Original Article

Topical treatment of *Klebsiella pneumoniae* B5055 induced burn wound infection in mice using natural products

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Abstract

Background: Burn wound infection remains the principal cause of death in burn patients. Efficacy of honey and aloe vera gel was evaluated in the treatment of burn wound infection induced with *Klebsiella pneumoniae* B5055 and their efficacy was compared with an isolated and well-characterized *Klebsiella* specific phage Kpn5.

Methodology: A full thickness burn wound was induced in mice and infected with *K. pneumoniae* B5055 via topical route. The efficacy of natural antimicrobial agents (honey and aloe vera gel) topically applied daily was compared with the efficacy of phage Kpn5 suspended in hydrogel applied topically a single time on the burn wound. Efficacy of these antimicrobial agents was assessed on the basis of the percentage of infected mice that survived following treatment.

Results: In comparison to untreated control mice, those treated with a single dose of phage Kpn5 at MOI of 200 showed significant reduction in mortality ($P < 0.001$). Daily application of honey and aloe vera gel provided significant protection ($P < 0.001$), but in combination with phage, no additional advantage was observed ($P > 0.05$) compared to the use of honey and aloe vera gel alone.

Conclusions: The results of this study strongly suggest that phage Kpn5 has therapeutic value in treating burn wound infection in mice as a single topical application of this phage was able to rescue mice from infection caused by *K. pneumoniae* B5055 in comparison to multiple applications of honey and aloe vera gel.

Key words: *Klebsiella pneumoniae*, honey, aloe vera gel, HPMC hydrogel

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Introduction

Skin serves as a barrier to water and various pathogens [1]. Thermal injury destroys this barrier that normally prevents invasion of bacteria, fungi and viruses [2]. The increased susceptibility to infection after major burns is dependent on increased access of pathogens and reduced immune competence [3]. Additionally, the risk of infection in severe burns is well known as the burn wound consisting of moist necrotic tissue represents an ideal culture medium for a wide variety of microorganisms [4]. It has been estimated that more than 75% of the mortality following burn injuries is related to infections rather than osmotic shock and hypovolemia [5]. Although advances in burn care have reduced the mortality rates, burns remain a major public health issue in terms of morbidity and long-term disability throughout the world, especially in developing countries where the risk of infection in severe burns is well known [6].

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 management [7]. Topical antimicrobial agents are essential adjuncts in the prevention and treatment of burn wound infections [8,9]. Various substances have been advocated as effective topical burn treatments, such as sodium hypochloride [10], povidone iodine [11,12], antibiotics [9,13,14] and metal ions [15,16]. The widespread use of these agents in hospital settings has led to the emergence of multidrug resistant organisms of low virulence, such as *Klebsiella*, causing serious opportunistic infections [17]. Currently used antimicrobial agents are not effective in treating such infections and fail to control many bacterial infections due to the development of super-resistant strains. For this reason the search is ongoing for new antimicrobial agents, either by design and synthesis of new agents or through the investigation of natural sources.

Herbal medications in particular have seen a revival of interest due to the perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals. Coupled with the reduced costs of plant preparations, this makes the search for natural therapeutics an attractive option. Natural antimicrobial agents such as honey, aloe vera, and bacteriophages (bacteria killing viruses) have been considered in the recent past for the topical treatment of burn wound infection. Honey has long been used to treat wounds and cutaneous ulcers and its healing properties have been recently rediscovered [18,19]. The high viscosity, acidic pH, inhibine factor, high osmolarity, and nutrient content of honey contribute to the inhibition of bacterial growth and promote wound healing [20,21]**.** Similarly, *Aloe barbadensis* Miller (*Aloe vera*) has a long history of use as a therapeutic agent with many reported medicinal properties. It has antibacterial activity due to the presence of anthraquinones such as aloin and emodin, saponins, and salicylic acid which increase blood flow to wounded areas [22], and cell growth stimulatory activity [23]. *Aloe vera* has been reported for years to be effective in treating various types of burns [24-26]. Another agent, bacteriophages or simply phages, can be the best answer to antibiotic resistance in the treatment of bacterial infections [27-29]. These phages are considered to be economical, safe, self-replicating and are able to kill bacteria [30,31].

Since all three agents, namely honey, aloe vera gel and bacteriophages, have been used for treating burn wound infection, a comparison of these antibacterial agents was made in this study to assess their potential when used alone or in combination in treating burn wound infection caused by *K. pneumoniae* B5055 in BALB/c mice.

Materials and methods

Bacterial strain and growth media

K. pneumoniae B5055 obtained from Dr. Matthias Trautmann, Department of Medical Microbiology and Hygiene, Ulm University Hospital, Steinhövelstraße 9, D-89075 Ulm, Germany, and maintained in the laboratory was used in this study. The strain was maintained on nutrient agar slants at 4^{o}C.

Honey

Honey marketed by Dabur Limited, village Billanwali Lavana, P.O. Baddi, Distt Solan (Himachal Pradesh, India) was used in this study. It was sterilized by autoclaving at 10 lb for 30 minutes and stored in clean, sterilized bottle at 4^{o}C until use.

Preparation of aloe vera gel

Thick succulent leaves of *A. vera* (*Aloe barbadensis*) plant obtained from Medicinal Plants Garden, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, were used. Aloe vera gel (AVG) is the mucilaginous jelly obtained from the centre (the parenchyma) of the plant leaf of *Aloe vera.* The gel portion of the plant was prepared by the method as described by Madan *et al.* [32]. In brief, leaves of *A. vera* were collected, washed with water and a mild chlorine solution and were finally cut transversely into pieces. With a vegetable peeler, the thick epidermis was selectively removed and the inner gel-like pulp in the center of the leaf was separated with a spoon, minced, and homogenized in a mixer or blender. The resulting mucilaginous homogenate (AVG) was sterilized by autoclaving at 10 lb for 30 minutes and stored in a clean, sterilized bottle at 4^{o}C until use.

Before use, unautoclaved honey and AVG were tested for their sterility by streaking on nutrient agar plates. The plates were incubated at 37^{o}C overnight and checked for any microbial growth. Honey and AVG were found to be sterile as no growth was observed on any of the media employed.

Antibacterial activity of honey and AVG against K. pneumoniae *B5055*

The antibacterial activity of honey and AVG against *K. pneumoniae* B5055 was determined as follows: 1.0 ml of *K. pneumoniae* B5055 was mixed with 1.0 ml of either autoclaved or unautoclaved honey or AVG, achieving a final titer of 10^8 CFU/ml. The contents were mixed homogeneously. Mixtures were stored at 37^{o}C for a period of seven days. The bacterial count was checked every day by plating different dilutions on nutrient agar plates.

Animals

Adult BALB/c mice, six weeks old, weighing 20-25 ± 5g were obtained from the Central Animal House, Panjab University, Chandigarh. All animals were given an antibiotic-free diet (Hindustan Liver limited, Mumbai) and water *ad libitum*. The animal study was conducted following approval of protocols by the Institutional Animals Ethical Committee. All the experiments were conducted in triplicate. The error bars in graphs are representative of the standard deviation in each experiment.

Murine burn wound model

A third-degree burn wound infection model was developed in mice using *K. pneumoniae* B5055 via topical route following the method of Dale *et al.* [33]. Briefly, the skin was denuded with a commercially available hair removing cream. Mice were anesthetized with ether fumes and burn was induced by applying heated brass bar (10 ×10 ×100 mm) for 45 seconds. Immediately after the burn, all the mice were injected intraperitoneally (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 in drinking water as a post-burn analgesic. Bacterial inoculum was prepared by incubating *K. pneumoniae* B5055 in nutrient broth at 37^{o}C overnight followed by repeated centrifugation (10,000 rpm for 10 min) and washing, with a final resuspension in normal saline. To determine the LD_{100} (Lethal dose causing 100% mortality) value of *K. pneumoniae* culture, doses ranging from 10^2 to 10^{10} colony forming unit/ml (CFU/ml) were evenly applied topically on the burn site, after a waiting period of 30 minutes. Burned mice were inoculated topically with phosphate buffer saline (PBS, pH 7.2) and acted as controls. Burned mice inoculated with bacteria and PBS 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.

Phage isolation

Klebsiella bacteriophages Kpn5, Kpn12, Kpn13, Kpn17 and Kpn22 were isolated from sewage samples from different sources in and around Chandigarh area. Phage titer was determined by the soft agar overlay method described by Adams [34]. Efficacy of *Klebsiella* phages to treat burn wound infection caused by *K. pneumoniae* B5055 in compromised mice was evaluated by delivering phage intraperitoneally and Kpn5 phage was found to be the most effective ($P < 0.001$) resulting in a substantial decrease in bacterial load in different organs as compared to other phages [35]. Therefore, phage Kpn5 was used in the present study for the topical treatment of burn wound infection caused by *K. pneumoniae* B5055 in mice.

Hydrogel preparation

Hydrogels were prepared to suspend Kpn5 for topical application. Gels with a remarkable ability to absorb water or aqueous solvents, usually more than 20% of their own weight, are called hydrogels. It is a gel-like material, capable of holding a large amount of water within its structure. Hydrogels were prepared according to the method of Cooper and Guns [36]. Hydroxy propyl methyl cellulose (E464, or HPMC) was used to prepare hydrogel. Hydrogels were prepared using various concentrations of polymers, *e.g.* 1%, 2% and 3%. Briefly, required amount of polymers were dissolved in warm water and stirred with a mechanical stirrer at a speed of 100 rpm for 15 minutes. When the gel became homogenous 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 appropriate viscosity for the topical application on mouse skin and hence in further experiments 3% HPMC hydrogel was used. Hydrogel prepared as above was sterilized by autoclaving at 10 lb for 30 minutes and checked for viscosity with the help of viscometer. No change in the viscosity of hydrogel preparations upon autoclaving was seen, as the viscosity curve, which is a plot of viscosity versus shear rate, showed a straight line for the hydrogel preparation (data not shown). The flow curve, which is a plot of shear rate versus shear stress (τ), 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 newtonean fluid.

Toxicity of hydrogel on mouse skin

Three percent HPMC Hydrogel was tested for its toxicity by employing a skin irritation test in mice using the methods described by Basketter *et al.* [37] for single and three-patch application tests.

The single patch application test (S-PAT) was conducted to confirm that hydrogel was not a skin irritant even under highly exaggerated exposure conditions. In the single application patch test, test material was applied once to the shaved skin of mice using a small patch. The patch was kept in place for 4, 24 and 48 hours. Once a product or material was shown to be non-irritating in a single application

patch test, the next step was to confirm that the product would not irritate on exposures of longer duration (three 24-hour applications in a five-day period). For this, the three-patch application test (3-PAT) was performed. In this experiment, five groups each containing six mice were taken. The skin was shaved with the help of commercially available hair removing cream. No hydrogel was applied to group I, which acted as the control group. In groups II, III and IV, a 0.5 ml sample of the hydrogel was applied for 4 hours, 12 hours and 24 hours (a 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 applied for 24 hours (three applications over a five-day period). A double gauze layer was applied onto the skin and 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 (the development of a rash, inflammation, swelling, scaling, and abnormal tissue growth in the affected area) over the next five days.

Stability of K. pneumoniae *specific phage Kpn5 in 3% HPMC hydrogel, honey and AVG*

Stability of *Klebsiella* specific phage Kpn5 was checked in 3% HPMC hydrogel, honey and AVG for varying periods of time. A 1.0 ml phage sample was taken and mixed with 1.0 ml of either of 3% HPMC hydrogel, honey or AVG to obtain a count of a 10^8 PFU/ml. The mixture was stored at 37^{o}C for a period of seven days. The viable phage was quantitated in this ointment daily by using the plaque assay method [34].

Efficacy of topical application of phage Kpn5, honey and AVG in treating Klebsiella *induced burn wound infection*

Efficacy of phage Kpn5, honey and AVG was evaluated in treating burn wound infection caused by *K. pneumoniae* B5055 in BALB/c mice. Seven groups of mice (10 mice in each) were taken. A full thickness burn was induced in all groups and challenged with LD_{100} of *K. pneumoniae* culture directly on the burn site as described earlier. In group I, all the burned mice challenged with bacterial inoculum acted as controls. In groups II and III, mice were burned, infected and treated with a single application of 0.5 ml of Kpn5 phage at 10^8 PFU/ml (low titer 1.0 MOI) and 10^{10} PFU/ml (high titer 200 MOI) respectively, and mixed with hydrogel. In groups IV and V, mice were treated with a daily topical application of 0.5 ml each of honey and AVG immediately after the burn/bacterial challenge. In groups VI and VII, burned/infected mice were treated with 0.5 ml each of honey + phage Kpn5 and AVG + phage Kpn5 (daily application) respectively. All mice were monitored for seven days for any signs of morbidity and mortality.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Student's t test for direct mean comparison, and one-way analysis of variance (ANOVA) followed by Boneferroni test for multiple comparisons were performed with Graph Pad Instat Software (Version 3.00, GraphPad Software, San Diego, California, USA). Difference with $P \leq 0.05$ was considered statistically significant.

Results

Antibacterial activity of honey against K. pneumoniae *B5055*

The results as illustrated in Figure 1 show that honey inhibited the growth of *K. pneumoniae* B5055. Both autoclaved and unautoclved honey showed similar antibacterial activity. A significant 4 log cycle decrease in viable bacterial count was observed in both cases on the third day, followed by complete elimination of bacteria (8 log cycle decrease) on the seventh day ($P < 0.001$).

Antibacterial activity of AVG against K. pneumoniae *B5055*

The AVG showed loss of antibacterial activity upon heating as no significant decrease in viable bacterial count ($P > 0.05$) was observed in autoclaved AVG over a seven-day period (Figure 2). In unautoclaved AVG, a significant decrease in bacterial count (3 log cycle decrease) was observed on the third day followed by complete elimination of bacteria (8 log cycle decrease) on the seventh day ($P < 0.001$).

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 (the development of a rash, inflammation, swelling, scaling, and abnormal tissue growth in the affected area) when observed for a period of seven days after single and multiple hydrogel applications on shaved mouse skin. Thus, due to its nontoxicity, 3% HPMC hydrogel preparation was selected as ointment for the topical application on burn wounds.

Figure 1. Antiibacterial activity of autoclaved and unautoclaved honey against *K. pneumoniae* B5055 *in vitro.*

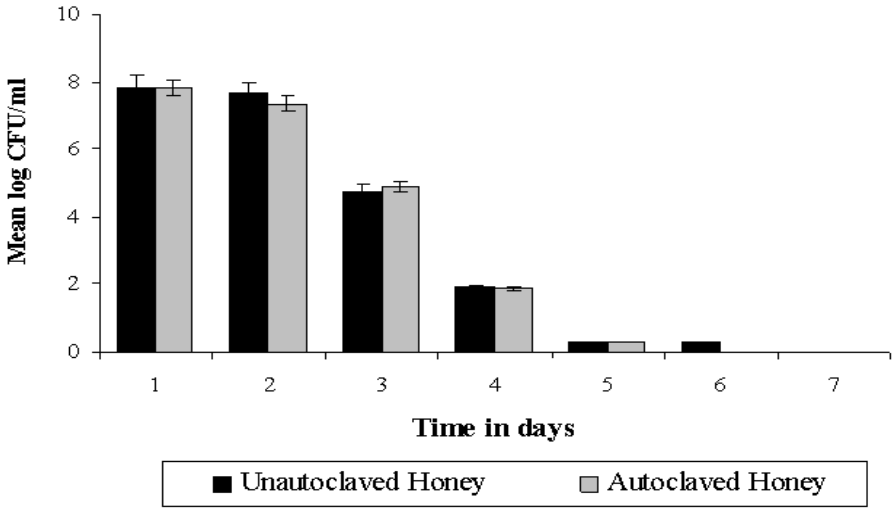
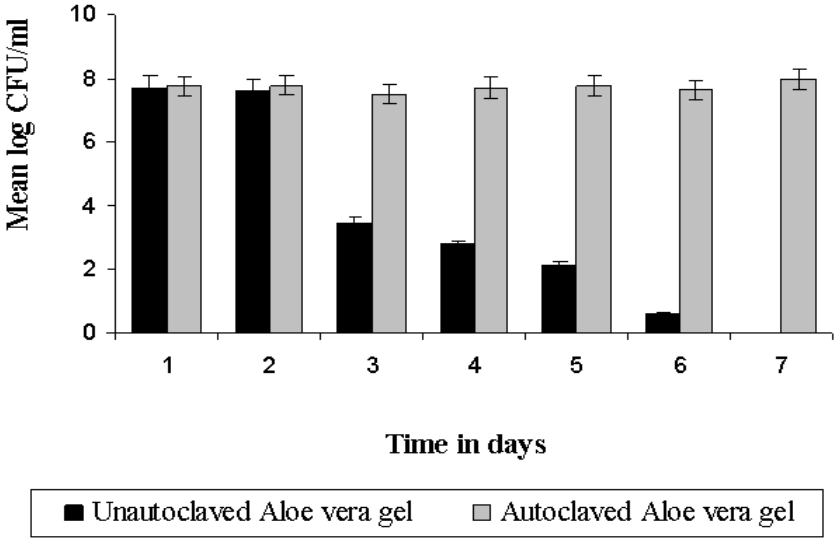


Figure 2. Antibacterial activity of autoclaved and unautoclaved AVG against *K. pneumoniae* B5055 *in vitro.*



Establishment of a full thickness burn wound infection via topical route

A full thickness burn wound was established by applying heat for 45 seconds. For establishing the infection via topical route, LD_{100} dose of *K. pneumoniae* B5055 was determined. A dose of 10^8 CFU was found to be sufficient to cause burn wound infection and all the animals succumbed to infection within 48-72 hours following bacterial challenge in burned mice. All burned mice that received PBS (pH 7.2) only (controls) did not show any sign of illness (Figure 3).

Topical treatment of burn wound infection caused by K. pneumoniae *B5055 in mice using honey and AVG*

The results presented in Figure 4 show that burned/infected mice treated with a daily topical application of undiluted honey and AVG received protection as a 100% survival rate was observed in these animals compared to 93% survival in the untreated control group on the first post-infection day (P >0.05). On the second day, as infection progressed, a significantly higher number of animals survived (96.66 and 86.66 % respectively) in the honey and AVG treated groups compared to 16.66% survival in the untreated control group ($P < 0.001$). A

Figure 3. Lethality of *K. pneumoniae* B5055 at a dose of 10^8 CFU in burn wound mouse model. A score of 5 indicates normal health, while 0 indicates death (see the text for full description of the scales). All animals were dead within 48 - 72 h.

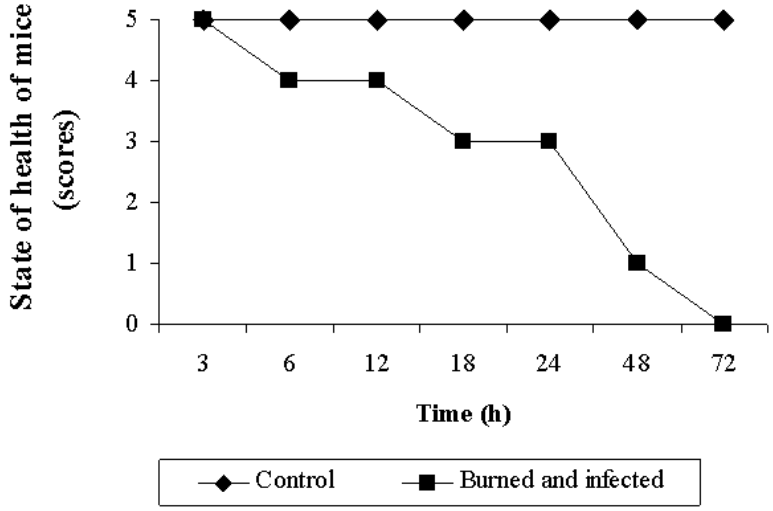
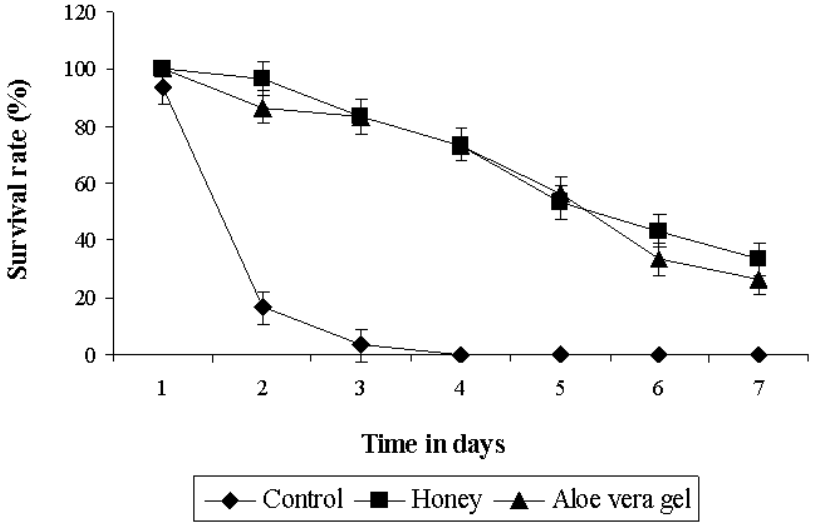


Figure 4. Topical treatment of *K. pneumoniae* B5055 induced burn wound infection in mice with honey and AVG.



gradual decrease in the percent survival of honey treated and AVG treated mice was observed on the third day onwards and the decline continued until seven days past the treatment period. When topically applied daily on the burned and infected area, honey and AVG still resulted in significantly higher survival rates of 33.33% and 26.66% ($P < 0.001$) compared to 0% survival observed in control mice on the seventh day.

Determination of phage Kpn5 stability in 3% HPMC hydrogel, honey and AVG in vitro

Results showed a non significant decrease in phage titer ($P > 0.05$), over a period of seven days indicating 100% stability of phage in 3% HPMC hydrogel preparation and two natural antimicrobial agents, *i.e.* honey and AVG (data not shown).

Efficacy of phage Kpn5 in burn wound infection model

The results in Figure 5 show that, when applied topically, phage Kpn5 suspended in 3% hydrogel provided protection on the first day, as a survival rate of 86.66% and 100% was observed at low multiplicity of infection (MOI = 1.0) and at high MOI of 200 respectively as compared to 86.66% survival in the phage untreated (control) group ($P > 0.05$). A survival ($P < 0.001$) of 80% and 96.66 % at low MOI and high MOI respectively was observed in comparison to mortality in the control group (83.34

Figure 5. Efficacy of phage Kpn5 in terms of percent survival of phage treated mice (with low and high phage titer) following topical application.

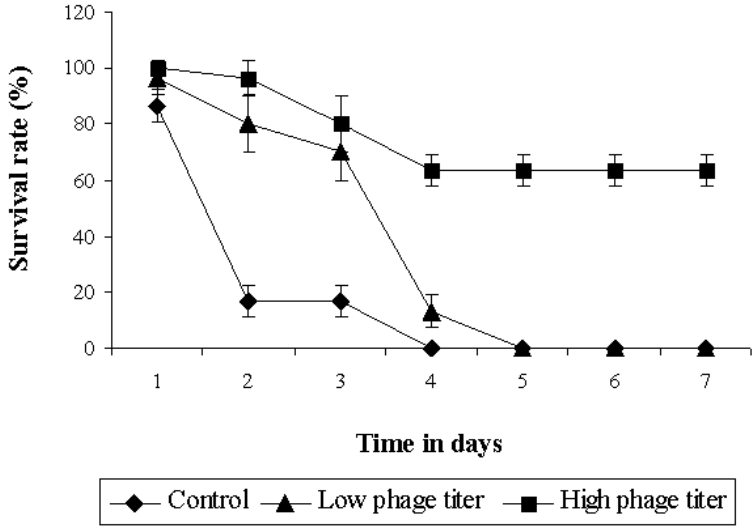
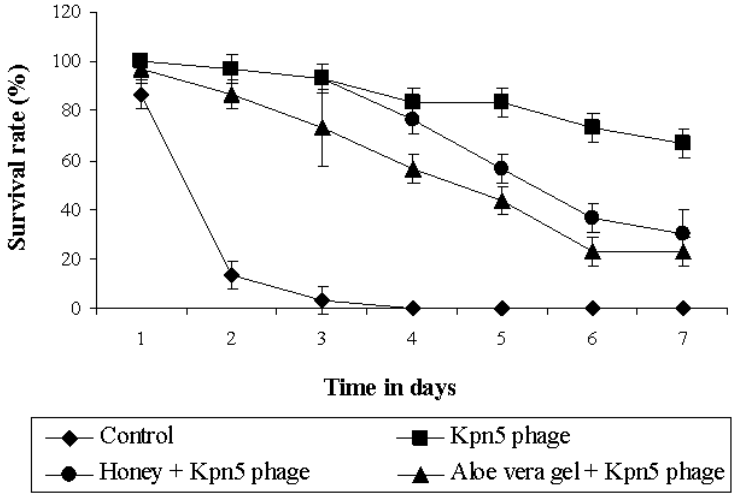


Figure 6. Protective ability of phage Kpn5 on use in combination with honey and AVG.



%) on the second day post-phage treatment. With time, percent survival went on decreasing in both the phage treated groups. However, the high titer phage treated group showed a high level of protection (66.66%) and this difference was statistically significant ($P < 0.001$) compared to the untreated (control) group (0% survival). All the animals in the low titer phage treated group (0% survival) succumbed to infection.

Topical treatment of burned/infected mice with phage Kpn5 in combination with honey and AVG

Phage Kpn5 alone as well as in combination with honey and AVG provided protection on the first day (Figure 6) with survival rates of 100%, 100% and 96.66% respectively in comparison to 86.66% survival in untreated control mice ($P > 0.05$%). The survival rate in the groups treated with phage Kpn5 alone or in combination with honey was similar on the third post-infection day. The difference in the two groups became significant with time and on the

seventh day, the percent survival in the honey + phage treated group was less than half of the survival seen in the group treated with phage Kpn5 alone. Similarly, no added advantage was observed in the group treated with AVG + phage Kpn5 as the survival rate decreased in mice from day one.

Discussion

In spite of recent advances in treatment, burns are still a major threat to life due to infection, particularly in developing countries such as India [38,39]. The local interruption of blood flow associated with burns makes prophylaxis of systemic infection difficult, and thus topical antimicrobial therapy is important. The purpose of antimicrobial agents is to reduce the microbial load (bioburden) in wounds, and hence protect against infection. In the present study, a mouse burn wound model was used to evaluate the therapeutic potential of honey and AVG in comparison to phage Kpn5 in the treatment of infection caused by *K. pneumoniae* B5055.

Honey is the most ancient wound dressing known, but as a bioactive dressing it is also the most modern type of wound dressing [40]. It acts as an autolytic debriding agent where the wound has broken down and where there is necrotic tissue. It is the most famous rediscovered remedy that had been used earlier to promote wound and burn healing and also to treat infected wounds [41,42]. Many studies have shown that honey has antibacterial activity *in vitro*, and this observation is supported by clinical case studies in which the application of honey to severely infected cutaneous wounds not only helped clear infection from the wound but also improved healing [43,44]. It is non-irritant, non-toxic, easily available, and inexpensive [38]. Because of its high viscosity it forms a physical barrier, creating a moist environment which appears to accelerate wound healing despite its water activity (*i.e.*, amount of "free" water) being very low. It does not cause dehydration of tissues because its osmotic effect draws fluid through the wound tissue from the underlying circulation [45]. Thus it creates a film of honey under the dressing, which prevents the dressing from adhering to the wound. This in turn prevents tearing away of newly formed tissue and no pain is felt while changing the dressing. Research in the early 1960s confirmed that wounds kept moist heal quickly. Unfortunately a moist environment also promotes bacterial growth; thus a successful dressing material must also be anti-bacterial. The antimicrobial activity of bee honey has been attributed to several of its properties, including osmotic effect, low pH, ability to produce hydrogen peroxide and the presence of phenolic acids, lysozyme, and flavanoids [46]. As a result, honey is known to act against different bacteria including those that are highly resistant to antibiotics [47]. In this study, an attempt was made to utilize honey as topical antimicrobial agent for the treatment of burn wound infection caused by *K. pneumoniae* B5055 in mice. First, honey was checked for its sterility and found to be completely sterile as no growth was seen on any of the media employed. The antibacterial activity of honey was found to be heat stable; that is, it remained active even after autoclaving. The undiluted honey showed antibacterial activity against *K. pneumoniae* B5055 *in vitro* and mice with burn wounds treated with a daily topical application of honey had a significantly higher survival rate of 33.33% ($P < 0.001$) as compared to 0% survival observed in control mice on the seventh day of treatment.

Many treatments discovered by early civilizations were based on the use of local plants [48]. The majority of medicinal plant species are rich in biomolecule contents which possess antibacterial properties and are not associated with any health hazard. Among these medicinal plants, aloe vera or *Aloe barbadensis* has been used for therapeutic purposes for centuries. Numerous cosmetics and medicinal products are made from the mucilaginous tissue, called aloe vera gel (AVG), located in the center of the aloe vera leaf. AVG has been used for many clinical conditions since the Roman era or even long before. Burn wound healing is one of the major indications of AVG use in many countries [49]. AVG has been shown to have direct benefit in the treatment of burns and skin abrasion injury [50]. AVG is known to promote wound healing due to the presence of active components such as anthraquinones and chromones [51]. Wounds treated with AVG heal faster than other wounds not so treated. The reason may be that it contains not only vitamins E and C as well as zinc, but also polysaccharides which reduce inflammation and stimulate fibroblast and epidermal growth and therefore the repair process. AVG has been shown to exhibit some wound healing effects, including the encouragement of granulation tissue, and aloe polysaccharides have demonstrated some positive effects in preventing burns. It is both antimicrobial and anti-inflammatory [52]. For burns and other wounds, AVG is particularly effective as it activates the macrophages which fight bacterial

infection while at the same time it results in increasing circulation to the area which finally is responsible for accelerated healing. The potential of AVG was also evaluated in this study as a topical antimicrobial agent for the treatment of burn wound infection caused by *K. pneumoniae* B5055 in mice. AVG was also checked for its sterility and, like honey, was found to be completely sterile as no growth was seen on any of the media plates. AVG was also heat sterilized by autoclaving but it did not retain its antibacterial activity against *K. pneumoniae* B5055 *in vitro.* The antibacterial activity of unsterilized AVG was observed against *K. pneumoniae* B5055 *in vitro* and when applied topically daily at the burn site, a significantly higher percent survival of 26.66% ($P < 0.001$) was seen in treated mice in comparison to the untreated control group (0%) on the seventh day post treatment. The results indicate the feasibility of using AVG as an antibacterial agent against burn wound infections.

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 the phages can be applied externally (topically) and are, therefore, less likely to be removed by the immune system. Therefore, the therapeutic potential of phage Kpn5 as a topical agent was evaluated for the treatment of burn wound infection caused by multidrug resistant bacterial pathogens. HPMC hydrogel was used as an ointment base for the topical application of phage Kpn5 because of its numerous positive features. Hydrogels (also called aquagels) are highly absorbent and contain over 99% water. Natural or synthetic polymers also possess a degree of flexibility very similar to that of natural tissue due to their significant water content. These gels possess many properties that make them preferable wound dressings as they are biodegradable, sterile, not antigenic, not allergenic, easy to store and use, protect against excessive loss of body fluids, and form an efficient antiseptic and particle barrier which absorbs wound excreta as well [53]. Because of these properties, 3% HPMC hydrogel was prepared and used as a wound dressing and ointment base for the topical application of phage Kpn5.Three percent HPMC hydrogel was found to be non-irritant on the shaved skin of normal mice when tested for short as well as long periods. Phage Kpn5 (at high MOI 200) provided protection when mixed with 3% HPMC hydrogel and applied topically to treat burn wound infection in mice caused by *K. pneumoniae* B5055. A significantly higher percent survival of 66.66% ($P < 0.001$) was obtained as compared to 0% survival in PBS treated control mice when observed over a seven-day period. This protection possibly might be due to the release of phage Kpn5 from 3% hydrogel which was used as ointment (mixed with phage). These phages were able to locate bacteria in the body before the animal succumbed to bacteremia and septic shock.

The efficacy of the topical application of phage Kpn5 in combination with natural products such as honey and AVG was also evaluated for treating *K. pneumoniae* B5055 induced burn wound infection in mice. The stability of phage Kpn5 was checked in honey and AVG *in vitro*. The results showed that when phage Kpn5 was mixed with honey and AVG, no significant decrease in phage count ($P > 0.05$) was observed over a seven-day period, which confirmed 100% stability of phage Kpn5 in these two natural antimicrobial agents. The results showed that phage Kpn5, when used in combination with honey and AVG, resulted in survival rates of 30% and 23.33% respectively in burned mice as compared to 0% in the untreated control group, but the protection level was almost similar to that obtained with honey and AVG when used alone in treating *Klebsiella* induced burn wound infection in mice. These results indicate that no added advantage was observed on combining phage Kpn5 with AVG or honey from day one onward. It appears that possibly due to the high viscosity of honey and AVG, the lytic phage was not able to enter into the wound and thus eradicate bacteria. As it remained entrapped in these natural products, it is suggested that, in future, diluted honey or AVG may be used along with hydrogels to allow the phage to enter into the burn wound and thus act against invading bacteria at the wound site.

This study demonstrates the importance of honey, AVG and phage Kpn5 to control 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 honey and AVG in the treatment of burn wound infection caused by *K. pneumoniae* B5055 in BALB/c mice; however, the potential of these natural antimicrobials to treat burn wound infection caused by nosocomial pathogens was also confirmed.

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