Large variations in clinical antibiotic activity against *Staphylococcus aureus* biofilms of periprosthetic joint infection isolates

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Abstract

Staphylococcus aureus biofilms have a high tolerance to antibiotics, making the treatment of periprosthetic joint infection (PJI) challenging. From a clinical perspective, bacteria from surgical specimens are cultured in a planktonic state to determine antibiotic sensitivity. However, *S. aureus* exists primarily as established biofilms in PJI. To address this dichotomy, we developed a prospective registry of total knee and hip arthroplasty PJI *S. aureus* isolates to quantify the activity of clinically important antibiotics against isolates grown as biofilms. *S. aureus* planktonic minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed using clinical laboratory standard index assays for 10 antibiotics (cefazolin, clindamycin, vancomycin, rifampin, linezolid, nafcillin, gentamicin, trimethoprim/sulfamethoxazole, doxycycline, and daptomycin). Mature biofilms of each strain were grown *in vitro*, after which biofilm MIC (MBIC) and biofilm MBC (MBBC) were determined. Overall, isolates grown as biofilms displayed larger variations in antibiotic MICs as compared to planktonic MIC values. Only rifampin, doxycycline, and daptomycin had measurable biofilm MIC values across all *S. aureus* isolates tested. Biofilm MBC observations complemented biofilm MIC observations;

rifampin, doxycycline, and daptomycin were the only antibiotics with measurable biofilm MBC values. 90% of *S. aureus* biofilms could be killed by rifampin, 50% by doxycycline, and only 15% by daptomycin. Biofilm formation increased bacterial antibiotic tolerance nonspecifically across all antibiotics, in both MSSA and MRSA samples. Rifampin and doxycycline were the most effective antibiotics at killing established *S. aureus* biofilms.

Introduction

Staphylococcus aureus is a common organism responsible for orthopaedic related infections[1,2]. *S. aureus* in implant related infections regularly form and primarily exist as established biofilms[3,4,5] These infections are difficult to treat because bacterial cells in biofilms have a high tolerance to traditional antibiotics[2]. This antibiotic tolerance of biofilms is also seen in many other types of bacteria[6–8]. A variety of drug tolerance mechanisms have been proposed, including impaired antibiotic penetration, quorum-sensing regulation, and altered metabolic states[9].

Although biofilms are the primary state for bacterial cells in infections, standard antibiotic susceptibility testing uses bacteria grown as planktonic cultures[10,11]. This accurately quantifies antibiotic genetic resistance but fails to assess antibiotic activity against bacteria in a biofilm state. Antibiotic susceptibility and tolerance is much less understood when the bacteria are cultured as biofilms. There is a significant decrease in antibiotic sensitivity between planktonic bacteria and bacteria cells in biofilms because of the high tolerance of biofilms to antibiotics[12,13]. Therefore, testing of *in vitro* bacteria cultured as biofilms from patient isolates would provide a more accurate determination of antibiotic susceptibility by directly observing the bacteria phenotype that exists during infection.

There are limited studies that have evaluated the activity of antibiotics against *S. aureus* biofilms specifically using PJI clinical isolates. To address this, we developed a prospective clinical isolate registry of total knee arthroplasty (TKA) PJI samples to quantify the sensitivity of different antibiotics to clinical isolates of both methicillin sensitive *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) *in vitro* cultured biofilms. Both planktonic and biofilm MIC and MBC of a panel of commonly administered antibiotics were quantified across all isolates. The objective was to determine the extent of variation in biofilm antibiotic sensitivity to clinically administered antibiotics.

Methods

Culture conditions and bacterial strains—All strains were inoculated from frozen stocks into 5 ml Tryptic Soy Broth (TSB, Bectin Dickinson and Company) in a 15 ml conical tube overnight at 37° C with shaking at 250 rpm. 16 hours later, all strains were diluted in Mueller Hinton Broth (MHB; Bectin Dickinson and Company) to a final concentration of 0.5×10^6 CFU/ml using the 0.5 MacFarland Standard (GFS Chemicals) and an Infinite M200 Spectrophotometer (Tecan). Assays were performed utilizing high throughput methods using sterile, tissue culture treated flat bottom 96 well plates (Thermo-Fisher Scientific). All experiments were performed at least in triplicate at three separate times with freshly inoculated cultures. Two lab strains, USA300[14] and SH1000[15], as well as

10 MRSA clinical isolates and 8 MSSA clinical isolates were tested. Clinical isolates from PJI patients were obtained from the clinical microbiology laboratory which were cultured on TSB agar slants. *S. aureus* clinical isolates were expanded in TSB in a shaking overnight culture and banked in cryotubes at −80°C in TSB with 10% glycerol. Institutional Review Board guidelines and regulations were followed in completing this study, IRB approval # PRO15070263.

Planktonic Culture MIC and MBC—All lab strains and clinical isolates of *S. aureus* were cultured planktonically at a concentration of 0.5×10^6 CFU/ml in sterile, tissue culture treated flat bottom 96 well plates with fold dilutions of six antibiotics (vancomycin, rifampin, gentamicin, trimethoprim/sulfamethoxazole, doxycycline, and daptomycin) at a final volume of 100 ul. MSSA samples were additionally tested with cefazolin and nafcillin, while MRSA samples were additionally tested with clindamycin and linezolid. Antibiotic concentrations of 125, 62, 31, 16, 8, 4, 2, 1, 0.5, 0.25, 0.13 μg/ml as well as untreated controls were tested. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed following clinical laboratory standard institute assays[10,11]. MIC was assessed by staining treated cultures with PrestoBlue viability assay (Thermo-Fisher Scientific)[16] according to manufacturer's instructions using a SynTek microplate reader. A clear and definitive color change occurred, and plates were quantified after 10 minutes of staining. MBC was determined by serial dilution of treated wells and plating on TSA II with 5% sheep blood CS100 plates. After an overnight incubation at 37° C, colony forming unit (CFU) analysis on plates was performed; a 99.9% reduction in CFU's of the original plating density represented the MBC[17].

Mature Biofilms MIC and MBC—For bacterial biofilms treatment assays, *S. aureus* strains were cultured planktonically at a concentration of 0.5×10^6 CFU/ml in sterile, tissue culture treated flat bottom 96 well plates at a volume of 100 ul. *S. aureus* strains were statically grown for 24 hours, at which time fresh MHB was exchanged. After another static incubation for 24 hours, wells were washed with dPBS to remove planktonic bacteria, and the 48-hour mature biofilms were treated with the same panel of antibiotics used in planktonic assays. Antibiotic concentrations were raised to 2000, 1000, 500, 250, 125, 62, 31, 16, 8, 4, 2 μg/ml and diluted in MHB. After 24 hours of treatment, the antibiotic supplemented MHB was removed, biofilms were washed with dPBS, and 100 μl of dPBS was added to biofilms in wells. The 96 well plates were then manually scrapped for 1 minute in the dPBS to homogenize biofilms for minimum biofilm inhibitory concentration (MBIC) analysis. For scraping, sterile and autoclaved 0.1–10 μl micropipette tips were kept in holder within the hinged box container and taped securely in place. Pipette tips were inserted simultaneously into all 96 wells and the wells were scraped vigorously. MBIC was assessed using PrestoBlue viability assay according to manufacturer's instructions using a SynTek microplate reader. A clear and definitive color change took longer in biofilm wells and occurred after 1 hour of staining at which time plates were quantified. For minimum biofilm bactericidal concentration (MBBC), scrapped biofilms well contents were plated directly onto blood agar plates and CFU analysis was performed. MBBC was determined as 99.9% reduction of 48-hour biofilm CFU.

Statistical Analysis—Antibiotic susceptibility and tolerance between planktonic and established biofilms of PJI clinical isolates were collected and compared. All graphical and statistical analysis was performed using Prism 7.0 (GraphPad, La Jolla, CA). Data was analyzed using the D'Agostino & Pearson normality test. Since data was non-parametric, when comparing two groups, a Mann-Whitney test was performed. Matched sample analysis was performed using a Wilcoxon rank test, and multiple group variance testing was performed using a Kruskal-Wallis test with a Dunn's multiple comparisons posttest.

Results

Variations in antibiotic activity against *S. aureus* planktonic cultures and mature biofilms

Clinical laboratory standard index protocol and a mature biofilm *in vitro* model was used to quantify variations in planktonic MIC, planktonic MBC, biofilm MIC, and biofilm MBC of different antibiotics across *S. aureus* isolates. Gentamicin, trimethoprim/sulfamethoxazole, and vancomycin displayed larger variations (~1.5 log spread) in planktonic MICs across all isolates, while rifampin, doxycycline and daptomycin displayed smaller variations (~0.5 log spread) (Fig 1A-blue). Alternatively, planktonic MBCs of all antibiotics displayed a large amount of variation (2 log spread) across all isolates (Fig 1B-blue). Rifampin showed superior antimicrobial action against planktonic cultures compared to other antibiotics, with MBC values ranging from 0.13 to 8 μg/ml. Next, we determined variations in MBIC and MBBC of antibiotics tested against all isolates. Most antibiotics showed a large variability (2–3 log spread) in MBIC across all isolates, except for daptomycin which displayed a variation of only one order of magnitude (Fig 1A-red). Many isolate biofilms showed no sensitivity to trimethoprim/sulfamethoxazole, gentamicin, or vancomycin despite normal sensitivity observed in planktonic cultures. Only rifampin, doxycycline, and daptomycin could kill the 48-hour mature biofilms, with biofilm MBCs ranging from 80 to 2000 μg/ml (Fig1B-red).

Both MSSA and MRSA biofilms display decreased sensitivity across all antibiotics

We assessed planktonic MIC and biofilm MIC (MBIC) of additional antibiotics cefazolin and nafcillin for MSSA isolates, as well as clindamycin and linezolid for MRSA isolates. 78% of MSSA isolate biofilms displayed sensitivity to cefazolin under our limit of detection (Fig 2A-red), while ~83% MRSA isolate biofilms displayed no sensitivity to clindamycin (Fig 2B-red). MIC values for established biofilms were more variable across isolates compared to planktonic cultures. In both MSSA and MRSA isolates, rifampin MIC for all 20 *S. aureus* strains was 0.125 μg/ml, while the MBICs ranged dramatically from 2–2000 μg/ml (Fig 2A and B). Daptomycin was the only antibiotic for which biofilm MIC did not display dramatic variation in sensitivity across all isolates. In every antibiotic tested, there was a significant increase in MBIC compared to planktonic MIC in both MSSA (Fig 2A) and MRSA (Fig 2B) isolates. Rifampin, doxycycline, and daptomycin could inhibit growth in all isolate biofilms at the doses tested. Lab strains SH1000 and USA300 also showed and statistically significant increase in 48- hour biofilm MIC compared to planktonic cultures. Matched sample analysis confirmed that the MIC for all strains did significantly increase in the biofilm phenotype compared to planktonic for both MSSA and MRSA (Fig 2C and D).

Cefazolin fails to eliminate MSSA biofilms and vancomycin fails to eliminate MRSA biofilms

Planktonic MBC and biofilm MBC (MBBC) of isolates were determined. Cefazolin, nafcillin, clindamycin, linezolid, trimethoprim/sulfamethoxazole, gentamicin, and vancomycin could not eliminate any isolate biofilms up to our limit of detection (Fig 3A and B-red). In all antibiotics tested, there was a significant increase in MBBC compared to planktonic MBC in MSSA (Fig 3A) and MRSA (Fig 3B) isolates. Measurements of biofilm MBC complements MIC observations; rifampin, doxycycline, and daptomycin were best at killing biofilms *in vitro*. 90% of *S. aureus* biofilms could be eliminated by rifampin, 50% by doxycycline, and only 15% by daptomycin. MBBC values of each antibiotic against both MSSA (Fig 3A) and MRSA (Fig 3B) were compared to determine which antibiotic eliminated biofilms most effectively. Lab strain SH1000 and USA300 biofilms also showed similar tolerance to antibiotics compared to planktonic cultures. In MSSA biofilms, rifampin and doxycycline MBBC values were significantly lower than MBBCs of cefazolin, nafcillin, trimethoprim/sulfamethoxazole, gentamicin, and vancomycin (Fig 3C). In MRSA biofilms, only rifampin MBBC values were significantly lower across all antibiotics tested (Fig 3D).

Discussion

S. aureus is a virulent and extremely challenging pathogen to treat in orthopaedic implant associated infections. The major criterion for antibiotic selection in the treatment of infection is based on susceptibility testing of clinical isolates cultured in a planktonic state[11]. However, in a clinical infection, *S. aureus* primarily exists as established biofilms[18]. Based on this dichotomy, we were interested in determining variations in antibiotic sensitivity between clinical *S. aureus* isolates when cultured as established biofilms compared to the typical planktonic culture. We observed a remarkably large variation in biofilm MIC as compared to planktonic MIC in the clinically important antibiotics cefazolin, vancomycin, and rifampin.

The large decrease in antibiotic sensitivity of *S. aureus* biofilms as compared to planktonic cells across all classes of drugs tested demonstrated that this tolerance mechanism was nonspecific. Rifampin, daptomycin, and doxycycline demonstrated a superior ability to inhibit biofilm growth compared to other antibiotics tested based on relative comparison of MIC. This is in close agreement with other groups showing a clear tolerance to traditional antibiotics by bacterial biofilms[12,19]. Rifampin is the only traditionally administered antibiotic that has shown high and reliable anti-biofilm activity in *S. aureus*[5]. However, rifampin monotreatment is avoided clinically since *S. aureus* can develop rapid resistance to this drug. In periprosthetic joint infections, rifampin is only used in combination with another antibiotic such as cefazolin or vancomycin[20,21]. Daptomycin is a more recently developed lipopeptide which disrupts the cellular membrane of bacteria, rapidly lysing cells independent of metabolic activity. This antibiotic has previously been shown to be highly effective against a panel of MRSA clinical isolate biofilms[19,22]. Recently, suppressive doxycycline therapy in a small and high risk PJI cohort showed reasonable effectiveness and tolerability of the antibiotic for successful treatment[23].To our knowledge, our work is the

first-time doxycycline has been demonstrated to be as effective as rifampin or daptomycin at killing *S. aureus* mature biofilms from PJI patients.

Comparison of antibiotic activity in mature biofilms and planktonic cultures is complicated by intra-strain growth and biofilm characteristic differences. Our observations were likely limited due to possible differences in biophysical properties of the secreted extracellular matrix, metabolic output, and the population density within biofilms grown from each individual isolate, however these and other factors were not measured in this study. *S. aureus* bacterial biofilms have been shown to become more susceptible to antibiotics with increased exposure time from one to five days[24]. While this study did not address the effect of treatment time on biofilm drug susceptibility, we acknowledge this is an important factor to consider. Despite these limitations, we clearly show bacterial biofilms remained after treatment with extremely high doses of clinically important antibiotics. This study displays a clear loss of antibiotic activity against bacterial biofilms compared to planktonic cells. Rifampin, the optimal anti-biofilm antibiotic screened, could only effectively kill biofilms *in vitro* at doses not achievable in human patients secondary to overt toxicity.

Established bacterial biofilms have a remarkable tolerance to antibiotics. Our data suggests that antibiotic treatment in *S. aureus* knee and hip PJI and other orthopaedic infections are a critical but incomplete part of treatment. Surgical debridement and the host immune system play vital roles in successful treatment of orthopaedic related biofilm infections. Our results should not be interpreted in the context that standard planktonic antibiotic sensitivity testing should be replaced. Standard clinical laboratory standards institute testing for MIC provide invaluable clinical data on genetic antibiotic resistance. This work does suggest that there is a phenotypic and non-specific change in tolerance to antibiotics that occurs between planktonic and biofilm phenotypes of bacteria. Further, it suggests that from an *in vitro* perspective, rifampin, daptomycin, and doxycycline may have a stronger ability to control implant associated infections. Further clinical studies are warranted to confirm these results.

Acknowledgements

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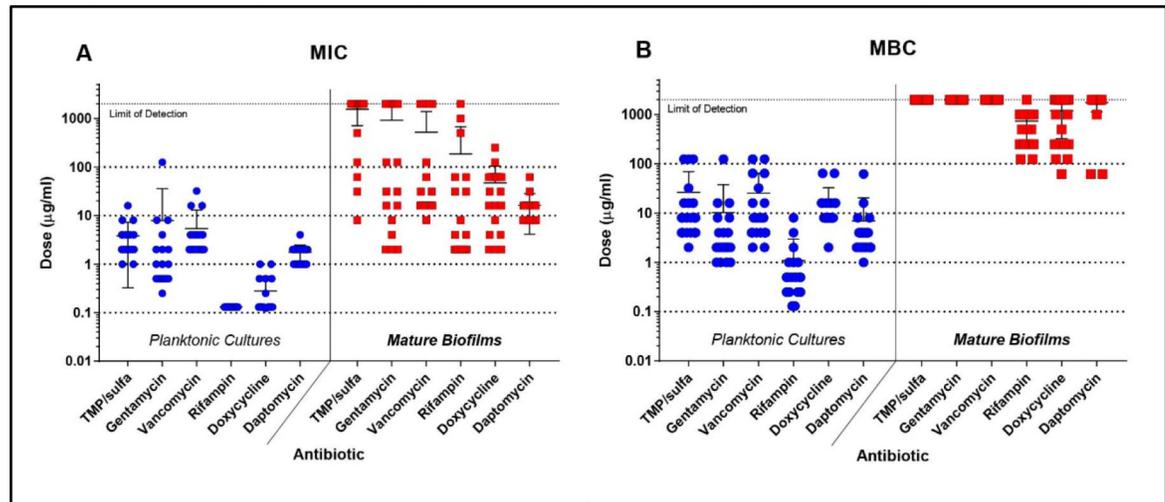
Figure 1. PJI *S. aureus* biofilms show decreased antibiotic sensitivity and increased tolerance to killing.

Across all clinical isolates and lab strains, antibiotic planktonic MICs (blue) and antibiotic mature biofilm MICs (red) were determined using a PrestoBlue viability assay (A). Antibiotic MICs of mature biofilms showed a much larger variation across all isolates compared to planktonic cultures. Across isolates, antibiotic planktonic MBCs (blue) and antibiotic mature biofilm MBCs (red) were determined by CFU analysis using blood agar plates (B). Clinical isolate biofilms and lab strains were all highly tolerant to antibiotics.
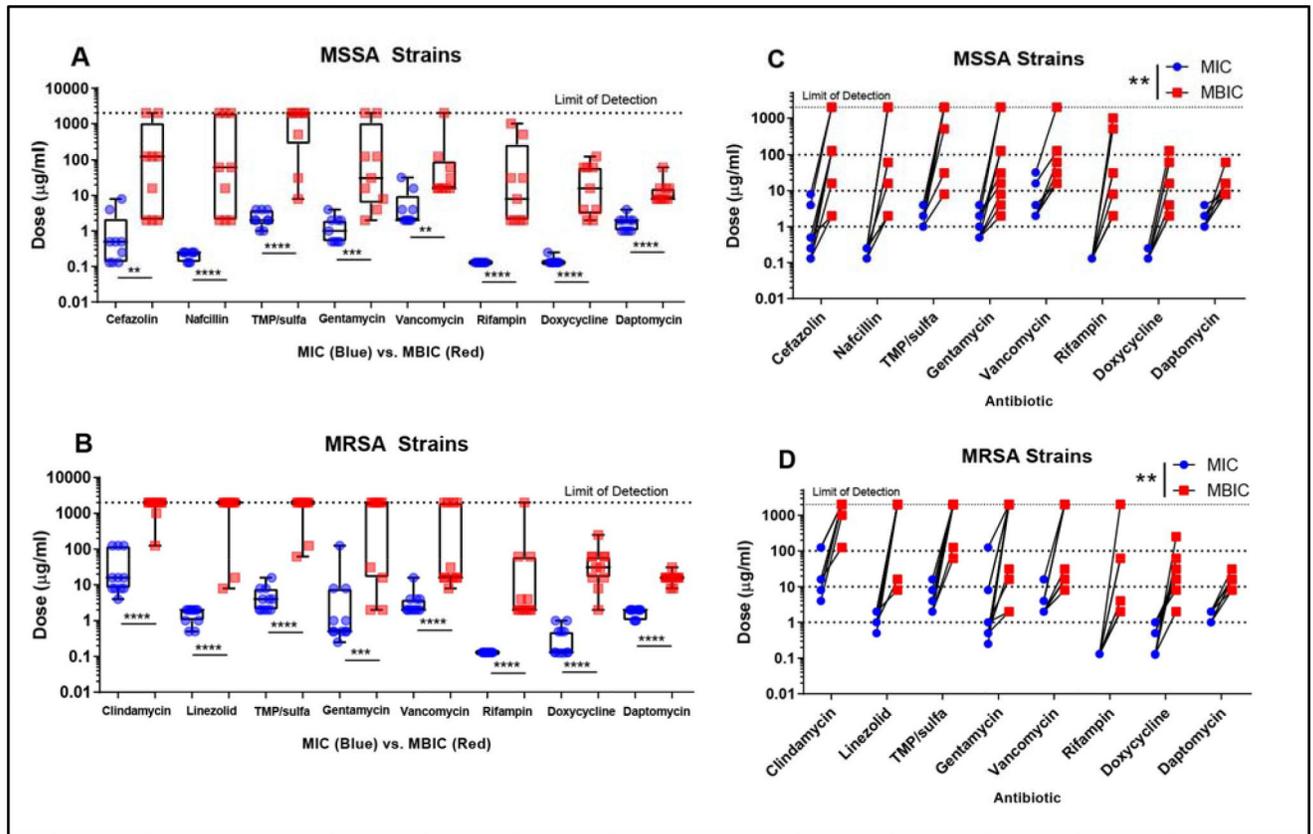
Figure 2. Both MSSA and MRSA biofilms demonstrate decreased sensitivity across all clinically used antibiotics.

MSSA and MRSA biofilms both show less antibiotic sensitivity with mature biofilm MICs (MBICs) significantly higher (p<0.001 ****, p<0.005*** p<0.01 **) than planktonic MIC in between every antibiotic tested using a Mann-Whitney Test (A-B). Matched analysis of *S. aureus* planktonic and biofilm MICs confirms a statistically significant increase (p<0.01 **) in MBIC compared to MIC in every clinical and laboratory strain tested using a Wilcoxon Test (C-D).
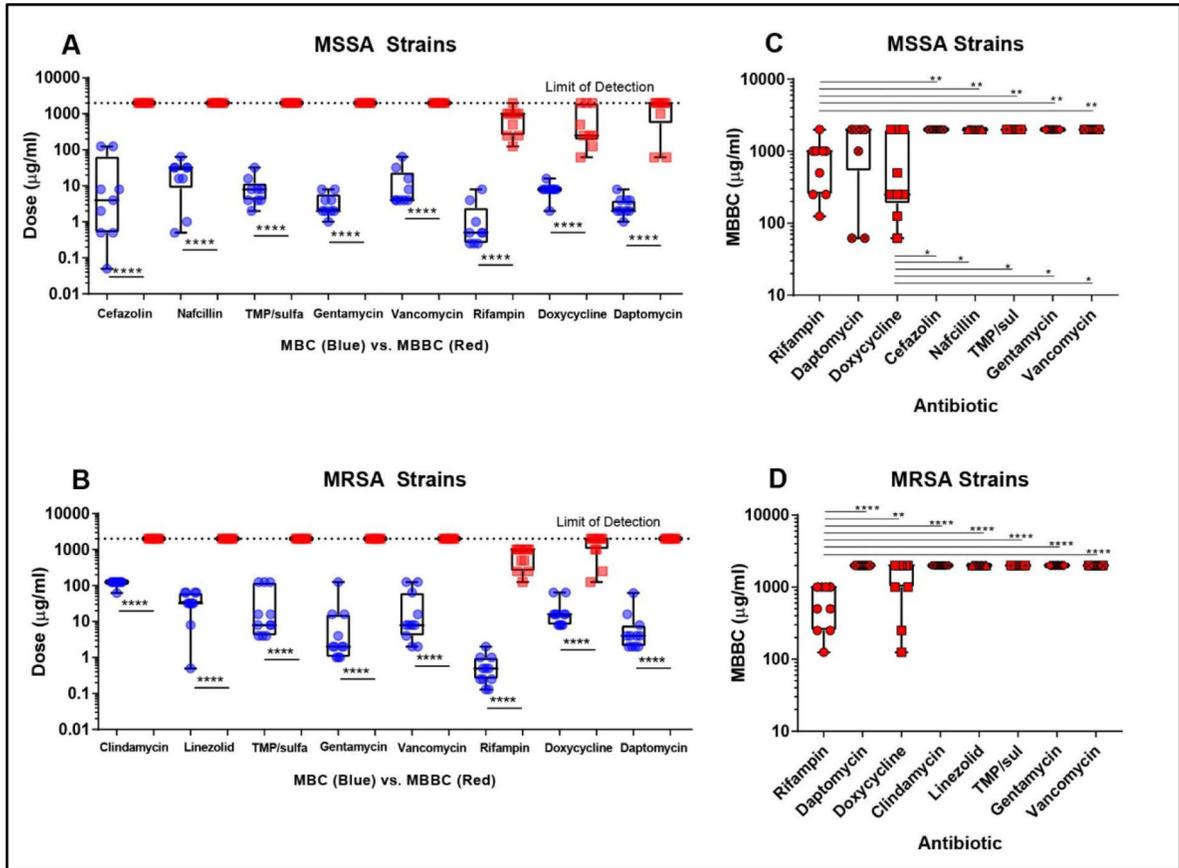
Figure 3. Clinically important antibiotics are unable to effectively kill MSSA and MRSA *S. aureus* biofilms.

S. aureus planktonic minimum bactericidal concentration (MBC) was compared to minimum biofilm bactericidal concentration (MBBC) for MSSA (A) and MRSA (B). All clinical and laboratory strains show increased tolerance to all antibiotics tested in our panel with MBBC values all statistically significantly higher (p<0.0001****) than the MBC values (A-B). In MSSA isolate biofilms, the rifampin and doxycycline treatment groups had a significantly lower MBC (p<0.05 *, p<0.0001 ****) compared to the remaining antibiotics (C). In MRSA isolate biofilms, only the rifampin treatment group had significantly lower MBC (p<0.0001 ****) compared to remaining antibiotics (D).