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Nebulized Bacteriophages for Prophylaxis of Experimental Ventilator-Associated Pneumonia Due to Methicillin-Resistant *Staphylococcus aureus*

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Objectives: There is a need for alternative strategies to combat and prevent antibiotic-resistant bacterial infections. Here, we assessed the potential for bacteriophage prophylaxis in the context of experimental ventilator-associated pneumonia due to methicillin-resistant *Staphylococcus aureus* in rats.

Design: Nebulized phages (aerophages) were delivered to the lungs of rats using a modified vibrating mesh aerosol drug delivery system. Animals were intubated and ventilated for 4 hours, at which point they were infected with methicillin-resistant S. *aureus* strain AW7 via the endotracheal tube, extubated, and then monitored for 96 hours.

Setting: Ventilator-associated pneumonia.

Subjects: Male Wistar rats.

Interventions: A single application of aerophages prior to ventilation at one of two concentrations (~1010 plaque forming units/mL or ~1011 plaque forming units/mL).

Measurements and Main Results: 1) Animal survival at 96 hours, 2) enumeration of bacteria and phages in the lungs and spleen, and 3) lung tissue histopathology. Animals that received aerophages prior to ventilation and methicillin-resistant *S. aureus* challenge showed a higher survival rate compared with untreated controls (60% for animals that received 3×10^{10} plaque forming units; 70% for animals that received 3×10^{11} plaque forming units; 0% for controls; $p < 0.01$ for each treatment versus untreated). Surviving animals that received aerophage prophylaxis had fewer methicillin-resistant *S. aureus* in the lungs compared with untreated control animals that succumbed to pneumonia (1.6×10^6 colony forming units/g vs 8.0×10^8; $p < 0.01$).

Conclusions: Prophylactically administered nebulized bacteriophages reduced lung bacterial burdens and improved survival of methicillin-resistant *S. aureus* infected rats, underscoring its potential in the context of ventilator-associated pneumonia. (*Crit Care Med* 2020; XX:00–00)

Key Words: antibiotic resistance; nosocomial infections; phage therapy

Mechanically ventilated patients are at risk of contracting bacterial infections, most notably ventilator-associated pneumonia (VAP). In light of this, patients often receive parenteral antibiotics in response to early signs of inflammation or even as means of prevention for at-risk patients (1). At least for *Staphylococcus aureus*, a frequent contributor to infection in this setting (2, 3), systemic antibiotic administration does not effectively reduce the prevalence of VAP, likely due to the limitations of anti-staphylococcal antibiotics, such as poor lung penetration and limited effectiveness against bacterial biofilms (1, 4, 5). Additionally, topical administration of antibiotics and disinfectants have produced mixed results and have been associated with the spread of antibiotic resistance (5). Clearly, antibiotic alternatives warrant investigation for the prevention of VAP due to *S. aureus*.

One strategy is the application of bacterial viruses known as phage therapy, which has been rediscovered for the treatment of many diverse, antibiotic-resistant infections (for a recent review, see reference [6]). In the context of VAP, intravenously administered phages have been shown to be as effective as standard of care antibiotics for the treatment of pneumonia due to methicillin-resistant *S. aureus* (MRSA) in an experimental model of VAP in rats (7). Phages have the added benefits of effectiveness against bacterial biofilms, and mechanisms of action that are distinct from typical antibiotics, which limits the threat of antibiotic resistance (8). Important

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questions relating to phage therapy remain, including when, how and how many bacteriophages should be delivered (8, 9). In the current report, we have assessed the utility of nebulized bacteriophages ("aerophages") for prophylaxis of VAP due to MRSA in an experimental rat model.

MATERIALS AND METHODS

All animal experiments were approved by the Cantonal Committee on Animal Experiments of the State of Bern, Switzerland (approval BE 53/19). We first addressed the bioavailability of aerophages over a 24-hour period in uninfected Wistar rats (male, 300–400 g). Twelve animals were administered 3 mL of a multiphage cocktail (consisting of phages K, 3A, 2002 and 2003 at final concentration of 1×10^{10} plaque forming units [PFUs] per mL) using a modified vibrating mesh aerosol drug delivery system (Aerogen Solo, Aerogen, Ireland). Animals anesthetized with sevoflurane (Fi 1–3%) were connected to the nebulizer via a facemask and aerophages were delivered inside an enclosed induction chamber. Following aerophage application, animals were returned to their cages. Animals were euthanized at 0, 2, 6, and 24 hours ($n = 3$ per time point) at which point the lungs were homogenized and phages were quantified in double-layer agar plates as described previously (7). To determine if increasing the number of aerophages would improve experimental outcomes, the phage cocktail was concentrated via isopycnic ultracentrifugation through a caesium chloride ($CsCl_2$) gradient, the residual $CsCl_2$ was removed via filtration (Amicon Ultra Centrifugal Filters; Merck, Germany), and the phages were resuspended in 0.9% sodium chloride to a final concentration of 1×10^{11} PFU/mL. To address bioavailability, animals ($n = 4$) were administered 3 mL of the 1×10^{11} PFU/ml cocktail, euthanized, and phages in the lungs were quantified as described above.

We next employed our established model of severe MRSA VAP essentially as described elsewhere (7). Briefly, animals ($n =$ 10 per treatment group) received a single dose of either 3×10^{10} PFU or 3×10^{11} PFU of aerophages as described above and were returned to their cages. After 2 hours, animals were ventilated for 4 hours on ventilators for small animals (VentElite; Harvard Apparatus [Holliston, MA]; 10 mL/kg tidal volume, 5 cm H_2O of positive end-expiratory pressure, 50 breaths/min with F_{IO_2} 0.35). Anesthesia was maintained with inhaled sevoflurane (Fi 3–4%) with additional subcutaneous fentanyl (20 µg/kg) delivered every 30–60 minutes. A total of 6 hours after aerophage prophylaxis, animals were inoculated with $\sim 8 \times 10^9$ colony forming units of an alpha-toxin producing clinical MRSA strain (AW7) via the endotracheal tube and were then extubated. Animal welfare was determined at least three times per day over the course of 96 hours, and the severity of illness was scored using an animal welfare scoring system (**Supplemental Table 1**, Supplemental Digital Content 1, http://links.lww.com/CCM/F449). Animals were euthanized with pentobarbital (150 mg/kg) if they scored greater than "1," or if their weight decreased by more than 20% (classed as "non-survivors") (7). Remaining animals were euthanized at 96 hours and classed as "survivors." Quantification of MRSA burdens in the lungs and spleen was performed following tissue homogenization as described previously (7). Histopathological analyses were performed as described previously (7), and a histopathological score was calculated for lung tissues according to parameters detailed in **Supplemental Table 2** (Supplemental Digital Content 1, http://links.lww.com/CCM/F449). Infected and untreated animals ($n = 7$) were included as controls to validate the lethality of the model.

RESULTS

A single application of 3×10^{10} PFU of aerophages to the airways of uninfected rats resulted in moderate phage loads in the lungs immediately after administration (1×10^6 PFU/g; **Fig. 1*A***), and a phage lung burden was still apparent at 24 hours (4×10^4 PFU/g; **Fig. 1*B***). Application of a higher dose (3×10^{11} PFU) to uninfected animals resulted in an average phage load for the lungs of 6×10^6 PFU/g soon after application (Fig. 1*A*).

In the context of experimental VAP due to MRSA, all control animals showed severe symptoms and succumbed to infection within 48 hours (**Fig. 1*C***). A single application of 3×10^{10} PFU of aerophages prior to ventilation and infection prevented mortality in 60% of animals (Fig. 1*C*; $p < 0.01$ compared with untreated controls; log-rank test). Administration of 3×10^{11} PFU prior to ventilation rescued 70% of animals, which was higher than for untreated animals ($p < 0.01$; log-rank test), but survival was not statistically different compared with those that received the lower dose of phages ($p = 0.66$).

Mortality in experimental models of VAP due to MRSA correlates with high bacterial loads in the lungs (7). In support of this, surviving animals treated with prophylactic aerophages had ~500 times less MRSA in the lungs compared with either untreated controls, or treated animals that succumbed to infection (**Fig. 2*A***; $p < 0.01$ using one-way analysis of variance [ANOVA]; and $p < 0.01$ for each comparison using Tukey multiple comparisons test). When lung bacterial loads were compared between control animals and each phage treatment group, no significant differences were determined, likely owing to the high MRSA loads for the nonsurviving animals present in each group (**Fig. 2*B***; $p = 0.17$; one way ANOVA). There was a positive correlation between bacterial burden in the lungs and histopathological scoring of the lung tissue for animals that received phage prophylaxis (**Fig. 2*C***; $r = 0.80$; $p < 0.0001$; Pearson two-tailed correlation test). MRSA loads for the spleen, which we use as a marker for the systemic spread of infection, were not different between surviving and nonsurviving animals (**Fig. 2*D***; $p = 0.24$; one-way ANOVA) or between treatment groups (**Fig. 2*E***; $p = 0.88$; one-way ANOVA). Phages were recovered from the lungs of most of the phage treated animals after euthanasia (17/20; **Fig. 2*F***), and were infrequently detected in the spleen (1/20 animals) suggesting aerophages remain localized in the lung tissue and do not readily spread to other organs.

DISCUSSION AND CONCLUSIONS

A single, prophylactic application of a nebulized phage cocktail prior to ventilation prevented lethal infection for the

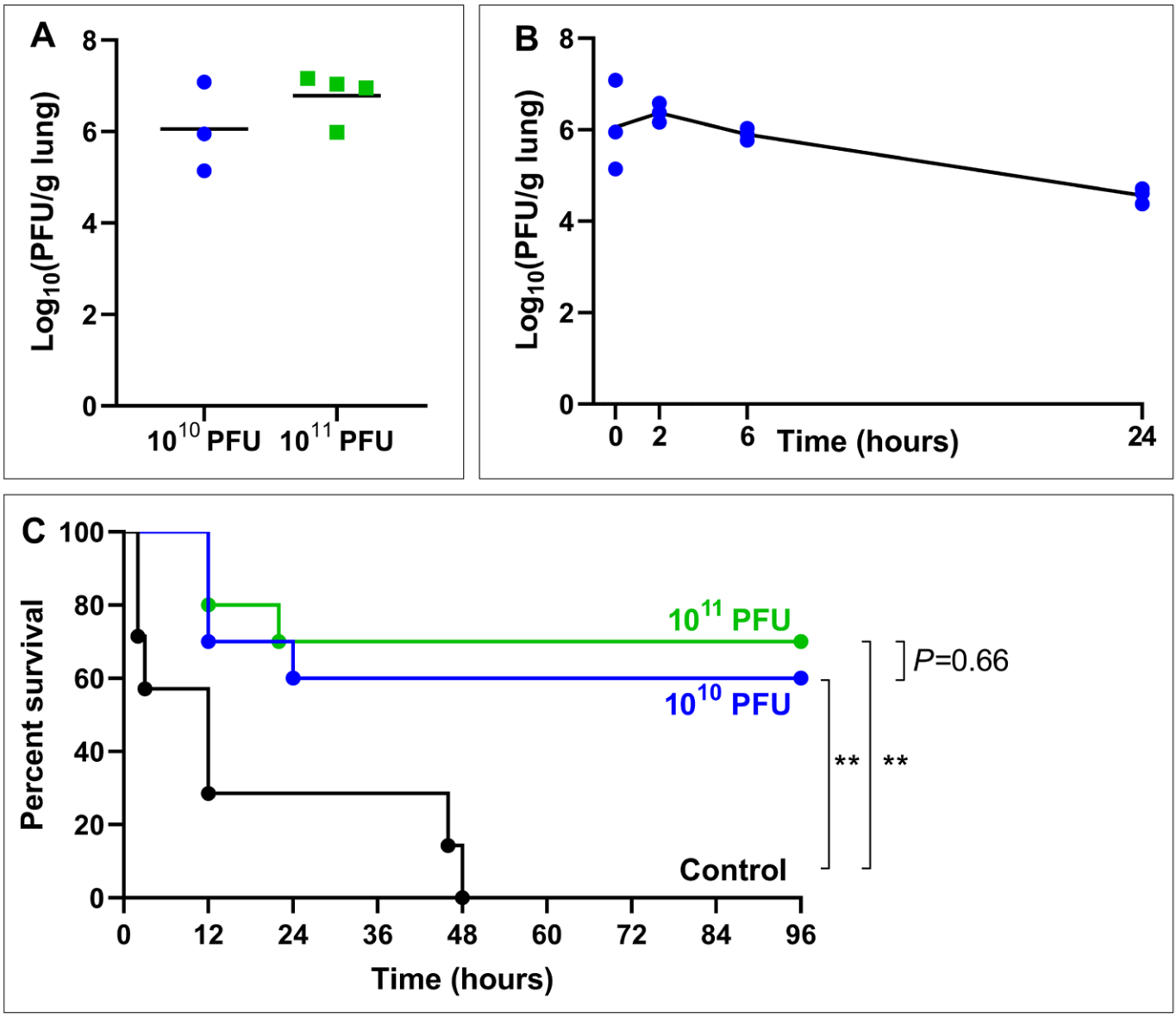

Figure 1. Aerophages for prophylaxis of experimental ventilator-associated pneumonia due to methicillin-resistant *Staphylococcus aureus* (MRSA). **A**, Bioavailability of phages in the lungs immediately after nebulized delivery of either 3×10^{10} plaque forming units (PFUs) (*blue symbols*) or 3×10^{11} PFU (*green symbols*) of a phage cocktail (aerophage) and (**B**) over the course of 24 hr (only for 3×10^{10} PFU of aerophages). **C**, Kaplan-Meier survival curves for MRSA infected animals with and without (*black line*) aerophage prophylaxis. Significance was determined using log-rank tests, $^{**}p < 0.01$.

majority of animals in an experimental model of VAP due to MRSA (Fig. 1). Survival was associated with substantial decreases in bacterial burden in the lungs and reduced lung tissue damage (Fig. 2). The results of the current report provide an important proof-of-concept for phage prophylaxis in the context of VAP. Although we observed promising results for laboratory animals in a controlled study, the distribution of phages and effectiveness of prophylaxis in severely damaged and heterogeneously ventilated lungs of human patients remains to be determined.

Phage prophylaxis joins a small but expanding list of appealing antibiotic alternatives that may be effective for VAP prevention. Generally, probiotics have shown promise in mechanically ventilated patients, whereby inoculation with *Lactobacillus* proved to be effective at reducing the prevalence of VAP in a placebo-controlled trial, irrespective of the causative organism (10). When focusing on MRSA, monoclonal antibodies that can neutralize *S. aureus* toxins have shown promise for the prevention of pneumonia in animal models (11), they are safe and well-tolerated in healthy human adults (12), and are being investigated in an interventional clinical trial for the prevention of pneumonia in high-risk patients (NCT02296320).

Our current findings pave the way for future phase I/II trials that will address safety, dosing and tolerability of phages in patients at risk of MRSA VAP including those colonized with *S. aureus*, and expected to require prolonged ventilation (i.e., longer than 48 hr).

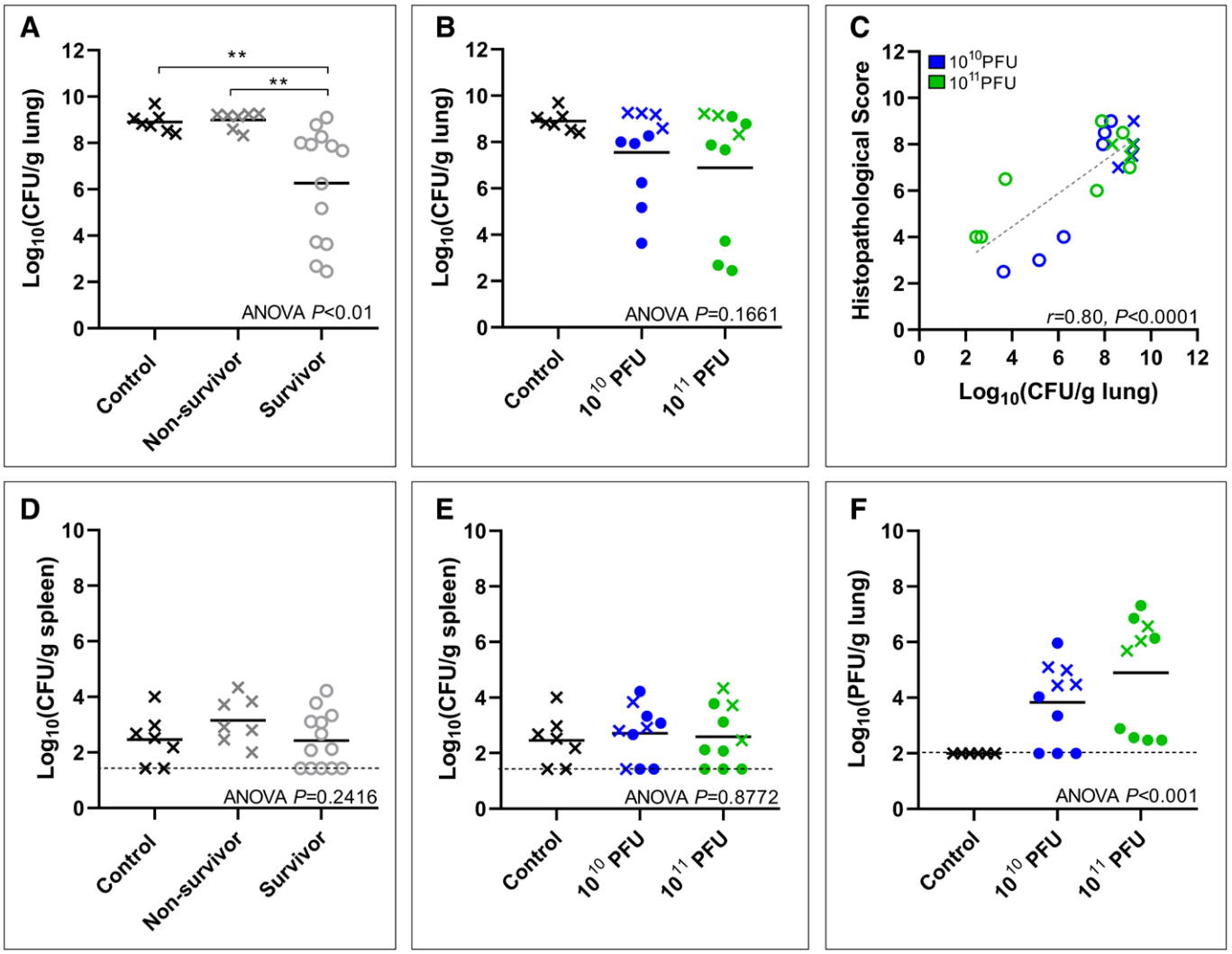

Figure 2. Bacterial and phage loads in the organs of animals with ventilator-associated pneumonia (VAP). **A**, Methicillin-resistant *Staphylococcus aureus* (MRSA) counts from the lungs of surviving and nonsurviving animals. Significance was determined using one-way analysis of variance (ANOVA) with multiple comparisons using Tukey test, ** $p < 0.01$. **B**, Comparison of MRSA counts from the lungs of animals treated with either a prophylactic dose of 3×10^{10} plaque forming units (PFUs) (*blue symbols*) or 3×10^{11} PFU of aerophages (*green symbols*). **C**, Correlation between lung bacterial density and histopathological scoring of the lung tissue. Significance was determined using Pearson two-tailed correlation test. The *gray line* represents the line of best fit. **D**, MRSA loads in the spleen for surviving and nonsurviving animals with MRSA VAP. **E**, Comparison of MRSA loads from the spleen of animals with VAP treated with either of 3×10^{10} PFU or 3×10^{11} PFU of aerophages. **F**, Phage counts for the lungs of animals treated with either 3×10^{10} PFU or 3×10^{11} PFU of aerophages. For each panel, nonsurviving animals are represented by *crosses*, and surviving animals by *circles*.
For **D–F**, *dotted/broken black lines* represent the limit of detection. CFU = colony forming unit.

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