



ORIGINAL ARTICLE

Efficacy of combination oral antimicrobial agents against biofilm-embedded methicillin-resistant *Staphylococcus aureus*

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KEYWORDS

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Background: The combination of fusidic acid and rifampicin has a demonstrated synergistic effect against methicillin-resistant *Staphylococcus aureus* (MRSA), including planktonic and biofilm-related organisms. However, the *in vitro* efficacy of other combinations of oral anti-MRSA antibiotics in biofilm models has not been established.

Methods: The antibacterial activity of fusidic acid, linezolid, rifampicin, and minocycline against 33 biofilm-embedded MRSA isolates in low susceptibility and high resistance breakpoint concentrations was investigated using the 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide staining method. The compounds were further examined to determine their antibacterial efficacies in combination. The optical density ratio (ODr) was used to evaluate the antibacterial effects of these antibiotics, and the results indicate higher survival rates of MRSA on biofilm. A biofilm-positive phenotype (determined using the crystal violet stain) was defined as an optical density ≥ 0.17 at 492 nm, and strong biofilm formation was defined as an optical density ≥ 1.0 .

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Results: One-third of the MRSA isolates demonstrated weak biofilm formation, and two-thirds demonstrated strong biofilm formation. At low concentrations, linezolid alone lowered the ODR to 0.55 and was effective against biofilm-embedded MRSA ($p < 0.001$). The activity of minocycline was concentration-dependent and more effective against MRSA isolates that demonstrated weak biofilm formation. The effect of minocycline seems to be further enhanced when used in combination with either fusidic acid or linezolid at low concentrations, with the obtained results equal to those obtained with rifampicin-based regimens ($p < 0.001$). Rifampicin plus minocycline was also effective against MRSA in biofilm.

Conclusion: In comparison with monotherapy, minocycline-based combinations exhibit highly effective bactericidal effects against biofilm-embedded MRSA.

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes a variety of infections, including bacteremia, septic arthritis, osteomyelitis, and artificial graft infections such as those that occur in artificial joints.^{1–3} When infected artificial grafts are retained, biofilm-associated infections are very difficult to treat.⁴ Consequently, long-term combination oral antibiotic therapies are needed that not only effectively treat biofilm-related infections but also demonstrate few side effects. Some *in vitro* studies on the antibacterial effects of combination therapies have been performed on biofilms.^{4–12} Among these, rifampicin has a demonstrated synergistic effect against MRSA, including planktonic and biofilm-related organisms.^{3,11,13} In a review of the literature, it was found that minocycline alone is highly active—even more effective than other anti-MRSA antibiotics—against biofilm-embedded MRSA isolates.^{14,15} Considering the hepatotoxicity of rifampicin,¹⁶ the development of a minocycline-based combination therapy (such as those that incorporate fusidic acid or linezolid) may be especially important for overcoming the present problem of treating biofilm-associated MRSA infections that require long-term oral antibiotics. In this *in vitro* study, we examined the efficacy of minocycline, in combination with other available oral anti-MRSA agents, for reducing the bacterial burden on biofilms.

Materials and methods

Bacterial isolates

Thirty-three MRSA isolates, including those from blood ($n = 18$), joint fluid ($n = 7$), pus ($n = 5$), and other aseptic specimens ($n = 3$), were randomly obtained from patients with clinical infections from the clinical microbiology laboratory of Chi-Mei Foundational Hospital (Tainan, Taiwan). *Staphylococcus* species were identified by colonial morphology, Gram staining, and coagulase testing. MRSA was further confirmed by tube coagulase testing and growth on a 6 µg/mL oxacillin salt-agar screening plate. The organism was stored at -70°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, Lancashire, England) before use.

Antibiotics and minimum inhibitory concentrations

The antibiotics that were tested included rifampicin and minocycline (Sigma, St. Louis, MO, USA), linezolid (Pfizer, New York, NY, USA), and fusidic acid (Leo, Ballemp, Denmark). The minimum inhibitory concentrations (MICs) were determined by agar dilution, according to the recommendations from the Clinical and Laboratory Standards Institute (CLSI).¹⁷ Interpretation criteria for susceptibility testing were based on the guidelines from CLSI or the British Society for Antimicrobial Chemotherapy (BSAC).^{17,18} The inoculum was 5.0×10^5 colony-forming units (CFU)/mL. The inoculated plates were incubated in ambient air at 37°C for 24 hours. The MIC was defined as the lowest concentration of antibiotic that yielded no visible growth after overnight incubation. *S. aureus* ATCC 29213 was included in each run as the standard quality control strain.

Biofilm formation

Isolates were cultured for 1 day at 37°C in 5 mL of tryptic soy broth that was supplemented with 1% D-glucose (TSBGlc). The cultures were diluted to 1:1000 in TSBGlc, and 200 µL aliquots were added to each well of a 96-well tissue culture-treated polystyrene plate. After 24 hours of growth at 37°C , the plates were vigorously washed three times with phosphate-buffered saline (PBS) to remove any unattached bacteria and then dried for 1 hour at 60°C prior to staining with 0.4% crystal violet solution. The optical density (OD) was used as an index of bacterial adherence to the surface and biofilm formation. Experiments were performed in triplicate, the results were averaged, and standard deviations were calculated. To compensate for background absorbance, OD readings of the sterile medium with both the fixative and dye were averaged and subtracted from all of the experimental values. A biofilm-positive phenotype was defined as $\text{OD} \geq 0.17$ at 492 nm (OD_{492}). Strong biofilm formation was classified as $\text{OD}_{492} \geq 1.0$, and weak biofilm formation was classified as OD_{492} between 0.17–1.0.¹⁹

Biofilm staining method

A 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium-bromide (MTT) assay was performed using the method

described by Kairo et al with minor modifications.^{20,21–23} The MTT method can be used to evaluate the antibacterial activities of different drugs in the biofilm model and has been reported in the literature^{23–26} but not by CLSI. Briefly, at the endpoint of the treatment of the biofilms with antibiotics, the wells were emptied and washed three times with 200 μ L of sterile PBS. Then, 100 μ L PBS with 1% MTT (Sigma) solution was added and allowed to incubate for 2 hours at 37°C. The MTT solution was replaced with 100 μ L dimethyl sulfoxide and allowed to incubate for 15 minutes at room temperature. Viable bacteria reduced the tetrazolium salt to a purple water-soluble formazan product. The numbers of surviving bacteria are determined before and after a 90-minute incubation by measuring their ability to reduce the yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a purple formazan product. This reaction, which is mediated by bacterial dehydrogenases, can be detected colorimetrically by reading the plates on a microtiter plate reader at 570 nm. Higher OD values indicate an increased number of surviving MRSA isolates in the biofilm.

Minimum biofilm eradication concentration

The antibacterial activity of each drug in the biofilm was measured using the minimum biofilm eradication concentration (MBEC) assay.²⁷ MBEC indicates the lowest concentrations of the antimicrobial agent leading to clear wells in the 96-well ELISA platforms. The assay involved biofilm formation on the plastic pegs of the lid of the MBEC device. These biofilms were then exposed to the tested antibiotics for a defined period of time in a 96-well plate and incubated overnight. The MBEC value corresponds to the lowest dilution that prevents the regrowth of bacteria on the treated biofilm. This assay was validated in terms of reproducibility and accuracy by the standard reference isolates, according to the methods specified by CLSI. MBEC₅₀ and MBEC₉₀ are the minimum concentrations that inhibit 50% or 90% of the isolates from forming on the biofilm, respectively.

Antimicrobial effects of the antimicrobial agents on the biofilms

The biofilms of each isolate were prepared in two separated 24-well culture plates, as described above. The medium-free biofilm on the plate was incubated with fusidic acid, linezolid, rifampicin, and minocycline (either alone or in combination) for 1 day at 37°C. The biofilm of the wells in one plate was stained with MTT. The wells of the other plates were refilled with fresh dilutions of antimicrobial agents every day for 5 consecutive days and then stained with MTT. Two concentrations of antimicrobial agents—the susceptibility and resistance breakpoints—were used in accordance with CLSI guidelines. Group 1 included antibiotics at low concentrations (fusidic acid: 1 μ g/mL; linezolid: 4 μ g/mL; rifampicin: 1 μ g/mL; minocycline: 4 μ g/mL), and group 2 included antibiotics at high concentrations (fusidic acid: 2 μ g/mL; linezolid: 8 μ g/mL; rifampicin: 4 μ g/mL; minocycline: 16 μ g/mL). To account for the individual biofilm formation of each isolate, the ratio of the biofilm OD of

the isolate that was incubated with the antibiotics was calculated in relation to the biofilm OD of the same isolate without antibiotics (native biofilm). The baseline of the untreated biofilm was set to 1. This optical density ratio (ODr) was used to measure changes in the viable MRSA on each biofilm. The average color intensity of soluble formazan, including the effects of the single agents and the combination therapies on the 33 MRSA isolates on the biofilm, was compared with every control group on the fifth day and is presented in terms of the ODr values. A lower ODr indicates a greater inhibitory effect on the MRSA biofilm. All experiments were performed in triplicate, and all studies were repeated three times.

Colony counting

To count the number of colonies formed, 10 out of 33 MRSA isolates were randomly selected. After the 5-day incubation period with the antibiotics, the biofilm-containing wells were sonicated using a water table sonicator (VWR International, model 250HT) for 5 minutes. The disrupted biofilm was serially diluted, plated, and incubated overnight at 37°C for viable cell counting. The detection limit of the plate counting method was 100 CFU/mL.

Statistical analysis

Data were recorded as the mean \pm standard deviation. Because of the small sample size and the abnormal distribution of ODr values, the Mann-Whitney U test was used to compare differences between the two groups, and the Kruskal-Wallis H test followed by the Dunn's test was used for multiple comparison analysis. Statistical significance was set to $p < 0.05$. The Bonferroni correction rule was applied when multiple comparisons were performed. Data were analyzed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Of the 33 MRSA isolates, biofilm formation was weak in one-third (11 isolates) of the isolates and strong in the remaining two-thirds (22 isolates). The MIC and MBEC_{50/90} values of the different antibiotics against planktonic and biofilm MRSA are shown in Table 1. Of the isolates studied, only 39% of the planktonic isolates were susceptible to minocycline, whereas 21% and 6% of the biofilm-embedded isolates were susceptible to rifampicin and minocycline, respectively. Moreover, for each drug there were no significant differences in terms of the antibacterial activities against the antimicrobial-susceptible and -resistant isolates that were embedded in the biofilms.

When each of the four antibiotics were used alone at susceptibility breakpoint concentrations (SBCs) (Fig. 1), linezolid was more effective against biofilm-embedded MRSA (ODr = 0.55) in comparison with the control group ($p < 0.001$). When minocycline was used in a combination with the other three antibiotics, the combination of all four were also more effective than the control and reduced the ODr to around 0.29 or 0.47 ($p < 0.001$). When used in combination with rifampicin, fusidic acid, minocycline, and

Table 1 Antimicrobial susceptibilities, minimum inhibitory concentrations (MIC_{50/90}), and minimum biofilm eradication concentrations (MBEC_{50/90}) of four antibiotics against 33 planktonic or biofilm-embedded MRSA isolates

| Drugs | Planktonic MRSA | | | Biofilm-embedded MRSA | | |
|--------------|---------------------------|---------------------------|----------------|----------------------------|----------------------------|----------------|
| | MIC ₅₀ , µg/mL | MIC ₉₀ , µg/mL | Susceptible, % | MBEC ₅₀ , µg/mL | MBEC ₉₀ , µg/mL | Susceptible, % |
| Fusidic acid | 0.12 | 4 | 88 | 8 | > 256 | 0 |
| Linezolid | 2 | 2 | 100 | 64 | > 256 | 0 |
| Minocycline | 8 | 16 | 39 | 16 | 64 | 6 |
| Rifampicin | < 0.06 | < 0.06 | 100 | 8 | > 256 | 21 |

linezolid reduced the ODr to approximately 0.24–0.31, yielding greater antimicrobial effects than the control ($p < 0.001$). The minocycline- and rifampicin-based combination regimens also exhibited greater antimicrobial effect over fusidic acid, linezolid, minocycline, and rifampicin monotherapy ($p < 0.05$). One exception is that the combination of linezolid plus minocycline seems to be more effective than minocycline alone. However, there was no obvious statistical difference when compared with linezolid alone because linezolid monotherapy is effective against biofilm-embedded MRSA. Besides, there was no obvious statistical difference ($p > 0.05$) between the given rifampicin- and minocycline-based combinations.

When the same antibiotics were used alone at resistance breakpoint concentrations (RBCs) (Fig. 2), the antibacterial effects of linezolid and minocycline were more apparent (ODr = 0.37 and 0.27, respectively) against biofilm-embedded MRSA on the fifth day ($p < 0.001$) in comparison with the control group. On the other hand, the ODr on the fifth day was lower than the ODr on the first day for linezolid and minocycline. However, the ODr gradually increased from the first through the fifth days for rifampicin

and fusidic acid. Regarding the minocycline-based combination, all three antibiotics seemed to be more effective than the control and reduced the ODr to approximately 0.18 or 0.23 ($p < 0.001$). The rifampicin-based combination also seemed to be more effective than the control and reduced the ODr to 0.18, 0.27, and 0.37 for minocycline, linezolid, and fusidic acid, respectively ($p < 0.001$). Comparing the antibacterial effects of the different concentrations of antibiotics, the ODr of the RBC was generally lower than the SBC ($p < 0.05$) of the minocycline-based group. However, there was no significant difference in terms of the antibacterial effects of the minocycline- and rifampicin-based regimens at RBC on biofilm-embedded MRSA ($p > 0.05$).

Regarding the antibacterial effects of the single and combination regimens on the strong and weak biofilm-forming groups (Fig. 3), minocycline decreased the ODr to 0.43 and seemed to be more effective against the weak biofilm-forming MRSA group in comparison with the strong biofilm-forming group (ODr = 1.00) ($p < 0.05$). When fusidic acid was added to minocycline, the ODr of the weak biofilm-forming group was decreased to 0.25 in comparison

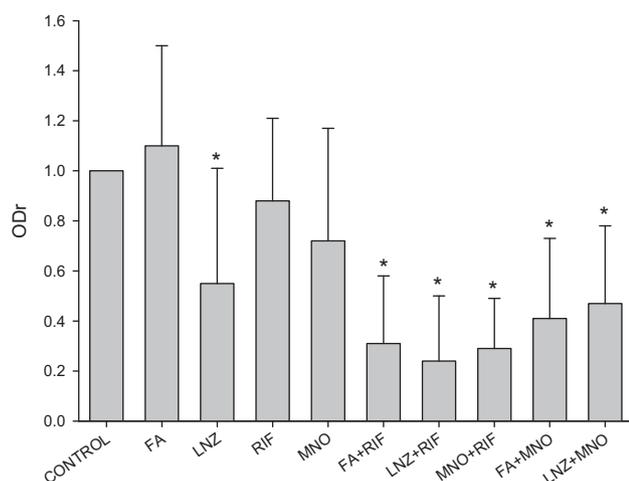


Figure 1. Optical density ratios (ODr) of the four antibiotics used (either alone or in combination) to treat 33 MRSA isolates in biofilm. The isolates were exposed to susceptibility breakpoint concentrations of the following antibiotics for 5 days: fusidic acid (FA), 1 µg/mL; linezolid (LNZ), 4 µg/mL; rifampicin (RIF), 1 µg/mL; and minocycline (MNO), 4 µg/mL. The optical density of MRSA growth on the biofilm, without treatment by antibiotics, was used as the control. Data are shown as the mean values \pm standard deviations. *Indicates a p value < 0.0001 .

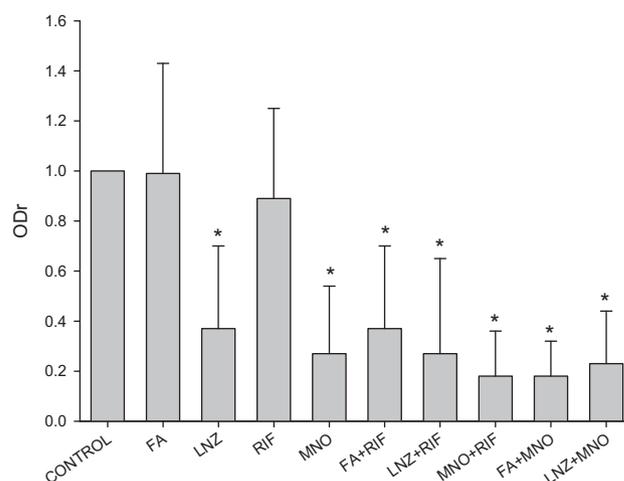


Figure 2. Optical density ratios (ODr) of the four antibiotics used (either alone or in combination) to treat 33 MRSA isolates in biofilm. The isolates were exposed to resistance breakpoint concentrations of the following antibiotics for 5 days: fusidic acid (FA), 2 µg/mL; linezolid (LNZ), 8 µg/mL; rifampicin (RIF), 4 µg/mL; and minocycline (MNO), 16 µg/mL. The optical density of MRSA growth on the biofilm, without treatment by antibiotics, was used as the control. Data are shown as the mean values \pm standard deviations. *Indicates a p value < 0.0001 .

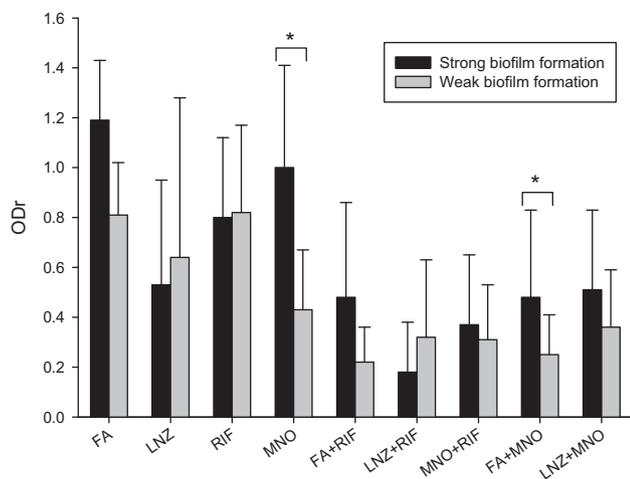


Figure 3. Comparisons of the antibacterial activities of the four antibiotics used (either alone or in combination) to treat MRSA isolates with strong and weak biofilm-forming groups. These groups were exposed to susceptibility breakpoint concentrations of the following drugs for 5 days: fusidic acid (FA), 1 $\mu\text{g}/\text{mL}$; linezolid (LNZ), 4 $\mu\text{g}/\text{mL}$; rifampicin (RIF), 1 $\mu\text{g}/\text{mL}$; and minocycline (MNO), 4 $\mu\text{g}/\text{mL}$. The optical density ratios (ODr) are shown below and compared with the control (defined as 1.0). Data are shown as the mean values \pm standard deviations. *Indicates a p value < 0.05 .

with the strong biofilm-forming group (ODr = 0.05) ($p < 0.05$).

We also wanted to compare the accuracies and differences between ODr and colony counting for measuring the survival of MRSA on biofilms (Fig. 4). For colony counting, 10 of the 33 MRSA isolates were randomly selected, and 8 of these 10 MRSA isolates were resistant to minocycline. Using

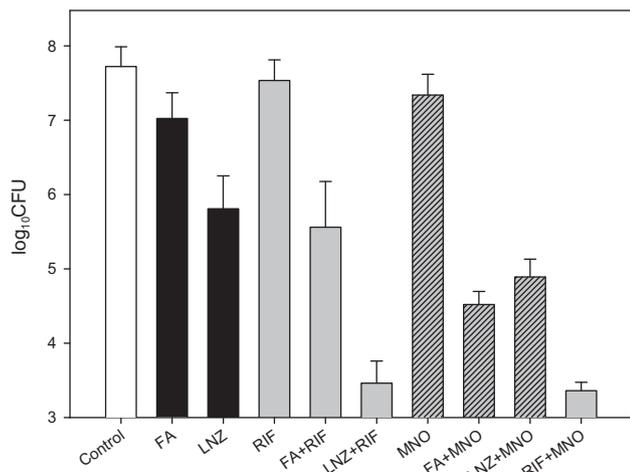


Figure 4. Antibacterial activities of the four antibiotics used (either alone or in combination) to treat 10 MRSA isolates on biofilm. These isolate were exposed to susceptibility breakpoint concentrations of the following drugs for 5 days: fusidic acid (FA), 1 $\mu\text{g}/\text{mL}$; linezolid (LNZ), 4 $\mu\text{g}/\text{mL}$; rifampicin (RIF), 1 $\mu\text{g}/\text{mL}$; and minocycline (MNO), 4 $\mu\text{g}/\text{mL}$. Colony counts are shown as the means \pm standard deviations.

SBC and CFU counts instead of ODr to measure the survival of MRSA on biofilm, linezolid was shown to decrease the colony count by about 2 \log_{10} -fold by the fifth day compared with the control group. Regarding the combination groups, minocycline- and rifampicin-based regimens all demonstrated high antibacterial effects with no obvious differences between them ($p > 0.05$). Minocycline plus rifampicin decreased the colony count by approximately 4 \log_{10} -fold, which seems to be the most effective combination regimen, exhibiting an enhanced antibacterial effect in contrast with monotherapy. The results of the two methods are comparable and the correlation coefficient was 0.86 ($p = 0.0014$).

Discussion

In this study, we evaluated the antibacterial effects of combinations of oral antibiotics on 33 biofilm-embedded MRSA isolates. Linezolid at SBC seemed to be effective. Rifampicin was initially inhibitory, but there was bacterial regrowth at two different concentrations (data not shown). This result is comparable with that the results reported by Raad et al,¹⁵ which showed that rifampicin initially causes a significant decrease in MRSA colonization on biofilms. However, after repeated daily exposure to rifampicin, most of MRSA isolates develop resistance to this antibiotic.¹⁵

According to a previous study, rifampicin is a constituent of all of the combinations that are active against MRSA and is a part of any antibiotic therapy that is directed against biofilms formed by these organisms.¹¹ In our study, rifampicin plus linezolid was effective and demonstrated an enhanced antibacterial effect compared with monotherapy. This result is comparable with those of a previous study.¹⁵ Compared with the study by Aboltins et al,¹³ fusidic acid plus rifampicin seems to be as effective as it was in our biofilm model. Such a rifampicin-based regimen is useful and critically important because oral formulations need to be available for long-term therapy.

Our study demonstrates that minocycline plus linezolid or fusidic acid at SBC is as effective as rifampicin-based regimens and this combination, when applied at the higher RBC, was even more effective, even in cases where 80% of the MRSA isolates were resistant to minocycline. In our model, a low concentration of minocycline alone was ineffective. However, minocycline-based combinations demonstrated enhanced antibacterial effects. The antibacterial effect of minocycline in this study was not as strong as that reported in a previous study,¹⁵ in which minocycline exhibited a greater antibacterial effect than linezolid did in the biofilm model. These discordant results may be related to differences in the experimental methodology between the two studies.

The antibacterial effect of minocycline seems to be greater in the weak biofilm-forming group. However, the effects of the other three antibiotics on both groups were the same. Therefore, according to our results, the thickness of the biofilm does not seem to be related to the antibacterial effect, except in the case of minocycline. Fusidic acid plus minocycline exhibited an obviously enhanced antibacterial effect on the weak biofilm-forming MRSA isolates. Clinically, we expect that the invasive MRSA

biofilm intensity can be used to predict the success rate of minocycline combination therapies.

Because high concentrations of antibiotics are necessary to treat biofilm-related infections, according to the findings published in the literature and our results,¹⁵ we must prescribe higher doses of antibiotics to achieve effective serum levels. However, high-dose antibiotics are not always practical *in vivo* because high concentrations increase the toxicity and introduce related side effects. According to our results, low-concentration combination therapies that use minocycline plus fusidic acid, linezolid, or even rifampicin seem to effectively eradicate biofilm-related MRSA. The combination of minocycline and either linezolid or fusidic acid seems to be an alternative for patients who cannot tolerate rifampicin.

Minocycline, when used in combination with either fusidic acid, linezolid, or rifampicin, is a good choice for clinicians because all of these antibiotics are available in oral form. The role of minocycline is more important than rifampicin, especially in patients with liver cirrhosis where long-term treatment with rifampicin is intolerable. Long-term use of linezolid may lead to bone marrow suppression. In this situation, the role of fusidic acid plus minocycline becomes relatively more important and useful, especially for treating weak biofilm-forming MRSA.

Because the concentrations of different antibiotics in the bones, synovial membranes, and joint spaces are variable, and because *in vitro* study results cannot completely explain or represent biofilm-related infections, further *in vivo* and clinical investigations are required to verify the potential advantage of minocycline when used in combination with other antistaphylococcal antibiotics to treat biofilm-related infections.

In conclusion, we have described the significant antibacterial activities of minocycline-based combination regimens, i.e., minocycline in combination with either fusidic acid, linezolid, or rifampicin, in a biofilm model of MRSA infection. However, their clinical significance requires further study.

Ethics approval

Ethics approval was not required for this study.

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Competing interests

The authors have no competing interests to declare.

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