

RESEARCH ARTICLE

Combinatory antibiotic therapy increases rate of bacterial kill but not final outcome in a novel mouse model of *Staphylococcus aureus* spinal implant infection

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Abstract

Background

Management of spine implant infections (SII) are challenging. Explantation of infected spinal hardware can destabilize the spine, but retention can lead to cord compromise and biofilm formation, complicating management. While vancomycin monotherapy is commonly used, *in vitro* studies have shown reduced efficacy against biofilm compared to combination therapy with rifampin. Using an established *in vivo* mouse model of SII, we aim to evaluate whether combination therapy has increased efficacy compared to both vancomycin alone and infected controls.

Methods

An L-shaped, Kirschner-wire was transfixed into the L4 spinous process of 12-week-old C57BL/6 mice, and inoculated with bioluminescent *Staphylococcus aureus*. Mice were randomized into a vancomycin group, a combination group with vancomycin plus rifampin, or a control group receiving saline. Treatment began on post-operative day (POD) 7 and continued through POD 14. *In vivo* imaging was performed to monitor bioluminescence for 35 days. Colony-forming units (CFUs) were cultured on POD 35.

Results

Bioluminescence peaked around POD 7 for all groups. The combination group had a 10-fold decrease in signal by POD 10. The vancomycin and control groups reached similar levels on POD 17 and 21, respectively. On POD 25 the combination group dropped below baseline, but rebounded to the same level as the other groups, demonstrating a biofilm-associated infection by POD 35. Quantification of CFUs on POD 35 confirmed an ongoing infection in all three groups.

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Conclusions

Although both therapies were initially effective, they were not able to eliminate implant biofilm bacteria, resulting in a rebound infection after antibiotic cessation. This model shows, for the first time, why histologic-based, static assessments of antimicrobials can be misleading, and the importance of longitudinal tracking of infection. Future studies can use this model to test combinations of antibiotic therapies to see if they are more effective in eliminating biofilm prior to human trials.

Introduction

Spinal implant infections (SII) are a disastrous complication of spine surgery, requiring lengthy hospitalizations, repeat surgeries, and protracted courses of intravenous and oral antibiotics. These infections often result in considerable morbidity for the patient, including neurological compromise, disability, and possibly death. In addition, economic costs are significant, with treatment of a single case averaging approximately \$900,000.[1] Despite advances in aseptic surgical technique and perioperative antibiotic management, the rate of SII is still reported at 3–8% in elective spine surgery.[2–6] For multilevel or revision surgeries, rates of infection have been reported to be 65% higher than for primary cases.[7] As the demand for spine surgery continues to rise, so will the prevalence of these devastating infections, making their prevention and treatment critical.[8]

SII remains a unique challenge in orthopedics, as hardware removal is often not an option due to the potentially destabilizing effects of spinal hardware explantation prior to bony fusion, risking catastrophic surgical failure.[9] In addition, instrumentation itself results in 28% higher infection rates when compared to spinal surgery without the use of implants, as the avascular surface provides a nidus for microbial growth and eventual biofilm formation.[10] Once a biofilm layer is established on the implant surface, bacterial susceptibility to antibiotic and immune cell penetrance is reduced 1000-fold.[11] Since the current standard of treatment for SII relies on IV antibiotics +/- surgical debridement, the selection of antibiotic is critical.[9,12–15]

The optimal antibiotic regimen for biofilm treatment remains controversial. These infections typically arise from *Staphylococcus* species like *S. aureus*, the leading causative agent found in approximately 50% of cases of SII.[10] *S. epidermidis* is also a common cause and is associated with spinal implants. Given the established efficacy of the glycopeptide class against *Staphylococcus*, many surgeons choose to treat SII with IV vancomycin monotherapy.[9] However, *in vitro* studies have shown that vancomycin has reduced efficacy in the presence of biofilm, requiring concentrations of antibiotic that would be deemed toxic in humans.[16] Alternatively, the ability of rifampin to penetrate biofilm has been previously espoused as a rationale to utilize it against implant infections.[17–19] The Infectious Disease Society of America (IDSA) has recommended combination therapy with rifampin for acute staphylococcal SII.[20] This recommendation has been supported by *in vitro* studies, which show a synergistic effect when combination rifampin therapy is used in the setting of biofilm formation.[17–19,21–23] An optimized antibiotic therapy regimen for SII could lead to improved clearance of bacterial biofilm from implants, decreasing the need for repeated surgical debridement and thus reducing morbidity associated with SII.

An *in vivo* mouse model of SII in which bioluminescent *S. aureus* is inoculated onto a metallic orthopedic implant inserted into the L4 spinous process has recently been developed.

[24] As there is a clinical impetus to improve the antibiotic treatment for SII and a lack of *in vivo* data, this model has the power to provide a rapid and cost effective platform through which to compare efficacies of antibiotics and combination therapies *in vivo* before transitioning to large-scale studies in human subjects. Thus, we used this established mouse model of SII to evaluate whether combination therapy with vancomycin plus rifampin has increased efficacy compared with vancomycin monotherapy in the treatment of a *S. aureus* SII with retention of the implant. We hypothesize that a combined therapy of rifampin and vancomycin will be synergistic in its treatment of bacterial biofilm and result in improved bacterial kill when compared to vancomycin alone.

Materials and methods

Ethics statement

All animals were handled in strict accordance with good animal practice as defined in the federal regulations as set forth in the Animal Welfare Act (AWA), the 1996 Guide for the Care and Use of Laboratory Animals, PHS Policy for the Humane Care and Use of Laboratory Animals, as well as our institution's policies and procedures as set forth in the Animal Care and Use Training Manual, and all animal work was approved by the Chancellor's Animal Research Committee (ARC#: 2012-104-11D).

Staphylococcus aureus bioluminescent strain

The bioluminescent *S. aureus* strain Xen36 (PerkinElmer, Hopkinton, MA) was used in all experiments. The strain was derived from the parental strain ATCC 49525 (Wright), a clinical isolate obtained from a patient with *S. aureus* bacteremia.[25] Xen36 possesses a gram-positive optimized luxABCDE operon modified from the bacterial insect pathogen *Photobacterium luminescens* in a stable bacterial plasmid that is maintained in all progeny. Live, actively metabolizing Xen36 bacteria emit a blue-green light with a maximal emission wavelength at approximately 490 nm.

Preparation of *S. aureus* for inoculation of the spine implant

S. aureus Xen 36 possesses a kanamycin resistance marker on the lux operon. Bacteria were streaked onto tryptic soy agar plates (tryptic soy broth [TSB] plus 1.5% Bacto agar [BD Biosciences, Franklin Lakes, NJ]) containing 200ug/ml kanamycin (Sigma-Aldrich) and grown overnight at 37°C to select for Xen36 as previously described.[26] Single colonies of Xen36 were cultured in TSB and grown overnight at 37°C in a shaking incubator (240 rpm) (MaxQ 4450; Thermo, Waltham, MA). Mid-logarithmic phase bacteria were obtained after a 2-hour subculture of a 1:50 dilution of the overnight culture. Bacterial cells were pelleted, resuspended and washed 3 times in phosphate buffered saline (PBS). Bacterial inocula (1×10^3 CFU/ml) were estimated by measuring the absorbance at 600 nm (Biomate 3; Thermo).

Mice

Twelve-week old male C57BL/6 mice were used in all experiments.[27,28] Animals were kept at 3 mice per cage in standard cages with a 12-hr light and dark cycle. They were fed a standard pellet diet with free access to bottled water. Assessments were carried out daily by veterinary staff to ensure the well being of all animals throughout the experiment.

Mouse surgical procedures

The institution's Animal Research Committee approved all procedures. Mice were anesthetized via inhalation isoflurane (2%). The surgical procedure for this mouse model of spine

implant infection was performed as previously described.[24] In brief, a custom, L-shaped, orthopaedic-grade 0.1 mm diameter stainless steel Kirschner-wire (Modern Grinding, Port Washington, WI) was press-fit into the L4 spinous process with the short arm of the implant in the spine and the long arm oriented longitudinally along the spine heading cranially. An inoculum of Xen36 (1×10^3 CFUs) in 2 μ l PBS was pipetted onto the 90-degree bend of the implant. Deep 5–0 Vicryl sutures were used to close the muscle layer and a running 5–0 Vicryl was used to approximate the skin. For analgesia, non-sustained release buprenorphine (0.1 mg/kg given in 100 μ l of volume) (Par Pharmaceuticals, Spring Valley, NY) was injected subcutaneously prior to surgery and every 12 hours postoperatively for 7–14 days as needed. A high-resolution AP radiograph (Faxitron LX-60 DC-12 imaging system) was taken immediately after surgery to ensure proper placement of the implant.

Antibiotic therapy

Prior to surgery, mice were randomized to one of two treatment groups or a control group ($n = 10$ per group). Mice in the vancomycin group were subcutaneously administered a therapeutic dose of vancomycin (110 mg/kg twice daily) (Mylan, Cannonsburg, PA), which approximated the area under the curve (AUC) of 440 μ g·h/ml for typical human exposure for vancomycin (1g twice daily).[29–31] Mice in the combination therapy group were administered a therapeutic subcutaneous mouse dose of rifampin (25 mg/kg daily) (Fresenius Kabi, Lake Zurich, IL) in addition to vancomycin therapy (110 mg/kg twice daily).[32] This rifampin dose was chosen based on previously published studies of various mouse models of staphylococcal infection.[32–36] All antibiotic therapy and sham injections of sterile saline were initiated on postoperative day 7 and continued through postoperative day 14 to mimic the treatment of an established SII. The MICs for Xen36 were ≤ 0.5 μ g/ml for vancomycin and ≤ 0.5 μ g/ml for rifampin.

In vivo bacterial burden as measured by *in vivo* bioluminescence imaging

To obtain noninvasive measurement of bacterial burden, mice were anesthetized with inhalation isoflurane (2%), the dorsal region of the mouse was shaved, and *in vivo* bioluminescence imaging was performed using the Xenogen IVIS Lumina II (PerkinElmer, Hopkinton, MA) on post-operative days (POD) 0, 1, 3, 5, 7, 10, 14, 17, 21, 25, 28, 32, and 35 as previously described.[26,37–39] The Xen36 bioluminescent signals were obtained with a 5-minute acquisition time, a 13 cm field of view (FOV), and medium binning settings. Data are presented on a color scale overlaid on a grayscale photograph of mice and quantified as total flux (photons per second (s) per cm^2 per steradian (sr) [photons/s/ cm^2 /sr]) within a circular region of interest (1×10^3 pixels) using Living Image software (PerkinElmer, Hopkinton, MA).

Quantification of bacteria adhering to the implants and in the peri-implant tissue

To confirm that the *in vivo* bioluminescence signal accurately represented the bacterial burden *in vivo*, mice were euthanized on POD 35 using carbon dioxide exposure and secondary confirmation by cervical dislocation. Traditional colony-forming unit (CFU) counts were then performed from bacteria adherent to the surgical implant and in the tissue surrounding the implant. Bacteria in the peri-implant tissue were isolated by homogenizing the surrounding soft tissue and infected bone of the spinous process (Pro200 Series homogenizer; Pro Scientific, Oxford, CT). Bacteria adherent to the implants were detached by sonication in 1 ml 0.3% Tween-80 in TSB for 10 min followed by vortexing for 5 minutes. Serial dilutions were plated

and cultured overnight as previously described.[26] The number of bacterial CFUs obtained from the peri-implant tissue and the implants was determined by counting CFUs after overnight culture of plates.

Statistical analysis

Each group had 10 mice based on previous studies from our group showing that 10 animals/group was necessary to attain statistical significance.[24,26,40] Data between two groups was compared using Student's t-test (one or two-tailed where indicated), while data between three or more groups was compared using a one-way ANOVA. All data are expressed as mean \pm standard error of the mean (SEM). Values of $p < 0.05$ were considered statistically significant.

Results

Efficacy of systemic antibiotic therapy on *in vivo* bioluminescent signals

Control mice treated with sterile saline had bioluminescent signals that peaked on POD 10 ($3.35 \times 10^6 \pm 9.14 \times 10^5$ photons/s/cm²/sr) and remained above 1.00×10^5 photons/s/cm²/sr throughout the duration of the experiment (35 days), modeling a chronic SII (Fig 1). A significant reduction in bioluminescent signal (approximately 2 fold) was observed with vancomycin monotherapy when compared to control mice from POD 10–21 ($p < 0.03$). After this time point there was no difference in signal between the two groups. Combination therapy with vancomycin plus rifampin resulted in a rapid initial reduction in bioluminescent signal compared to controls on POD 10 (approximately 20 fold), which then remained lower until POD 28 ($p < 0.01$), after which time there was no difference in signal between the combination and control groups. There was also a reduction in bioluminescent signal when comparing combination therapy to vancomycin monotherapy from POD 10–17 (approximately 2 fold) ($p < 0.05$), after which there was no difference between the two groups. At final follow up (POD35), there were no differences in bacterial burden between any of the three groups.

Quantification of CFUs from the implant and peri-implant bone and soft tissue

On POD 35, peri-implant bone and soft tissue as well as the implants themselves were harvested, and bacterial burden was quantified using CFU counts (Fig 2). There was no difference in the CFU counts between control, vancomycin monotherapy, and combination therapy mice ($2.62 \times 10^2 \pm 1.34 \times 10^2$ CFUs, $2.76 \times 10^2 \pm 1.38 \times 10^2$ CFUs, and $2.82 \times 10^2 \pm 1.25 \times 10^2$ CFUs, respectively). This corroborates the bioluminescent data showing no difference between the three groups in terms of bioluminescent signal at POD 35.

Discussion

The selection of antibiotic therapy for SII represents a significant challenge, as hardware typically must be retained to prevent potentially devastating destabilization of the spine. Biofilm has been shown to form as soon as 1 day after infection, with a robust biofilm present at 7 days.[41] Successfully treating biofilm infection necessitates the use of antibiotics that are effective against senescent bacteria. Although there is *in vitro* data indicating that combination therapy with rifampin is more effective against biofilm, there is a lack of *in vivo* data.[17–19,23] We thus used a previously established mouse model of SII to evaluate the efficacy of rifampin combination therapy when compared with vancomycin monotherapy in combating infection.[24]

The results of this study indicate that both vancomycin monotherapy and combination therapy with rifampin are not sufficient to eliminate a SII. Although combination therapy

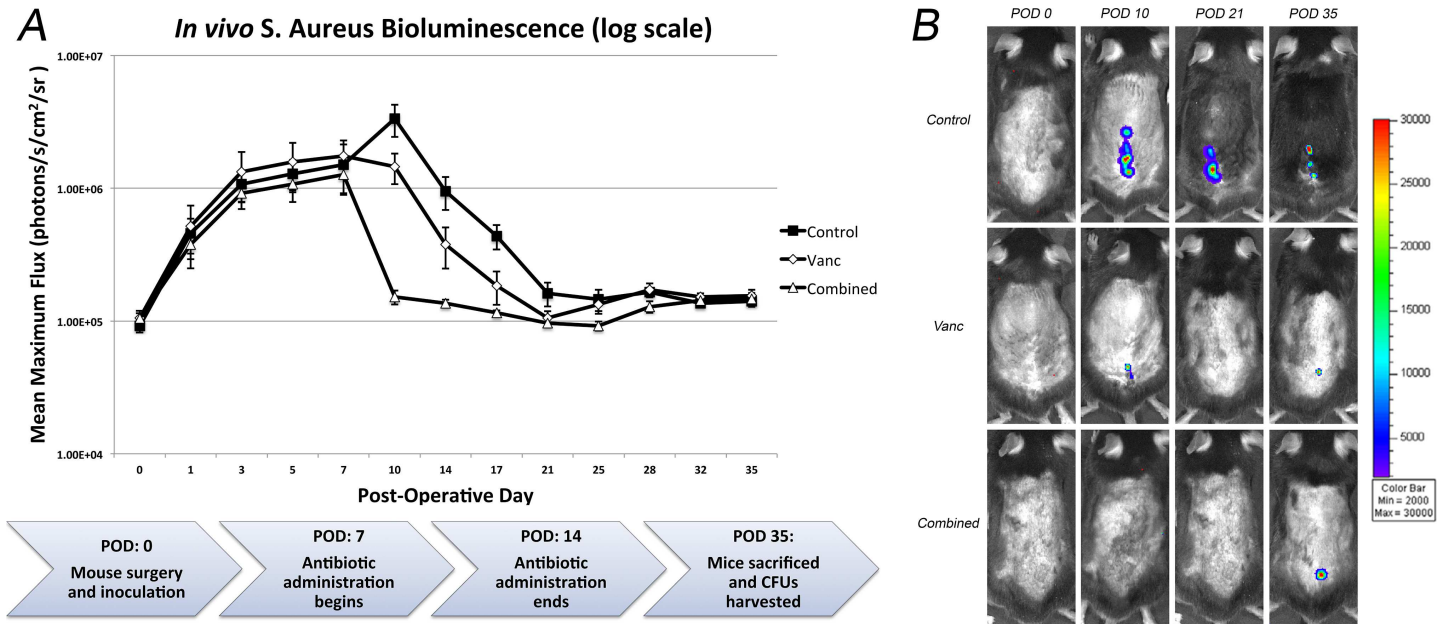


Fig 1. Measurement of bacterial burden using *in vivo* bioluminescence to POD 35. 1×10^3 CFU of *S. aureus* possessing the bioluminescent construct in a stable plasmid (Xen36) were inoculated into the L4 spinous process of mice ($n = 10$ mice per group) in the presence of a stainless steel implant. (A) Bacterial counts as measured by *in vivo S. aureus* bioluminescence (mean maximum flux [photons/s/cm²/sr] \pm sem [logarithmic scale]), with a flow diagram of the experimental protocol below. On POD 7, antibiotic administration began with vancomycin, a combination of vancomycin and rifampin or a sterile saline control. Antibiotic administration was stopped on POD 14. On POD 35, mice were sacrificed and CFUs from the implant and surrounding tissue were measured. (B) Representative *in vivo S. aureus* bioluminescence on a color scale overlaid on top of a grayscale image of mice.

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resulted in a considerably more rapid initial rate of kill compared to vancomycin alone, both treatments did result in a return to a baseline level of bioluminescent signal by POD 21. Yet after POD 21, and following the cessation of antibiotic therapy on POD 14, the signals for both groups increased back up to the level of control mice, indicating the persistence of a biofilm on the implant. Thus, while the rate of bactericidal activity was increased by combination therapy, neither regimen achieved eradication of the biofilm. This was corroborated by the *ex vivo* CFUs, which were present and not significantly different between the three groups at POD 35.

When interpreting this data, it is imperative to take into consideration the distinct pharmacokinetics of vancomycin and rifampin in humans versus mice, which can result in different levels of antibiotic exposure and efficacy. Firstly, serum protein binding differs between humans and mice for vancomycin (~35–50% versus ~25%) and rifampin (~97% versus ~90%), indicating that the level of active, unbound antibiotic is higher in mice than humans.[42,43] In addition, when humans are compared with mice, there is a longer half-life of vancomycin in humans (7.7h versus 2h), but a shorter half-life of rifampin (4h versus 12h).[44–47] This difference in half-life may result in a decreased rate of kill of vancomycin and increased rate of kill of rifampin therapy in this mouse model when compared to humans. These differences in the pharmacokinetics of the antibiotics highlight the difficulty of approximating human antibiotic exposures in mice. In future studies a range of antibiotic doses tested against a variety of *S. aureus* strains with varying antibiotic susceptibilities would ensure more robust pharmacokinetic and pharmacodynamic data that could more accurately evaluate the efficacy of these antibiotic therapies against SII in humans.

It is important to note that rifampin monotherapy is not used clinically due to a well documented rapid emergence of bacterial resistance, and was thus not tested in our mouse model. [48–50] While it could certainly be interesting to evaluate the curve dynamics for rifampin

CFUs On Implant and Surrounding Tissue

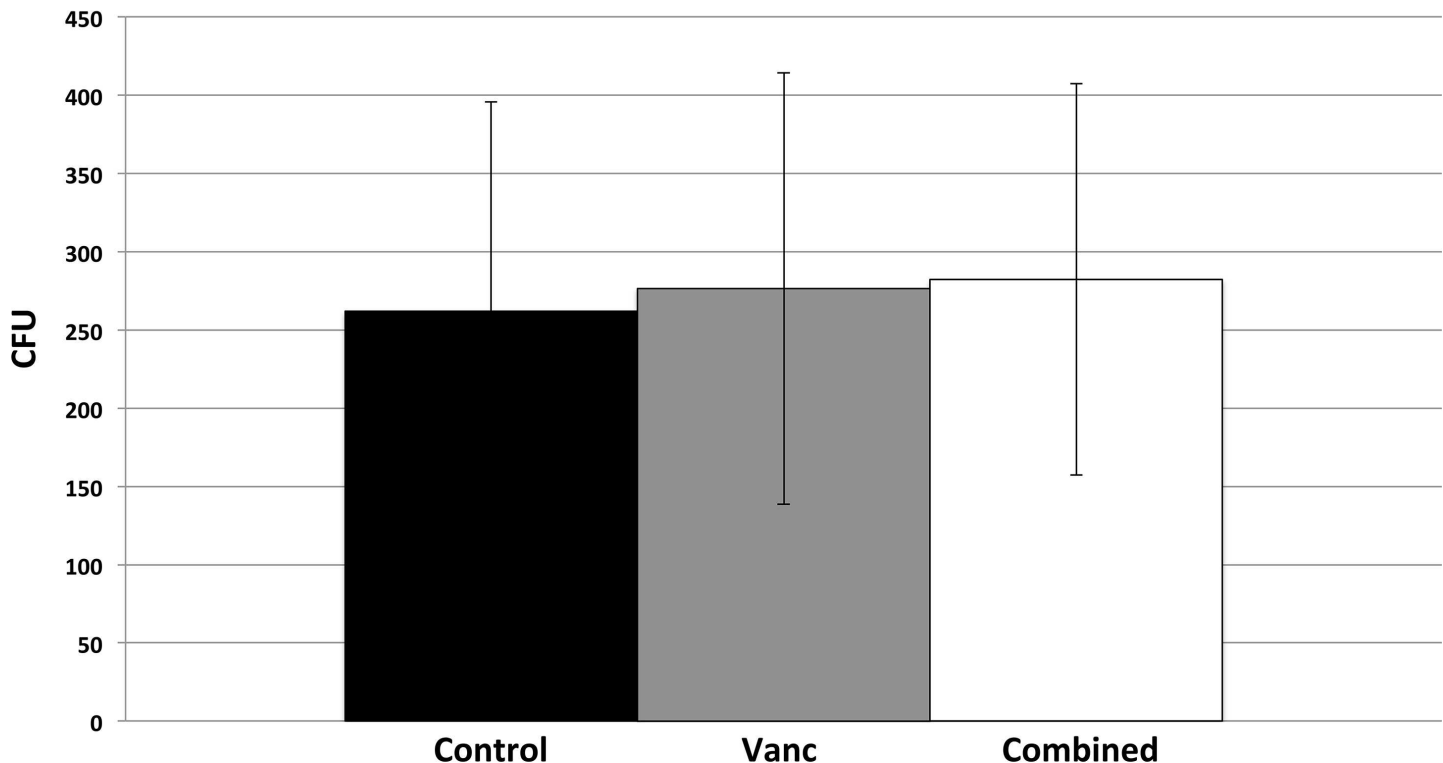


Fig 2. Confirmation of bacterial burden using CFU counts. At POD 35, mice were sacrificed, pins were sonicated, tissue was homogenized and bacteria were cultured and counted.

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monotherapy, this data would not be clinically relevant and could give dangerous false-confidence in an otherwise well-negated drug regimen. Yet this study does confirm prior *in vitro* data indicating the utility of rifampin as an adjunctive agent.[23,51–53] Studies have shown that rifampin is able to penetrate biofilm, improve diffusion of a companion agent through biofilm, and reduce bacterial adherence to foreign body surfaces.[17–19,23] Differences in the mechanism of action of vancomycin and rifampin may also partially explain the increased efficacy of combination therapy. While vancomycin acts by disrupting cell wall synthesis, rifampin acts by inhibiting bacterial DNA-dependent RNA synthesis.[54,55] As bacteria in biofilms appear to have increased cell wall thickness, this may render vancomycin less effective when compared to its activity against planktonic bacteria, but may also improve rifampin penetrance into these thickened cell walls by interfering with their synthesis.[56] In spite of this, the synergy of these two bactericidal antibiotics was not sufficient to completely eliminate all of the bacteria in the biofilm, resulting in a return in the infection after antibiotic therapy had been stopped.

Looking at the curves for each of the three treatment groups in this experiment, the power of this longitudinal model of SII becomes apparent. Other infection models require sacrifice of animals and rely on CFU counts to measure bacterial burden, providing data at a single time point rather than tracking the course of the infection over time.[57–59] Taking this experiment as an example, if sacrifice and CFU counts were performed statically at POD 10, POD 21 or POD 35, completely different conclusions about the efficacy of vancomycin monotherapy versus combination therapy with rifampin would result (Fig 1). At POD 10, one would interpret that combination therapy is considerably more effective than vancomycin monotherapy.

Interpreted at POD 21, it would appear that both treatment regimens were equally effective, and significant when compared to control animals. Finally, with counts taken at POD 35, it would appear that neither therapy was effective compared to control animals. Instead, by having bioluminescence as a surrogate for bacterial burden, the course of the infection can be tracked non-invasively and longitudinally without increased animal sacrifice, resulting in substantially more data and accuracy about the *in vivo* efficacy of these antibiotics in treating SII over time.

In conclusion, it is clear that both vancomycin monotherapy and combination therapy with rifampin are insufficient to clear a SII in this model. Even though it may appear that the infection has resolved, biofilm-associated bacteria remain on the implant. Thus, once antibiotics are discontinued, as was evidenced in this experiment, the infection persists at a quantifiable level. Using the power of a longitudinal model of SII, the progression of the infection can be traced, revealing more about the efficacy of antibiotic treatment over time than traditional infection models that are only capable of producing data at a single time point. In the future, this model can be used to examine different combinations of other antistaphylococcal drugs such as linezolid, tetracyclines (doxycycline, minocycline or tigecycline) or newer glycopeptides (daptomycin, telavancin, dalbavancin or oritavancin) to see if they perform better against SII than the currently accepted options of vancomycin monotherapy or combination therapy with rifampin. In addition, the course of antibiotic treatment could be studied longitudinally to help optimize the duration of suppressive therapy protocols. Expanding this model would result in a valuable preclinical system to screen different combinations of antibiotic therapy and duration before pursuing trials in humans.

Supporting information

S1 File. Bioluminescence and colony-forming unit data.
(XLSX)

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