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Nebulization as the mode to administer therapeutic bacteriophages to resolve Acinetobacter pneumonia in rabbits

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ABSTRACT

Introduction: Multidrug-resistant strains of *Acinetobacter baumannii*, a significant hospital pathogen, have developed resistance to virtually all available antibiotics. Carbapenems antibiotics are among the most commonly used antibiotics against Acinetobacter infections, but they can be rendered ineffective by the metallo-beta-lactamase enzyme. In this study, bacteriophage isolated against bla_{SPM-1} producing *Acinetobacter baumannii* was evaluated for its therapeutic potential in the rabbit pneumonia model.

Materials and Methods: *Acinetobacter baumannii* was isolated from the sputum and was speciated as per the standard microbiological techniques. Bacteriophage specific for bla_{SPM-1} producing *Acinetobacter baumannii* from the sewage water. Pneumonia was introduced in the rabbits as per Esposito Pennington method and efficacy of the bacteriophage in resolving pneumonia was evaluated. For in-vivo experiments, five groups of rabbits were used including infection-free, phage control, bacteria-infected control, and the other two groups infected with *Acinetobacter baumannii* and treated either with an antibiotic, colistin (2.5 mg/kg, twice a day intraperitoneally) or Acinetobacter phage (3×10^9 PFU/mL, given once through nebulizer). The experimental animals were monitored for 72 hours for mortality, and the surviving rabbits were killed for bacteriological and histopathological analysis

Results: In the infected group, pneumonia was developed within 48 hours, and 6/10 animals were dead after 72 hours. All the animals in the antibiotic group survived but showed signs of pneumonia, and there was up to 4 log CFU/g±0.24 reduction in the bacterial count. In phage treated group, all the animals survived at the end of 72 hours and all the animals were healthy with no signs of pneumonia.

Conclusions: The experiment showed new insights into the application of bacteriophage through nebulization, a non-invasive method of phage delivery to rescue rabbits from pneumonia caused by bla_{SPM-1}-producing *Acinetobacter baumannii*.

1. Introduction

Pneumonia ensues recurrently in immunocompromised patients and regularly shows a complicated progression of the disease when compared to immunocompetent persons. The type of etiology involved is directly linked with age, and the type of immunosuppression.[1] Gram-negative bacteria are the most common bacterial etiology of hospital-acquired pneumonia. In the period of increasing antimicrobial resistance, Acinetobacter, a gram-negative bacillus stands

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out as one of the most resistant bacteria responsible for increased morbidity and mortality in the hospital. The cause of resistance in *Acinetobacter baumannii* can be due to declined outer membrane permeability, efflux pumps, and secretion enzymes.[2,3] Different classes of beta-lactamase enzymes have been identified and demonstrated.[3] As per the Ambler classification, Metallo-beta lactamases are classified in group B. Further B group is divided into three subclasses: BI, BII, and BIII. According to the molecular structure, the BI subclass is divided into four categories: the IMP, VIM, GIM, and SPM types.[3] The first Metallo-beta lactamases identified was IMP-1 which was found in S. marcescens in Japan (1991), VIM-1 originally detected in Italy (1997), SPM-1 in Brazil (1997), and finally GIM detected in Germany (2002).

An innovative method to combat the harmful effects of enhanced rates of antimicrobial resistance is needed. With a diminution in the innovation of novel antibiotics and based on scientific literature, bacteriophages which are written off as a forgotten cure in the 19th century are one of the best hopeful substitutes to antibiotics for clinical use.[4,5] Phages are viruses that occur in nature that can kill the bacterial host, including those that are resistant to antibiotics.

The present study aimed to evaluate the efficacy of bacteriophages applied through nebulization in rescuing a rabbit from pneumonia caused by *blaSPM*-1 - producing *Acinetobacter baumannii.*

2. Materials and Methods

2.1. Bacterial isolates

The study protocol was approved by the Institutional Ethics Review Board and Institutional Animal Ethics Committee of the S.S. Institute of Medical Sciences and Research Centre. Consent from patient was taken before enrolling in the research. Acinetobacter baumanii strain used in this study was isolated from the pneumonia patient admitted to the Intensive Care Unit (ICU) at the S.S. Institute of Medical Sciences & Research Centre, Davangere, India.sputum sample was collected from the patient and bacteria were isolated using the standard procedures.[6,7] The VITEK, Biomerieuxsystem was used for the identification, confirmation of bacteria and to screen for antibiotic susceptibility,[6,7] The antibiotics tested include construedas per CLSI guidelines.[6] In addition, 16S rRNA sequencing was performed using universal primers. The polymerase chain reaction products sequenced and BLAST hit with >98% similarities were deemed as sufficient for bacterial identification.[8]

To detect the presence of Metallo-Beta Lactamase (MBL) genes, PCR was performed for five genes; bla$_{KPC}$, bla$_{NDM}$, bla$_{OXA-48}$, bla_{SPM-1} and bla_{IMP}. The primers and PCR conditions were previously reported by van der Zwaluw et al.[9,10] The PCR products were sequenced and BLAST analysis was performed to identify the similarities in nucleotide sequences.

2.2. Isolation of bacteriophage

The bacteriophage against bla_{SPM-1} producing *Acinetobacter baumannii* was isolated from sewage samples collected at a municipal sewage treatment plant, Davangere by the method of Smith and Huggins.[4] Briefly, 30 mL of sewage water sample was mixed with 10 mL of bacterial culture and incubated overnight at 37°C without shaking. The mixture was centrifuged at 6000 × g for 15 min and the supernatant was collected. A 2 mL of the collected supernatant was filtered through 0.22-micron syringe filters and tested for the presence of phage. To perform the double agar overlay method, 200 μL of bacterial culture and 100 μL of the filtrate were added to 3 mL of soft agar (0.75%). The contents were mixed gently and poured onto pre-prepared Mueller-Hinton agar plates. The plates were incubated for 18 hours at 37°C and the presence of phages was determined by visualizing the plaques. The phages were carefully removed from the individual plaques using a needle and propogated in the presence of host bacteria. Now, the samples were filtered using a 0.22-micron syringe filter and the filtered phage samples were precipitated using polyethylene glycol (PEG) and NaCl. The overnight precipitated samples were centrifuged at 15,000 × g for 30 min and the pellet was mixed with buffer. The precipitated phages were extracted against chloroform at a 1:1 ratio to remove bacterial debris and stored at -20°C.

2.3. Phage morphology

The phages isolated against bla_{SPM-1} producing *Acinetobacter baumannii* were negatively stained by 2% uranyl acetate and morphology was studied under transmission electron microscope.[11] Briefly, 5 μL of phage filtrate was placed over the copper grid and allowed to settle for 10 min. To the dried grid surface, 2 μL of uranyl acetate was added and allowed to stain for 1 min and remove. The excess dye was washed using distilled water by placing 5 μL of water on the grid and removed immediately. The copper grid was allowed to dry for 30 min and visualized under TEM.

2.4. Host bacterial lytic activity test

Time-kill kinetic assay was conducted to study the kinetics of phage against bla_{SPM-1} producing Acinetobacter baumanii. Briefly, the 10^8 CFU/mL of bacterial culture was infected with 10^9 PFU/mL of bacteriophages and incubated at 37°C, without shaking. The viable counts were determined at 0, 4, 8, 16, and 24 h by plating 100 μL of cultures onto Mueller Hinton agar plates. The viability was reported as log10 (CFU/mL) values and the experiment was

repeated twice for statistical significance.[6,11]

2.5. Minimum infective dose of bla_{SPM-1} producing Acinetobacter baumannii in rabbits

The rabbits were randomly divided into 6 groups of five animals each. A modification of the Esposito and Pennington model[12] was used for the induction of pneumonia infection. Each group received 50ml of bacterial suspension in different densities (10^4-10^9CFU). The animals were monitored for 48 h and were scored for their state of health on a scale of 5 to 0, based on the advancement of disease which is reflected by several clinical signs.[12] A normal and unremarkable condition was scored at 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 respiratory symptoms, specifically crackles, was scored as 2; a moribund state was scored as 1, and death was scored as 0. After 48 hours of observation, all the surviving rabbits were killed by intraperitoneal administration of 5 % sodium thiopental. All the rabbits (dead and killed) were analyzed immediately after their death. Qualitative bacterial culture and histopathological study of the lungs were done to assess the intensity of the infection.

2.6. Effect of pH and temperature on stability of Acinetobacter baumannii phage

To study the effect of pH, two types of buffer solutions were prepared, i.e. Clark and Lubs buffer solutions (pH 3–8) and alkaline buffer solutions (pH 9–11). The buffers were used at a final concentration of 50 mM: HCl for pH 3–4, potassium hydrogen phthalate for pH 5, KH_2PO_4 for pH 6–8, Tris(hydroxymethyl)-aminomethane for pH 9, and NaOH for pH 10–11. Buffer (500 ml) was stored in a pyrex glass beaker. As the buffer solutions did not contain NaCl, they are unlikely to affect the inactivation process.[5] The experiments were conducted in triplicate

Temperature effect studies, the buffers were kept at 32^0C, 34^0C, 36^0C, 38^0C, 40^0 and 42^0C. A 1 ml aliquot of 3.72 X10^9PFU of *Acinetobacter baumannii* phage stock solution was added to the various samples at time zero.[5,10] At time intervals of every 20 minutes, 2-ml samples were assayed to determine concentrations of *Acinetobacter baumannii* phage by agar overlay technique.[10] The experiments were conducted in triplicate.

2.7. Maintenance and induction of pneumonia in rabbits

Rabbit weighing 2-2.5 g, bred locally in the animal house of S.S. Institute of Medical Sciences and Research Centre, Davangere was selected for the study. The animals were housed in regulation cages and given free access to food and water.[4,11]

A modification of the Esposito and Pennington model[12] was used for the pneumonia infection. The rabbits were anesthetized by an intraperitoneal injection of 5% sodium thiopental. Pneumonia was induced by introducing 50 μL of a 10^8 CFU/mL of bla_{SPM-1} producing Acinetobacter baumanii bacterial suspension obtained from 18 hours of culture in trypticase soy broth (Hi-Media, India) at 37°C.

2.8. Effectiveness of Acinetobacter phages

To evaluate the effectiveness of the Acinetobacter phage, the animals were divided into five groups;

Group I (Placebo)**:** The rabbits were injected with PBS.

Group II (phage-only, control)**:** The rabbits were not infected and received 3×10^9PFU/mL of phage by nebulization. This group was included to study whether phages can cause some sort of hypersensitivity in rabbits.

Group III (Infection control)**:** The pneumonia was induced in rabbits (10^8 CFU/mL) and was not treated with antibiotics or challenged with phages.

Group IV (Antibiotic treated)**:** Pneumonia was induced in rabbits and pneumonia-infected rabbits were treated with colistin, 2.5 mg/kg, twice a day intraperitoneally. The antibiotic was administered 48 hours after inoculation. The antibiotic was given for three days.

Group V (Phage treated)**:** Pneumonia was induced in rabbits and pneumonia-infected rabbits were treated with Acinetobacter phage (3×10^9PFU/mL) administered by nebulization.[13] Phage was administered 48 hours after inoculation. The bacteriophage was given only once.

After 48 hours of observation, all the surviving rabbits were killed by intraperitoneal administration of 5 % sodium thiopental. All the rabbits (dead and killed) were analyzed immediately after the death. Qualitative bacterial culture and histopathological study of the lungs were done to assess the intensity of the infection. The results were expressed as mean ± SD of the log CFU/g of the lung.

2.9. Estimation of neutralizing antibodies

A plaque reduction assay was used to estimate the presence of neutralizing antibodies. The dilution of serum neutralizing the phage was estimated by observing a decrease in the PFU number in double-agar overlay method.[14]

2.10. Statistical analysis

All the experiments were repeated twice for statistical significance. The survival graphs were plotted using the Kaplan-Meier method and any differences in survival rates were calculated using the log-rank test (GraphPad Prism software 7.0). $P < 0.05$ was considered as statistically significant (log-rank test).

3. Results

3.1. Characterization of bla_{SPM-1} producing Acinetobacter baumannii clinical isolates

The isolated *Acinetobacter baumannii* strain was found to be multi-drug resistant. Antibiotic susceptibility test showed that the *Acinetobacter baumannii* strain was resistant to imipenem (MIC, > 256 μg/ml), meropenem (MIC, > 256 μg/ml), cefepime (MIC, 16 μg/ml), piperacillin-tazobactam (MIC, ≥ 128 μg/ ml), ampicillin (MIC, ≥ 32 μg/ml), ampicillin/sulbactam (MIC, ≥ 32 μg/ml), ceftriaxone (MIC, ≥ 64 μg/ml), cefazolin (MIC, ≥ 64 μg/ml), nitrofurantoin (MIC,128 μg/ml) and ceftazidime (MIC, ≥ 64 μg/ml), ciprofloxacin (MIC, ≥ 8 μg/ml), aztreonam (MIC, ≥ 128 μg/ml), amikacin (MIC, ≥ 64 μg/ml), levofloxacin (MIC, ≥ 0.25 μg/ml) but was susceptible to colistin (MIC, 2 μg/ml). PCR and sequencing study showed that bla_{SPM-1} was the only carbapenemase-encoding gene carried by the isolate. (Gene accession number OM0105578)

3.2. Characterization of phage infecting bla_{SPM-1} producing Acinetobacter baumannii

Bacteriophage was isolated against bla_{SPM-1} producing *Acinetobacter baumannii* from the sewage treated-effluent sample. The phage produced tiny, clear plaques on the double agar overlay plate (Figure 1A). Transmission electron microscopy (TEM) analysis showed a phage with the icosahedral head measuring about 80 ± 0.5 nm in diameter and a 100 ± 0.5 nm long non-contractile tail. Thus, morphologically the phage belongs to the Siphoviridae family (Figure 1B).

3.3. Effect of pH and temperature on the viability of phages

At pH of 3 to 5, no phages were recovered at different temperatures. At pH 6, the maximum number of phages isolated was less than the initial concentration (9.5415 log10 PFU). The maximum phage recovered at temperature 32^0C is 8.3579 log10 PFU, at 34^0C is 8.7180 log10 PFU, at 36^0C is 9.2562 log10 PFU, at 38^0C is 9.0539 log10 PFU, at 40^0 C is 8.4186 log10 PFU and at 42^0C is 8.2504 log10 PFU (Table 1 , Figure 2).

At pH 7, the maximum number of phages (10.4966 log10 PFU) was isolated at 38^0C. It was more than the initial concentration (9.5415 log10 PFU). The phages isolated at other temperatures are; at temperature 32^0C is 8.8115 log10 PFU, at 34^0C is 8.9882 log10 PFU, at 36^0C is 9.9447 log10 PFU, at 40^0 C is 9.6569 log10 PFU and at 42^0C is 8.4639 log10 PFU (Table 1 , Figure 2).

At pH 8, the maximum number of phages isolated was less than the initial concentration (9.5415log10 PFU). The maximum phage recovered at temperature 32^0C is 7.4487 log10 PFU, at 34^0C is 7.8269 log10 PFU, at 36^0C is 7.9304 log10 PFU, at 38^0C is 9.5051 log10 PFU, at 40^0 C is 9.8325 log10 PFU and at 42^0C is 8.4742 log10 PFU. At pH 9 to 12 the phages were not recovered.

3.4. Lethality of bla_{SPM-1} producing Acinetobacter baumannii in the rabbit model

Figure 3 shows lethality of the minimum infective dose of bla_{SPM-1} producing *Acinetobacter baumannii* in an experimental pneumonia rabbit model. Rabbits inoculated bla_{SPM-1} producing *Acinetobacter baumannii* with 10^8 CFU established infection within 48 h. In Figure 3, each bar indicates the state of health of a single rat, a score of 5 indicates normal health, while 0 indicates death (see the material and methods for a full description of the scale). For all experimental work, 10^8 CFU of bla_{SPM-1} producing *Acinetobacter baumannii* was taken as an infective dose to evaluate the therapeutic utility of phages in the rabbit model.

3.5. Lysis kinetics of Acinetobacter baumannii infected with phage

The results of the time-kill assay are presented in Figure 4. The data showed that there is a 4-fold reduction in the concentration of viable count at the 4^{th} hour after colistin (2.5 μg/mL) treatment and this gradual decrease in bacterial count was observed till 16^{th} hour and further the bacterial load started increasing at 24 hours. In the case of bacteriophage challenge assay (3×10^9 PFU/mL), a 6-fold reduction in the viable count was seen at the 4^{th} hour. Interestingly, no bacterial regrowth was seen at the end of 16 hours for bacteriophage (Fig.4).

3.6. Acinetobacter phage exhibited comparable efficacy with Colistin in treating acute pneumonia with less organ toxicity

In the control groups, PBS and phage-only, the survival rate was 100% which clearly showed that the phage preparations were free from toxic substances. In the infected group, i.e. bacteria-only, out of 10 rabbit, six were dead and four animals were moribund at the end of 72 hours of inoculation. Bacterial count in dead rabbit was 14.64 log CFU/g of lung±0.34, which is 6 logs higher than the minimum lethal dose and bacterial count in killed rabbit was 11.22 log CFU/g of lung±0.66 which was 3 logs higher than the minimum lethal dose used in the experiment.

Among the Colistin-treated group, all the rabbits survived (n=10) at the end of the 72 hours but the surviving rabbit showed signs of pneumonia. The bacterial enumeration showed there was up to 4 log CFU/g±0.24 reduction in the lungs (Table 2) which caused the pneumonia symptoms even after six doses of colistin. In bacteriophage-treated rabbits, all the rabbits survived (n=10) at the end of 72 hours and the rabbits were

healthy with no signs of pneumonia. None of the rabbits showed bacterial growth from the lung samples, indicating bacteria were cleared from the lung by the phage (Table 2). Histopathological study showed that the rabbit infected with bla_{SPM-1} producing *Acinetobacter baumannii* had changes specific to pneumonia. Accordingly, the lungs showed acute inflammation characterized by diffuse and/or focal effects on all lobes with severe inflammatory infiltration of polymorphonuclear cells (Figure 5). None of the animals from the groups showed the presence of neutralizing antibodies against the bacteriophage used in the study.

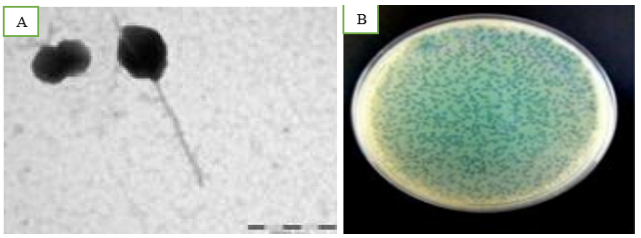

Fig. 1: A: Morphology of plaques formed on the double agar-overlay plate; **B:** Electron microgram of Klebsiella phage (The scale bar represents 200 nm). The morphological structure shows that the phage consists of a head (80±0.5 nm) and a long non-contractile tail (100±0.5 nm); therefore the phage belongs to the Siphoviridae family.

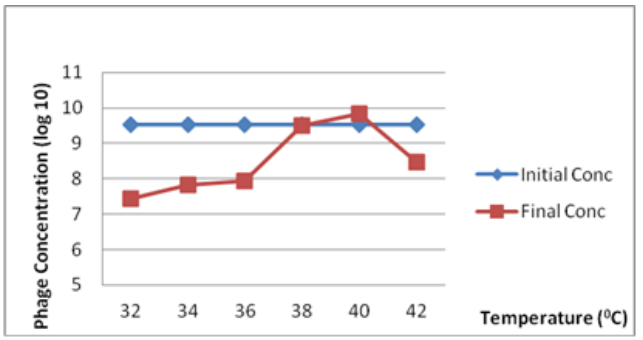

Fig. 2: Different concentration of Acinetobacter baumannii phages produced at different temperature at pH 7. Initial concentration of phage tested is 9.6414 log 10. The maximum concentration of phage obtained at temperature of 36^0C is 11.99493 log 10.

4. Discussion

Acinetobacter baumannii is an opportunistic pathogen in humans, affecting people with compromised immune systems, and is becoming ever more important as a hospital-acquired infection.[15,16] Multidrug-resistant *Acinetobacter baumannii* causes infections such as pneumonia, meningitis, wound infections, bacteremia, and urinary tract infections. Antimicrobial resistance in Acinetobacter species poses great limits for therapeutic options in infected patients, especially if the isolates are resistant

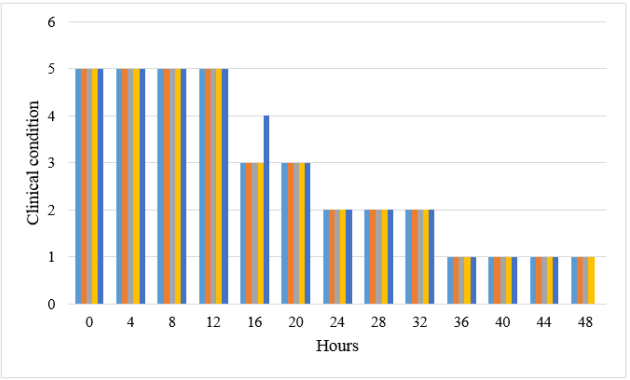

Fig. 3: Lethality of the Minimum infective dose of bla_{SPM-1} producing *Acinetobacter baumannii* in rabbit model (Esposito and Pennington model). Each group of rabbits received inoculation of 50ml aliquots of bacterial suspension in different densities (10^4-10^9CFU). The animals were observed for 48 h. Rabbits were scored for their state of health on a scale of 5 to 0, based on progressive disease states reflected by several clinical signs. A normal and unremarkable condition was scored at 5; slightly 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 respiratory symptoms, specifically crackles, was scored as 2; a moribund state was scored as 1; and death was scored as 0. All the rabbits were moribund within 48 h for the bacterial dose of 10^8 CFU.

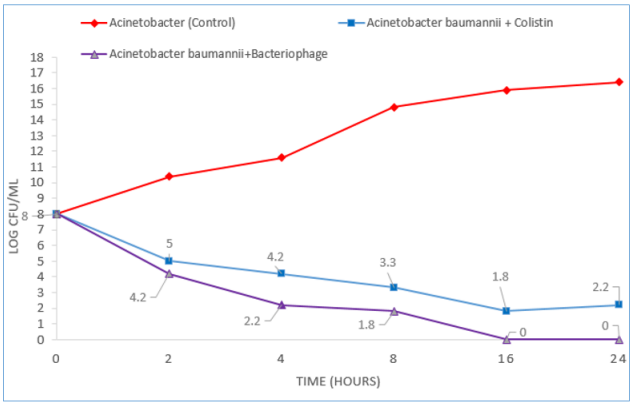

Fig. 4: Lysis kinetics of bla_{SPM-1} producing *Acinetobacter baumannii* treated with colistin and challenged with phage. The bla_{SPM-1} producing *Acinetobacter baumannii* was incubated in MH broth (red), with antibiotic (blue), and with phage (purple), at 37°C and bacterial counts were performed at regular time intervals. The values indicated are the means of two independent experiments.

Table 1: Initial and the final concentration of *Acinetobacter baumannii* phages obtained at pH 6, 7 & 8 at different temperature

Temp (^{0}C)	pH 6		7		8	
	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)	Initial Concentration (log 10)	Final Concentration (log 10)
32	9.541579	8.357935	9.541579	8.811575	9.541579	7.448706
34	9.541579	8.718003	9.541579	8.988291	9.541579	7.826981
36	9.541579	9.256208	9.541579	9.944713	9.541579	7.930440
38	9.541579	9.05395	9.541579	10.49661	9.541579	9.505164
40	9.541579	8.418633	9.541579	9.656998	9.541579	9.832509
42	9.541579	8.250435	9.541579	8.463902	9.541579	8.474225

Table 2: Efficacy and safety of Colistin and *Acinetobacter baumannii* phage on the survival rates of rabbits and the clearance of bla $_{SPM-1}$ producing *Acinetobacter baumannii* from their lungs.

Treatment group	Number of rabbit (N)	Number of Rabbit Survived		Number of Rabbit died	
		N (%)	Bacterial count (Log CFU/g of lung × ± SD)	N (%)	Bacterial count (Log CFU/g of lung × ± SD)
Pneumonia infected	10	*4 (40)	11.22±0.66	6 (60)	14.64±0.34
Colistin treatment	10	*10 (100)	4.42±0.24	0	-
Phage treatment	10	*10 (100)	0	0	-

*The surviving rabbits were killed 12 hours after the last dose in the treatment groups.

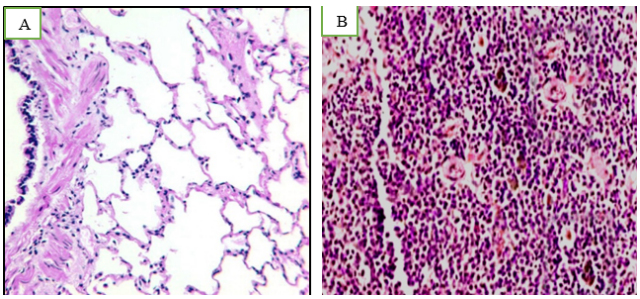

Fig. 5: Rabbit lung histology (Haematoxylin and eosin staining); **A:** normal lung, and **B:** bronchopneumonia with inflammatory cells and debri.

to the carbapenems.[13,16–18] In recent years, Metalla beta-lactamase enzymes have been identified from clinical isolates with increasing frequency across the world, and strains that produce these enzymes have been accountable for prolonged treatment and acute infections. Since there is decrease in discovery of new antibiotics and emergence of extensively resistant bacterial isolates has challenged the scientific world to look for other alternative therapy to tackle the drug resistance menace. Bacteriophage therapy is one such alternative which can be used as an anti-therapeutic agent.

Bacteriophage (phage) therapy is regaining attention as a potential treatment option for bacterial infections, including those caused by multidrug-resistant bacteria. Phage therapy utilizes obligatory lytic phages to kill its host bacteria. The species-specific nature of phages is of particular interest as a targeted treatment can limit the unintended adverse impacts on the patient's microbiome which is commonly observed in antibiotic treatment. Phages have been used for the treatment of various clinical conditions. Depending on the target site of bacterial infection, different routes have been used in the administration of therapeutic phages to the patient.[5] Ideally, routes for phage therapy are chosen to ensure the effective delivery of the phage to the target site of infection.[19] However, despite the prospective benefits, the use of phages for the treatment of pulmonary bacterial infections has been fairly underexplored. In the present study, the therapeutic effect of Acinetobacter phage against bla $_{SPM-1}$ producing *Acinetobacter baumannii* was evaluated in the rabbit model in which pneumonia was induced and the phages were introduced through nebulization. The findings from this study suggest that bacteriophage isolated against bla $_{SPM-1}$ producing *Acinetobacter baumannii* was effective in resolving pneumonia in rabbits. That is, a single dose of bacteriophage was sufficient to clear bacteria from the infected lungs compared to the antibiotic colistin which was given twice a day for three days. One of the previous studies also showed that a single dose of intraperitoneal phage administration immediately after infection could rescue 100% of pneumonia-infected animals.[20] In the case of pneumonia, the route of phage administration is always challenging but both intraperitoneal and intranasal administration proved to be effective.[21] A study by Cao et al. showed 100% recovery of infected animals with intranasal phage administration when animals were treated 2 hours after the infection.[21] Our study is one of the

rarest to show the 100% effectiveness of single-dose phage administered by using a nebulizer in pneumonia developed in a rabbit model compared to the organ toxic colistin treatment. Phages were very effective in clearing bacterial from the lungs.

Temperature and pH plays a very important role in the survival and proliferation of bacteriophages and the success of the phage therapy. The optimum pH and temperature for the phage isolated against bla $_{SPM-1}$ producing *Acinetobacter baumannii* was 7 and 38^0C. The maximum plaque forming units was obtained at this temperature and pH. For successful of phage therapy, the isolated bacteriophage should be stable and should able to propagate efficientyly at body temperature and the pH.

In the in vitro time-kill assay, when the bacteria were challenged with colistin, the bacterial load decreased up to 18 hours of incubation, and then the bacterial load started increasing. But, in the phage challenged test, a 6-fold reduction in the viable count was seen after 4 hours and no bacterial regrowth was seen at the end of 8 hours for bacteriophage. Similar observations were made in the previous studies to prove that the bacterial doubling time was higher during phage treatment.[21] Neutralizing antibody titre was evaluated to estimate the possible antibody production against the bacteriophages in the acute condition and also to assess the possible exposure of the rabbit to the specific bacteriophage tested. No observable antibody titres were seen in the present study indicating the rabbit were not exposed to the specific bacteriophage earlier and also antibodies were not formed during the treatment of acute infection. During phage therapy, the lysis of bacteria can cause immune responses but it was shown that there was no overstimulation of inflammatory response in the in vivo pneumonia treatment.[22]

Hundreds of thousands of deaths occur every day due to previously treatable infections such as lower respiratory and bloodstream infections because the bacteria that cause them have become resistant to treatment.[2,3] Carbapenemase-producing *Acinetobacter baumannii* strains represent a challenge for clinical practitioners due to their increasing prevalence in hospital settings and antibiotic resistance bla $_{SPM-1}$ Metallo-β-lactamase is one of the antimicrobial resistance factors causing the greatest concern because its global spread has been rapid and it is frequently associated with other resistance genes.[3,6] Sixteen variants of SPM-1 enzyme have been discovered in different countries since the identification of bla$_{SPM-1}$ in India. In this study, the *bla*$_{SPM-1}$ producing MDR *Acinetobacter baumannii* was isolated from pneumonia patients and found to be susceptible to bacteriophage that could clear the bacterial load in the rabbit models. Bacteriophage was administered through nebulization in which phage aerosol were generated and rabbit was made to inhale the bacteriophage which is found to be very effective in treating pneumonia. This proof-of-concept study shed light on the use of phage as a therapeutic agent to cure acute pneumonia caused by MDR *Acinetobacter baumannii*.

5. Acknowledgement

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6. Conflicts of interest

The authors have no conflict of interest to declare.

7. Source of Funding

None.

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