Chapter - 6
Summary and Conclusion
The present investigation was planned to isolate, classify and characterize bacteriophages specific for *Klebsiella pneumoniae* B5055 and *Pseudomonas aeruginosa* PAO and to study the efficacy of isolated phages in treating burn wound infection induced by these pathogens. The salient features of the present study entitled “Therapeutic potential of bacteriophage(s) in burn wound sepsis model induced by common nosocomial pathogens” are as follows:

1. 40 sewage samples were processed for the phage isolation following the enrichment method. A total of 27 phages specific to *K. pneumoniae* B5055 and *P. aeruginosa* PAO were isolated from sewage samples in and around Chandigarh.
   a. Out of 27 phages, five phages each having specificity for *K. pneumoniae* B5055 and *P. aeruginosa* PAO respectively were purified. All the phages were screened for lytic activity and plaque formation. *Klebsiella* lytic phages were named as Kpn5, Kpn12, Kpn13, Kpn17 and Kpn22 and *Pseudomonas* phages as Pa29, Pa30, Pa31, Pa33 and P34.
   b. *K. pneumoniae* B5055 specific phages formed clear, triple layered plaques of approximately 6.5-7.8 mm in diameter and *P. aeruginosa* PAO specific phages formed clear, single layered plaques of approximately 3.2 - 4.0 mm in diameter.

2. Following purification, the phages specific for *K. pneumoniae* B5055 and *P. aeruginosa* PAO respectively were tested for their cross sensitivity. Out of 5 phages specific for *K. pneumoniae* B5055, phage Kpn12 showed sensitivity to *P. aeruginosa* PAO as well whereas all the 5 selected *Pseudomonas* phages showed sensitivity towards *K. pneumoniae* B5055.

3. Factors affecting phage yield of *K. pneumoniae* B5055 and *P. aeruginosa* PAO specific phages were studied. The results showed:
   a. Optimum temperature for plaque assay for *K. pneumoniae* B5055 and *P. aeruginosa* PAO specific phages was found to be 37°C.
b. Agar concentration of 0.75% was found to be optimum for maximum phage yield for all the phages.

c. Phage yield was found to be maximum using 4-6 hours old Indicators cells of both *K. pneumoniae* B5055 and *P. aeruginosa* PAO in their exponential phase (lag phase).

4. Sensitivity of these isolated phages to different physical and chemical agents was determined.
   a. Sensitivity to chloroform showed that all the *K. pneumoniae* B5055 and *P. aeruginosa* PAO specific phages were resistant to the action of chloroform confirming that all the phages were non-enveloped.
   b. All the *Klebsiella* and *Pseudomonas* specific phages were found to be heat labile as these got inactivated either at 60°C or 70°C.
   c. UV- inactivation studies showed complete inactivation on exposure to UV light between 80 to 100 minutes and 60 to 100 minutes respectively for *Klebsiella* and *Pseudomonas* specific phages.

5. Growth characteristics of all the isolated phages were checked.
   a. Absorption rate of *Klebsiella* and *Pseudomonas* specific phages was determined and it was found to be approximately 5-10 minutes and 5-12 minutes respectively for the phages of both the bacteria.
   b. One step growth curve of all the isolated phages was determined. Klebsiella specific phages showed eclipse period of 10-25 minutes, latent period of 20-35 minutes and burst size of 100-140 PFU/bacterial cell whereas *Pseudomonas* specific phages depicted eclipse period of 10-20 minutes, latent period of 20-25 minutes and burst size of 100-140 PFU/bacterial cell.

6. Isolated phages were classified on the basis of morphological features, structural proteins analysis and nucleic acid characteristics.
   a. Transmission electron micrograph of potassium phosphotungstate stained *Klebsiella* and *Pseudomonas* specific phages showed that
phages possessed icosahedral heads and short non contractile stumpy tails. Based on this information, these phages were assigned to the order Caudovirales and family Podoviridae.

b. Structural protein composition of all the isolated phages was analyzed by SDS-PAGE. All the *Klebsiella* and *Pseudomonas* specific phages assigned to *Podoviridae* family showed similar structural protein pattern.

c. All the 5 *Klebsiella* phages harbored major structural proteins of 20 and 29 kDa. Phage Kpn12 showed 3 minor structural proteins of 39, 43 and 55 kDa and phages Kpn13, Kpn17 and Kpn22 showed one more major protein band of 22 kDa. Phage Kpn13 showed 2 minor proteins of 39 and 43 kDa. Phage Kpn17 showed only 1 minor protein band of approximately 43 kDa. Phage Kpn22 showed 2 minor bands of 43 and 47 kDa.

d. Five *Pseudomonas* phages exhibited 4 major structural protein bands of approximately 20, 22, 27 and 45 kDa and 1 minor structural protein band of molecular weight approximately 67 kDa.

e. Genome size of *Klebsiella* and *Pseudomonas* specific phages was found to be in the range of 23.0 – 24.0 kb.

f. All the *Klebsiella* and *Pseudomonas* specific phages were found to harbor double stranded DNA as genetic material.

g. In order to construct the genetic fingerprint of the isolated phages, and to distinguish them genetically, RAPD PCR using six commercially available primers was performed with purified phage DNA of respective phages. Different banding patterns obtained with *Klebsiella* and *Pseudomonas* specific phages confirmed genetic variation among the isolated phages.

7. Toxicity, survival and stability of phages were assessed in mice. The results showed:
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a. Absence of illness in thermally injured mice injected with purified preparations of *Klebsiella* and *Pseudomonas* phages confirmed non toxic nature of these phages.

b. Survival and stability of *Klebsiella* and *Pseudomonas* specific phages in blood, peritoneal fluid and different organs (lungs and skin) of phage treated mice was determined. The results showed that in case of *Klebsiella* phage Kpn5, maximum phage count in blood, peritoneal fluid, lungs and skin was obtained at 6 hours post phage inoculation. No phage could be isolated in peritoneal lavage fluid, blood, lungs and skin at 48 hours post phage injection. *Pseudomonas* specific phages exhibited shorter survival in mice in comparison to *Klebsiella* specific phages as maximum phage count in blood, peritoneal fluid, lungs and skin was observed at 3 hours post injection. No phage could be isolated in peritoneal fluid, blood, lungs and skin samples at 36 hours after phage injection.

8. In order to assess the potential of isolated and well characterized phages in treating infection, a murine model of full thickness contact burn wound infection was established employing standard strains of *K. pneumoniae* B5055 and *P. aeruginosa* PAO.

a. On the basis of histopathological evaluation of the burnt skin, the time required to establish a full thickness or third degree burn was found to be 45 sec.

b. LD$_{100}$ dose values for *K. pneumoniae* B5055 via subcutaneous and topical route were found to be $10^6$ and $10^8$ CFU respectively whereas lethal dose causing 100% infection with *P. aeruginosa* PAO via subcutaneous and topical route were estimated to be $10^7$ and $10^9$ CFU respectively.

9. Efficacy of different *Klebsiella* phages (Kpn5, Kpn12, Kpn13, Kpn17 and Kpn22) individually as well as in cocktail, to prevent *K. pneumoniae* infection was examined in mouse model of thermal injury in terms of
increased survival rate and decreased bacterial counts in different organs at 24, 48 and 72 hours as compared to untreated control group. The results showed:

a. 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.

b. All of burned and infected mice died within 48 - 72 hours with 5.53% survival rate whereas all burned infected and phage treated mice survived, showed 80-100% protection ($P<0.001$) at 24, 48 and 72 hours post inoculation.

c. 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 hours.

10. Among all *Klebsiella* specific phages tested, phage Kpn5 was found to be most effective ($P<0.001$), hence, detailed study on phage Kpn5 was conducted to assess its potential in burn wound treatment.

a. The lytic activity of phage Kpn5 against *K. pneumoniae* B5055, *in vitro*, was checked which demonstrated that phage Kpn5 had potent lytic activity against *K. pneumoniae*. Emergence of resistant mutants of Kpn5 was also observed.

b. Phage Kpn5 at a MOI ≥1.0 was found to give 100% survival with minimal signs of illness. Therefore phage dose at MOI of 1.0 was selected as optimum dose for treating burned and infected mice.

c. To examine the limitation of this phage therapy model, phage was administered after 6, 12, 18 and 24 hours following bacterial challenge in burn site via s.c. route. The results showed that a single i.p. injection of phage could rescue 73.33 % of the animals even when treatment was delayed up to 6 hours after burn/
bacterial challenge (P<0.001) as compared to a delay of 24 hours in which 100% mortality in control group was observed.

d. To check whether phage rescue of mice with *K. pneumoniae* B5055 bacteremia required functional phage or whether phage rescue was associated with a non specific immune activation response, efficacy of viable phage to treat burn wound infection was compared with heat inactivated phage. A significantly high (P<0.001) percent survival was observed in mice injected with viable plaque forming phage in comparison to PBS treated control mice and mice injected with heat inactivated phage.

e. The levels of pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-10) cytokines in the serum samples and lungs of phage treated and untreated control mice was evaluated at different time intervals. The results revealed that phage treated mice showed significantly lower level of cytokine IL-1β and TNF-α and IL-10 as compared to control untreated group at 24, 48 and 72 hours of phage treatment.

f. Efficacy of phage Kpn5 in treating burn wound infection in mice caused by *K. pneumoniae* B5055 was also assessed histopathologically in terms of regeneration of damaged skin layers. Recovered skin (11th day and 20th day post burn) of burned and infected mouse following treatment with phage showed regeneration of epidermis and reappearance of hair follicles and sweat glands, comparable to normal mouse skin.

11. The therapeutic potential of *Pseudomonas* phages (Pa29, Pa30, Pa31, Pa33 and Pa34) to treat *P. aeruginosa* PAO induced infection in burned mice was evaluated. All *Pseudomonas* specific phages were injected i.p. at a MOI ranging from 0.001 to 900, in burned and *P. aeruginosa* PAO infected mice. Protection was not observed with either of *P. aeruginosa*
PAO specific bacteriophages belonging to family Podoviridae upto 96 hours post phage treatment.

12. Phage Kpn12 which showed sensitivity and lytic activity to both *K. pneumoniae* B5055 and *P. aeruginosa* PAO was used for the treatment of mixed infection of burn wound caused by these two bacteria. Phage treated group showed no protection and results were similar to that seen in untreated control mice which gave 100% mortality (P>0.05).

13. Efficacy of phage Kpn5 in the treatment of burn wound infection caused by *K. pneumoniae* B5055 was also checked via topical route.
   a. The prepared hydrogel was tested for its toxicity on shaved skin on the back of normal mice for different time period following single and multiple applications. No signs of irritation were found in any of the mice confirming non toxic nature of hydrogel.
   b. 3% HPMC hydrogel was prepared and stability of phage kpn5 was checked in this hydrogel. It was found to be 100% stable with no decrease in phage titer over a period of 7 days.
   c. Phage Kpn5 suspended in 3% hydrogel was tested for the treatment of burn wound infection caused by *K. pneumoniae* B5055 in mice via topical route. The results showed that phage Kpn5 provided protection (66.66%) at a MOI of 200 (P<0.001) as compared to untreated control mice.

14. Different antimicrobial agents such as silver nitrate (AgNO3), gentamicin, honey and aloe vera gel were also tested along with phage Kpn5 for the treatment of burn wound infected with *Klebsiella pneumoniae* B5055 via topical route in mice.

15. Efficacy of silver nitrate and gentamicin (mixed with 3% hydrogel) was checked for topical treatment of burn wound infection caused by *K. pneumoniae* B5055 in mice. Both the antimicrobial agents did not show
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any protection at lower concentrations whereas at higher concentration, 0.5% silver nitrate and 0.1% gentamicin treated mice showed significantly higher protection (P<0.001) as compared to control on 7th day of treatment. On comparison, phage Kpn5 on single application was found to be slightly more effective than multiple applications of silver nitrate and gentamicin (Once per day).

16. Natural products such as undiluted honey and aloe vera gel were also tested for their antibacterial activity against *K. pneumoniae* B5055 induced burn wound infection in mice. The results showed that burned/infected mice when treated daily by applying honey and aloe vera topically showed protection of 33.33% and 26.66% respectively in comparison to untreated control group.

17. Stability of phage Kpn5 was tested in honey and aloe vera gel and phage Kpn5 was found to be 100% stable when observed for a period of 7 days.

18. Phage Kpn5 alone was highly effective in treating burn wound infection caused by *K. pneumoniae* B5055 in mice following topical application whereas its combination with honey and aloe vera did not provide any additional advantage to the phage *in vivo*.

It is concluded from the study that phages against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* can be isolated from environmental samples: these phages can be easily stored, classified and characterized. The isolated *K. pneumoniae* specific bacteriophages individually as well as in cocktail, demonstrates potential to treat burn wound infection as ascertained on the basis of increased percentage survival, lower bacterial load in blood, peritoneal fluid, lungs and skin and decreased levels of inflammatory cytokines in serum and lung homogenates of phage treated mice. Unfortunately, *Pseudomonas* specific phages belonging to *Podoviridae* family order *Caudovirales* did not provide any protection. Further studies employing large number of clinical strains of *K. pneumoniae* and evaluation of *Pseudomonas* specific phages other than *Podoviridae* family as therapeutic agents is warranted.