Increased copy number of 23S ribosomal RNA gene with point mutation in MRSA associated with linezolid resistance in a patient treated with long-term linezolid

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Research Article

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Abstract

Background

Methicillin-resistant Staphylococcus aureus (MRSA) infection is one of the most difficult infections we have to treat. Linezolid is one of the effective treatment options for refractory MRSA infections. There are cases where we are forced to use long-term linezolid treatment for refractory MRSA infections.

Objective

To discuss the evolution of Linezolid resistance factors in clinical isolates of MRSA.

Methods

We investigated 16 MRSA isolated from a patient treated with linezolid for a long period of 75 days. We performed antibiotic susceptibility test, 23S rRNA genes sequencing analysis, Pulsed-field gel electrophoresis.

Results

MRSA isolates were susceptible to linezolid before the start of treatment, but became less susceptible by prolonged treatment. The 23S rRNA sequencing analysis of linezolid-resistant strains that appeared 17 days after the start of treatment with linezolid revealed that all resistant MRSA had the G2576T substitution (Escherichia coli 23S rRNA gene number). The number of copies of this mutation increased with the use of linezolid.

Conclusion

Long-term use of linezolid in a patient or reuse of linezolid in a patient who has been previously treated with linezolid can lead to the emerging of linezolid-resistant MRSA in the host.

Background

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are one of the most difficult infections for us to treat. We usually treat MRSA infections with glycopeptide antibiotics such as vancomycin and teicoplanin, and oxazolidinone antibiotics such as linezolid. Among these, linezolid is one of the most promising therapeutic options in clinical use, and is effective against most Gram-positive bacteria, including MRSA and vancomycin-resistant enterococci. Linezolid binds to the domain V region of the 23S ribosomal RNA (rRNA) and exhibits antimicrobial activity by inhibiting protein synthesis in susceptible
cells and by inactivating the function of the 50S ribosomal subunit [1, 2]. Linezolid was first approved for clinical use in the United States in 2000. In 2001, shortly after the start of clinical use, the emergence of linezolid-resistant MRSA was reported in North America [3].

The most common mechanism of linezolid resistance is a single base substitution in chromosomal DNA encoding the domain V region of 23S rRNA. The most frequent mutation associated with linezolid resistance in clinical strains of *S. aureus* is the G2576T substitution (*Escherichia coli* 23S rRNA gene number) [3–5, 7]. In *vitro*, the frequency of linezolid resistance was very low, proving to be less than 10<sup>-9</sup>, and the emergence of linezolid-resistant bacteria was considered to be low. [7]. This mutation was confirmed not only in a single copy of the 23S rRNA gene, but also in multiple copies [8]. Other mutations found in linezolid-resistant MRSA include a T2500A substitution in the domain V region of the rRNA gene [9]. Besides mutations in the 23S rRNA gene, two other mechanisms of linezolid resistance have been reported. One is the expression of the chloramphenicol florfenicol resistance (*cfr*) gene, which encodes 23S rRNA methyltransferase [10], and the other is genetic mutation of the 50S ribosomal subunit proteins L3 and L4 (referred to as *rplC* and *rplD*, respectively) [11, 12].

The *S. aureus* chromosome encodes five to six independent rRNA genes (*rrn*) or operons [13]. When the G2576T mutation accumulates in different copies of the 23S rRNA gene in one cell, it can be imagined that the level of resistance to linezolid gradually increases. Indeed, *in vitro* studies showed that stepwise passaging of linezolid-sensitive cells into medium containing progressively higher concentrations of linezolid resulted in mutants with progressively higher minimum inhibitory concentrations (MICs) of linezolid [5]. Analysis of such mutants showed that they accumulated the G2576T mutation in multiple copies of the 23S rRNA gene, and the number of mutations roughly correlated with the level of resistance [14]. However, the evolution of resistance factors over time in a single strain is not clear. In the present study, we investigated MRSA isolated from a patient treated with linezolid for a long period of 75 days. The isolates were susceptible to linezolid before the start of treatment, but became less susceptible by prolonged treatment. The evolution of resistance factors in these clinical isolates of MRSA was discussed.

**Methods**

**Bacterial strains**

A Japanese 65-year-old man with refractory pyothorax after lung cancer surgery was treated with linezolid for 75 days. A total of 16 MRSA strains isolated from sputum, airway secretions, pleural fluid, and wound pus of the patient before and during the treatment with linezolid were analyzed. These 16 clinical isolates were named as KUB3961 to KUB3976. We also used the strain ATCC29213 as a control strain of *S. aureus* and the strain ATCC29212 as a control strain of *E. faecalis*. (Table 1) This patient had not been previously treated with Linezolid.

**Antibiotic susceptibility test**
We performed antibiotic susceptibility test of 16 clinical isolates for linezolid (LZD), oxacillin (MPIPC), vancomycin (VCM), teicoplanin (TEIC), daptomycin (DAP), rifampicin (RFP) and cefoxitin (CFX). The MIC of each antimicrobial agent was determined by the agar dilution method according to the protocols of the Clinical and Laboratory Standards Institute (CLSI) [15]. We used *S. aureus* strain ATCC29213 and *E. faecalis* strain ATCC29212 as control strains for antibiotic susceptibility test as well.

### 23S rRNA genes sequencing analysis

The genomic DNA was extracted from 16 isolates of *S. aureus* by the QIAGEN DNeasy Blood & Tissue kits (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instruction [18]. Chromosomal genes encoding the 23S rRNA of five independent operons were amplified by polymerase chain reaction (PCR) using the primers reported previously [8]. Briefly, long-range PCR with product sizes of 5.6 to 6.5 kbp was performed to amplify each operon of 23S rRNA gene using primer sets (*rrn1* to *rrn5*) [8]. The purified 23S rRNA gene fragment was then used as a template to amplify the domain V region. Next, the PCR products were sequenced (Takara Bio, Mie, Japan or Nihon Gene Research Laboratories, Miyagi, Japan) and aligned with the corresponding nucleotide sequences obtained from linezolid-susceptible *S. aureus* strain NCTC8325 (GenBank accession no. X68425) [18].

### Pulsed-field gel electrophoresis (PFGE)

Chromosomal DNA was extracted from 16 isolates of *S. aureus* and then digested with *Sma* I according to the method described by Bannerman et al. [16, 17]. PFGE was carried out with a CHEF DRIII electrophoresis cell (Bio-Rad) at 6 V/cm for 20 h at 14°C, with initial and final pulses conducted for 5.3 and 34.9 s, respectively. The gel was stained with GelRed (Biotim) according to the manufacturer's manual and visualized under a 254-nm ultraviolet light [18].

## Results

### Antimicrobial susceptibility of 16 clinical isolates

The antimicrobial susceptibility of 16 clinical isolates are shown in Table 1. All 16 isolates had oxacillin (MPIPC) MIC of 128 µg/mL, which together with the results of identification of *S. aureus* indicated that they were MRSA. Of these 16 strains, KUB3970, KUB3971, KUB3972, KUB3974, and KUB3976 had linezolid (LZD) MICs of 8, 8, 8, >16, and >16 µg/mL, respectively. Vancomycin (VCM) MICs for KUB3970 to KUB3976 were 1 µg/mL. These strains are classified as linezolid-resistant vancomycin-sensitive MRSA. All isolates were susceptible to teicoplanin (TEIC), daptomycin (DAP), and rifampicin (RFP).

### PFGE typing of 16 clinical isolates

To determine whether these linezolid-resistant MRSA strains were derived from different or similar clones, DNA typing by PFGE of *Sma* I restricted DNA was performed (Fig. 1). The results showed that the 16 clinical isolates were 100% identical, indicating a single clone.
23S rRNA domain V sequencing of 16 clinical isolates

We performed 23S rRNA domain V sequencing of linezolid-susceptible strain KUB3961 and linezolid-resistant strains KUB3970, KUB3971, KUB3972, KUB3974 and KUB3976. The linezolid-susceptible strain KUB3961 (Linezolid MIC of 2 µg/mL) showed no mutations in the ribosomal operon at mutation sites G2447T, T2500A, T2571C, and G2576T, which are reported to be point mutations for linezolid resistance. On the other hand, three linezolid-resistant strains KUB3970, KUB3971 and KUB3972 with linezolid MIC of 8 µg/mL showed G2576T mutation in ribosomal operons 4 and 5. Two linezolid-resistant strains KUB3974 and KUB3976 with linezolid MIC of >16 µg/mL showed G2576T mutation in ribosomal operons 1, 3, 4, and 5. These results indicated that the copy number of G2576T mutation in 23S rRNA gene of MRSA isolates increased with the use of linezolid. (Table 2) The above-mentioned gene mutation was not found in the 23S rRNA gene of *E. faecalis* wild-type strain ATCC2912.

**Discussion**

This is the first report showing that MRSA isolated from one long-term linezolid-treated patient gradually became linezolid-resistant with an increase in the number of copies of the 23S rRNA point mutation.

Previous reports have suggested that the cause of linezolid resistance in *Staphylococcus aureus* is mainly a base substitution of G2576T in the domain V of 23S rRNA gene. In fact, Yurika Ikeda-Dantsuji et al. [18] and Yoshida et al. [19] reported that the clinical linezolid-resistance MRSA strains were found to have the G2576T mutation in the 23S rRNA gene domain V.

*S. aureus* is well known to have five to six copies of the 23S rRNA operon [8, 13]. Yurika Ikeda-Dantsuji et al. [18] reported that all clinical linezolid-resistant MRSA strains showed G2576T mutation in the 23S rRNA gene of at least one operon, which was found to be a possible cause of linezolid resistance. A previous *in vitro* study revealed that accumulation of G2576T mutation in different operons can lead to a stepwise increase in linezolid resistance levels [5], indicating that the number of mutant operons may correlate with the linezolid resistance levels of MRSA.

In this study, linezolid-resistant MRSA was isolated from pus 17 days after treatment with linezolid was started. Fifty-two days after the start of treatment, strains with increased linezolid-tolerance were isolated from pus. These linezolid-resistant strains had the same PFGE pattern as the linezolid-sensitive strains before the start of treatment, thus they were considered to be the same strains. In other words, a single strain may have become more linezolid-resistant by continued exposure to linezolid. These strains did not change to be resistant to antibiotics other than linezolid. The G2576T mutations in the 23S rRNA gene were found in all linezolid-resistant MRSA isolates. The strains with mutant operon numbers 2 and 4 showed MIC of 8 and >16 µg/mL, respectively. Therefore, it is suggested that the longer the exposure to linezolid *in vivo*, the higher the number of mutant operons of 23S rRNA of infected MRSA and the increased resistance of MRSA to linezolid.
We believe that there are three limitations of our study. First, we investigated only one patient in this study and could not confirm if the same phenomenon was always seen in any patient. Second, patients with linezolid-resistant isolates were treated for 75 days, but the relationship between the duration of linezolid treatment and the risk of G2576T mutation could not be known by this study alone. Third, linezolid-resistant strains were isolated only from pus, and it was unclear why MRSA isolated from other sites did not show linezolid resistance. The resistant strains can only appear in the areas where the organisms are growing.

From this study, long-term use of linezolid carries the risk of developing linezolid-resistant MRSA.

**Conclusion**

Long-term use of linezolid in a patient or reuse of linezolid in a patient who has been previously treated with linezolid can lead to the emerging of linezolid-resistant MRSA in the host.

**Declarations**

**Data availability**

Not applicable.

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This study was not financially supported.

**Contributions**

All authors reviewed and approved the final manuscript. All authors read and approved the final manuscript.

**Conflict of interest**

All authors declare no conflict of interest.

**Consent to participate**

Written consent to participate was obtained for enrolled subjects.

**Ethics approval and consent to participate**

All participants provided written informed consent. In addition, informed consent was approved by the ethics committee.

**References**


**Tables**

Table 1 and 2 are available in the Supplementary Files section.

**Figures**

**Fig. 1**

![Image of agarose gel electrophoresis](image-url)
Figure 1

Pulsed-field gel electrophoresis (PFGE) patterns of chromosomal DNAs extracted from clinical MRSA isolates. Chromosomal DNA was digested with the *Sma* I restriction endonuclease. Lanes 1, strain KUB3961; 2, strain KUB3962; 3, strain KUB3963; 4, strain KUB3964; 5, strain KUB3965; 6, strain KUB3966; 7, strain KUB3967; 8, strain KUB3961; 9, strain KUB3969; 10, strain KUB3970; 11, strain KUB3971; 12, strain KUB3972; 13, strain KUB3973; 14, strain KUB3974; 15, strain KUB3975; 16, strain KUB3976; L, bacteriophage lambda concatamer molecular size marker.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.pdf
- Table2.pdf
- SupplementaryFig1.pdf