European Journal of Pharmaceutics and Biopharmaceutics 125 (2018) 124–130

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Nebulization effects on structural stability of bacteriophage PEV 44

Ariel Astudillo[a,1], Sharon Shui Yee Leung[a,1], Elizabeth Kutter[b], Sandra Morales[c], Hak-Kim Chan[a,*]

[a] Faculty of Pharmacy, University of Sydney, Sydney, NSW 2006, Australia
[b] The Evergreen State College, Olympia, WA 98502, USA
[c] AmpliPhi Biosciences AU, 7/27 Dale Street, Brookvale, Sydney, NSW 2100, Australia

ARTICLE INFO

Keywords:
Bacteriophage
TEM
Nebulizer
Plaque assay
Viable respirable fraction

ABSTRACT

Reduced infectivity of phage due to the nebulization process has been reported previously, but no visual evidence on structural change upon nebulization has been established, or whether these structural changes can be attributed to the titer reduction. In this study, transmission electron microscopy (TEM) was employed to assess the impact of three different types of nebulizers, air-jet, vibrating-mesh and static-mesh nebulizers, on the structural stability of a *Myoviridae* phage, PEV44, active against *Pseudomonas aeruginosa*. The morphology of the phage in the nebulized samples was categorized into two groups: "whole" (the capsid and tail held together) and "broken" (the capsid separated from the tail) phages. The "whole" phage group was further divided into three sub-groups: (1) intact, (2) contracted tail and (3) empty capsid. The starting stock suspension was found to contain considerable portions of "broken" phages (35.5 ± 6.7%), "whole" phages with contracted tails (9.9 ± 5.4%) and empty capsids (19.3 ± 8.9%). The fraction of "broken" phages was significantly increased after nebulization, with the air-jet nebulizer (83%) being more pronounced than the mesh type nebulizers (50–60%). While the amount of intact phages (2–10%) and whole phages with contracted tails (0–3%) were significantly decreased, the proportion of phages with empty capsids (15–36%) were not significantly different. Phages with broken features obtained by TEM quantification were 92.9 ± 3.2%, 74.8 ± 10.4% and 71.2 ± 11.0% for the jet, vibrating-mesh and static-mesh nebulizers, respectively. These results were found to be comparable with the titer loss obtained by the conventional plaque assay results. The *in vitro* aerosol performance and viable phage delivery of the three nebulizers was also assessed. The Omron nebulizer achieved a significantly higher viable respirable fraction (VRF) than the SideStream and Aeroneb Go (15.1 ± 5.8%, 2.4 ± 2.0%, 4.1 ± 2.7% respectively). In conclusion, this study identified various changes in the phage structure and viability of phage from different types of nebulizers. Understanding these effects and the phage tolerance to nebulization stresses can potentially improve our choice of the delivery method for inhaled phage therapy.

1. Introduction

Bacteriophages (phages) are viruses that typically infect a narrow spectrum of bacterial hosts. The therapeutic use of phages for the treatment of bacterial infections was clinically implemented in the first half of the 20th century, in France and Eastern Europe, proving to be successful in many areas of medicine including dermatology, ophthalmology, urology, stomatology, otolaryngology and surgery [1,2]. Though it was largely forgotten in the Western medicine due to the advent of antibiotics, the emergence of bacterial multi-drug resistance (MDR) has re-established interest in phage therapy as an alternative or adjunct to antibiotics [3]. Phages are able to coevolve with bacteria to minimize resistance development. Even when bacterial resistance is developed, it usually just takes weeks to find new phages to combat the resistant strains, compared with years of development for new antibiotics [4]. The capability of phage targeting MDR bacteria has been demonstrated in recent *in vitro* infection [5–7] and animal models [8,9], and cases studied in human [10–12].

Recently, as discussed below, inhaled phage therapy has received increasing efforts to target respiratory infections caused by *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex and *Mycobacterium tuberculosis*. Pulmonary delivery of phage can be achieved using nebulizers, pressurized metered dose inhalers, or dry powder inhalers. Among them, nebulizers have been a popular choice for early studies to deliver phage into the lung, as they require only limited formulation development and can achieve a reasonable phage

* Corresponding author at: A15 – Pharmacy and Bank Building, Faculty of Pharmacy, University of Sydney, Sydney, NSW 2006, Australia.
E-mail address: kim.chan@sydney.edu.au (H.-K. Chan).
[1] Equal contribution.

https://doi.org/10.1016/j.ejpb.2018.01.010
Received 29 August 2017; Received in revised form 11 December 2017; Accepted 13 January 2018
Available online 17 January 2018
0939-6411/

Nomenclatures

ANOVA	one-way analysis of variance
dsDNA	double-stranded deoxyribonucleic acid
E	predetermined precision
MDR	multi-drug resistance
MSLI	multi-stage liquid impinger
n_1, n_2	number of particles
pfu	plaque formation unit
PSD	particle size distribution
PTA	phosphotungstic acid
RF	respirable fraction
SMB	salt-magnesium buffer
TEM	transmission electron microscopy
USP	United State Pharmacopeia
VRF	viable respirable fraction
X	mean quantity measured

lung dose [13].

Golshahi et al. [14] successfully aerosolized a *Burkholderia cepacia* specific *Myoviridae* phage (2.15×10^8 pfu/ml), KS4-M, using a Pari LC star jet nebulizers and an eFlow vibrating mesh nebulizer. They showed that both nebulizers caused less than 1 $\log_{10}$ titer loss and were capable of delivering a phage lung dose in the order of 10^7 pfu. Cooper et al. [15] nebulized a cocktail of three bacteriophages (GL-1, GL-$1_{2.5}$ and LP-M_{10} of 10^{11} pfu/ml) active against *Pseudomonas aeruginosa* using a Porta-neb jet nebulzer and reported that the loss of infectivity was minimal after nebulization. Recently, Sahota et al. [10] reported a reduction in viability on the nebulization of two *Myoviridae* phages (PELP20 and PELI40) active against *P. aeruginosa* using an air-jet nebulizer and a mesh nebulizer. They showed that air-jet was more effective than mesh nebulization for those two phages, though the comparison did not consider individual nebulizer outputs.

Other nebulization studies working with *Siphoviridae* phage D29 (phage with flexible uncontractile tail) have also reported a reduction in the viability of their phage preparations [16,17]. It has been speculated that potential damages to the phage structure caused by the physical stresses of nebulization are related to the phage viability reduction. Turgeon et al. [18] compared the effect of aerosolization on the infectivity of five tail-less bacteriophages using three nebulizers including the Aeroneb Lab, similar to the one used in this study. They reported that a 1000-fold more genome copies were found in their nebulized product compared with pfu values. This suggested that aerosolization by those nebulizers strongly affects the structural integrity of phages. However, there was no direct evidence showing structural changes in the phages.

More recently, Groulx et al. [19] nebulized a *Myoviridae* phage PP01 against *Escherichia coli* using a single jet atomizer and imaged the aerosol droplets using transmission electron microscopy (TEM) to study viral charge (number of phages) in single aerosol particles. Phage debris, such as phage capsids, phage tails, and full phages with empty capsids and contracted tails were observed in the nebulized droplets. However, it was unclear whether the presence of these phage morphologies was due to the nebulization process. The present study aimed to determine nebulization effects on the phage structural stability by direct visualization of the nebulized phage samples and initial phage suspension using TEM. A *P. aeruginosa* specific *Myoviridae* phage, PEV44, was used as the model phage. The morphology of PEV44 consists of a hexagonal head/capsid containing the genomic material, and a contractile tail use for bacterial cell recognition, attachment and subsequent injection of genome. The basic morphology of PEV44 allows changes to its structure to be easily recognised under TEM. Three commonly used nebulizers, an air-jet nebulizer, a vibrating-mesh nebulizer and a static-mesh nebulizer, were examined in this study. The *in vitro* aerosol performance of these three nebulizers in delivering PEV44 was also assessed. A relationship between structural changes obtained in TEM with titer loss was established.

2. Materials and methods

2.1. Bacteriophage suspension

Phage PEV44 was isolated from the sewage treatment plant in Olympia, WA, USA by the Evergreen State College Phage Laboratory. A PEV44 stock stored in salt-magnesium buffer (SMB – 5.2 g/L sodium chloride, 2 g/L magnesium sulphate, 6.35 g/L Tris-HCL, 1.18 g/L Tris-base, with adjusted pH to 7.4) with an initial titer of 4.63×10^{10} pfu/mL was supplied by AmpliPhi Biosciences AU and used in this study without further purification.

2.2. Phage nebulization

A volume of 3 mL phage stock suspension was nebulized from three different nebulizers, a SideStream air-jet nebulizer with a Porta-neb compressor operated at 8 L/min, an Aeroneb Go vibrating-mesh nebulizer and an Omron NE-U22 static-mesh nebulizer. Nebulized samples were collected by a method detailed in Cipolla and Gonda [20] for collection of nebulized proteins (Fig. 1). Briefly, the nebulized aerosol was drawn into an ice-cooled test tube placed inside an aspiration flask through a Tygon tubing connected with a 2.0 mL plastic pipette at 8 L/min. For the two mesh-type nebulizers, the collection apparatus was operated without the compressed air supply. The sample collected in the test tube was used for TEM analysis and plaque assay. All nebulizations were done in triplicate.

The aerosol collection efficiency of the apparatus was estimated by the volume of suspension collected at the test tube and deposition at connecting tubings was washed out with the collected aerosols. The recovery of phage suspension was ≥80% for the air-jet nebulizer and ≥90% for the mesh nebulizers. The lower collection efficiency of the air-jet nebulizer could be because a significant fraction of fine aerosol droplets remained entrained in the airstream and escaped collection

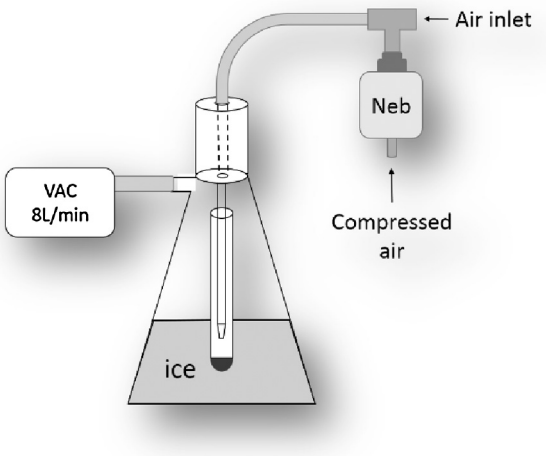

Fig. 1. Apparatus for trapping nebulized phages from the SideStream air-jet nebulizer.