

RESEARCH ARTICLE

Isolation and Characterization of a “phiKMV-Like” Bacteriophage and Its Therapeutic Effect on Mink Hemorrhagic Pneumonia

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Citation: Cao Z, Zhang J, Niu YD, Cui N, Ma Y, Cao F, et al. (2015) Isolation and Characterization of a “phiKMV-Like” Bacteriophage and Its Therapeutic Effect on Mink Hemorrhagic Pneumonia. PLoS ONE 10(1): e0116571. doi:10.1371/journal.pone.0116571

Academic Editor: Jamunarani Vadivelu, University of Malaya, MALAYSIA

Received: April 19, 2014

Accepted: December 11, 2014

Published: January 23, 2015

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: YPX received a grant supported by the National High Technology Research and Development Program (Grant No. 2013AA102805), URL: <http://www.863.gov.cn/>. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

The objective of this study was to investigate the potential of using phages as a therapy against hemorrhagic pneumonia in mink both *in vitro* and *in vivo*. Five *Pseudomonas aeruginosa* (*P. aeruginosa*) strains were isolated from lungs of mink with suspected hemorrhagic pneumonia and their identity was confirmed by morphological observation and 16S rDNA sequence analysis. Compared to *P. aeruginosa* strains isolated from mink with hemorrhagic pneumonia in 2002, these isolates were more resistant to antibiotics selected. A lytic phage vB_PaeP_PPA-ABTNL (PPA-ABTNL) of the *Podoviridae* family was isolated from hospital sewage using a *P. aeruginosa* isolate as host, showing broad host range against *P. aeruginosa*. A one-step growth curve analysis of PPA-ABTNL revealed eclipse and latent periods of 20 and 35 min, respectively, with a burst size of about 110 PFU per infected cell. Phage PPA-ABTNL significantly reduced the growth of *P. aeruginosa* isolates *in vitro*. The genome of PPA-ABTNL was 43,227 bp (62.4% G+C) containing 54 open reading frames and lacked regions encoding known virulence factors, integration-related proteins and antibiotic resistance determinants. Genome architecture analysis showed that PPA-ABTNL belonged to the “phiKMV-like Viruses” group. A repeated dose inhalational toxicity study using PPA-ABTNL crude preparation was conducted in mice and no significantly abnormal histological changes, morbidity or mortality were observed. There was no indication of any potential risk associated with using PPA-ABTNL as a therapeutic agent. The results of a curative treatment experiment demonstrated that atomization by ultrasonic treatment could efficiently deliver phage to the lungs of mink and a dose of 10 multiplicity of infection was optimal for treating mink hemorrhagic pneumonia. Our work demonstrated the potential for phage to fight *P. aeruginosa* involved in mink lung infections when administered by means of ultrasonic nebulization.

Introduction

The mink industry has been growing in production with more than 50 million mink pelts harvested globally in 2010 (European Fur Breeder's Association (EFBA) Annual Report 2010). However, the high animal density commonly associated with mink farming presents a great opportunity for the spread of diseases [1]. In particular, hemorrhagic pneumonia, caused by *Pseudomonas aeruginosa* (*P. aeruginosa*), has been one of the most costly infectious diseases among farmed mink [2,3]. Hemorrhagic pneumonia in mink is characterized by an acute and often fatal course of infection and very severe pathological changes in the lungs [4]. Mink of all ages are affected with mortality approaching 50% in some affected farms [5].

Antibiotics such as gentamycin, polymyxin and penicillins are mainly used to treat hemorrhagic pneumonia in mink [6]. However, with emergence and increase of antibiotic resistant *P. aeruginosa*, antimicrobial treatment is becoming less and less effective [7]. Though, multivalent vaccines against mink hemorrhagic pneumonia were available, application of these commercial vaccines was limited because of their exorbitant price and short protection period [5]. Other therapeutic approaches are being explored, as new antibacterial compounds are scarce [8].

Bacteriophages (phages) are viruses that specifically target and kill bacteria [9]. They represent one of the most abundant biological entities in nature and have long been recognized for their potential use as therapeutic agents [10]. *P. aeruginosa*-infecting phages have been shown to be effective in the treatment of bacterial infection in dogs [11] and human [12]. However, there are no reports on the use of phages to mitigate *P. aeruginosa* infection in mink. Therefore, the aim of this study was to evaluate the effects of a lytic phage for the treatment of hemorrhagic pneumonia in mink.

Materials and Methods

Ethics statement

This study was performed according to the National Guidelines for Experimental Animal Welfare (Ministry of Science and Technology of China, 2006) and approved by the Institutional Animal Care and Use Committee at Dalian University of Technology. The animals were treated humanely and all efforts were made to minimize suffering. All farmed mink experiments were done at Dalian Mingwei Fur Farm Co. Ltd. (Dalian, China), which was founded in 1958 and accredited by Fur Professional Committee of China Leather Association. Other experiments were done at Animal Biotechnology and Nutritional Laboratory in School of Life Science and Biotechnology, Dalian University of Technology. No specific permissions were required for this location. No endangered or protected species were involved in the study.

Animals and housing conditions

Mink were housed in open-sided sheds with roofing panels, which provided normal temperature and light conditions, while protecting against direct sunlight, wind and rain. Mink were kept individually in wire mesh pens (L×W×H: 60×40×40 cm) with straw-bedded nest boxes. Feed and fresh water were available *ad libitum*. Mice were kept in a temperature controlled animal room, with a 12 h light/dark cycle. Feed was provided *ad libitum* with exception of a 24 h fasting period immediately before sacrifice. Fresh water was available *ad libitum*.

Isolation of *P. aeruginosa*

Five lung samples from dead farmed Jinzhou Black mink (a strain derived from a cross between male American Black mink and female Denmark Black mink) that were suspected of suffering

hemorrhagic pneumonia were kindly provided by Dalian Mingwei Fur Farm Co. Ltd. All these samples were homogenized individually in PBS buffer and 100 μ l of the liquid were added to the Bile Lactose Broth (Guangdong Huankai Microbial Sci. &Tech., Co., Ltd, Guangzhou, China) and incubated at 37°C with shaking at 140 rpm for 12 h. The enriched culture was serially diluted and plated onto selective agar plates (Cetrimid Agar, Guangdong Huankai Microbial Science&Technology Co., Ltd, Guangzhou, China) in order to isolate *P. aeruginosa*. After the plates were incubated at 37°C for 24 h, single colonies were picked and streaked onto Luria-Bertani (LB) agar plates a minimum of three times in order to obtain pure bacterium. Five colonies (PA1-1, PA5-1-1, PA5-1-2, PA5-2-1, PA5-2-2) suspected of being *P. aeruginosa* were further confirmed using 16S rDNA sequence analysis.

16S rDNA sequence analysis

Genomic DNA was extracted from each isolate using the thermal cracking method. One milliliter of *P. aeruginosa* culture at log phase was heated at 100°C for 10 min and centrifuged at 3,000 g for 5 min. The supernatant containing genomic DNA was harvested and served as template for PCR. Then 16S rDNA was amplified by PCR using the universal 16S rDNA primers 27f (AGAGTTTGTGATCCTGGCTCAG) and 1492r (GGTTACCTTGTACGACTT). The reaction conditions were 94°C for 4 min, 32 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1.5 min, followed by 72°C for 10 min. PCR products electrophoresed through 1% agarose gels and DNA bands corresponding to 1450 bp of the 16S rDNA amplicon were extracted and sent to TaKaRa Biotechnology (Dalian) Co., LTD (No.19, Northeast Second Street, Economic and Technological Development District, Dalian, Liaoning Province, China) for DNA sequencing. All isolated bacterial 16S rDNA sequences were submitted to the GenBank database and compared to similar sequences by BLAST analysis. These five sequences were aligned with the most similar sequences and the sequences of other representative bacteria using Cluster W [13]. A phylogenetic tree of the 16S rDNA sequences was constructed with MEGA 4.0 software using the neighboring joining [14].

Antibiotic resistance of *P. aeruginosa* isolates

Antibiotic susceptibility of *P. aeruginosa* was evaluated using the Kirby-Bauer disc diffusion method and the results were interpreted according to criteria by Clinical and Laboratory Standards Institute of the American Clinical Laboratory [15]. Twelve antibiotics including gentamicin (10 μ g), ciprofloxacin (50 μ g), ticarcillin/clavulanic acid (75/10 μ g), ceftazidime (30 μ g), sulfamethoxazole/trimethoprim (23.75/1.25 μ g), cefazolin (30 μ g), aztreonam (30 μ g), piperacillin (100 μ g), amikacin (30 μ g), meropenem (10 μ g), polymyxin B (300 IU) and penicillin (10 IU) (Beijing Tiantan Drug Biotechnology Development Company, Beijing, China) were selected. From these, nine were recommended by the above institute and three others were selected during the process of mink-rearing in the light of instructions from veterinary practitioners. *P. aeruginosa* ATCC 27853 purchased from American Type Culture collection (10801 University Blvd, Virginia, USA) was used as the quality control strain.

P. aeruginosa infecting phages isolation

Sewage sample of approximately 500 ml was collected from the sewerage system in the Affiliated Zhongshan Hospital of Dalian University, Dalian, China. CaCl₂ was added to the sewage at the final concentration of 1 mmol/L. The sewage was centrifuged at 10,000 g for 10 min and supernatant (10 ml) were mixed with 10 ml of 2 \times LB and 200 μ l of early log phase *P. aeruginosa* (optical density at 600 nm = 0.4). The mixture was then incubated for 12 h at 37°C with shaking at 140 rpm. The enriched culture was then centrifuged at 10,000 g for 10 min at

4°C, and the supernatant was filtered through a 0.22 µm syringe filter (EMD Millipore Co., Billerica, MA, USA). Spot tests were carried out to detect the presence of phage [16]. Phage was repeatedly purified using the method of the double-layer agar plate [17], until the plaques were homogeneous. Phages were collected by adding 5 ml of SM buffer (NaCl 5.8 g/L, MgSO₄·7H₂O 2 g/L, 1M Tris HCl (pH7.5) 50 ml/L and 2% gelatin 5 ml/L) to each plate and leaving overnight at 4°C on a rotating platform, filtered by a 0.22 µm Millipore filter and stored at 4°C. *P. aeruginosa* strain PA5-1-1 was used as a host bacterium for isolation and propagation of the phage.

Propagation and purification of the phage

Large-scale propagation and purification of the phage were performed as described by Sambrook and Russel [18] with slight modifications. Briefly, early log phase cultures of *P. aeruginosa* PA5-1-1 strains (OD₆₀₀ = 0.4, 50 ml) were infected with phage filtrates at a multiplicity of infection (MOI) of 0.1. The phage infected cultures were incubated at 37°C with shaking at 140 rpm for 5 h until complete lysis occurred. The lysed culture was treated with DNase I (1 µg/ml, Roche Molecular Biochemicals, Indianapolis, IN, USA) and RNase A (1 µg/ml, Sigma-Aldrich Co., St. Louis, MO, USA) at 37°C for 1 h. Sodium chloride was added in the lysate to achieve final concentration of 1 M and incubated on ice for 1 h. The treated lysates were then centrifuged at 10,000 g at 4°C for 10 min. Supernatants were collected and treated with polyethylene glycol (PEG) 8000 at final concentration of 10% (w/v). After incubation in an ice-bath for 1 h, the culture was centrifuged at 4°C at 12,000 g for 10 min. Pellets containing phages were dissolved in 1 ml of SM buffer. The solution was named after the crude phage preparation.

Subsequently, the crude phage preparation was layered on a CsCl step gradient (1.33, 1.45, 1.50 and 1.70 g/ml) and centrifuged at 140,000 g for 3 h. Phage was collected at the interface between the gradients of 1.45 and 1.50 g/ml [19] and dialyzed three times each for 30 min against 300 volumes of gelatin-free SM buffer for extracting of phage genomic DNA.

Examination of the phage morphology

The CsCl purified phage was spotted onto a carbon-coated copper acid grid, stained with 0.5% (w/v) uranyl acetate, and examined by JEOL-1200EX transmission electron microscopy (JEOL USA, Inc., Peabody, MA, USA) at an accelerating voltage of 80 kv.

Host range of the phage

Host range of PAA-ABTNL was determined by pipetting 5 µl droplets of crude phage preparation stocks (10¹² PFU/ml) on lawns of indicated bacterial strains prepared on LB plates. Plates were incubated for 12 h at 37°C and observed whether plaques formed. Clear plaques indicated high host sensitivity, turbid plaques or no plaques indicated a nonhost. Details of bacterial strains used in the study were listed in [Table 1](#).

One-step growth curve

A one-step growth curve was performed as described by Pajunen *et al.* [20] with some modifications. Briefly, a culture of PA5-1-1 was grown to mid-exponential phase, harvested by centrifugation (10,000 g, 5 min, 4°C) and re-suspended in fresh LB medium to adjust the density approximately to 10⁸ cells/ml. Phages were added to achieve an MOI of 0.1 and allowed to adsorb for 5 min at 37°C. The phage-host mixture was centrifuged (10,000 g for 5 min at 4°C), and the pellets were suspended in 10 ml of fresh LB medium. This suspension was incubated at

Table 1. Bacterial stains used to determine the host range of phage PPA-ABTNL.

Strains	Source	Year	Lysed or not
<i>P. aeruginosa</i> 0212 ¹	Mink with hemorrhagic pneumonia	2002	Y
<i>P. aeruginosa</i> 0205 ¹	Mink with hemorrhagic pneumonia	2002	Y
<i>P. aeruginosa</i> 0206 ¹	Mink with hemorrhagic pneumonia	2002	Y
<i>P. aeruginosa</i> 1-1 ²	Mink with hemorrhagic pneumonia	2012	Y
<i>P. aeruginosa</i> 5-1-1 ²	Mink with hemorrhagic pneumonia	2012	Y
<i>P. aeruginosa</i> 5-1-2 ²	Mink with hemorrhagic pneumonia	2012	Y
<i>P. aeruginosa</i> 5-2-1 ²	Mink with hemorrhagic pneumonia	2012	Y
<i>P. aeruginosa</i> 5-2-2 ²	Mink with hemorrhagic pneumonia	2012	Y
<i>P. aeruginosa</i> c-1 ³	Human with burn	2012	N
<i>P. aeruginosa</i> c-2 ³	Human with burn	2012	Y
<i>P. aeruginosa</i> c-3 ³	Human with burn	2012	Y
<i>P. aeruginosa</i> c-4 ³	Human with burn	2012	Y
<i>P. aeruginosa</i> c-5 ³	Human with burn	2012	Y
<i>P. aeruginosa</i> 10104 ⁴	ChinaMedicalCulture Collection	— ⁶	Y
<i>P. aeruginosa</i> ATCC 27853 ⁵	The American Type Culture Collection	— ⁶	Y
<i>Klebsiella</i> 6*-1 ²	Mink	— ⁶	N
<i>P. mirabilis</i> G1 ²	Mink	— ⁶	N
ETEC O157:H7 ATCC 35150 ⁵	The American Type Culture Collection	— ⁶	N
<i>S. aureus</i> ATCC 29213 ⁵	The American Type Culture Collection	— ⁶	N
<i>S. typhimurium</i> CMCC 20115 ⁴	China Medical Culture Collection	— ⁶	N
<i>L. monocytogenes</i> ATCC 19111 ⁵	The American Type Culture Collection	— ⁶	N

¹Gifted by institute of special animal and plant sciences of Chinese Academy of Agricultural Science

²isolated from the lung samples of dead mink

³isolated from clinical samples obtained from burn patients at the first hospital of Dalian Medical University

⁴purchased from ChinaMedicalCulture Collection

⁵purchased from the American Type Culture Collection

⁶means that no data is available.

doi:10.1371/journal.pone.0116571.t001

37°C with shaking at 140 rpm. Samples were taken at 10-min intervals over a period of 2 h, immediately diluted and plated for phage titration using double-agar layer technique.

Genome sequencing and annotation

Phage genomic DNA was extracted from the CsCl-purified phage lysates using the method of SDS-proteinase K protocol [18]. The highly purified DNA was sheared by nebulization and subsequently fractionated by gel filtration. Fractions with fragment sizes between 2.0 and 2.5 kb were ligated into pUC18 cleaved with *HincII* cleavage and electroporated into *Escherichia coli* HST08 Premium Electro-Cells (TaKaRa Biotechnology (Dalian) Co., LTD, Dalian, China). From the sub-clones, plasmid DNA was prepared using TaKaRa MiniBEST Plasmid Purification Kit Ver.3.0 (TaKaRa Biotechnology (Dalian) Co., LTD, Dalian, China). The inserts in the isolated plasmids were sequenced on an ABI PRIMER 3730XL DNA Analyzer using BigDye Terminator V3.1 cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). One hundred and eighty-six clones were sequenced using M13-47 forward and reverse sequencing primers. Based on these sequences, primer walking was used with 17-mer oligonucleotide primers and pure PPA-ABTNL DNA was used as template until all the contigs could be assembled into a single sequence. The terminal redundancies were identified by analogy with previously published sequence data and verified by primer extension sequencing.

Initial genome annotation was completed using myRAST [21]. The Geneious v5.4 program (Biomatters Ltd., Auckland, New Zealand) was used to visually scan the sequence for potential genes. All translated proteins were scanned for homologues using BLASTP and Psi-BLAST [22,23] at <http://www.ncbi.nlm.nih.gov>. Rho-independent terminators were identified using WebGeSTer at <http://pallab.serc.iisc.ernet.in/gester/rungester.html> [24]. Transfer RNA (tRNA)-encoding genes were screened using Aragorn <http://130.235.46.10/ARAGORN/> [25] and tRNAScan at <http://greengene.uml.edu/programs/FindtRNA.html> [26]. Promoters were identified by visual inspection and by Martin Reese's neural network prediction program at http://www.fruitfly.org/seq_tools/promoter.html. Transmembrane domains were predicted by TMHMM 2.0 at <http://www.cbs.dtu.dk/services/TMHMM-2.0/> [27], Phobius at <http://phobius.sbc.su.se/> [28] and SPLIT 4.0 at <http://split.pmfst.hr/split/4/> [29].

In vitro inhibition of bacterial growth by phage

The phage's ability to inhibit bacterial growth was determined by optical densitometry against PA5-1-1 strain. Briefly, wells of a flat bottomed 96-well micro-titer plates were filled with 100 μ l of inoculated double strength LB and 100 μ l of prepared phage dilutions in SM buffer to provide a final bacterial count 10^7 CFU/ml in each well and phage counts 10^6 , 10^7 and 10^8 PFU/ml in various wells. Consequently, phage *in vitro* lytic efficiency was examined at several doses of MOI (0.1, 1 and 10), and each phage-host combination at specific MOIs was performed in triplicate wells. Controls for plate sterility, phage suspension sterility and bacterial growth without phage addition were also included. The plates were incubated at 37°C for 12 h and optical density at 600 nm was measured using a Micro-titer Plate Reader (Sunrise, Tecan Group Ltd., Austria) at 30-min intervals. Each independent trial was repeated three times.

Toxicity trial

Before experimentation with mink, the crude phage preparation was tested in mice to determine acute toxic effects. Twelve healthy male and 12 healthy female BALB/c mice (20.5 ± 1.2 g) of 7 weeks of age were provided by Laboratory Animal Center of Dalian Medical University. The mice were randomly selected and arbitrarily assigned into two groups of six males and six females per group. The crude phage preparation or physiological saline (pH = 7.0) was administered intra-nasally using a pipette once a day for a three-day period. Mice in phage-treated group were administered with 60 μ l of crude phage preparation (10^{12} PFU/ml) while mice in non-phage treated group (control group) received 60 μ l of physiological saline.

Experimental animals were monitored every four hours during the first three days and every six hours after then over a period of 8 days. Body weights were daily recorded pre-test and before sacrifice. At day 8, all surviving animals were sacrificed by cervical dislocation under deep anesthesia with ether. The samples of lung, liver and spleen were collected to examine their gross pathology and then preserved in 10% neutral buffered formalin until histopathological analysis.

Phage therapeutic study

The therapeutic potential of phage PPA-ABTNL was evaluated for its ability to treat hemorrhagic pneumonia in mink. A Yuehua Ultrasonic Nebulizer (model WH-2000, Guangdong Yuehua Medical Instrument Factory Co, Ltd., Shantou University Road, Rongsheng Science and Technology Park, Guangdong, China) was used in this experiment. Thirty-two, healthy, 6-month-old, male Jinzhou Black mink (2108 ± 143 g) were randomly assigned to four groups: three phage treated groups and one non-phage treated group with eight mink in each group. Mink in all groups were anesthetized by intra-peritoneal injection of 1.5 ml of 40% (w/v)

chloral hydrate followed by intra-nasally challenge with 20 μ l of PBS containing 1×10^8 CFU *P. aeruginosa* PA5-1-1. Choral hydrate was irritant to the peritoneal cavity for a few minutes until anaesthesia intervened. Two hours after challenge, all mink in each group were placed into a plexiglass chamber (L×W×H: 120 × 50 × 40 cm), and the sprayer was run for 3 min at 3 ml/min to atomize the phages at MOI 1, 10, 100 or PBS. Then, mink were kept in the chamber for an extra 3 min.

Experimental animals were monitored every four hours during the first three days and every six hours after then. At 24-h post phage administration, three mink from each treatment were euthanized by carbon dioxide asphyxiation which has been shown an appropriate method of euthanasia for mink according to the previous studies [30,31]. Each mink was anaesthetized by intra-peritoneal injection of 1.5 ml of 40% (m/v) chloral hydrate and placed individually in a wooden-glass chamber (L×W×H: 60 × 30 × 35 cm). While mink was asleep, the chamber was filled with CO₂ for 5 min to ensure death. The lungs were removed from each animal, weighed, suspended in 2 ml of sterilized PBS (pH = 7.2) and homogenized using a mechanical homogenizer (Ultra-TurraxT10, IKA-Labortechnik Co., Staufen, Germany). The viable number of PA5-1-1 cells (CFU/g tissue) was determined by serial dilution and plating on LB agar plates. The titer (PFU/g tissue) of the phages recovered from the lungs was determined by the soft agar overlay technique. The survival rates for each group were recorded at 1 d intervals until the end of the experiment (12 d). The humane endpoint was defined with troubling breath accompanied by slow action, having running noses and screaming. Mink when killed for humane reasons was counted as a death.

Statistical analysis

The Kolmogorov-Smirnov test was used for testing the normality of the distribution. Two-tailed paired t-test was performed to determine the effect of atomization on the phage titer. One-way ANOVA followed by Tukey test was used to compare quantifications of bacteria and phages in the lung sample. A log rank test was performed to test the significance of the difference between the survival curves. Error bars represented the standard deviation.

Results

Isolations of *P. aeruginosa*

In the LB agar medium, the purified bacterial isolations formed colonies which showed characteristics of round shape, convex surface, smooth margin, viscous and moist texture. They all produced a blue-green pigment in LB liquid medium. These isolates were named after their sample numbers, such as PA1-1, PA5-1-1, PA5-1-2, PA5-2-1 and PA5-2-2. 16S rDNA fragments from all isolates were amplified by PCR using universal primers. The gel electrophoresis showed that all bacterial PCR products were approximately 1450 bp (data not shown). BLAST analysis showed that the partial 16S rDNAs of the above five strains were more than 98% identical with that of *P. aeruginosa* strains RHH13, RHH13, JL 091016, D1, JL091016 (GenBank accession number: HQ143612.1, HQ143612.1, HM224410.1, KF113578.1 and HM224410.1), respectively.

To determine the homologous relationships among these isolates and other pathogenic bacteria, a phylogenetic tree based on the information of 16S rDNA was constructed (S1 Fig.). According to their morphological characteristics and 16S rDNA sequence analysis, all isolates were identified as being *P. aeruginosa*. The 16S rDNA sequences of strains PA1-1, PA5-1-1, PA5-1-2, PA5-2-1 and PA5-2-2 were submitted to GenBank with accession numbers KF977856, KF977857, KF977858, KF977859 and KF977860, respectively.

Table 2. Antibiotic susceptibility of *P. aeruginosa* from mink.

Antibiotics	% Sensitive isolate	
	Isolated in 2002*	Isolated in 2012*
Piperacillin	100	60
Ceftazidime	100	100
Gentamicin	100	40
Amikacin	100	60
Aztreonam	100	40
Ciprofloxacin	100	80
Meropenem	100	100
Ticarcillin/Clavulanic acid	100	100
Polymyxin B	60	40
Penicillin	0	0
Cefazolin	0	0
Sulfamethoxazole/Trimethoprim	0	0

The content per antibiotics impregnated discs, 100 µg of piperacillin, 30 µg of ceftazidime, 10 µg of gentamicin, 30 µg of amikacin, 30 µg of aztreonam, 50 µg of ciprofloxacin, 10 µg of meropenem, 75/10 µg of ticarcillin/clavulanic acid, 300IU of polymyxin B, 10IU of penicillin, 30 µg of cefazolin, 23.75/1.25 µg of sulfamethoxazole/trimethoprim.

*n = 5

doi:10.1371/journal.pone.0116571.t002

Antibiotic resistance of *P. aeruginosa* isolates

The antibiotic susceptibilities of *P. aeruginosa* isolated from mink indicated that all strains isolated in 2002 and 2012 were sensitive to ceftazidime, meropenem and ticarcillin, and resistant to penicillin, azolin and sulfamethoxazole. However, the antibiotic susceptibilities of the 2012 isolates were lower than those of isolates in 2002. These isolates were becoming resistant to piperacillin, gentamicin, amikacin, aztreonam, ciproxacin and polymyxin to some extent (Table 2).

Isolation and characterization of a phage specific to *P. aeruginosa*

A *P. aeruginosa* phage was isolated from hospital sewage by the spot test and named vB_PaeP_PPA-ABTNL (PPA-ABTNL). Structurally, PPA-ABTNL had an icosahedral head of about 40 nm in diameter and a tail about 15 nm in length (Fig. 1), indicating it is likely to belong to the family of *Podoviridae*. Host range assay showed that the phage was able to infect 93% (14/15) of the tested *P. aeruginosa* strains (Table 1), suggesting that PPA-ABTNL is a broad host-range phage. The one-step growth curve of the phage propagated on the PA5-1-1 strain in LB broth revealed that the latent and rise periods were approximately 25 and 35 min (Fig. 2). The average burst size was estimated as 115 PFU per infected cell.

The effect of the phage on the *in vitro* growth of *P. aeruginosa* strain PA5-1-1 was shown in Fig. 3. With an increase of MOI, the optical density of the bacterial solution dropped rapidly. There were no significant difference between the phage treated groups and the control group through one hour after incubation ($P > 0.05$). However, compared to the control group, all phage treated group significantly inhibited bacterial growth starting one hour after incubation continuing to the end of the experiment ($P < 0.05$).

Genome description

The PPA-ABTNL genome comprised 43227 bp that included direct terminal repeats (DTRS) of 453 bp and has an average G+C content of 62.4%, which was similar to that of the lytic

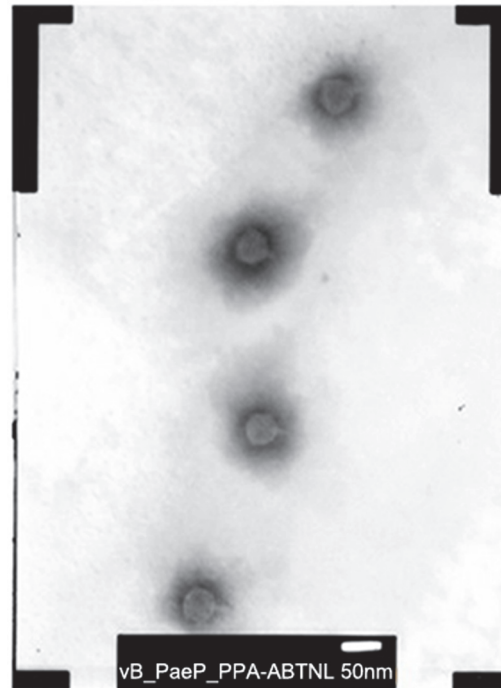


Figure 1. Transmission electron micrograph of phage PPA-ABTNL negatively stained with uranyl acetate. Scale bar represents 50 nm.

doi:10.1371/journal.pone.0116571.g001

P. aeruginosa phages LKD16 and LKA1 [32]. As shown in S1 Table, 54 open reading frames (ORFs) equal or longer than 100 bp were defined as potential genes. The majority of these genes had an ATG initiation codon, while seven started with GTG and one with TTG. All predicted ORFs were situated on the sense strand of the genome. Bioinformatic analysis and annotation showed that there were no similarities between genes or proteins of PPA-ABTNL and genes or proteins or other factor known to play a virulent role in the acute or chronic infection of *P. aeruginosa* [33].

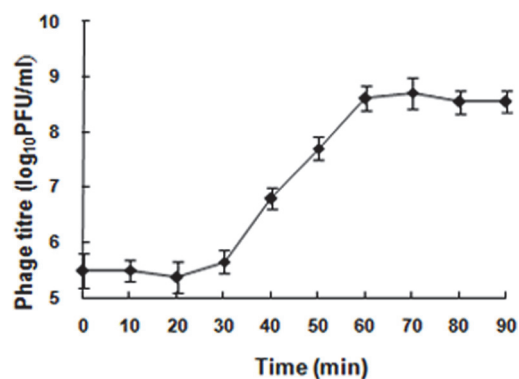


Figure 2. One-step growth curve of phage PPA-ABTNL. PPA-ABTNL phage was co-incubated with culture of PA5-1-1 to absorb for 5 min at 37°C. The mixture was centrifuged to remove non-absorbed phage. The re-suspended pellets were incubated at 37°C and sampled at 10 min interval over a period of 2 h. Phage titer was measured. Data represent mean±SD from three independent experiments. n = 3.

doi:10.1371/journal.pone.0116571.g002

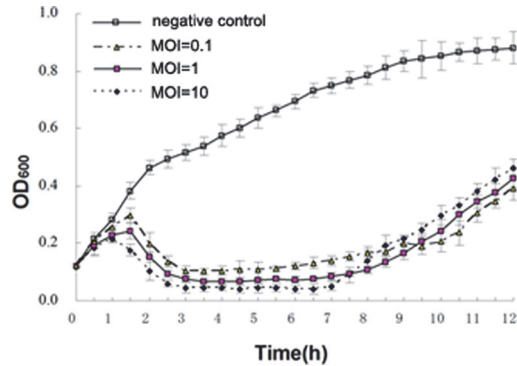


Figure 3. Antibacterial curve of phage PPA-ABTNL at different multiplication of infection *in vitro*. PA5-1-1 strain was lysed by the phage PPA-ABTNL in LB medium at 37°C. OD600 development of uninfected control culture and parallel cultures infected with phage at different multiplicities of infection (MOI) was measured. n = 3.

doi:10.1371/journal.pone.0116571.g003

Similar to *Pseudomonas* phage phiKMV [34], there were early, middle and late regions, marked by the position of ATP-dependent DNA ligase and DNA-dependent RNA polymerase in the PPA-ABTNL genome (Fig. 4). These three regions comprised the host conversion, DNA metabolism genes and the genes encoding structural and lysis proteins, respectively. Thus, phage PPA-ABTNL was categorized as a “phiKMV-like Virus”. The sequence and annotation of phage PPA-ABTNL were deposited in GenBank with the accession number KM067278.

Repeated dose toxicology study

Intranasal administration of a high dose of phage PPA-ABTNL for three consecutive days, followed by a five-day recovery period in male and female BALB/c mice, indicated no effects attributable to the crude phage preparation. No deaths were recorded in the study. There were no differences in average body weight and food consumption between the two groups (data not shown), although the weights of mice in the control and phage-treated groups decreased slightly by 1.6% and 1.4% compared to their initial weights, respectively, perhaps caused by the stress response of anesthesia during the first three days. Additionally, all mice behaved normally,

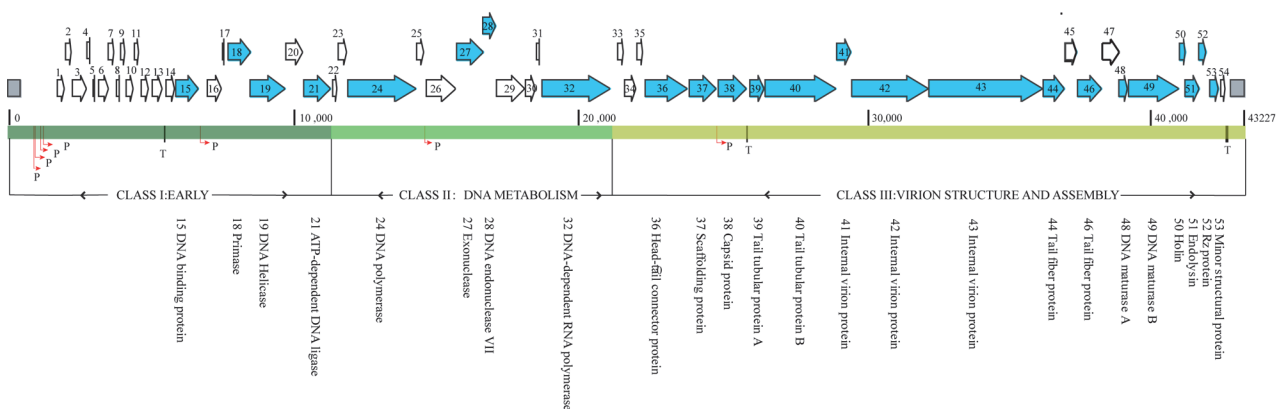


Figure 4. Overview of the PPA-ABTNL genome sequence analysis. Predicted open reading frames (ORFs) are numbered. ORFs with significant homology to known genes and to the identified proteins are in dark blue. Putative genome organization. Putative promoters (P), terminators (T) are indicated. Predicted and presumed proteins and corresponding ORF number are marked.

doi:10.1371/journal.pone.0116571.g004

Table 3. Incidence of histomorphological observations.

Dose Group	Control		Phage	
Sex	M ^a	F ^b	M	F
Number of animals/group	6	6	6	6
Lung Examined/Normal	6/6	6/6	6/6	6/6
Spleen Examined/Normal	6/6	6/6	6/5	6/6
Liver Examined/Normal	6/6	6/6	6/5	6/6

^aMale

^bFemale.

doi:10.1371/journal.pone.0116571.t003

with no symptoms of lethargy, ruffling, hunching or irregular breathing, except during the period of recovery from the anesthesia.

Gross pathology results showed that the organs of all the animals were normal with no obvious pathological changes (data not shown). Minor hemorrhage in spleen and slight inflammatory cells infiltration in liver were found in one male mouse intra-nasally administered with the phage as observed in histopathology analysis (see Table 3). The lungs, spleens and livers of all other mice were normal. Thus, it was concluded that administration of a crude preparation of phage PPA-ABTNL had no significant effect on the health of animals.

Phage therapeutic study

A pilot experiment showed that atomization by ultrasonic treatment did not affect the titer of phage ($P > 0.05$) (Fig. 5A). As shown in Fig. 5B, compared to 5.51 log₁₀ CFU/g of *P. aeruginosa* recovered from lungs in non-phage treated group (the control group), 5.23 log₁₀ CFU/g ($P > 0.05$), 4.44 log₁₀ CFU/g ($P < 0.05$), and 4.07 log₁₀ CFU/g ($P < 0.05$), respectively, were recovered from lungs in phage-treated mink with the MOI of 1, 10 or 100 after 24 h of infection. Notably, no significant difference in amount of bacteria between mink administered with phage of MOI 10 and MOI 100 was observed, indicating that an MOI 10 of phage is optimal for this clinical trial. At the same time, we measured the amount of phage in mink after 24 h of infection (Fig. 5C). As expected, no phage was detected in mink of the non-phage treated group. Viable phages were detected in the lungs of mink administered with the indicated dose

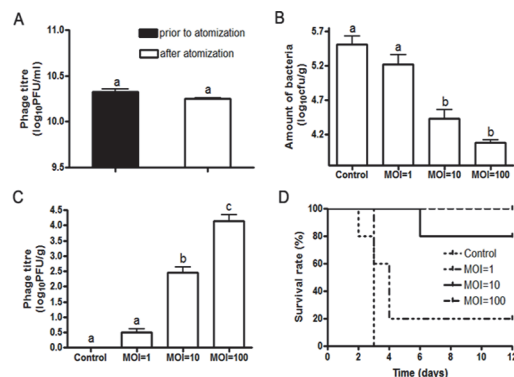


Figure 5. Phage PPA-ABTNL cures mink hemorrhagic pneumonia caused by a clinical *P. aeruginosa* strain. (A) Effect of atomization on the titer of phage. (B) Effect of different multiplicities of infection (MOIs) on the amount of bacteria in the lung of mink. (C) Effect of different MOIs on the amount of phage in the lung of mink. (D) Survival curves of mink infected with PA5-1-1 strain and treated with phage PPA-ABTNL. n = 5. Means with different superscripts differ significantly from each other ($P < 0.05$).

doi:10.1371/journal.pone.0116571.g005

of phage. As shown in Fig. 5C, the amount of viable phages in phage-treated groups with the MOI of 1, 10 or 100, respectively, were 0.50 log₁₀ PFU/g ($P > 0.05$ vs the control group), 2.47 log₁₀ PFU/g ($P < 0.05$ vs the control group) and 4.13 log₁₀ PFU/g ($P < 0.05$ vs the control group).

In the control group, one mink developed the clinical symptoms (difficult breathing and slow action) and was euthanized for humane reasons at day 2. Two mink actually died and two mink with symptoms of breathing trouble and slowing action were euthanized for humane reasons at day 3. Lung hemorrhage was seen on gross observation in all mink of the control group. In the MOI 1 group, one mink actually died and one with the same symptoms described above was euthanized for humane reason at day 3, and two mink actually died at day 4. In the MOI 10 group, one mink was euthanized for humane reasons at day 6. In the MOI 100 group, no mink died for 12 days. The results of phage therapeutic study were summarized in Fig. 5D. Compared to the non-phage treated group (the control group), MOI 10 and MOI 100 phage significantly increased ($P > 0.05$ vs the control group) the survival rate of mink infected with *P. aeruginosa*. Thus a dose of phage at MOI 10 is optimal for treating mink hemorrhagic pneumonia using ultrasonic atomization.

Discussion

In species other than mink, *P. aeruginosa* is known as a conditioned pathogen that only causes mild, opportunistic infections, such as soft tissue abscesses, *otitis media* and *otitis externa*, particularly in immune-compromised hosts or those susceptible to developing an infection in other ways [35,36,37]. Mink are the only animal species known to develop acute, contagious and fatal hemorrhagic pneumonia caused by *P. aeruginosa*. However, to date, no underlying reasons have been identified [38].

Hemorrhagic pneumonia can be a major cause of mortality in farmed mink in the fall [39]. High mortality rate of the disease has caused huge economic losses during the course of mink production [40]. The main measure to treat hemorrhagic pneumonia is still the application of antibiotics [7]. However, *P. aeruginosa* has natural and acquired mechanisms as well as the ability to form biofilm resistant to multiple groups of antimicrobial agents [41,42]. Pedersen *et al.* [7] reported that all 39 strains of *P. aeruginosa* isolated from Danish mink were only sensitive to two antibiotics, colistin and gentamicin, among 17 most used antibiotics for mink, showing the high level of resistance to antimicrobials. Therefore, antibiotics are playing a negligible role in the treatment of hemorrhagic pneumonia in mink. Furthermore, clinical trials in humans showed a dearth of effective antibiotics to treat *P. aeruginosa* and a shortage of new agents passing through the development pipeline [43]. These studies suggest that there is an urgent need to develop new antimicrobial agents to treat the infection caused by *P. aeruginosa* in the field of both veterinary and human clinics.

Phages are natural enemies of bacteria and are essentially ubiquitous, with an estimated 10³¹ phage particles on earth [44]. As antibacterial agents, phages have many advantages over antibiotics, such as target specificity, self-replication, overcoming resistance, synergistic activity in a phage cocktail or in combination with other antibiotics, and are easy to isolate and propagate [45]. In the past few decades, phages have been widely applied as antibacterial agents in the field of food production, aquaculture industry as well as in veterinary and human clinics to control undesirable bacteria [46].

Compared to other bacterial phages, phages specific for *P. aeruginosa* have advantages such as limited geographical variation, availability, lytic nature and lack of gene transfer and associated toxins [47]. There has been evidence of the efficacy of phage therapy in treating a number

of *P. aeruginosa* infections in animals [11,48] and humans [12]. Therefore, we proposed to apply a phage to control hemorrhagic pneumonia caused by *P. aeruginosa* in mink.

In this study, the predominant bacterial strains in lung samples of mink with suspected hemorrhagic pneumonia were isolated and identified as *P. aeruginosa* based on morphological observation and 16S rDNA sequence analysis. The overuse and misuse of antibiotics has led to newly isolated strains showing more resistance to antibiotics than those of ten years ago. The situation is the same in Denmark [7], which is the world's largest producer of mink. Subsequently, phage PPA-ABTNL was isolated from hospital sewage using one of the isolated strains as a host. Sewage is a rich source of phages that infect pathogenic bacteria such as *E. coli*, *P. aeruginosa* and *Salmonella* [49]. Thus, it is an appropriate sample source for conventional bacterial phage isolation.

Morphological and genomic analysis showed that PPA-ABTNL was a member of the "phiKMV-like" phages, which encodes its own RNA-polymerase and has significant homology to the *Pseudomonas* phiKMV phage, belonging to the subfamily Autographivirinae, family Podoviridae [50]. Like other "phiKMV-like" phages, the genes of PPA-ABTNL could be categorized into three classes including early (class I), a gene cluster dealing with DNA metabolism (class II) and a third group (class III) containing structural proteins, assembly proteins and the endolysin responsible for host lysis [19,32,34].

PPA-ABTNL was a strictly lytic phage, based on the appearance of phage plaque and its genome, which lacked genes encoding generalized transduction and virulence factors, without exhibiting lysogenic characteristics. Randomly chosen clinical strains revealed high activity in 14 of 15 analyzed strains, including eight strains isolated from mink, four strains isolated from human clinics and two standard reference strains. The high infectivity rate may be explained by a good adsorption to its host surface [51] and the absence of many restriction enzyme recognition sites in the PPA-ABTNL genome (data not shown). Meanwhile, PPA-ABTNL exhibited a relative high specificity, and did not target other bacterial species, such as *Listeria Monocytogenes* (*L. monocytogenes*), ETEC O157:H7, *Staphylococcus aureus* (*S. aureus*), and *Salmoella typhimurium* (*S. typhimurium*). These results showed that phage PPA-ABTNL meet the main prerequisites for a candidate phage in phage therapy, which should exhibit lytic activity and broad host range against a number of strains of the targeted host bacteria [52].

During phage therapy, safety is the major concern for their therapeutic application on farms or in the food chain. Early phage preparations were often prepared simply by filtering the lysates, such that they still contained bacterial toxins at the point of human delivery [53]. Effective purification of the phage can be achieved by means of ammonium sulfate precipitation, CsCl gradient centrifugation [54], ultrafiltration and two-step chromatography [55]. However, these studies showed the phage preparations with low amounts of endotoxin obtained as well as lost 97–99% of phages during the purification [53]. Therefore, the above purification methods were not efficient [53].

There were no reports of serious side effects from endotoxin in the early literature concerning experimental phage therapy [56]. Therefore, in the present study, we used a crude phage preparation to test its safety, which was prepared by centrifugation and PEG precipitation to remove bacterial debris [18]. No morbidity, mortality or significant histopathological changes in mice related to the PPA-ABTNL phage crude preparation were observed. One explanation may be that the mink respiratory tract seems to have low sensitivity to endotoxin applied by inhalation, similar to the human gut when endotoxin is applied orally [57].

Curative treatment appears to be of more interest as a clinical application than preventive treatment [58]. Therefore, the study initially evaluated the feasibility of applying phage to treat hemorrhagic pneumonia of mink. Previous studies showed that the route of administration affected the phage bacteriostatic action. Ryan *et al.* [59] concluded that the parenteral route was

the most successful delivery route for the treatment of systemic infections while local delivery was the method of choice in the treatment of topical infection and inhalation for the treatment for lung infections. Pulmonary drug delivery has significant advantages in the treatment of respiratory diseases because of its direct target treatment [60].

A nebulizer has been widely used drug delivery means for the treatment of asthma and respiratory disease worldwide [61]. Golshahi *et al.* [62] showed that phage administered by means of nebulization is effectively distributed in the lung. Our primary studies demonstrated that atomization by ultrasonic treatment did not significantly affect the titer of the phage. Therefore, we used a natural route for the infection of the lungs of the experimental mink and the phage treatment was delivered by inhalation in order to demonstrate that hemorrhagic pneumonia of mink can be treated by phages. The results showed that the more phages reached the lung site and the fewer bacteria remained. Therefore, mink can survive a lethal bacterial challenge. Also, nebulization administration may be an effective route to deliver phage in other species with respiratory diseases.

Conclusions

According to our knowledge, this research is the first systematic *in vitro* and *in vivo* investigation and evaluation of the capability of a “phiKMV-like” phage as a therapeutic application against mink hemorrhagic pneumonia. Our work supports the potential use of phage to fight *P. aeruginosa* involved in mink lung infection, administered by means of ultrasonic nebulization. We hope the results presented here will lead to novel treatments for hemorrhagic pneumonia in mink, decrease the amount of antibiotics used in the production of mink and enhance economic benefit of mink-rearing farms.

Supporting Information

S1 Fig. Phylogenetic tree showing the positions of the suspected *P. aeruginosa* isolates among the representative pathogenic bacteria. The phylogenetic tree is based on a Cluster W alignment of 16S rDNA sequences. Scale bar represents 0.02 substitution per nucleotide position.

(TIF)

S1 Table. Feature of phage PPA-ABTNL gene products and their functional assignments.

(DOC)

Author Contributions

Conceived and designed the experiments: ZHC LJJ YPX. Performed the experiments: ZHC JCZ FC ZL. Analyzed the data: YDN NZC YSM. Contributed reagents/materials/analysis tools: JCZ YDN. Wrote the paper: ZHC YDN.

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