

Subinhibitory concentrations of telithromycin, clarithromycin, and azithromycin reduce
methicillin-resistant *Staphylococcus aureus* coagulase *in vitro* and *in vivo*

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ABSTRACT

Background: Subinhibitory levels of clarithromycin (CAM) and azithromycin (AZM) have been shown to reduce the activity of bacterial virulence factors, but few studies have examined the effects of subinhibitory levels of telithromycin (TEL). Here, we examined the effects of TEL, CAM, and AZM on methicillin-resistant *Staphylococcus aureus* (MRSA) coagulase *in vitro*. We also examined the effects of these antibiotics on bacterial survival in murine model of pulmonary infection where the number of bacteria in the lung correlates with the coagulase titer.

Methods: The coagulase titer in MRSA strain NUMR 101, a clinical isolate, was measured after a 16-h treatment with TEL, CAM, or AZM at the minimal inhibitory concentration (MIC; 512 mg/L) and 1/2, 1/4, 1/8, and 1/16 of the MIC. In addition, we examined the effect of these drugs in a murine model of pulmonary infection induced by intravenous injection of *S. aureus* enmeshed in agar beads. Prior to infection, mice were pretreated once a day for 7 days by oral administration of 10 or 100 mg/kg of TEL, CAM, or AZM, and the number of viable bacteria in the lungs was counted 24 after the injection of the bacteria.

Results: The coagulase titers in mice treated with 1/8 of the MIC of TEL, CAM, and AZM and in the control were 8, 4, 8, and 32, respectively. In the mouse model of infection, the log colony forming units/lung (mean \pm SEM; n=5 or 6) were 6.62 \pm 0.81, 4.79 \pm 0.41, 6.15 \pm 0.38, and 8.41 \pm 0.30 for mice treated with 100 mg/kg/day of TEL, CAM, and AZM and for controls, respectively (P <0.05 for all groups vs. control).

Conclusions: Subinhibitory concentrations of TEL inhibit MRSA coagulase *in vitro*. In addition, the *in vivo* results indicate that pretreatment with TEL, CAM, or AZM can reduce the bacterial load in a murine model of pulmonary infection.

INTRODUCTION

Telithromycin is the first ketolide antibacterial to be approved for clinical use. The ketolides represent a novel class of antibacterial agents structurally related to the macrolides, and they were developed to treat a wide spectrum of upper and lower respiratory tract infections caused by common and atypical pathogens, including strains that are resistant to currently used antibiotics. The ketolides are semisynthetic erythromycin A derivatives that have a 3-keto group in place of the L-cladinose moiety at the C-3 position of the lactone ring¹.

Some reports have shown that subinhibitory levels of macrolides inhibit the activity of bacterial virulence factors. For example, subinhibitory levels of azithromycin reduce exotoxin A, total protease, elastase, and phospholipase C production by *Pseudomonas aeruginosa* without affecting growth or total protein production². Also, subinhibitory concentrations of erythromycin reduce the haemolytic activity of pneumolysin³. Furthermore, we previously demonstrated that subinhibitory concentrations of clarithromycin and azithromycin reduce pneumolysin of high-level Macrolide resistant *Streptococcus pneumoniae* both *in vitro* and *in vivo*⁴. However, to our knowledge, the effects of subinhibitory levels of telithromycin on bacterial virulence factors have not been examined.

Staphylococcus aureus produces many extracellular products that may act as virulence factors, and of these, staphylocoagulase has been considered one of the most important. We previously found that coagulase plays a role in the development of blood-borne staphylococcal pneumonia^{5,6}. In the current study, we examined the effect of telithromycin on staphylocoagulase in methicillin-resistant *S. aureus* (MRSA) *in vitro* and *in vivo*, and we compared the effects of telithromycin with clarithromycin and azithromycin.

MATERIALS AND METHODS

Bacterial strain

MRSA strain NUMR101 was a clinical isolate obtained from blood samples of patient at Nagasaki University Hospital. The bacteria were stored at -70°C in brain-heart infusion broth (BBL Microbiology System, Cockeysville, MD) supplemented with 10% (v/v) glycerol and 5% (w/v) skim milk (Yukijirushi Co., Tokyo, Japan) until use. MRSA NUMR101 was cultured on a trypticase soy agar (BBL Microbiology System)-based sheep blood agar plate for 24 h at 37°C . The MIC of each agent was determined by the microplate dilution technique using Muller-Hinton medium, with an inoculum size of 5×10^5 colony forming units (cfu)/mL. The MIC was defined as the lowest concentration of the test agent that inhibited visible growth of bacteria after 18 h at 37°C . The MIC of telithromycin, clarithromycin and azithromycin for NUMR101 was $512 \mu\text{g/mL}$.

Effect of antibiotics on coagulase production in vitro.

The *Staphylococcus aureus* NUMR101 strain was cultured in the presence of antibiotics at the minimal inhibitory concentration (MIC) and 1/2, 1/4, 1/8, and 1/16 of the MIC. Coagulase levels were determined using a modification of the method reported by Jordens al.⁷. Overnight cultures in BHI broth (BBL) were diluted two-fold in fresh sterile BHI to a total volume of 100 mL. Next, 0.5 ml of 1:20 fresh-frozen dry rabbit plasma (Eiken Chemical Co., Tokyo, Japan) in BHI broth was added, and clot formation was assessed after 2 h at 37°C . The highest dilution giving a definite clot was considered the coagulase titer.

Laboratory animals

Six-week-old, male, ddY, specific pathogen-free mice (25-30 g body weight) were purchased from Shizuoka Agricultural Cooperative Association Laboratory Animals (Shizuoka, Japan). All animals

were housed in a pathogen-free environment in the Laboratory Animal Centre for Biomedical Science at Nagasaki University and received sterile food and water *ad libitum*. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation at our institution.

Inoculation of mice with bacteria

The method of inoculation was described previously^{5,6}. Bacteria were suspended in endotoxin-free sterile saline and harvested by centrifugation (3000 × g; 4°C; 10 min). Briefly, the microorganisms were resuspended in cold sterile saline and diluted to approximately 2×10^9 to 4×10^9 cfu/ml, as estimated by turbidimetry. The concentration of bacteria was then verified by inoculating duplicates of serial dilutions onto blood agar plates and counting the cfu after 48 h at 37°C. The suspension was warmed to 45°C, and 10 mL of the suspension was mixed with 10 mL of 4% (w/v) molten Noble agar (Difco Laboratories, Detroit, MI) at 45°C. The agar-bacterium suspension (1.0 mL) was placed in a 1.0-mL syringe and then rapidly injected through a 26-gauge needle into 49 mL of rapidly stirred ice-cooled sterile saline. This resulted in solidification of the agar droplets into beads approximately 200 µm in diameter. The final concentration of agar in this suspension was 0.04% (w/v), and the final number of bacteria was 2×10^7 to 4×10^7 cfu/mL. Each mouse was injected in the tail vein with 0.20 to 0.25 ml of the bacteria-agar beads (10 ml/g of body weight) suspended in saline. Treatments with drugs were initiated 1 day before inoculation with bacteria.

Bacteriological analysis

Prior to infection, mice were pretreated once a day for 7 days by oral administration of 10 or 100 mg/kg of telithromycin, clarithromycin or azithromycin. Each group of animals was sacrificed by cervical dislocation 6 days after infection. After exsanguination, the lungs were dissected and removed under aseptic conditions. Organs used for bacteriological analyses were homogenized, serially diluted, and cultured on blood agar plates.

Statistical analysis

Bacteriological data were expressed as means \pm SEM. Differences between groups were examined for statistical significance using an unpaired t-test. A *P* value less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Effect of subinhibitory concentrations of antibiotics on coagulase activity in vitro

At concentrations of 1/2, 1/4, and 1/8 of the MIC, telithromycin , clarithromycin , and azithromycin inhibited coagulase production by *S. aureus* (Table 1). These concentrations did not, however, affect the number of bacteria.

Therapeutic effects of antibiotics

We next examined the effect of telithromycin , clarithromycin , and azithromycin on the number of viable bacteria in a murine model of hematogenous pulmonary infection. According to the previous reports, the peak concentrations in lung are 7 (10mg/kg) and 40-72.8 (100mg/kg) ug/ml^{8,9} We previously examined the role of coagulase in a murine model of hematogenous pulmonary infection with MRSA^{5,6}, and we found a significant correlation between the coagulase titer and the number of viable bacteria recovered from the lung⁵. Here, we found that treatment with low dose of telithromycin (10 mg/kg) did not cause a change in the number of viable bacteria in the lungs in comparison with the control (7.75±0.45 and 8.23±0.21log₁₀ cfu/lung [n=6], respectively; Table 2). In contrast, a high dose of telithromycin (100 mg/kg) significantly reduced the number of viable bacteria compared with control (6.62±0.81 log₁₀ cfu/lung [n=6]; *P*=0.0167 vs. control; Table 2). Similarly, treatment with low dose of clarithromycin or azithromycin (10 mg/kg) did not change the number of viable bacteria in the lungs, whereas a high dose of these drugs (100 mg/kg) significantly reduced the number of viable bacteria (Table 2).

DISCUSSION

In this study, we demonstrated that subinhibitory concentrations of telithromycin , clarithromycin , and azithromycin reduce the level of MRSA coagulase *in vitro* and *in vivo*. Our results suggest that telithromycin and macrolides can be used as a new therapeutic option for

preventing infection by resistant bacteria. Specifically, we showed that TEL and macrolides reduce the level of coagulase protein *in vitro* and significantly lowered the number of viable MRSA *in vivo*. We previously reported that the inhibition of staphylocoagulase by a short interfering RNA could be an effective means of controlling MRSA infection⁶. The current results further show that subinhibitory concentrations of telithromycin, clarithromycin, and azithromycin are effective against infection by MRSA *in vivo*. According to the previous reports, the peak concentrations in lung are 7 (10mg/kg) and 40-72.8 (100mg/kg) ug/ml^{8,9}. These data suggested that the high dose (100mg/kg) of antibiotics should have sub-MIC effect against MRSA. We already reported the the number of bacteria recovered from the lung tissue correlated with the titre of staphylocoagulase⁵. Thus, we decided the *in vivo* inhibition of coagulase induced the lower bacteria number.

Previous reports indicated that telithromycin has effects against Gram-positive cocci and *Helicobacter pylori* at sub-MIC concentrations^{10,11}. Telithromycin has also been reported to reduce the number of viable bacteria during *P. aeruginosa* infection by via inhibiting biofilm formation¹². These reports suggest that telithromycin has effects at sub-MIC concentrations. Furthermore, many investigators have reported that sub-MIC concentrations of macrolides can reduce pathogenic factors *in vitro* and *in vivo*. Here, we showed that telithromycin had a similar effect as macrolides. Finally, the antibiotics did not have an effect in the murine model of infection when administered 24 h after injection of bacteria (data not shown), indicating that this treatment is only effective prior to infection.

In conclusion, we showed that, like clarithromycin and azithromycin, a subinhibitory concentration of telithromycin reduces the level of MRSA coagulase. The *in vivo* results further revealed that pretreatment with telithromycin, clarithromycin, or azithromycin can reduce the bacterial load in a murine model of pulmonary infection.

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TRANSPARENCY DECLARATIONS

None to declare.

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Table 1. Effect of subinhibitory concentrations of antibiotics on coagulase titer of *S. aureus*

Antibiotic	Coagulase titer				
	No drug	1/2 MIC	1/4 MIC	1/8 MIC	1/16 MIC
TEL	32	0	4	8	32
CAM	32	0	2	4	32
AZM	32	0	4	8	32

Table 2. Effect of antibiotics on bacteria numbers *in vivo*

Antibiotic	Log ₁₀ cfu/lung of <i>S. aureus</i> (n=5 or 6)*	
	10 mg/kg	100 mg/kg
TEL	7.75 ± 0.45	6.62 ± 0.81 [#]
CAM	7.65 ± 0.59	4.79 ± 0.41 [¶]
AZM	7.55 ± 0.83	6.15 ± 0.38 [§]
Control	8.23 ± 0.21	8.41 ± 0.30

*Values are means ± SEM

[#] P=0.0167 vs control

[¶] P=0.0001 vs control

[§] P=0.0014 vs control