Linezolid Alone or Combined with Rifampin against Methicillin-Resistant *Staphylococcus aureus* in Experimental Foreign-Body Infection

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We investigated the activity of linezolid, alone and in combination with rifampin (rifampicin), against a methicillin-resistant *Staphylococcus aureus* (MRSA) strain in vitro and in a guinea pig model of foreign-body infection. The MIC, minimal bactericidal concentration (MBC) in logarithmic phase, and MBC in stationary growth phase were 2.5, >20, and >20 μg/ml, respectively, for linezolid; 0.01, 0.08, and 2.5 μg/ml, respectively, for rifampin; and 0.16, 0.63, >20 μg/ml, respectively, for levofloxacin. In time-kill studies, bacterial regrowth and the development of rifampin resistance were observed after 24 h with rifampin alone at ≥4× or ≥8× the MIC and were prevented by the addition of linezolid. After the administration of single intraperitoneal doses of 25, 50, and 75 mg/kg of body weight, linezolid peak concentrations of 6.8, 12.7, and 18.1 μg/ml, respectively, were achieved in sterile cage fluid at ~3 h. The linezolid concentration remained above the MIC of the test organism for 12 h with all doses. Antimicrobial treatments of animals with cage implant infections were given twice daily for 4 days. Linezolid alone at 25, 50, and 75 mg/kg reduced the planktonic bacteria in cage fluid during treatment by 1.2 to 1.7 log10 CFU/ml; only linezolid at 75 mg/kg prevented bacterial regrowth 5 days after the end of treatment. Linezolid used in combination with rifampin (12.5 mg/kg) was more effective than linezolid used as monotherapy, reducing the planktonic bacteria by ≥3 log10 CFU (P < 0.05). Efficacy in the eradication of cage-associated infection was achieved only when linezolid was combined with rifampin, with cure rates being between 50% and 60%, whereas the levofloxacin-rifampin combination demonstrated the highest cure rate (91%) against the strain tested. The linezolid-rifampin combination is a treatment option for implant-associated infections caused by quinolone-resistant MRSA.

Implanted devices are increasingly used in modern medicine to alleviate pain or improve a compromised function. Implant-associated infections represent an emerging complication caused by organisms which adhere to the implant surface and grow embedded in a protective extracellular polymeric matrix, known as a biofilm (7, 8, 41). In addition, the microorganisms in biofilms enter a stationary growth phase and become phenotypically resistant to most antimicrobials, frequently causing treatment failure. In such cases, surgical removal of the implant is often required, causing high morbidity and substantial health care costs (5, 14, 32). *Staphylococcus aureus* is the most common pathogen causing implant-associated infections (5, 7). Successful treatment of these infections includes early surgical intervention and antimicrobial treatment with bactericidal drugs that also act on surface-adhering microorganisms. Rifampin (rifampicin) is bactericidal against stationary-growth-phase staphylococci, as demonstrated in vitro, in experimental animal models, and in clinical studies (9, 43). However, when it is used as a single agent, the rapid emergence of rifampin resistance occurs (37). Therefore, the use of antimicrobial combinations to prevent the development of rifampin resistance during treatment have been investigated (34, 36, 39). Rifampin in combination with quinolones has successfully been used for the treatment of orthopedic implant-related infections (9, 25, 35). However, the increasing prevalence of quinolone-resistant staphylococci has urged investigations for alternative drugs for use in combination with rifampin treatment (4, 30). In particular, methicillin-resistant *staphylococci* represent an increasing challenge due to their resistance to a broad variety of antimicrobials (23, 33).

The oxazolidinone linezolid is active against gram-positive cocci, including methicillin-resistant *Staphylococcus aureus* (MRSA) (11, 20, 24, 28). Limited data on the use of the linezolid-rifampin combination for the treatment of implant-associated MRSA infections are available. In vitro time-kill experiments showed a potential additive effect between linezolid and rifampin against MRSA (12). However, only case reports or small case series describing the treatment of implant-associated infections with linezolid and rifampin exist (2, 16, 19, 26).

In the study described here, we investigated the activity of linezolid, alone and in combination with rifampin, against one reference MRSA strain in vitro and in an established foreign-body infection model. The cage-associated infection model in guinea pigs has been validated for testing the activities of antimicrobial agents and their combinations against implant-associated infections in preclinical studies (38, 42).

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Materials and Methods

Study organism. MRSA strain ATCC 29213 was susceptible to levofloxacin and rifampin, and was used for in vitro and in vivo antimicrobial testing. Methicillin-susceptible S. aureus strain ATCC 29213 was used as the indicator organism for the agar diffusion bioassay. The strains were stored at −70°C by use of a cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). One cryovial bead was cultured overnight on Columbia sheep blood agar plates (Becton Dickinson, Heidelberg, Germany). Inocula were prepared from subcultures of two to three colonies, which were resuspended in 5 ml of Trypticase soy broth (TSB) and incubated overnight at 37°C without shaking.

Antimicrobial agents. Linezolid was provided as a purified powder by the manufacturer (Pfizer AG, Zurich, Switzerland); stock solutions of 2.5 mg/ml were prepared in sterile pyrogen-free water. Levofloxacin hemihydrate injectable solution (5 mg/ml; Aventis Pharma AG, Zurich, Switzerland) and rifampin (Sanofi AG, Steinhausen, Switzerland) were purchased from the respective manufacturer.

In vitro antimicrobial susceptibility. The in vitro susceptibility of the MRSA strain to linezolid, levofloxacin, and rifampin was determined in triplicate by using a standard inoculum of 10^6 to 5 x 10^6 CFU/ml, adjusted to an optical density of 0.1 at 600 nm (E600). Each antimicrobial agent was tested at 10-fold serial dilutions of the 20-mg/liter linezolid solution, 300 µg/ml levofloxacin, and 75 mg/kg rifampin. Solution (5 mg/ml; Aventis Pharma AG, Zurich, Switzerland) and rifampin (Sanofi AG, Steinhausen, Switzerland) were purchased from the respective manufacturer. Each 10-mg tube of MHB was suspended in sterile pyrogen-free water, and the mixture was boiled at 100°C in a water bath for 30 min. After the medium was cooled to 50°C, it was inoculated with the overnight culture of the indicator organism (300 µl/400 ml medium), and poured into large assay plates (30 by 30 cm). Aliquots of appropriate dilutions on Mueller-Hinton agar (MHA). The concentration of rifampin was expressed as the median and interquartile range of the MIC.

In vitro antimicrobial resistance studies. An assay was developed to evaluate the rate of in vitro emergence of rifampin resistance. The rate of resistant to total colony counts was assessed after 24 h of incubation of the MRSA strain in 10 ml MHB containing rifampin alone or rifampin and linezolid at 1× the MIC. The 24-h bacterial cultures were serially diluted 10-fold, 50-µl aliquots were plated on MHA containing rifampin (1 µg/ml) and no antibiotic, and the colonies were counted after 48 h of incubation at 37°C. The results were expressed as a ratio between the rifampin-resistant log_{10} CFU/ml and the total log_{10} CFU/ml. Experiments were performed in triplicate.

In vitro time-kill studies. The antimicrobial activities of linezolid and rifampin, alone and in combination, against the MRSA strain were evaluated by time-kill studies with inocula of 10^6 to 5 x 10^6 CFU/ml, as described previously (15). Antibiotic solutions with 1× and 4× the MIC of the test strain were prepared in 10 ml of MHB. Growth in the absence of antibiotics served as the control. Colony counts were determined after 0, 6, and 24 h of incubation at 37°C by plating aliquots of appropriate dilutions on Mueller-Hinton agar (MHA). The 10-fold serial dilutions allowed accurate colony counts in the range of 10 to 250 CFU per plate. Growth in the absence of antibiotics served as the control. Colony counts were determined after 0, 6, and 24 h of incubation at 37°C by plating aliquots of appropriate dilutions on Mueller-Hinton agar (MHA). The 10-fold serial dilutions allowed accurate colony counts in the range of 10 to 250 CFU per plate and minimized the effects of drug carryover. The quantification limit was set equal to 200 CFU/ml (>10 CFU in 50 µl of a 10-fold dilution). Killing over time was expressed as the mean reduction in the log_{10} CFU/ml ± the standard deviation (SD). Synergy was defined as a 100-fold increase in the level of killing at 24 h with the combination in comparison with the level of killing achieved with the most active single drug. Antagonism was defined as a 100-fold decrease in the level of killing at 24 h with the combination of both drugs compared to the level of killing achieved with the most active single drug (15).

Cultures of the MRSA strain that were exposed to rifampin alone or in combination with linezolid and that showed visible growth after 24 h of incubation were tested for rifampin resistance. The cultures were adjusted to a standardized inoculum corresponding to a McFarland 0.5 standard, spread on MHA containing rifampin (1 µg/ml), and assessed for growth. Experiments were performed in triplicate.

Animal model. A foreign-body infection model in guinea pigs was used, as described previously (3, 38, 40, 42). Guinea pigs were kept under specific-pathogen-free conditions in the Animal House of the Department of Biomedicine, University Hospital Basel, and animal experimentation guidelines according to the regulations of Swiss veterinary law were followed. The study protocol was approved by the Institutional Animal Care and Use Committee. In brief, four sterile polytetrafluoroethylene (Teflon) cages (32 mm by 10 mm) perforated with 130 regularly spaced holes of 1 mm in diameter (Angst-Pfister AG, Zurich, Switzerland) were subcutaneously implanted in the flanks of male albino guinea pigs (Charles River, Sulzfeld, Germany) under aseptic conditions. Animals weighing 550 to 600 g were anesthetized with an intramuscular injection of ketamine (20 mg/kg of body weight) and xylazine (4 mg/kg). Two weeks after surgery and healing of the surgical wounds, the sterility of the cages was verified by culture of the aspired cage fluid. Contaminated cages were excluded from further studies. Sterile cages were used for the pharmacokinetic studies. For the treatment studies, the trapezoid were infected by percutaneous inoculation of 200 µl containing 2 x 10^8 CFU of the MRSA strain (day 0). Before inoculation, overnight bacterial cultures were washed twice, resuspended in 5 ml of sterile pyrogen-free normal saline, and diluted 1:1,000. The establishment of infection was confirmed 24 h later by quantitative culture of aspired cage fluid.

Pharmacokinetic studies. Cage fluid was aspirated from noninfected animals over 24 h (1, 2, 4, 6, 8, 10, 12, and 24 h) following intraperitoneal administration of the dose of linezolid at 25, 50, and 75 mg/kg. Guinea pigs; therefore, 12 cages were used to relate the pharmacokinetic parameters to the antimicrobial treatment efficacy results. At each time point, 150-µl aliquots of cage fluid were aspirated from two cages from each animal (i.e., six replicates per time point and drug dose). The collected fluid was centrifuged (2,100 × g for 7 min), and the supernatant was stored at −20°C until further analysis. Determination of drug concentrations. Linezolid concentrations in cage fluid were determined by an agar plate diffusion bioassay with S. aureus strain ATCC 29213 as the indicator organism. Antibiotic medium 1 (Difco, BD, Le Pont de Claix, France) was suspended in sterile pyrogen-free water, and the mixture was boiled at 100°C in a water bath for 30 min. After the medium was cooled to 50°C, it was inoculated with the overnight culture of the indicator organism (300 µl/400 ml medium), and poured into large assay plates (30 by 30 cm). The cultures were adjusted to a standardized inoculum corresponding to a McFarland 0.5 standard, spread on MHA containing rifampin (1 µg/ml), and assessed for growth. Experiments were performed in triplicate.

Animal treatment studies. Antimicrobial treatment was initiated 24 h after infection (day 1). At least three animals were randomized into each of the following treatment groups: control (saline); linezolid at 25, 50, and 75 mg/kg (alone or in combination with rifampin at 12.5 mg/kg); and levofloxacin at 10 mg/kg in combination with rifampin at 12.5 mg/kg (22). All antibiotics were administered intraperitoneally every 12 h over 4 days (i.e., a total of eight doses).

Efficacy of treatment against planktonic bacteria. planktonic bacteria in the aspired cage fluid was determined before and after the duration of antimicrobial treatment (day 1), on the fourth day of treatment and before administration of the last antimicrobial dose (day 4), and 5 days after the end of treatment (day 10).

Bacterial counts were expressed as the median and interquartile range of the log_{10} CFU/ml. The quantification limit of the planktonic bacteria was set at 1,000 CFU/ml (>10 CFU in 50 µl from dilutions ≥10-fold). Thus, negative cage fluid cultures were assigned a value of 3 log_{10} CFU/ml for calculation of the log_{10} reduction in bacterial numbers for statistical analysis. The efficacy of the treatment against planktonic bacteria was expressed as (i) the difference in bacterial counts in cage fluid (Δlog_{10} CFU/ml = log_{10} CFU/ml [day 4 or 10] − log_{10} CFU/ml [day 1]) and (ii) the rate of culture-negative cage fluid samples, i.e., the number of cage fluid samples without the detectable growth of the MRSA strain divided by the total number of cages in the treatment group.
TABLE 1. In vitro susceptibility of MRSA strain ATCC 43300

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
<th>MBC\text{log} (µg/ml)</th>
<th>MBC\text{stat} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linezolid</td>
<td>2.5</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.01</td>
<td>0.08</td>
<td>2.5</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.16</td>
<td>0.63</td>
<td>&gt;20</td>
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**RESULTS**

**In vitro antimicrobial susceptibility.** Table 1 shows the in vitro susceptibility of the MRSA strain to linezolid, rifampin, and levofloxacin. Linezolid inhibited bacterial growth at 2.5 µg/ml, whereas a bactericidal effect was not achieved up to 20 µg/ml either in the logarithmic growth phase or in the stationary growth phase. Rifampin exerted a low MIC (0.01 µg/ml) and was bactericidal in the logarithmic and the stationary growth phases (MBC\text{log} and MBC\text{stat}, 0.08 and 2.5 µg/ml, respectively). Levofloxacin had a MIC of 0.16 µg/ml and exhibited bactericidal activity only against bacteria in the logarithmic growth phase (MBC\text{log}, 0.63 µg/ml) and not those in the stationary phase (MBC\text{stat}, >20 µg/ml).

**In vitro time-kill studies.** In vitro time-kill studies were performed with inocula of 1 × 10^6 to 5 × 10^6 CFU/ml to investigate the synergism or antagonism of linezolid and rifampin. In the controls, the bacterial counts increased by 1.7 log_{10} CFU/ml after 24 h. In the presence of linezolid at 1× the MIC, the bacterial counts remained unchanged, while at 4× MIC they decreased by 1.7 log_{10} CFU/ml at 24 h. Rifampin at both 1× and 4× the MIC similarly decreased the bacterial counts after 6 h (0.5 log_{10} CFU/ml); however, regrowth to counts similar to those for the growth controls occurred after 24 h (Fig. 1). Bacteria exposed to rifampin alone showed regrowth after 24 h and were resistant to rifampin. When rifampin was combined with linezolid at either 1× or 4× the MIC, the bacterial counts at 24 h were decreased by 1.6 and 1.8 log_{10} CFU/ml, respectively. Due to the development of rifampin resistance during exposure to rifampin alone, it was not possible to evaluate whether a potential synergistic or antagonistic interaction between rifampin and linezolid existed, as described above.

**In vitro antimicrobial resistance.** With an MRSA inocula of 1 × 10^6 to 5 × 10^6 CFU/ml, 94% ± 3% of total colony counts developed rifampin resistance 24 h after exposure to rifampin alone at 1× the MIC. In contrast, no rifampin-resistant colonies were detected after 24 h incubation with the rifampin-linezolid combination at 1× the MIC.

**Pharmacokinetic studies.** Figure 2 shows the concentration-time profile in cage fluid after the administration of a single intraperitoneal dose in noninfected animals. The calculated values of the pharmacokinetic parameters are summarized in Table 2. The C_{max} of linezolid after the administration of a single intraperitoneal dose of 25, 50, or 75 mg/kg were 6.8, 12.6, and 18.1 µg/ml, respectively, which were achieved at ~3 h after dosing. The linezolid concentration remained above the MIC of the test organism for 12 h (C_{min}, 2.8 to 3.3 µg/ml), as did the rifampin and levofloxacin concentrations (C_{min}, 0.14 µg/ml and 0.27 µg/ml, respectively). The C_{max} of rifampin in the cage fluid reached almost 100× the MIC (C_{max}, 0.98 µg/ml), whereas this ratio was considerably lower for linezolid and levofloxacin (5× and 9× the MIC, respectively).

![FIG. 1. Time-kill curves for 1× and 4× the MIC of linezolid (closed circle, dashed line), their combination (open circles) against MRSA. Values are means ± SDs. LZD, linezolid; RIF, rifampin.](http://aac.asm.org/)

![FIG. 2. Pharmacokinetics of linezolid in cage fluid after the administration of single intraperitoneal doses of 25 mg/kg, 50 mg/kg, and 75 mg/kg. The mean values of six measurements at each time point are shown; error bars represent SDs. The horizontal dotted line indicates the MIC of linezolid for the MRSA test strain.](http://aac.asm.org/)
Antimicrobial treatment studies. Cage fluid sterility was confirmed prior to infection. At 24 h after infection, the median concentration of the bacteria enumerated in the cage fluid was 6.5 log_{10} CFU/ml. In control animals receiving saline, the bacterial counts in the cage fluid were 7.1 and 7.9 log_{10} CFU/ml after 4 and 10 days, respectively, which correspond to increases of 0.6 and 1.4 log_{10} CFU/ml, respectively. No spontaneous cure of the cage-associated infection occurred in the untreated animals. 

Efficacy of treatment against planktonic bacteria. Table 3 shows the counts of planktonic bacteria and the rates of culture-negative cage fluid samples during and after treatment. During treatment (day 4), the bacterial counts in the cage fluid of animals treated with linezolid alone at 25, 50, and 75 mg/kg were decreased by median values of 1.4, 1.2, and 1.7 log_{10} of animals treated with linezolid alone at 25, 50, and 75 mg/kg, respectively (Table 3), which correspond to increases of 1.0 and 0.8 log_{10} CFU/ml compared to the level of growth on day 1 (Fig. 3B). Linezolid at 75 mg/kg prevented bacterial regrowth in cage fluid on day 10, and the bacterial counts remained comparable to the values on day 4. In animals treated with the combination of linezolid and rifampin, the bacterial counts remained at the levels measured on day 4, independent of the linezolid dose (P > 0.05). No differences in treatment efficacy were observed between the three linezolid doses (P > 0.05) when they were combined with rifampin. The cure rates for animals treated with the linezolid-rifampin combination ranged from 75% to 95%, and rifampin resistance did not emerge.

Efficacy of treatment against adherent bacteria. No cure of cage-associated infections was observed with linezolid alone (Fig. 4). The use of linezolid in combination with rifampin showed cure rates of 50% to 60%. All linezolid-rifampin combinations exhibited significantly better activities than linezolid alone against adherent bacteria (P < 0.001). For comparison, the efficacy of the combination levofloxacin plus rifampin was tested and demonstrated a cure rate of 91%.

In vivo antimicrobial resistance studies. No rifampin-resistant MRSA strains were detected within positive cultures of cages from animals treated with rifampin alone or in combination with linezolid.

DISCUSSION

In this study, we investigated the activity of linezolid alone and in combination with rifampin against MRSA in vitro and in a guinea pig implant-associated infection model. The test
organism was inhibited by linezolid at 2.5 g/ml. However, a reduction of 99.9% CFU/ml was not achieved at concentrations up to 20 g/ml in either the logarithmic or the stationary growth phase. This is in agreement with the bacteriostatic activity of linezolid against staphylococci (13). On the basis of this characteristic, linezolid monotherapy does not seem to be appropriate for the treatment of staphylococcal implant-associated infections.

In the in vitro time kill-curve studies, rifampin resistance was detected after 24 h of incubation in all cultures exposed to rifampin alone (1 × and 4 × the MIC). In contrast, the use of rifampin in combination with linezolid never resulted in the emergence of rifampin resistance (11, 12, 29). Thus, we can conclude that in vitro the combination linezolid-rifampin did not display any synergism or antagonism against the strain tested, and it was difficult to interpret whether there was any additive effect because of bacterial regrowth at 24 h of incubation with rifampin alone. However, the combination was effective in completely preventing the development of rifampin resistance.

In the pharmacokinetic studies, the peak linezolid concentrations in cage fluid increased linearly with increasing doses between 25 and 75 mg/kg, whereas the increase in the AUC0–24 was not proportional to the dose due to the faster elimination of linezolid from the cage fluid at higher doses. The peak linezolid concentration and the AUC0–24 reached in the cage fluid with the 75-mg/kg dose were comparable to the values reported by Gee et al. (10) in the inflammatory blister fluid of healthy volunteers receiving 600 mg linezolid every 12 h. The 25-mg/kg and the 50-mg/kg linezolid doses administered to guinea pigs more likely simulate the doses achieved with the 400-mg and 600-mg single-dose regimens, respectively. The three doses of linezolid chosen guaranteed that the antimicrobial concentration in cage fluid remained above its MIC for the test organism (2.5 g/ml) for 12 h and, thus, during the entire treatment. Andes et al. (1a) showed that a plasma AUC0–24/MIC ratio of linezolid between 50 and 100 was predictive of a successful outcome of staphylococcal infections in the thigh muscle model. In our studies, the AUC0–24/MIC was only approximately 50 and was achieved with the highest linezolid dose (75 mg/kg). However, these values are difficult to interpret since we investigated a different compartment (cage fluid) and a different type of infection (an infection associated with an implant) compared to those used by Andes et al. (1a).

The rifampin dose of 12.5 mg/kg was chosen as described previously (31, 34). The peak levels in tissue fluid were equal to or less than the maximal concentrations reached in humans (1), and the rifampin concentration in cage fluid was greater than the MIC during 12 h after administration.

The cage fluid from the MRSA-infected cages implanted in guinea pigs demonstrated continuous bacterial growth for 10 days and no spontaneous cure. Linezolid induced a significant reduction in the counts of planktonic bacteria during treatment (day 4) both when it was given alone and when it was given in...
combination with rifampin \((P < 0.05)\) (Fig. 3A). During treat-
ment, no difference between the linezolid monotherapies was 
observed, but in combination with rifampin, bacterial killing 
was significantly improved \((P < 0.05)\). Five days after the end 
of treatment (day 10), bacterial regrowth occurred with lin-
ezolid doses of 25 and 50 mg/kg, whereas the counts remained 
suppressed after the linezolid dose of 75 mg/kg (Fig. 3B), even 
though the AUC\(_{0-24}\) was only slightly higher (Table 2). As 
shown previously (20), linezolid is able to induce a postantibi-
totic effect in \(S.\) aureus in a dose-dependent manner in vitro. 
Thus, it is likely that the postantibiotic effect was induced by 
the highest linezolid dose (75 mg/kg) but not by the two lower 
doses. In addition, the accumulation of linezolid may have 
occurring with the highest dose, delaying its time of clearance 
from the cage fluid. All combinations of linezolid with rifampin 
inhibited bacterial regrowth 5 days after administration of the 
last dose.

None of the treatment regimens with linezolid monothera-
pies eradicated the cage-associated MRSA infections, while 
the combinations of linezolid with rifampin achieved cure rates 
of between 50% and 60%, which is not significantly different 
from that achieved with rifampin monotherapy. The combina-
tion of rifampin and levofloxacin showed the highest cure rate 
(91%). Treatment failures were related to a lack of efficacy in 
the killing of bacteria when they were embedded in the biofilm 
matrix. The emergence of rifampin resistance did not occur in 
vivo with any of the rifampin regimens tested.

In conclusion, linezolid monotherapies showed bacteriosta-
tic activity against the MRSA strain tested and were not able to 
eradicate the adhering bacteria. Thus, linezolid should not be 
used alone for the eradication of implant-associated infections 
caused by MRSA. In vitro studies demonstrated the potential 
of the linezolid-rifampin combination for the treatment of 
MRSA infections, and these findings were confirmed in the 
animal foreign-body infection model. However, levofloxacin-
rifampin combinations achieved higher cure rates than the 
linezolid-rifampin combination against the quinolone-suscep-
tible MRSA strain tested (91% and 50 to 60%, respectively). In 
contrast to our previous recommendations (41), the quinolone-
rifampin combination seems to be a valid option for the treat-
ment of MRSA infections, whereas linezolid-rifampin regi-
mens may be used for the treatment of quinolone-resistant 
MRSA implant-associated infections.

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