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Efficacy of Bacteriophage Treatment in Murine Burn Wound Infection Induced by *Klebsiella pneumoniae*

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In the present study, the therapeutic potential of purified and well-characterized bacteriophages was evaluated in thermally injured mice infected with *Klebsiella pneumoniae* B5055. The efficacy of five *Klebsiella* phages (Kpn5, Kpn12, Kpn13, Kpn17, and Kpn22) was evaluated on the basis of survival rate, decrease in bacterial counts in different organs of phage-treated animals, and regeneration of skin cells as observed by histopathological examination of phage-treated skin. Toxicity studies performed with all the phages showed them to be non-toxic, as no signs of morbidity and mortality were observed in phage-treated mice. The results of the study indicate that a single dose of phages, intraperitoneally (i.p.) at an MOI of 1.0, resulted in significant decrease in mortality, and this dose was found to be sufficient to completely cure *K. pneumoniae* infection in the burn wound model. Maximum decrease in bacterial counts in different organs was observed at 72 h post infection. Histopathological examination of skin of phage-treated mice showed complete recovery of burn infection. Kpn5 phage was found to be highly effective among all the phages and equally effective when compared with a cocktail of all the phages. From these results, it can be concluded that phage therapy may have the potential to be used as stand-alone therapy for *K. pneumoniae* induced burn wound infection, especially in situations where multiple antibiotic-resistant organisms are encountered.

Keywords: Sewage samples, bacteriophages, phage therapy, burn wound infection, *Klebsiella pneumoniae*, histopathological examination

The burn wound is considered as one of the major health problems in the world, the infection of which results in severe complications in patients who have sustained burns [15]. It has been estimated that 50% of all deaths following thermal injuries are related to infection [9, 28]. The infection is due to the combined effect of impairment of the host natural defense system, colonization of the burn wound site, and systemic dissemination of the colonizing organisms. *Klebsiella* and *Pseudomonas aeruginosa* are two major organisms frequently responsible for bacteremia in burn patients [23]. However, *Klebsiella* is the leading pathogen in Gram-negative bacillary invasive burn wound infections [10, 15, 24]. The most important species of this genus is *Klebsiella pneumoniae*, which is an opportunistic pathogen that primarily attacks immunocompromised individuals and is commonly associated with hospital-acquired urinary tract infections (UTI), pneumonia, septicemia, and wound infections [11, 27]. The occurrence of *Klebsiella* strains displaying multiple-drug resistance has greatly complicated burn therapy [7]. Because of this problem, attention of the scientists has been directed towards developing new therapeutic and prophylactic strategies for controlling such infections, especially in burn patients who do not respond to conventional antibiotic therapies.

An alternative therapy is the use of bacterial viruses (bacteriophage) to treat bacterial infections as a supplement to antibiotic chemotherapy [13, 18, 20, 31, 32]. Treatment with bacteriophage is associated with numerous benefits, as therapy is very economical, safe (host specific and don't disturb the microflora of body), and effective (at single dose owing to self-replicating nature) [20]. Although bacteriophage therapy has been successfully used in the treatment of various diseases in animals [6, 12, 17, 33, 35] and humans [5, 14, 25, 30, 34], scanty reports exist in the literature with regard to phage treatment of burn wound infections. We previously isolated five *Klebsiella* phages (Kpn5, Kpn12, Kpn13, Kpn17, and Kpn22) from sewage samples, and purified and characterized these phages morphologically, genetically, and structurally (manuscript communicated).

In the present study, the therapeutic potential of these well-characterized bacteriophages has been evaluated in a burn wound infection model in animals. The murine model of thermal injury used for burn wound generation and infection with *K. pneumoniae* B5055 was initiated by injecting bacterial culture subcutaneously (s.c.) directly under the anterior end of the burn. The efficacy of phage therapy was evaluated in this model on the basis of survival rates and

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bacterial counts detected in the blood, peritoneal fluid, lungs, and skin of different phage-treated mice.

MATERIALS AND METHODS

Bacteria

Klebsiella pneumoniae B5055, provided by Dr. M. Trautman, Department of Medical Microbiology and Hygiene, Ulm, Germany and maintained in our laboratory, was used. The strain was maintained on nutrient agar slants at 4±1°C.

Phage Isolation and Preparation

Five *Klebsiella* bacteriophages were isolated from sewage samples from different sources in and around Chandigarh area. The method of Cerveny *et al.* [8] was adopted for the isolation of phages from sewage samples, specific for *K. pneumoniae* B5055. Phage titer was determined by the soft agar overlay method described by Adams, [1]. The phages were numbered Kpn5, Kpn12, Kpn13, Kpn17, and Kpn22 and characterized on the basis of morphological, genomic, and structural analysis. High titer was prepared by adding phages to host culture at an MOI (multiplicity of infection) of 0.1 and incubating at 37°C, until complete clearance was achieved. The clear phage suspensions were centrifuged (10,000 rpm for 10 min) and filtered through a 0.45-μm pore size Millipore filter to remove any bacterial contaminants (Sambrook *et al.*, 1989). High titer of these phages was estimated and expressed as plaque forming unit (PFU)/ml. Phage suspensions were stored at 4°C for routine use.

Animals

Adult BALB/c mice, six weeks old, weighing 20–25 g, were obtained from Central Animal House, Panjab University, Chandigarh. All animals were given antibiotic free diet (Hindustan Liver Limited, Mumbai) and water *ad libitum*. Animal study was conducted following protocols approved by the Institutional Animals Ethical Committee of the University. Proper care of animals was taken during the study period. All the experiments were carried out in triplicate. The error bars in graphs are representative of the standard deviation in each experiment.

Murine Burn Wound Model

A full-thickness murine burn wound infection model was induced using *Klebsiella pneumoniae* B5055 following the method of Dale *et al.* [11]. Briefly, the hair was clipped from the back of anesthetized mice and the skin was denuded with a commercially available hair remover cream. Mice were anesthetized with ether fumes and burn was produced with the help of a heated brass bar (10×10×100 mm) for 45 seconds (s) and the extent of burn injury was confirmed by histopathological examination. Immediately after the burn, all the mice were injected i.p. with 0.5 ml of sterile physiological saline for fluid replacement to prevent overt shock and acetaminophen (0.25 mg/ml) was given as post burn analgesic in drinking water. Bacterial inoculum was prepared by incubating *K. pneumoniae* in nutrient broth at 37°C overnight followed by repeated centrifugation (10,000 rpm for 10 min) and washing, finally resuspending in normal saline. To determine the LD_{100} (lethal dose causing 100% lethality) value of *K. pneumoniae* culture, doses ranging from 10^2 to 10^8 CFU/ml (colony forming unit/ml) were injected s.c. directly under the anterior end of the burn in mice, after a waiting period of 30 min. Burned mice injected s.c. with phosphate buffer saline (PBS, pH 7.2) acted as controls. Mice inoculated with bacteria were scored for their state of health on a scale of 5 to 0, based on progressive disease state reflected by several clinical signs. A normal and unremarkable condition was scored as 5; slight illness, defined as lethargy and ruffled fur, was scored as 4; moderate illness, defined as severe lethargy, ruffled fur, and hunched back, was scored as 3; severe illness, with the above signs plus exudative accumulation around partially closed eyes, was scored as 2; a moribund state was scored as 1; and death was scored as 0. The dose giving 100% lethality was taken as the optimum LD_{100} dose.

Toxicity Testing of Phages

The toxicity of all *Klebsiella* phages was investigated in burned (compromised) mice according to the method of McVay *et al.* [21]. The burned but uninfected mice were injected i.p. with 0.25 ml phage suspensions of 10^8 PFU/ml (plaque forming unit/ml). The mice were scored for their state of health for 48–72 h. An arbitrary scale of 0–2 was used to score the state of health of mice at different hourly intervals after i.p. administration of the phage suspension. A score of 2 indicated normal unremarkable health, 1 for slight illness/lethargy/abnormal health, and 0 for death.

Treatment with Phages

The efficacy of *Klebsiella* phages to treat burn wound infection caused by *K. pneumoniae* B5055 in compromised mice was evaluated in two separate experiments.

In the first experiment, the survival rate of burned and bacterial challenged mice after treatment with different phages, individually as well as in cocktail, was evaluated. Seven groups of mice (12 mice in each) were used. A full-thickness burn was induced in all groups followed by challenge with LD_{100} of *K. pneumoniae* culture s.c. directly under the anterior end of the burn as described earlier. In group I, all the burned mice were challenged with bacterial inoculum and acted as control. In groups II-VII, burned and infected mice were treated with single injections of the five *Klebsiella* phages individually as well as in cocktail, administered i.p. at MOI of 1.0 immediately after burn/bacterial challenge. The state of the health of these animals was monitored for 10 days and survival rates for control and phage-treated groups were recorded.

The second experiment was performed to compare the bacterial counts in different organs of control and phage-treated mice at different time intervals. Seven groups of mice (12 mice in each) were used. In all the groups, full-thickness burn was induced in mice, followed by challenge with LD_{100} of *K. pneumoniae* culture s.c. directly under the anterior end of the burn, and all five phages individually as well as in cocktail were administered i.p. at MOI of 1.0 immediately after the bacterial challenge, as described earlier. One hundred μl of blood sample was collected periodically from the retro-orbital plexus of mice in tubes containing 0.05 M EDTA. Two mice each at 24, 48, and 72 h were sacrificed by cervical dislocation. Peritoneal lavage fluids and lungs were harvested aseptically. Lung sample 1 g was homogenized in 1 ml of phosphate buffer saline (pH 7.4) with the help of a hand-held glass homogenizer. A section of burned and infected skin (5×5 mm) was obtained and homogenized. The number of CFU in each sample was determined by making serial dilutions of the homogenate and plating it on nutrient agar plates.

Histopathological Examination

Extent of injury caused by burn and regeneration of skin cells were assessed on the basis of histopathological analysis of the 45 sec burned

skin and recovered skin (11th and 20th days after burn, bacterial challenge, and phage treatment) and compared with the normal mouse skin. Skin samples preserved in 10% formalin were dehydrated in ascending series of alcohol (70–100%). The tissue was embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (Hi-Media, Mumbai) [4].

Statistical Analysis

Data are expressed as means±standard deviation (SD) of the mean, and statistical analysis was performed with Graph Pad Instat Software (Version 3.00; GraphPad Software, San Diego, CA, U.S.A.) using the Student's *t* test for calculations of mean and standard deviation, whereas one-way analysis of variance (ANOVA) followed by Boneferroni test was used for multiple comparisons. Difference with $p \leq 0.05$ was considered statistically significant.

Results and Discussion

Isolation and Screening of Bacteriophages Specific to *K. pneumoniae*

Bacteriophages specific to *K. pneumoniae* B5055 (Kpn5, Kpn12, Kpn13, Kpn17, and Kpn22) were isolated from sewage samples collected from different locations and screened for their lytic activity. All phages reduced turbidity of the culture and complete lysis was obtained in 3–6 h. The ability of lytic phages to rapidly kill and lyse infected bacteria, the specificity of phages for particular bacteria, and the ability of phages to increase in number during the infection process make phages excellent potential diagnostic and therapeutic agents for fighting bacterial diseases. All the phages exhibited potent lytic activity and thus were selected for *in vivo* studies. These five phages were characterized on the basis of morphology, genetic material, and structural protein composition. The morphology of viral particles was observed by transmission electron microscopy and all the five phage particles possessed icosahederal heads and very short stumpy tails, assigning them to family *Podoviridae*, order *Caudovirales*. As all the phages were members of family *Podoviridae*, morphotype C and subdivision C1, all of them displayed similar protein electrophoretic migration patterns. All *Klebsiella* phages harbored double-stranded DNAs and these were genetically distinct, as determined on the basis of restriction digestion and randomly amplified polymorphic DNA (RAPD) analysis (manuscript submitted). These different *Klebsiella* phages were then selected for further *in vivo* studies.

Establishment of Full-Thickness Burn Wound Infection in Mice with *K. pneumoniae* B5055

In this study, we developed a burn wound mouse model to determine the potential of phages in curing *K. pneumoniae* induced burn wound infection in mice. To develop a murine model of thermal injury, burn time of 45 s was found to be optimum for the establishment of full-thickness third-degree burn wound, which is the same as reported earlier [11]. The LD_{100} value of bacteria administered *via* s.c. route at the burn site, was determined and optimized as 10^6 CFU. This dose was found to be sufficient to cause burn wound infection in mice, resulting in 90–100% mortality within 48–72 h post infection, which corroborated with the findings of McVay and coworkers [21]. A probable reason for such high mortality might be due to the rapid proliferation and quick systemic spread of *Klebsiella pneumoniae* from skin to underlying tissue, as higher bacterial counts were detected in the blood, peritoneal lavage fluid, and tissues (lungs and skin) at 24 h post infection. Our finding is supported by an earlier study by Rumbaugh *et al.* [26], where 10^4 $PAO1^{Rif}$ CFU per gram of liver and spleen was detected within 24 h post inoculation. All burned mice receiving PBS only (control) did not show any signs of bacteremia or slight illness as shown in Fig. 1.

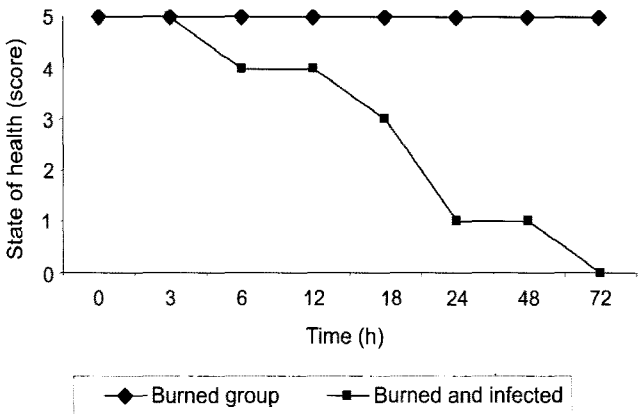

Fig. 1. Lethality of 10^6 CFU/ml of *K. pneumoniae* B5055 in burned mice. 5, Normal health; 4, slight illness, defined as lethargy and ruffled fur; 3, moderate illness, defined as severe lethargy, ruffled fur, and hunched back; 2, severe illness, with the above signs plus exudative accumulation around partially closed eyes; 1, a moribund state; 0, death.

Toxicity Testing of Phages in Compromised Mice

One of the criteria for the phage therapy is its non-toxicity. Therefore, selected phages were tested for their toxicity in compromised (thermally injured) mice. Toxicity testing data suggested that all phage-treated mice (without *K. pneumoniae* infection) survived with score of 2 at arbitrary scale, indicating that the phages were not toxic to thermally injured mice. The non-toxicity of the phage may be attributed to the absence of any bacterial contamination in the phage suspension so that the phage preparations were safe enough to be administered to compromised mice and thus were considered for further *in vivo* use [2, 21].

Phage Protection Study

Once phages were confirmed to be non-toxic in compromised mice, the ability of *K. pneumoniae* phages, individually as well as in cocktail, to prevent *K. pneumoniae* infections

Table 1. Protection studies: Efficacy of phage therapy on *K. pneumoniae* B5055 induced burn wound infection in mice.

Groups	Survival rate (percentage)[a]		
	24 h	48 h	72 h
Control	55.87±9.367	19.44±4.815	05.53±9.619
Kpn5-treated group	100.00±0	97.22±4.815	97.22±4.815
Kpn12-treated group	97.22±4.815	94.44±4.815	88.88±4.809
Kpn13-treated group	94.44±4.815	88.88±9.624	83.22±8.166
Kpn17-treated group	88.88±4.809	88.88±4.809	80.55±9.619
Kpn22-treated group	91.66±8.335	86.10±9.619	86.10±9.619
Cocktail	97.22±4.815	94.44±4.815	94.44±4.815

[a]The average percent survival of animals in three experiments was determined as mean±standard deviation. The rate of survival of animals receiving the phages individually as well as in cocktail was significantly greater ($P<0.001$) when compared with untreated infected control animals at 24, 48, and 72 h post infection time periods, respectively.

was examined in a mouse model of thermal injury in terms of survival rate and decreased bacterial counts in different organs at 24, 48, and 72 h. The five different phages were found to be effective in treating burn wound infection in mice following a single i.p. injection of individual phage or in a cocktail of five phages at a MOI of 1.0. Intraperitoneal route of phage administration was chosen in the present study because the most significant protection (87%) was observed *via* this route in comparison with intramuscular (i.m). and s.c. route as reported in the literature [19, 21, 22]. Moreover, a single dose of these phages resulted in complete eradication of bacterial infection in the burn wound model. Our finding is supported by an earlier study, where Benedict and Flamiano [2] showed that a single i.p. injection of 0.5 ml of each of the phage lysate was enough to rescue all mice back to normal health from *Escherichia coli*-induced lethal bacteremia. These results also corroborate the results of an earlier study by Biswas and coworkers [3], in which a single i.p. injection of phage ENB6 rescued 100% of vancomycin-resistant *Enterococcus faecium* (VRE) bacteremic

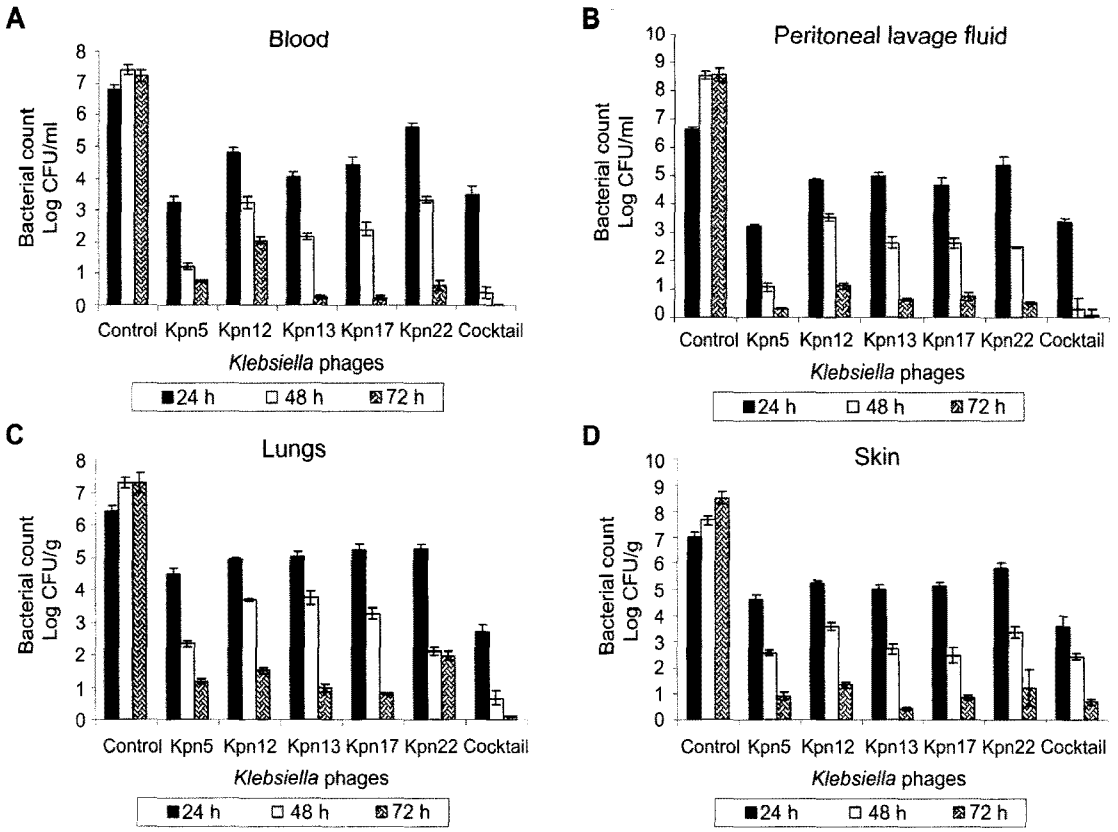

Fig. 2. Comparison of bacterial load in the different organs of burned, infected, and treated mice with different *Klebsiella* phages, individually as well as in cocktail at 24, 48, and 72 h post inoculation.

A. Bacterial load CFU/ml in blood. **B.** Bacterial load CFU/ml in peritoneal lavage. **C.** Bacterial load CFU/g of lungs. **D.** Bacterial load CFU/g of skin. Values represent mean±standard deviation (n=3).

mice even if treatment was delayed up to 5 h. This has been explained on the basis of a population dynamics model, which demonstrates that the exponential growth in numbers of phage particles and their self-replicating nature enabled a single injection of phage to be superior to multiple injections of antibiotics [16]. Additionally, another study supports this observation where mice inoculated with lethal intramuscular or intracerebral injection of *E. coli* were rescued by using a single injection of a coliphage preparation [29].

Different *Klebsiella* phages were evaluated for their efficacy in terms of survival rate and decrease in bacterial load in different organs of phage-treated groups at different time intervals. All of burned and infected mice died within 48–72 h with a 5.53% survival rate, whereas all burned, infected, and phage-treated mice survived, showing 80–100% protection ($p<0.001$) at 24, 48, and 72 h post inoculation. Among all phages, phage Kpn5 showed significant highest percentage survival (97.22%) at 72 h post inoculation ($p<0.001$), as shown in Table 1. A significant decrease ($p<0.001$) in bacterial counts in blood, peritoneal fluid, lungs, and skin was observed in all *Klebsiella* phage-treated groups individually when compared with control at 72 h (Fig. 2). On comparison of effectiveness of different phages, it was observed that phage Kpn5 was the most effective ($p<0.001$), resulting in substantial decrease in bacterial load in different organs as compared with other phages used in this study. The results in terms of percentage survival and bacterial load in different organs obtained with phage Kpn5 were comparable with the animal groups that were treated with a cocktail of all the five phages. The protection was due to significant decrease in bacterial load, indicating that viruses were able to locate and kill *K. pneumoniae in vivo* before the animal succumbed to bacteremia and septic shock.

Histopathology

These results were also confirmed on the basis of histopathological examination of burned and recovered skin. A full-thickness burnt skin showed complete loss of skin layers, whereas recovered skin after 11th day post phage treatment showed reappearance of epidermis, although still showing edema in the dermis, without sweat glands and hair follicles. A fully recovered skin (20th day post phage treatment) showed complete regeneration of skin layers,

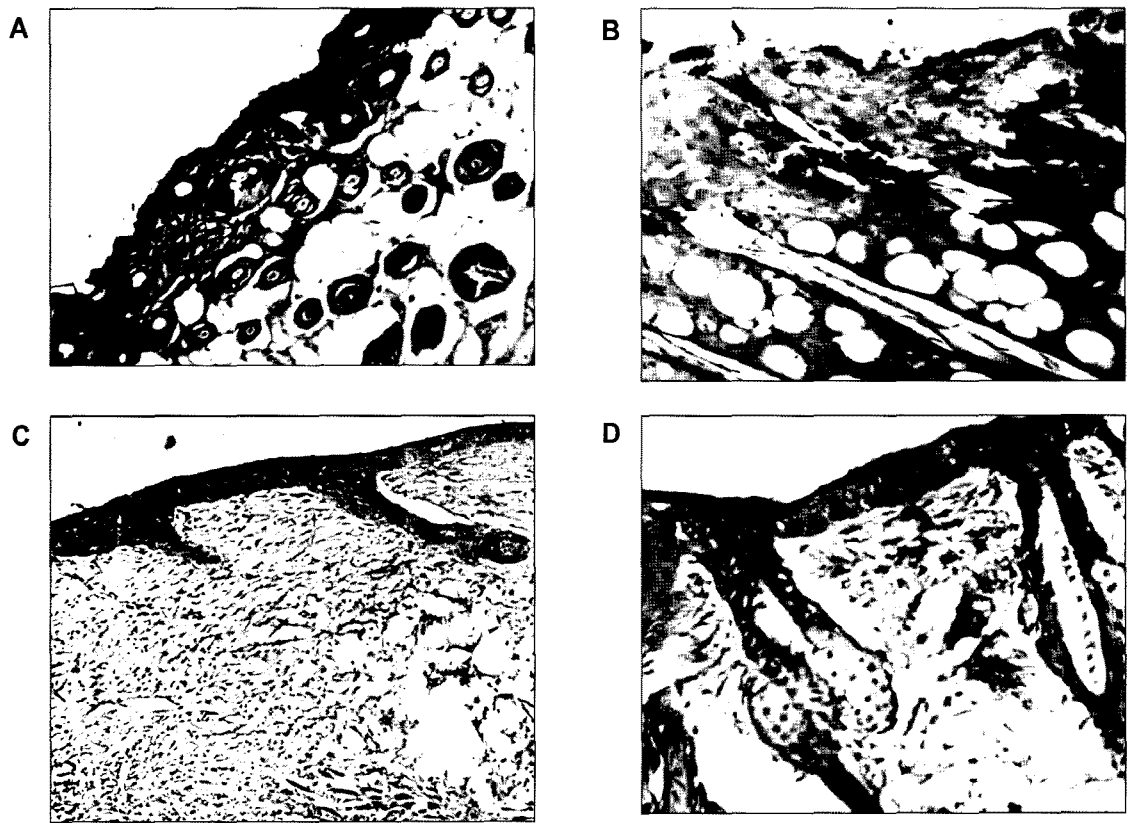

Fig. 3. Histopathological features of normal, thermally injured, and recovered skin of mice.
A. Normal skin of mouse with multiple layers of squamous cells in the epidermis. The dermal layers contain hair follicles, sweat glands, and deeper layer of muscles. **B**. Full-thickness burned skin showing complete destruction of superficial skin layers, which has been coagulated into an eosinophilic mass. Dermal collagen and elastic tissues have been converted into a hyaline coagulated mass in which some fat cells are present. **C**. 11th day recovered skin showing the regenerated epidermis and inflammation and fibrosis in the dermis. The hair follicles and sweat glands have disappeared. **D**. 20th day recovered skin showing the regenerated epidermis and reappearance of hair follicles and sweat glands.

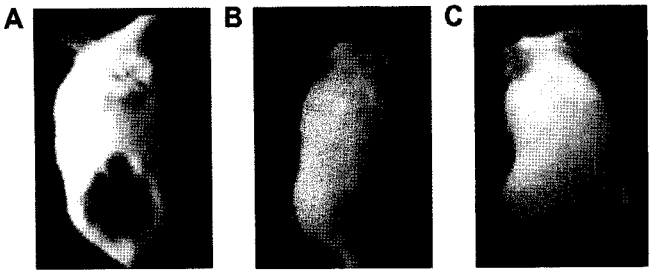

Fig. 4. Photographs of mice showing phage treatment and recovery of skin from burn wound infection. **A.** A mouse showing full-thickness burn. **B.** Recovered mouse on 20th day post burn showing complete recovery. **C.** Normal mouse.

hair follicles, and sweat glands comparable to the normal mouse skin (Fig. 3). The process of regeneration of skin cells and healing of burn wound leading to appearance of normal fur at the site of burn wound was observed (Fig. 4).

On the basis of these results, we can conclude that bacteriophages were found to be safe and effective in the complete elimination of *Klebsiella* infection from a burn wound site in the murine model. This current study therefore reinforces the view that phage therapy can be used to treat bacterial infections of burnt sites, especially in situations where resistance to antibiotics hinders the routine line of treatment.

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