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Bacteriophages Improve Outcome in Experimental *Staphylococcus aureus* Ventilator Associated Pneumonia

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Authors Contribution

JP, GR, YAQ, MH conceived the project; JP, MI, DRC, JS performed experiments; CG performed histology; JP, MI, DRC, DG, SJ, GR, JT, SL, YAQ, MH analysed the data; JP, DRC, GR, YAQ, MH wrote the paper; all authors reviewed and edited the manuscript.

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At a Glance Commentary

Scientific knowledge on the subject

Antibiotic resistance challenges current practice. New antimicrobials approved for clinical application are scarce, and this underscores the urgent need for alternative strategies. Phage therapy is a promising approach for the treatment of antibiotic resistant bacteria, however infection specific, proof-of-concept experimental studies are currently missing.

What this study adds to the field

We assessed the efficacy of phage therapy for the treatment of experimental ventilator associated pneumonia caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in rats. Phage treatment improved survival compared to placebo and was equally as effective as traditional antibiotics in controlling MRSA infection. Combination of phages with antibiotics did not further improve outcome in this experimental setting.

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Abstract:

Rationale: Infections caused by multidrug resistant bacteria are a major clinical challenge. Phage therapy is a promising alternative antibacterial strategy.

Objective: To evaluate the efficacy of intravenous phage therapy for the treatment of ventilator associated pneumonia due to methicillin-resistant *Staphylococcus aureus* in rats.

Methods: A randomized blinded controlled experimental study compared intravenous teicoplanin (3mg/kg, n=12), a cocktail of four phages (2-3 x 10^9 plaque forming units/ml of 2003, 2002, 3A and K, n=12) and combination of both (n=11), given two, 12 and 24 hours after induction of pneumonia, then once daily for four days. The primary outcome was survival at day four. Secondary outcomes were bacterial and phage densities in lungs and spleen, histopathological scoring of infection within the lungs and inflammatory biomarkers in blood.

Measurements and Main Results: Treatment with either phages or teicoplanin increased survival from 0% to 58% and 50% respectively ($p<0.005$). Combination of phage with antibiotics did not further improve outcome (45% survival). Animal survival correlated with reduced bacterial burden in the lung (1.2 x 10^6 CFU/g of tissue for survivors versus 1.2 x 10^9 CFU/g for non-surviving animals, $p<0.0001$), as well as improved histopathological outcomes. Phage multiplication within the lung occurred during treatment. IL-1β increased for all treatment groups over the course of therapy.

Conclusions: Phage therapy was as effective as teicoplanin in improving survival and decreasing bacterial load within the lungs of rats infected with methicillin-resistant *S. aureus*. Combining antibiotics with phage therapy did not further improve outcomes.

Key Words: bacteriophage; antibiotic resistance, microbial; pneumonia, ventilator associated.

Abstract word count without key words: **244**

INTRODUCTION

Ventilator associated pneumonia (VAP) is common in mechanically ventilated patients (1-3). The rapid development of antibiotic resistance in this infection setting both challenges treatment efficacy and is associated with increased morbidity and mortality (4-6). *Staphylococcus aureus* is consistently being reported as one of the top three pathogens isolated in this particular setting (7, 8). Although the rate of methicillin-resistance is decreasing worldwide, methicillin-resistant *S. aureus* (MRSA) still accounts for a significant portion of total VAP cases (5-15%) (2, 7, 9); this proportion varies across continents and even across hospitals within the same country (2, 9, 10).

The use of bacterial viruses (bacteriophages or phages) as antimicrobials is being actively re-evaluated as an alternative/complementary strategy to antibiotics, mainly in response to the rise in multidrug resistant infections (11, 12). The lack of trials conducted according to standards of good practices explains why phages are not yet part of the western pharmacopeia. Hence, studies ranging from experimental proof-of-concept to randomized controlled clinical trials are necessary to evaluate the potential of phage therapy for the treatment of multidrug resistant infections. The first phase I/II multicenter randomized controlled study (PhagoBurn) evaluating GMP-phage preparations has recently been published (13). PhagoBurn evaluated phage therapy for the treatment of burn wound infections due to *Pseudomonas aeruginosa* and while phages were well tolerated, their efficacy was challenged by innate bacterial immunity to phages and low phage titres within the study treatment cocktails (14, 15).

To date, many questions are yet to be investigated pertaining to phage therapy, especially in the context of pneumonia. For example, no clinical trial has evaluated phage therapy for the treatment of pneumonia. Additionally, there is a lack of preclinical data addressing (i) phage pharmacokinetics in the lung infection environment, (ii) frequency and mechanism of phage resistance selection in vivo, (iii) the effectiveness and appropriateness of phage-antibiotic combinations and (iv) the use of clinically validated administrative routes.

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Accordingly, the goal of the present work was to evaluate the efficacy of phage therapy alone and in combination with teicoplanin for the treatment of experimental VAP due to methicillin-resistant *S. aureus* (MRSA) in an experimental rat model. Emergence of phage resistance was monitored and data documenting phage pharmacokinetics in infected and non-infected animals were collected.

MATERIAL AND METHODS

The ventilator-associated pneumonia model. Male Wistar rats (Crl:WI(Han), Charles River, Germany), aged 9-10 weeks (280 – 330g) were housed in specific pathogen-free rooms (12h light/dark conditions, 23°C ± 1°, water and nutrition ad libitum). Animal protocols were run in accordance with the guidelines of the Swiss Animal Protection Law and were approved by the Cantonal Committee on Animal Experiments of the State of Bern (approval BE 83/17).

The VAP model published by Wu et al. (16) was adapted as follows (Fig. 1A). Animals were anaesthetized with sevoflurane followed by intraperitoneal injection of 20 µg/kg fentanyl. The anaesthetized animals were intubated with a G14 angiocath (Braun, Vasofix) (t_{-4}). Correct placement of the endotracheal tube was confirmed using capnometry. A polyurethane catheter (PU-065-50, SAI Infusion Technologies) was inserted in the jugular vein and connected to a harness with a Luer valve (QuickConnect, SAI Infusion Technologies) for therapeutic interventions and blood samplings. Rats were ventilated for four hours (10 mL/kg tidal volume, 5 cmH_2O of positive end-expiratory pressure, 50 breaths/min with FiO_2 0.35) on ventilators for small animals (VentElite, Harvard Apparatus). After ventilation, animals were infected by instillation of 0.15 mL inoculum containing 6-8 x 10^9 colony forming units (CFU, LD_{100}) of stationary phase *S. aureus* strain AW7 (17) (See online data supplement) into the endotracheal tube (t_0). Strain AW7 is a clinical MRSA strain producing α-hemolysin, a critical mediator of *S. aureus* pneumonia (18, 19). Immediately after inoculation, animals were extubated and placed into separate cages.

Treatment protocol. Two hours (t_2) after bacterial challenge, animals were randomly divided into four groups (n=10-12) and received: (i) 0.3ml of 0.9%NaCl (control); (ii) an intravenous phage cocktail consisting of equal titers (~2-3 x 10^9 PFU/ml) of phages 2003, 2002, 3A and K; (iii) intravenous teicoplanin (3mg/kg); (iv) a combination of both. Each treatment was further applied at t_{12}, t_{24}, t_{48} and t_{72} (Fig. 1A). We choose a glycopeptide antibiotic as the control treatment, as it is the standard of care according to current guidelines (3, 20). We preferred

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teicoplanin to vancomycin, because besides being equivalent to vancomycin (21, 22), its pharmacokinetic profile better matched our phage treatment regimen.

The respective therapies were given blinded (see online data supplement). Seven animals instilled with 0.15ml NaCl 0,9% after ventilation (t_0) served as the SHAM group and seven uninfected animals were administered phages (t_2, t_{12}, t_{24}, t_{48} and t_{72}) for pharmacokinetic studies (SHAM+phage).

Blood sampling was performed at catheter insertion (t_0), at the start of therapy (t_2), t_{24} and t_{96}. In non-surviving animals, blood was collected from animals immediately after death or euthanasia (see online data supplement), dissections were performed and lungs and spleens were collected. Surviving animals were euthanized at t_{96} with pentobarbital (150 mg/kg) before dissection.

Outcomes. Survival at t_{96} was the primary outcome. The secondary outcomes were 1) bacterial and phage loads in the lungs and spleen; 2) histopathological scoring of pneumonia (23); 3) quantification of inflammation biomarkers in the blood and 4) assessment of phage resistance. Methods describing secondary outcomes can be in the online data supplement.

Statistics. Log-rank tests were used to assess survival. Animals were divided into two mortality groups, Survivors and Non-survivors. Differences between the groups were determined using t test, Mann Whitney test, ordinary one- or two-way ANOVA. Corrections for multiple pairwise comparisons were achieved using the methods of Tukey (one-way) or Holm-Sidak (two-way). Analyses were performed using GraphPad Prism (v7) with a significance level of $p<0.05$.

RESULTS

Phage therapy significantly improves survival in rats with experimental VAP.

The experimental model of VAP was rapidly lethal, with 80% of untreated animals succumbing to infection within 12 hours and 100% mortality by 86 hours (Fig. 1B). To assess the efficacy of phage therapy in this infection setting, we developed a treatment cocktail consisting of four phages with complimentary host-range profiles, which infected 91.6% of *S. aureus* isolates tested (Fig. E1A, online data supplement). Importantly, the combination of multiple phages was shown to limit the development of phage resistance *in vitro* (Fig. E1B, online data supplement). Application of the cocktail improved survival compared to placebo ($p< 0.001$, log-rank test). All animals treated with phages survived for at least 12 hours following infection and 58% of the animals survived until the end of the experiment. This was comparable to the effect of the standard of care treatment teicoplanin (50% survival, $p=0.85$).

The combination of phages and antibiotic did not further improve outcome compared to phage or antibiotic alone (45% survival, $p=0.57$ and 0.74, respectively. Fig 1B). In support of this, we did not observe synergy or antagonism for teicoplanin and the phage cocktail *in vitro* (Fig. E2, online data supplement).

Phage therapy controls bacterial load within infected lungs of surviving rats.

Mortality correlated with high bacterial load within the lung (Fig. 2A). Animals that succumbed to infection had 1,000-times more MRSA in the lung when compared to those that survived (1.2×10^9 CFU/g verses 1.2×10^6 CFU/g, respectively, $p <0.0001$, t test). When compared with teicoplanin, phages were equally as effective at controlling bacterial loads within the lung of infected rats, and combination of phages and antibiotic did not further reduce lung bacterial burden (Fig. 2B, no difference between treatment groups, $p=0.72$, two-way ANOVA). Metastasis to the spleen was infrequently detected (13 of 42 spleens tested) and there was no difference in spleen bacterial loads between groups based on time-to-mortality (Fig. 2C, $p = 0.30$, Mann Whitney test).

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In the absence of pneumonia, intravenous application of phages did not lead to phage accumulation within the lung. However, phages were detected in the lungs of rats with pneumonia treated with the phage cocktail, suggesting either amplification and/or persistence of phages at the site of infection (Fig. 2D). The average phage load for the lung was higher in non-surviving animals, however this was not statistically significant (Fig. 2E, p=0.30, Mann Whitney test). Similarly, a positive correlation between CFU/g of lung and PFU/g of lung was observed, but this did not reach statistical significance (Fig 2F,-r = 0.33, p = 0.12, Pearson two-tailed correlation test). In the absence of infection, phages were sequestered by the spleen (Fig. 2D).

Despite bacterial load reductions within the lungs, phage treatment did not eradicate the infection (Fig. 2B). To evaluate selection of phage resistance, which may explain MRSA persistence, we performed phage cross-streaking assays using a total of 100 bacterial isolates taken from 10 infected lungs of animals treated with phages, and 10 infected lungs from animals treated with a combination of phages and antibiotics. All of the MRSA strains tested remained susceptible to phage lysis irrespective of rat mortality, suggesting that persistence was not due to the evolution of resistance (Fig. 3).

Phage and/or antibiotic therapy is associated with reduced lung damage in surviving rats.

Experimental MRSA VAP resulted in considerable lung damage (Fig. 4A). The lungs of ventilated but uninfected rats received a histopathological score of “1” indicating mild injury with mild to moderate perivascular and peribronchiolar inflammation and oedema. Application of phages in uninfected rats did not result in additional lung damage. All infected rats that succumbed to infection early had an average total histopathological score of 2.65 indicating moderate to severe disease (Fig. 4B). In contrast, survival was associated with a lower histopathological score compared to rats that did not survive (average of 2.06, p> 0.0001, representative image Fig. 4C). The score for surviving animals was still higher than that of uninfected rats (p= 0.056). More specifically, surviving rats treated with either phages, or

teicoplanin displayed a reduction in lung damage compared to treated rats that did not survive (Fig. 4D, $p < 0.05$ for both, Holm-Sidak test). The difference between histopathological score for survivors and non-survivors treated with a combination of phages and teicoplanin however was not significant (Fig. 4D, $p = 0.32$). Given the importance of staphylococcal exotoxins in this infection setting, we also analyzed necrosis independent of other histopathological factors. MRSA induced significant necrosis when compared to uninfected SHAM controls. For infected animals, high scores for necrosis correlated with poor animal survival (Fig. E3A) and there were no differences in necrosis scores between each treatment group (Fig. E3B). To characterise this further, we performed rabbit red blood cell (RBC) lysis assays for filtered supernatants from phage-exposed cultures as a functional measure of exotoxin production. In support of the necrosis data (Fig. E3B), phages had no discernible impact on RBC cell lysis *in vitro* (Fig. E4).

Lung inflammation correlated with increased cytokines in blood.

The cytokine profile in blood of MRSA infected rats was different to that of uninfected controls ($p < 0.01$, two-way ANOVA, Fig. 5A). MRSA infection resulted in an increase in two of the six pro-inflammatory cytokines tested. IL-6 was increased in MRSA infected rats compared to SHAM controls ($p < 0.01$), and whilst TNFα levels were below the limit of detection in uninfected rats, levels were elevated for 22 of the 25 MRSA infected rats. IL-1β levels increased significantly in each treatment group over the course of therapy including uninfected animals treated with phages (SHAM+phage), which suggests that the IL-1β response is induced by the phage in the absence of bacterial infection (Fig. 5B). IL-18, an additional inflammasome marker was not detectable in samples after 96 hours of treatment (data not shown). IL-1α increased only in MRSA infected animals treated with phages (with or without antibiotic). The levels of TNFα and IL-6 returned to baseline levels following 96 hours of treatment (Fig. 5B). IL-10, and IL-17A levels did not vary over the course of treatment (data not shown).

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DISCUSSION

Phage therapy is a promising alternative and/or complementary strategy to overcome the rising problem of multi-resistant bacteria. Although an increasing amount of data has been generated in the last five years, strong pre-clinical evidence and milestone clinical studies are still lacking to support its use in human medicine (24, 25). We recently reported a positive effect of a cocktail of bacteriophages for the treatment of *P. aeruginosa* experimental endocarditis (26) as part of the Phagoburn clinical trial (13); in the current report, we systematically evaluated the efficacy of a new anti-*S. aureus* phage cocktail for the treatment of VAP in rats.

To better mimic the clinical setting, we adapted a model of VAP previously described by Wu et al. by using higher oxygen fraction (0.35 vs 0.21) and by lowering minute-ventilation rates. This first enabled us to generate the typical ventilator-induced injuries observed in ICU patients as demonstrated by the mild to moderate, perivascular and peribronchiolar inflammation reported in ventilated but not infected animals and secondly, these adaptations increased the severity of the experimental model, with infected but untreated animals harbouring high bacterial loads within the lungs (Fig. 2A).

Our study has some important implications. First, we could demonstrate that phage therapy significantly reduced mortality compared to placebo in a lethal model of staphylococcal pneumonia (Fig. 1B). This effect was comparable to that of the standard of care. Similar results have been reported in other pneumonia models caused by gram-negative pathogens including *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *P. aeruginosa* (14, 15, 27, 28). Nevertheless, our report, focusing on a troublesome clinically relevant antibiotic-resistant gram-positive pathogen, better emulates the clinical setting, in the way that we first ventilated the animals, before infecting them via the endotracheal tube – mimicking bronchoaspiration – and then treating them with phages given intravenously as opposed to via intraperitoneal injection.

Phages were detected in infected lungs and were not detected in the absence of infection (Fig. 2B) highlighting the capacity for phages administered in the bloodstream to become

concentrated at the site of infection. Additionally, we observed sequestration of phages in the spleen of uninfected animals, as has been described in other experimental models (29-31), the physiological significance of which remains to be determined. Phage titers did not explain early death (ie. one might expect that low titers early on may lead to poor outcomes), however this result is confounded by lower bacteria (hosts for phage replication) in the lungs of surviving animals.

Moreover, our observation that phage-treated non-infected animals displayed some increase in IL1β-production raises some concern about the use of phage therapy without a diagnosis or high suspicion of VAP. This phenomenon has also been observed in other studies, using other experimental settings and different phages (32), which highlights the need for a thorough reassessment of the exact impact of induced inflammation upon the clinical course of the disease. One obligatory step would be the use of highly purified, toxin free phage preparations produced using Good Manufacturing Practices (GMP).

Although phage therapy resulted in a reduction of bacterial densities within the lungs, it neither cleared nor cured the infection. This partial microbiological response might be due to either limited time of treatment or emergence of bacterial immunity against phages. We did not observe the emergence of resistance during short course therapy *in vivo*, which supports our previous observations using an anti-*Pseudomonas* phage cocktail for the treatment of endocarditis (26). In the absence of resistance, survival of bacteria following phage treatment may be attributable to a subpopulation of phage-tolerant persister cells; a hypothesis that we are currently investigating. Equally concerning, is the observation that the standard-of-care antibiotic chosen did not eliminate bacteria from the lung of infected animals. This supports claims that glycopeptides, may not be the best antibiotic choice in this infection setting, and that others, such as linezolid, which showed clear synergism with phages *in vitro* (Fig. E2), should be considered (33).

In a previous study, Yilmaz and colleagues reported synergy between teicoplanin and phages for the treatment of MRSA biofilms in an experimental implant-related infection model in rats

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(34). In the current report, we did not observe improved outcomes for rats treated with a combination of anti-*S. aureus* phages and teicoplanin for the treatment of pneumonia. This correlates with research from others focusing on pneumonia due to *K. pneumoniae* (27), suggesting that synergism between phage and antibiotics is perhaps infection setting dependent.

In addition, we found that mortality correlated not only with bacterial density within the lung (Fig. 2A), but also with lung tissue necrosis (Fig. E3). It is apparent that phage administration did not prevent or limit tissue necrosis or limit exotoxin production (Fig. E4), raising the question as to whether treatment outcomes could be further improved using adjunctive anti-toxin strategies such as protein synthesis inhibitors or anti-alpha toxin immunotherapeutics.

Our study has some limitations. First, we used a rapidly lethal model of pneumonia, whereas the overall mortality of ventilator associated pneumonia in humans is much lower at around 10 – 17% (35). As such, we plan to evaluate phage therapy in the future in less acute models of pneumonia, settings where longer courses of treatment are possible, which will likely provide further insights into phage pharmacodynamics and the development of bacterial phage immunity *in vivo*.

Second, despite what we perceived to be good bioavailability of phages in the lung, we could not eradicate the infection. It is not yet clear how often phages should be given. We administered them once daily, since we expected phage amplification at the site of infection to be enough to decrease bacterial load. It is possible that we did not reach the optimal multiplicity of infection (MOI) appropriate to combat the high bacterial burden within the lungs. Further experiments are needed to evaluate the potential benefit of more frequent phage delivery and/or the application of more highly concentrated phage cocktails.

In addition to more frequent application of phages, the route of administration could be optimised. For instance, aerosolisation could be used to deliver more phages to densely infected lung tissues. Aerosols are currently being used in mechanically ventilated patients to treat pneumonia (36) and previous studies succeeded in delivering anti-*Pseudomonas* phages

to the lung (28). Currently, no such report exists for anti-*S. aureus* phages. Moreover, the encapsulation of anti-Gram negative bacteriophages in phospholipid vesicles (37) and polymeric microparticles (28) effectively concentrated phages in the lung and these processes should be evaluated for anti-*S. aureus* phages.

A well-known limitation of phage therapy is its narrow spectrum of activity (38). This caveat has clearly been demonstrated in the PhagoBurn study, where patients harbouring a resistant strain at the beginning of the treatment could not be cured from the wound infection. In the case of VAP, which often begins with prior colonisation (39), we may have the benefit of a time window of opportunity to personalize the phage treatment for the patients strain prior to the initiation of therapy.

Conclusions

Phage therapy significantly improved outcome compared to placebo and was equivalent to antibiotic in controlling MRSA-ventilator associated pneumonia. This study was the first to explore phage therapy for the treatment of MRSA experimental VAP. However, many questions remain unanswered, which preclude its immediate clinical implementation. Further studies are needed to assess whether alternative administration approaches, such as phage aerosolisation or other antibiotic-phage combinations (ie. using protein synthesis inhibitors as opposed to cell wall inhibitors), could further improve the efficacy of this therapy.

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FIGURES

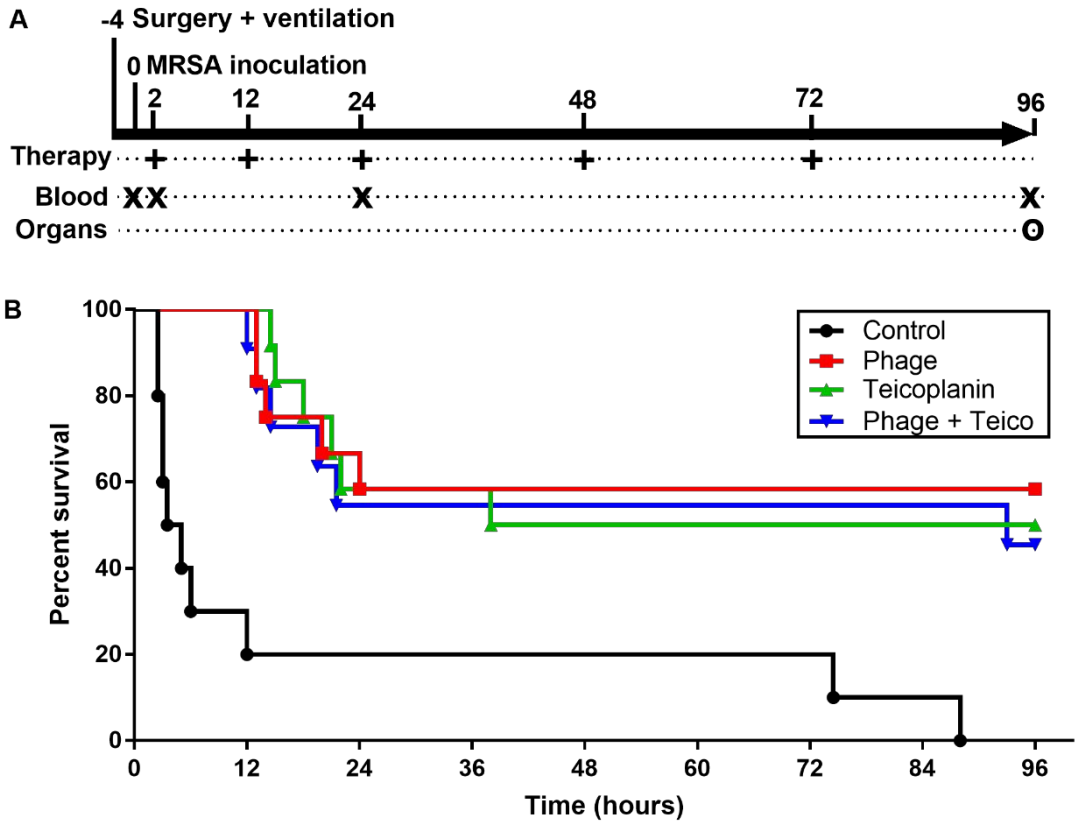

Figure 1. Survival of rats with MRSA VAP treated with phage, antibiotics or a combination of both. Study design of the VAP model with sampling time points **(A)**. Kaplan-Meier survival curves for animals with MRSA VAP that were untreated (n= 10), treated with the phage cocktail (n= 12), treated with the conventional antibiotic, teicoplanin, (n= 12) or a combination of both (n= 11) **(B)**.

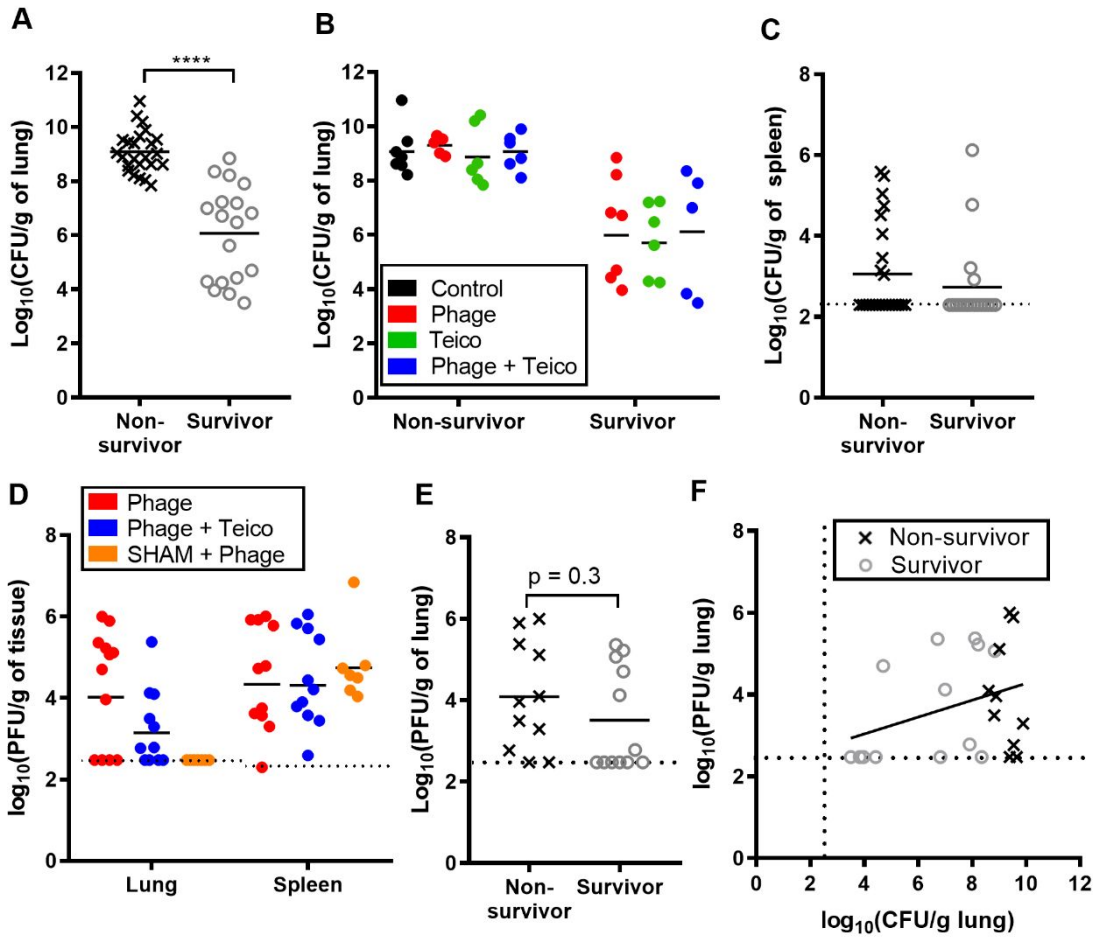

Figure 2. Bacterial and phage loads in the lung and spleen of animals with experimental MRSA VAP. Animals were divided into two groups based on mortality: non-survivors and survivors. Bacterial burden for the lungs of rats infected with MRSA. Significance was determined using t test, **** $p < 0.0001$ **(A)**. Comparative analysis of lung bacterial load for rats treated with phages, teicoplanin, or a combination of both **(B).** Bacterial burden for the spleen of rats infected with MRSA **(C)**. Phage titres for the lung and spleen **(D).** Comparative analysis of phage burden for the lung of non-surviving and surviving rats. The p-value was determined using t test **(E)**. Correlation analysis between lung phage titres and bacterial loads. Pearson 2-tailed correlation test, $r = 0.33$, $p = 0.12$. Line of best fit represented in black **(F)**. For each panel, data are summarised using the mean (solid black lines), and the limit of detection for each organ is represented by dotted black lines. Abbreviations: CFU, colony forming units; PFU, plaque forming units; Teico, Teicoplanin.

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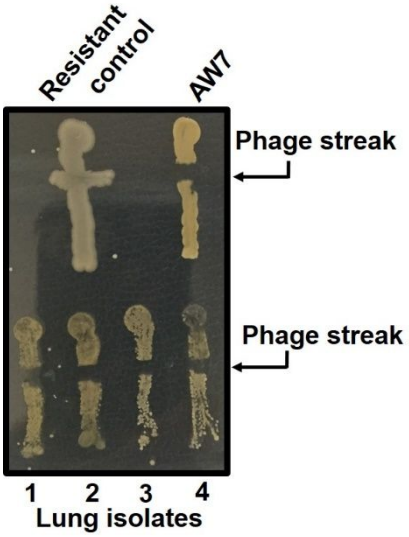

Figure 3. Determination of phage susceptibilities for bacteria isolated from the lungs of phage treated rats. One hundred bacterial colonies were tested for phage resistance using cross streak assays. All isolates were susceptible to the phage based on the absence of growth in the phage streak. Four representative colonies are shown.

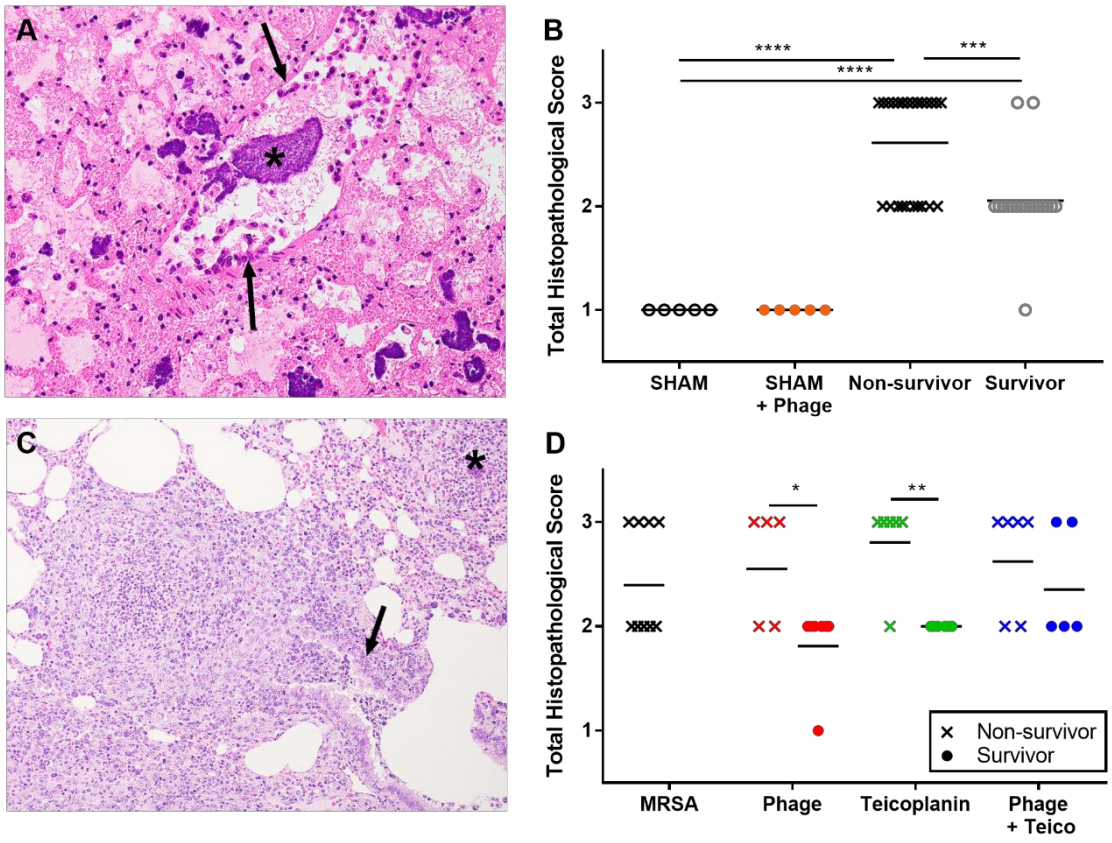

Figure 4. Histopathogical scoring of lung tissue following MRSA VAP. Rat lung inoculated with MRSA (untreated control) showing a necrotic bronchiolus (arrows) with intraluminal bacterial colonies (*), which invade surrounding, necrotic alveoli **(A)**. Scoring for lung histopathology was published by Montgomery et al(23). A score of 1, 2, 3 represents mild, moderate or severe pneumonia, respectively. Significant differences were determined using one-way ANOVA with multiple comparisons using the method of Tukey. *** $p < 0.0005$, **** $p < 0.0001$ **(B)**. Lung 96 hours after MRSA inoculation followed by phage therapy displaying a hyperplastic bronchiolus (regeneration, arrow) filled with and surrounded by many inflammatory cells (neutrophilic granulocytes, macrophages) that display a good immune response and few bacterial micro-colonies (*) **(C)**. Comparative analysis of non-surviving and surviving animals following treatment. Significant differences were determined using two-way ANOVA with multiple comparisons using the method of Holm and Sidak. As none of the untreated animals survived, the MRSA group was excluded from the two-way ANOVA. * $p < 0.05$, ** $p < 0.01$. **(D).** A and C are representative images of 200x HPF (high power field) magnification. For B and D, animals that died are represented by crosses, and those that survived are represented by closed circles. Black lines represent the mean.

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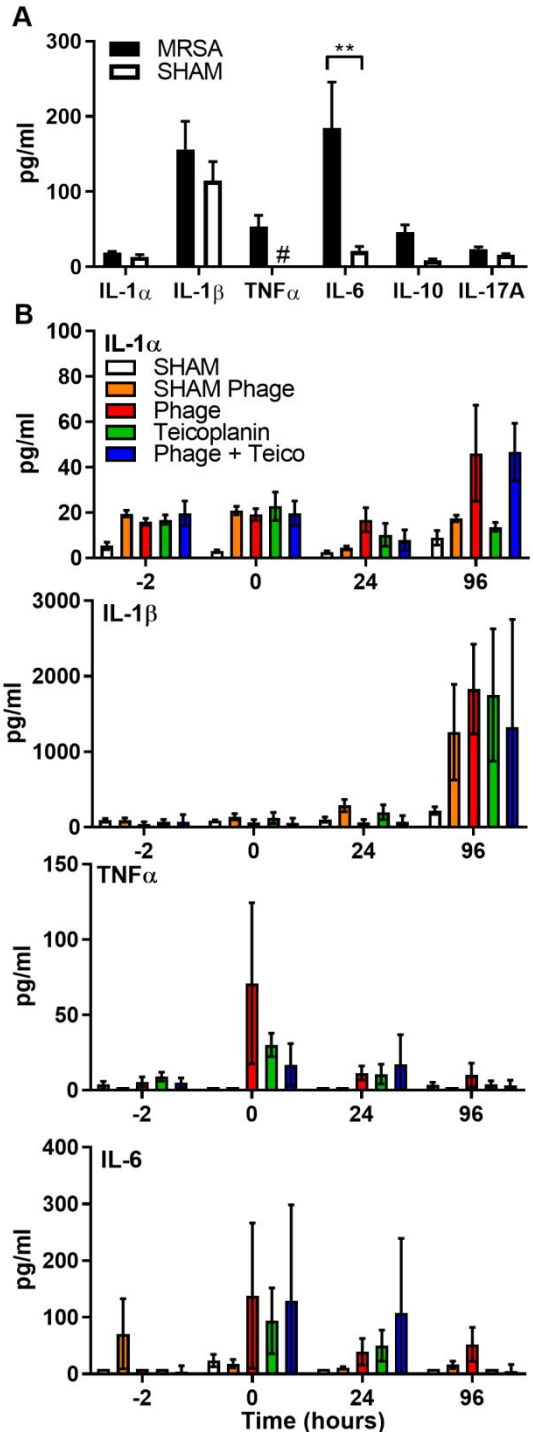

Figure 5. Evaluation of inflammatory biomarkers for rats with experimental MRSA VAP. Concentration of select cytokines in the blood of rats following two hours of MRSA pneumonia (black bars, n=25) compared to uninfected controls (SHAM, white bars, n=12). Significant differences were determined using two-way ANOVA with multiple comparisons using the method of Holm and Sidak, ** $p < 0.01$. # indicates that levels were below the limit of detection **(A)**. Cytokine concentration dynamics in the blood of late death/surviving rats following MRSA VAP (t_0) and treatment with phages (n=7) or teicoplanin (n=6) or combination of both (n=5), as

well as uninfected controls (SHAM, n=5), and uninfected controls treated with phages (SHAM+phage, n=7) **(B)**. For each panel, data represent the mean and standard error.

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Online Data Supplement

Bacteriophages Improve Outcome in Experimental *Staphylococcus aureus* Ventilator Associated Pneumonia

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MATERIAL AND METHODS

Bacteria and Phages

Bacterial strain and growth conditions. MRSA strain AW7 was isolated from a patient with bacteremia (1-3). The strain is representative of sequence type 247 as determined by whole-genome sequencing (CC8, SCC*mec*I [1B], *hlgABC*$^+$, *lukDE*$^+$, *sea*$^+$, *scn*$^+$, PVL$^-$). Importantly, AW7 produces α-hemolysin, which is a critical mediator of *S. aureus* pneumonia (4-6). In addition to beta lactam resistance, the genome harbors resistance determinants for aminoglycosides, tetracyclines, macrolides and chloramphenicol. AW7 was stored frozen in Tryptic Soy Broth (TSB) containing 10% (v/v) glycerol at -80^{o}C and sub-cultured on Tryptic Soy Agar (TSA) plates to ensure purity before testing. For liquid culture, TSB was inoculated with at least five single colonies and incubated for 24 hours with agitation (200 rpm) at 37^{o}C.

Bacteriophages. Phages K and 3A were purchased from the University of Laval, Québec (https://www.phage.ulaval.ca/en/phages-catalog/). Phage 2003 was isolated from the staphylococcal phage product of the Eliava Institute of Bacteriophages, Microbiology and Virology, Tbilissi, Georgia. Briefly, serial dilutions of the phage product were mixed with *S. aureus* strain 8325-4 and grown in double-layer TSA plates. A single plaque was excised, resuspended in 2 mL of sterile saline, and then passed through a 0.45 µm syringe filter. The method was repeated three times to ensure isolation of a single phage. Phage 2002 was isolated from sewage water (Vidy wastewater treatment plants, Lausanne, Switzerland). Briefly, a sample of wastewater was added to a mixture of five different *S. aureus* strains (S144-1, S47-1, Bk, G04 and Jn) in liquid culture then plated in double-layer TSA plates. A single plaque was excised and the phage was purified as described above for Phage 2003. To produce large quantities of phage, amplification was performed in double-layer agar plates using *S. aureus* strain 8325-4 as a susceptible host. For each agar plate, the soft layer containing phages was scraped and suspended in 10 ml of sterile saline, homogenized by vortex then centrifuged at 4000 rpm for 40 minutes. The supernatant containing phages was then passed through a 0.45 µm filter. The concentration of the phage preparation was determined using double-layer assays. The host-range of each phage from the cocktail was determined in double layer-assays for 83 diverse *S. aureus* isolates.

Assessment of phage resistance development. *S. aureus* AW7 was infected with each of the four phages independently, as well as with a multiphage cocktail at a multiplicity of infection (MOI) of 0.1 in 96-well plates. Plates were incubated at 37°C for 24h. Optical density (OD_{595}) was recorded every ten minutes.

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Checkerboard assay. Phage-antibiotic synergy was determined using checkerboard assays. Briefly, two-fold dilutions of teicoplanin beginning at 32 µg/ml, and ten-fold dilutions of the phage cocktail starting at a concentration of ~$2x10^7$ PFU/ml were prepared in sterile TSB. Decreasing concentrations of teicoplanin (50 µl volumes) were distributed along columns of a 96 well plate, and decreasing concentrations of phage (also 50 µl volumes) were distributed along rows. A suspension of *S. aureus* AW7 (~$1x10^6$ CFU/ml) was prepared in sterile TSB, and 100 µl was distributed into each well (total volume 200 µl in each well, final inoculum $5x10^5$ CFU/ml). Each well from one column served as a growth control (no antibiotic or phage), and a second column was not inoculated with bacteria (no growth control). The lowest concentration of phage, antibiotic and the combination of both to inhibit bacterial growth was determined by assessing turbidity with an unaided eye, as defined by European Committee on Antimicrobial Susceptibility Testing guidelines.

Rabbit red blood cell lysis assay. *S. aureus* AW7 was grown to mid-exponential phase, at which point cells were treated with sub inhibitory concentrations (1/4 of the MIC) of antibiotics and phages alone or in combination for 6 hours (teicoplanin 0.125 ug/ml, linezolid 0.25 ug/ml, phages at an MOI of 0.001). Cultures were centrifuged and supernatants were filter sterilized. Rabbit red blood cell lysis experiments using filtered supernatants were then performed as described previously (7). Four biological replicates were performed for each experimental condition. Linezolid reduces exotoxin production in *S. aureus* and was included as a control.

Experimental model of ventilator associated pneumonia in rats (additional information)

Power calculation. In order to perform a power calculation, we hypothesized that treatment with phages and standard antibiotic therapy would increase survival from 20% to 80% within 4 days (primary outcome). These estimates, with an alpha=0.05 and a power (1-β) = 0.8 required a sample size of 13 per group (Power analysis performed with SigmaPlot 12.0, module “Sample Size for Proportion”). Not all of the animals were fit enough to be randomized following surgery as determined by the Cantonal Committee on Animal Experiments of the State of Bern (approval BE 83/17), thus the sample sizes for each group were 10-12 animals.

Blinding Procedure. Treatment syringes (phages, antibiotic or a combination of both) as well as placebo control syringes (0.9% NaCl) were prepared and four doses were packaged into unmarked envelopes. Envelopes were randomly delivered to the surgeon prior to the initiation of therapy by an independent researcher who did not participate further in the study.

Criteria for Euthanasia. Animal welfare was determined at least three times per day using an in-house Welfare Score Sheet for Rodents, which is summarised in Table 1. Animals were excluded from randomisation if they showed persistent respiratory instability, missing reflexes as measured by a failure to withdraw from painful stimulus, or an inability to remain upright two hours after anesthesia. Animals were euthanized if the total score was greater than '1' or if body weight decreased by more than 20%.

Table 1. Welfare Score Sheet for Rodents

Observation	Details	Score
Attitude	BAR (bright/active/responsive)	0.0
	Burrowing or hiding, quiet but rouses when touched	0.1
	Markedly diminished exploratory activity when assessed for neurobehavioral score, vocalizes or aggressive when touched	0.4
Porphyrins	None	0.0
	Mild around eyes and/or nostrils	0.1
	Obvious on face and/or paws	0.4
Gait and Posture	Normal	0.0
	Mild incoordination when stimulated, hunched posture, mild piloerection	0.1
	Obvious ataxia or head tilt, hunching, drags one or both limbs, severe piloerection	0.4
Weight	Weight gain or weight loss < 5%	0.0
	Weight loss 5 – 10 %	0.1
	Weight loss > 10 %	0.4

Secondary outcomes

Bacterial and phage loads in tissues and blood. Bacterial loads were determined from heparinized blood and organs. Organs were first mechanically homogenized in weight-adapted volumes of 0.9% NaCl (2 ml for spleens, 3 ml for lungs). Samples were serially diluted and plated onto TSA. Plates were incubated at 37°C and colonies were counted the following day. Phage loads were determined using double-layer agar plates, whereby the top agar layer consisted of 100 µl of diluted sample and 3.0 x 10^7 CFU of MRSA AW7. Plates were incubated at 37°C and plaques were counted the following day.

Histopathology and histopathological grading. Cranial and caudal tissue samples of the right and left lung from each rat were fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin. Blocks were sectioned and slides were stained with hematoxylin and eosin (H&E). On H&E-stained sections, morphologic changes were recorded as previously

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described Montgomery et al(8). Microscopic evaluation was performed in a blinded fashion without knowledge of treatment group by a European board certified veterinary pathologist (CG).

Inflammatory Cytokine Analysis. Heparinized blood was centrifuged at 2,000 g for 5 minutes, and the plasma was frozen at –80°C for future analysis. Plasma concentrations of inflammation biomarkers interleukin (IL)–6, IL-1α, IL-1β, IL-10, IL-17A, IL-18 and tumor necrosis factor (TNF)–α were determined by microsphere-based multiplex immunoassay (BioRad, BioPlex pro Luminex Assay USA) on the Luminex platform according to the recommendations of the manufacturer.

Phage resistance assays. Phage cross streak assays were performed to assess phage resistance as described previously by Duerkop et al.(9). Briefly, 50 µl of the phage cocktail was streaked in a straight line across the surface of a TSA plate. Single colonies harvested from lung tissue homogenates were resuspended in 20 µl of sterile PBS then 5µl of the suspension was streaked across the plate intersecting the phage in a perpendicular arrangement. Plates were incubated overnight at 37°C and susceptibility was determined by the absence of bacterial growth within the phage streak. Five colonies from each of ten lungs (n=50) from animals treated with phage, and the same from animals treated with a combination of phage and antibiotics were tested.

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Online Data Supplement (figures)

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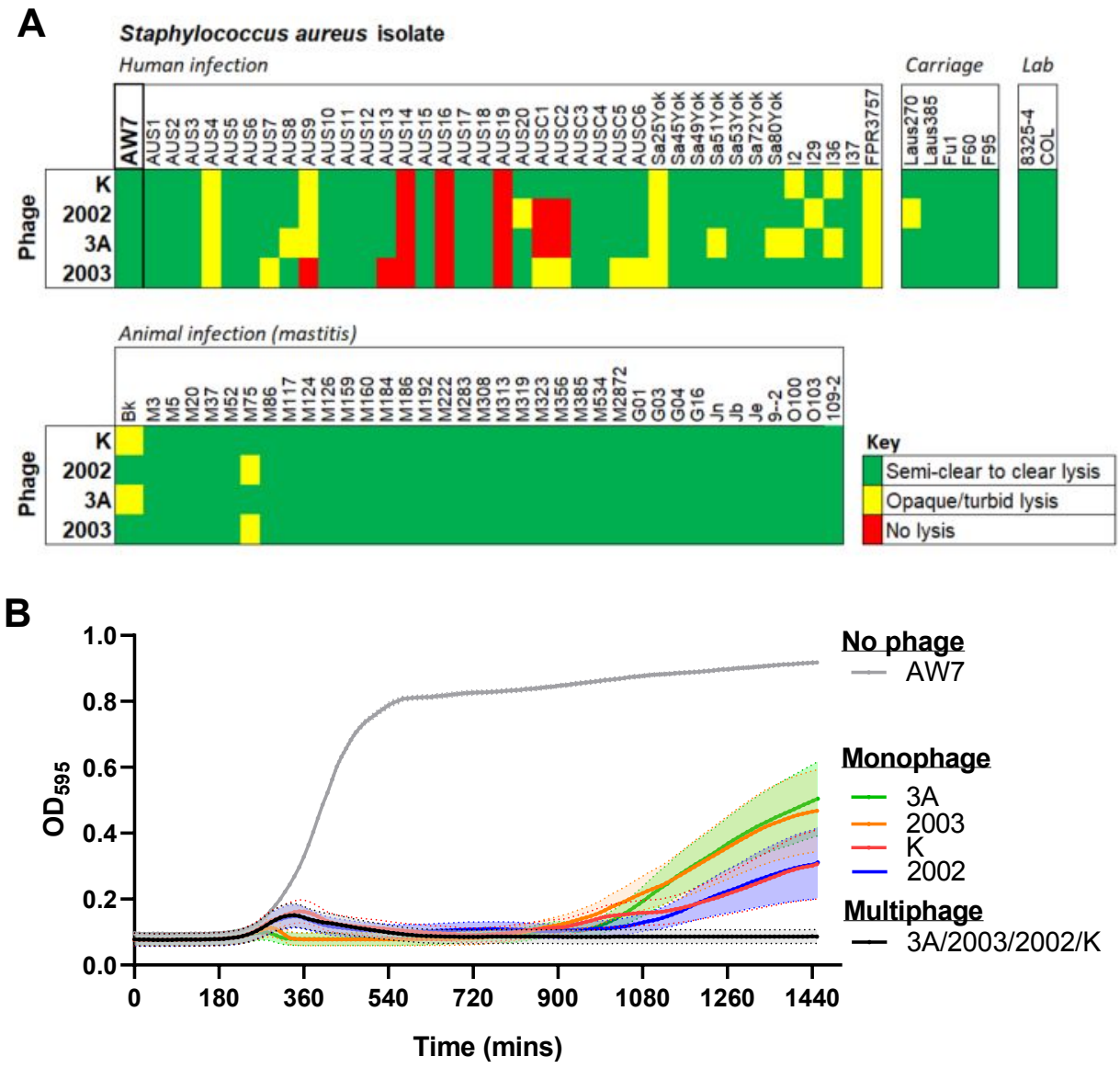

Figure E1. Characterization of the anti-*Staphylococcus aureus* phages used in this study. Host-range determination for each phage against 83 staphylococcal isolates from diverse sources **(A)**. *In vitro* assessment of phage resistance development. *S. aureus* AW7 was infected with each of the four phages independently, as well as with a multiphage cocktail at a multiplicity of infection (MOI) of 0.1. Optical density (OD_{595}) was recorded every ten minutes. Data are presented as the mean (line) ± SEM (fill) from 4-5 replicates **(B)**.

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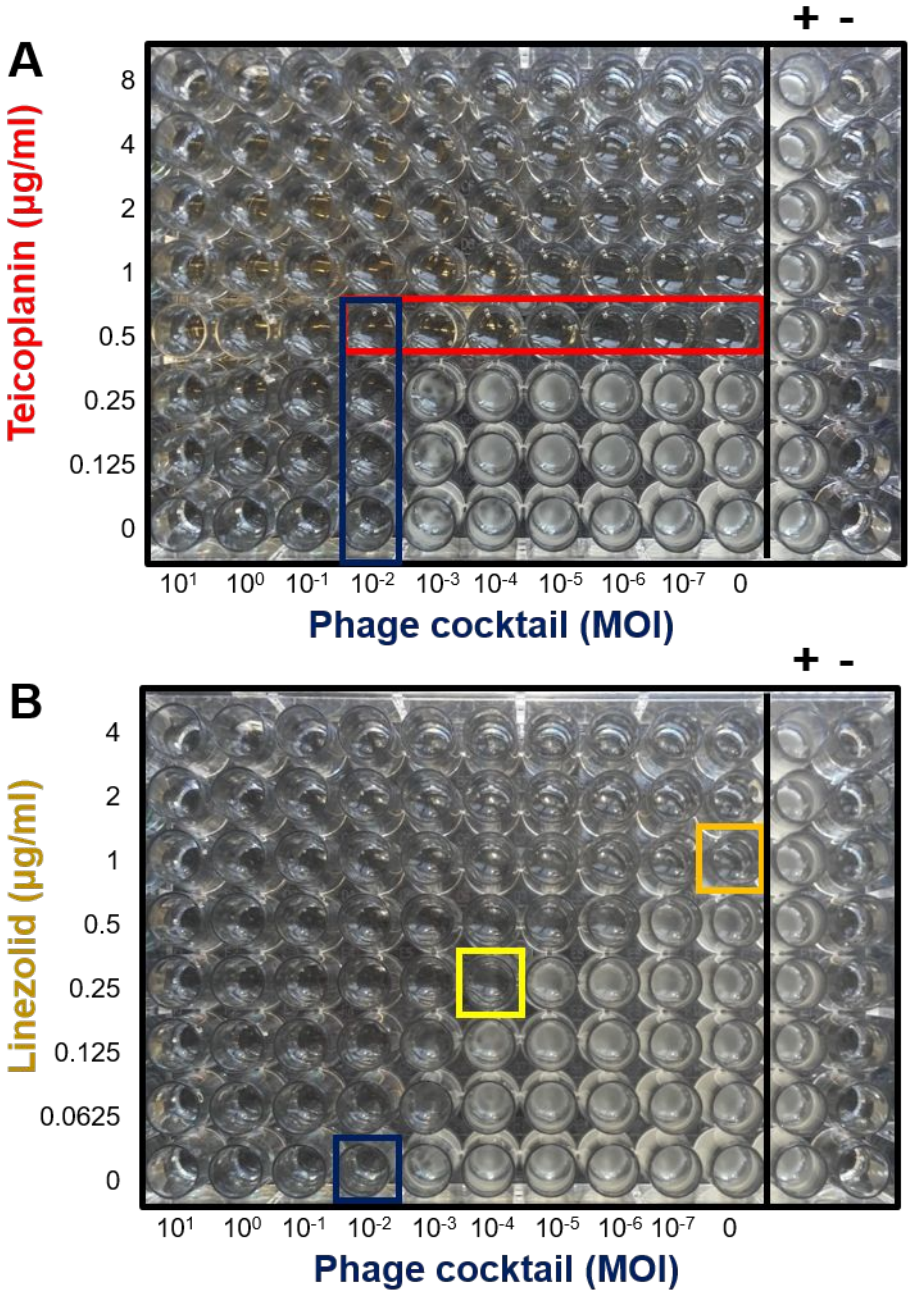

Figure E2. Determination of phage-antibiotic synergism/antagonism for *Staphylococcus aureus* strain AW7. The minimum inhibitory concentration (MIC) for teicoplanin was 0.5 µg/mL (red box) and the minimal inhibitory multiplicity of infection (MIMOI) for the phage cocktail was 0.01 (blue box). No synergy between phages and teicoplanin was observed **(A)**. The MIC for Linezolid was 1.0 µg/mL (orange box). The combination of phages at an MOI of 0.0001 and linezolid at 0.25X the MIC inhibited growth (yellow box), indicating synergism **(B)**. +, growth control; -, uninoculated control; MOI, multiplicity of infection.

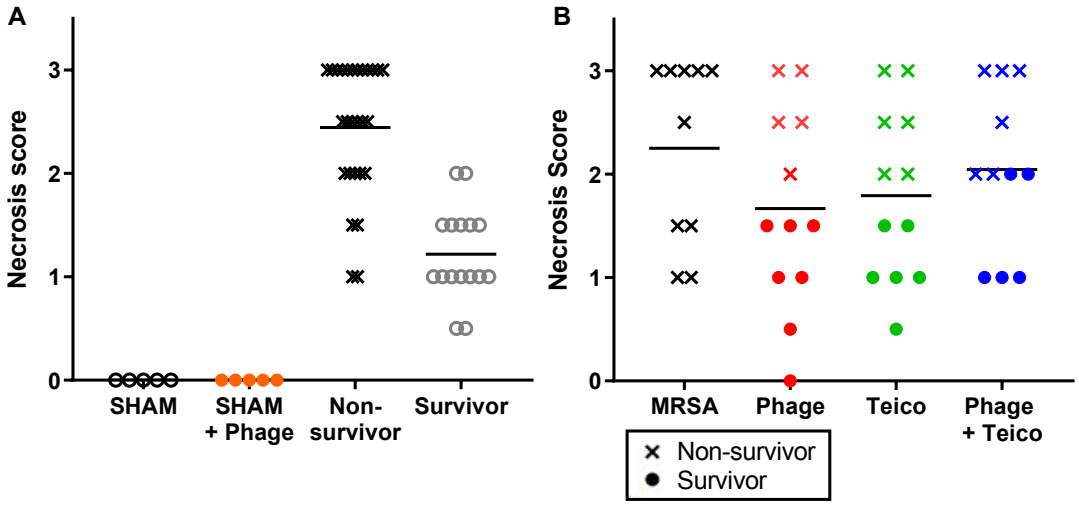

Figure E3. Necrosis scoring of lung tissue following MRSA VAP. Necrosis of lung tissue was scored as follows: 0, no necrosis; 1, mild necrosis (1-9%); 2, moderate necrosis (10-50% of the sample); 3, severe necrosis (>50% of the sample) **(A).** Comparison of Necrosis scores for the lungs of animals treated with phages, teicoplanin or a combination of both **(B)**. Bars represent the mean.

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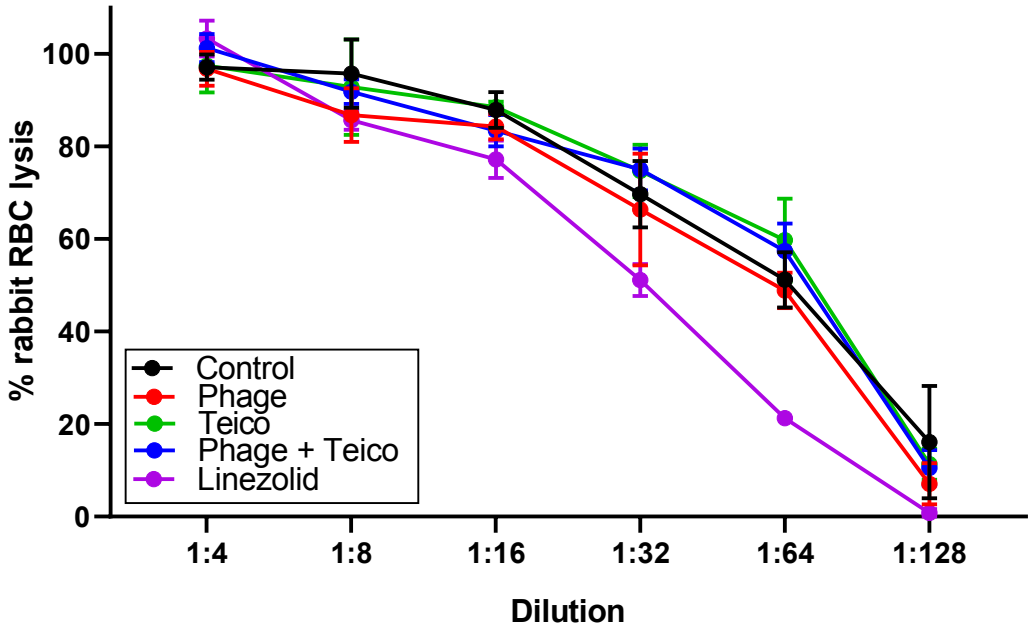

Figure E4. Functional assessment of exotoxin activity as inferred from rabbit red blood cell lysis. Cultures of *S. aureus* AW7 were exposed to sub inhibitory concentrations of antibiotics and/or phages for six hours. Serial dilutions of filtered supernatants were then added to rabbit red blood cells at various dilutions and lysis was monitored by assessing optical density (550nm). Linezolid, which reduces exotoxin production in *S. aureus*, was included as a control. Data are represented by the mean and standard error of four biological replicates.