Bacteriophage versus antimicrobial agents for the treatment of murine burn wound infection caused by Klebsiella pneumoniae B5055

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INTRODUCTION

Mainly because of the larger area involved and longer duration of patient stay in hospital, burns provide a suitable site for bacterial multiplication and are a more persistent, richer source of infection than surgical wounds (Ozumba & Jiburum, 2000; Mayhall, 2003). The risk is further exacerbated by immunosuppression associated with burn injury (Cook, 1998). In addition, the burn surface, which contains a large amount of necrotic tissue and protein-rich wound exudate, provides a rich growth medium (Erol et al., 2004; Tanuja et al., 2004) and hence infection remains the principal cause of death in burn patients (Heggars et al., 1991; Pruitt et al., 1998).

Third-degree burns, which are the most severe form of burn, usually need dressing with appropriate medication in order to prevent infection (Panjeshahin et al., 2001). Topical antimicrobial agents are essential adjuncts in the prevention and treatment of burn wound infections (Ollstein & McDonald, 1980; Noronha & Almeida, 2000). Several groups of topical medications and antibiotics such as silver nitrate, gentamicin and polymyxin B have been used in the treatment of wound infection of burns (Moyer et al., 1965; Fox, 1968; Snelling et al., 1978; Steen, 1993; Ward & Saffle, 1995). Because systemic antibiotics are ineffective in reducing bacterial counts in granulation wounds, the use of suitable topical antibacterial agents may substantially decrease wound sepsis and benefit overall burn wound management (Manafi et al., 2008).

The widespread use of antimicrobial agents in hospital settings has led to the emergence of multidrug-resistant organisms of low virulence such as Klebsiella causing serious opportunistic infections (Shukla et al., 2004). Beside this, concerns about problems such as high cost of treatment and inability to restore initial appearance of skin have resulted in research on newer agents for the treatment of burn wounds (Church et al., 2006; McVay et al., 2007). Bacteriophages or simply phages can be the best answer to antibiotic resistance in the treatment of bacterial infections (Matsuzaki et al., 2005; Hanlon, 2007; Skurnik et al., 2007). These phages are considered to be economical, safe, self-replicating and effective bactericidal agents (Inal, 2003; Bradbury, 2004). In our earlier studies, we have reported the efficacy of phage therapy in treating various infections when injected systemically (Chhibber et al., 2008; Bedi et al., 2009; Kumari et al., 2009; Malik & Chhibber, 2009). In the present study, the efficacy of topical application of silver nitrate and gentamicin was evaluated and compared with that of a well-characterized Klebsiella-specific phage, Kpn5, for treating K. pneumoniae B5055 induced burn wound infection in BALB/c mice.
METHODS

Bacterial strain and growth media. *K. pneumoniae* B5055, obtained from Dr Matthias Trautmann, Ulm University Hospital, Germany, and maintained in the laboratory, was used in this study. The strain was maintained on nutrient agar slants at 4 °C.

Antimicrobial agents. Silver nitrate marketed by E. Merck India and gentamicin (sulfate) marketed by Nicholas Piramal India were used in this study.

Animals. Adult BALB/c mice, 6 weeks old, weighing 20–25 ± 5 g were obtained from Central Animal House, Panjab University, Chandigarh. All the animals were given an antibiotic-free diet (Hindustan Lever) and water ad libitum. The animal study was conducted following approval of the Institutional Animals Ethical Committee. 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 burn wound infection was established in mice using *K. pneumoniae* B5055 via the topical route (Dale et al., 2004). Briefly, skin was denuded with a commercially available hair-removing cream and mice were anesthetized with ether fumes. A third-degree burn was induced by applying a heated brass bar (10 × 10 × 100 mm) for 45 s. Immediately after the burn, all the mice were injected intraperitoneally (i.p.) with 0.5 ml sterile physiological saline for fluid replacement to prevent overt shock, and acetaminophen (0.25 mg ml⁻¹) was given as postburn analgesic in drinking water. The bacterial inoculum was prepared by incubating a loopful of *K. pneumoniae* B5055 in nutrient broth at 37 °C overnight followed by repeated centrifugation (10 000 r.p.m. for 10 min) and washing. Finally, the organisms were suspended in normal saline. To determine the LD₁₀₀ (lethal dose causing 100 % mortality) value of the *K. pneumoniae* culture, doses ranging from 10⁵ to 10¹⁰ c.f.u. ml⁻¹ were applied topically evenly on the burn site, after a waiting period of 30 min. Burned mice injected subcutaneously with PBS (pH 7.2) acted as controls. Burned mice inoculated with either bacteria or PBS were monitored for any signs of morbidity and mortality. The dose giving 100 % lethality within a period of 48–72 h was taken as the optimum LD₁₀₀ dose.

Phage isolation. *Klebsiella*-specific phage Kpn5 was isolated from a sewage sample. Its utility in treating *K. pneumoniae* B5055 induced burn wound infections on i.p. injection has been established (Kumari et al., 2009). In this study, phage Kpn5 was evaluated for topical treatment of burn wound infection.

Hydrogel preparation. Hydroxypropylmethylcellulose (E464; HPMC) hydrogel was prepared according to the method of Cooper (1989) to suspend phage Kpn5 for topical application. Hydrogels were prepared with various concentrations of polymers, e.g. 1 %, 2 % and 3 %. Briefly, the required amount of polymer was dissolved in warm water and stirred with a mechanical stirrer at a speed of 100 r.p.m. for 15 min. When the gel became homogeneous in consistency, it was kept in a vacuum oven at room temperature to remove entrapped air. The gel was stored at 8–15 °C for further use. HPMC at 3 % concentration provided the appropriate viscosity required for the topical treatment of burn wounds on mouse skin. Before application, it was sterilized by autoclaving at 68.94 kPa for 30 min and checked for any change in viscosity.

Toxicity of hydrogel on mouse skin. A skin irritation test was conducted according to the method of Baskettet et al. (2004) to confirm that hydrogel was not a skin irritant even under highly exaggerated exposure conditions for varying periods of time.

In this experiment, five groups each containing six mice were taken. All the mice were shaved with the help of commercially available hair-removing cream. In group I, no hydrogel was applied and these mice acted as controls. In groups II, III and IV, a 0.5 ml sample of the hydrogel was applied for 4, 12 and 24 h (single time), respectively, onto the shaved back of each mouse in a 1.0 square inch area. In group V, a 0.5 ml sample of the hydrogel was repeatedly applied and each application was left for a minimum of 24 h (three applications in a 5 day period). A double gauze layer was applied on the skin, the patches were covered with a non-reactive tape and the entire test site was wrapped with a binder. The test site was observed for signs of irritation (development of a rash, inflammation, swelling, scaling and abnormal tissue growth in the affected area) over the next 5 days.

Effect of hydrogel on MICs of gentamicin or silver nitrate. Gentamicin or silver nitrate was mixed with hydrogel preparation in different proportions for different time intervals and the effect of hydrogel on these two antimicrobial agents was determined.

Efficacy of topical application of silver nitrate in treating *Klebsiella* induced burn wound infection. The MIC of the silver nitrate was determined for *K. pneumoniae* B5055 by the tube dilution method of Atkinson (1980). Three groups of mice (10 mice in each) were taken. A full-thickness burn was induced in all the groups and challenged with the LD₁₀₀ of *K. pneumoniae* culture topically directly on the burn site as described earlier. In group I, all the burned mice were challenged with bacterial inocula and acted as controls. In group II and group III, using a sterile spatula burned and infected mice were treated by applying the MIC or 0.5 % of silver nitrate mixed with hydrogel topically (daily) immediately after burn/bacterial challenge. The state of health of these animals was monitored up to 7 days and the survival rate for control and treated groups was recorded during this period.

Efficacy of topical application of gentamicin in treating *Klebsiella* induced burn wound infection. Three groups of mice (10 mice in each) were taken and a full-thickness burn wound infection was induced in all the groups as described earlier. In group I, all the burned mice were challenged with bacterial inocula and acted as controls. In group II and group III, the wound was treated daily with gentamicin by applying either the MIC (determined by the tube dilution method) or 1000 mg l⁻¹ mixed with hydrogel with the help of a sterile spatula immediately after burn/bacterial challenge. All the animals were monitored for any morbidity and mortality and the survival rate for control and treated groups was recorded.

Stability of phage Kpn5 in 3 % HPMC hydrogel. The stability of *Klebsiella*-specific phage Kpn5 was checked in 3 % HPMC hydrogel for varying periods of time. One millilitre of phage Kpn5 was taken and mixed with 1.0 ml 3 % HPMC hydrogel to attain a titre of 10⁹ p.f.u. ml⁻¹. Mixtures were stored at 37 °C for a period of 7 days. Every day, phage number was quantified in this ointment by using the plaque assay method (Adams, 1959).

Efficacy of topical application of phage Kpn5 in treating *Klebsiella* induced burn wound infection. The efficacy of phage Kpn5 in treating burn wound infection in mice was evaluated in each group. Burn wound infection was induced in two groups (10 mice in each) as described earlier. In group I, all the burned mice were challenged with bacterial inoculum and acted as controls. In group II, burned/infected mice were treated with a single injection of 0.5 ml Kpn5 phage (10¹⁰ p.f.u. ml⁻¹) mixed with hydrogel at an m.o.i. (i.e. the ratio of phage count to bacterial count) of 200. The state of the health of these animals was monitored for 7 days and survival rate for control and treated groups was recorded.

Statistical analysis. Data are expressed as means ± standard deviation of the mean. Statistical analysis was performed with GraphPad Instat Software (version 3.00) using Student’s t-test for calculations of the mean and standard deviation while one-way
hydrogel preparation. Results showed no change in the viscosity of hydrogel preparations upon autoclaving, as the viscosity curve, which is a plot of viscosity versus shear rate, showed a straight line for the hydrogel preparation (data not shown). Therefore, prepared hydrogel was described as a Newtonian fluid. According to this, viscosity is dependent upon temperature but independent of the applied shear rate (forces acting upon it). The flow curve, which is a plot of shear rate versus shear stress, of both autoclaved and unautoclaved hydrogel preparations was found to be linear and passed through the origin (data not shown). On the basis of all the parameters, the prepared hydrogel preparation was described as a Newtonian fluid.

Toxicity testing of 3 % HPMC hydrogel on mouse skin

The mice in all hydrogel-treated and untreated groups did not show any sign of irritation when observed for a period of 7 days after single and multiple hydrogel applications on shaved mouse skin. Therefore, the 3 % HPMC hydrogel preparation was found to be non-toxic to mouse skin and selected as ointment for the topical treatment of burn wound infection in mice.

Establishment of a third-degree burn wound infection via the topical route

The results presented in Fig. 1 show that a dose of \(10^8\) c.f.u. was found to be sufficient to cause burn wound infection in mice. This resulted in sickness and all the animals succumbed to infection within 48–72 h following bacterial challenge in burned mice. All burned mice (control) receiving PBS (pH 7.2) only did not show any sign of illness.

Determination of lack of activity of hydrogel on silver nitrate and gentamicin

Silver nitrate and gentamicin were found to be stable when mixed with hydrogel, suggesting a lack of any activity on the MICs of both the antimicrobial agents (data not shown).

Efficacy of silver nitrate as an antibacterial agent

The untreated control mice showed 100 % mortality whereas, during the same period, silver nitrate provided protection in terms of increased percentage survival of burned and infected mice. As illustrated in Fig. 2, a percentage survival of 93.33 % and 96.66 %, respectively, was observed in groups treated with either 0.5 % silver nitrate or 0.0005 % silver nitrate (MIC) after 24 h as compared to untreated control group with 93.33 % survival (\(P>0.05\)). Thereafter, a decline in survival rate was observed. A survival of 56.66 % with 0.5 % silver nitrate as compared to untreated control (0 % survival) and 0.0005 % silver nitrate (16.66 %) was observed after 7 days.

Efficacy of gentamicin as an antibacterial agent

A high percentage survival of 93.33 % was observed in all the groups following treatment with gentamicin at 7 mg l\(^{-1}\) (MIC) and 1000 mg l\(^{-1}\) after 24 h (\(P>0.05\)). From the third day onwards, percentage survival in all treated groups decreased. A significantly higher survival (\(P<0.001\)) of 53.33 % in the group treated with 1000 mg gentamicin l\(^{-1}\) was seen as compared to untreated control (0 % survival) and mice treated with 7 mg gentamicin l\(^{-1}\) (13.33 %) over a 7 day period (Fig. 3).

Efficacy of phage Kpn5 in topical treatment of burn wound infection

A non-significant decrease in phage titre (\(P>0.05\)) over a period of 7 days indicated 100 % stability of phage in the 3 % HPMC hydrogel preparation. This preparation was therefore used, and the results in Fig. 4 show that phage Kpn5 provided protection on the first day, as 100 % survival was observed at an m.o.i. of 200 as compared to 86.66 % survival in the phage-untreated (control) group (\(P>0.05\)). With time, percentage survival went on decreasing in both the control and phage-treated groups. However, the phage-treated group showed the highest level of protection (63.33 %), which was statistically significant.
Comparison of the antibacterial activity of silver nitrate, gentamicin and phage Kpn5 in topical treatment of burn wound infection

A comparison of the results obtained with the two antimicrobial agents and phage Kpn5 showed that Kpn5 was found to be most effective followed by 0.5 % silver nitrate and 1000 mg gentamicin l$^{-1}$, respectively. Lower concentrations of silver nitrate and gentamicin did not afford any protection in terms of percentage survival. The percentage survival was 63.33 %, 56.66 % and 53.33 %, respectively, with phage Kpn5, silver nitrate (0.5 %) and gentamicin (1000 mg l$^{-1}$); this difference was not statistically significant ($P > 0.05$).

DISCUSSION

Different antibacterial agents have been used over the years for the treatment of burn wound infections. Antibiotics have been the mainstay of treatment and gentamicin is the drug commonly employed for topical treatment. However, development of resistance towards antibiotics prompted scientists to look for alternative regimes for burn wound treatment. Attention was directed towards silver nitrate and, based on the results of different studies, silver is regarded as a broad-spectrum alternative (O’Neill et al., 2003; Lansdown, 2005; Ulku¨r et al., 2005; Ip et al., 2006; Chopra, 2007). In one of the studies from our laboratory, multidrug-resistant *K. pneumoniae* was found to be sensitive to very low concentrations (5–10 p.p.m.) of silver nitrate (Kapoor et al., 1989). However, its *in vivo* application has shown some limitations for using its products in burn treatment (Dunn & Edwards-Jones, 2004). In recent years, phage therapy has been considered another alternative for the treatment of various infections including those of burns because of its associated advantages (Wills et al., 2005; Wang et al., 2006; Vinodkumar et al., 2008; Górski et al., 2009). In the present study, all three modes of treatment were compared in the burn wound model established in BALB/c mice using *K. pneumoniae* B5055. The results showed the

(P<0.001) when compared with the untreated (control) group (0 %).
effectiveness of both gentamicin and silver nitrate in affording protection against infection in terms of increased survival rate among the treated animals. In both cases, the concentrations of the two compounds did influence the outcome of treatment. Both the compounds were ineffective at the MIC level, but restricted microbial infection at doses that were higher than the MIC but well within the recommended levels.

It seems unlikely that phage therapy will ever replace antibiotics; however, with the increasing incidence of antibiotic-resistant bacteria, there is a clear potential for it to be used in a complementary fashion. This is particularly true in cases where phages can be applied externally (topically) and are, therefore, less likely to be removed by the immune system. Keeping this in mind, the therapeutic potential of phage Kpn5 as a topical agent was evaluated for the treatment of burn wound infection. Phage Kpn5 (at high m.o.i. 200) provided protection when mixed with 3% HPMC hydrogel and applied topically to treat infection caused by K. pneumoniae B5055. A significantly higher percentage survival of 63.33 % (P<0.001) was obtained as compared to 0% survival in PBS-treated control mice when observed over a 7 day period. On the basis of this observation, it can be speculated that phage Kpn5 was able to release itself from 3% hydrogel and penetrate the wound area. The phage was able to locate bacteria in the body before the animal succumbed to bacteraemia and septic shock.

This study highlights the importance of silver nitrate, gentamicin and phage Kpn5 for controlling burn wound infections caused by nosocomial pathogens such as K. pneumoniae. A single application of phage Kpn5 was found to be superior to multiple applications of silver and gentamicin in the treatment of burn wound infection caused by K. pneumoniae B5055 in BALB/c mice. Studies using these compounds in combination to treat burn wound infection are warranted. This will not only increase the survival of the infected animals but such a strategy will also keep a check on the development of resistant mutants, a problem frequently encountered by clinicians. In a recent study, we have demonstrated such an effect on treating biofilms of K. pneumoniae B5055 with a combination of ciprofloxacin and phage (Verma et al., 2009).

ACKNOWLEDGEMENTS

Ms Seema Kumari acknowledges the fellowship received from University Grant Commission (UGC), New Delhi. We are grateful to Dr V. R. Sinha, Professor (Pharmaceutics), University Institute of Pharmaceutical Sciences, Punjab University, Chandigarh, for his help in carrying out hydrogel preparations.

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