Efficacy of Azithromycin in a Mouse Pneumonia Model against Hospital-Acquired Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT The use of macrolides against pneumonia has been reported to improve survival; however, little is known about their efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) pneumonia. In this study, we investigated the effect of azithromycin (AZM) and compared it with that of vancomycin (VCM) and daptomycin (DAP) in a murine model of MRSA pneumonia. Mice were infected with MRSA by intratracheal injection and then treated with AZM, VCM, or DAP. The therapeutic effect of AZM, in combination or not with the other drugs, was compared *in vivo*, whereas the effect of AZM on MRSA growth and toxin mRNA expression was evaluated *in vitro*. *In vivo*, the AZM-treated group showed significantly longer survival and fewer bacteria in the lungs 24 h after infection than the untreated group, as well as the other anti-MRSA drug groups. No significant decrease in cytokine levels (interleukin-6 [IL-6] and macrophage inflammatory protein-2 [MIP-2]) in bronchoalveolar lavage fluid or toxin expression levels (α -hemolysin [Hla] and staphylococcal protein A [Spa]) was observed following AZM treatment. *In vitro*, AZM suppressed the growth of MRSA in late log phase but not in stationary phase. No suppressive effect against toxin production was observed following AZM treatment *in vitro*. In conclusion, contrary to the situation *in vitro*, AZM was effective against MRSA growth *in vivo* in our pneumonia model, substantially improving survival. The suppressive effect on MRSA growth at the initial stage of pneumonia could underlie the potential mechanism of AZM action against MRSA pneumonia.

KEYWORDS azithromycin, methicillin-resistant *Staphylococcus aureus*, murine model, pneumonia

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of nosocomial pneumonia and is associated with significant morbidity and mortality (1, 2). Despite its frequency, nosocomial pneumonia due to MRSA (MRSA-NP) is difficult to distinguish because MRSA colonizes the lower respiratory tract, making it difficult to determine the most appropriate treatment (3–5). Guidelines for nosocomial pneumonia recommend vancomycin (VCM) and linezolid as empirical anti-MRSA therapies in at-risk patients (6, 7). The recent elevation of MICs with VCM has caused an increase in the use of other anti-MRSA drugs, such as daptomycin (DAP), tedizolid, oritavancin, or dalbavancin (8). Moreover, given concerns about the overuse of anti-MRSA drugs, clindamycin and trimethoprim-sulfamethoxazole have been recently considered potential second-choice drugs against MRSA pneumonia (9–12). Overall, the therapeutic options against MRSA pneumonia are still limited and controversial, which is, at least in part, due to the difficulty of an accurate diagnosis.

Combination therapy with β -lactam antibiotics and macrolides has been reported to help decrease mortality in community-acquired pneumonia, including pneumococcal pneumonia (13, 14), and is recommended as empirical therapy for community-acquired

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FIG 1 Survival rates (A) of mice infected with MRSA by an intratracheal inoculum containing 2.5×10^7 to 1×10^8 CFU/mouse. Survival rates decreased depending on inoculated bacterial load (* $P < 0.05$, ** $P < 0.005$ versus control group). MRSA loads in the lungs (B) and blood (C) of mice infected with 1×10^8 CFU/mouse. Bacterial counts in the lungs increased significantly between 4 and 16 h after infection, while no significant increase was observed in the blood. Bars represent mean bacterial counts. The broken horizontal line represents the lower limit of detection ($1.7 \log_{10}$ CFU/ml of blood or lung specimen). ** $P < 0.005$; NS, not significant. Similar results were obtained from two independent experiments.

pneumonia in the guidelines (15). In addition, several reports have shown that intravenous clarithromycin (CAM) administration for 3 to 4 consecutive days reduces the illness period and mortality of ventilator-associated pneumonia or acute lung injury (16–18). To date, there exist only a limited number of reports describing the effect of macrolides against MRSA pneumonia. In a case report, Grobost et al. described the successful use of long-term azithromycin (AZM) prophylaxis against two cases of recurrent *S. aureus* infection, which were resistant to AZM (19). At the same time, long-term, low-dose AZM treatment was reported to reduce incidence but increase macrolide resistance of *S. aureus* in Danish cystic fibrosis patients (20). Overall, the detailed mechanism underlying AZM-treatment against MRSA-NP remains poorly understood.

Herein, we sought to investigate the effect of AZM in a murine model of pulmonary infection caused by hospital-acquired MRSA (HA-MRSA) via transtracheal infection. As a comparison, we also investigated the effect of VCM and DAP; the former is a first-choice drug against MRSA-NP, whereas the latter is less effective due to inactivation by alveolar surfactant (21). Additionally, we examined the effect of AZM on MRSA virulence factors, such as mRNA expression of protein A (Spa) and α -hemolysin (Hla). These factors are relevant for the pathogenesis of MRSA pulmonary infection and are deployed by most *S. aureus* strains (22).

RESULTS

Establishment of the MRSA-NP model by intratracheal infection. First, we established the MRSA-NP model by intratracheal infection with the HA-MRSA strain. As shown in Fig. 1A, lethal pneumonia was induced by tracheal injection of strain HUYM

FIG 2 Survival rates (A) and bacterial loads in the lungs (B) of MRSA-infected mice treated with AZM. AZM was injected intraperitoneally once after infection (treatment protocol) or twice (24 h before and 2 h after infection; prophylactic protocol). The AZM prophylactic group showed significantly improved survival rates and decreased viable bacterial counts compared with the untreated group (* $P < 0.05$ versus control group). Bars represent mean bacterial counts. The broken horizontal line represents the lower limit of detection ($1.7 \log_{10}$ CFU/ml of blood or lung specimen). Similar results were obtained from two independent experiments.

at 2.5×10^7 to 1×10^8 CFU/mouse ($n = 10$ in each group). In preliminary experiments, an inoculum of at least 5×10^6 CFU/mouse appeared necessary to cause lethal lower respiratory infection. With an inoculum of 1×10^8 CFU/mouse, the mean bacterial count in the lungs of infected mice increased significantly between 4 and 16 h after infection (Fig. 1B), whereas no significant increase was observed in the blood (Fig. 1C) ($n = 8$ in each group). These results confirmed the successful establishment of the MRSA-NP model, which requires a relatively large bacterial inoculum during the initial phase of infection. As bacteremia was not induced even with the highest bacterial load, lethal MRSA pneumonia in our model was caused mainly by proliferation in the lungs. Therefore, bacterial counts in the lungs were validated and applied for further experiments.

Effect of AZM treatment versus prophylactic AZM therapy *in vivo*. Before validating the effect of AZM in relation to established anti-MRSA drugs, we compared the efficacy of AZM single-administration (2 h after infection) versus double-administration (24 h before and 2 h after infection). The former corresponded to a treatment protocol, and the latter to prophylactic therapy. We used a relatively small inoculum of 4×10^7 CFU/mouse because a higher bacterial load induced too severe pneumonia and was, thus, unsuitable for validating mild antibiotic treatment. As shown in Fig. 2A ($n = 12$ in each group), survival of the prophylactic therapy group was significantly longer than that of the untreated group ($P < 0.05$). The number of viable MRSA in the lungs 24 h after infection is shown in Fig. 2B ($n = 8$ in each group). Accordingly, the mean bacterial count was significantly suppressed in the prophylactic group compared with the untreated group. From these results, prophylactic AZM therapy seemed to have a greater effect on MRSA-NP than the treatment protocol. For further experiments, we used the prophylactic AZM protocol to examine the potential effect of AZM against MRSA-NP.

FIG 3 Survival rates (A) and bacterial loads in the lungs (B) of MRSA-infected mice treated with AZM, VCM, and DAP. AZM was injected intraperitoneally following the prophylactic protocol (24 h before and 2 h after infection). Bacterial counts were validated 20 h after infection. Survival rates and bacterial counts in the lungs improved significantly following treatment. Bars represent the mean of bacterial counts (* $P < 0.05$, ** $P < 0.005$ versus untreated group). Changes in inflammatory cytokines IL-6 (C) and MIP-2 (D) in bronchoalveolar lavage fluid of MRSA-infected mice treated with AZM, VCM, and DAP. Data are expressed as means and the error bars as SEM. In the VCM group, all cytokine levels were significantly decreased compared with the untreated group (** $P < 0.005$). Hla (E) and Spa (F) transcript levels 20 h postinoculation in MRSA-infected mice treated with AZM, VCM, and DAP. Each mRNA expression was significantly decreased in DAP group compared with the untreated group (* $P < 0.05$, ** $P < 0.005$). Data are representative of two separate experiments and represent the mean $\pm$ SEM of 12 mice.

Prophylactic effect of AZM compared with VCM and DAP *in vivo*. To validate the effect of AZM on MRSA-NP, we performed treatment experiments using the MRSA-NP mouse model with an inoculum of 4×10^7 CFU/mouse. As shown in Fig. 3A, the survival rate improved significantly in all the treatment groups compared with the untreated

FIG 4 Effect of AZM, VCM, and DAP on MRSA growth (A) and virulence factor expression (B, C) *in vitro*. MRSA was cultured in TSB broth containing 100 μ g/ml AZM, 10 μ g/ml DAP, or 0.75 μ g/ml VCM. MRSA counts were significantly decreased in all treatment groups in late log phase ($n = 6$ in each group) ($*P < 0.05$). Hla (B) and Spa (C) transcript levels showed no significant decrease in any group. Data represent the mean $\pm$ SEM of four-five replicates from three independent experiments.

group. Indeed, 168 h after infection, the survival rate was 93.3% in the VCM group, 66.7% in the DAP group, and 46.7% in the AZM group, whereas all untreated mice were dead 152 h after infection ($n = 12$ in each group). Similarly, the mean bacterial count in lungs 24 h after infection ($n = 9$ in each group) was also significantly lower in all treatment groups than in untreated animals, and it stood at 3.9, 6.1, and 6.8 log₁₀ CFU/lungs in the VCM, DAP, and AZM groups, respectively (Fig. 3B). In contrast, the levels of inflammatory cytokines interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2) in bronchoalveolar lavage fluid (BALF) ($n = 7$ in each group) decreased significantly only in the VCM group (Fig. 3C and D). The total cell counts (see Table S1 in the supplemental material) were significantly higher in the DAP and VCM groups than in the untreated group. Finally, an examination of toxin transcript levels indicated that Hla/Gyrb and Spa/Gyrb were significantly lower in the DAP group than the untreated group (Fig. 3E and F).

For the experiments of toxin transcript levels, data are representative of two separate experiments and represent the mean $\pm$ SEM of 12 mice.

Effect of AZM on bacterial growth and virulence factor expression *in vitro*. To examine the effect of AZM on bacterial growth of HA-MRSA *in vitro*, MRSA was cultured in tryptic soy broth (TSB) containing VCM, DAP, or AZM, and bacterial counts in late log phase and stationary phase was determined. The concentration of AZM was adjusted to 100 μ g/ml, whereas DAP and VCM were adjusted to 2/3 MICs, that is 10 μ g/ml for DAP and 0.75 μ g/ml for VCM. As shown in Fig. 4A ($n = 6$ in each group), MRSA counts decreased significantly in all treatment groups in late log phase compared with untreated cells; whereas only the VCM group showed a significant drop in stationary phase. To examine the effect of antibiotics on Hla and Spa expression, we assessed Hla/Gyrb and Spa/Gyrb ratios by relative quantitative real-time PCR. Neither Hla/Gyrb (Fig. 4B) nor Spa/Gyrb (Fig. 4C) transcript levels displayed any significant reduction

FIG 5 Synergistic effect of AZM and VCM or DAP on MRSA growth (A) and virulence factor expression (B, C) *in vitro*. MRSA were cultured in TSB broth containing VCM+AZM, DAP+AZM, or VCM and DAP alone. AZM was adjusted to 100 $\mu\text{g/ml}$, whereas DAP and VCM were adjusted to 2/3 MICs (10 $\mu\text{g/ml}$ for DAP and 0.75 $\mu\text{g/ml}$ for VCM). MRSA counts decreased significantly in the VCM and VCM+AZM groups in late log phase compared with the untreated group. No synergistic effect of AZM was detected. *Hla* (B) and *Spa* (C) transcript levels displayed no significant decrease in any group in late log phase. Data represent the mean $\pm$ SEM of three replicates from three independent experiments (* $P < 0.05$).

upon antibiotic treatment *in vitro*. For examination of *Hla* and *Spa* expression, data represent the mean $\pm$ SEM of four to five replicates from three independent experiments.

Effect of combination therapy with AZM and VCM or DAP *in vitro*. To examine the synergistic effect of AZM with VCM or DAP on bacterial growth of HA-MRSA *in vitro*, MRSA was cultured in TSB with VCM+AZM and DAP+AZM or VCM and DAP alone. Bacterial counts in late log phase and stationary phase were validated ($n = 6$ in each group). VCM or DAP were used at 2/3 MICs and were supplemented with 100 $\mu\text{g/ml}$ of AZM. AZM addition was compared with growth in the presence of VCM or DAP alone. As shown in Fig. 5A, MRSA counts decreased significantly in the VCM and VCM+AZM groups in late log phase compared with the untreated group. However, no significant synergistic effect was observed between VCM versus VCM+AZM or DAP versus DAP+AZM. We also examined the synergistic effect of AZM with VCM or DAP on *Hla*/*GyrB* and *Spa*/*GyrB* transcript levels in late log phase *in vitro*. Again, no significant synergistic effect of AZM was observed relative to *Hla*/*GyrB* (Fig. 5B) or *Spa*/*GyrB* (Fig. 5C) transcript levels. For examination of *Hla* and *Spa* expression, data represent the mean $\pm$ SEM of three replicates from three independent experiments.

Effect of combination therapy with AZM and VCM or DAP *in vivo*. Finally, we examined the synergistic effect of AZM with VCM or DAP *in vivo*. To validate the effect of AZM addition on anti-MRSA drug treatment, an inoculum of 1×10^8 CFU/mouse was used. As shown in Fig. 6A ($n = 12$ in each group), the survival rate was significantly improved following treatment with VCM+AZM and DAP+AZM compared with the untreated group. However, the improvement was generally minor, and no significant

FIG 6 Survival rates (A) and bacterial loads in the lungs (B) of MRSA-infected mice treated with VCM+AZM and DAP+AZM or VCM and DAP alone. VCM and DAP were injected intraperitoneally once a day 2 h after infection, with or without AZM prophylactic therapy (100 mg/kg intraperitoneally 24 h before and 2 h after infection). Bacterial counts were validated 20 h after infection. The survival rate was significantly improved following VCM+AZM or DAP+AZM treatment; however, no significant change was observed with bacterial counts in the lungs. Bars represent the mean of bacterial counts (* $P < 0.05$ versus untreated group). Hla (C) and Spa (D) transcript levels 20 h postinoculation in MRSA-infected mice treated with VCM+AZM and DAP+AZM or VCM and DAP alone. Hla/Spa mRNA expression was significantly decreased in the VCM group compared with the untreated group (* $P < 0.05$). Data are representative of two separate experiments and represent the mean $\pm$ SEM of 8 mice.

synergistic effect of AZM on VCM or DAP was detected. Moreover, no significant difference was observed in terms of mean bacterial counts in the lungs 24 h after infection ($n = 8$ in each group) between treatment groups and untreated mice (Fig. 6B). There was also no significant synergistic effect of AZM on Hla/GyrB (Fig. 6C) or Spa/GyrB (Fig. 6D) transcript levels. For the experiments of toxin transcript levels, data are representative of two separate experiments and represent the mean $\pm$ SEM of 8 mice.

DISCUSSION

In the present study, we established a MRSA-NP murine model and then investigated the effect of AZM on MRSA-NP. AZM was effective against MRSA growth in our pneumonia model, improving the survival period. *In vitro* experiments demonstrated that AZM suppressed bacterial growth in late log phase.

So far, MRSA pneumonia models have often been investigated using virulent strains that produce Pantone-Valentine leukocidin (PVL) or via hematogenous pulmonary infection (23–25). Epidemiologically, most MRSA strains identified in NP cases belong to staphylococcal cassette chromosome *mec* (SCC*mec*) types I, II, and III, whereas most community-acquired MRSA strains belong to SCC*mec* types IV and V (26). Given that HA-MRSA is substantially less virulent, it is difficult to establish a severe pneumonia model by transtracheal infection using HA-MRSA. A few studies have attempted to use a murine transtracheal pneumonia model of HA-MRSA (27–29) by inoculating a high load of MRSA (up to 10^7 to 10^9 CFU/mouse) via tracheal injection. At least 2×10^9 CFU/mouse was necessary to induce lethal MRSA pneumonia, even in a neutropenic

mouse model treated with cyclophosphamide (29). In contrast, in our neutropenic mouse model subjected to cyclophosphamide (see Table S2 in the supplemental material), we could reproducibly induce lethal MRSA pneumonia after inoculating 2.5×10^7 to 1×10^8 CFU/mouse via tracheal intubation with a 22-gauge catheter. Thus, our method was less invasive and more suitable for inoculating higher bacterial loads directly into the lower respiratory tract. Nevertheless, we still needed to inoculate a relatively large amount of bacteria to induce lethal MRSA pneumonia. Moreover, as shown in Fig. 1A, a small decrease in bacterial loads in the inoculum was reflected by clear changes in survival rates. On the basis of these results, we suggest that a greater bacterial load might be necessary to achieve pathogenesis in MRSA pneumonia. This conclusion is supported by a previous clinical report, whereby quantitative cultivation yielding more than 10^6 CFU/ml had been an independent predictor of the pathogenicity of MRSA-NP (3). Interestingly, DAP was previously shown to possess an equivalent therapeutic effect as VCM in a hematogenous pulmonary infection model of MRSA (23). Here, treatment of MRSA pneumonia was less effective with DAP than with VCM (Fig. 3), which might reflect inactivation of DAP by alveolar surfactant, as our MRSA infection model involves passage through the airways. Taken together, we believe that our MRSA pneumonia model reproduces well the clinical features of MRSA-NP under immunosuppressive conditions.

In this study, we used a clinically isolated MRSA strain with SCCmec type III. Recently, an increased prevalence of MRSA with SCCmec type III has been reported in Middle Eastern and Asian countries, particularly in nosocomial infections (30, 31). Chu et al. reported that 81.3% of MRSA strains isolated from lower respiratory tract specimens ($n = 103$) at Shanghai Pulmonary Hospital were SCCmec type III, suggesting that this gene type may favor respiratory tract infections (31). Although MRSA-NP with SCCmec type III is rare in Japan, our strain possessed relatively strong virulence during respiratory tract infection.

We show that AZM was effective against MRSA pneumonia by significantly suppressing bacterial proliferation both *in vivo* and *in vitro*, although the impact was not as large as that achieved by VCM. As shown in Fig. 4A, the suppressive effect of AZM on MRSA growth *in vitro* was observed at an early stage (late log phase) but not later on in stationary phase. This finding indicates that AZM possesses a partial anti-MRSA effect, which is limited to the earlier stage of bacterial growth. *In vivo*, the reduction of bacterial load in the lungs of MRSA-infected mice treated with AZM might be due to the above partial anti-MRSA effect. Considering that the partial anti-MRSA effect could not be tested for MICs, antibiotics having a partial anti-MRSA effect, including AZM, could be candidate therapeutic agents for MRSA pneumonia, irrespective of conventional drug susceptibility tests. In addition, bacterial growth during the initial phase of pneumonia is also important for the pathogenesis of MRSA pneumonia because a minor suppression of bacterial growth can substantially affect the severity of the disease.

The mechanisms of action of macrolides have been shown to include immunomodulatory effects, suppression of bacterial activity with quorum-sensing or biofilm formation, and inhibition of mucus hyperplasia (32–35). The present results revealed no apparent suppressive effect against MRSA toxin expression or an immunomodulatory effects by AZM. Importantly, however, we show that AZM is effective against the earlier stage of MRSA growth, a finding that had not been reported previously. In this study, we applied prophylactic AZM administration. AZM is known to concentrate and persist in lung tissue, such as alveolar cells, epithelial lining fluid, polymorphonuclear leukocytes (PMNs), and sputum (36). The concentration of AZM in the lungs of BALB/c mice was previously reported to be approximately 10-fold higher than in serum (37). Accordingly, we believe that AZM administration 24 h prior to infection (prophylactic protocol) might ensure AZM persistence in the lungs until infection, resulting in a slightly better outcome than a single AZM treatment. Because the therapeutic effect of AZM was mild, AZM monotherapy against MRSA-NP could not be fully recommended. Nevertheless, our results support the potential prophylactic effect of AZM against

MRSA-NP. As with the treatment of ventilator-associated pneumonia patients with CAM (16, 17), AZM administration might help reduce the occurrence of MRSA-NP.

Combination therapy with AZM and other anti-MRSA drugs revealed a slight improvement in the survival rate of the VCM+AZM and DAP+AZM group compared with the VCM and DAP group (Fig. 6A). This finding contrasts with Hla and Spa expression, which remained elevated in AZM+VCM and AZM+DAP groups compared with VCM or DAP groups (Fig. 6C and D). These results could not support a clear synergistic effect of AZM on the other anti-MRSA drugs. Hence, further studies are necessary to validate the synergistic effect of AZM on anti-MRSA drugs and its application in combined therapy.

The present study has several limitations. First, we examined the effect of AZM on MRSA-NP using a single strain. As the strain in this study does not produce virulent toxins, such as PVL or toxic shock syndrome toxin (TSST), the effect of AZM on toxin expression should be validated in other strains. Second, we administered VCM and DAP only once after inoculation. Accordingly, the observed suppressive effect of AZM against bacterial proliferation in the early phase of MRSA-NP might not fully correlate with clinical therapeutic effects against MRSA-NP, as well as VCM nor DAP. Third, we used a neutropenic mouse model, which is not the most appropriate when examining the immunomodulatory effect of AZM. In spite of these failings, we believe that the results of our study indicate the potential therapeutic benefit of AZM and anti-MRSA drugs on MRSA-NP.

In conclusion, our results provide novel evidence that AZM possesses treatment potential against MRSA-NP by suppressing early stage MRSA growth, which might also be important for understanding the pathogenesis of MRSA-NP.

MATERIALS AND METHODS

Bacterial strains. MRSA strain HUYM, which was clinically isolated at Hokkaido University School of Medicine, was used in the present study. The strain was SCCmec type III and Panton-Valentine leukocidin (PVL)-toxic shock syndrome toxin (TSST) negative, which were genetically analyzed at Nagasaki University Hospital, by using the previously reported methods (38). The antimicrobial susceptibility profile of this isolate showed MICs of 0.75 µg/ml to VCM and DAP and >100 µg/ml to AZM. MICs were determined by the broth microdilution method, according to the reference procedure recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Mice. Eight-week-old male BALB/c specific-pathogen-free mice were obtained from SLC Japan Inc., Shizuoka, Japan. The mice were housed in a pathogen-free environment and received sterile food and water in the Laboratory Animal Center for Biomedical Research, Hokkaido University School of Medicine.

Ethics. All mouse experiments were performed according to the guidelines of our institution, which were approved by the Ethic Committee on Animal Research of Hokkaido University (approval number 17-0134).

Intratracheal infection procedure. The MRSA strain was cultured on a blood agar plate (Becton, Dickinson Co., Ltd., Fukushima, Japan) for 24 h at 37°C, then scraped and suspended in tryptic soy broth (TSB), cultured with shaking for 7 h at 37°C and 250 rpm, and harvested by centrifugation at 3,000 rpm for 10 min. Cells were resuspended in normal saline at a final concentration of 10⁸ to 10⁹ CFU/ml according to optical density measurements. Mice were anaesthetized with ketamine-xylazine, and 0.05 ml of the bacterial suspension was inoculated via a 22-gauge catheter inserted into the trachea. The bacterial suspension was prepared for each experiment. Before infection, mice were administered intraperitoneal cyclophosphamide (150 mg/kg of body weight on day -4 and 100 mg/kg on day -1).

In vivo treatment protocol. AZM (Pfizer, Groton, CT), VCM (Wako, Osaka, Japan), and DAP (MSD, Tokyo, Japan) were administered intraperitoneally at 100 mg/kg once after infection. A dose of 100 mg/kg for AZM and VCM corresponds to the recommended dosage for humans, which are 1 g/body for VCM and 500 mg/body for AZM (23, 39). For DAP, a dose of 100 mg/kg corresponds to 12 mg/kg of body weight in humans and has been used to validate the therapeutic potential of DAP against MRSA pneumonia (40, 41).

For prophylactic therapy with AZM, an equivalent dose of AZM was administered twice at 24 h before infection and 2 h after infection. For combination therapy, AZM was administered simultaneously with VCM or DAP after infection, as well as 24 h before infection. Placebo-treated mice (control group) received the same volume of sterile physiological saline as that used to dissolve AZM, VCM, and DAP.

Bacteriological examination. Each group of animals was sacrificed at specific time intervals by cervical dislocation. After exsanguination, the lungs and blood were dissected and removed under aseptic conditions. The lungs used for bacteriological analyses were transferred into 7-ml Precellys homogenization tubes (CK28; ceramic beads of 2.8 mm; Bertin Technologies, Rockville, MD) with 2,000 µl sterile saline and then processed at 5,500 rpm for 2 × 20 s in a Precellys 24 high-throughput homogenizer (Bertin Technologies). The homogenized suspension was quantitatively inoculated onto blood agar plates by serial dilution.

Bronchoalveolar lavage and cytokine enzyme-linked immunosorbent assay. Bronchoalveolar lavage (BAL) was performed as described previously (42, 43). Recovered fluid fractions were pooled for each animal. Total cell counts were determined with a hemocytometer. For differential cell counts, cells were centrifuged at 850 rpm for 2 min onto slides that were then stained with Diff-Quik stain. Differential cell counts were performed by counting 100 cells. To validate the effect of antibiotics on inflammatory cytokines in this MRSA pneumonia model, we examined the concentration of two previously validated mouse cytokines (27, 28, 44), macrophage inflammatory protein-2 (MIP-2) and interleukin-6 (IL-6) using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Effect of antibiotics on MRSA growth in late log or stationary phase *in vitro*. MRSA growth was assessed in TSB containing AZM, VCM, or DAP. The MRSA culture was initiated from 2×10^5 CFU/ml until late log phase (7 h) or stationary phase (12 h), at which point bacterial colonies were counted following serial dilutions on agar plates.

Hla/Spa transcript levels in MRSA exposed to anti-MRSA drugs or AZM *in vitro*. Transcript levels of Hla/Spa in MRSA grown in TSB were assessed by quantitative real-time PCR. Total RNA was extracted from MRSA using NucleoSpin RNA kits (TaKaRa Co., Tokyo, Japan), according to the manufacturer's instructions. Total RNA (1 μ g) was reverse-transcribed into cDNA using TaqMan reverse transcription reagents and reverse transcription reaction mix (Thermo Fisher Scientific, Waltham, MA) on an ABI 2720 thermal cycler (Thermo Fisher Scientific). The resulting cDNA was used as a template for real-time PCR on an ABI Prism 7300 sequence detection system (Thermo Fisher Scientific), using the LightCycler DNA master SYBR green I kit (Roche, Basel, Switzerland). To quantify the expression of the Hla/Spa gene, the following sets of PCR primers were used: for GyrB (45), TTATGGTGTGGGCAAATACA and CACCATGTA AACCACAGATA; for Spa (46), TGGTTGTGCTGTTGCTTCTTA and GCAAAAGCAAACGGCACTAC; and for Hla (46), TTGTGATTTCTTTCTTTTCCCA and AAGCATCCAAACAACAACAAT. Data are presented as a ratio of Hla or Spa against GyrB.

Lung Hla/Spa transcript levels in mice infected with MRSA *in vivo*. Lung Hla/Spa transcript levels in mice were examined 24 h after MRSA inoculation, with or without antibiotics therapy. RNA extraction was performed as described previously (47). Each group of animals was sacrificed at specific time intervals, and whole lungs were aliquoted into a 7-ml Precellys homogenization tube (CK28) with 2 ml RNAlater (Thermo Fisher Scientific) and processed at 5,500 rpm for 2×20 s. The homogenized suspension was then transferred into 2-ml Precellys homogenization tubes (CK01; ceramic beads of 0.1 mm; Bertin Technologies) and processed at 6,000 rpm for 2×30 s. Using 100 μ l of the homogenized suspension, total RNA was extracted using NucleoSpin RNA kits according to the manufacturer's instructions. After RNA extraction, cDNA synthesis was carried out and mRNA transcript levels of Hla, Spa, and GyrB were determined by quantitative real-time PCR, as described above.

Statistical analysis. All data are expressed as means and standard errors of the mean (SEMs). Differences between two groups were evaluated using the Mann-Whitney U test; differences between the untreated group and therapy groups were evaluated using Steel's multiple-comparison test. Survival analysis was performed using the log-rank test, and the survival rates were calculated using the Kaplan-Meier method. Results with *P* values of <0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00149-19>.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

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