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**Original Article**

# Protection with bacteriophage KØ1 against fatal *Klebsiella pneumoniae*–induced burn wound infection in mice

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**Background and purpose:** Bacteriophages, viruses that cause lysis of bacteria, can serve as an effective tool for the control of infections, especially those caused by multiple drug–resistant organisms. *Klebsiella pneumoniae* is one of the most predominant pathogens associated with burn wound infections, causing considerable morbidity and mortality. This study investigated the protective effect of *K. pneumoniae*–specific bacteriophage KØ1 isolated from the environment in a mouse model of burn wound infection induced by *K. pneumoniae*.
**Methods:** The toxicity of the bacteriophage and its in vivo survival and stability in mice was assessed. The fatal dose of *K. pneumoniae* was ascertained in third-degree burn wound infection. The protective effect of the bacteriophage administered via the subcutaneous or intraperitoneal route was investigated.
**Results:** A substantial decrease in the bacterial load of blood, peritoneal lavage, and lung tissue was noted following treatment with the bacteriophage preparation. The decrease in microbial count was evident when the bacteriophage was administered either via the subcutaneous or intraperitoneal route.
**Conclusion:** These results suggest that bacteriophages have the potential to modulate the course of burn wound infection caused by *K. pneumoniae*.

**Key words:** Bacteriophages; Burns; *Klebsiella pneumoniae*

## Introduction

The increasing incidence of antibiotic-resistant bacteria and the lack of development of new types of antibiotics to control infections caused by these organisms has renewed the interest of researchers in bacteriophage therapy. Harnessing bacteriophages as bioagents for the treatment of bacterial disease was originally introduced approximately 80 years ago by Felix d'Herelle [1], the discoverer of bacteriophages. In the past, the efficacy of bacteriophages for treating bacterial diseases has been demonstrated by using animal models for *Vibrio vulnificus* [2], *Staphylococcus aureus* [3,4], *Escherichia coli* [5-11], *Salmonella enterica* serovar Typhimurium [12], and *Pseudomonas aeruginosa* [13,14]. Although it is unlikely that bacteriophages will ever replace antibiotics, they may be used when no other effective treatment is available, either alone or in conjunction with antibiotics.

Secondary infections with Gram-negative bacteria cause considerable morbidity and occasional mortality in patients with burns. Bacterial infections in patients who are admitted to hospital for burn injuries rapidly lead to overwhelming bacteremia, resulting in shock and subsequent death. Within 24 hours of a burn injury, patients are vulnerable to opportunistic bacterial attacks, which can vary from simple infection that is easily treated by antibiotics to more complicated infections caused by multiple drug–resistant bacteria. As an alternative to treating bacterial infections by antibiotics, bacteriophages have been in use in some parts of the world, including Georgia and Poland [15-20]. This approach to treatment of infection is now becoming widely recognized.

*Klebsiella* spp. are major causes of nosocomial infections, leading to morbidity and mortality among several patient populations. Of these groups, patients with burn injuries are particularly susceptible to life-threatening infections [21]. The occurrence of *Klebsiella*

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strains displaying multiple drug–resistance has greatly complicated therapy [22].

To examine the potential usefulness of bacteriophages and to contribute to the understanding of the situations in which bacteriophage therapy may be appropriate, this study investigated the therapeutic potential of bacteriophages in burn wound infections caused by *Klebsiella pneumoniae*.

## Methods

### Bacterial strain and bacteriophage

*K. pneumoniae* B5055 used in an earlier study [23] was obtained from the Department of Medical Microbiology and Hygiene, Ulm University Hospital, Ulm, Germany. The organism was stored on nutrient agar slants at 4°C and in glycerol stocks at –80°C. Purified bacteriophage KØ1 particles were stored in SM buffer (sodium chloride 100 mM, magnesium sulfate 8 mM, 2,3-dibromopropyl-chlorinate 50 mM [pH 7.5]) at –20°C.

### Animals

Male Swiss Webster LACA (Laboratory Animal Centre Albino) mice, aged 6 to 8 weeks and weighing 20 to 25 g, were obtained from the Central Animal House, Panjab University, Chandigarh, India. All animals were fed an antibiotic-free diet (Hindustan Lever Limited, Mumbai, India) and given food and water ad libitum.

### Bacteriophage titration

Bacteriophages were titrated using the soft agar overlay technique as described previously [24]. Nutrient broth containing 0.75% (w/v) agar was used for the overlay method. In brief, 100 µL of serially diluted bacteriophage was mixed with soft agar overlay containing 100 µL of bacterial suspension and poured over an agar plate. The plates were incubated at 37°C overnight and examined for the number of plaques.

### Animal model of burn wound infection

A burn model was established in mice following the method described previously [25]. To establish the infection, a fatal dose of bacteria, sufficient to cause systemic burn wound infection, was standardized. Three groups of 8 mice were anesthetized and their backs were cleansed and shaved. To induce burn in the backs of these animals, a brass bar ($10 \times 10 \times 100$ mm) was heated in boiling water for 15 min. The end of the heated bar was then applied to the shaved backs of the mice for 45 sec. Immediately after the burn was administered, each mouse was given an intraperitoneal bolus of 0.9% sodium chloride 0.5 mL as fluid replacement therapy, and acetaminophen 0.25 mg/mL in drinking water as an analgesic. After 30 min, 100 µL of the bacterial inoculum was injected subcutaneously into the burn site on the animal's back. The mice were then allowed to recover in a warm dry cage, given water and food ad libitum, and were monitored daily for symptoms and death.

A third-degree burn was confirmed by histopathology testing of the burned skin. Burned skin and normal skin (for comparison) was removed and fixed in 10% buffered formalin. Fixed tissue was dehydrated in different grades of alcohol and then dipped in molten wax. A 5-µm section of the tissue was cut with a fine razor attached to a Spencer microtome. The sections were placed on slides, fixed with Mayer's adhesive, and stained with hematoxylin and eosin stain.

### Determination of phage toxicity in mice

The toxicity of the bacteriophage to mice was investigated following the method described previously [11]. Briefly, 5 male LACA mice were injected intraperitoneally with 100 µL of pure bacteriophage preparation containing $10^{10}$ to $10^{11}$ plaque-forming units (PFU)/mL. Mice were then examined for signs of illness or lethargy. The rectal temperature of the mice was noted every hour for 5 h on the first day and once for the next 4 days. Mice injected with sterile phosphate-buffered saline (PBS) 100 µL acted as controls.

### Determination of bacteriophage survival in mice

The survival and stability of the bacteriophage in mice was determined following the method described previously [2]. Briefly, 8 uninfected mice were injected intraperitoneally with $10^8$ PFU of bacteriophage KØ1. At 1, 3, 6, and 24 h postinjection, the mice were euthanized, and the peritoneal cavity was lavaged with PBS 4 mL. The lavage fluid was collected by needle and syringe. Cardiac blood 100 µL was collected in ethylenediaminetetraacetic acid (EDTA) 0.05 M. A sample of lung was removed, weighed, and homogenized in PBS. All the samples were examined for PFU on *K. pneumoniae* B5055, as described above.

### Determination of 100% lethal dose of *Klebsiella pneumoniae* in a burn wound model

The 100% lethal dose ($LD_{100}$) of *K. pneumoniae* in a burn wound model inoculated via the subcutaneous route was determined by using the method described

previously [26]. Briefly, a burn wound was established in 3 groups of 8 mice each, and the mice were inoculated with different doses of *K. pneumoniae* ($10^4$, $10^6$, and $10^8$ colony-forming units (CFU)/mL). The percent survival was measured in each group for 10 days. The dose that resulted in 100% mortality by day 10 was considered the $LD_{100}$.

### Treatment of burn wound infection

To determine the effectiveness of bacteriophage for treatment of a burn wound infection, a burn wound was established in 4 groups of 8 mice each, as described above. The mice were subcutaneously infected with 10 times $LD_{100}$ of *K. pneumoniae* B5055. For treatment of infection by bacteriophage via the subcutaneous route, the mice were treated with the bacteriophage injected subcutaneously at the site of the burn wound 30 min and 6 h postinfection. The same protocol was followed for the treatment with the bacteriophage via the intraperitoneal route. After 1, 3, 6, and 24 h, the bacterial load in the blood, lung, and peritoneal cavity was assessed. A group of 5 mice that were infected but untreated with the bacteriophage acted as a control.

### Quantitative analysis of bacteria in tissue

The mice were euthanized and the peritoneal cavity was lavaged with PBS 4 mL. The lungs were removed, weighed, and homogenized in sterile PBS at a pH of 7.2. Cardiac blood 100 µL was collected in EDTA 0.05 M. Appropriate dilutions of the samples were plated on MacConkeys agar plate for the determination of CFU/mL or CFU/g of tissue.

### Statistical analysis

The results were analyzed by using Student's *t* test and calculating the *p* values. Tests were considered statistically significant at *p* values of <0.05.

## Results

Table 1 summarizes the characteristics of the bacteriophage KØ1. The toxicity of bacteriophage KØ1 when injected into non-infected mice via the intraperitoneal route was assessed on the basis of rectal temperature. There was no significant difference in the body temperature of the mice when compared with the body temperature of mice injected with PBS.

The maximum bacteriophage count in the blood and lungs was obtained 6 h postinjection, although maximum bacteriophage count in peritoneum was obtained 3 h postinjection (Fig. 1). The PFU/g of lung was approximately 0.1% and the PFU/mL of blood was 5.73 at 1 h postinjection. A significant decrease of approximately 4-log cycles was seen 24 h postinjection in all the mice ($p = 0.002$).

**Table 1.** Characteristics of the bacteriophage KØ1.

| Variable | Bacteriophage KØ1 |
|---|---|
| Morphology | Icosahedral head and short stubby tail |
| Order | Caudovirales |
| Family | Podoviridae |
| Head length (nm) | 77.91 ± 3.99 |
| Head width (nm) | 67.70 ± 3.25 |
| Tail length (nm) | 29.16 ± 1.15 |
| Genetic material | dsDNA |
| Size of phage genome (Kb) | 56.1 |
| Adsorption rate (min) | 4-5 |
| Latent period (min) | ~25 |
| Eclipse period (min) | ~20 |
| Burst size (vp) | 110-120 |

The $LD_{100}$ value via the subcutaneous route was found to be $10^6$ for *K. pneumoniae.* When the selected $LD_{100}$ was administered subcutaneously, death occurred by postinfection days 7 to 8. No mortality was noted in the non-infected burn control mice.

Fig. 2 shows the histopathological analysis of the burned skin compared with healthy skin. Complete destruction of the superficial skin layers, epithelial blistering, sloughing, and complete loss of cellularity was seen after a third-degree burn injury.

The results of the therapeutic efficacy of the bacteriophage in the blood, lungs, and peritoneal fluid are shown in Fig. 3. There was no significant decrease in the bacterial load in the blood 1 h post-treatment with

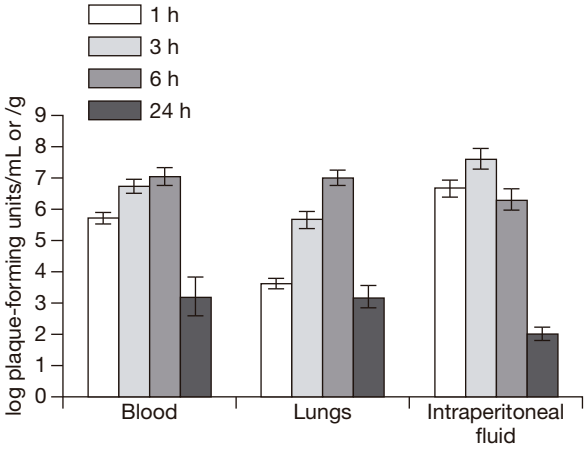


**Fig. 1.** Phage counts in blood, lungs, and peritoneum at different time intervals.

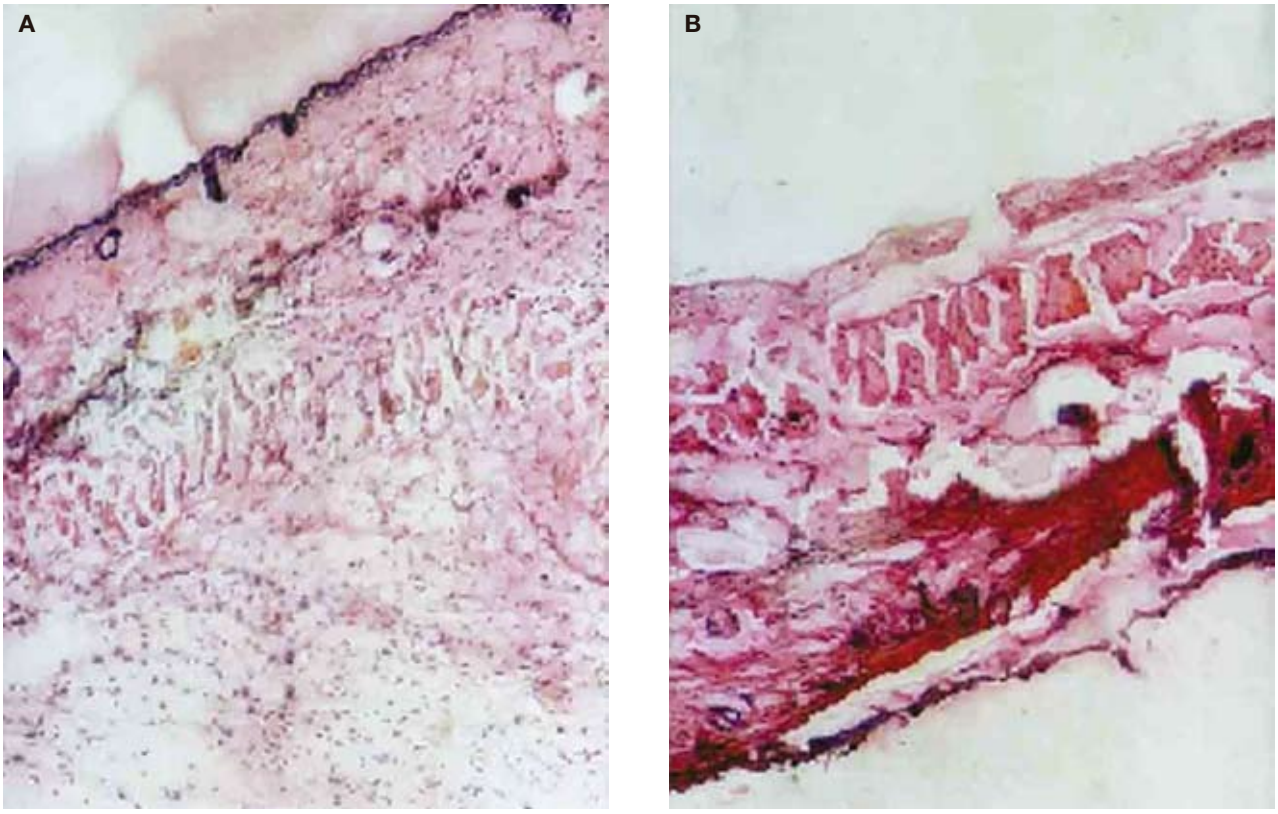


**Fig. 2.** Photomicrograph of (A) normal skin showing the intact epithelial layers and intact hair follicles and (B) skin burned for 45 sec showing complete destruction of superficial skin layers, epithelial blistering, sloughing, and complete loss of cellularity (hematoxylin and eosin stain, × 10).

bacteriophage KØ1 when given via the subcutaneous or intraperitoneal routes (Fig. 3A). However, after 3, 6, and 24 h, a significant decrease in the bacterial load of blood was observed ($p < 0.05$). A similar trend was observed in the lungs (Fig. 3B), although a significant decrease in the bacterial load of the lungs was only observed 6 h post-treatment via either route ($p < 0.004$). The level of protection continued until 24 h post-treatment. No change in the bacterial load was observed in the peritoneum 1 h post-treatment (Fig. 3C), but a significant decrease in the bacterial load was observed after 3 h, which remained stable until 24 h.

## Discussion

Bacteriophage therapy, which was discovered before antibiotics, was abandoned due to a paucity of knowledge about the classification and characterization of bacteriophages [1]. In recent years, renewed interest in this approach to treatment has been noted. Bacteriophage therapy can be effective for some conditions and has unique advantages over antibiotics. Interest in this therapy is increasing as a result of the continuing rise in the incidence of multiple antibiotic–resistant pathogenic bacteria [27]. With the deficit in the development of new classes of antibiotics to counteract these bacteria, there is a need to research new therapies, including bacteriophages for a range of infections.

Bacteriophage persistence in the mammalian host has been proposed to have an impact on the efficacy of bacteriophage treatment [10]. Rapid elimination of bacteriophages from the mammalian organism might reduce the number of bacteriophages to a level that is insufficient to combat the infecting bacteria. Hence, the stability and survival of bacteriophages in the host becomes an important issue for using bacteriophages as a therapeutic agent. In this study, complete clearance of the bacteriophage from the 3 sites was observed 36 h after injection. Similar findings are available in the literature [28,29]. Appelmans first reported that bacteriophages injected into the bloodstream of uninfected rabbits disappeared rapidly from the blood and internal organs, and persisted longest in the spleen [28]. This observation was confirmed in a number of later studies, such as that by Evans [29].

 

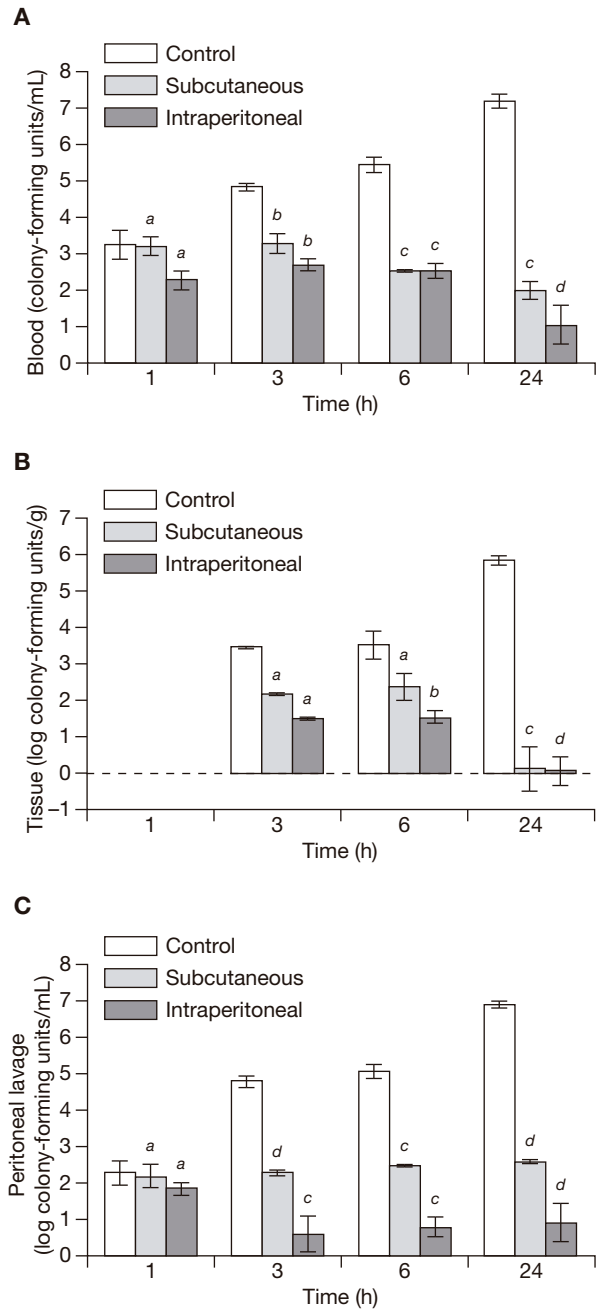


**Fig. 3.** Bacterial counts in the (A) blood, (B) lungs, and (C) peritoneum of experimental mice receiving treatment with bacteriophage KØ1 administered via the subcutaneous and intraperitoneal routes at different time intervals.
[a] $p > 0.05$.
[b] $p < 0.05$.
[c] $p < 0.01$.
[d] $p < 0.001$.

Studies of experimentally infected animals have shown that bacteriophages enter the bloodstream within 2 to 4 h, and are recoverable from the internal organs (liver, spleen, kidney) 10 h after injection [30,31].

To assess the ability of bacteriophages to treat burn wound infection, a full-thickness murine model of contact burn wound infection was made in LACA mice [19] using *K. pneumoniae* B5055, which is capsular type 2. This capsule type is the most common type isolated from patients with pneumonia, urinary tract infections, or bacteremia associated with burns [32]. The results of this investigation show that it is possible to protect the mice from lethal burn wound infection caused by *K. pneumoniae* by administering non-toxic bacteriophages via the subcutaneous or intraperitoneal routes. To check the therapeutic efficacy of bacteriophages, various researchers have used both these routes of administration in the past [3,10]. In a study conducted by Merril et al, the intraperitoneal route of bacteriophage administration was used to treat mice infected with *E. coli* strain CMR1 [10]. Matsuzaki et al have also used the intraperitoneal route of bacteriophage administration to ascertain the protection of mice against fatal *S. aureus* infection by novel bacteriophage ØMR11 [3]. Some researchers have also tried the intravenous route of administration [2]. In this study, the efficacy of the bacteriophage introduced via both the routes was assessed in terms of percentage survival of the experimental animals and decrease in the bacterial load in blood, lungs, and peritoneum at various intervals. There was 100% survival of experimental animals compared with 100% mortality for positive control animals (burnt and not treated), when treated with bacteriophage KØ1 alone. Evaluation of the hourly bacterial load of blood, lungs, and peritoneum following administration of bacteriophage via the subcutaneous or intraperitoneal routes showed no significant decrease in the bacterial load 1 h post-treatment with bacteriophage KØ1 at all 3 sites. However, decreases were significant 3 h after bacteriophage administration via both routes. A significant decrease was seen 6 h post-treatment in the lungs; this may be due to the additional time required for the bacteriophages to travel to the lungs. Since bacteriophages were able to transverse into the peritoneum and blood faster than into the lungs, a decrease in bacterial load was seen after 3 h. On the basis of these results, the intraperitoneal route of bacteriophage administration was thought to be better than the subcutaneous route.

This study highlights the importance of bacteriophages for the control of infections occurring as a sequela of burn wounds. Bacteriophages can be used effectively for the treatment of post-burn infections,

particularly the ubiquitous opportunistic pathogen *K. pneumoniae,* which is known to be resistant to a variety of antibiotics. Subcutaneous or intraperitoneal bacteriophage treatment of burn wound mice infected with *K. pneumoniae* can prevent mortality.

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