
0.04	3.5×10 ²	+
0.05	4.0×10 ²	+
0.10	6.0×10 ³	+
0.15	4.0×10 ⁴	+
0.18	3.2×10 ⁴	+
0.20	3.6×10 ⁴	+
0.30	1.2×10 ⁵	+
0.34	2.0×10 ⁴	+
0.40	3.6×10 ⁵	+
0.50	5.8×10 ⁵	+

OD: Optical density, PEG: Polyethylene glycol, CFU: Colony-forming unit

densities (OD: 0.01–0.5). In case of culture suspension having densities 0.01, the CFU per PEG of 1.2×10^2 was obtained. Maximum cell count was 5.6×10^5 CFU/PEG when a culture density was 0.5 at 560 nm. All cell densities (0.01–0.5) could able to form a biofilm on PEGs within 24 h; therefore, condition such as cell density and the time period influenced the formation of biofilm [Table 5].

Dispersion of Biofilm

In this study, the effectiveness of lytic phage in the dispersion of biofilm which was formed under a static condition was studied. The amount of biofilm on the PEGs after phage treatment was assessed. In this experiment, the number of phages applied was 2.5×10^2 PFU. Phages in terms of PFU in the well and on the PEGs were assessed after 24 h [Figure 2]. Before phage treatment, the cell count

on the PEG was reduced to almost 2–4 log cycles. Phage counts on the PEGs were increased by 2 log cycles. This proved that phage is efficient in dispersing biofilm formed on the PEG.

DISCUSSION

In this study, hospital isolates of *K. pneumoniae* were resistant to a broad spectrum of antibiotics, chemicals, and disinfectants. Bacteria within the biofilm are more resistant to antibiotics and to the host immune system than planktonic bacteria. Several mechanisms are thought to be responsible; first, phagocytes cannot penetrate the biofilm efficiently; second, through adsorption and neutralization, the matrix of protein and polysaccharide that constitute the intercellular milieu and capsule of the biofilm prevents antibiotics and other blood constitute from reaching microbial target; and third, the bacteria within a mature biofilm are relatively metabolically inactive, a condition known as the persistent state. Factors that are implicated in the virulence of *K. pneumoniae* included the capsular serotype, lipopolysaccharide, iron-scavenging system, and fimbrial and non-fimbrial adhesions.

The abundant polysaccharides that typically surround *K. pneumoniae* protect against the bactericidal action of serum and impair phagocytosis and regarded as the most important virulence determinant of *K. pneumoniae*. Furthermore, many β -lactamase genes have been found in *K. pneumoniae* plasmid including extended-spectrum β -lactamase, AmpC

β -lactamase, inhibitor-resistant TEM β -lactamase, and metalloenzymes. These enzymes confirm the resistant to various antimicrobial agents including the third and fourth generations of cephalosporins and cephamycins (Sanchez et al., 2013). Nonmicrobial particles from the host or environment influenced the structure of biofilm. The fibrin capsule surrounding the biofilm will protect the organisms from the leukocytes of the host, leading to infective endocarditis (Donlan and Costerton, 2002).

Biofilms develop in urinary catheters containing uropathogens which release urease that hydrolyzes urea in the urine to form free ammonia leading to rise the pH of urine at the biofilm liquid interface, resulting in the precipitation of calcium phosphate and magnesium ammonium phosphate (struvite) (Lin and Scott, 2012). Biofilm can be entrapped by these minerals and cause encrustation of the catheter, as a consequence, the catheter became completely blocked by this mineral buildup (Donlan, 2002).

Phage specific for isolates of *K. pneumoniae* had been isolated from river water and shown the potential to disperse preformed biofilm. Thus, bacteriophages are used as an alternative treatment against the antibiotic-resistant microorganisms (Sillankorva et al., 2004). Lytic bacteriophages instruct the machinery in the host cell to make more bacteriophages. Fully viable progeny bacteriophages burst out and kill the bacteria. The released bacteriophages attack new bacteria. Each of these cycles takes an average of 30 min and produces about 50–400 phages. This process continues until all the bacteria are eliminated from the system. The lysogenic bacteriophages attach their strands of genetic instructions to the DNA of the bacteria. Thus, only the lytic phages are a good choice for developing therapeutic phage preparations. Hence, phage therapy is possible in all bacterial infections. Because phages are so specific to the bacteria they infect, they will not harm to other beneficial bacteria present in the intestine and other parts of the body and will not affect the microbial ecosystem in the body. There is no chance of superinfection with other bacteria.

There are numerous researches reporting on phage-based treatment of infections around the world. A similar study about using phage therapy against *K. pneumoniae* has been conducted (Stahlhut et al., 2012). Other research study using phage against other pathogenic bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Campylobacter jejuni* were proposed (Morita et al., 2002; Atterbury et al., 2003; and Ceyskens and Lavigne, 2010).

In this study, biofilm was formed under static condition, which is related to the total number of cells and also to the

supply of fresh nutrient. Each cell densities ranging from 0.01 to 0.5 could be able to form biofilms on the PEGs of microtiter plate PEG lids within 24 h, and culture was supplemented with fresh sterile nutrient broth; this helped the sessile bacterial cell to multiply in the biofilm. Biofilm is composed of pores and channels, through which nutrients as well as phages are able to circulate and by this way reach and absorbed into the cells of different layers of biofilm. Phage application shows that highest colony count of biofilm growth was 5.6 CFU/PEG which is reduced to 2.0 CFU/PEG after the treatment of phage having a 2.5 PFU/PEG of initial count similarly which is increased to about 5.8 PFU/PEG after the phage treatment. This indicates that the phage application is effective to control the biofilm formation.

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