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Therapeutic effectiveness of bacteriophage in the treatment of pneumonia caused by NDM-4 producing *Klebsiella pneumoniae* in a mouse model

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ABSTRACT

Introduction: Exploration for an alternative to antibiotics to treat bacterial diseases is becoming more important due to the emergence of antibiotic resistance. Bacteriophage or phage has been used for the treatment of superficial infections.

Materials and Methods: In this study, *Klebsiella* phage isolated from the environment was used as a therapeutic agent against NDM-producing *Klebsiella pneumoniae* causing respiratory disease in an experimental mouse model. The isolated *Klebsiella* phage was found to belong to the *Siphoviridae* family based on morphology. For *in vivo* experiments, five groups of mice were used including infection-free, phage-only, bacteria-infected, and the other two groups infected with *K. pneumoniae* and treated either with an antibiotic, levofloxacin (50 mg/kg, twice a day intraperitoneally) or *Klebsiella* phage (3×10^9 PFU/mL). All the animals were observed for 72 hours for mortality, and the surviving mice were killed for analysis.

Results: In the infected group, pneumonia was developed after 48 hours, and 8/10 animals were dead after 72 hours. When pneumonia infected mice were treated with an antibiotic, levofloxacin all the animals survived but showed the signs of pneumonia, and there was up to 6 log CFU/g±0.82 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 biology of the broad host range of phage, demonstrating that phage has prospects for the treatment of pneumonia caused by the NDM-producing *Klebsiella pneumoniae*.

1. Introduction

Pneumonia is an acute respiratory infection that affects the lungs and kills more than 700,000 children worldwide under the age of 5 in 2019.[1] Pneumonia is caused by viruses, bacteria, parasites, and fungi. *Klebsiellapneumoniae* a Gram-negative bacillus is the most common bacterial aetiology of hospital-acquired pneumonia. As per the World Health Organization, *Klebsiellapneumoniae* is one of the multi-drug resistant pathogens identified as an urgent threat to human health.[2] Some of the lineages of *Klebsiellapneumoniae* carry gene coding for carbapenemase, as well as other acquired antimicrobial

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determinants which are responsible for outbreaks in different countries.[3] Colistin, a polymyxin E is the drug of choice for the treatment of MDR *Klebsiella* p*neumoniae*, but reports of colistin-resistant *Klebsiella* isolates have been reported from different parts of the world.[4,5] Infection with multidrug-resistant *Klebsiella pneumoniae* may increase the duration of hospital stay and cost of management and may cause additional morbidity and mortality. Today, resistance has rendered most of the original antibiotics obsolete for many infections. The emergence of pathogenic bacteria resistant to most, if not all, currently available antimicrobial agents has become a critical problem in modern medicine, particularly because of the concomitant increase in immuno-suppressed patients. With a decrease in the discovery rate of novel antibiotics and based on scientific literature, bacteriophages which are labelled as a forgotten cure in the 19^{th} century are one of the most promising alternatives to antibiotics for clinical use in the prevailing conditions.[6,7]

Phage therapy is a renewed concept in the 21^{st} century that uses live bacteriophages to treat bacterial infections.[8] Many notable bacterial infections were cured using phage therapy (either natural or engineered) in recent times.[9,10] With an increasing antibiotic resistance crisis, it is important to find an immediate alternative cure to treat infections that are irresponsive to antibiotics. Though the studies on therapeutic bacteriophages are growing fast, it is necessary to have proper pre-clinical studies to prove their efficacy. The present study aimed to evaluate the effectiveness of bacteriophages in rescuing mice from pneumonia caused by New Delhi Metallo-beta-lactamase (NDM) producing *Klebsiella pneumoniae*. This is a proof-of-concept study in which severely pneumonia infected murein models were treated with *Klebsiella* phage and observed the reduction in the severity of infection over time.

2. Materials and Methods

2.1. Bacterial isolates

The *K. pneumoniae* 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, Devangere, India. The sputum sample was collected from the patient and bacteria were isolated using the standard procedures. The VITEK system was used for the identification of bacteria and to screen for antibiotic susceptibility.[11] The antibiotics tested include imipenem, meropenem, cefepime, piperacillin-tazobactam, ampicillin, ampicillin/sulbactam, ceftriaxone, cefazolin, nitrofurantoin, ceftazidime, ciprofloxacin, aztreonam, amikacin, colistin and levofloxacin. The results were interpreted according to CLSI guidelines.[12] In addition, 16S rRNA sequencing was performed using universal primers, 27F and 1492R. The polymerase chain reaction (PCR) products sequenced and BLAST hit with >96% similarities were deemed as sufficient for bacterial identification.

To detect the presence of Metallo-Beta Lactamase (MBL) genes, PCR was performed for five genes; bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{VIM} and bla_{IMP}. The primers and PCR conditions were previously reported by van der Zwaluw et al.[13] 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 *K. pneumoniae* was isolated from sewage samples collected at a municipal sewage treatment plant, Davangere by the method of Smith and Huggins.[7] 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 (MH) hard 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 multiplied 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 is 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

To study the morphology of the isolated bacteriophage, the phages were negatively stained using 2% uranyl acetate and visualized under Transmission Electron Microscope.[14] The electron microscopic study was carried out at the National Institute of Mental Health and Neuro-Sciences, Bangalore. 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 lytic activity of phage against NDM-producing *K. pneumoniae*. Briefly, the bacterial culture (MH broth) at 10^8 CFU/mL was infected with bacteriophages at 10^9 PFU/mL 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 MH agar plates. Plates were incubated at 37°C for 48 h, and the bacterial colonies were counted. The viability was reported as log10 (CFU/mL) values and the experiment was repeated twice for statistical significance.[7,14]

2.5. Phage therapy in the mouse infection model

2.5.1. Maintenance and induction of pneumonia in mice

Female mice weighing 15-18g, 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.[15]

A modification of the Esposito and Pennington model[16] was used for the pneumonia infection. The mice were anaesthetized by an intraperitoneal injection of 5 % sodium thiopental. Mice were suspended vertically and the trachea was then cannulated with a blunt-tipped metal needle. The feel of the needle tip against the tracheal cartilage confirmed the intra-tracheal location. A microlitre syringe (BD, USA) was used for inoculation. After the inoculation, the mice remained in a vertical position for 3 min and then in a 30-degree position until awake.[16] Pneumonia was induced by introducing 50 μL of a 10^8 CFU/mL of *Klebsiella pneumoniae* bacterial suspension obtained from 18 hours of culture in trypticase soy broth (Hi-Media, India) at 37°C.

2.5.2. Effectiveness of Klebsiella phages

To evaluate the effectiveness of the *Klebsiella* phage, the animals were divided into five groups; Group I: (control) The mice were injected with PBS. Group II: (phage-only, control) The mice were not infected and received 3×10^9 PFU/mL of phage intraperitoneally. This group was included to study whether phages can cause some sort of hypersensitivity in the mouse. Group III: (Bacteria-only, infection control) The pneumonia was induced in mice (10^8 CFU/mL) and was not treated with antibiotics or challenged with phages. Group IV: (bacteria + antibiotic, treatment) Pneumonia was induced in mice and pneumonia infected mice were treated with levofloxacin, 50 mg/kg, twice a day intraperitoneally. Antibiotic was administered 48 hours after inoculation. The antibiotic was given for three days. Group V: (bacteria + phage, treatment) Pneumonia was induced in mice and pneumonia infected mice were treated with Klebsiella phage (3×10^9 PFU/mL) intraperitoneally.[17] Phage was administered 48 hours after inoculation. The bacteriophage was given only once. The experimental setup is shown in fig.2A.

After treatment, the mice were observed for 72 hours for mortality, and the surviving mice were killed 12 hours (≈84 hours after initiating the treatment) after the last dose in the treatment groups and at the same time in the control group, by intraperitoneal administration of 5% sodium thiopental. All the mice (dead and killed) were analysed immediately after death. After the animals' death, thoracotomy was carried out. The heart and lungs were extracted together and the lungs were later separated on a sterile Petri plate. The lungs were processed for quantitative culture,[12] after being homogenized in 2 mL of sterile saline solution. After ten-fold dilution, aliquots of 100 μL were plated on sheep blood agar plates for 24 hours at 37°C. The results were expressed as mean ± SD of the log CFU/g of the lung. Lung samples of all the groups were processed for histological studies.

2.5.3. Estimation of neutralizing antibodies

A plaque reduction assay was used to estimate the presence of neutralizing antibodies. Accordingly, serum collected from mice was heat-inactivated at 56°C for one hour in a water bath to inactivate the complement. Serum was diluted serially in normal saline from 1:10, 1:100, 1:1000 and 1:2000. Diluted serum (450μL) was allowed to react with phage (50μL) for 30 min at 37°C in a water bath. The phage titre used as antigens was 10^6 PFU/mL. The mixture was diluted up to 1000 times with TMG (tris-magnesium-gelatin) buffer after incubation and subjected to PFU determination by the double-agar overlay method.[18] The dilution of serum neutralizing the phage was estimated by observing a decrease in the PFU number.

2.6. 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 NDM-producing K. pneumoniae clinical isolates

The isolated *K. pneumoniae* strain was found to be multi-drug resistant. Antibiotic susceptibility test showed that the *K. pneumoniae* 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), but was susceptible to colistin (MIC, 2 μg/ml), levofloxacin (MIC, ≤ 0.25 μg/ml). PCR and sequencing study showed that bla_{NDM-4} was the only carbapenemase-encoding gene carried by the isolate. (Gene accession number OM025089)

3.2. *Characterization of phage infecting K. pneumoniae*

Bacteriophage was isolated against NDM-4 producing *K. pneumoniae* 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 65 ± 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. *Lysis kinetics of K. pneumoniae infected with phage*

The results of the time-kill assay are presented in Figure 1C. The data showed that there is a 5-fold reduction in the concentration of viable count at the 4^{th} hour for levofloxacin (0.25 μg/mL), but bacterial regrowth was noted at 24 hours. In the case of bacteriophage challenge assay (3×10^9 PFU/mL), a 7-fold reduction in the viable count was seen at the 4^{th} hour. Interestingly, no bacterial regrowth was seen at the end of 8 hours for bacteriophage (Figure 1C).

3.4. *Klebsiella phage exhibited comparable efficacy with levofloxacin 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 mice, nine were dead and one animal was moribund at the end of 72 hours of inoculation (Fig.2B). Bacterial count in dead mice was 12.32 log CFU/g of lung±1.88, which was 4 logs higher than the minimum lethal dose and bacterial count in killed mice was 10.68 log CFU/g of lung±1.08 which was 2 logs higher than the minimum lethal dose used in the experiment.

Among the levofloxacin-treated group, all the mice survived (n=10) at the end of the 72 hours but the surviving mice showed the signs of pneumonia. The bacterial enumeration showed there was up to 6 log CFU/g±0.82 reduction in the lungs (Table 1) which caused the pneumonia symptoms even after six doses of levofloxacin. In bacteriophage treated mice, all the mice survived (n=10) at the end of 72 hours and the mice were healthy with no signs of pneumonia. None of the mice showed bacterial growth from the lung samples, indicating bacteria were cleared from the lung by the phage (Table 1). Histopathological study showed that the mice inoculated with NDM-4 producing *K. pneumoniae* 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 (data not shown). None of the animals from the groups showed the presence of neutralizing antibodies against the bacteriophage used in the study.

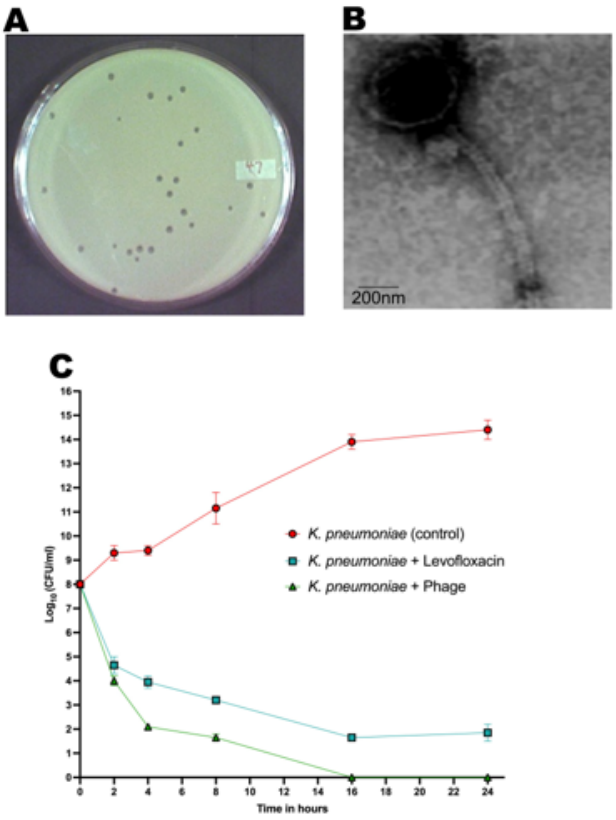

Fig. 1: Biological characteristics of *Klebsiella* phage. (A) Morphology of plaques formed on the double agar-overlay plate, (B) Electron micrograph of *Klebsiella* phage (The scale bar represents 200 nm). The morphological structure shows that the phage consists of a head (65±0.5 nm) and a long non-contractile tail (100±0.5 nm); therefore the phage belongs to the *Siphoviridae* family, and (C) Lysis kinetics of *K. pneumoniae* treated with levofloxacin and infected with *Klebsiella* phage. The *K. pneumoniae* was incubated in MH broth (red circles), with antibiotic (blue squares), or with phage (green triangles), at 37°C and bacterial counts were performed at regular time intervals. The values indicated are the means of two independent experiments.

4. Discussion

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

Table 1: Efficacy and safety of levofloxacin and *Klebsiella* phage on the survival rates of mice and the clearance of *Klebsiella pneumoniae* from their lungs.

Treatment group	Number of mice (N)	Survival		Death	
		N (%)	Log CFU/g of lung × ± SD	N (%)	Log CFU/g of lung × ± SD
Pneumonia infected	10	*2 (20)	10.68±1.08	8 (80)	12.32±1.88
Levofloxacin treatment	10	*10 (100)	2.12±0.82	0	-
Phage treatment	10	*10 (100)	0	0	-

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

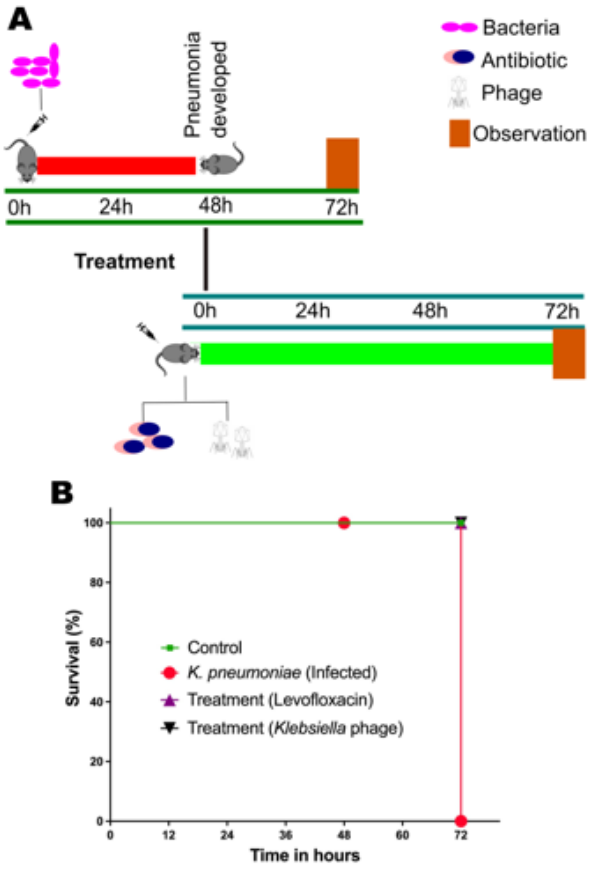

Fig. 2: Experimental design and survival of mice in each group. (A) Schematic representation of *in vivo* experimental setup, (B) Pathogenicity of NDM-4 producing *K. pneumoniae* strain in mice and efficacy of *Klebsiella* phage in pneumonia mice models. Each group consisted of 10 mice. Survivability of mice infected with bacteria (10^8CFU/mL) and treated either with antibiotic, levofloxacin (50 mg/kg twice a day, i.p.) or *Klebsiella* phage (3×10^9PFU/mL). The survival curves were plotted using the Kaplan-Meier, and the log-rank test was used to analyse the difference in survival rates in GraphPad Prism 7.0. A statistically significant difference ($P < 0.05$) was observed in the treatment groups.

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.[13,19] Ideally, routes for phage therapy are chosen to ensure the effective delivery of the phage to the target site of infection.[20] However, despite the prospective benefits, the use of phages for the treatment of pulmonary bacterial infections has been fairly under explored. In the present study, the therapeutic effect of *Klebsiella* phage against NDM-4 producing K. pneumonia was evaluated in the mice model in which pneumonia was induced. The findings from this study suggest that bacteriophage isolated against NDM-4 producing *K. pneumoniae* was effective in resolving pneumonia in the infected mouse. That is, a single dose of bacteriophage was sufficient to clear bacteria from the infected lungs compared to antibiotic levofloxacin 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.[21] In the case of pneumonia, the route of phage administration is always challenging but both intraperitoneal and intranasal administration proved to be effective.[21,22] 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.[22] Our study is one of the rarest to show the effectiveness of single-dose phage administration in pneumonia developed murein models and compared the organ toxicity against levofloxacin treatment which showed the efficacy of phage treatment.

In the in vitro time-kill assay, when the bacteria were challenged with levofloxacin, the bacterial load decreased up to 18 hours of incubation, and then the bacterial load started increasing. But, in the phage challenged test, a 7-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.[22,23] 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 mice to the specific bacteriophage tested. No observable antibody titres were seen in the present study indicating the mice 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 over stimulation of inflammatory response in the *in vivo* pneumonia treatment.[23]

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.[1,2] Carbapenemase-producing *K. pneumoniae* strains represent a challenge for clinical practitioners due to their increasing prevalence in hospital settings and antibiotic resistance. New Delhi Metallo-β-lactamase (NDM) 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.[7,13] Sixteen variants of NDM enzymes have been discovered in different countries since the identification of NDM-1 in India. In this study, the NDM-producing MDR *K. pneumoniae* was isolated from pneumonia patients and found to be susceptible to the isolated *Klebsiella* phage that could clear the bacterial load in the murein models. Therefore, further characterization and molecular analysis of the phage will prove to be effective in treating pneumonia.

5. Conclusions

Since the onset of the antibiotic era, the escalation of antibiotic-resistant pathogens is causing jolts in health care and food-producing facilities worldwide. In the pursuit of new therapeutics, re-evaluation of bacteriophage therapy, to tackle infections, is gaining interest. The bacteriophage isolated against NDM-4 producing *Klebsiella pneumoniae* was effective in clearing bacteria from the lung in a much shorter duration compared to the susceptible antibiotic used in a pneumonia mice model. Even though bacteriophages were used in the early 19^{th} century, the use of phages as a therapeutic agent faded due to the discovery of antibiotics and the scarcity of well-documented phage research. But the emergence of multidrug-resistant bacteria has opened renewed interest in bacteriophage as an alternative therapeutic agent. This proof-of-concept study will shed light on the use of phage therapy to cure acute pneumonia caused by *K. pneumoniae*.

6. Acknowledgement

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7. Ethical Statement

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.

8. Conflicts of Interest

The authors have no conflict of interest to declare.

9. Source of Funding

Not applicable

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