Bacteriophage Treatment of Burn Wound Infection Caused by *Pseudomonas aeruginosa* PAO in BALB/c Mice

Seema Kumari¹, Kusum Harjai¹ and Sanjay Chhibber¹*

¹Basic Medical Sciences Building, Department of Microbiology, Panjab University, Chandigarh-160014, India.

*Corresponding author
Dr. Sanjay Chhibber
Professor
Basic Medical Sciences Building
Department of Microbiology
Panjab University
Chandigarh –160014, India.
Phone: 91-0172-2534141, 91- 0172-2541770
Fax: 91-0172-2541499;
E-mail: sanjaychhibber8@sify.com

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Abstract

Therapeutic potential of five purified and well characterized bacteriophages (Pa29, Pa30, Pa31, Pa33 and Pa34) was evaluated in thermally injured and *Pseudomonas aeruginosa* PAO infected mice. Efficacy of five *P. aeruginosa* phages was assessed on the basis of percentage survival of phage treated mice. Toxicity evaluation of all the phages, showed them to be non toxic as no sign of morbidity and mortality was observed in phage treated mice. Survival and stability of *Pseudomonas* specific phages was evaluated in mice and maximum phage count in various organs was obtained at 3 h which persisted till 24 h and no phage could be detected at 36 h post inoculation. The results of the study indicate that a single dose of phages intraperitoneally (i.p.) at highest multiplicity of infection (MOI) of 900, did not provide any protection from *P. aeruginosa* PAO induced burn wound infection in mice. Since a single application of *Pseudomonas* specific phages did not restrict the infection in thermally injured mice by *P. aeruginosa* PAO, the experiment was repeated by administering these phages at 24 h intervals, starting immediately after the infection up to 72 h at above mentioned MOI, even then no protection was observed in phage treated groups and results were comparable to control groups with 100% mortality (*P*<0.05). The results of the present study show that, phages belonging to *Podoviridae* family, order *Caudovirales* were not effective in protecting *P. aeruginosa* infected thermally injured mice.

Keywords: *Pseudomonas aeruginosa*, bacteriophages, multiplicity of infection, *Podoviridae*, *Myoviridae*, *Caudovirales*.
1.0 Introduction

*Pseudomonas aeruginosa* is a gram negative, versatile, opportunistic pathogen found along with other *Pseudomonas* spp. as part of normal flora of human skin [1, 2]. It rarely causes infections in healthy individuals but can cause serious infections in immunocompromised hosts [3] which include patients with severe burn wounds [4, 5], cystic fibrosis patients [6], cancer patients [7] and patients with human immunodeficiency viral infection [8, 9]. To initiate infection, *P. aeruginosa* usually requires a substantial breach in first-line defenses. Burn causes a breach in the protective skin barrier which suppresses the immune system, rendering the patients highly susceptible to bacterial infection and this opportunistic bacterium can then quickly colonize and infect the burn wound site [10]. 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 [11]. The denatured protein of the burn wound provides nutrition for the organisms. 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 [12]. Rapid colonization of burn site with *P. aeruginosa* PAO and its dissemination into distant organs via bloodstream often leads to bacteremia, endotoxic shock and sepsis [13]. In case studies of burn patients who developed *P. aeruginosa* septicemia, mortality rate greater than 75% was observed [14, 15]. Antibiotics that are administered orally are generally ineffective against most serious skin and soft tissue infections by *P. aeruginosa* [15]. Treatment of such infections is confounded by the innate and acquired resistance of *P. aeruginosa* to many antimicrobials agents [16]. Hence, the development of new therapeutic and prophylactic agents for the control of bacterial infection in patients with burn wounds is needed. An alternative to antibiotic therapy is phage therapy which involves the use of bacterial viruses to target bacterial infections [17-25].

Earlier we reported isolation, purification and characterization of bacteriophages specific to *Pseudomonas aeruginosa* PAO from sewage samples collected from areas in and around Chandigarh, India [26]. In continuation of that work, the present study was planned to evaluate the efficacy of *Pseudomonas* specific phages in the experimental treatment of burn wound infection caused by *P. aeruginosa* PAO.

2.0 Materials and Methods

**Bacterial strain and growth media-** Standard strain of *Pseudomonas aeruginosa* PAO obtained from Dr. Barbara H. Igleski, University of Rochester, New York (U.S.A) and maintained in our laboratory was used in this study. This organism was stored in 60% glycerol at -80°C and when necessary, maintained on nutrient agar slants at 4°C.

**Phage isolation-** Five *Pseudomonas* bacteriophages were isolated from sewage samples from different sources in and around Chandigarh area. The method of Cerveny et al. [27] was adopted for the isolation of phages from sewage samples. Phage titer was determined by soft agar overlay method as described by Adam [28]. The phages were purified, numbered Pa29, Pa30, Pa31, Pa33, and Pa34 and characterized on the basis of morphological, genomic studies and structural protein analysis [26].

**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. 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 third degree burn wound infection model was developed in mice using *P. aeruginosa* PAO following the method of Dale et al. [15]. 5 groups of mice (6 mice in each) were taken. Briefly, hair was clipped from the back of anesthetized mice and skin was denuded with a commercially available hair removing cream. Mice were anesthetized with ether fumes and burn was induced with the help of
heated brass bar (10 × 10 × 100 mm) for 45 seconds (s). 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 inoculating P. aeruginosa in nutrient broth, incubating at 37°C overnight followed by repeated centrifugation (10,000 rpm for 10 min) and washing, finally resuspending in normal saline. To determine LD$_{100}$ (Lethal dose causing 100% mortality), value of P. aeruginosa culture, in group I - IV, doses ranging from 10$^2$ to 10$^8$ CFU/ml (colony forming unit/ml) were injected subcutaneously (s.c.) directly under the anterior end of the burn in mice, after a waiting period of 30 min. In group – V, burned mice were injected s.c. with Phosphate buffer saline (PBS, pH 7.2) and acted as control. Burned mice inoculated with bacteria and PBS were scored for their state of health on a arbitrary 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 Pseudomonas aeruginosa phages was evaluated in burned (compromised) mice according to the method of McVay et al. [5]. 5 groups of mice (6 mice in each) were taken. Each of these groups (burned but uninfected mice) were injected i.p. with 0.25 ml of Pseudomonas specific phages of 10$^8$ PFU/ml (plaque forming unit/ml) individually. 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 time intervals after i.p. administration of the phage suspension. A score of 2 indicated normal unremarkable health, 1 slight illness/lethargy/ abnormal health, and 0 for death.

**Survival and stability of phages** - Survival and stability of Pseudomonas phages was measured in mice according to the method of Cerveny et al. [27]. Five groups of mice (12 mice in each) were taken and each group (uninfected or normal mice) was injected i.p. with one of the specific phage (10$^8$ PFU/ml). At 1, 3, 6, 12, 24, 36, 48 and 72 h, blood was collected in screw capped vials containing 0.05 M EDTA. Then mice were sacrificed and peritoneal fluid, skin and lungs were aseptically removed and subjected to phage count by plaque assay by employing double agar overlay technique [28].

**Treatment with Pseudomonas phages** - The efficacy of Pseudomonas phages to treat burn wound infection was evaluated in two separate experiments.

(a) **Single phage dose:** The therapeutic potential of phages, specific for P. aeruginosa PAO was evaluated for their ability to resolve burn wound infection in mice. Eleven groups of mice (10 mice in each) were used. Briefly, a full thickness burn was induced in mice and challenged with LD$_{100}$ dose of P. aeruginosa culture s.c. directly under the anterior end of burn as described earlier. In group I-X, all the burned/infected mice were treated immediately with a single i.p. injection of P. aeruginosa PAO specific phages administered at various MOI (0.001, 0.01, 0.1, 1.0, 10, 100, 300, 500, 700 and 900). In group XI, burned and infected mice without any phage treatment were kept as control. Survival rate for control and phage treated groups was recorded up to 72 h. Similar set of experiment was repeated for every phage separately.

(b) **Multiple doses:** In the second experiment, burned / bacterial challenged mice were injected i.p. with Pseudomonas specific phages (at highest MOI of 900) after every 24 h up to 72 h and effect of multiple doses of phages on the survival of animals was ascertained. 6 groups of mice (10 mice in each) were taken. Briefly, a third degree burn was induced in each group and challenged with LD$_{100}$ dose of P. aeruginosa s.c. directly under the anterior end of burn as described earlier. In group I-V, all the burned/infected mice were treated immediately with a single i.p. injection of P. aeruginosa PAO specific phage individually administered at highest
MOI (900). In group V, burned /bacterial challenged mice without any phage treatment were kept as control. Survival rate for control and phage treated groups was recorded up to 72 h.

Statistical analysis - Data are expressed as means ± standard deviation (SD) of mean and statistical analysis was performed with Graph Pad Instat Software (Version 3.00, GraphPad Software, San Diego, California, USA) using student’s t test for calculations of mean and standard deviation while one-way analysis of variance (ANOVA) followed by Boneferroni test for multiple comparisons. Difference with P ≤ 0.05 was considered statistically significant.

3.0 Results

Determination of lethality of *P. aeruginosa* in thermally injured mice - Burned mice were challenged with bacterial doses ranging from 10² to 10⁸ CFU/ml. All burned mice inoculated s.c with 10⁷ CFU/ml died within 72 h as shown in Fig. 1. This dose was recorded as LD₁₀₀ dose of bacteria and was used in all the experiments for the induction of infection. Death was attributed to septic shock due to the presence of organisms in the circulation of mice upon challenge with bacteria. All burned mice receiving PBS treatment only (control) did not show any signs of bacteraemia or slight illness over for a period of 72 h.

Toxicity testing of *P. aeruginosa* phages in mice - *Pseudomonas* phages did not manifest any illness and all the animals survived with a score of 2 (Fig. 2). This indicates that the either endotoxin present in the phage lysate was in non lethal concentration or the preparation was devoid of endotoxin suggesting that each individual phage was not toxic to thermally injured mice.

Survival and stability of *Pseudomonas* phages in mice - The survival and stability of isolated *Pseudomonas* specific phages, Pa29, Pa30, Pa31, Pa33 and Pa34 was determined in various body compartment of mice following i.p. injection (3.0 ×10⁸ PFU/ml) of each phage. The phage count was measured in blood, peritoneal fluid, lungs and skin of phage treated mice. Maximum phage count in blood, peritoneal fluid, lungs and skin was obtained at 3 h post injection.

Figure 1. Lethality of 10⁷ CFU/ml of *P. aeruginosa* PAO 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.
in all the groups (Fig.3a-e). The phage count in peritoneal fluid of all *Pseudomonas* phage injected mice was slightly higher as compared to that in blood, lungs and skin samples. With increase in time, phage titer kept on decreasing in all body compartments. The phage count showed a significant decrease of 6-7 log units at 12 h and negligible counts were obtained at 24 h after injection. No phage could be isolated in peritoneal fluid, blood, lungs and skin samples at 36 h after phage treatment.

![Graph showing state of health of mice (Scores) over time](image)

**Figure. 2.** Toxicity of the phage treatments; 2 = normal or unremarkable condition, 1 = slight illness, 0 = Death.

**Efficacy of *Pseudomonas* phages in the treatment of *P. aeruginosa* PAO induced burn wound infection in mice** - To optimize the phage dose for the treatment of *P. aeruginosa* induced burn wound infection in mice, single i.p. dose of each *P. aeruginosa* PAO specific phage (Pa29, Pa30, Pa31, Pa33 and Pa34) was injected at varying MOI ranging from 0.001 to 900, in separate groups of burned and *P. aeruginosa* PAO infected mice. At lower MOI (0.001 - 700), none of the *Pseudomonas* phages was able to protect burned mice from bacteraemia. Even at very high MOI (900), a non significant (\(P>0.05\)) difference in survival rate of all phage treated groups was observed as compared to burned/ infected mice treated with only nutrient broth (control) at 72 h post infection.

Even when multiple doses of phages were injected at every 24 h interval following burn infection, similar pattern of survival rates was observed in all phage treated and untreated control groups. The results obtained with even at highest tested MOI (900) were quite discouraging. A non significant difference (\(P>0.05\)) between survival rates of control and phage treated groups was observed (Fig. 4).

**4.0 Discussion**

Therapeutic potential of five well characterized phages of *Pseudomonas aeruginosa* PAO [26] isolated from sewage samples was evaluated in this study for the treatment of burn wound infection. There are reports available in literature where phages have been used to treat variety of bacterial infections in animal model systems [29-37], the information in this regard to treat burn wound infection is scanty. Soothill [38] demonstrated the ability of phages to prevent rejection of skin grafts in guinea pigs. On this basis it was suggested that bacteriophages can play a role in the treatment of burn wound...
infection by *P. aeruginosa*. Later, using cocktail of three different *P. aeruginosa* specific phages, Mcvay *et al.*, [5] showed protection against fetal infection with *P. aeruginosa* in mice. Clinical trials have been initiated on the basis of this information where a cocktail of phages targeted against *P. aeruginosa* and *Staphylococcus aureus* is under way in Belgium [39].

**Figure.** 3. Phage count in blood, peritoneal fluid, lungs and skin at varying time periods after administration of *P. aeruginosa* PAO specific phages. (a) Phage Pa29; (b) Phage Pa30; (c) Phage Pa31; (d) Phage Pa33; (e) Phage Pa34 (each having titer of $10^8$ PFU/ml) in BALB/c mice.

Figure 4. Percentage survival of burned and *P. aeruginosa* PAO infected mice following treatment with different phages at MOI of 900.

*Pseudomonas* phages in this study when tested in thermally injured mice were found to be non toxic, devoid of any bacterial contamination in phage suspension and hence phage preparation was considered safe enough to be administered to compromised mice [40, 5]. The other critical parameters that affect phage therapy were also evaluated in terms of phage adsorption rate, burst size, latent period and initial phage dose. All the phages selected were found to have shorter latent period and burst size making them most appropriate agents for phage therapy. In addition, the clearance of the phage particles from the body fluids by the reticuloendothelial system (RES) is also crucial in determining their potential as therapeutic agents. Although phages entered into the blood stream after 1 h but they achieved the maximum count at 3 h post injection. In earlier studies also, it has been observed that phages take a maximum of 2 – 4 h to reach their peak in the blood stream [41, 42]. The phage count however significantly declined at 24 h leading to total absence of phage particles at 36 h post injections in all the body compartments of mice.

None of the five phages was found to be capable of clearing bacteria from the burn wound site even when used at a high MOI of 900 inspite of their potential to inhibit bacteria *in vitro*. In our laboratory, using *Klebsiella pneumoniae* B5055 phages, we were able to restrict the growth of bacteria in the same model of burn wound at a MOI of 1.0 [43]. These results indicate that their exist an intricate molecular mechanism in the phage / bacterium system. In fact, according to Skurnik and Strauch [44], all phage /host systems are not likely to behave identically *in vivo*. The differences in their action may arise due to number of reasons. It is likely that *in vivo* the bacterium may become resistant to phages due to mutation thereby losing the receptors for phage on bacteria. Since attachment to the receptors is the initial step in the phage infection, hence such loss of receptors may influence the *in vivo* susceptibility of the pathogens to bacteriophages. However, no alteration in the sensitivity of *P. aeruginosa*, PAO isolated from different organs to phages was observed in this study indicating absence of such mutants *in vivo*. In addition, a phage may also
become resistant due to lysogeny which renders the bacterium not only immune to the original phage but also to related phages [44]. According to these workers, phage resistance may also be due to horizontal acquisition of a restriction modification system that degrades the injected phage nucleic acid or due to mutation in gene whose product is essential for phage replication assembly. These observations point toward the complexity of the phage / bacterium interactions in the animal model systems. In fact mathematical model adopted by Weld et al. [45] to monitor phage growth in rats failed to predict their growth in vivo. The inability of Pseudomonas specific bacteriophages to control P. aeruginosa induced infection in thermally injured mice may also be attributed to biofilm forming property of this bacteria. P. aeruginosa has tendency to form biofilm consisting of bacterial communities embedded in a mucoid exopolysaccharide matrix [46]. Biofilms are resistant to antimicrobial agents as well as to host defense mechanisms and hence are difficult to eradicate. Failure of phage therapy possibly due to inability of these phages to penetrate into deeper layers of the thicker biofilms [47] may be another reason which did not restrict the bacterial growth in this animal model system. It is known that biofilms from time to time shed these protected bacterial populations in the system thereby aggravating the clinical choice of disease [48].

The results of this study therefore suggest that future research should concentrate on to answer the intricate mechanisms making phages ineffective or effective in vivo, which in turn influence bacterial growth. Such information will help in the development of therapeutic phages against different organisms, an approach which has received lots of attention of scientists the world over to tackle the menace of drug resistance among pathogens.

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References

33. Vinodkumar, C.S.; Neelagund, Y.F.; Kalsurmath, S. Bacteriophage in the treatment


