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Original article

Therapeutic effect of the YH6 phage in a murine hemorrhagic pneumonia model

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Abstract

The treatment, in farmed mink, of hemorrhagic pneumonia caused by multidrug-resistant *Pseudomonas aeruginosa* strains has become increasingly difficult. This study investigated the potential use of phages as a therapy against hemorrhagic pneumonia caused by *P. aeruginosa* in a murine hemorrhagic pneumonia model. An N4-like phage designated YH6 was isolated using *P. aeruginosa* strain D9. YH6 is a virulent phage with efficient and broad host lytic activity against *P. aeruginosa*. No bacterial virulence- or lysogenesis-related ORF is present in the YH6 genome, making it eligible for use in phage therapy. In our murine experiments, a single intranasal administration of YH6 (2×10^7 PFU) 2 h after D9 intranasal injections at double minimum lethal dose was sufficient to protect mice against hemorrhagic pneumonia. The bacterial load in the lungs of YH6-protected mice was less than 10^3 CFU/g within 24 h after challenge and ultimately became undetectable, whereas the amount of bacteria in the lung tissue derived from unprotected mice was more than 10^8 CFU/g within 24 h after challenge. In view of its protective efficacy in this murine hemorrhagic pneumonia model, YH6 may serve as an alternative treatment strategy for infections caused by multidrug-resistant *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*; Pneumonia model; Bacteriophage; Mink

1. Introduction

In recent years, the global prevalence of antibiotic-resistant bacteria has become a major challenge to antibiotic therapy. *Pseudomonas aeruginosa* is one of the chief multidrug-resistant bacteria. This pathogen is not only intrinsically resistant to many antimicrobial agents [32,42], but it also acquires resistance to the most effective antimicrobial agents [44]. *P. aeruginosa* is a common Gram-negative opportunistic pathogen that is widely present in a variety of environments. *P. aeruginosa* causes acute, life-threatening or chronic infections in patients with cystic fibrosis (CF) or severe burns, and in patients who are immunosuppressed [8]. In animals, *P. aeruginosa* causes endometritis in horses [21], mastitis in cows and other ruminant animals [29], otitis media and otitis interna in chinchillas [18]. *P. aeruginosa* is also the major cause of hemorrhagic pneumonia in farmed mink [31], which results in high mortality and morbidity [20,35,38]. The primary measure for treating infections caused by *P. aeruginosa* involves the

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application of antibiotics. However, treatment of these infections has become increasingly difficult given the prevalence of multidrug-resistant *P. aeruginosa* strains. In addition, there is a shortage of new antimicrobial agents passing through the development pipeline [40]. Therefore, there is an urgent need for novel therapeutic agents for treating infections caused by multidrug-resistant *P. aeruginosa* in the fields of both human and veterinary medicine.

Lytic bacteriophages are viruses that specifically infect and lyse bacteria [9]. Since the early 1920s, phage therapy has been explored as an antimicrobial agent for the treatment of bacterial infectious diseases. However, the development of this therapy has been hampered by the advent of antibiotics. Phage therapy has recently been resurrected primarily due to the continuing worldwide increase in antibiotic resistance [27]. Phage therapy may be a supplementary or viable alternative to conventional antibiotic therapy because it was previously demonstrated to be advantageous and more specific than antibiotics in certain circumstances [23]. Another advantage of using phages over antibiotics is that phages can replicate at the site of infection, thus increasing in numbers at the site of infection [39]. In addition, several recent and well-controlled animal studies have demonstrated the potential of phages in antibacterial therapy [11,22].

Phage therapy is expected to be useful as an alternative therapeutic measure for treating drug-resistant *P. aeruginosa* infections [16]. *P. aeruginosa*-specific bacteriophage KPP12 eye drops may be a novel adjunctive or alternative therapeutic agent for treating infectious keratitis caused by antibiotic-resistant strains [11]. Phage therapy has also been used for the control and treatment of multidrug-resistant *P. aeruginosa* lung infections in mice and in cystic fibrosis airway lung cells [1]. Cao et al. evaluated the potential for phage against a mink hemorrhagic pneumonia model when administered by means of ultrasonic nebulization [4].

In the present study, YH6, a newly-isolated *P. aeruginosa* phage, was shown to possess broad host lytic activity against *P. aeruginosa* strains originating from mink. Sequencing and analysis of the YH6 genome indicated the lack of bacterial-virulence or lysogenesis-related ORFs, thus making it eligible for use in phage therapy. Additionally, our positive results suggest that phage treatment may provide an effective approach for controlling hemorrhagic pneumonia caused by *P. aeruginosa* infections in mink.

2. Materials and methods

2.1. Ethical statements

All animal studies were conducted according to the National Guidelines for Experimental Animal Welfare (Ministry of Science and Technology of China, 2006) and approved by the Animal Welfare and Research Ethics Committee at Jilin University. The animals were treated humanely, and all efforts were made to minimize suffering. Animal experiments were done at the College of Veterinary Medicine, Jilin University, China. No specific permission was required for this location. No endangered or protected species were involved in the study.

2.2. Animals

Animal experiments were performed on 20–22 g female BALB/c mice purchased from the Experimental Animal Center of Jilin University. Mice were maintained in a temperature-controlled animal room with a 12-h light/dark cycle. Feed and fresh water were available ad libitum.

2.3. Isolation and identification of P. aeruginosa

The lung, feces and feed samples from dead farmed American Black mink that were suspected of having died from hemorrhagic pneumonia were kindly provided by mink farms, listed in Table 1. All of these samples were homogenized individually in phosphate-buffered saline (PBS, Takara, Dalian, China), and the homogenate was serially diluted and plated onto cetrimide agar plates (Guangdong Huankai Microbial Sci. & Tech. Co., Ltd., Guangzhou, China) to isolate *P. aeruginosa*. After the plates were incubated at 37 °C for 18 h, single colonies were selected and streaked onto Luria–Bertani (LB, Becton, Dickinson and Company, American) agar plates for a minimum of three times to isolate individual bacteria.

Several colonies suspected of being *P. aeruginosa* were further confirmed using 16S rDNA sequence analysis after amplification with universal primers Pa16S-F (GGGGGATCTTCGGACCTCA) and Pa16S-R (TCCTTAGAGTGCCCACCCG) [37]. The serotypes of the *P. aeruginosa* isolates were detected using a *P. aeruginosa* serotyping kit as described previously [19].

Bacterial genomic DNA from the clinical strains as well as from the reference strains *P. aeruginosa* CMCC10102 and ATCC27853 was amplified by randomly amplified polymorphic DNA analysis (RAPD), as previously described with some modification [26,14]. The RAPD mixture (25 μL) consisted of 10 × PCR buffer, 3 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, 1 U of ExTaq polymerase (Takara, Dalian, China), 40 pmol of oligonucleotide (5′-AGCGGGCCAA-3′), 40 ng of genomic DNA and sterile distilled water. RAPD products were then separated by electrophoresis in 1% (w/v) agarose gels containing ethidium bromide (0.5 g/ml) and analyzed using BioNumerics (Applied Maths, St. Martens-Latem, Belgium).

2.4. Antibiotic resistance of the P. aeruginosa isolates

The antibiotic susceptibility of the *P. aeruginosa* isolates was evaluated using the Kirby–Bauer disk diffusion method, and the results were interpreted according to the criteria of the Clinical and Laboratory Standards Institute of the American Clinical Laboratory [7]. Eighteen antibiotics were selected for this study (all from the Hangzhou Binhe Microorganism Reagent Development Company, Hangzhou, China): cefepime (30 μg), ofloxacin (5 μg), tetracycline (30 μg), tobramycin (10 μg), lomefloxacin (10 μg), ciprofloxacin (5 μg),