Utility of lytic bacteriophage in the treatment of multidrug-resistant *Pseudomonas aeruginosa* septicemia in mice

CS Vinodkumar¹, Suneeta Kalurmath², YF Neelagund³

¹ Department of Microbiology, SS Institute of Medical Sciences and Research Centre, Davanagere, India
² Department of Physiology, SS Institute of Medical Sciences and Research Centre, Davanagere, India
³ Department of Microbiology, Gulbarga University, Gulbarga, Karnataka, India

Correspondence Address:
C S Vinodkumar
Department of Microbiology, SS Institute of Medical Sciences and Research Centre, Vidhyanagar Post Box-1, NH-4, Bypass, Davanagere - 577 005, Karnataka
India

Abstract

Drug resistance is the major cause of increase in morbidity and mortality in neonates. One thousand six hundred forty-seven suspected septicemic neonates were subjected for microbiological analysis over a period of 5 years. Forty-two *P. aeruginosa* were isolated and the antibiogram revealed that 28 *P. aeruginosa* were resistant to almost all the common drugs used (multidrug-resistant). The emergence of antibiotic-resistant bacterial strains is one of the most critical problems of modern medicine. As a result, a novel and most effective approaches for treating infection caused by multidrug-resistant bacteria are urgently required. In this context, one intriguing approach is to use bacteriophages (viruses that kill bacteria) in the treatment of infection caused by drug-resistant bacteria. In the present study, the utility of lytic bacteriophages to rescue septicemic mice with multidrug-resistant (MDR) *P. aeruginosa* infection was evaluated. MDR *P. aeruginosa* was used to induce septicemia in mice by intraperitoneal (i.p.) injection of 10⁷ CFU. The resulting bacteremia was fatal within 48 hrs. The phage strain used in this study had lytic activity against a wide range of clinical isolates of MDR *P. aeruginosa*. A single i.p. injection of 3 × 10⁹ PFU of the phage strain, administered 45 min after the bacterial challenge, was sufficient to rescue 100% of the animals. Even when treatment was delayed to the point where all animals were moribund, approximately 50% of them were rescued by a single injection of this phage preparation. The ability of this phage to rescue septicemic mice was demonstrated to be due to the functional capabilities of the phage and not to a nonspecific immune effect. The rescue of septicemic mice could be affected only by phage strains able to grow in vitro on the bacterial host used to infect the animals and when such strains are heat-inactivated, they lose their ability to rescue the infected mice. Multidrug-resistant bacteria have opened a second window for phage therapy. It would seem timely to begin to look afresh at this approach. A scientific methodology can make phage therapy as a stand-alone therapy for infections that are fully resistant to antibiotics.

Introduction

*Pseudomonas aeruginosa*, a Gram-negative bacterium is one of the most important causative agents in neonatal septicemia. [1] Several outbreaks of infection caused by *P. aeruginosa* isolates that are simultaneously resistant to broad-spectrum cephalosporins and aminoglycosides have been reported. Some of these multidrug-resistant isolates produce “Extended spectrum β-Lactamases” that are able to hydrolyze expanded spectrum cephalosporins, aztreonam and related oxyimino-β-lactam. [2] Colonization of such multidrug-resistant *P.*
Utility of lytic bacteriophage in the treatment of multidrug-resistant *P. aeruginosa*. [3] According to National Neonatal Perinatal Database 2000, the incidence of neonatal sepsis caused by *P. aeruginosa* is 18%. [4] The potential clinical significance of *P. aeruginosa* in neonatal sepsis continues to increase as medical therapy involves more invasive intervention procedures. Emergences of *P. aeruginosa* as one of the leading nosocomial pathogens in neonatal intensive care units, especially in high-risk babies and the widespread occurrence of multidrug-resistant among them pose a therapeutic problem. [1]

With the rising prevalence of antibiotic-resistant bacteria, alternatives to treatment with antibiotics are receiving increased attention. One such alternative is the possible therapeutic use of bacteriophages - viruses that parasitize and kill bacteria. [5] The suggestion of administering phages as pharmaceutical agents has been mooted for more than 85 years. The relative simplicity and economy of phage therapy should have gained much momentum and magnitude earlier. This has not happened, probably because of a poor understanding of mechanism of bacterial pathogenesis and of the nature of phage-host interactions and the absence of animal models of diseases and also due to badly designed and executed experiments and field trials which led to failure in using phages in therapy. [5],[6],[7],[8],[9] Another reason was due to the fact that the scientists concentrated on escalated production of newer and newer antibiotics of commercial importance. However, the increasing incidence of multidrug-resistant bacteria and a deficit in the development of new chemotherapeutics to counteract bacteria, [2],[4],[10] have rekindled the interest in phage therapy. This work was carried out to investigate the possibility of use of lytic phage in the treatment of experimental septicemic mice, which are infected with MDR *P. aeruginosa*.

**Materials and Methods**

**Ethical clearance**

The institutional ethical review board at Gulbarga University, Gulbarga approved all the study procedures.

**Bacterial strain and antibiotic sensitivity pattern**

*P. aeruginosa* strain was isolated from the blood of a neonate with septicemia and was designated as YFN-58 in our nomenclature. Antibiotic susceptibility testing by Kirby-Bauer's method [10] revealed that the YFN-58 strain was resistant to most of the commonly used drugs. *Staphylococcus aureus* (ATCC no. 25923) and *Escherichia coli* (ATCC no. 25922) were used as control strains in antibiotic susceptibility testing.

**Establishing the minimum lethal dose (MLD) of *P. aeruginosa* in the mouse model**

One-month-old BALB/c male mice (four per group) free from specific pathogen, belonging to the same race, weighing 22.0 ± 1.5 g, caged singly and maintained on a proper diet were used for infection experiments. All the mice were housed in a pathogen-free environment within the animal-care facility at Gulbarga University. Bacterial inocula were prepared by culturing *P. aeruginosa* in brain-heart infusion overnight and centrifuging it at 2,000 x g for 10 min, washing, centrifuging it twice and then resuspending it in saline to various densities. Each group of mice received intraperitoneal inoculation of 400µl aliquots of bacterial suspension in different densities (10² -10⁷ CFU). The animals were observed for 100 hrs. Mice inoculated with bacteria were scored for their state of health on a scale of 0 to 5, based on progressive disease states reflected by several clinical signs. [11],[12],[13],[14] 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 exudative accumulation around partially closed eyes, was scored as 2; a moribund state was scored as 1; and death was scored as 0. Scores were determined by two independent observers.

**Isolation and purification of phage strains for *P. aeruginosa***

The *P. aeruginosa* phage was isolated from raw sewage at a municipal sewage treatment plant, Gulbarga by the method of Smith and Huggins. [8] Sewage water (50 ml) was collected in a sterile conical flask and treated with a few drops of chloroform. To this, an equal volume of sterile nutrient broth and 1 ml of the 24-hour-old broth culture of *P. aeruginosa* YFN-58 were added. The sample inoculated with bacterial pathogens was incubated at 37°C for 12-24 hrs in shaker water bath. After 12-24 hrs, the lysate was shaken with few drops of chloroform for about 10 min, centrifuged at 10,000 rpm for 10 min and the supernatant was filtered through 0.22 µ pore size acrodisk membrane filters (PALL, German Laboratory) to remove the bacteria. [11] and subjected to plaque-forming unit (PFU) assay using the double-layer agar method described by Smith and Huggins. [8] The phage was denoted as CSV-31.

**In vitro confirmation of bacteriophage activity on *P. aeruginosa***

The bacterial lawn was prepared on nutrient agar plates employing 1.0 ml of 24 hrs *P. aeruginosa* YFN-58 culture by flooding and draining out the excess. Wells were dug into the agar by employing a sterile cork borer and the 20µl phage suspension (3 x 10⁹ PFU/ml) was loaded into each of the well. Sterile distilled water served as the control. The plates were incubated at 37°C for 24 hrs. Thereafter, the zone of inhibition, if any, was recorded. [12],[13]

**Treatment with phage**

The efficacy of phage therapy was evaluated in two separate experiments using MDR *P. aeruginosa* bacteremic mouse model. The first experiment was to examine the effect of phage dose on the ability of phage to rescue mice from MDR *P. aeruginosa* bacteremia.
second study, on the outcome of delaying treatment for various periods. In the dose-ranging study, five groups of mice (four mice in each) were challenged by i.p. injection of the MLD of YFNI58. Each of these groups was treated with a single injection of phage CSV-31 administered i.p. 45 min after the bacterial challenge at $3 \times 10^9$, $3 \times 10^8$, $3 \times 10^4$ and 0 PFU. As an additional control, a sixth group (two mice) was not challenged with bacteria, receiving only the injection of phage (at the highest dose). The state of health of these animals was monitored for 20 days. [14]

In the delayed-treatment study, treatment (a single injection of phage at the highest dose) was initiated at 5, 8, 12, 16 and 24 hrs after the bacterial challenge with the MLD P. aeruginosa YFNI58. The state of health of these animals was also monitored for 20 days. [10],[13],[15]

Effects of heat-inactivated phage

A sample of phage with a titer of $3 \times 10^9$ PFU/ml was heat-inactivated by incubation at 80°C. Phage that had been heated for a total of 20 min, at which time no viable phage was detectable, was used to determine whether phage rescue mice with MDR Pseudomonas bacteremia requires functional phage or whether the rescue might be associated with nonspecific immune activation. [16],[17] The mice in this study were divided into two groups of four each. All of the mice were challenged by i.p. injection of $3 \times 10^9$ PFU of phage CSV-31, 45 min after the bacterial challenge. The second group was treated with i.p. injection with $3 \times 10^9$ PFU of heat-inactivated phage particles 45 min after the bacterial challenge.

Results

Antibiotic susceptibility testing

Antibiotic sensitivity testing revealed that out of 42 P. aeruginosa isolated from neonatal septicemia, 28 isolates were multidrug-resistant. All these 28 isolates were extended spectrum β-lactamase producers.

Lethality of MDR P. aeruginosa bacteremia

As seen in [Figure 1], all mice inoculated i.p. with MLD 10 7 CFU of the clinical isolate MDR P. aeruginosa YFNI-58 died within 48 hrs.

Phage strain antibacterial activity

Of the phage strains isolated in the present study, phage CSV-31 was found to form plaques on 66% of the MDR Pseudomonas clinical isolates and inhibited bacterial growth of an additional 8% of the strains, thus exhibiting an antibacterial effect against 74% of the strains in our collection.

Ability of the phage preparation to rescue mice from MDR Pseudomonas bacteremia

A single dose of phage CSV-31 was administered i.p. 45 min after the challenge with the MLD of bacteria. By 24 hrs, a dose effect on the state of health of the infected animals was clearly visible. At higher doses of phage, 100% of the animals survived and only minimal signs of illness (mild lethargy) were seen (in the first 24 hrs). As the phage dose decreased, the animals became critically ill, with survival rates of 40% and 60%, respectively, at day 6 and beyond [Figure 2]. All the mice that were alive and healthy at day 6 remained so for an additional 20 days, at which time the experiment was terminated.

Effect of delay in treatment on the ability of the phage preparation to rescue mice from MDR Pseudomonas bacteremia

In this experiment, the MLD of MDR Pseudomonas strain YFNI-58 was injected i.p. to induce fatal bacteremia. At various intervals thereafter, ranging from 2 to 24 hrs, the mice received a single i.p. injection of the high dose ($3 \times 10^9$ PFU) of phage CSV-31. The results of treatment at the time points are illustrated in [Figure 3]. The state of health of these mice was monitored for 20 days following bacterial infection. The experiments demonstrate that a single injection of phage can rescue 100% of the animals, even when treatment is delayed until 5 hrs after lethal bacterial challenge and if treatment is delayed beyond that point, morbidity increases and mortality begins to appear. However, even with delay of 18 and 24 hrs, at which point all the mice are moribund, approximately 50% of the animals are rescued and recovered completely.

Effects of heat-inactivated phage

An experiment was performed to determine whether phage rescue of mice with multidrug-resistant Pseudomonas bacteremia requires phage that can grow on the bacterial host or whether phage rescue might be associated with nonspecific immune activation response. Heating at 80°C for 5 min decreased the phage titer by 1000-fold and no viable phage was detected after heating for 15 to 20 min. As illustrated in [Figure 4], only mice inoculated with plaque-forming phage had enhanced survival, with 75% survival at 4 days and with 10% of the mice injected with heat-inactivated phage surviving.

Rescue is associated with a significant decrease in bacterial titer

In a similar experiment, blood was obtained by cardiac puncture during a rescue experiment in order to compare bacterial titers from two groups of four mice each, 20 hrs after i.p. inoculation with $3 \times 10^7$ CFU of YFN-58. Forty-five minutes after the bacterial inoculation of the
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We focused our efforts on MDR Pseudomonas because we anticipated that positive results would demonstrate the potential of this form of resistant Enterococcus infection. A single i.p. injection of the phage strain was sufficient to rescue 100% of the animals. Even in the Pioneering study by Shankar Adhya indicated that bacteriophage therapy could be used in gastrointestinal colonization with vancomycin protection, suggesting that acute infections might also be amenable to phage treatment. Phage CSVI31 persisted long enough in the tissues to be effective when administered to the mice a day or two before challenge with the P. aeruginosa. This was also reported previously with chicken [20],[21] and suggests that there are possibilities for prophylaxis. More interestingly, it was possible to delay administration of the phage until signs of disease were evident in the mice and yet retain considerable protection, suggesting that acute infections might also be amenable to phage treatment.

Pioneering study by Shankar Adhya indicated that bacteriophage therapy could be used in gastrointestinal colonization with vancomycin-resistant Enterococcus infection. [11] A single i.p. injection of the phage strain was sufficient to rescue 100% of the animals. Even in the present study, we could demonstrate that viable phage were able to rescue 75% of the septicemic mice.

We focused our efforts on MDR Pseudomonas because we anticipated that positive results would demonstrate the potential of this form of therapy in situations where few alternatives are available today. It is tempting to advocate research investigations into many bacterial...
infections for which animal models are available and for which phages may be isolated. The potential of phage therapy has been the subject of several recent reviews, [15],[16],[17],[18] and the present study reinforces the view that this therapy is worth exploring.

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