Inhaled bacteriophage therapy in a porcine model of pneumonia caused by Pseudomonas aeruginosa during mechanical ventilation

Antoine Guillon, Jeoffrey Pardessus, Guillaume L'hostis, Cindy Fevre, Céline Barc, Emilie Dalloneau, Youenn Jouan, Elsa Bodier-Montagutelli, Yonatan Perez, Camille Thorey, et al.

Title:

Inhaled bacteriophage therapy in a porcine model of pneumonia caused by *Pseudomonas aeruginosa* during mechanical ventilation.

Short running title:

Inhaled bacteriophage therapy

Authors:

Antoine Guillon[1,2,3] *, Jeoffrey Pardessus[1,2]*, Guillaume L'Hostis[4], Cindy Fevre[4], Celine Barc[5], Emilie Dalloneau[1,2], Youenn Jouan[1,2,3], Elsa Bodier-Montagutelli[1,2], Yonatan Perez[1,2,3], Camille Thorey[1,2], Laurent Mereghetti[2,6,7], Maria Cabrera[1,2], Mickaël Riou[5], Laurent Vecellio[1,2], Sandrine Le Guellec[1,2,8], and Nathalie Heuzé-Vourc'h[1,2#]

Affiliations:

INSERM, Centre d'Etude des Pathologies Respiratoires, U1100, Tours, France

Université de Tours, Tours, France

CHRU de Tours, Service de médecine intensive réanimation, Tours, France

Pherecydes Pharma, Research and Development, Romainville, France.

INRAE, UE-1277 Plateforme d'infectiologie expérimentale (PFIE), Centre Val de Loire, F-37380 Nouzilly, France.

INRAE, UMR1282 Infectiologie et Santé Publique, Centre Val de Loire, F-37380 Nouzilly, France.

CHRU de Tours, Service de Bactériologie-Virologie, Tours, France

DTF-Aerodrug, Faculté de Médecine, Bâtiment M, 10 ter bd Tonnellé, F-37032, Tours, France

 # Corresponding author:

INSERM, Centre d'Etude des Pathologies Respiratoires, U1100, Université de Tours, 10 Boulevard Tonnellé, F-37032 Tours, France. E-mail address: nathalie.vourch@med.univ-tours.fr (N. Heuzé-Vourc'h).

*Contributed equally to this work.

Email:

Antoine Guillon <antoine.guillon@univ-tours.fr>

Jeoffrey Pardessus <jeoffrey.pardessus@univ-tours.fr>

Guillaume L'Hostis <guillaume.lhostis@pherecydes-pharma.com>

Cindy Fevre <cindy.fevre@pherecydes-pharma.com>

Celine Barc <celine.barc@inrae.fr>

Emilie Dalloneau <emilie.dalloneau@univ-tours.fr>

Youenn Jouan <youenn.jouan@univ-tours.fr>

Elsa Bodier-Montagutelli <elsa.bodier@univ-tours.fr>

Yonatan Perez <yonatperez@gmail.com>

Camille Thorey <camillethorey@gmail.com>

Laurent Mereghetti <mereghetti@univ-tours.fr>

Maria Cabrera <maria.cabrera@univ-tours.fr>

Mickaël Riou <mickael.riou@inrae.fr>

Laurent Vecellio <vecellio@med.univ-tours.fr>

Sandrine Le Guellec <sandrine.leguellec@med.univ-tours.fr>

Nathalie Heuzé-Vourc'h <nathalie.vourch@univ-tours.fr>

Funding: This project was supported by a public grant overseen by the Direction Générale des Armées (DGA)- Project RAPID – Pneumophage grant.

Conflict of Interest disclosures: AG, JP, GLH, CF, ED, YJ, YP, CT, MC, MR, LV, SLG, EBM, NHV report grant from DGA (Direction Générale des Armées). CF and GLH are employed by Pherecydes Pharma. LV was employed by Nemera Company. NHV is co-founder and scientific expert for Cynbiose Respiratory. ED is presently employed by Cynbiose Respiratory. SLG is employed by DTFmedical and is the coordinator of Aerodrug, the Aerosol R&D department of DTFmedical, located in Tours.

Declaration of transparency and scientific rigour: This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Contributions: AG, JP, GLH, CF, LV, SLG, NHV designed the experiments. AG, JP, CB, ED, YJ, YP, CT, MC, EBM, MR, SLG, NHV performed the experiences. AG, JP, GLH, CF, CB, ED, YJ, YP, CT, LM, MC, MR, LV, SLG, EBM; NHV analyzed the results. AG, JP, NHV wrote the manuscript. AG, JP, GLH, CF, CB, ED, YJ, YP, CT, LM, MC, MR, LV, SLG, EBM, NHV read and approved the final manuscript. AG and NHV take responsibility for the integrity of the work as a whole.

Acknowledgements: Many thanks to Stéphane Abrioux and Edouard Guitton, directors of the Experimental Infectiology Platform (UE-1277 PFIE, INRAE Centre Val de Loire, Nouzilly, France, https://doi.org/10.15454/1.5535888072272498e12), to the zootechnical staff of the "Zone Confinée" group, and particularly to Olivier Boulesteix (Leader of the "Zone Confinée" group), Jérémy Pezant,

Anne Pinard, Alexis Pléau, Noémie Perrot and Alain Deslis (zootechnicians) for expert and dedicated help with the pig experiments and to Nathalie Kasal-Hoc (DMV of PFIE) for the authorizations of the deliverable of veterinary drugs. We thank Déborah Le Pennec, Thomas Baranek, and Charlotte Petitjean for their technical assistance. We sincerely thank Jérôme Gabard, Laure Saujet, and Emmanuelle Guillot-Combe for their helpful exchanges. We also thank Dr Gianluigi Li Bassi (Division of Animal Experimentation, Department of Pulmonary and Critical Care Medicine, Thorax Institute, Hospital Clinic, Barcelona, Spai) for the scientific exchanges on VAP models in piglets. The manuscript has been professionally corrected by a native English speaker.

Availability of data: The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Bullet point summary:

'What is already known'

- Lytic bacteriophages are natural bacterial-specific antimicrobials.
- *Pseudomonas aeruginosa* is a main cause of ventilation-associated pneumonia with drug-resistant bacteria.

'What this study adds'

- We used an infected and mechanically ventilated pig model with high translational value.
- We determined the optimal conditions to ensure efficient phage delivery by aerosol during mechanical ventilation.

'Clinical significance'

- We demonstrated that inhaled bacteriophages induced a 1.5-Log reduction of bacterial load in focal pneumonia.
- We provide a strong rationale for evaluating nebulized phage in ventilated patients with pneumonia.

Abstract /250w

Background and Purpose. *Pseudomonas aeruginosa* is a main cause of ventilator-associated pneumonia (VAP) with drug-resistant bacteria. Bacteriophage therapy has experienced resurgence to compensate for the limited development of novel antibiotics. However, phage therapy is limited to a compassionate use so far, resulting from lack of adequate studies in relevant pharmacological models. We used a pig model of pneumonia caused by *P. aeruginosa* that recapitulates essential features of human disease to study the antimicrobial efficacy of nebulized-phage therapy.

Experimental Approach. (*i*) Lysis kinetic assays were performed to evaluate *in vitro* phage antibacterial efficacy against *P. aeruginosa* and select relevant combinations of lytic phages. (*ii*) The efficacy of the phage combinations was investigated *in vivo* (murine model of *P. aeruginosa* lung infection). *(iii)* We determined the optimal conditions to ensure efficient phage delivery by aerosol during mechanical ventilation. (*iv*) Lung antimicrobial efficacy of inhaled-phage therapy was evaluated in pigs, which were anesthetized, mechanically ventilated and infected with *P. aeruginosa*.

Key Results. By selecting an active phage cocktail and optimizing aerosol delivery conditions, we were able to deliver high phage concentrations in the lungs, which resulted in a rapid and marked reduction in *P. aeruginosa* density (1.5 Log reduction, $p<0.001$). No infective phage was detected in the sera and urines throughout the experiment.

Conclusion and Implications. Our findings demonstrated: (*i*) the feasibility of delivering large amounts of active phages by nebulization during mechanical ventilation, (*ii*) rapid control of *in situ* infection by inhaled bacteriophage in an experimental model of *P. aeruginosa* pneumonia with high translational value.

Abbreviations

BAL: Broncho-Alveolar Lavage

CFU: Colony Forming Unit

EOP: Efficiency Of Plating

ETT: Endo Tracheal Tube

FiO2: Fraction of inspired oxygen

ICU: Intensive Care Unit

MMAD: Mass Median Aerodymanic Diameter

MOI: Multiplicity Of Infection

NaF: sodium fluoride

OD: Optical Density

PA: *Pseudomonas aeruginosa*

PEEP: Positive End-Expiratory Pressure

PFU: Plaque-Forming Unit

RR: Respiratory Rate

VAP: Ventilation-Associated Pneumonia

Vt: Tidal Volume

Introduction

The world mortality and economic burden of respiratory infections is huge and expected to increase due to antimicrobial resistance (Welte et al., 2012; Gibson et al., 2013). *Pseudomonas aeruginosa* (PA) is one of the most important drug-resistant bacteria for which there is an urgent need for new treatments, being listed as a critical priority in the *Priority Pathogen List* recently published by the World Health Organization (retrieved from: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed. PA is causing frequent opportunistic pneumonia in the community and hospital, either as an acute infection or associated to chronic respiratory infections. For instance, PA is the most frequent Gram-negative bacillus associated to nosocomial ventilation-associated pneumonia (VAP). VAP due to PA has a high attributable mortality (13%), which doubles in patients infected with bacteria multi-resistant to standard-of-care antibiotics (Luyt et al., 2018).

Lytic bacteriophages are natural bacterial-species-specific antimicrobials, for which there is increasing interest as an alternative/complementary approach to compensate for the limited development of novel antibiotics and a treatment for multidrug-resistant bacteria (Bodier-Montagutelli et al., 2017; Hesse and Adhya, 2019). Bacteriophages are viruses with a very narrow bacterial host range, self-propagating in the presence of their host, but unable to infect other bacteria species or eukaryotic cells, greatly limiting their toxicity and impact on natural microbiota (Aslam and Schooley, 2019; Kortright et al., 2019). During an acute infection, like VAP, bacteriophages may replicate to the site of infection until the disappearance of their host bacteria. Although resistance to bacteriophages may occur during the course of the treatment, it may be avoided by mixtures containing several phages. The route of administration may also influence the effectiveness of bacteriophage therapy in respiratory tract infections (Bodier-Montagutelli et al., 2017; Kortright et al., 2019). Indeed, in a murine model of acute *P. aeruginosa* lung infection, pulmonary delivery of bacteriophages (compared to systemic administration) improved bacterial clearance and survival rate (Bodier-Montagutelli et al., 2017).

A rising number of preclinical results and case reports on the effects of anti-PA phage therapy have been published, with remarkably positive outcomes (Debarbieux et al., 2010; Chang et al., 2018; Abd El-Aziz et al., 2019; Hesse and Adhya, 2019; Maddocks et al., 2019; McCallin et al., 2019). However, up to now, anti-PA phage therapy is limited to a compassionate use and mostly in combination with antibiotics. This may be partly explained by the lack of adequate studies in relevant experimental models to evaluate the real impact of phage therapy itself. Indeed, most animal models used for application of phage therapy are poorly reproducing the features of PA-infections in humans, so far.

Herein, we developed a model of focal pneumonia caused by PA in ventilated piglets, which have a lung anatomy and physiology close to that of humans and are relevant for aerosol administration

under mechanical ventilation (Guillon et al., 2018; Sécher et al., 2020a). Strikingly, our results show that the pig model recapitulates essential features of human pneumonia. Then, applying a translational approach, we: (i) selected a relevant combination of lytic phages and (ii) determined optimal delivery conditions by inhalation to ensure efficient delivery of inhaled phages in the pig model, (iii) and *in fine*, assessed the lung antimicrobial efficacy of inhaled-phage therapy in mechanically ventilated pigs with *P. aeruginosa* pneumonia.

Material and methods

Bacterial strain and phage mixture

The PAO1 *P. aeruginosa* strain was purchased from the BCCM/LMG12228 Bacteria Collection. It was used to produce all phages and to titer the PP1450 and PP1777 phages. The NAR71 strain is a clinical lung isolate from Pherecydes Pharma (Romainville, France, https://www.pherecydes-pharma.com/en/) own collection. It was used to titer the PP1792 and PP1797 phages.

The phage mixture against PA was provided by Pherecydes Pharma and contains a combination of 5 strictly lytic phages: 3 *Myoviridae* pseudomonal phages and 2 *Podoviridae* pseudomonal phages designated PP1450, PP1777, PP1902, PP1792 & PP1797, respectively. The 5 phages did not contain any toxin or antibiotic resistant genes. The phage titer was determined using a slight modification of the plaque spot assay, which detects only infective virus. Briefly, phage titration was performed by spotting and spreading 30µL of the phage dilution on the surface of 30mL LB-0.75%-agar containing 0.5mL of an overnight PA culture, of the PAO1 or NAR71 strain. Once dried, the Petri dishes were inverted and incubated 18 to 20h at 37°C. Areas exhibiting at least 30 plaques (or less in case of no dilution) were used to calculate phage titer. The phages were produced by the same process used for compassionate treatment. Briefly, the phages were produced by transducing 1L of an early phase or mid-exponential culture of the PAO1, depending on the phage. After 6h or overnight incubation, the cell debris were removed by depth filtration, then the phages were concentrated and purified to remove host cell impurities and process impurities using tangential flow filtration and to reach a concentration of 1 $x10^{10\pm1}$ PFU/mL. The endotoxin concentration, varying from 0.02 and 3.3 EU/mL for $1x10^{10}$ PFU/mL depending on the phage type and batch, is low enough to allow human i.v. administration without dilution according to the 5.1.10 Guidelines of the European Pharmacopoeia, which define a maximum of 5 EU/kg.

PAK-lux is derived from PAK, a clinical non-cystic fibrosis strain from the international panel, (De Soyza et al., 2013) and resistant to beta-lactams and tetracycline. To obtain PAK-Lux, PAK was transformed with a mini-Tn7T transposon that enables the constitutive expression of the LuxCDABE operon. The strain is highly susceptible to the phages with an *in vitro* efficiency of plating (EOP)>0.1. In a kinetic assay in liquid culture the phages can control the bacterial growth for up to 20h.

Bactericidal effect of phages

Lysis kinetic assay was performed to evaluate phage antibacterial efficacy regarding PAK-lux. Briefly, a fresh culture of PAK-Lux was incubated overnight in 15 mL of Miller's Luria-Bertani (LB) broth at 37 °C under shaking, then ten-fold diluted in Miller's LB and incubated until the optical density at 600 nm (OD_{600}) reached 0.6. The assay was performed with a final volume of 150µL of Miller's LB into a 96-well plate where $1.5x10^7$ Colony Forming Unit (CFU) PAK-Lux were dispensed, then $1.5x10^7$ and

$1.5x10^8$ Plaque Forming Unit (PFU) were added corresponding to a Multiplicity Of Infection (MOI) 1 and MOI10, respectively. For the kinetic assay with MIX A and MIX B the plates were seeded with $7.5x10^3$ CFU, then $7.5x10^3$, $7.5x10^4$ and $7.5x10^5$ PFU were added corresponding to MOI 1, MOI 10 and MOI 100. The plates were then covered and incubated into the plate reader (Multiskan GO, Thermo Scientific or Infinite® 200 Pro, Tecan) and continuously shaken during the assay. OD_{600} was recorded every 30 minutes or hour.

Animals and PA infection

Bioluminescent murine model of acute respiratory infection due to PA

Mice experiments were approved by the Ethics Committee of the Val de Loire and the Ministry of Higher Education and Research (Notification: APAFIS #2920-2015113011225044V3). Balb/cJRj male mice, 10-12 week-old (Janvier Labs, https://www.janvier-labs.com/en/) were anesthetized with 3% isoflurane and infected with a freshly prepared inoculum, as previously described (Sécher et al., 2019). The infectious dose was $1x10^7$ CFU PAK-Lux resuspended in 40 µL of PBS ($2.5x10^8$ CFU/mL) and the luminescent bacteria instilled orotracheally using a Harvard Apparatus optic fiber system (Sécher et al., 2019.) Each inoculum was checked for accuracy by direct plating on fresh agar plates. Two hours after bacterial infection, the bioluminescence was recorded to check proper lung signal and then, the bacteriophage treatment was given.

The course of the infection was followed by bioluminescence imaging (IVIS-Lumina XR, Perkin Elmer, France) 2 hours, 15 hours and 48 hours after the bacterial challenge, as previously described (Debarbieux et al., 2010). The animals' mortality and body weight were monitored daily. Along the experiment or at 48 hours post-infection, moribund animals, animals having lost >25% of body weight or surviving animals were euthanized by an overdose of injectable anesthetics.

Model of PA pneumonia in mechanically ventilated pigs

Large white piglets (17 females, 23.3 ± 0.4 kg, 8.6 ± 0.1 weeks old) were housed in BSL2 containment at the PFIE (Plateforme d'infection Expérimentale, UE-1277 PFIE, INRAE, Nouzilly, France (https://doi.org/10.15454/1.5535888072272498e12), under the accreditation number for animal experimentation D37-1753) and handled according to EU guidelines and French regulations. All experimental procedures were evaluated and approved by the Ethics Committee of the Val de Loire and the Ministry of Higher Education and Research (Notification: APAFIS#10687-2017072015069332764 3-V7).

Animal welfare was determined by assessing the following parameters: general condition, body temperature, heart rate, respiratory rate, mucous color, nasal discharge, and weight. The

experimental settings are summarized in supplemental Figure 1. Antibiotic prophylaxis with amoxicillin-clavulanic acid (NOROCLAV® P, injectable 1mL/20kg, BAYER Santé) was administered the day before the anesthesia, to prevent oropharyngeal co-infections, without affecting subsequent PA infection (PAK-Lux being resistant to this antibiotic). Piglets were anesthetized, subjected to buccal decontamination with povidone iodine solution (Betadine® green 10%, mouthwash), intubated (6 to 6.5 mm inner diameter, Rusch® Safety Clear®) and mechanically ventilated (Fabius® Tiro or Primus® Ventilator (Dräger, Telford), as previously described (Guillon et al., 2015). Main settings were: tidal volume (Vt) of 8 mL/kg, adapted respiratory rate and FiO_2 to maintain a $PaCO_2$ and PaO_2 of approximately 40 mmHg and 100 mmHg, respectively, and 5 cmH_2O positive end-expiratory pressure. The femoral venous and artery were cannulated for continuous analgesic/anesthetic infusion (midazolam, 0.3 to 1 mg/kg/h, Accord Healthcare and fentanyl (FENTADON®) 5 to 10 µg/kg/h, DECHRA Veterinary, France) and blood sampling. A venous line (G24 catheter) was placed and a solution containing glucose 5%, potassium chloride 0.2% and sodium chloride 0.4% (POLYIONIQUE G5 MACO-PHARMA) was infused (20 mL/h from H0 to H10 post-op, 10 mL/h from H10 to H24 and then 3 mL/h). The minimum flow rate was set to 3 mL/h to avoid catheter blockage. Noradrenaline (NORADRENALINE, Mylan) was used to control arterial pressure. Ventilator settings were evaluated on a regular basis and adjusted to maintain blood gases acceptable. Ventilated animals were infected in the right lower lung (see supplemental figure 1) by flexible bronchoscopic instillation of 15 mL inoculum containing $1.5x10^8$ CFU of exponential phase PA strain PAK-Lux. Each inoculum was checked for accuracy by direct plating on fresh agar plates. In final, the pigs were euthanized by an overdose of Dolethal® (Vetoquinol, 182.2 mg/kg).

Blood cells were counted with a MS9-5 Hematology Counter® (digital automatic haematological analyser, Melet Schloesing Laboratories) (Chevaleyre et al., 2016). Twenty-nine parameters were analyzed, which characterized three categories of blood cells: 1- total white blood cells (lymphocytes, monocytes; neutrophils; eosinophils; basophils and others white cells); 2- red blood cells and 3- platelets. All pigs were terminated if the mean blood pressure was below 50 mmHg and/or heart rates were below 50 beats per minutes for more than 2 hours despite corrective measures and at a distance from induction of anesthesia (6 hours).

To evaluate inhaled phage therapy, animals were euthanized 21 hours after bacterial challenge. After sacrifice, the lungs were excised and washed with 2x40 mL of PBS 1X. The resulting lavages were pooled and correspond to broncho-alveolar lavage (BAL). Lungs were dissected – a total of six pieces were taken from the right (1 piece at the site of infection and 3 pieces elsewhere in the right lower lung) and left (2 pieces) lower lungs (see supplemental figure 1) and homogenized for microbiological analysis. To avoid *ex vivo* lysis of PAK-Lux by phages, lung tissues and BAL were kept on ice and processed quickly. In some cases, 3 additional pieces were dissected, fixed and stained for

pathological examination (see supplemental figure 1). Infectious phage titers were measured by plaque assay (lower limit of detection 33 PFU/mL) in the respiratory samples (pulmonary tissues and BAL), blood, and urine. The count of PA has been measured after plating 25µl of samples on Petri dishes containing *Pseudomonas* isolation medium (Pseudomonas Isolation Agar, Sigma-Aldrich, France). The detection of other bacteria has been performed using 5% sheep blood agar plate (Columbia Agar with 5% Sheep Blood, bioMérieux, France). Once dried, Petri dishes were incubated for 24h at 37°C. PA was the main bacteria found in the lung, other pathogens included: *Escherichia coli* and *Enterococcus hirae*. To determine bacteremia, blood samples were collected in vacutainer vials filled with appropriate medium to detect aerobic and anaerobic bacteria (BD BACTEC™ Plus Aerobic/F , BD BACTEC™ Lyctic/10 Anaerobic F, Becton Dickinson, France) and according to the supplier's recommendations at 10h, 16h and 20h post-infection and then incubated up to 5 days.

Phage delivery

In anesthetized mice, 40 µL of phage mixtures containing 3 (MIX A - PP1450, PP1777 and PP1902) or 5 phages (MIX B - PP1450, PP1777, PP1902, PP1792 & PP1797) were instilled orotracheally, using a Harvard optical fiber Apparatus System, 2 hours after the bacterial challenge at a dose corresponding to a MOI of 10 or 100 (Sécher et al., 2019). Mixture corresponded to equal titers of $8.3x10^8$ or $8.3 x10^9$ PFU/mL of the 3 phages for MIX A and $5 x10^8$ or $5 x10^9$ PFU of the 5 phages for MIX B, for MOI 10 or MOI 100, respectively. Each inoculum was checked for accuracy by plaque spot assay.

In piglets, inhaled phage therapy was given 2 and 11 hours after bacterial challenge: animals (n=4) received 6 mL of a phage combination, consisting of equal titers of ~$1.1 x10^{10}$ PFU/mL of three *Myoviridae* (PP1450, PP1777 and PP1902), which are highly active against PAK-Lux. Inocula were checked by plaque spot assay. Phage treatment was delivered by inhalation through a static-mesh prototype nebulizer (Diffusion Technique Française – DTFmedical, https://www.dtf.fr/), France) connected to an inhalation chamber (CombiHaler®, OptimHal-ProtecSom®,France) on the inspiratory branch of the ventilation circuit. Duration of nebulization was 14.1 ± 1.9 minutes (mean±SEM). Animals were euthanized 21 hours after bacterial challenge.

Aerosol metrology and resistance of phages to aerosolization for phage delivery in VAP-piglets

An *in vitro* experimental set-up (supplemental figure 2) was performed to measure the phage aerosol characteristics delivered by a static-mesh prototype nebulizer (from DTFmedical), during simulated piglet mechanical ventilation. A passive lung model (Dual adult lung simulator TTL, Michigan-instruments, MI, US) with a compliance settled at 0.05 mL/mmH_2O, was mechanically ventilated by the Primus® Ventilator (Dräger Telford, UK) through an endotracheal tube (ETT) and a ventilator circuit (same references and models as used *in vivo*). Ventilator was settled to reproduce piglet

ventilation parameters (Vt = 200 mL, RR = 25 c/min, I:E = 1:2, PEEP = 5 cmH_2O, FGF = 3 L/min). An absolute filter (Anest-Guard®, Teleflex Medical) was connected between ETT and lung model (supplemental figure 2-A). Several previous ventilation sessions validated the respiratory circuit was air-tight and the passive lung model responsiveness was checked. Three measurements of the aerosol were conducted (n=3 for each): aerosol output delivery (or inhalable mass), particle size-mass distribution and phage viability, at the ETT outlet. Aerosols were produced and administered through the inspiratory branch during ventilation sessions by the static-mesh prototype nebulizer connected before the Y-piece.

For experiments A and B (supplemental figure 2), the nebulizer was loaded with 6 mL of 2.5 % sodium fluoride (NaF) (European standard norme, 2009b). Inhalable mass (aerosol output) was assessed to predict the phage quantity produced at the ETT outlet and able to deposit in piglet lungs, by collecting the nebulized NaF in the absolute filter during ventilation session. In order to optimize phage administration *in vivo* to the piglet lung, the use of the inhalation chamber CombiHaler® as an interface between the nebulizer and the inspiratory branch was tested for inhalable mass, in comparison to a standard T-piece. For both experiments (n=3 for each interface), nebulization was started after the establishment of ventilation in the respiratory circuit and experiment stopped at the end of aerosol production. NaF collected in the filter was then rinsed with 20 mL of 2% Total Ionic Strength Adjustement Buffer IV (TISAB IV). Concentration of fluoride ions was measured using a daily calibrated ionometer (SevenGo pro™ - SG8, Metler Toledo).

Mass median aerodymanic diameter (MMAD) and respirable fractions were determined using a cascade impactor (NGI, Copley Scientific, UK) connected to ETT instead of the filter (supplemental figure 2-B). Herein, a derivation set-up was established to connect the cascade impactor with a vacuum pump (15 L/min) and ensure in the same time correct passive lung ventilation. NaF aerosol was collected in the cascade impactor (previously cooled at 5°C, according to European Pharmacopeia protocol) during 3 minutes of nebulization and ventilation. At the end of the experiment, NGI parts (trachea, plates and terminal filter) were imaged rinsed using 10 mL of 2 % TISAB IV for each part, then fluoride ion concentrations were measured as previously described by E-Cam gamma camera, during 2 minutes. Correction factors were applied on all activities counted in cascade impactor to assess the real activities deposited with respect to NGI cut-off diameters. Cumulative particle size distribution traced with the percent of fluoride activities deposited was assessed to determine the MMAD of the phage aerosol delivered at the ETT outlet. Fine particle fraction (FPF) < 5µm and < 2µm were then deducted to predict respectively the phage whole lung deposition and the phage alveolar deposition.

For phage viability assessment (experiment C, supplemental figure 2), nebulizers were loaded with 6 mL of the MIX A phage combination (equal titers of ~1.1 $x10^{10}$ PFU/mL of three *Myoviridae* (PP1450,

PP1777 and PP1902). Phage aerosols were collected with a BioSampler™ (SKC), an aerosol collector dedicated for nebulized biologics, suitable to gently collect droplets (D_{50}< 0.3µm) in a 150 mM KCl vortex (McDonagh and Noakes, 2014), with a collection efficiency was 89% ± 7% (at the outlet of the nebulizer). The mean duration of nebulization was 16.2±0.5 minutes. Viable/infectious phages were determined by spot plaque assay. Phage viability (which reflected their resistance to aerosolization) was defined as the number of viable phages retrieved in the aerosol divided by the theoretical phage count (taking into account the nebulization yield and the collection efficiency of the BioSampler™). Sodium (chloride), which was the diluent of phage suspensions, was used as a tracer to determine the amount of aerosol deposited in our collection system. Briefly, sodium concentrations of aerosols collected with the BioSampler™ were measured using an iCAP™ Inductively-Coupled Plasma – Optical Emission Spectrometer (ICP-OES) (ThermoFisher Scientific, Waltham, MA) with an axial wavelength of detection of 818.3 nm. Phage/sodium concentration ratios of the suspensions loaded in the nebulizers were then used to calculate the theoretical amounts of phages expected in the BioSampler™.

Statistical analysis

The results are expressed as the mean ± SEM. The different statistical tests are mentioned in the legend of the figures and significance was considered for p≤0.01 (**) or p≤0.001 (***).

Results

Bacteriophage preparation and selection

Bacteriophages have variable host range going up to 70% or may be specific of few strains within the same species (Loc-Carrillo and Abedon, 2011; Kortright et al., 2019). Accordingly, a broad-species-range phage mixture containing 5 phages (PP1450, PP1777, PP1902, PP1792 and PP1797) was developed by Pherecydes Pharma against an international representative panel of PA.(De Soyza et al., 2013) The five phages of the combination were tested separately on PAK-Lux to characterize host susceptibility and phage time-to-lysis. Among them, 3 (PP1450, PP1777 and PP1902) out 5 bacteriophages showed a rapid bactericidal effect on PAK-Lux *in vitro* in the lysis kinetic assay (figure 1A). PP1792 and PP1797 showed a limited activity on PAK-Lux with a longer time-to-lysis. These results are coherent with the EOP of 0.5, 1.5 and 0.7 obtained for PP1450, PP1777 and PP1902, respectively, while no plaque was seen with PP1792 and PP1797. The activity of phage combinations – either the whole cocktail (MIX B) or a mixture containing only phages forming plaques on PAK-Lux (PP1450, PP1777 and PP1902 (MIX A) – were tested in the kinetic assay at MOI 1, 10 and 100. Whatever the mix and the dose, all showed a complete inhibition of the growth of PAK-Lux. To

explore further bacteria-phage dynamics, we investigated the efficacy of these phage combinations, *in vivo*, in a murine model of acute respiratory PA infection (Figures 1 B and 1C).(Debarbieux et al., 2010) The phages were delivered oro-tracheally for a reliable delivery and high deposition in the lungs of small animals (Guillon et al., 2018; Ehrmann et al., 2020). As shown on the Kaplan-Meier curves (figure 1C), a single dose of MIX A (MOI 10 or 100) delivered two hours after the bacterial challenge, rescued 83% to 100% of the infected mice, whereas the whole combination (MIX B) was nearly ineffective. At the end of the experiment, bacterial loads were similar in the lung compartment for the two groups that received MIX A (MOI 10 or 100). MIX A -containing PP1450, PP1777 and PP1902- was thus considered for further investigations.

Establishment of *P. aeruginosa* pneumonia model in mechanically ventilated piglets

Animal models help bridge the gap between *in vitro* and clinical studies. With a lung anatomy and physiology close to that of humans, pigs are a relevant model for aerosol administration under mechanical ventilation (Guillon et al., 2018; Sécher et al., 2020a). Thanks to the remarkable work of Torres-Bassi's group on animal models of VAP (Luna et al., 2007, 2009; Sibila et al., 2007; Li Bassi et al., 2014), we established a model of PAK-Lux pneumonia in mechanically ventilated piglets (Supplemental Figure 1A). As shown in Figure 2, ventilated pigs were challenged with PAK-Lux and ultimately developed a fatal severe experimental bronchopneumonia with clinical symptoms: fever, progressive deterioration of gas exchange and positive microbial cultures (Figures 2A-B). The mean survival of piglets was 22.7 hours, and most animals survived longer than 20 hours along the experimental practice. The infection was associated with a change in white blood cell count, with a peak of leukocytes, mainly attributable to lymphocytes and neutrophils, between 7 and 14 hours after bacterial challenge (Figure 2C). PA density was $1.4x10^5$ $\pm 2.4x10^4$ CFU/g at the inoculation site and $8.3x10^7$ $\pm$ $7.8x10^7$ CFU/mL in the BAL (Figure 2D). Although the instillation was selective (right lobe), the infection was disseminated to the right and left lungs (at least 10^3 CFU/g in each lobe, see Supplementary Figure 3). Thanks to systemic antibiotic pre-treatment and buccal decontamination prior to intubation, only few other micro-organisms were detected in the right and left lung tissues after animal sacrifice, making secondary infections due to other bacteria unlikely. No PA or other bacteria were detected in the blood (Figure 2D). PA infection resulted in focalized pneumonia (limited to the right lungs) with marked lung inflammation, characterized by a massive polymorphonuclear leukocyte infiltration (most likely neutrophils) within the bronchiolar and alveolar lumen, leading to non-aerated peripheral lung-tissue segments at the site of bacterial inoculation (Figure 2E). There were only few erythrocytes in the same areas. Overall, the resulting pneumonia caused by PA in intubated and ventilated piglets closely reproduced the features of focal pneumonia in humans.

Determination of optimal dose and set-up for inhaled phage therapy in mechanically ventilated piglets

Inhalation during mechanical ventilation exposes to specific challenges for optimal and reliable drug delivery (Ari and Fink, 2016; Dhand, 2017; Edge and Butcher, 2019). Thus, it is important to determine the inhalation conditions to ensure efficient phage delivery in the PA-associated VAP model in pigs.

In the clinic, aerosols are often produced by nebulizers, which are appropriate for inhaled phage delivery (Bodier-Montagutelli et al., 2017). Herein, we used a mesh nebulizer, a type of device commonly used to deliver aerosolized drugs in patients under mechanical ventilation. The static-mesh prototype nebulizer had a low residual volume (<0.05 mL) and was placed in the ventilator circuit, on the inspiratory branch, proximal to the ventilator, as suggested to maximize drug delivery (supplemental figure 2) (Dugernier et al., 2017). To optimize further aerosol delivery, we evaluated two interfaces – a T-piece and an inhalation chamber (CombiHaler®) - to connect the mesh-nebulizer to the circuit. Using pig ventilator parameters, we found that connecting the mesh-nebulizer to circuit with an inhalation chamber resulted in a slightly higher output rate (75.2 ± 3.3%) at the end of the endotracheal tube (Figure 3A). Cascade impactor characterization of this aerosol revealed a MMAD of 1.13 ± 0.03 µm, which is compatible with a high delivery at the pneumonic site ($FPF_{2\mu m}$ = 91.7 ± 1.2 %), in the deep lung (Figure 3B).

To take into account a reduction of phage titer due to their instability during nebulization(Bodier-Montagutelli et al., 2017; Leung et al., 2019), we investigated the viability of phages contained in MIX A after nebulization in the experimental conditions (see supplemental Figure 2, set-up C). As shown in Figure 3C, the proportion of infectious phages (as measured by plaque spot assay) dropped to 5.3 ±1.9 % for MIX A after nebulization. This is equivalent to 1.3 Log-reduction in infective phage concentration upon nebulization.

Considering the bacterial inoculum ($1.5x10^8$ CFU) instilled in piglets, the aerosol output rate and $FPF_{2\mu m}$ fraction, and the phage viability obtained using the settings described above, we estimated that loading app. $2x10^{11}$ PFU (corresponding to 6 mL of equal titer of the 3 phages at ~$1.1 x10^{10}$ PFU/mL) of phage MIX A in the nebulizer reservoir was necessary to obtain a MOI comprised between 10 and 100 at the site of infection in piglets, which should be effective based on mouse results (Figure 1C).

Inhaled phage therapy in the PA-associated pneumonia model in ventilated pigs.

Phage therapy was given two hours and eleven hours after bacterial challenge by inhalation at the dose and following the procedure defined above (Supplemental Figure 1B). In accordance with the mean survival of infected animals (Figure 2A), the animals were euthanized 21 hours post-infection

(Supplemental Figure 1B). Both clinical parameters and gas exchanges were similar in the control and treated groups during the study period (Figure 4A). Treated animals displayed a different blood leukocyte pattern than control animals, with a marked and constant increase of lymphocytes over time (Figure 4B). Remarkably, phage-treated animals had a 1.5-Log reduction ($p<0.001$) of bacterial load in the pneumonic foci along with a marked bacterial reduction in BAL compared to untreated piglets (Figure 5A), suggesting that inhaled phage therapy was able to contain PA pneumonia. It is noteworthy that a decrease of the bacterial load was observed in each section of the right lower lung (supplemental figure 3) and, although it should be taken with caution considering the group sizes, co-infection events seemed less frequent in the treated group. Lung histology did not reveal major differences between groups: there was a marked inflammation in the right lower lungs characterized by infiltration of neutrophils within the bronchiolar and alveolar lumen, alveolar and interstitial edema, increased alveolar macrophages, increased cellularity within alveolar septa and vascular/perivascular inflammation. Focal and multifocal hemorrhage was observed in some samples. At the time of sacrifice, phages were relatively homogeneously distributed in the right and left lower lungs (supplemental Figure 3). Phage densities reached $1.8x10^9 \pm 9.5x10^8$ PFU/mL in the broncho-alveolar lavage and $4.1x10^7 \pm 1.2x10^7$ PFU/g in the consolidated pneumonia (Figure 5B). These amounts were higher than the expected calculated density (5.8×10^6 PFU/g of lung tissue) based on the dose loaded twice in the nebulizer, predicted phage viability and aerosolization yield (1.58×10^9 PFU viable phages entering the whole lungs) and lung weights (271.5 ± 9.9 g), assuming a homogeneous aerosol distribution in the lungs. No (infective) phage was detected in the sera and urines throughout the experiment.

Discussion

VAP is a serious complication during mechanical ventilation contributing to life-threatening lung dysfunction, prolonged ICU stay and increased hospitalization costs. Moreover, it is increasingly associated with multidrug-resistant bacteria, urging the necessity of new therapeutic strategies to overcome antibiotic limits. Over the past decade, bacteriophage therapy has experienced resurgence for the treatment of *Pseudomonas aeruginosa* infections in different clinical conditions, but evidences of efficacy in VAP are poor. Herein, we developed a clinically-relevant pig model of pneumonia caused by PA in mechanically-ventilated animals to explore bacteriophage therapy and demonstrated that inhaled bacteriophages induced a 1.5-Log reduction of bacterial load in the consolidated pneumonia.

Advanced animal models are critical to translate preclinical findings towards clinical trials. VAP is characterized by a pneumonia occurring within 4 days (early-onset) or over 4 days (late-onset) of intubation. In humans, common features of VAP that help diagnosis are signs of systemic infection associating fever and leukocytosis, infiltrates in the lungs (by chest radiography), purulent tracheobronchial aspirations with detection of pathogens and gas exchange degradation. Here, intubated and ventilated piglets infected with PA experienced a severe compartmented pneumonia without massive disruption of the alveolar-capillary barrier, with progressive deterioration of gas exchange, fever, systemic inflammation, mainly attributable to an increase in lymphocytes and neutrophils, without bacteremia. Contrary to most murine models of acute respiratory infection caused by PA(Sécher et al., 2019, 2020b), this model presents a high translational value, closely mimicking human pneumonia and allowing to evaluate aerosol therapy in clinical conditions. Models of VAP in piglets usually rely on whole-lung instillation of a specific pathogen, leading to a severe lung infection eventually aggravated by other infections resulting from prolonged mechanical ventilation, which even occurred in intubated and ventilated healthy piglets(Goldstein et al., 2002; Luna et al., 2007, 2009; Sibila et al., 2007)(Marquette et al., 1999). Although oro-pharyngeal co-infections may be anecdotal when evaluating large spectrum antibiotics, it was critical to prevent them considering the species-limited host range of bacteriophages. Applying prophylactic antibiotherapy and buccal decontamination prior to intubation, we were able to limit co-infections, thereby developing a severe respiratory infection mostly restricted to PA and sensitive to the phage mixture.

Overall, our model may help bridge the gap in translating preclinical findings of novel antimicrobials against PA into clinical applications since pig physiology and anatomy are similar to those of humans (Guillon et al., 2018, 2019; Sécher et al., 2020a). Besides, pigs offer the possibility to use human respiratory devices (*i.e.* ventilator, nebulizer) and settings used in the clinics for aerosol delivery.

Pseudomonas aeruginosa is frequently associated to VAP, which are increasingly difficult to eradicate due to fast increasing antimicrobial resistance (Riou et al., 2010). Although no drug product is already approved, bacteriophages have a tremendous opportunity to benefit to patients with PA-caused pneumonia. Pherecydes Pharma has selected anti-PA phages that show the highest host range complementarity and minimum number of phages combined with manufacturing advantages and stability over time when kept individually within a common formulation. When tested on the international PA panel by plaque spot assay and kinetic assay (De Soyza et al., 2013), 95% of the strains were susceptible to at least one of the selected phages (not shown). *In vitro*, PAK-Lux was more sensitive to 3 out of 5 bacteriophages. *In vivo*, our results showed that a mixture containing the 5 phages (MIX B) was markedly less active than a combination of the 3 active phages (MIX A). Although the concentration of active phages is slightly lower in MIX B than MIX A, it may not explain the differences observed *in vivo*. Interestingly, the *in vitro* kinetic assay showed similar efficacy of both mixtures, which suggests that interference between phages may not be the cause of the *in vivo* lack of activity of MIX B. Our findings emphasize the importance to further explore the *in vitro* predictability of antimicrobial efficacy in preclinical and clinical settings.

Nebulization of bacteriophages to treat pneumonia during mechanical ventilation is challenging. First, phages are highly sensitive to nebulization, in particular *Myoviridae* that can be structurally damaged during nebulization rendering them inactive.(Bodier-Montagutelli et al., 2017; Leung et al., 2019) Second, several parameters influence aerosol deposition in intubated and mechanically ventilated subjects (Ari and Fink, 2016; Dhand, 2017; Edge and Butcher, 2019). Finally, the deposition of nebulized drugs in poorly ventilated area is often questioned and may constitute a major limit for antimicrobials inhalation to treat VAP (Goldstein et al., 2002; Bodier-Montagutelli et al., 2017). Herein, we measured high phage titers, which correspond to bioactive virus (as plaque assay detects infective phages), throughout the lungs. Interestingly, high titers of bioactive virus were measured within the consolidated pneumonia, demonstrating the ability of nebulized bacteriophages to efficiently reach poorly aerated lung parenchyma. Moreover, we measured a higher phage titer in the respiratory tract after animal euthanasia than expected, suggesting phage replication *in situ*. Despite the definite sensitivity of phages to aerosolization under ventilation conditions, our findings support the feasibility to deliver large amounts of infectious phages by nebulization in the lungs and in pneumonia with loss of aeration. This may be attributable to the non-inert and self-replicating nature of phages that may migrate after intrapulmonary delivery, and/or the aerodynamical properties of the aerosol generated by the mesh-nebulizer connected to the ventilation circuit that may greatly deposit into the deep lung.

High phage concentrations in the lungs resulted in a rapid and marked reduction in PA density, indicating that inhaled phages were able to control the infection. We observed a slight increase of leukocytes, which may be mainly attributable to lymphocytes, in the systemic circulation after inhaled phage therapy and may sign for an anti-PA immune response. However, as a whole, the timescale for analysis (which is imposed by the complexity of the animal model) limits conclusion drawing on the ability of inhaled phage therapy to ultimately clear or cure PA infection. On the one hand, the short treatment time course may not be adequate to observe the benefits of these self-amplifying antimicrobials. On the other hand, the activity of phage monotherapy may not be sufficient to eradicate the infection. Even though a synergism between phages and antibiotic remains debated (Abedon, 2019; Segall et al., 2019), it would be worth testing inhaled phage therapy combined with standard-of-care antibiotic in this model.

No phage was detected in sera or urine throughout the experiment (plaque assay – LLOQ= 30 PFU/mL). Our findings suggest poor translocation of infective phages from the lungs into the bloodstream in intubated piglets, contrary to what was observed in acute PA infection models in mice. This is probably attributable to the maintenance of alveolar-capillary barrier integrity observed in the piglet model of pneumonia.

Our study has some limitations. First, PAK-Lux infection in intubated and mechanically-ventilated piglet model resulted in a rapid fatal pneumonia, which does not reproduce VAP kinetics in the clinic. Moreover, VAP is often multifocal. Although, we did not observe multiple foci after selective instillation (right lobe), the infection was disseminated in the whole lungs. Further efforts would be required to optimize the model, thereby making it more suitable to investigate the multi-facets of antimicrobial and host responses. Second, one hurdle of phage therapy is the debated ability of phages to induce immune responses that may add to the excessive and uncontrolled inflammation associated to pneumonia pathophysiology, thereby threatening lung stability and patient outcome (Loc-Carrillo and Abedon, 2011; Dufour et al., 2019). Although we did not search specifically for lung tissue remodeling, the uninfected lungs did not show any significant cellularity differences (neutrophils, alveolar macrophages), as examined by a pathologist, within the alveoli and bronchioles in the control and treated groups, suggesting that highly purified and low-endotoxin phage preparations were well-tolerated with no notable increase of local inflammation. Third, our results cannot drive conclusions on bacteria-phage dynamics in the respiratory tract. The amounts of phages detected into the lungs were compatible with the expected deposited dose, but cannot foresee *in situ* phage replication. It would be worth investigating further host-pathogen interactions, in particular evolution of bacteria resistance to phage, in this complex system (Loc-Carrillo and Abedon,

2011; Kortright et al., 2019). It is noteworthy that resistance to antimicrobials is inevitable; although it often appears quickly *in vitro* with single bacteriophages, it may not be predictive to what may happen *in vivo* with products containing several phages. Moreover, except for intestinal infection or colonization, phage resistance is often associated to loss of virulence (Oechslin, 2018). Finally, VAP is often associated to several concomitant pathogens (Kollef et al., 2017). In the studied model, PA was the prevailing bacteria in the lungs of piglets. It would be valuable to develop a co-infection piglet model and develop phage mixtures combining phages targeting the most common VAP pathogens.

Recently, Maddocks et al.(Maddocks et al., 2019) reported positive results of phage therapy, in the clinics, given concomitantly by i.v and locally, as adjunctive therapy to antibiotics (i.v) in the management of a patient with extensive ventilator-associated pseudomonal pneumonia. Our study has some important implications since it is the first one showing that local phage therapy, given as a monotherapy, led to the containment of PA respiratory infection under conditions closely mimicking clinical practice and demonstrated the feasibility to deliver large quantities of phages by inhalation during mechanical ventilation. Our findings provide a strong rationale for evaluating the efficiency of nebulized phage to treat PA pneumonia in ventilated patients. Beyond our results and to help transfer phage therapy to the clinic routine, it is critical to develop both phage mixtures designed to treat VAP with various bacteria and assays for rapid identification of bacterial susceptibility to lytic phages.

References

Abd El-Aziz, A.M., Elgaml, A., and Ali, Y.M. (2019). Bacteriophage Therapy Increases Complement-Mediated Lysis of Bacteria and Enhances Bacterial Clearance After Acute Lung Infection With Multidrug-Resistant Pseudomonas aeruginosa. J. Infect. Dis. *219*: 1439–1447.

Abedon, S.T. (2019). Phage-Antibiotic Combination Treatments: Antagonistic Impacts of Antibiotics on the Pharmacodynamics of Phage Therapy? Antibiotics (Basel) *8*:.

Ari, A., and Fink, J.B. (2016). Differential Medical Aerosol Device and Interface Selection in Patients during Spontaneous, Conventional Mechanical and Noninvasive Ventilation. J Aerosol Med Pulm Drug Deliv *29*: 95–106.

Aslam, S., and Schooley, R.T. (2019). What's Old Is New Again: Bacteriophage Therapy in the 21st Century. Antimicrob. Agents Chemother. *64*:.

Bodier-Montagutelli, E., Morello, E., L'Hostis, G., Guillon, A., Dalloneau, E., Respaud, R., et al. (2017). Inhaled phage therapy: a promising and challenging approach to treat bacterial respiratory infections. Expert Opin Drug Deliv *14*: 959–972.

Chang, R.Y.K., Chen, K., Wang, J., Wallin, M., Britton, W., Morales, S., et al. (2018). Proof-of-Principle Study in a Murine Lung Infection Model of Antipseudomonal Activity of Phage PEV20 in a Dry-Powder Formulation. Antimicrob. Agents Chemother. *62*:.

Chevaleyre, C., Riou, M., Bréa, D., Vandebrouck, C., Barc, C., Pezant, J., et al. (2016). The Pig: A Relevant Model for Evaluating the Neutrophil Serine Protease Activities during Acute Pseudomonas aeruginosa Lung Infection. PLoS One *11*: e0168577.

De Soyza, A., Hall, A.J., Mahenthiralingam, E., Drevinek, P., Kaca, W., Drulis-Kawa, Z., et al. (2013). Developing an international Pseudomonas aeruginosa reference panel. Microbiologyopen *2*: 1010–1023.

Debarbieux, L., Leduc, D., Maura, D., Morello, E., Criscuolo, A., Grossi, O., et al. (2010). Bacteriophages can treat and prevent Pseudomonas aeruginosa lung infections. J. Infect. Dis. *201*: 1096–1104.

Dhand, R. (2017). How Should Aerosols Be Delivered During Invasive Mechanical Ventilation? Respir Care *62*: 1343–1367.

Dufour, N., Delattre, R., Chevallereau, A., Ricard, J.-D., and Debarbieux, L. (2019). Phage Therapy of Pneumonia Is Not Associated with an Overstimulation of the Inflammatory Response Compared to Antibiotic Treatment in Mice. Antimicrob Agents Chemother *63*:.

Dugernier, J., Ehrmann, S., Sottiaux, T., Roeseler, J., Wittebole, X., Dugernier, T., et al. (2017). Aerosol delivery during invasive mechanical ventilation: a systematic review. Crit Care *21*: 264.

Edge, R., and Butcher, R. (2019). Vibrating Mesh Nebulizers for Patients with Respiratory Conditions: Clinical Effectiveness, Cost-Effectiveness, and Guidelines (Ottawa (ON): Canadian Agency for Drugs and Technologies in Health).

Ehrmann, S., Schmid, O., Darquenne, C., Rothen-Rutishauser, B., Sznitman, J., Yang, L., et al. (2020). Innovative preclinical models for pulmonary drug delivery research. Expert Opin Drug Deliv *17*: 463–478.

European standard norme (2009). European standard norme NF EN 13544-1+A1:2009: Respiratory therapy equipment - Part 1: Nebulizing systems and their components EN ISO 27427:2019: Anaesthetic and respiratory equipment - Nebulizing systems and components (ISO 27427:2013). p.

Gibson, G.J., Loddenkemper, R., Lundbäck, B., and Sibille, Y. (2013). Respiratory health and disease in Europe: the new European Lung White Book. Eur. Respir. J. *42*: 559–563.

Goldstein, I., Wallet, F., Nicolas-Robin, A., Ferrari, F., Marquette, C.-H., and Rouby, J.-J. (2002). Lung deposition and efficiency of nebulized amikacin during Escherichia coli pneumonia in ventilated piglets. Am. J. Respir. Crit. Care Med. *166*: 1375–1381.

Guillon, A., Mercier, E., Lanotte, P., Haguenoer, E., Darrouzain, F., Barc, C., et al. (2015). Aerosol Route to Administer Teicoplanin in Mechanical Ventilation: In Vitro Study, Lung Deposition and Pharmacokinetic Analyses in Pigs. J Aerosol Med Pulm Drug Deliv *28*: 290–298.

Guillon, A., Preau, S., Aboab, J., Azabou, E., Jung, B., Silva, S., et al. (2019). Preclinical septic shock research: why we need an animal ICU. Ann Intensive Care *9*: 66.

Guillon, A., Sécher, T., Dailey, L.A., Vecellio, L., Monte, M. de, Si-Tahar, M., et al. (2018). Insights on animal models to investigate inhalation therapy: Relevance for biotherapeutics. Int J Pharm *536*: 116–126.

Hesse, S., and Adhya, S. (2019). Phage Therapy in the Twenty-First Century: Facing the Decline of the Antibiotic Era; Is It Finally Time for the Age of the Phage? Annu. Rev. Microbiol. *73*: 155–174.

Kollef, M.H., Ricard, J.-D., Roux, D., Francois, B., Ischaki, E., Rozgonyi, Z., et al. (2017). A Randomized Trial of the Amikacin Fosfomycin Inhalation System for the Adjunctive Therapy of Gram-Negative Ventilator-Associated Pneumonia: IASIS Trial. Chest *151*: 1239–1246.

Kortright, K.E., Chan, B.K., Koff, J.L., and Turner, P.E. (2019). Phage Therapy: A Renewed Approach to Combat Antibiotic-Resistant Bacteria. Cell Host Microbe *25*: 219–232.

Leung, S.S.Y., Carrigy, N.B., Vehring, R., Finlay, W.H., Morales, S., Carter, E.A., et al. (2019). Jet nebulization of bacteriophages with different tail morphologies - Structural effects. Int J Pharm *554*: 322–326.

Li Bassi, G., Rigol, M., Marti, J.-D., Saucedo, L., Ranzani, O.T., Roca, I., et al. (2014). A novel porcine model of ventilator-associated pneumonia caused by oropharyngeal challenge with Pseudomonas aeruginosa. Anesthesiology *120*: 1205–1215.

Loc-Carrillo, C., and Abedon, S.T. (2011). Pros and cons of phage therapy. Bacteriophage *1*: 111–114.

Luna, C.M., Baquero, S., Gando, S., Patrón, J.R., Morato, J.G., Sibila, O., et al. (2007). Experimental severe Pseudomonas aeruginosa pneumonia and antibiotic therapy in piglets receiving mechanical ventilation. Chest *132*: 523–531.

Luna, C.M., Sibila, O., Agusti, C., and Torres, A. (2009). Animal models of ventilator-associated pneumonia. Eur. Respir. J. *33*: 182–188.

Luyt, C.-E., Hékimian, G., Koulenti, D., and Chastre, J. (2018). Microbial cause of ICU-acquired pneumonia: hospital-acquired pneumonia versus ventilator-associated pneumonia. Curr Opin Crit Care *24*: 332–338.

Maddocks, S., Petrovic Fabijan, A., Ho, J., Lin, R.C., Ben Zakour, N.L., Dugan, C., et al. (2019). Bacteriophage Therapy of Ventilator-associated Pneumonia and Empyema caused by Pseudomonas aeruginosa. Am. J. Respir. Crit. Care Med.

Marquette, C.H., Wermert, D., Wallet, F., Copin, M.C., and Tonnel, A.B. (1999). Characterization of an animal model of ventilator-acquired pneumonia. Chest *115*: 200–209.

McCallin, S., Sacher, J.C., Zheng, J., and Chan, B.K. (2019). Current State of Compassionate Phage Therapy. Viruses *11*:.

McDonagh, A., and Noakes, C.J. (2014). A comparison of the sampling efficiency of bioaerosol samplers and particle counters in natural and controlled environments.

Oechslin, F. (2018). Resistance Development to Bacteriophages Occurring during Bacteriophage Therapy. Viruses *10*:.

Riou, M., Carbonnelle, S., Avrain, L., Mesaros, N., Pirnay, J.-P., Bilocq, F., et al. (2010). In vivo development of antimicrobial resistance in Pseudomonas aeruginosa strains isolated from the lower respiratory tract of Intensive Care Unit patients with nosocomial pneumonia and receiving antipseudomonal therapy. Int J Antimicrob Agents *36*: 513–522.

Sécher, T., Bodier-Montagutelli, E., Guillon, A., and Heuzé-Vourc'h, N. (2020a). Correlation and clinical relevance of animal models for inhaled pharmaceuticals and biopharmaceuticals. Adv. Drug Deliv. Rev.

Sécher, T., Bodier-Montagutelli, E., Guillon, A., and Heuzé-Vourc'h, N. (2020b). Correlation and clinical relevance of animal models for inhaled pharmaceuticals and biopharmaceuticals. Adv Drug Deliv Rev *167*: 148–169.

Sécher, T., Dalonneau, E., Ferreira, M., Parent, C., Azzopardi, N., Paintaud, G., et al. (2019). In a murine model of acute lung infection, airway administration of a therapeutic antibody confers greater protection than parenteral administration. J Control Release *303*: 24–33.

Segall, A.M., Roach, D.R., and Strathdee, S.A. (2019). Stronger together? Perspectives on phage-antibiotic synergy in clinical applications of phage therapy. Curr Opin Microbiol *51*: 46–50.

Sibila, O., Agustí, C., Torres, A., Baquero, S., Gando, S., Patrón, J.R., et al. (2007). Experimental Pseudomonas aeruginosa pneumonia: evaluation of the associated inflammatory response. Eur. Respir. J. *30*: 1167–1172.

Welte, T., Torres, A., and Nathwani, D. (2012). Clinical and economic burden of community-acquired pneumonia among adults in Europe. Thorax *67*: 71–79.

Figure legends:

Figure 1: Sensitivity of PAK-Lux to bacteriophages and selection of an adapted bacteriophage combination. A. On the upper panel, PAK-Lux sensitivity to phages (PP1777, PP1902, PP1450, PP1792 and PP1797) was measured in a lysis kinetic assay by incubating the bacteria with each phage at MOI 1 or 10, during 12 hours at 37°C. On the lower panel, PAK-Lux sensitivity to phage combination MIX A (PP1777, PP1450 and PP1902) or MIX B (PP1777, PP1450, PP1902, PP1797 and PP1792) was measured at MOI 1, MOI 10 and MOI 100 during 22 hours at 37°C. B. According to the kinetic assay, two mixtures of phages: MIX A containing the 3 phages with a rapid bactericidal effect on PAK-Lux and MIX B containing all phages. C. Kaplan-Meier curves of mice infected with PAK-Lux and treated two hours post-infection with MIX A or MIX B at MOI 10 or MOI 100. Statistical comparisons between groups (n=6 per group) was assessed using a Mantel-Cox test. **$p \leq 0.01$. ***$p \leq 0.001$.

Figure 2: Establishment of a PA-associated VAP model in piglets. A. Percent of animals (n=9) surviving to PAK-Lux infection by range of hours post-infection. B. Clinical parameters (heart rate, body temperature and PaO_2/FiO_2) of piglets infected with PAK-Lux up to 42 hours. C. Measurement of total leukocytes, lymphocytes, neutrophils and monocytes ($10^3/mm^3$) in the blood of infected piglets over time. Results are expressed in mean ± SEM. D. After sacrifice or death, bacteria were measured in the lung tissue (CFU/g) and broncho-alveolar lavage (CFU) to determine bacterial load. Urines and blood were collected and bacteria measured (CFU/mL) along the experiment to assess infection dissemination. Box plots express results in mean, IQR, min and max. E. Photographs of hematoxylin/eosin staining of the infected right lower lung (upper) and non-infected left lower lung (lower).

Figure 3: Optimization of administration of inhaled bacteriophage in piglets under mechanical ventilation (set-up described in supplemental figure 2). A. Prediction of the aerosol output (inhalable mass/dose) in piglets under mechanical ventilation when connecting the static-mesh prototype nebulizer through either a T-piece or an inhalation chamber to the inspiratory branch of a ventilation circuit. B. Aerodynamical properties of the aerosol measured by cascade impaction and generated during mechanical ventilation using pig breathing parameters. FPF: fine particle fraction. MMAD: median mass aerodynamical diameter C. Predicted phage viability after aerosolization in piglets under mechanical ventilation. Aerosols were collected in a Biosampler™ connected at the end of the ventilation circuit. The results are expressed in mean ± SEM.

Figure 4: Clinical and hematological parameters of infected piglets after bacteriophage inhalation. A. Along the experiment, body temperature, heart rate and PaO_2/FiO_2 were monitored in piglets infected with PA and treated (inhaled phages) or not (control) with inhaled phages. B. Measurement of total leukocytes, lymphocytes, neutrophils and monocytes ($10^3/mm^3$) – along the experiment - in the blood of infected piglets treated (inhaled phages) or untreated (control). The results are expressed in mean ± SEM.

Figure 5: Administration of inhaled bacteriophages controlled PA infection in a VAP-model. A. Bacterial load in the infected right lower lung (CFU/g), broncho-alveolar lavage (CFU), serum (CFU/mL) and urine (CFU/mL) in piglets infected with PA and treated with inhaled phages (n=4) or untreated (control, n=4). B. Bacteriophage titers in the infected right lower lung (PFU/g), broncho-alveolar lavage (PFU), serum (PFU/mL) and urine (PFU/mL) in piglets infected with PA and treated with inhaled phages (n=4). Box plots express results in mean, IQR, min and max. Statistical comparisons between groups was assessed using a Mann-Whitney test. **p ≤ 0.01. ***p ≤ 0.001.

Supplemental figure 1: Schematic of experimental settings for VAP model in piglets. A. Chronogram of the experimental procedure for the establishment of a PA-associated VAP model in pigs. B. Chronogram of the experimental procedure for inhaled-phage therapy in the PA-associated VAP model. BAL: broncho-alveolar lavage, PA: *Pseudomonas aeruginosa*, MOI: multiplicity of infection, CFU: colony-forming unit.

Supplemental figure 2: Schematic of optimization of aerosol delivery in piglets under mechanical ventilation. The inhalable mass/dose, the particle sizes and the phage viability in phage aerosols in ventilated-pig breathing conditions measured using different metrological methods. Vt: tidal volume, RR: Respiratory Rate, I: inspiration, E: expiration, PEEP: Positive End Expiratory Pressure, FGF: Fresh Gas Flow, I.D: Inner diameter.

Supplemental figure 3: Density of phages and bacteria in the different pieces of the right and left lower lungs in the VAP model, in the control group and the animals treated with inhaled phages. The results are expressed as the mean ± SEM of CFU/g or PFU/g in each lung pieces ad. There is no

statistical differences in the quantity of phages in the different pieces (Kruskal-Wallis non-parametric test).

Figure 1. Sensitivity of PAK-Lux to bacteriophages and selection of an adapted bacteriophage combination

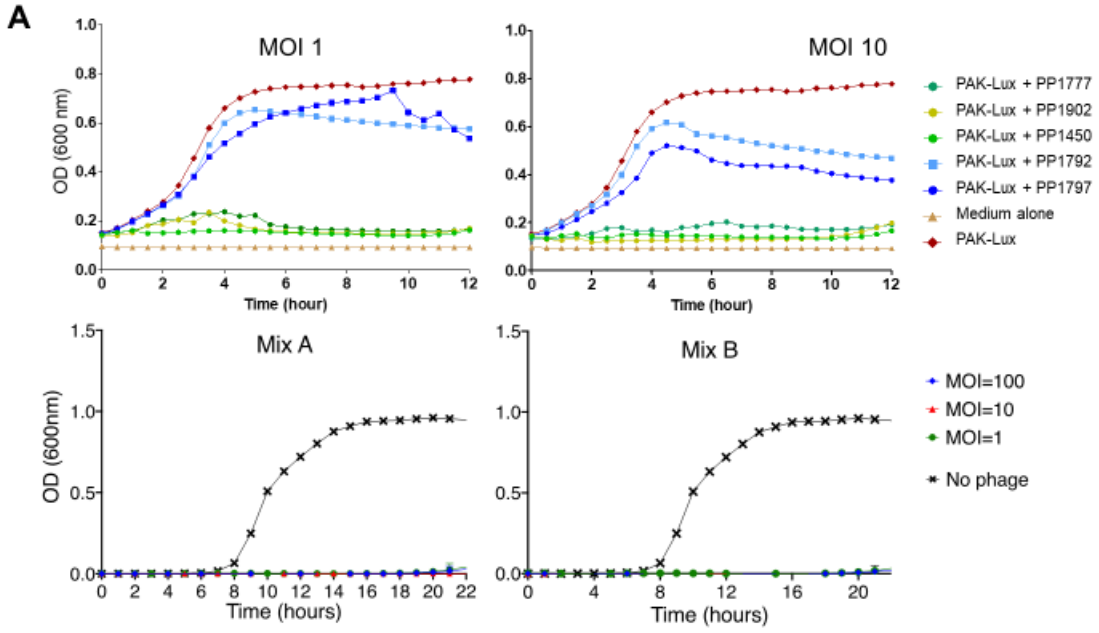


Figure 1. Sensitivity of PAK-Lux to bacteriophages and selection of an adapted bacteriophage combination

B

MIX B : 5 phages

MIX A : 3 phages

PHAGES	PP1777	PP1450	PP1902	PP1797	PP1792
PAK-Lux strain, sensitivity	+	+	+	+/-	+/-

C

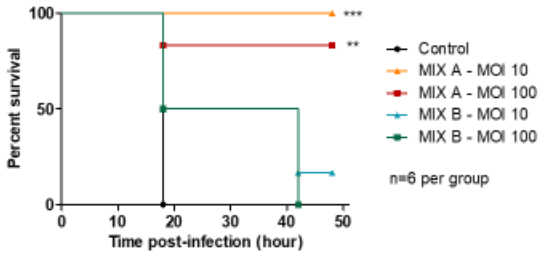


Figure 2. Establishment of a PA-associated VAP model in piglets

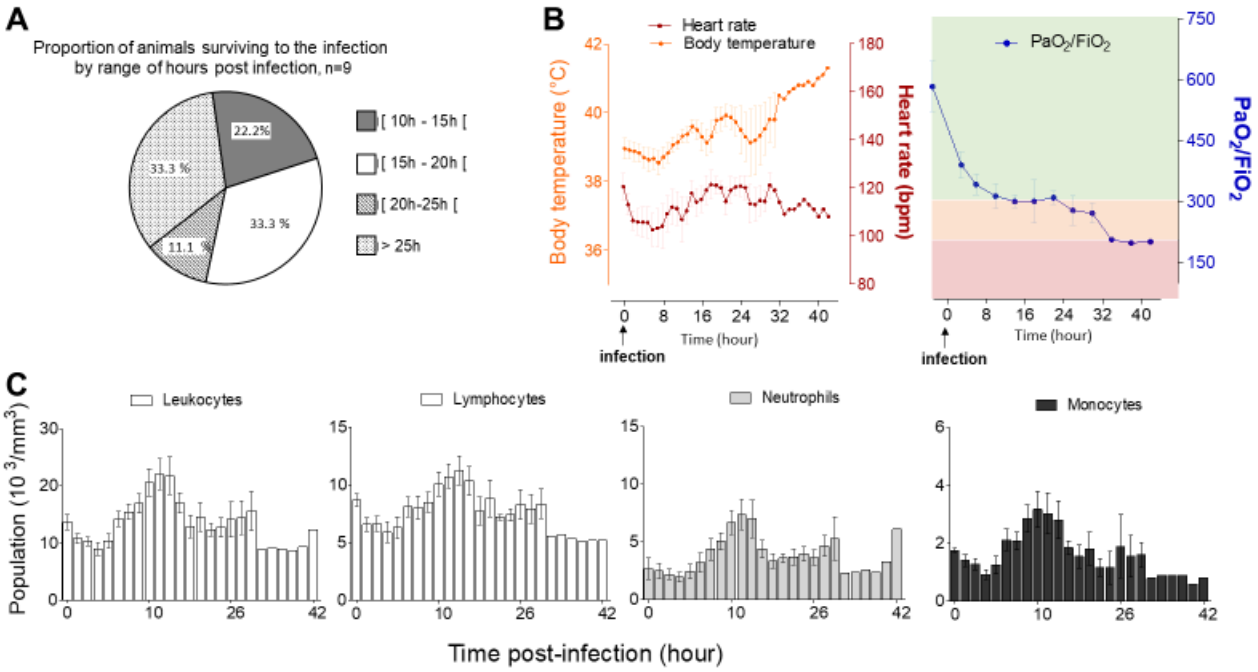


Figure 2. Establishment of a PA-associated VAP model in piglets

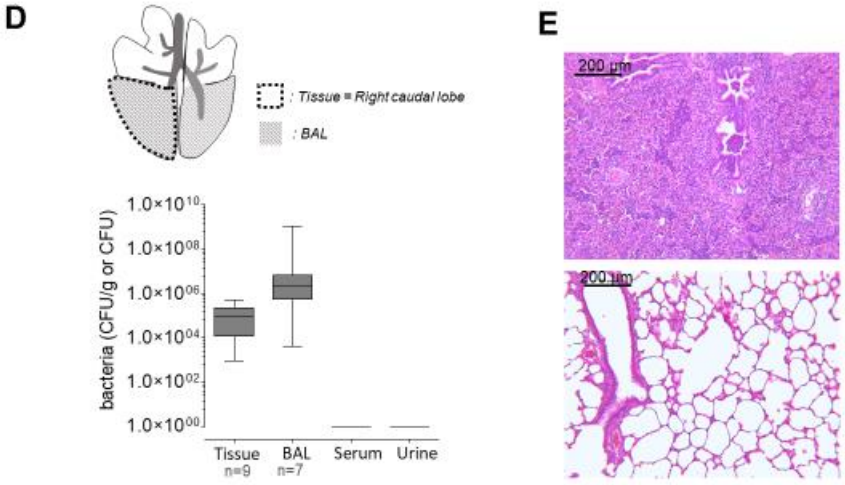


Figure 3. Optimization of administration of inhaled bacteriophage in piglets under mechanical ventilation

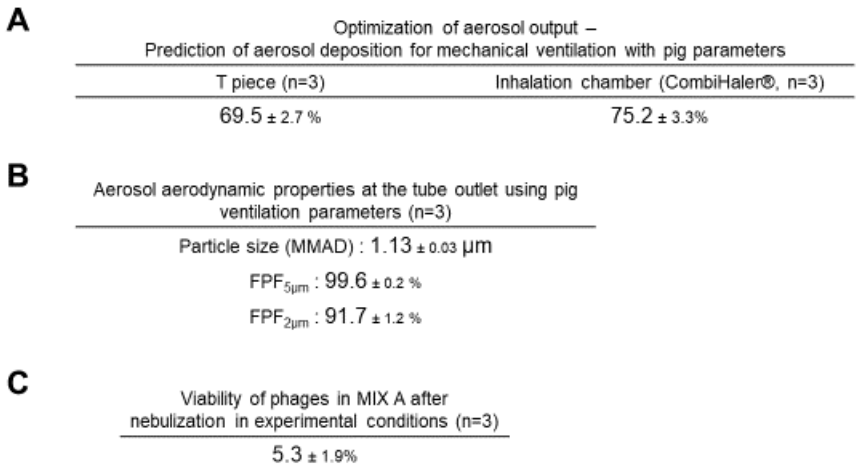


Figure 4. Clinical and hematological parameters of infected piglets after bacteriophage inhalation

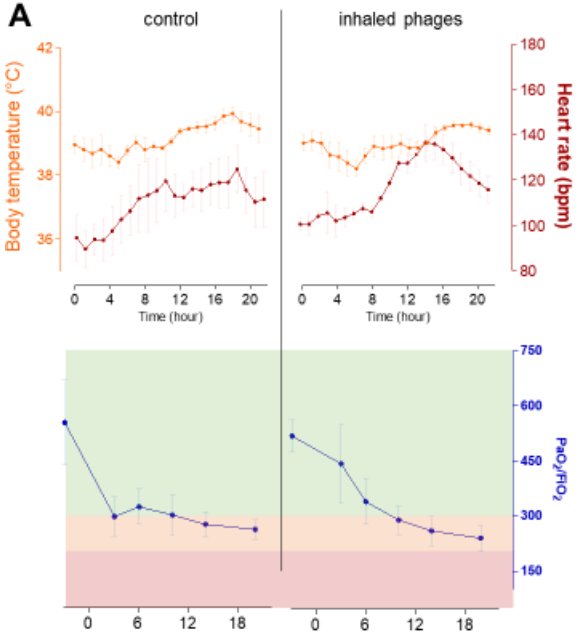


Figure 4. Clinical and hematological parameters of infected piglets after bacteriophage inhalation

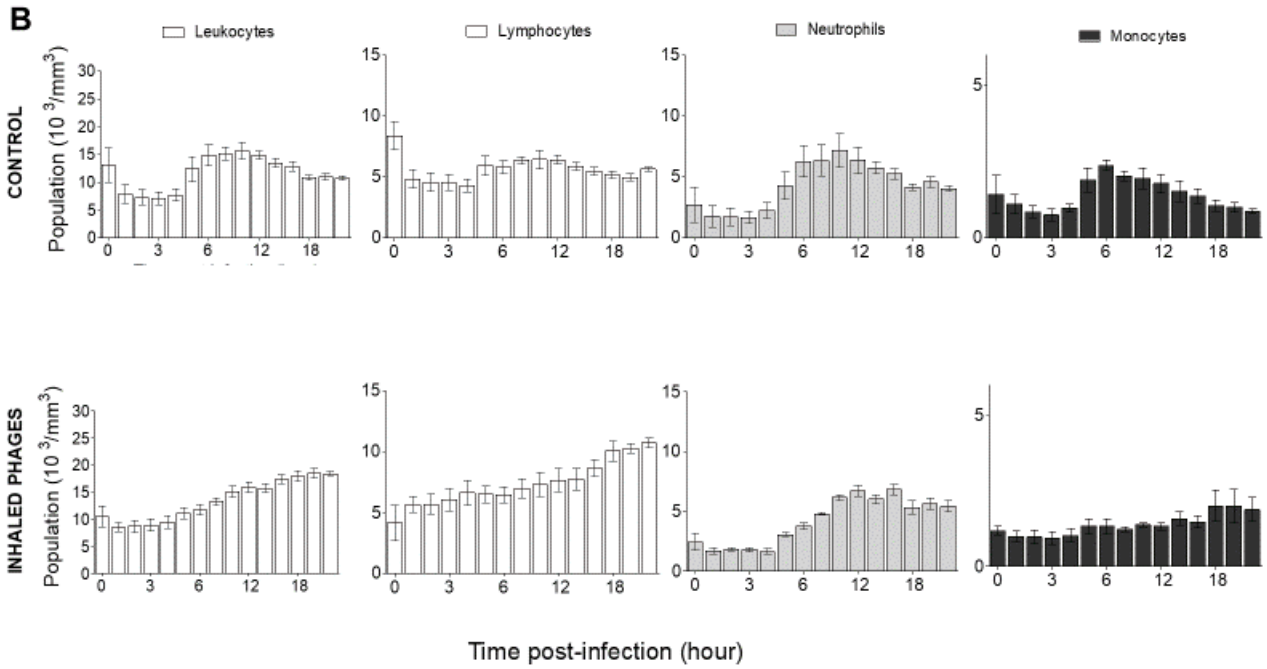
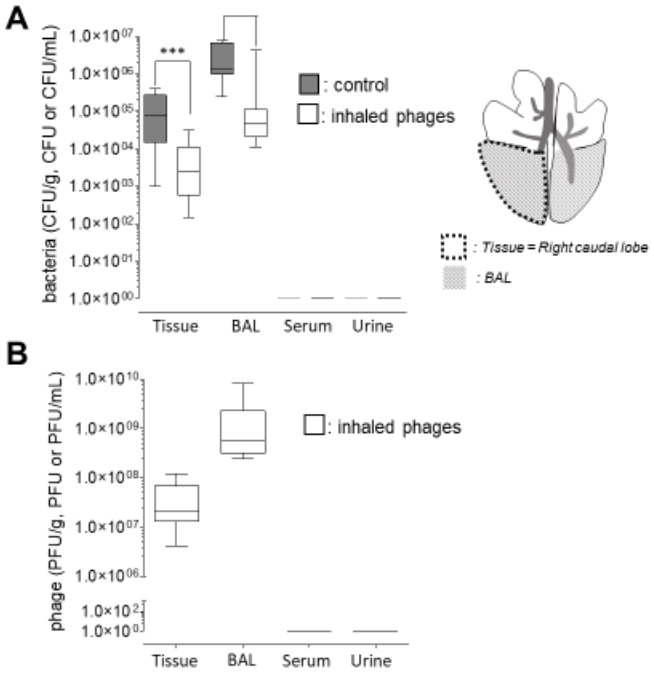
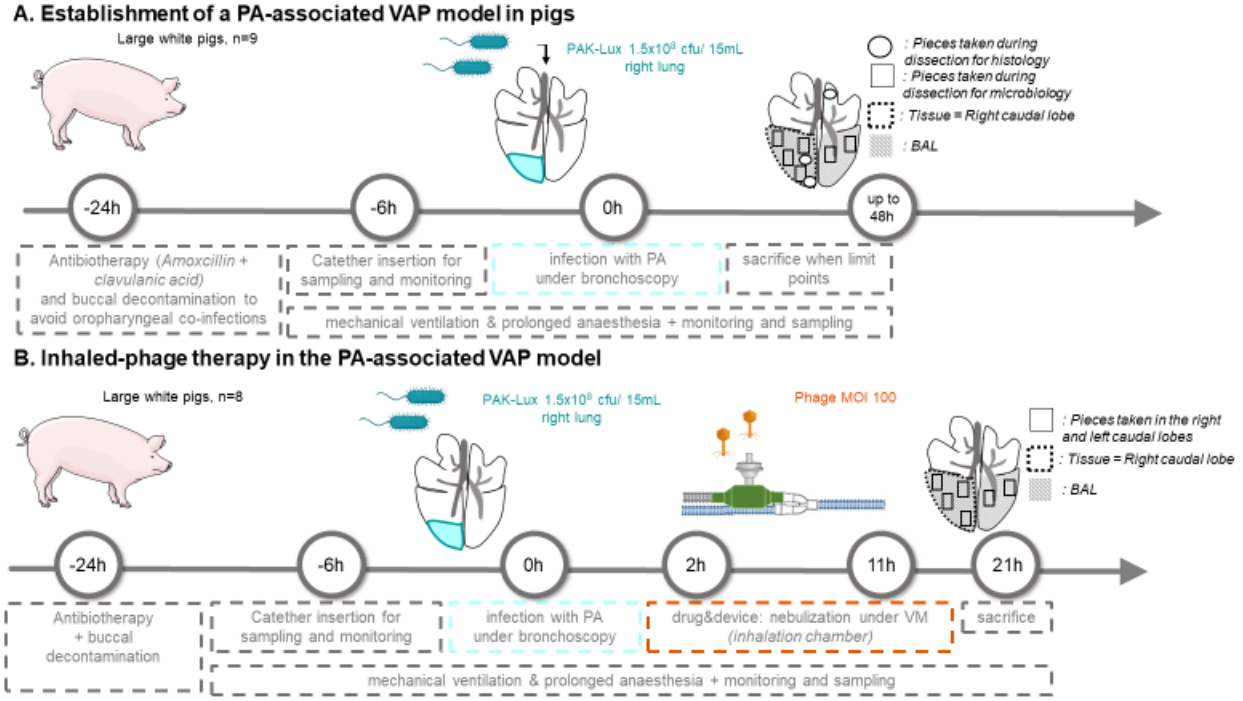


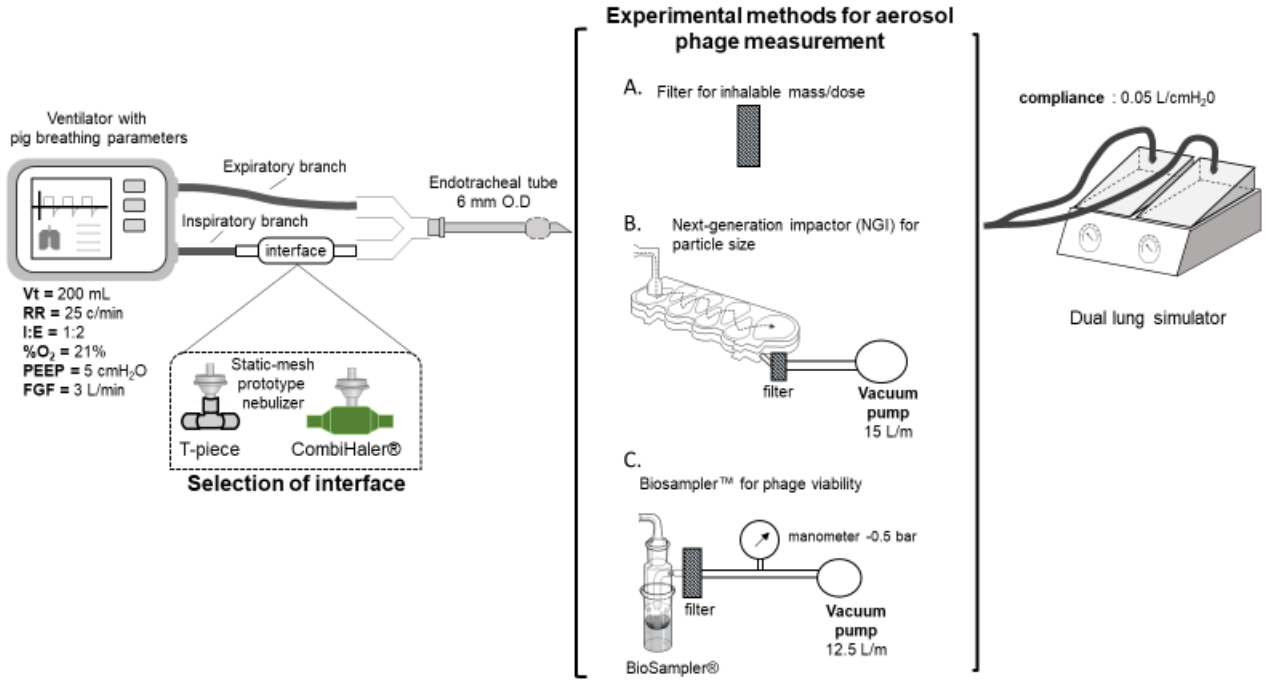
Figure 5. Administration of inhaled bacteriophages controlled PA infection in a VAP-model



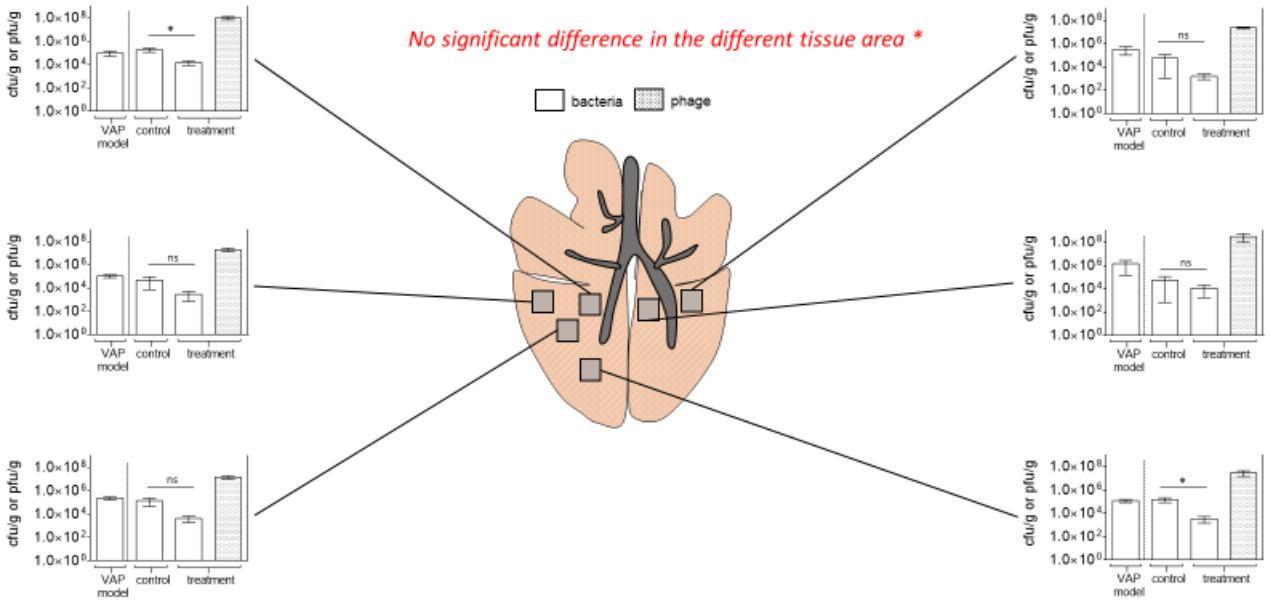
Supplemental Figure 1. Schematic of experimental settings for VAP model in piglets



Supplemental Figure 2. Schematic of optimization of aerosol delivery in piglets under mechanical ventilation



Supplemental Figure 3. Density of phages and bacteria in the different pieces of the right and left lower lungs in the VAP model, in the control group and the animals treated with inhaled phages



**Kruskal-Wallis test*