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■ **INFECTION**

# Recalcitrant methicillin-resistant *Staphylococcus aureus* infection of bone cells: Intracellular penetration and control strategies

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**Aims**
To characterize the intracellular penetration of osteoblasts and osteoclasts by methicillin-resistant *Staphylococcus aureus* (MRSA) and the antibiotic and detergent susceptibility of MRSA in bone.

**Methods**
Time-lapse confocal microscopy was used to analyze the interaction of MRSA strain USA300 with primary murine osteoblasts and osteoclasts. The effects of early and delayed antibiotic treatments on intracellular and extracellular bacterial colony formation and cell death were quantified. We tested the effects of cefazolin, gentamicin, vancomycin, tetracycline, rifampicin, and ampicillin, as well as agents used in surgical preparation and irrigation.

**Results**
MRSA infiltrated bone-resident cells within 15 to 30 minutes. Penetration was most effectively prevented with early (i.e. 30 minutes) antibiotic administration. The combined administration of rifampicin with other antibiotics potentiated their protective effects against MRSA-induced cytotoxicity and most significantly reduced extracellular bacterial bioburden. Gentamicin-containing compounds were most effective in reducing intracellular MRSA bioburden. Of the surgical preparation agents evaluated, betadine reduced in vitro MRSA growth to the greatest extent.

**Conclusion**
The standard of care for open fractures involves debridement and antibiotics within the first six hours of injury but does not account for the window in which bacteria penetrate cells. Antibiotics must be administered as early as possible after injury or prior to incision to prevent intracellular infestation. Rifampicin can potentiate the capacity of antibiotic regimens to reduce MRSA-induced cytotoxicity.

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## Article focus
■ The most common causative agent of musculoskeletal infection is *Staphylococcus aureus* (*S. aureus*). The spread of bacteria to the intracellular compartment significantly reduces antimicrobial sensitivity and can cause recalcitrant infection.
■ We hypothesized that the ability of methicillin-resistant *S. aureus* (MRSA) to penetrate bone resident cells contributes to antibiotic resistance, and that combining antibiotic treatment with cell-penetrating antibiotics would more effectively inhibit MRSA growth within bone cells than single antibiotic regimens.

## Key messages
■ MRSA achieves intracellular penetration of osteoblasts and osteoclasts in shorter timeframes (i.e. 30 minutes) than antibiotics may be administered for open fractures (i.e. within six hours).

- Early antibiotic administration significantly reduces the extent of MRSA infection and MRSA-induced cytotoxicity in bone.

## Strengths and limitations

- Through time-lapse confocal microscopy, we tracked the intracellular penetration of murine osteoblasts and osteoclasts by MRSA strain USA300 and observed a significant reduction in MRSA bioburden and cytotoxicity with earlier gentamicin administration.
- A limitation of the experiments described is the small sample sizes involved.

## Introduction

Bone infection is difficult to treat, with high rates of recurrence and treatment failure.[1] Bone necrosis, soft-tissue injuries, contamination, the presence of foreign bodies and/or implants, and conditions such as diabetes and peripheral vascular disease increase susceptibility to infection.[2] Basic infection control paradigms include prophylactic antibiotics, irrigation, and surgical debridement for open fracture- and implant-associated infections. Despite these practices, infection persists. Characterizing the ability of bacteria to penetrate bone cells is critical to developing effective preventive and therapeutic strategies that maximize antimicrobial efficacy against both extracellular and intracellular musculoskeletal infection.

*Staphylococcus aureus* (*S. aureus*) is the main causative agent of chronic osteomyelitis (OM) worldwide; 50% to 80% of cases have been attributed to *S. aureus*.[2,3] *S. aureus* can survive within phagocytes such as monocyte-derived macrophages, as well as neutrophils and osteoblasts.[4] Upon entering cells, bacteria become less sensitive to antibiotics and immune to washout. Differentiation of bone marrow-derived osteoclast precursors into macrophages capable of secreting proinflammatory cytokines was observed following *S. aureus* infection.[5] This inflammatory milieu promotes osteoclastogenesis and enhances the bone resorption capacity of infected osteoclasts.[5] Infected osteoblasts release viable *S. aureus*, enabling further infection within bone.[6] Staphylococci species possess quorum-sensing systems for cell-to-cell communication and regulated colonization and virulence factor expression.[7] *S. aureus* was found to persist within various cell lines for weeks in a set of in vitro and in vivo murine models of chronic *S. aureus* infection and achieved intracellular penetration of human osteocyte-like cells.[8] Colonization of the osteocytic-canalicular system by *S. aureus* was observed in murine models of OM and tissue samples from a patient with chronic osteomyelitis, representing another potential mechanism of infection persistence.[9,10]

Various agents have been investigated for their activity against intracellular and biofilm-forming *S. aureus*. Deng et al[11] infected wound models with resistant bacterial species, including methicillin-resistant *S. aureus*

(MRSA), then performed irrigation and debridement with normal saline (0.9%), castile soap, benzalkonium chloride, bacitracin, or ethylenediaminetetraacetic acid (EDTA) until negative wound cultures were obtained. EDTA treatment yielded fewer irrigation and debridement procedures for infection eradication.[11] Dusane et al[12] infected SAOS-2 osteoblast-like cells with *S. aureus*, then treated these cultures with gentamicin, after which bacterial survival was quantified. Though extracellular planktonic bacteria were killed by gentamicin, bacterial seeding from infected osteoblasts and biofilm formation were observed after treatment ceased. Vancomycin alone was ineffective in reducing intracellular bacterial bioburden, but the combination of piperine or carbonyl cyanide m-chlorophenylhydrazine (CCCP) with vancomycin significantly reduced intracellular *S. aureus* levels.[12] In vivo and in vitro studies have shown that rifampicin-containing regimens are effective against implant-associated staphylococcal infections and potentiate the bactericidal activity of other antibiotics, potentially due to the ability of rifampicin to enter and concentrate within cells.[13,14]

The ability of bacteria to evade antimicrobial treatments suggests that conventional methods of infection prevention and management for bone infections must be revised. We hypothesized that early targeting of intracellular bacteria with antibiotics, particularly cell-penetrating antibiotics such as rifampicin, would effectively reduce bacterial bioburden in and around bone cells and protect against the infection of bone.

## Methods

**Bacterial cultures.** The USA300 MRSA strain (ATCC BAA-171) was obtained from the American Type Culture Collection (Manassas, Virginia, USA). The USA300 strain is sensitive to gentamicin. A single bacteria colony from a Luria-Bertani (LB) agar plate was inoculated into 3 ml LB medium and shaken at 250 rpm overnight at 37° C. After 48 hours, the bacteria culture was diluted 1,000-fold in 1 ml fresh LB medium and grown at 37°C. The optical density (OD) at 600 nm (OD600) was monitored until the density of the bacterial culture reached 1.0. The bacteria were washed once with sterile phosphate-buffered saline (PBS) and resuspended in 1 ml sterile PBS. The resultant bacteria stock was determined at $2.5 \times 10^8$ colony-forming units (CFU)/ml.

**MRSA-induced cytotoxicity assays.** Mouse primary osteoblasts were derived from a five-week-old male C57BL/6 mouse, as described by Soong et al.[15] Bone marrow-derived osteoclasts (BMOCs) were differentiated from bone-marrow-derived progenitors from these mice.[16] Briefly, the bone marrow progenitors were treated with recombinant 50 ng/ml human macrophage colony-stimulating factor (M-CSF) (Shenandoah Biotechnology, Warwick, Pennsylvania, USA) for two days and 50 ng/ml M-CSF and 10 ng/ml recombinant human receptor activator

of nuclear factor kappa-B ligand (RANKL) (Shenandoah Biotechnology) for an additional three days. Mouse primary osteoblasts and osteoclasts were grown on 12-well plates in Minimal Essential Medium (MEM) (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) containing 10% heat-inactivated foetal calf serum (FCS) (Gemini Bio-Products, West Sacramento, California, USA) and infected with wild-type USA300 at a multiplicity of infection (MOI) of 10. The medium was changed to MEM containing gentamicin (50 μg/ml) 15 minutes, 30 minutes, one hour, two hours, and four hours postinfection. The supernatant was aspirated and spun down. An amount of 25 μl was used to measure lactate dehydrogenase (LDH) activity using the Roche Cytotoxicity Detection Kit (LDH) (Sigma-Aldrich, St. Louis, Missouri, USA). Relative cell death is presented as the percentage of lysis buffer-induced death.

**Antibiotic and antimicrobial treatment of infected osteoblasts.** The bactericidal effect of various extracellular and cell-penetrating antibiotics was investigated in mouse osteoblast cell line MC3T3-E1 cells exposed to USA300 green fluorescent protein (GFP)-labelled MRSA at a MOI of 10 for three hours. The cells were then treated with vancomycin (10 μg/ml), gentamicin (50 μg/ml), cefazolin (100 μg/ml), rifampicin (4 μg/ml), or ampicillin (50 μg/ml) for 22 hours. The supernatants were collected and assayed for LDH the next day, as previously described.

To test the bactericidal effects of agents commonly used in surgical preparation, disinfection, and wound irrigation, USA300 MRSA was inoculated into LB broth at a ratio of 1:100. After the culture grew to log phase (OD 1.0), 0.3% betadine, 7% ethanol, or 0.3% hydrogen peroxide ($H_2O_2$) was added. The cultures were incubated at 37°C at 250 rpm for two to seven hours. Optical density at 625 nm was observed five and seven hours after treatment.

The protective effects of combined antibiotic regimens against MRSA-induced cytotoxicity were evaluated in MC3T3-E1 cells, which were infected with GFP-labeled USA300 at a MOI of 10. Three hours postinfection, they were treated with: 10 μg/ml vancomycin; 100 μg/ml cefazolin; 50 μg/mL gentamicin; 4 μg/ml rifampicin; 50 μg/ml gentamicin and 10 μg/ml vancomycin; 50 μg/ml gentamicin and 100 μg/ml cefazolin; 4 μg/ml rifampicin and 10 μg/ml vancomycin; 4 μg/ml rifampicin and 100 μg/ml cefazolin; or left untreated.

Antibiotic concentrations and combinations were chosen based on studies that investigated the effects of varied concentrations on susceptible and resistant *S. aureus* species.[17,18] Cytotoxicity was assessed 24 hours after treatment.

**Live cell imaging of MRSA infection.** A total of four groups of mCherry-labelled osteoblasts were left uninfected, infected with GFP-labelled USA300 and left untreated (i.e. no antibiotics), or infected with GFP-labelled USA300 and treated with 50 μg/ml gentamicin 30 minutes or four hours postinfection. Time-lapse confocal microscopy

was performed over a 14-hour period. mCherry-labelled osteoclasts were also exposed to GFP-labelled USA300 and visualized under confocal microscopy over 14 hours.

**Intracellular and extracellular bacteria quantification.** Red fluorescent protein (RFP)-labelled MC3T3-E1 cells and osteoclasts were cultured in 12-well plates. The medium was replaced with fresh Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific) prior to beginning each experiment. GFP-labelled USA300 was added to three wells at a MOI of 10. A single well was left uninfected and served as a control (t = 0). At t = 0, 15, 30, and 60 minutes, the supernatant was removed and placed in a 1.5 mL microcentrifuge tube. The well was washed three times with sterile PBS and imaged using confocal microscopy, after which it was scraped using a Falcon Corning Cell Scraper (Corning Incorporated, Corning, New York, USA). Following this, 1 mL PBS was added to the well, and the cell mixture was transferred to a 1.5 mL microcentrifuge tube, with 10 μl from each cell mixture tube added to a counting slide. Cell counts were performed using a TC20 Automated Cell Counter (Bio-Rad Laboratories Inc., Hercules, California, USA), before $1 \times 10^6$ cells were transferred from each cell mixture tube to a microcentrifuge tube. These tubes were centrifuged at 5000 rpm for ten minutes. The supernatants were removed and 100 μl of mammalian protein extraction reagent (M-PER) (ThermoFisher Scientific) were added to lyse the harvested cells. After five minutes, the M-PER was removed and the tubes were recentrifuged at 5000 rpm for ten minutes. The supernatants were removed and the cell layers were resuspended in 100 μl of PBS, and 100 μl of either supernatant or lysed cell contents were plated on LB agar.

To quantify intracellular and extracellular bacterial bioburden following gentamicin administration, RFP-labelled MC3T3-E1 cells were infected with GFP-labelled USA300 at a MOI of 10. The cells were treated with 50 μg/ml gentamicin at t = 15, 30, 60, 120, and 240 minutes after infection, or left untreated. The methods of intracellular and extracellular bacteria quantification described above were performed five hours postinfection.

Intracellular and extracellular bacteria counts were performed following different antibiotic therapies. RFP-labelled MC3T3-E1 cells were infected with GFP-labelled USA300 at a MOI of 10. At three hours postinfection, the cells were treated with 10 μg/ml vancomycin, 100 μg/ml cephalexin, 50 μg/ml gentamicin, 50 μg/ml ampicillin, 50 μg/ml gentamicin and 10 μg/ml vancomycin (VG), 50 μg/ml gentamicin and 100 μg/ml cephalexin (CG), 4 μg/ml rifampicin, 4 μg/ml rifampicin and 10 μg/ml vancomycin (VR), 4 μg/ml rifampicin and 100 μg/ml cephalexin (CR), or left untreated. Intracellular and extracellular bacteria quantification was performed as described one hour after treatment. These concentrations and timepoints of antibiotic therapy were chosen to complement the cytotoxicity experiments previously described.
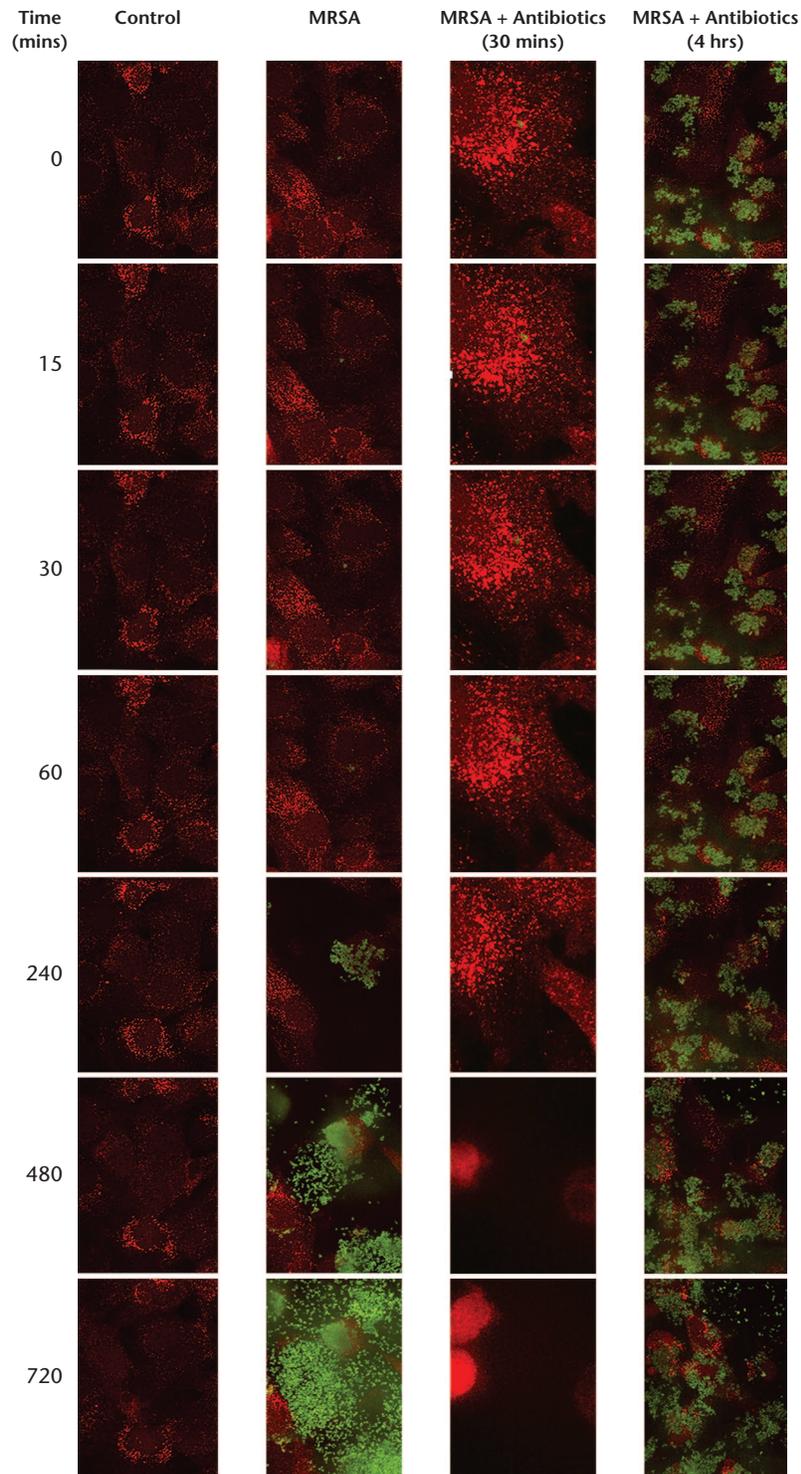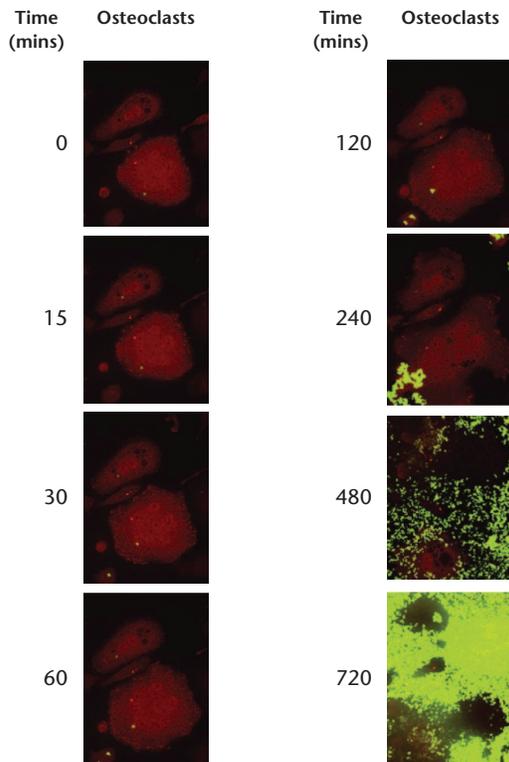
**Fig. 1**

Time-lapse microscopy images of mCherry-labelled osteoblasts (OBs) exposed to green fluorescent protein (GFP)-labelled USA300 over 14 hours. Rapid bacterial proliferation was observed in USA300-infected OBs between the first and second hours of infection. Administration of 50 µg/ml gentamicin occurred either 30 minutes or four hours postinfection. Gentamicin treatment within the first 30 minutes of infection significantly reduced bacterial burden relative to the untreated and four-hour postinfection treatment groups by one-way, repeated measures ANOVA (p = 0.025). Bacterial burden was reduced in USA300-infected OBs treated with gentamicin four hours postinfection, but to a lesser extent than that in the OBs treated within 30 minutes of infection. ANOVA, analysis of variance. MRSA, methicillin-resistant *Staphylococcus aureus*.

All experiments were performed in triplicate and bacterial growth was quantified after 48 hours. Plates were imaged using the ChemiDoc MP Imaging System (Bio-Rad Laboratories Inc.). The images were exported with Image Lab 6.0 (Bio-Rad Laboratories Inc.) and analyzed using ImageJ (National Institutes of Health, Bethesda,

**Fig. 2**

Intracellular penetration of mCherry-labelled osteoclasts by green fluorescent protein (GFP)-labelled USA300 captured via time-lapse microscopy over 14 hours. Rapid bacterial proliferation was observed to begin between two and four hours postinfection.

Maryland, USA) to generate CFU counts, which were averaged for each condition.

**Statistical analysis.** Statistical significance was determined using a Student's *t*-test (two-tailed) for all experiments other than the bacterial quantification experiments. A value of $p < 0.05$ was considered significant. For the CFU count experiments, one-way analysis of variance (ANOVA) was performed. Tukey post-hoc tests were performed for statistically significant ANOVA results. All statistical analyses were performed in GraphPad Prism 8 (GraphPad Software, San Diego, California, USA).

## Results

**Intracellular invasion of bone cells and bone colonization.** Time-lapse microscopy of GFP-labelled MRSA revealed that MRSA enters osteoblasts within 15 to 30 minutes of infection (Figure 1). MRSA also penetrated osteoclasts (Figure 2). Over the first 60 minutes of infection, a statistically significant increase was observed in intracellular osteoblast USA300 levels by one-way, repeated measures ANOVA ($F_{(3,24)} = 10.66$; $p < 0.001$) (Figure 3a). Tukey post-hoc comparisons revealed a statistically significant increase in intracellular bacteria levels between the 15-, 30-, and 60-minute timepoints relative to the control (mean 505.1 (SD 78.2); $p = 0.004$; mean 561.4 (SD 78.2); $p = 0.010$;

and mean 430.7 (SD 78.2); $p = 0.002$, respectively). There was no statistically significant difference in intracellular bacteria counts between the 15-, 30-, and 60-minute timepoints. An increase was also observed in extracellular USA300 colony counts at each timepoint with respect to the control by one-way, repeated measures ANOVA ($F_{(3,8)} = 5.67$; $p = 0.022$), although this difference only achieved statistical significance 30 minutes postinfection by Tukey post-hoc comparisons (mean 11,405 (SD 1974.5); $p = 0.015$ (Figure 3a). Differences in CFU counts between the intracellular and extracellular osteoblastic compartments were not statistically significant. A significant increase in extracellular osteoclast USA300 levels over time was observed by one-way ANOVA ($F_{(3,20)} = 16.36$; $p < 0.001$ (Figure 3b). Differences in bacterial load measured from the intracellular compartment of infected osteoclasts over time and in bacterial bioburden between the intracellular and extracellular compartments were not statistically significant.

**Bacterial colony formation after early and delayed antibiotic treatment.** Early administration of antibiotics conferred protective effects upon infected osteoblasts. Treatment of infected osteoblasts with gentamicin across all timepoints reduced both intracellular and extracellular bacterial concentrations compared with untreated, infected controls by one-way ANOVA ($F_{(5,13)} = 20.89$; $p < 0.001$ and $F_{(5,10)} = 68.83$; $p < 0.001$, respectively) (Figure 4a). Application of 50 µg/ml gentamicin 30 minutes after infection reduced bacterial burden compared with the group treated four hours postinfection by two-way student's *t*-test ($p = 0.036$) (Figure 1). Gentamicin treatment within the first two hours of infection significantly reduced MRSA-induced cytotoxicity by two-way student's *t*-test ($p = 0.042$ (Figure 4b).

**Combinatorial antibiotic regimens to eradicate intracellular bacteria.** The ability of antibiotics and surgical preparation and irrigation solutions to control bacterial growth and prevent *S. aureus*-induced osteolysis in vitro was evaluated. As single agents, gentamicin and rifampicin demonstrated the greatest protective effects against MRSA-induced cytotoxicity (Figure 5a). Rifampicin conferred greater protection than gentamicin, although this difference was not statistically significant. Combined vancomycin and cefazolin treatment with gentamicin or rifampicin more substantially reduced cytotoxicity than pure vancomycin or cefazolin (Figure 5b). Combined vancomycin and rifampicin therapy demonstrated the greatest reduction in cytotoxic activity by USA300. Observed differences between groups were not statistically significant.

One-way ANOVA revealed significant reductions in both intracellular and extracellular bacterial concentrations with antibiotic treatment ($F_{(9,45)} = 6.08$; $p < 0.001$ and $F_{(9,45)} = 5.82$; $p < 0.001$, respectively) (Figure 5c). Tukey post-hoc comparisons revealed significant differences in
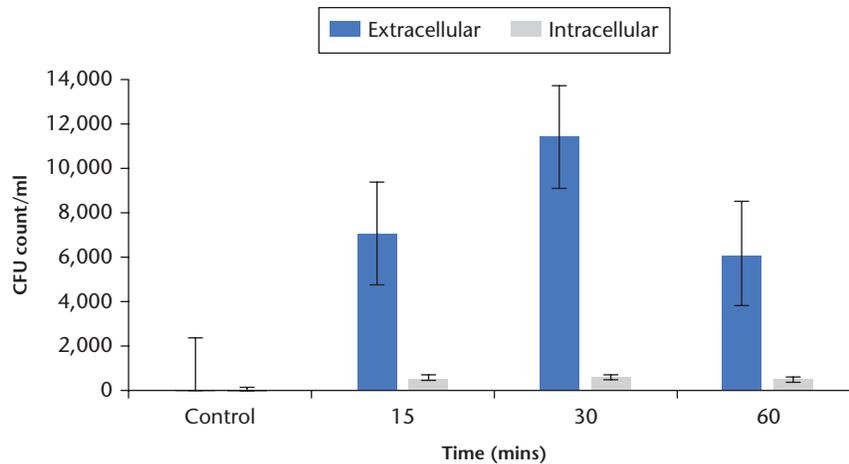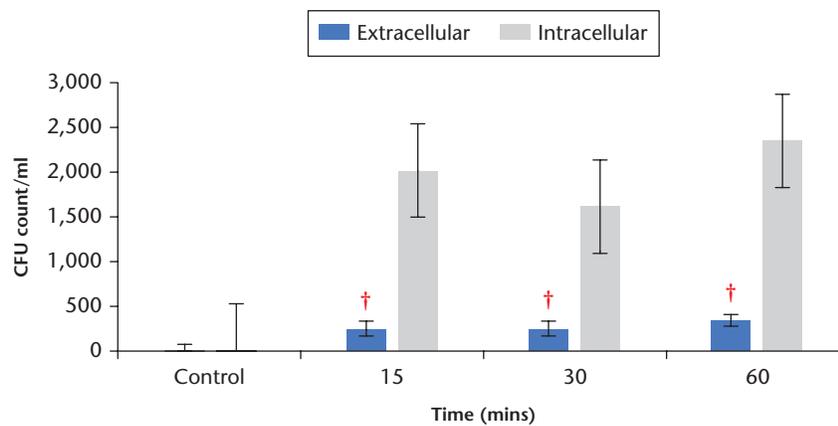
**Fig. 3a**



**Fig. 3b**

a) Intracellular and extracellular bacterial concentrations following in vitro USA300 infection of murine osteoblasts and osteoclasts. Differences in bacterial bioburden between the intracellular and extracellular compartments of both cell types were not statistically significant. Intracellular and extracellular osteoblast USA300 levels increased over time relative to the control. Differences in both intracellular and extracellular bacterial concentrations between timepoints were not statistically significant. b) Univariate, repeated measures ANOVA analysis identified a statistically significant increase in extracellular USA300 levels from infected osteoclasts over time (F (3,20) = 16.36; p < 0.001), but failed to demonstrate a significant difference in intracellular osteoclast bacterial load over time. ANOVA, analysis of variance.

intracellular bacterial concentrations between the vancomycin and gentamicin (p = 0.002), VG (p < 0.001), and CG (p = 0.003), gentamicin and ampicillin (p = 0.008), and ampicillin and VG groups (p = 0.002), as well as between the gentamicin and VR (p = 0.042), rifampicin and VG (p = 0.046), VG and VR (p = 0.010), and CG and VR groups, (p = 0.049) (Figure 5c). Tukey post-hoc tests revealed significant differences in extracellular bacterial concentrations between the control and rifampicin (p < 0.001), VR (p < 0.001), and CR (p < 0.001) groups, vancomycin and rifampicin (p = 0.006), and vancomycin and CR groups (p = 0.009), as well as between the control and VG (p = 0.012), control and CG (p = 0.014), and vancomycin and VR groups (p < 0.001) (Figure 5c). Overall, gentamicin-containing treatments most effectively reduced intracellular bacterial levels three hours postinfection, however, this difference was not statistically significant compared

with untreated, MRSA-infected controls (Figure 5c). Incubation with rifampicin alone or in combination with vancomycin or cephalexin for one hour most effectively reduced extracellular bacterial concentrations (Figure 5c).

**Antibacterial effects of skin preparation solutions.** Betadine hindered USA300 growth in vitro to the greatest extent at both timepoints, as indirectly assessed through optical density (Figure 5d). Although betadine, ethanol, and hydrogen peroxide all reduced USA300 proliferation in vitro, these differences were not statistically significant (Figure 5d).

## Discussion

The persistence of infection after antibiotic treatment represents a complex issue in clinical practice. Although the incidence of OM has largely been reduced due to modern antibiotics, infection remains a major concern in
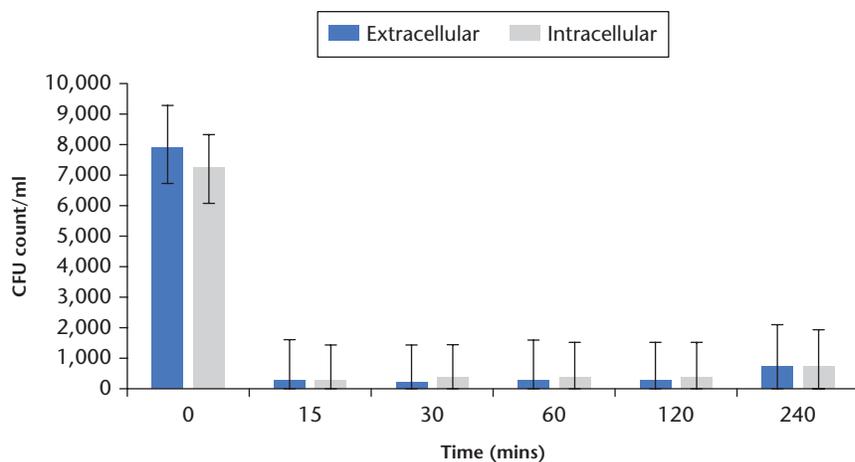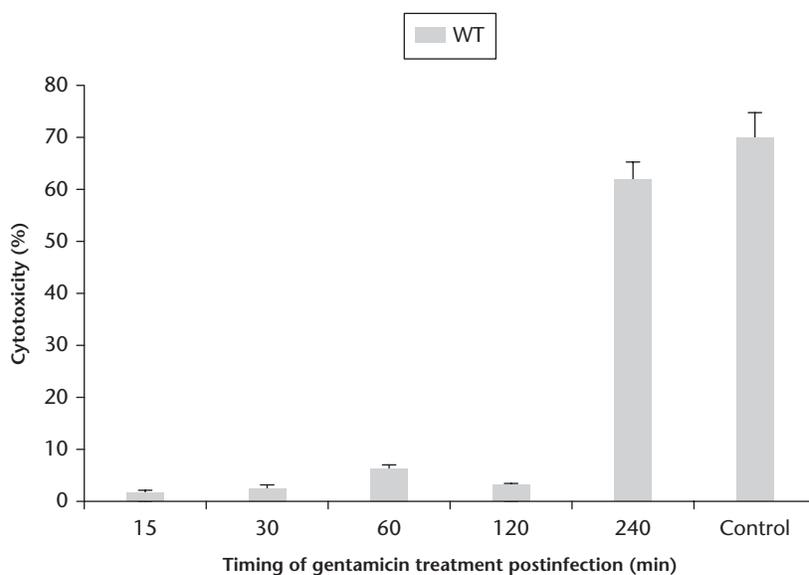
**Fig. 4a**



**Fig. 4b**

a) Time dependence of antibiotic protection against methicillin-resistant *Staphylococcus aureus* (MRSA) infection in vitro. Treatment of infected osteoblasts with gentamicin reduced both intracellular and extracellular bacterial concentrations compared with the control condition by one-way ANOVA (F (5,13) = 20.89; p < 0.001 and F (5,10) = 68.83; p < 0.001, respectively). b) Treatment of WT USA300 with 50 μg/ml gentamicin within two hours of infection significantly reduced MRSA-induced cytotoxicity in infected osteoblasts compared with untreated, infected osteoblasts by two-way student's t-test (p = 0.042). WT, wild type.

orthopaedic practice. Trauma-induced OM is the most common cause of chronic OM; open long bone fracture infection rates are between 4% and 64%.[19,20] The sequelae and costs of chronic OM necessitate investigation of the mechanisms of bacterial colonization, infection persistence, and damage to bone in order to improve treatment paradigms.

*S. aureus* retains viability after antibiotic treatment due to its intracellular persistence and other mechanisms of antibiotic resistance. Proinflammatory changes and osteolytic mechanisms of bone destruction have been described in response to *S. aureus*. Intracellular persistence within osteoblasts and osteoclasts may exacerbate the lytic changes observed in MRSA osteomyelitis. We not

only verified the intracellular invasion of osteoblasts and osteoclasts by *S. aureus*, but also demonstrated how MRSA grows within cells. *S. aureus* has a doubling rate of 20 minutes to 40 minutes and an average growth rate of 0.99h[-1].[21] Bacterial overgrowth can cause cell death within a short timeframe, but antibiotic administration during this window can protect cells from bacterial colonization. Antibiotic therapy for open fractures and prosthetic joint installation typically occurs beyond this early timepoint of bacterial susceptibility. Treatment of *S. aureus*-infected mouse osteoblasts with erythromycin and clindamycin at t = 0 inhibited *S. aureus* growth, however, treatment with these agents 12 hours postinfection was ineffective in curbing further *S. aureus* growth.[3] This
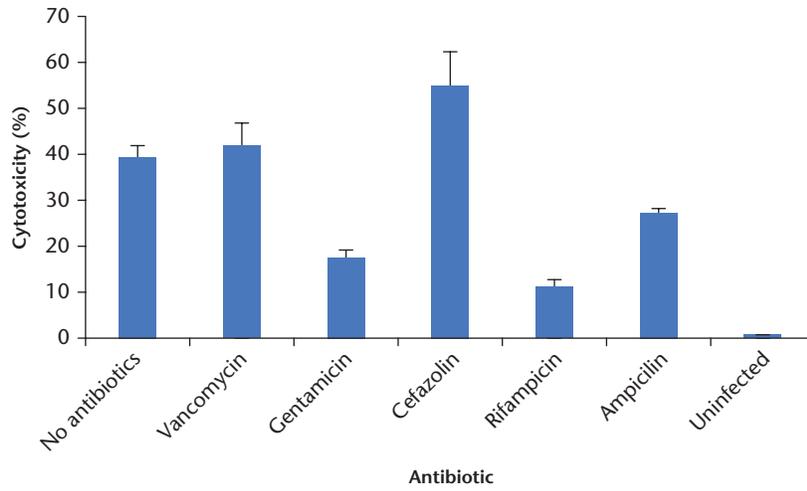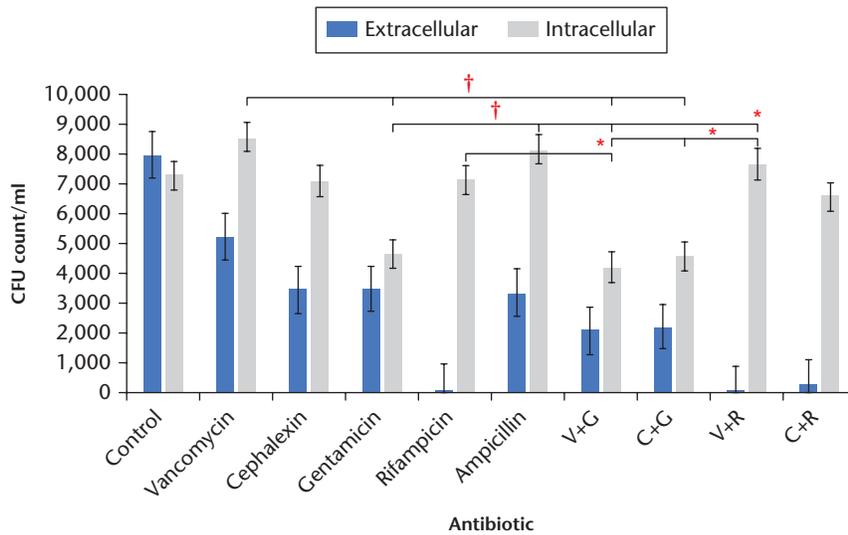
Fig. 5a
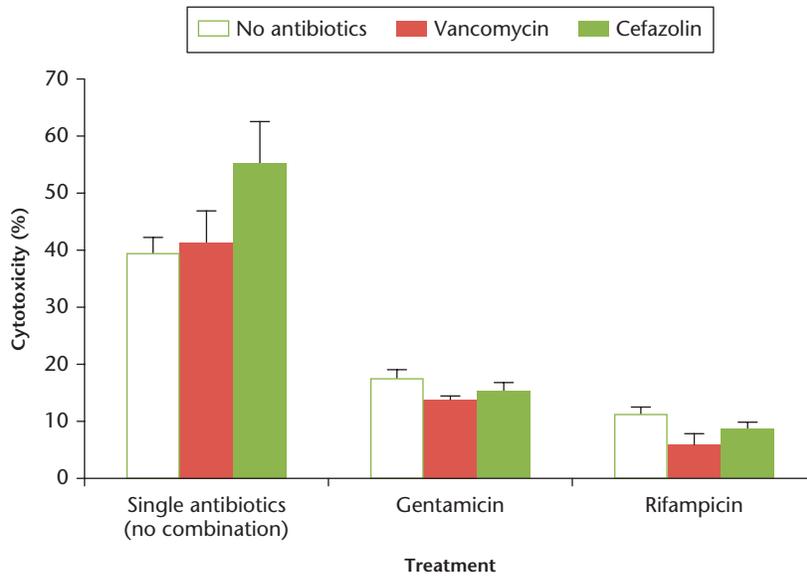


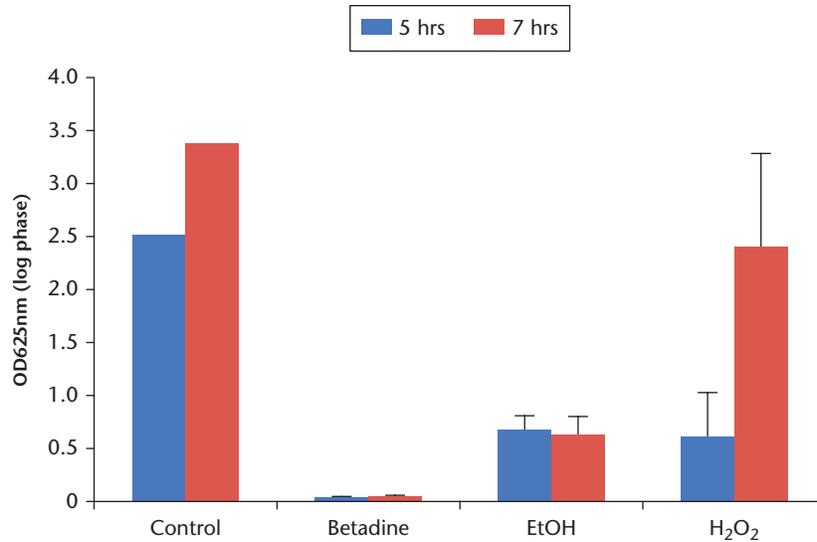Fig. 5b



Fig. 5c

*(Continued)*

**Fig. 5d**

a) Commonly used antiseptics were investigated for their efficacy against in vitro methicillin-resistant *Staphylococcus aureus* (MRSA) infection of murine osteoblast precursor cells. The ability of antibiotics to rescue USA300-infected MC3T3-E1 cells as single agents and in combination was assessed. As single agents, rifampicin and gentamicin demonstrated the greatest reductions in cytotoxicity, although these differences were not statistically significant. b) Inhibition of USA300-infected osteoblast death by antibiotics within 24 hours of infection. Greater reductions in cytotoxicity were observed when vancomycin and cefazolin were administered with gentamicin or rifampicin relative to pure vancomycin or cefazolin treatment. Combined vancomycin and rifampicin administration reduced USA300-induced cytotoxicity to the greatest extent. However, these differences were not statistically significant. c) Intracellular and extracellular bacterial concentrations obtained from infected osteoblasts were significantly lessened by antibiotic treatment three hours postinfection by one-way ANOVA (F $(9,45) = 6.08$; $p < 0.001$ and F $(9,45) = 5.82$; $p < 0.001$, respectively). After an antibiotic incubation period of one hour, gentamicin-containing treatments most effectively reduced intracellular bacterial level, although this difference was not statistically significant compared with untreated, infected controls. Rifampicin treatment alone or in combination with vancomycin or cephalexin for one hour most effectively reduced extracellular bacterial concentrations three hours postinfection relative to the untreated control and groups treated with other antibiotics. Tukey's post hoc comparisons demonstrated that gentamicin more effectively reduced intracellular USA300 concentrations compared to vancomycin ($p = 0.002$), ampicillin ($p = 0.008$), and the combination of vancomycin and rifampin (VR) ($p = 0.042$), while the combination of vancomycin and gentamicin (VG) and cefazolin and gentamicin (CG) more effectively reduced intracellular USA300 concentrations than vancomycin alone, $p = 0.000$ and $p = 0.003$, respectively. Tukey post hoc comparisons also showed that the combination of vancomycin and gentamicin reduced intracellular USA300 more than rifampicin ($p = 0.046$), and that the combined administration of cefazolin and gentamicin (CG) and vancomycin and gentamicin (VG) more effectively reduced intracellular USA300 concentrations compared to the combination of vancomycin and rifampicin (VR), $p = 0.049$ and $p = 0.010$, respectively. Optical density (OD) at 625 nm was observed following USA300 infection and betadine, ethanol, or hydrogen peroxide treatment after five and seven hours. All three agents reduced optical density, although betadine most effectively reduced bacterial proliferation and optical density in vitro. *$p < 0.050$, †$p < 0.010$. ANOVA, analysis of variance. CFU, colony-forming units; C, cephalexin; EtOH, ethanol; G, gentamicin; $H_2O_2$, hydrogen peroxide; R, rifampicin; V, vancomycin.

suggests that *S. aureus* can penetrate cells, adapt to the intracellular environment, and develop resistance to antibiotics within 12 hours of infection. Similar results were observed with rifampicin treatment; only a meager reduction in *S. aureus* proliferation was observed when administration was delayed to 12 hours.[3] Similar findings were obtained in our study, as gentamicin treatment 30 minutes postinfection significantly reduced bacterial bioburden compared with the untreated control and infected osteoblasts for which gentamicin treatment was delayed to four hours. Gentamicin treatment of infected osteoblasts reduced MRSA-induced cytotoxicity and bioburden at timepoints earlier than observed per the current standard of care for open fracture contamination, by which antibiotics are administered and surgical debridement is performed within the first three and six hours, respectively.

Systemic vancomycin or cefazolin treatment represents the current standard of care for OM treatment or prevention in the setting of wound contamination.

Combined vancomycin and rifampicin treatment demonstrated the greatest reduction in USA300-induced cytotoxic activity in infected osteoblasts. However, when antibiotics were administered three hours postinfection and allowed to incubate for one hour, gentamicin-containing treatments resulted in the greatest reduction of intracellular USA300 burden, while rifampicin-containing treatments resulted in the greatest reduction of extracellular USA300 levels. These findings may be partially attributable to the sensitivity of USA300 to gentamicin and potential differences between the optimal timeframes in which antibiotics may enter cells and act on bacteria. It is possible that gentamicin is capable of more rapid intracellular penetration and accumulation than rifampicin, such that antibiotic treatment over one hour did not fully capture the cell-penetrating effects and profiles of the antibiotics evaluated.[22] Aminoglycosides are believed to achieve low intracellular penetrance.[23,24] However, gentamicin has been proven capable of penetrating macrophages and killing intracellular bacteria such as *Listeria*

*monocytogenes* and *Yersinia pestis*, even with short incubation times.[25,26] Additional experiments investigating the antibiotic penetration of infected bone cells over time are needed. The bactericidal potency of rifampicin against USA300, as it considerably reduced extracellular USA300 bioburden and cytotoxicity, suggests that it is highly active against MRSA and protective in bony infection. Rifampicin is used to treat OM and preoperatively to kill bacteria at the early stage of growth and biofilm production.[3]

Early administration of cell membrane-penetrating antibiotics can mitigate damage to bone and soft tissue by acting on bacteria prior to their intracellular invasion of osteoblasts and osteoclasts. Standard open fracture management includes washout with antibiotic treatment (most commonly a cephalosporin-based regimen), surgical debridement and irrigation, fracture stabilization, and soft-tissue coverage. This protocol could be modified to include washout with rifampicin-containing solutions and early systemic antibiotic treatment. Additional research into earlier administration of antibiotics and surgical preparation solutions in the setting of MRSA musculoskeletal infection is warranted. A limitation of the experiments conducted is the small sample sizes used. Despite this, the therapeutic platform investigated herein can be applied to the management of contaminated open fractures, implants and revision arthroplasties, and limb salvage procedures with immunosuppressive therapy. We advocate for future initiatives to further examine the effect of intracellular *S. aureus* infection in vivo or in clinical trials.

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**Ethical review statement**
- This study was approved by the Columbia University Medical Center Institutional Review Board (ID: AAAM1606).