

## 1 **Expanding Clinical Phage Microbiology:**

### 2 **Simulating Phage Inhalation for Respiratory Tract Infections**

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4 Shira Ben Porat<sup>1,2\*</sup>, Daniel Gelman<sup>1,2,3,\*</sup>, Ortal Yerushalmy<sup>1</sup>, Sivan Alkalay-Oren<sup>1</sup>, Shunit  
5 Copenhagen-Glazer<sup>1</sup>, Malena Cohen-Cymerknoh<sup>4,5</sup>, Eitan Kerem<sup>4,5</sup>, Israel Amirav<sup>6</sup>, Ran Nir-  
6 Paz<sup>3,5,#</sup>, Ronen Hazan<sup>1,#</sup>

7  
8 <sup>1</sup>Institute of Biomedical and Oral Research (IBOR), Faculty of Dental Medicine, The Hebrew  
9 University of Jerusalem, Jerusalem, Israel.

10 <sup>2</sup>Department of Military Medicine, Faculty of Medicine, The Hebrew University of Jerusalem,  
11 Jerusalem, Israel.

12 <sup>3</sup>Department of Clinical Microbiology and Infectious Diseases, Hadassah-Hebrew University Medical  
13 Center, Jerusalem, Israel.

14 <sup>4</sup>Pediatric Pulmonology Unit and Cystic fibrosis Center, Hadassah Medical Center.

15 <sup>5</sup>Faculty of Medicine, Hebrew University of Jerusalem, Israel.

16 <sup>6</sup>Pediatric Pulmonary Unit, Dana-Dwek Children's Hospital, Tel Aviv.

17  
18 \*Equal contribution.

19 #Equal contribution.

20 - Address correspondence to Ronen Hazan, [ronenh@ekmd.huji.ac.il](mailto:ronenh@ekmd.huji.ac.il).

#### 21 22 **Take-Home Message:**

23  
24 Phage therapy can be used against infectious diseases if personally tailored. Using a 3D airways  
25 model, we show that phage delivery by inhalation to the respiratory tract is unpredictable and also  
26 requires a precise evaluation.

27

28 **Abstract**

29 Phage therapy is a promising antibacterial strategy for resistant respiratory tract infections. Phage  
30 inhalation may serve this goal; however, it requires a careful assessment of their delivery by this  
31 approach. Here we present an *in-vitro* model to evaluate phage inhalation.

32 Eight phages, most of which target CF-common pathogens, were aerosolized and administered to a  
33 real-scale CT $\square$  derived 3D airways model with a breathing simulator. Viable phage loads reaching the  
34 output of the nebulizer and the tracheal level of the model were determined and compared to the  
35 loaded amount.

36 Phage inhalation resulted in a diverse range of titer reduction, primarily associated with the  
37 nebulization process. No correlation was found between phage delivery to the phage physical or  
38 genomic dimensions. These findings highlight the need for tailored simulations of phage delivery,  
39 ideally by a patient-specific model in addition to proper phage matching, to increase the potential of  
40 phage therapy success.

41

42

## 43 **Introduction**

44 Phage therapy refers to the use of bacteriophages (phages), bacterial viruses, as antimicrobial agents.  
45 Lytic phages can propagate in the presence of their bacterial hosts while sequentially lysing proximal  
46 bacterial cells. This enables them to penetrate and destroy biofilm and co-evolve with bacterial targets  
47 [1]. The emerging threat of antimicrobial resistance has re-introduced this previously neglected  
48 method and its advantages to the clinical practice, with an increasing number of reports applying  
49 phages for infectious diseases in recent years [2–4].

50 A significant target of phage therapy is the treatment of life-threatening pulmonary infections [5]. In  
51 this regard, phages have been used in cases of cystic fibrosis (CF), an inherited life-shortening disease  
52 associated with recurrent and chronic lung infections [6–8]. Examples include the use of intravenous  
53 (IV) phages in a 26-year-old CF patient with (MDR) *Pseudomonas aeruginosa* pneumonia [7], a case  
54 of a 12-year-old lung-transplanted CF patient treated by inhaled phages and by direct phage  
55 administration via therapeutic bronchoscopy for *Achromobacter xylosoxidans* [8], and the treatment  
56 of six individuals with CF or non-CF bronchiectasis, treated by nebulized phages for *P. aeruginosa*  
57 [9]. Evidently, these published cases differ in treatment protocols and specifically in the method for  
58 phage delivery.

59 The current understanding of the importance of a personalized and accurate phage matching for  
60 therapy dictates the use of simulations and *in-vitro* predictions during phage therapy treatment design  
61 [10]. Phage inhalation is not exceptional in this manner, as differences in the biochemical and  
62 physical characteristics of phages may cause sub-optimal delivery to the target site and thus should be  
63 tested in advance.

64 Several *in-vitro* studies have previously compared phage recovery at the output of various nebulizers  
65 [11, 12]. However, phage concentration reaching the lower respiratory tract was usually not measured  
66 but only estimated, for instance, according to the aerodynamic diameter of the released particles [11].  
67 To bridge the gap, we applied a real-scale 3D model of patient's CT-derived airways. This model,  
68 which was previously used for *in-vitro* aerosol studies [13, 14] was adapted here for evaluation of  
69 phage delivery to the lungs by inhalation.

70

## 71 **Methods**

72 **Phages and bacteria.** Phages targeting the following seven bacterial strains, most of which are  
73 associated with CF, were used (Table 1): *P. aeruginosa* strains PA14 and PAR1, *Mycobacterium*  
74 *abscessus* MAC107, *Burkholderia cepacia* strains BCC129 and BCC378, *Staphylococcus aureus*  
75 SAR1 and *Staphylococcus epidermidis* SE52. Except for the laboratory strain *P. aeruginosa* PA14, all  
76 isolates were obtained from the Clinical Microbiology Department at the Hadassah Medical Center,  
77 Israel. *S. epidermidis* was grown in brain heart infusion (BHI) broth (Difco, Sparks, MD) and all  
78 other bacteria were grown in LB broth (Difco, Sparks, MD), all at 37 °C under aerobic conditions  
79 shaken on a rotary shaker at 0.85 rcf. BHI or LB agar (1.5%) plates were respectively used for  
80 isolation streaks and phage enumeration. All phages were isolated as part of the Israeli Phage Bank  
81 [15]. The phages were propagated on their respective target bacterial hosts as previously described  
82 [16].

83

84 **Phage enumeration.** Phage titers were determined by the double-layered agarose assay as previously  
85 described [17]. Briefly, agar plates were covered with pre-warmed agarose (0.5%), to which 300 µl of  
86 the relevant overnight bacterial culture was added. The phage lysates were serially diluted by 10-fold,  
87 and 5 µl drops of these dilutions were spotted on the double-layered plates which were then incubated  
88 overnight at 37 °C. The number of plaques on the plates was then counted to calculate the initial  
89 phage titers, presented as plaque-forming units (PFU)/ml.

90

91 **Phage recovery from filters.** Phages were recovered from filters placed across the model to evaluate  
92 phage viability at these sites. Following each run of the nebulizer, the used filter was left overnight in  
93 a sterile tube containing 15 ml of fresh media in 4° C, followed by phage enumeration.

94 To estimate the fraction of phage titer reduction caused by the used filters (*Fr* - filter reduction  
95 fraction), 0.2 ml of each phage in a known concentration were dropped directly on the pads. The  
96 filters were then left to dry, and later treated as describe above. For each phage, the *Fr* was calculated

97 as the proportion between viable phages retrieved from the filters to their original inserted titer (Table  
98 2).

99

100 **Phage nebulization.** The commonly clinically used Pari LC Sprint nebulizer (Pari GmbH, Starnberg,  
101 Germany), combined with Pari TurboBoy SX Compressor (Pari GmbH, Starnberg, Germany) was  
102 used for phage nebulization (Figure 1). In each run, 3 ml of phage lysate in a previously determined  
103 concentration were pipetted into the inhalation device reservoir, and the compressor was operated for  
104 15 minutes. For evaluation of phage viability following nebulization, a PARI filter/valve set (Pari  
105 GmbH, Starnberg, Germany) filled with single-use filter pads (Pari GmbH, Starnberg, Germany) was  
106 connected to the output of the nebulizer to collect aerosol particles (Figure 1A).

107

108 **3D face and airways model construction.** The ability of nebulized phages to reach the lower  
109 respiratory tract (lung delivery) was evaluated using a 3D reconstruction model of a face and  
110 respiratory tract airways [13, 14] (Figure 1B). For the face and airways model creation, CT (Brilliance  
111 CT, 64-channel scanner, Philips Healthcare, Best, The Netherlands) scans of 5-year-old children with  
112 no craniofacial anomalies were obtained for medical indications. The data were digitized and  
113 converted to electronic files. Technical acquisition parameters included: 64 x 0.625 collimation, 0.75-  
114 s pitch, 120 kV, 100 mA, and a 2-s rotation time. The thickness of a slice was 2.5 mm, with  
115 increments of 1.25 mm. Slices were stored in Digital Imaging and Communications in Medicine  
116 (DICOM) format. Subsequently, the slices were joined together to compose a 3-dimensional  
117 reconstruction image, which was stored in Standard Transformation Language format, and later  
118 transferred over local area network (LAN) for further analysis. The scans were reconstructed and  
119 stored in stereolithography (STL) format from which the model was then printed using rapid  
120 prototype development techniques. We constructed the model using a photopolymer resin (PolyJet  
121 FullCure 720, Objet Geometries Ltd., Sint-Stevens-Woluwe, Belgium), a transparent, stiff material  
122 widely used in rapid product development techniques on an Objet Eden 330 3D Printer (Objet  
123 Geometries, Rehovot, Israel). Printing layer thickness was 0.016 mm. Anatomical structures included  
124 were all air conducting parts from the nostrils to 5 mm below the glottis.

125 In addition, a breathing simulator (Harvard Apparatus Respirator Model 665, Harvard Apparatus,  
126 USA) was connected through the distal orifice of the model. The breathing simulator generated a  
127 standard waveform at a pre-set frequency ( $f=20$  breaths/min) and tidal volume ( $V_t=80$  ml),  
128 representing breathing patterns appropriate for the model size. A flow meter (TSI Model 4043, TSI  
129 Inc., USA) was connected to the breathing simulator and to the filter/valve set to ensure the flow  
130 characteristics through the model (Figure 1C).

131

132 **Lung delivery of phages.** The face and airways model was connected to the Pari LC Sprint nebulizer  
133 (Pari GmbH, Starnberg, Germany) by a tightly connected face mask (Pari GmbH, Starnberg,  
134 Germany) at the output of the nebulizer (Figure 1C). In each run, 3 ml of phage lysate were pipetted  
135 into the nebulizer reservoir, and the compressor was operated for 15 minutes. For evaluation of phage  
136 viability at the tracheal level, the PARI filter/valve set (Pari GmbH, Starnberg, Germany) was now  
137 placed at the distal portion of the airways model to collect aerosol particles reaching this site (Figure  
138 1C).

139

140 **Phage physical characteristics determination.** Transmission electron microscopy (TEM) was used  
141 for measurement of phage particles size. Phage samples were prepared as previously described [16].  
142 Briefly, 1 ml of phage lysate containing at least  $10^8$  PFU/ml was centrifuged at  
143 12,225 g (WiseSpin® CF10, Daihan Scientific) for 1.5 h at room temperature. The supernatant was  
144 discarded, and the pellet was resuspended in 200  $\mu$ l of 5 mM MgSO<sub>4</sub> and left for 24 hours at 4°C. For  
145 grid preparation, 10  $\mu$ l of the phage mixtures were added to 30  $\mu$ l of 5 mM MgSO<sub>4</sub>, and the grids  
146 were placed on the drops with the carbon side facing down. After a minute, the grid was placed on a  
147 30  $\mu$ l drop of NanoVan (Nanoprobes, NY, USA), followed by an additional incubation for 5-10 sec. A  
148 transmission electron microscope (Jeol, TEM 1400 plus) with a charge-coupled device camera (Gatan  
149 Orius 600) was used to capture images. Phage particle's size was determined as an average of  
150 measurements capturing 4-14 (median 8.5) virions for each phage, using ImageJ 1.53h software  
151 (<http://imagej.nih.gov/ij/>).

152

153 **Statistical analysis.** Phage titers were compared by an unpaired *t-test*. A significance level of 0.05  
154 was used to determine the statistical difference of viable phage loads due to nebulization or lung  
155 delivery. Pearson's correlation test was used for correlation plots. GraphPad prism 8.02 Build 263  
156 (GraphPad Software, Inc, USA) was used for statistical tests. Adobe Illustrator (CC release 21.0.2)  
157 was used for graph drawing.

158

## 159 **Results**

### 160 **The effect of inhalation on viable phage titers.**

161 We evaluated the delivery of eight different phages to the lungs using an anatomically accurate face  
162 and airways model with corresponding simulation of breathing patterns. The tested phages were  
163 selected due to their ability to target pathogens isolated from CF patients (Table 1). An additional  
164 phage targeting *S. epidermidis* was tested due to a remarkable difference in morphology.

165 The phages were nebulized and administered to the model. After phage titer correction according to  
166 the *Fr* (Table 2), we found a diverse range of titer drops during phage inhalation to the lungs between  
167 the various phages (Figure 2). This reduction ranged from 2.8- log for the phage Maco7 to only 0.2-  
168 log reduction with the phage SeAlphi. Overall, seven of the eight tested phages presented a significant  
169 titer drop at the tracheal level, simulating their deposition in the lower respiratory tract (Figure 2).

170

### 171 **Determination of phage titer drop during the process of nebulization.**

172 In order to better understand the factors associated with phage titer reduction during inhalation, we  
173 evaluated phage viability following the nebulization process alone. Viable aerosolized phages were  
174 now captured at the output of the nebulizer, representing their titer at the oropharyngeal level. We  
175 found that a significant reduction in viable phage loads was also observed following nebulization for  
176 all phages presenting decreased titers at the tracheal level (Figure 2). The nebulization process also  
177 led to a diverse range of phage titer reductions, between 2.45- log for the phage BCSR129 to only  
178 0.15- log for the phage SeAlphi. Furthermore, in all phages presenting a significant titer drop at the

179 tracheal level, no significant difference was found between their loads at the trachea and at the output  
180 of the nebulizer. This demonstrates that this reduction is highly due to the nebulization process itself.

### 181 **Effect of phage characteristics on titer drop during inhalation.**

182 Next, we turned to test for intrinsic phage characteristics which may affect their delivery. That way, if  
183 a correlation between specific phage characteristics and their ability to pass the model is found, it will  
184 allow to better predict the desired phages to administer and their required dosage. The tested traits  
185 included the size of the capsids, tail length, total phage length and genome size. However, no  
186 correlation was found between these tested characteristics and the phage deposition at the tracheal  
187 level (Figure 3).

188

## 189 **Discussion**

190 In this study we have simulated phage inhalation using a dedicated real size 3D reconstruction model  
191 of human airways, with an adjusted breathing simulator. To the best of our knowledge, this is a first  
192 of a kind simulation allowing to directly measure phage delivery to the lungs in realistic human  
193 aerodynamic characteristics. Using this model, we found that there is a high variation in the levels of  
194 phage titer reduction following inhalation among different phages. Moreover, this reduction was  
195 mostly attributed to the nebulization process itself and was significant in seven of eight tested phages.

196

197 Previous reports have evaluated the ability of phages to be aerosolized, usually by direct  
198 measurements of their titers at the output of the nebulization device. Sahota *et al.* described the  
199 recovery of specific anti-Pseudomonal phages following nebulization [11]. In their study, the total  
200 number of viable phages leaving a jet nebulizer ranged between 15% for one phage to 2% for another  
201 respectively to their original amount. Carrigy *et al.* have shown that the same anti-Tuberculosis phage  
202 presented highly diverse levels of titer reductions, ranging between 0.4- and 3.7- logs, when nebulized  
203 by different devices such as a vibrating mesh nebulizer, a jet nebulizer and a soft mist inhaler [12]. In  
204 another study, Astudillo *et al.* have supplied visual proof for the structural changes of phages upon



205 nebulization by different methodologies [18]. Using TEM, they visualized that nebulization of a  
206 specific anti-Pseudomonal phage by jet, vibrating-mesh or static-mesh nebulizers resulted in high  
207 levels of phage tail separation. As in the current study, these reports emphasize that phage titer may  
208 drop during nebulization, and that the most appropriate phages chosen for inhalation, as well as the  
209 nebulization methodology itself may vary.

210

211 Leung *et al.* have suggested that nebulization-induced titer loss was particularly correlated with the  
212 tail length of the phage [19]. In their study, viable phage titers dropped by 0.04- to 2- logs following  
213 jet nebulization, in correlation with phage morphology. However, in our study, using different phages  
214 with a wide range of different characteristics, including short-tailed Podoviridae as well as  
215 Myoviridae and Siphoviridae, no correlation to tail length was observed. Furthermore, we have also  
216 found no correlation between phage inhalation capabilities to phage size, genome size, taxonomy or to  
217 the bacterial host. Thus, we conclude that routine genomic and morphological characterization of the  
218 phages may not be sufficient to predict their nebulization and inhalation abilities. Accordingly,  
219 specific *in-vitro* evaluations should be performed to address phage delivery prior to inhalation  
220 treatment. This point was recently demonstrated by Guillon *et al.*, who measured phage delivery by  
221 nebulization *in-vitro*, to select the most appropriate inhalation interface and phage dosage before  
222 administering aerosolized phages to pigs suffering from pneumonia [20].

223 The current study has several limitations. Firstly, although the presented model supplies accurate  
224 anatomical characteristics, it does not include the complete physiological conditions found in the  
225 respiratory tract. These include the complex tissue environment, the mucocilliary structures and the  
226 immune system effects. Thus, an additional reduction in phage titers may be expected in the clinical  
227 setting. Accordingly, the evaluation of these parameters on phage inhalation requires additional  
228 studies using animal models, which will further expand the knowledge in the field [20, 21]. Secondly,  
229 even though many phages from diverse families were included in the study, a wider evaluation could  
230 potentially shed more light on specific characteristics associated with titer decrease during inhalation.

231 Finally, we have used only one nebulizer type, while other nebulizers may behave differently [12].  
232 However, this only reinforces our main conclusion, that prior testing of the phage and the inhalation  
233 device in a tailored model are important for proper treatment design.

234 In conclusion, here we have shown that as in many other cases, phages may act in a non-predictive  
235 way in terms of lung deposition following inhalation. Thus, analogously to the concept of phage  
236 matching which is based on accurate laboratory testing of phages against the bacterial target [10], *in-*  
237 *vitro* phage delivery assays should also be performed in a personalized manner, potentially by patient-  
238 specific airways models in the future. In this regard, proper phage inhalation requires not only the  
239 selection of the most suitable phage, but also the most appropriate inhalation device by empirical *in-*  
240 *vitro* assessments.

241

## 242 **Acknowledgements**

243 The authors thank Yossi Aldar for technical assistance, and Ori Inbar from the Cystic Fibrosis  
244 foundation in Israel for insightful ideas.

245

## 246 **Financial Support:**

247 A) The “United States - Israel Binational Science Foundation (BSF)” grant #2017123.

248 B) The Israel Science Foundation (ISF) IPMP grant #ISF\_ 1349/20.

249 C) The Rosetrees Trust grant A2232.

250 D) The Milgrom Family Support Program.

251

252 **Conflict of interests:** The authors declare no conflict of interest

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335

336 **Tables**

337

338 **Table 1. Characteristics of the phages used in the study.**

339

<b>Phage</b>	<b>Host</b>	<b>Taxonomy</b>	<b>Genome size (bp)</b>	<b>Genome Structure</b>	<b>GenBank accession</b>
<b>BCSR5</b>	<i>B. cepacia</i> BCC378	Myoviridae	227,351	Circular	MW460245.1
<b>SAOMS1</b>	<i>S. aureus</i> SAR1	Herelleviridae	140,135	Circular	MW460250.1
<b>BCSR129</b>	<i>B. cepacia</i> BCC197	Myoviridae	66,147	Circular	MW460247.1
<b>PASA16</b>	<i>P. aeruginosa</i> PA14	Myoviridae	66,127	Circular	MT933737.1c
<b>Itty13</b>	<i>P. aeruginosa</i> PARI	Podoviridae	61,818	Linear	MW460249.1
<b>Maco2</b>	<i>M. abscessus</i> c107	Siphoviridae	48,239	Circular	MW460248.1
<b>Maco7</b>	<i>M. abscessus</i> c107	Siphoviridae	47,836	Linear	MZ152914
<b>SeAlphi</b>	<i>S. epidermidis</i> SE52	Podoviridae	18,368	Circular	MZ152915

340

341 **Table 2. Filter reduction fraction (Fr) of each phage**

<b>Phage</b>	<b>Fr<sup>342</sup></b>
<b>BCSR5</b>	0.178 <sup>343</sup>
<b>SAOMS1</b>	0.222 <sup>344</sup>
<b>BCSR129</b>	0.132 <sup>345</sup>
<b>PASA16</b>	0.085
<b>Itty13</b>	0.053 <sup>347</sup> 348
<b>Maco2</b>	0.036 <sup>349</sup>
<b>Maco7</b>	0.015 <sup>350</sup>
<b>SeAlphi</b>	0.58 <sup>351</sup>

346

352

353 **Figure legends**

354

355 **Figure 1. Model for evaluation of phage nebulization and delivery.** (A) Demonstration and  
356 schematic representation of phage nebulization. Filter pads were placed at the output of the nebulizer  
357 to allow phage capture after each run of nebulization. (B) Anterior and lateral views of the 3D face  
358 and respiratory airways model. (C) Demonstration and schematic representation of phage inhalation.  
359 Phages were nebulized and delivered by a face mask to the face and respiratory tract model,  
360 connected to a breathing simulator. Phages were captured by filter pads placed at the distal orifice of  
361 the airways model.

362

363 **Figure 2. Titer reduction of phages following nebulization and inhalation to the airways model.**

364 For each phage, the initial phage titer inserted to the nebulizer, corrected according to the filter  
365 reduction fraction (Corrected Input), is compared to the phage titers retrieved from filters placed at the  
366 output of the nebulizer (Nebulization) and at the tracheal level of the respiratory tract model  
367 (Inhalation). Three separate runs were made for each phage in every condition. Asterisks represent  
368 significant difference ( $p < 0.05$ ).

369

370

371 **Figure 3. Phage characterization and its effect on inhalation fitness.** (A-D) Correlation plots  
372 representing phage inhalation efficacy, defined as the proportion between viable phages reaching the  
373 tracheal level and the corrected initial phage loads, according to (A) tail length, (B) capsid diameter,  
374 (C) total phage length and (D) phage genome size. (E) TEM images of the phages used in the study.  
375 All images are scaled according to the bar representing 200 nm.

376

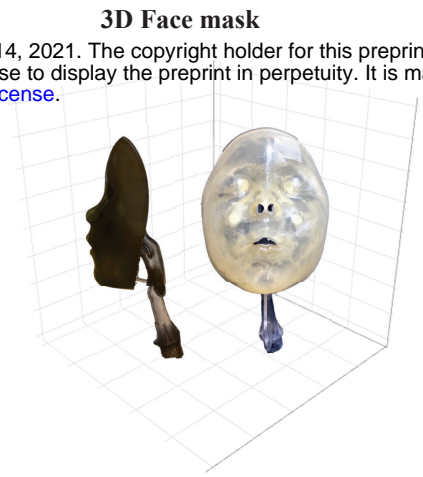


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**A**



**B**



**C**

