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

Yousif Abdullah AlBany\(^1\)*, Mohammad Ismail Al-Berfkani\(^2\), Mahde Saleh Assafi\(^3\)

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

**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 (Biswa 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. The plates were 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 um). 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 um. 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 | -     | +    | +    | +       | +                | +            | +          |

**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   |

**POLYTECHNIC JOURNAL ● VOL 9 ● NO 1 ● 2019 | 18**
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
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 constituent 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.

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 $2.5 \times 10^2$ 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**

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 cephemycins (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. pneumonia* 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 Ceyssens 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.

REFERENCES

Al-Berfkani, M. I., A. A. Muslim, A. M. Sherzad. 2016. The effect of climate temperature and daily water intake on the diversity of uropathogens causing urinary tract infections in adult hospital patients. Dijala J. Med. 11(1): 62-69.

Atterbury, R., P. L. Connerton, C. E. Dodd, C. E. Rees, and I. F. Connerton. 2003. Application of host-specific bacteriophages to the surface of chicken skin leads to a reduction in recovery of *Campylobacter jejuni*. Appl. Environ. Microbiol. 69: 6302-6306.

Bedi, M. S., V. Verma and S. Chhibber. 2009. Amoxicillin and specific bacteriophage can be used together for eradication of biofilm of *Klebsiella pneumoniae* b5055. World J. Microbiol. Biotechnol. 25(7): 1145-1151.

Biswas, B., S. Adhya, P. Washart, B. Paul, A. Trostel, B. Powell, R. Carlton and C. R. Merrill. 2002. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. Infect. Immun. 70(1): 204-210.

Ceyssens, P. J. and R. Lavigne. 2010. Bacteriophages of *Pseudomonas*. Future Microbiol. 5(7): 1041-1055.

Costerton, J. W., P. S. Stewart and E. Greenberg. 1999. Bacterial biofilms: A common cause of persistent infections. Science. 284(5418): 1318-1322.

Curtin, J. J. and R. M. Donlan. 2006. Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*, antimicrobial. Agents Chemother. 50: 1268-1275.

Donlan, R. M. 2002. Biofilms: Microbial life on surfaces. Emerg. Infect. Dis. 8(9): 881-890.

Donlan, R. M. and J. W. Costerton. 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15(2): 167-193.

Fox, J. 2000. Phage treatments yield healthier tomato, pepper plants. ASM News. 66(8): 455-456.

Godkar, P. G. D. 2003. Medical Laboratory Technology. 2nd ed. Bhalani Published House, Mumbai.

Hancockm, A. 1959. Bacteriophages. Interscience, New York.

Jagnow, J. and S. Clegg. 2003. *Klebsiella pneumoniae* mrkd-mediated biofilm formation on extracellular matrix and collagen-coated surfaces. Microbiology. 149(9): 2397-2405.

Lewis, K. 2001. Riddle of biofilm resistance. Antimicrob. Agents Chemother. 45(4): 999-1007.

Lin, G. G. and J. G. Scott. 2012. Role of biofilm in catheter associated
urinary tract infection. Am. J. Infect. Control. 100(2): 130-134.
Lu, T. K. and J. J. Collins. 2007. Dispersing Biofilms with Engineered Enzymatic Bacteriophage. Vol. 104. Proceedings of the National Academy of Sciences of the United States of America. pp11197-11202.
Riley, P. A. 2006. Phages: Their role in bacterial pathogenesis and biotechnology. J. Clin. Pathol. 59(9): 1003-1004.
Morita, M., Y. Tanji, K. Mizoguchi, T. Akitsu, N. Kijima and H. Unno. 2002. Characterization of a virulent bacteriophage specific for Escherichia coli O157: H7 and analysis of its cellular receptor and two tail fiber genes. FEMS Microbiol. Lett. 211: 77-83.
Otman, J., M. E. Perugini, M. C. B. Tognim and M. C. Vidotto. 2007. Atypical phenotypic characteristics of Klebsiella pneumoniae isolates from an outbreak in a neonatal intensive care unit in Brazil. Braz. J. Microbiol. 38(2): 273-277.
Parsek, M. R. and P. K. Singh. 2003. Bacterial biofilms: An emerging link to disease pathogenesis. Annu. Rev. Microbiol. 57: 677-701.
Russell, A. D., U. Tattawasart, J. Y. Maillard and J. R. Friday. 1988. Possible link between bacterial resistance and use of antibiotics. Antimicrob. Agents Chemother. 42: 2151-2152.
Sanchez, G. V., R. N. Master, R. B. Clark, M. Fyyaz, P. Duvvuri, G. Ekta and J. Bordon. 2013. Klebsiella pneumoniae antimicrobial drug resistance, United States, 1998-2010. Emerg. Infect. Dis. 19(1): 133-136.
Sillankorva, S., R. Oliveira, M. J. Vieira, I. Sutherland and J. Azeredo. 2004. Pseudomonas fluorescens infection by bacteriophage φs1: The influence of temperature, host growth phase and media. FEMS Microbiol. Lett. 241(1): 13-20.
Stahlhut, S. G., C. Struve, K. A. Krogfelt and A. Reisner. 2012. Biofilm formation of Klebsiella pneumoniae on urethral catheters requires either Type 1 or Type 3 fimbriae. FEMS Immunol. Med. Microbiol. 65: 350-359.