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Overcoming bacteriophage insensitivity in *Staphylococcus aureus* using clindamycin and azithromycinat sub-inhibitory concentrations

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Abstract:

Staphylococcus aureus is a pathogen of major concern in both acute infections as well as chronic conditions such as chronic rhinosinusitis (CRS). Bacteriophage (phage) therapy has been considered in Western countries as early as the 1920s and has recently regained interest for its potential to treat infections caused by antibiotic resistant strains including Methicillin Resistant *Staphylococcus aureus* (MRSA). However, bacteria can adapt and become resistant to phages. The ability to overcome phage resistance is considered critically important for phage therapy applications. Here we show that sub-inhibitory concentrations of Protein Synthesis Inhibitor (PSI) antibiotics clindamycin, erythromycin and azithromycin could sensitise phage-resistant*S. aureus* strains to phages *in vitro* and *in vivo*. All 3 antibiotics showed synergy when combined with multiple lytic phages against 9 *S. aureus* clinicalisolates in planktonic growth and 2 strains forming biofilms. The combination of clindamycin and phages was safe and could eradicate *S. aureus*sinonasal biofilms in a murine model of sinusitis. This data supports the potential use of phage-PSI antibiotic combination therapies in particular for difficult to treat infections with phage-resistant*S. aureus* and MRSA strains.

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Introduction

Staphylococcus aureus recurrent infections and biofilms are associated with recalcitrant chronic rhinosinusitis (CRS) (1-3). The rapid emergence of antibiotic resistant bacteria is making the treatment of recurrent infections and biofilm states more challenging (4) and drives the need for the development of alternative antimicrobial strategies. Bacteriophages (phages) are viruses that can infect and lyse specific bacterial species whilst leaving commensals and the human host unharmed. Phages can kill antibiotic resistant *S. aureus* strains (MRSA), making them an attractive target for treating antibiotic resistant infections (5). A primary weakness of the phage therapy concept however is the existence or emergence during treatment of phage resistant strains. Phage resistance can result from several mechanisms, including modification of cell-surface receptors and restriction-modification of incoming phage DNA (6). To delay the appearance of phage insensitive mutants during phage treatment, phage cocktails are preferred rather than phage mono-therapies (7). In addition, combination therapies with antibiotics can be beneficial to help in killing phage resistant mutants as they emerge during phage treatment thus improving the overall efficacy of bacterial killing (8). In both of these cases the emergence of resistance to one phage or antibiotic is often insufficient for bacterial survival potentially allowing the eradication of the infection.

Antibiotics have previously been shown to affect phage infectivity and promote prophage induction and virulence modulation of bacteria.As early as 1947 Price and Krueger had reported that more phage particles were produced from bacteria in the presence of penicillin (9, 10). There have also been reports of the stimulation of phage development in *Escherichia coli*(11)and *S. aureus*(12)by β-lactam antibiotics, and prophage induction and virulence modulation of *S. aureus* by ciprofloxacin and trimethoprim (13). More recently, some reports showed that low, sub-inhibitoryconcentrations of certain antibiotics can substantially stimulate the host bacteria's production of virulent phage, and this effect has been termed phage-antibiotic synergy (PAS) (8). PAS has been shown to facilitate the killing of *Pseudomonas aeruginosa*(14, 15) and the eradication of *E. coli* (16) and *S. aureus* biofilms (17).However, those strategies enhance phage activity when targeting phage sensitive strains and they do not sensitise phage-resistant*S. aureus*strains to phages.

Previously we have demonstrated the safety and efficacy of phage cocktails targeting *S. aureusin*

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vitro (17)and in a sheep model of sinusitis (18). We have subsequently used the anti-*S. aureus* phage cocktail AB-SA01 in a Phase 1 Human Clinical Trial (HCT) in *S. aureus* associated CRS patients. Topical AB-SA01 was safe and well tolerated with no dose-limiting side effects in any of the study participants (19). The same cocktail was also considered safe when applied intravenously in patients with severe *S. aureus*bacteraemia(20). In both studies, however, some patients were excluded because their *S. aureus* strains were found to be resistant to AB-SA01. The aim of the present study was to evaluate whether specific antibiotics could sensitise phage-resistant*S. aureus* isolates to phage infection*in vitro* and *in vivo*.

Materials and Methods

Phages, clinical isolates and antibiotics

The study was approved by The Central Adelaide Local Health Network (CALHN) Human Research Ethics Committee (HREC/15/TQEH/132) and only patients that had provided written informed consent were included in the study. *S. aureus* ATCC51650 and ATCC25923 were obtained from the American Type Culture Collection (Manassas, VA, USA) and used as reference strains. In addition, a total of 15 *S. aureus* clinical isolates (CIs) were isolated from the sinonasal cavities of CRS patients at The Queen Elizabeth Hospital (TQEH) (Woodville, SA, Australia) from April 2013 to November 2015. In each case, *S. aureus* was cultured by an independent diagnostic laboratory and the antibiotic susceptibility was determined by disc diffusion on Mueller-Hinton agar per Clinical and Laboratory Standards Institute (CLSI) recommendations (Adelaide Pathology Partners, Mile End, SA, Australia). Bacteria were isolated on Columbian horse blood agar plates, colistin nalidixic acid plates, or cystine lactose electrolyte–deficient plates (Oxoid, Thebarton, SA, Australia). Positive cultures were confirmed to be *S. aureus* using latex agglutination testing. Phages J-Sa36, Sa83 and Sa87 were obtained from AmpliPhi Australia (Brookvale, NSW, Australia).

Antibiotic sensitivity testing

The minimum inhibitory concentration (MIC) of amoxicillin, vancomycin, erythromycin, clindamycin and azithromycin against the *S. aureus* strains was assessed using the broth microdilution technique as previously described (21) and breakpoints were defined according to CSLI guidelines. Clinical strains resistant to at least one antibiotic in three or more antibiotic

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categories were defined as multidrug resistant (MDR).

Phage sensitivity test

The sensitivity of *S. aureus* isolates (15 clinical isolates and 2 ATCC strains) to phages J-Sa36, Sa83 and Sa87 was determined as described (17). Briefly, 0.5 McFarland Units of *S. aureus*CIs diluted 1:100 in 10 ml Tryptic soy broth (TSB) were grown overnight at 37°C with shaking. 200 µL of overnight broth cultures were spread onto Columbian blood agar plates evenly (Oxoid, Thebarton, SA, Australia). Plates were incubated at 37°C for 1 hour and 5 µL [10^6 plaque forming units (PFU)] of 3 phages were spotted in triplicates onto the plates and incubated at 37°C overnight. Phage buffer solution (SM buffer) alone was spotted in the centre of the plates as negative control. Phage sensitivity was determined as previously described(5). Confluent lysis zones observed on plates were defined as phage sensitive, semiconfluent lysis zones were defined as phage semi-sensitive and no plaques were defined as phage insensitive.

Determining the effect of phage J-Sa36, Sa83 and Sa87 combined with antibiotics on free-floating planktonic *S. aureusin vitro*

Initial screening was performed in 100 µl in 96 well plates, with follow up experiments in 10 mL culture volume. *S. aureus* strains were grown on 1.5 % Tryptone Soya Agar (TSA) at 37°C overnight. Single colonies were transferred to Tryptone Soya Broth (TSB) to generate a culture with a starting concentration of 5 x 10^5 CFU/ml. Phage J-Sa36, Sa83 and Sa87 were added to the cultures at multiplicity of infection (MOI) of 0.2 according to predetermined phage titers (described below). Antibiotics were added to the cultures at ½ MIC (according to the pretest MIC of antibiotics) if required. Optical density (OD) readings at 600 nm were recorded at 24 hours to assess the growth of bacteria co-inoculated with phage unless otherwise specified. Uninoculated, fresh media was included as a sterility control.

Determination of phagetiters

Phage titers were determined against *S. aureus* RN4220 using the double layer spot assay technique. Briefly, 100 µl of *S. aureus* (RN4220) overnight culture was mixed with 4 mL of 0.4% TSA and poured onto a 1.5 % TSA plate. After 20 minutes, serially diluted samples of phage J-Sa36, Sa83 or Sa87 were spotted onto the double layer agar plates in 5 µl spot volumes

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in triplicates. After the plates were incubated in a humidified incubator at 37°C overnight, plaques were counted, counts were averaged, and titers of the filtrate were calculated based on the dilution.

Determining phage replication by titer

A 100 µl sample was taken from a *S. aureus* culture (10 ml in TSB) after 0 and 48 hours following treatment with clindamycin, phage or a phage clindamycin co-incubation. The sample was treated with 25µl of chloroform, shaken for 30 seconds, centrifuged (3000 rpm, 5 minutes, room temperature) and the supernatant used to determine phage titers against the same isolate. Phage titers in plaque forming units [PFU]/ mL were recorded initially and at 48 hours to assess for phage replication. To allow for phage resistant bacterial isolates to show plaques, clindamycin was added to the soft agar overlay.

Determining the effect of phage J-Sa36, Sa83 and Sa87 in combination with clindamycin on *S. aureus* biofilms

The effect of J-Sa36, Sa83 and Sa87 combined with clindamycin was tested on *S. aureus* biofilms formed by ATCC51650 and CI11. Briefly, a 1.0 McFarland unit *S. aureus* culture suspended in 1:15 dilution in TSB was added to 96-well microtiter plates (Costar; Corning Incorporated, Corning, NY, USA). Plates were incubated for 48 hours at 37°C on a rotating platform at 70 rpm (3D Gyratory Mixer; Ratek Instruments, Boronia, VIC, Australia) to allow biofilm formation followed by washing with phosphate-buffered saline. Biofilms were then treated with 8 $x10^6$ plaque forming units (PFU) of J-Sa36, Sa83 or Sa87 and clindamycin added with a final concentration of 1 µg/ml, 0.5 µg/ml, 0.2 µg/ml, 0.1 µg/ml, or 0.05 µg/ml, in TSB. Wells were treated for 24 hours at 37°C with gentle shaking. The AlamarBlue viability assay (Thermo Fisher Scientific, Waltham, MA, USA) was used as previously described (22)to determine the anti-biofilm activity of the phages. Fluorescence was measured in a microplate reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany) and the percent reduction of biofilm after treatment determined relative to untreated controls.

Time-dependent effect of the interaction of phage J-Sa36 and clindamycin in ATCC51650 and CI11

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(1) ATCC51650 or CI11 was incubated with ½ MIC clindamycin in 10ml TSB, then phage J-Sa36 (1×10^5 PFU/ml) was added into the above at different times (1h, 2h, 3h, 3.5h, 4h, 6h and 24h) and incubated for 24 hours. OD600 readings were taken at phage addition and following 24-hour incubation.

(2) ATCC51650 or CI11 was incubated with either ½ MIC clindamycin or phage J-Sa36 (MOI=0.2) for 3.5 hours followed by the addition of phage J-Sa36 (1×10^5 PFU/ml, MOI=0.2) or ½ MIC clindamycin respectively and continuous incubation with both ½ MIC clindamycin and phage J-Sa36 for 24 hours. OD600 readings were taken at addition of either phage or clindamycin and following the 24-hour incubation.

The total amount of Phage J-Sa36 added into the culture at different time points was adjusted to maintain MOI=0.2, according to the number of bacteria measured via CFU counts at each time point.

***In vivo* experiments**

Animal procedures were conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes, and were approved by the Animal Ethics Committee of the University of Adelaide, Australia （M-2019-049). All rats were housed under standard conditions.

Selection and preparation of the *S. aureus* clinical isolates for inoculation

A *S. aureus* clinical isolate (CI3) was selected for *in vivo* experiments that was sensitive to both antibiotics to be used, insensitive to Sa87 phage on planktonic inhibition assays and could be sensitised to phage in the presence of both antibiotics *in vitro*. A fresh culture of CI3 on 1.5% TSA plates was prepared 2 days before inoculation. A single colony was resuspended in 0.9 % saline (ca. 2ml) to turbidity reading 0.5 McFarland Units then 1 in 100 dilution in TSB was cultured overnight one day before the *in vivo* experiment. CI3 pellets were harvested and resuspended in fresh TSB to reach $2.5*10^9$ Colony Forming Units (CFU)/ml.

***In vivo* safety study**

Nine Specific Pathogen Free (SPF) Sprague Dawley rats (6 weeks old, males) were administered (n=3 rats/group)(23)20ul (1) Sa87 ($0.5*10^{10}$ PFU/ml) + ½ MIC Clindamycin in saline, (b) Sa87

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213 ($0.5*10^{10}$ PFU/ml) + ½ MIC Azithromycin in saline or (3) saline. Treatments were administered
214 intranasally twice a day for 21 days, the general wellbeing of the rat—including its appetite,
215 behaviour, and any weight change—being closely monitored. The rats were then humanely
216 sacrificed and the sinus mucosal tissue, lungs, heart, liver, kidneys, spleen and brain harvested.
217 Tissue was divided and kept fresh or 10% formalin-fixed, the latter then processed to paraffin
218 wax, and 6 µm sections cut and stained with haematoxylin and eosin for histopathological
219 examination. Formalin fixed tissue was embedded in paraffin and processed to undergo
220 Haematoxylin and Eosin staining and histopathological analysis. The fresh sinus mucosa, lungs,
221 heart, liver, kidneys, spleen, and brain were all analysed for phage titers. Faecal samples were
222 collected from all the rats prior to the administration of the first treatment, as well as every day
223 thereafter for phage titers. 500 µl blood samples were collected from the rat tail vein prior to the
224 first treatment and again at 1, 2, 4 and 6 hours following the morning treatment on day 7 and 14
225 of treatment. This was repeated at 1, 2, 4, 6, 12, 24 and 48 hours following the last treatments on
226 Day 21.
227
228 **Phage titers in faeces and organ tissues**
229 1 g of feces wassuspended in 10 mL of SM buffer, centrifuged at 3214 × g for 10 minutes
230 (Eppendorf 5810 centrifuge, North Ryde,NSW, Australia), filtered (0.22 µm), and titrated for
231 phageconcentration.1g of rat faeces was collected from every rat at day 0 and daily after the first
232 treatment, and directly placed into 5 mL SM buffer. Further, following humane killing, the rat
233 frontal sinus mucosa was dissected and placed directly into 5 mL SM buffer. 1cm*1cm pieces of
234 organ were collected from the brain, lungs, heart, liver, kidney and spleen and were also placed
235 into 5 mL SM buffer. All samples were then transported to the lab on ice. Phage titers and
236 enrichment were performed as described by Drilling et al (17). Briefly, organ and sinus tissues
237 were ground in 5mL SM buffer using a Qiagen TissueRuptor (Qiagen, Hilden, Germany). All
238 tissue samples were then centrifuged at 3000 g for 10 minutes, filtered through a 0.22µm of
239 syringe filter (Pall Corporation, San Diego, USA), and titrated for phage. 1g of faeces was
240 suspended in 5 mL of SM buffer, centrifuged at 3214 × g for 10 minutes (Eppendorf 5810
241 centrifuge, North Ryde, NSW, Australia), filtered using a 0.22µm filter, and titrated for phage
242 concentration.
243 For phage enrichment, 100µL of 0.5 McFarland Units of *S. aureus* RN4220 diluted 1:100 with

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10 ml TSB was grown overnight at 37◦C with shaking. Samples (1ml tissue and faeces supernatants) were then incubated overnight with 5ml TSB and 50 µL of *S. aureus* RN4220 overnight culture. The enriched samples were then centrifuged and filtered using a 0.22µm filter, then titrated for phage. Phage titers were determined against *S. aureus* RN4220 using the double layer spot assay technique as described above.

Phage titer in the blood sample

500ul of blood was collected from the rat tail vein. Blood tubes were kept on ice and transported into the laboratory within one hour after harvest and centrifuged at 1000rpm for 20 minutes at 4°C. The plasma was then harvested and filtered through a 0.22µm syringe filter. Phage enrichment was then performed, as above.

Histopathology

Rat nasal tissues were fixed in 10% neutral buffered formalin. Tissue samples were then decalcified using 9.5% nitric acid in 1% ethylenediaminetetracetic acid (EDTA), processed to paraffin wax, and 6µm sections cut and stained with haematoxylin and eosin (H&E). The samples were examined by a veterinary pathologist (J.F.), blinded to the treatments given.

Efficacy arm protocol

An established murine rhinosinusitis model was used with modifications(24-26). Thirty Sprague Dawley rats (6 weeks old, males) underwent general anaesthesia and placement into the right nasal cavity of a 10mm × 0.5mm × 0.5mm sponge(Gyrus ACMI, MA, USA), soaked in a 20ul0.5*10^8 CFU *S. aureus*CI3 solution(sensitive to clindamycin and azithromycin but insensitive to Sa87), diluted in 0.9% saline. The sponges were placed at approximately 15 mm from the anterior nostril, located in the ostiomeatal complex.The rats were monitored for a nine-day period to allow for mucosal biofilms to form, and, on the tenth day, the sponges were removed. A two-day recovery was given to the rat before treatments begun. The rats were then randomised into one of 6 treatment groups (n=5 rats each) to receive 20 µl (1) Sa87 ($0.5*10^{10}$ PFU/ml) + ½ MIC Clindamycin in saline, (2) Sa87 ($0.5*10^{10}$ PFU/ml) + ½ MIC Azithromycin in saline, (3) Sa87 ($0.5*10^{10}$ PFU/ml in saline), (4) ½ MIC Clindamycin in saline, (5) ½ MIC

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Azithromycin in saline, (6) saline.Treatments were administered intranasally twice a day for 14 days.

Rats were monitored for weight and respiratory alterations (respiration rate; nasal discharge, hyperaemia or pruritus; and sneezing) during treatment, and, 14 days post-treatment initiation, the rats were humanely sacrificed. Their sinus mucosa was harvested for CFU counting, biofilm quantification, histopathology, and phage enumeration.

Colony-forming unit (CFU) counting of sinus mucosa

A fresh (0.2*0.2cm) piece of sinus mucosa was harvested and directly placed in 250µl 0.9% saline. The mucosa was then homogenized on the highest setting for 30 seconds, or underwent vortex mixing on the highest setting for two minutes. The homogenized supernatants were then serially diluted in 0.9% saline and spotted on sheep blood agar in triplicates (Beckton Dickenson, Franklin Lakes, NJ, USA) and incubated at 37 ^{0}C overnight for CFU counting of *S. aureus*.

Biofilm biomass quantification on sinus mucosa

The quantification of biofilm biomass on sinus mucosa was determined using an established protocol (27). Briefly, the frontal sinus mucosa was harvested and placed into Dulbecco's Modified Eagle's Medium (Life Technologies, Carlsbad, California, USA) on ice. One piece of mucosa from each frontal sinus was stained with a LIVE/DEAD BacLight Bacterial Viability Kit (Life Technologies Australia, Mulgrave, Victoria, Australia). The stained mucosa was then examined at 20X magnification using a confocal laser scanning microscope (Zeiss LSM700, Carl Zeiss AG, Oberkochen, Germany). The z-stack images (21 slices, interval 1 µm) were then taken from each sample and, COMSTAT Version 2.1 software was used to measure the biomass of biofilms, the threshold setting being applied so as to minimise background staining(28, 29).

Post-treatment sinus microbiology analysis

A fresh (0.2*0.2 cm) piece of mucosa from each rat sinus was harvested and homogenized in 0.9% saline. The supernatant was then harvested and transferred into a new tube without centrifugation. The collected supernatant was serially diluted and plated on blood agar plates in triplicates. After overnight incubation at 37^{0}C, different bacterial colonies (according to the morphology and haemolytic reaction)were subcultured on Mannitol salt agar. Positive colonies

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on the Mannitol salt agar were confirmed to be *S. aureus* with a Staphylase Test Kit (Oxoid, Scoresby, Australia).Other colonies with different morphologies were further identified using MALDI-TOF (Bruker, Preston, Vic, Australia).

Post-treatment sinus *S. aureus* Phage Sensitivity

S. aureus isolates, cultured from the sinuses of rats, were sub-cultured and tested for sensitivity to phage Sa 87 using phage sensitivity testas specified above.

Statistical analysis

GraphPad Prism (GraphPad Prism version 8.00; GraphPad Software, La Jolla, CA) was used to graph and analyse statistical significance of data. Statistical significance for in vitro and in vivo results was computed using a one-way analysis of variance (ANOVA) or mixed ANOVA. Significance was determined at a p-value < 0.05.

Results

1. Synergism of phages combined with antibiotics to kill *S. aureus in vitro*

Of the 17 *S. aureus* isolates [ATCC51650, ATCC25923 and 15 clinical isolates (CIs)], the two ATCC strains and 10 of the 15 CIs were sensitive or showed intermediate sensitivity to all five antibiotics tested (amoxicillin, vancomycin, erythromycin, clindamycin and azithromycin). A further 3 isolates exhibited resistance to only erythromycin, 2 isolates exhibited resistance to azithromycin and 2 exhibited multidrug resistance. The isolates were also tested for susceptibility to the 3 phages, J-Sa36, Sa83 and Sa87. J-Sa36 was active *in vitro* against only 2/17 strains tested (sensitive strains); 2/17 strains were semi-sensitive and 13/17 strains were insensitive (resistant). The *in vitro* activity observed for Sa83 and Sa87 was considerably higher; for Sa83, 11/17 isolates were sensitive or semi-sensitive and 6/17 insensitive and for Sa87, 12/17 isolates were sensitive or semi-sensitive and 5/17 insensitive **(Supplementary Table 1)**.

To evaluate if the combination of phage and antibiotics would be more effective than either treatment alone, we screened J-Sa36, Sa83 or Sa87 (MOI = 0.2) in combination with ½ MIC of all 5 antibiotics against 9 isolates. *S. aureus* ATCC51650 and CI's (CI2, CI3, CI4, CI6, CI7, CI8, CI9, CI11) were selected to represent a range of phage sensitivities. All 9 isolates were either

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resistant (7/9) or semi-sensitive (2/9) to phage J-Sa36 alone, whilst 4/9 and 5/9 were resistant or semi-sensitive to phages Sa83 and Sa87. A synergistic effect was defined as a statistically significant decrease in optical density (OD) that was larger than the combined decrease in OD of antibiotic and phage only controls. The combination of any phage with either of the cell wall actives (amoxicillin and vancomycin) did not show any significant additive or synergistic effects. In contrast, all 9 isolates showed synergism with the combination of a PSI antibiotic (erythromycin, clindamycin and azithromycin) and at least 1 phage. **Figure 1** illustrates the results for one representative *S. aureus* CI3 and Supplementary Figure 1 shows the results for all isolates.

Combination treatment of J-Sa36 with ½ MIC clindamycin and azithromycin showed synergistic effects against 6 of the 9 phage-insensitive and semi-sensitive isolates, with erythromycin showing synergistic effects in 5 of 9 isolates. For those isolates insensitive to Sa83 the addition of ½ MIC of erythromycin, clindamycin and azithromycin significantly improved antimicrobial activity in 3 out of 4 isolates. For isolates insensitive or semi-sensitive to Sa87, addition of ½ MIC erythromycin, clindamycin and azithromycin improved the antimicrobial activity in 4 out of 5 (for erythromycin) and 5 out of 5 (for clindamycin and azithromycin) insensitive and semi-sensitive isolates (**Table 1).** As clindamycin was the most effective antibiotic in combination with all 3 phages, further investigation was focused on this antibiotic.

2. Dose-dependence of clindamycin in combination with phage J-Sa36 to kill *S. aureus*

J-Sa36 semi-sensitive and insensitive isolates ATCC51650 (MIC=0.2μg/ml) and CI11 (MIC=0.2μg/ml) were each co-incubated with J-Sa36 and a range of clindamycin concentrations to determine the minimum dose of clindamycin required for facilitating phage J-Sa36 anti-microbial activity. Incubation with 0.025μg/ml (1/8 MIC), 0.05μg/ml (1/4 MIC) or 0.1μg/ml(1/2 MIC) in combination with phage J-Sa36 equally reduced OD values compared to positive controls after 24 hours incubation ($p<0.01$). The lowest effective concentration of clindamycin required to facilitate phage J-Sa36 killing of ATCC51650 and CI11 was 0.025μg/ml, $1/8^{th}$ the MIC. Lower (0.01μg/ml or 0.005μg/ml) clindamycin concentrations combined with phage J-Sa36 showed no significant reduction in OD compared to either the J-Sa36 only treatment or medium only control (**Figure 2**).

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3. Phage J-Sa36, Sa83 or Sa87 effect on preformed *S. aureus* biofilms with and without clindamycin

Clindamycin alone significantly reduced the viability of preformed ATCC51650 and CI11 biofilms at a concentration of 0.5 μg/ml (MIC=0.2 μg/ml) and above with lower concentrations having no significant effect on biofilm viability. Treatment with phages J-Sa36, Sa83 or Sa87 (PFU=$8x10^6$/ml) alone did not affect CI11 biofilm viability, and J-Sa36 did not affect ATCC51650 biofilm viability. However, the combination of ≥ 0.1μg/ml (1/2 MIC) clindamycin with J-Sa36, Sa83 or Sa87 significantly reduced the viability of ATCC51650 and CI11 biofilms, compared to untreated, clindamycin only and phage only controls. The combination reduced the required concentration of clindamycin to significantly reduce biofilm viability by five-fold **(Figure 3)**.

4. Clindamycin-dependent increase in phage infectivity in ATCC51650 and CI11 is constrained in time

To determine if phages were sensitising*S. aureus* isolates to clindamycin or if clindamycin treatment was facilitating phage infection of *S. aureus* phage insensitive and semi-sensitive strains, both ATCC51650 and CI11 in liquid culture were incubated with either ½ MIC clindamycin or phage J-Sa36 (MOI 0.2) for 3.5 hours. Following this the alternate treatment was added before continuous incubation with ½ MIC clindamycin and phage J-Sa36 for 24 hours. As shown in **Figure 4 (A-B)**, incubation with ½ MIC clindamycin or J-Sa36 only did not significantly affect bacterial viability at 24 hours. However, pre-incubation of ATCC51650 or CI11 with ½ MIC clindamycin for 3.5 hours, followed by incubation with J-Sa36 resulted in almost complete eradication of both ATCC51650 and CI11 after 24 hours. Reversing the sequence of challenge, namely incubation of the strains with J-Sa36 for 3.5 hours, followed by incubation with ½ MIC clindamycin for 24 hours resulted in a smaller but still significant reduction ($p < 0.01$) in OD compared with untreated control. To confirm time-dependent effects of this co-treatment, ATCC51650 or CI11 were incubated with ½ MIC clindamycin in 10ml TSB, then phage J-Sa36 was added into the above at different times (1h, 2h, 3h, 3.5h, 4h, 6h and 24h) (with the phage adjusted at the different time points to maintain MOI=0.2). Following 24 hours incubation, significantly reduced OD600 values were observed for cultures where J-Sa36

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was added up to 4 hours post-clindamycin challenge but not when J-Sa36 was added 6 and 24 hours after challenge **(Figure 4 C-D)**.

5. Sub-inhibitory (below MIC concentrations) clindamycin enhances phage activity

To determine whether clindamycin enhanced phage activity or induced an endogenous prophage, phage titers were determined from cultures of ATCC51650, CI11 and CI8 after incubation for 48 hours with ½ MIC clindamycin, phage or both. 100µl of the resulting culture was then chloroform treated to release phage and the supernatant used to determine phage titers usingATCC51650, CI11 and CI8 as host. The initial phage inoculum of 10^4 PFU/ml at 0 hours generated plaques only against ATCC51650 in the presence of clindamycin in the agar overlay but no plaques were observed for CI11 or CI8. This suggests that a high MOI might be required in addition to clindamycin to facilitate phage lysis in these strains. The supernatant of the isolates exposed to ½ MIC clindamycin only for ATCC51650, CI11 and CI8 produced no plaques in any of the strains after 48 hours incubation, indicating no intact prophages that could lyse those isolates were induced by clindamycin treatment. For the phage only treatment groups, J-Sa36 added to ATCC51650 produced no significant increase in phage titer and CI11 produced no plaques, supporting that J-Sa36 cannot infect and replicate in these isolates in the absence of clindamycin. Similar results were observed for phage Sa83 with phage only treatment of CI11 for 48 hours not producing any plaques. In contrast, co-treatment of ½ MIC clindamycin + phage led to increased phage titers in all cases. After 48 hours, ATCC51650 treated with J-Sa36 and ½ MIC clindamycin showed a concentration increase from ~10^4 PFU/ml to 7.73±2.14×10^6 PFU/ml. This result suggests that a clindamycin-mediated phage infection is capable of producing new phage particles, not simply leading to host cell death. The same result was observed for CI11 treated with J-Sa36 and ½ MIC clindamycin (1.9±1.3×10^9 PFU/ml), and Sa83 and ½ MIC clindamycin (6.4±2.6×10^7 PFU/ml). Whilst the same trend was also observed for CI8 with Sa83 and ½ MIC clindamycin a semi-confluent plaque was observed only at higher concentrations making quantification of titer impossible **(Supplementary Table 2)**.

6. Topical phage and PSI antibiotic combination *in vivo* safety evaluation

All animals survived the entire duration of the experiment, and the general wellbeing of the rats was uneventful and no different between the groups. No phages were detected in rat faeces at

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time point =0 or on day 1 and 2 post treatment initiation. Phage was only detected in faeces of phage-treated rats after enrichment protocols from day 3 onwards where low phage titers of < 10^4 PFU/ml were found **(Supplementary Table 3)**. Phage was not detected in the blood samples in any of the treatment groups at any of the time points (results not shown).21 days post-treatment, the rats were humanely sacrificed, and the rat nasal cavity, fresh mucosa, brain, lungs, heart, liver, kidneys, and spleen were harvested to investigate the titer of phage and histopathological changes. Phage was not detected in any of the organ samples from the different treatment groups; however, phage was detected in the nasal mucosa after enrichment in the phage-treated group only **(Supplementary Table 4)**. No clinically significant histopathological findings were noted in any of the organs or rat nasal mucosal samples **(Supplementary Figure 2)**.

7. Sub-inhibitory PSI antibiotics sensitise*S. aureus* insensitive strains to phage *in vivo*

From the 30 rats across the 6treatment groups, 27 completed the study protocol [1 rat from the azithromycin group and 2 rats from the saline control group died due to ketamine intoxication (diagnosed by the University of Adelaide Animal Welfare Officer)]. After 2 weeks of twice daily topical application of test treatments, control saline treated rats had similar *S. aureus* CFU counts/ml compared to rats treated with $0.5*10^{10}$ PFU/ml Sa87, ½ MIC clindamycin or ½ MIC azithromycin ($p > 0.05$). Sensitivity testing indicated similar sensitivity to the antibiotics and insensitivity to Sa87 for the post- and pre-treatment *S. aureus* isolates **(Supplementary Table 5)**. In contrast, no *S. aureus*were cultured in rats treated with $0.5*10^{10}$ PFU/ml Sa87 in combination with ½ MIC clindamycin or azithromycin and were significantly lower than control saline-treated rats or rats treated with Sa87 or ½ MIC clindamycin or azithromycin ($p=0.0086$) **(Figure 5A)**. However, bacterial colonies that were not *S. aureus* were grown from the nasal mucosa of those rats(CFU counts of <500 CFU/mL). MALDI-TOF analysis identified those bacteria to be *E. Coli* and *E. Faecalis***(Supplementary Table 6)**. Biofilm biomass quantitation showed similar biomass quantities in controls, Sa87 and ½ MIC clindamycin treated rats and a significant increase in biomass in ½ MIC azithromycin treated rats compared to control.In contrast, there was a significant reduction in biofilm biomass in rats treated with $0.5*10^{10}$PFU/mlSa87 in combination with ½ MIC clindamycin or azithromycin compared to control ($p<0.0001$) **(Figure 5B and 5C)**.

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Histopathology of the maxilloturbinates by a pathologist, blinded to the treatment given (J.F.) showed normal ciliated pseudostratified respiratory epithelium with no evidence of inflammation or glandular hyperplasia in rats treated with Sa87 in combination with ½ MIC clindamycin or azithromycin. In contrast, in rats treated with Sa87, ½ MIC clindamycin, ½ MIC azithromycin or saline only, there was evidence of cilial denudation and surface epithelial erosion, with desquamated necrotic luminal cellular debris admixed with neutrophils. The lamina propria was congested and oedematous and contained a more robust mixed inflammatory cell infiltrate, which also infiltrated the lining epithelium **(Supplementary Figure 3)**.

Discussion

To date no strategies have been reported that can sensitise*S. aureus* and MRSA phage resistant strains to phage.Consequently, patients infected withsuch strains are excluded from phage therapy with few or no therapeutic options remaining to treat those often-devastating infections. This study shows that sub-inhibitory (below MIC values) concentrations of PSI antibiotics clindamycin, erythromycin and azithromycin could sensitise phage resistant and semi-sensitive *S. aureus* clinical isolates to phage. Clindamycin and azithromycin were the most successful antibiotics with 6/9 isolates resistant and semi-sensitive to J-Sa36 and all 5/5 isolates resistant and semi-sensitive to Sa87sensitised to respective phages when combined with clindamycin or azithromycin. Sensitisation also occurred *in vivo* leading to eradication of phage-insensitive *S. aureus* biofilms in the presence of both antibiotics and Sa87.

To our knowledge this is the first report of PAS with clindamycin or azithromycin with previous studies demonstrating no synergistic effects and in fact a detrimental effect of clindamycin on phage production (30) and treatment efficacy (31). Both these studies used *S. aureus* isolates that were sensitive to phage however and combined phage with clindamycin treatments at concentrations at or above MIC values. Also, the Oduor et al (31) study used an *in vivo* MRSA sepsis model where MRSA was injected intravenously to test the effect of phage and clindamycin treatments. Hence the aim of both studies and thus their experimental designs are not comparable to the present study.

An important factor in the intractable nature of chronic relapsing bacterial infections in the context of chronic diseases like CRS are biofilm states that allow bacteria to resist antibiotic

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therapy. Often sub-inhibitory concentrations of antibiotics have been shown to induce biofilm formation as observed for the CA-MRSA strain USA300 LAC (32)making them detrimental to use in chronic infections. An increased biofilm biomass was also seen in this study *in vivo* in the presence of sub-inhibitory azithromycin. The addition of sub-inhibitory concentrations of PSI antibiotics, however, was necessary to mediate killing of *S. aureus* phage insensitive strains by phages, significantly expanding the scope and potential of phage therapy applications. Indeed, it has been recognised that the ability to overcome phage resistance at the onset or occurring during phage treatment represents the most valuable phenotype for a therapeutic phage candidate (33).

Previously it has been reported that PAS often involves antibiotics sensitizing bacteria to phage infection and increased phage production (9-13).To investigate if the PAS observed was due to increased phage activity, we determined whether the order of phage/ antibiotic addition was important and whether phage titers increased as a result of infection. Consistent with this model both co-incubation of phage and clindamycin and pre-incubation with clindamycin for up to 4 hours before phage addition allowed for a subsequent phage infection and bacterial killing. In contrast, pre-incubation with phage did not allow clindamycin-mediated killing. This suggests that clindamycin is able to effect a time-dependent change in the bacteria that facilitates phage infection, not the other way around. One possible explanation for this is reduction in *S. aureus* exoproteins. It has previously been reported that sub-inhibitory clindamycin and azithromycin can inhibit the production of *S. aureus* exoproteins (34, 35).This includes amongst others, protein A, one *S. aureus* exoprotein that has previously been demonstrated to protect *S. aureus* from phage by prevention of phage binding to the receptor protein, by masking phage receptor sites (36). Alternatively, clindamycin has also been shown to affect the expression of bacterial regulatory proteins and genes and therefore could also affect phage replication and/or assembly processes once inside the bacterial cell(34). The effect of co-treatment was no longer observed following a longer (> 6hr) delay before addition of the phage. Whilst the reason for this is not known, it might be due to activation of the SOS response of bacteria protecting from infection (37). Alternatively, clindamycin might undergo degradation after extended incubation in the presence of bacteria or their products thereby losing its antimicrobial activity (38). The exact mechanism of action of how PSI antibiotics promote *S. aureus*sensitisation to phage and supports a productive phage infection warrants further investigation.

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In this study, whilst phage in combination with sub-inhibitory clindamycin or azithromycin could eradicate a phage resistant *S. aureus* biofilm infection *in vivo*, *E. Coli* and/or *E. Faecalis*could be cultured from the mucosae of those rats, albeit at very low CFU counts. This is not unexpected however given we have used a specific pathogen-free rat model where a sponge was placed into the nasal cavity that might allow colonising bacteria or bacteria in the environment to thrive as a consequence of the presence of a foreign body. Importantly however, the mucosal biofilm biomass of those rats was low and histopathology showed no evidence of inflammation.

In conclusion, our findings show that the combination of antibiotics targeting protein synthesis and phages are safe and can be an effective treatment against *S. aureus* clinical isolates in both planktonic and biofilm states. In particular, clindamycin facilitated sensitisation of *S. aureus* phage resistant strains with increased sensitivity to phages at concentrations as low as 1/8 the MIC. This is a promising result and suggests combination therapy ofsub-inhibitory PSI antibiotics such as clindamycin or azithromycin and phages may be an effective approach against infections with difficult-to-treat planktonic and biofilm *S. aureus* infections with phage resistant strains.

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Author contributions: SL, YZ, AH conducted experiments, interpreted results and wrote the paper. KH, GZ, CB, HH conducted experiments. JF analysed data. SM, LS, AJP, KS interpreted results and wrote the paper. PJW designed the study, conducted experiments and wrote the paper. SV designed the study, interpreted results, wrote the paper.

Competing interests: SL, YZ, AH, KH, GZ, CB, HH JF, LS, AJP, KS, PJW & SV have no conflicts that are relevant to this work. SM has been an employee of Ampliphi.

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Table 1: Summary of screening the combination of bacteriophage J-Sa36, Sa83, Sa87 with erythromycin (E), clindamycin (C) and azithromycin (Az) to kill *Staphylococcus aureus* clinical isolates (CIs) from the sinonasal cavity of CRS patients and ATCC 51650. Effects were characterized as resistant (-) if no significant difference in OD was observed compared to untreated control, semi-sensitive (±) if a significant difference was observed but OD remained ≥ 10% of untreated control, sensitive (+) if growth remained < 10% of untreated control and synergistic (+) with shading if both sensitive and significantly reduced compared to antibiotic and phage only controls. Dark grey represents synergistic effects against phage-insensitive strains; light grey represents synergistic effects against semi-sensitive strains.

CIs	J-Sa36	Sa 83	Sa 87	E + J-Sa36	E + Sa 83	E + Sa 87	C + J-Sa36	C + Sa 83	C + Sa 87	Az + J-Sa36	Az + Sa 83	Az + Sa 87
2	±	-	-	+	+	+	+	+	+	+	+	+
3	-	-	-	+	+	+	+	+	+	+	+	+
4	-	+	+	+	+	+	+	+	+	+	+	+
6	-	+	±	-	+	+	-	+	+	-	+	+
7	-	-	+	-	+	+	-	+	+	-	+	+
8	-	+	±	+	+	+	+	+	+	+	+	+
9	-	-	-	-	-	-	-	-	+	-	-	+
11	-	+	+	-	+	+	+	+	+	+	+	+
ATCC 51650	±	+	+	+	+	+	+	+	+	+	+	+

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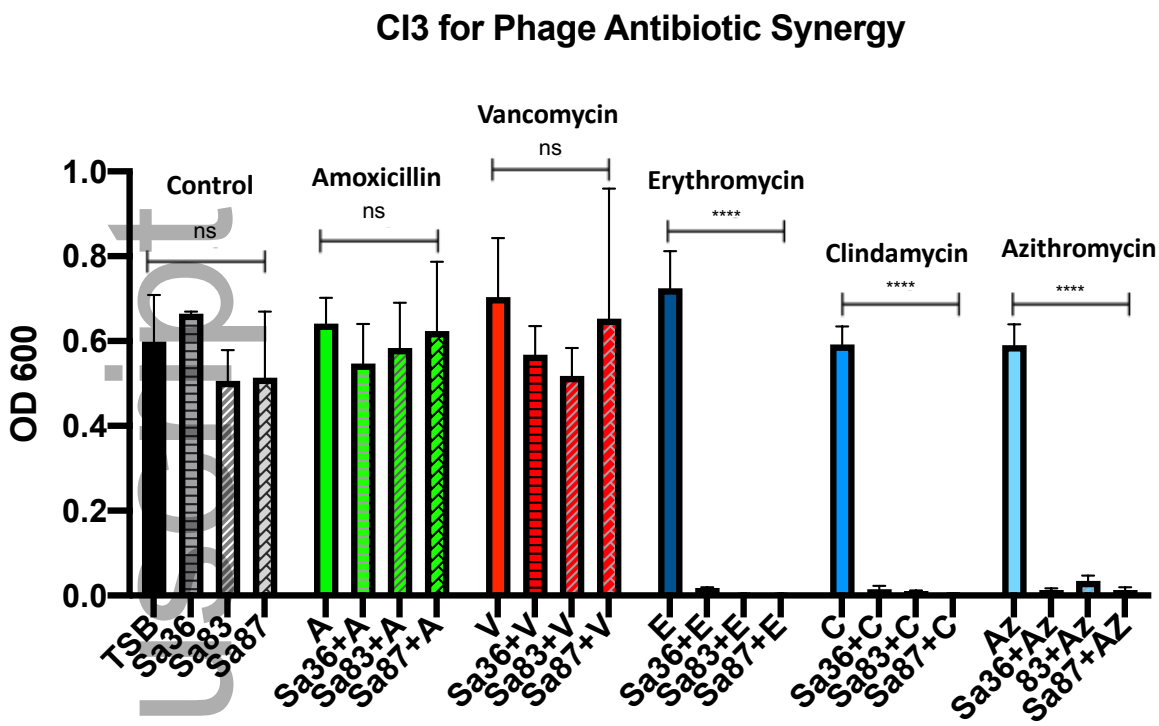

Figure 1. Protein synthesis inhibitors erythromycin (E), clindamycin (C) and azithromycin (Az), but not cell wall actives amoxicillin (A) and vancomycin (V), have synergistic effects with phage J-Sa36 (Sa36), Sa83 and Sa87 to reduce or eradicate CI3. OD600 values, representing bacterial viability, were measured after 24 hours in the absence (TSB) or presence of ½ MIC antibiotics alone, Phage alone (MOI = 0.2), or the combination of ½ MIC antibiotics and phage (MOI = 0.2). Significant reductions were observed compared to treatment with phage or antibiotic alone for all combinations of phage and protein synthesis inhibitors. n=3. Bars represent standard error of the mean (SEM). ns=not significant, ****, $p < 0.0001$.

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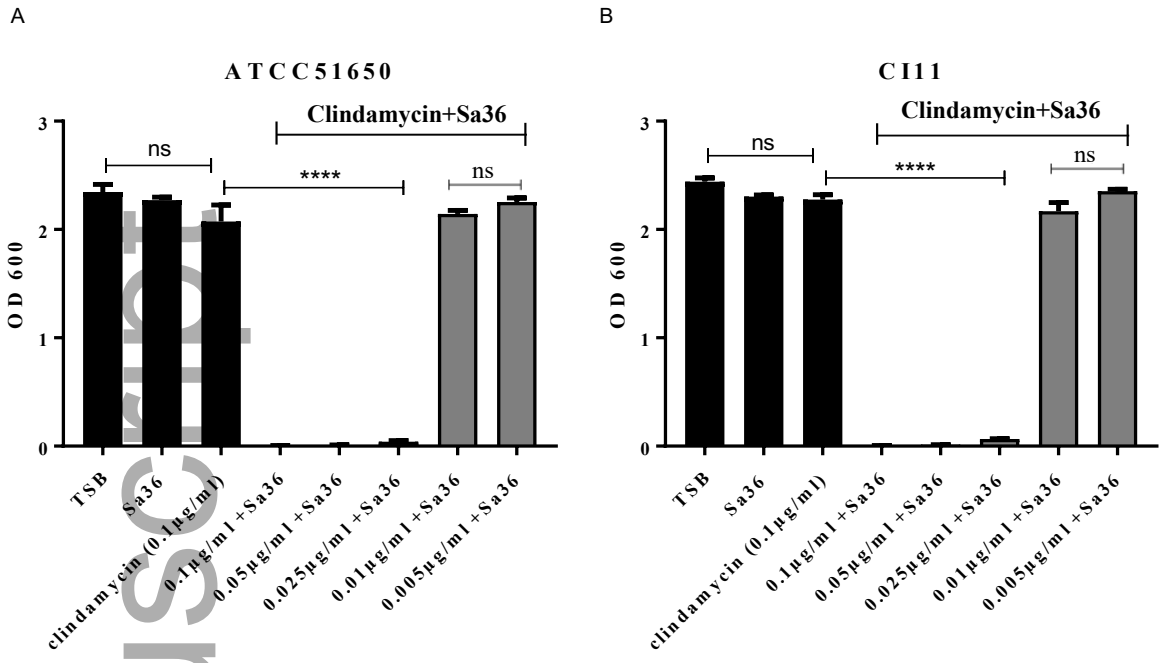

Figure 2: Low (1/8th MIC) doses of clindamycin in combination with J-Sa36 (Sa36) are effective against *S. aureus* ATCC51650 and CI11. OD 600 values represent bacterial growth of (A) ATCC51650 and (B) CI11 after 24 hours exposure to 10^5 PFU/ml J-Sa36 (MOI=0.2) and varying clindamycin concentrations (0.005 µg/ml -1/32 MIC- to 0.1µg/ml-1/2 MIC) or control (TSB). ns=not significant, ****, $p < 0.0001$, one-way ANOVA with Tukey's multiple comparisons test. n=3. Bars represent standard error of the mean (SEM). Black bars represent *S. aureus* ATCC51650 and CI11 treated with medium (TSB), J-Sa36 (Sa36) or Clindamycin (1/2 MIC or 0.1µg/ml) as control. Grey bars represent *S. aureus* ATCC51650 and CI11 treated with J-Sa36 combined with different concentrations of Clindamycin.

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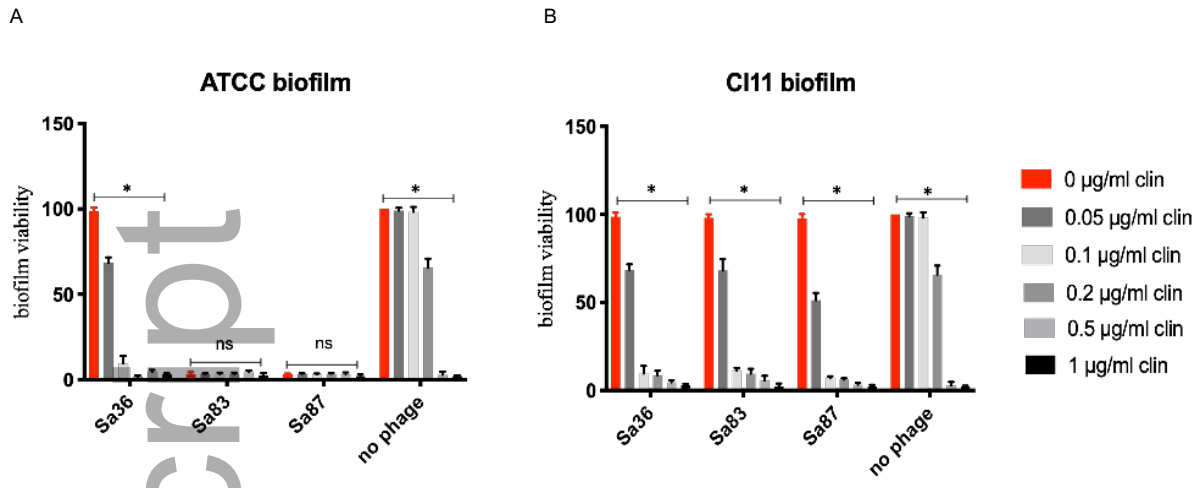

Figure 3. Phage J-Sa36, Sa83 or Sa87 combined with clindamycin reduces the viability of ATCC51650 and CI11 biofilms. Reduction of biofilm viability of *S. aureus* (A) ATCC51650 and (B) CI11 measured by Alamar Blue viability assay. All measurements were normalized and compared to untreated control (red bar). Concentrations of clindamycin ranged from (left to right) 1, 0.5, 0.2, 0.1, 0.05 and 0 (red) µg/ml. ns=not significant, *, $p < 0.05$, Significance was determined by one-way ANOVA with Dunnett's multiple comparisons test in comparison with phage only controls. n=3. Bars represent standard error of the mean (SEM).

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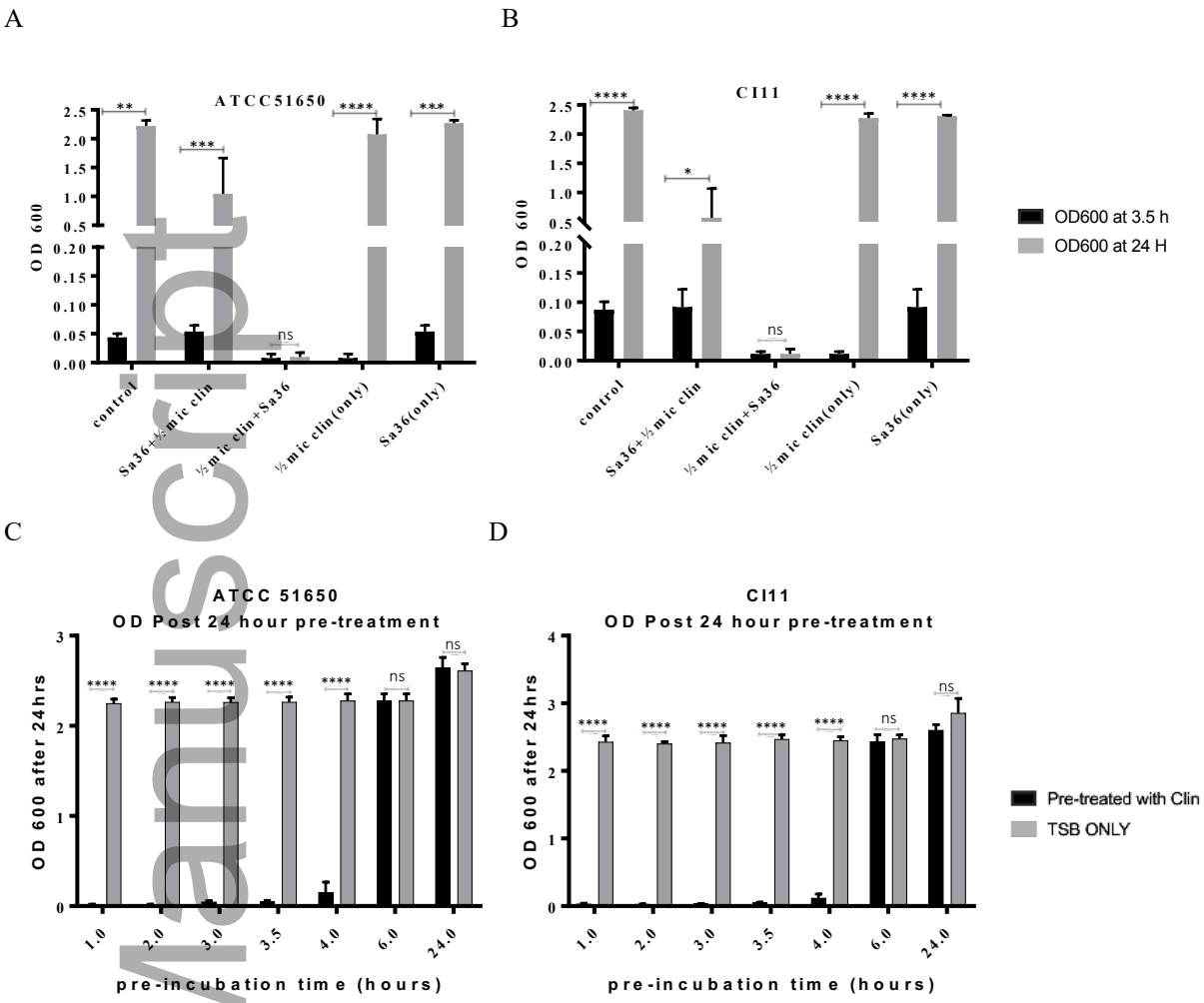

Figure 4: Time dependent effects on phage antibiotic synergy. (A, B) ATCC51650 or CI11 were grown in the absence (control) or presence of J-Sa36 (Sa36; MOI 0.2) or ½ MIC clindamycin for 3.5 hours, followed by the addition of ½ MIC clindamycin (Sa36+ ½ MIC clin added at 3.5 hrs) or phage J-Sa36 (½ MIC clin + J-Sa36 added at 3.5 hrs) with OD600 recorded at 3.5 (black bars) and 24 hours (grey bars). (C, D) ATCC51650 and CI11 respectively were pre-treated for different times with either ½ MIC clindamycin (black bar) or TSB only (grey) before J-Sa36 (MOI 0.2) was added at 1, 2, 3, 3.5, 4, 6 and 24 hrs. OD600 was recorded 24 hours later. n=3.ns=not significant, ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, mixed ANOVA with Tukey's multiple comparisons test. Bars represent standard error of the mean (SEM).

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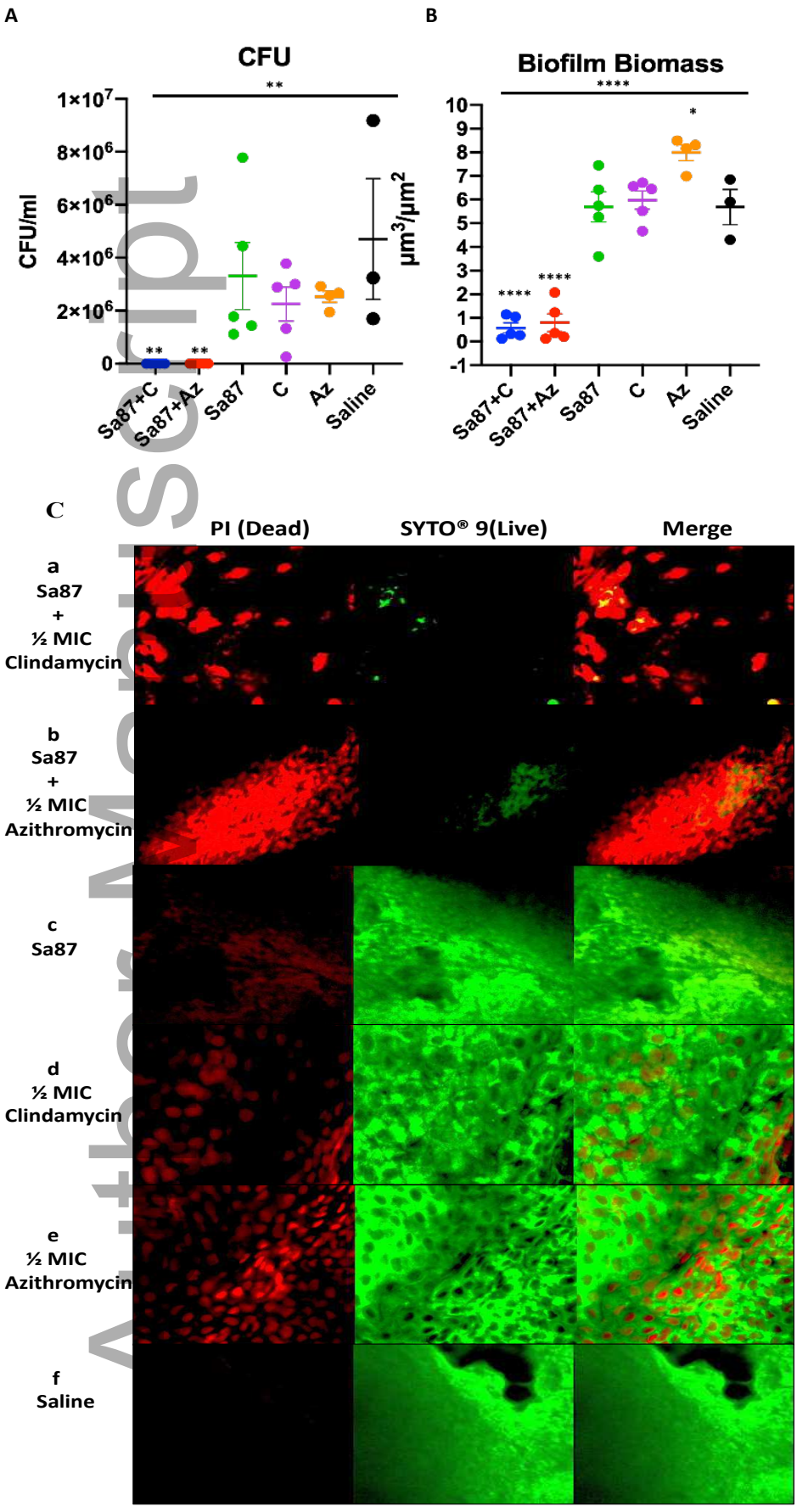

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Figure 5: Phage and PSI antibiotic combination *in vivo* efficacy test. Colony Forming Unit (CFU) counts (CFU/ml) (A) and biofilm biomass ($\mu m^3/\mu m^2$) (B and C) of a fresh piece of rat sinonasal mucosa for rats treated with $0.5*10^{10}$ PFU/ml Sa87 in combination with ½ MIC clindamycin [Sa87 + C, blue dots in A and B and line (a) in C)], $0.5*10^{10}$ PFU/ml Sa87 in combination with ½ MIC azithromycin [Sa87 + Az, red dots in A and B and line (b) in C)], $0.5*10^{10}$ PFU/ml Sa87 [Sa87, green dots in A and B and line (c) in C)], ½ MIC clindamycin [C, purple dots in A and B and line (d) in C)], ½ MIC azithromycin [Az, orange dots in A and B and line (e) in C)], saline [Saline, black dots in A and B and line (f) in C)]. Experiments were performed with 3 replicates for each rat, averaged and each rat represented by 1 dot. LIVE/DEAD® Bacterial Viability Kit was used in C showing dead (PI, red) and live (SYTO® 9, green) biofilm associated bacteria in samples representative of each treatment group). ****, $p < 0.0001$; **, $p < 0.01$; *, $p < 0.05$, One-way ANOVA with Dunnett's posthoc. Error bars represent standard error of the mean (SEM).

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