Molecular characterization of linezolid-resistant CoNS isolates in Japan

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Objectives: Linezolid has been reported to remain active against 98% of staphylococci with resistance identified in 0.05% of Staphylococcus aureus and 1.4% of CoNS. The objective of this study was to characterize the linezolid-resistance mechanisms in the linezolid-resistant CoNS strains isolated in Japan.

Methods: Staphylococcus capitis strains exhibiting linezolid MICs >8 mg/L isolated from inpatients between 2012 and 2014 were screened for cfr and mutations in 23S rRNA, L3 and L4 by PCR/sequencing. Isolates were also examined for mutations in the rlmN gene.

Results: S. capitis had six 23S rRNA alleles. Five S. capitis isolates displayed linezolid MICs of 8, 16 and 32 mg/L. G2576U mutations were detected in three, four or five copies of 23S rRNA in all isolates. In two isolates exhibiting the highest linezolid MIC (32 mg/L) there was a large deletion in a single copy of 23S rRNA. Repeated 10 bp sequences were found in both 16S and 23S rRNAs, suggesting deletion by recombination between the repeats. One isolate had the mutation Ala-142→Thr in the ribosomal protein L3. All linezolid-resistant isolates also demonstrated mutations in the gene encoding RlmN methyltransferase, leading to Thr-62→Met and Gly-148→Ser.

Conclusions: Multiple mechanisms appeared to be responsible for the elevated linezolid resistance in S. capitis isolates: a G2576U mutation in different numbers of copies of 23S rRNA, loss of a single copy of 23S rRNA and a mutation in the ribosomal protein L3, suggesting the accumulation of independent mutational events.

Keywords: S. capitis, ribosomal mutations, rlmN

Introduction

Linezolid is the first agent of the oxazolidinone class to be introduced clinically and to remain consistently active against MDR Gram-positive bacteria, including MRSA and VRE.1–3 Linezolid was first approved for clinical use in 2000 in the USA, Europe and other countries. Soon after its introduction to clinical use, in 2001, a linezolid-resistant MRSA recovered from a patient treated with this agent was reported in the USA.4 Although multifocal outbreaks of linezolid-resistant staphylococci have been reported,4–7 a recent article reviewing the literature concerning linezolid-resistant Staphylococcus infections mentioned that linezolid remains active against >98% of staphylococci, with resistance identified in 0.05% of S. aureus.8 In Japan, linezolid was approved for treating MRSA infection in 2006, but has been closely restricted to ensure control of hospital-acquired infections. Despite this restricted use, 11 linezolid-resistant MRSA strains were identified in clinical isolates collected at six hospitals during 2006–08.9 Furthermore, a case of linezolid-resistant MRSA isolated after long-term repeated use of linezolid at a hospital in Japan was reported in 2009.10

Methicillin-resistant CoNS and MRSA are major causes of both healthcare- and community-associated infections. Linezolid is not approved for treating patients with catheter-site or catheter-related bloodstream infections or infections where CoNS are commonly implicated, but the incidence of linezolid-resistant CoNS has increased. CoNS seem to develop linezolid resistance readily since linezolid-resistant strains have been identified in 1.4% of CoNS, but in only 0.05% of Staphylococcus aureus.8 Linezolid-resistant CoNS reported in Europe comprised nine different species, among which 76.4% were Staphylococcus epidermidis, 9.1% were Staphylococcus hominis and 8.8% were Staphylococcus haemolyticus.

To our knowledge, linezolid-resistant CoNS have not been clinically isolated in Japan. During the interval May 2012 to April 2014...
we isolated seven linezolid-resistant CoNS strains from inpatients at Chiba, Japan, including five strains of Staphylococcus capitis recovered from different patients. In the present study, the linezolid resistance mechanisms in these five clinical S. capitis isolates were characterized. Linezolid inhibits bacterial growth via protein synthesis inhibition by binding to the peptidyltransferase centre (PTC) in the 50S ribosomal subunit, thereby perturbing the correct positioning of aminoacyl-tRNA on the ribosome. Modification of the ribosome at the PTC, commonly by mutation of domain V in 23S rRNA, has primarily been related to linezolid resistance in staphylococci. However, a naturally occurring resistance gene cfr, encoding a Cfr methyltransferase that catalyses methylation at the C-8 position of A2503 in 23S rRNA, has more recently been reported in clinical isolates. Although rRNA methylation is a common mechanism for acquiring antimicrobial resistance, there is recent evidence that the endogenous RlmN that modifies the C-2 position of A2503 in 23S rRNA could be linked to linezolid susceptibility in S. aureus. In the S. capitis isolates studied here, we detected G2576U mutations in three, four or five copies of 23S rRNA. In two isolates with the highest MIC of linezolid, a large deletion in a single copy of 23S rRNA was found in addition to the G2576U mutation. In all linezolid-resistant isolates, we demonstrated mutations in the gene encoding the RlmN methyltransferase. The present results provide insight into the multiple mechanisms of linezolid resistance in S. capitis strains clinically isolated in Japan.

Materials and methods

Bacterial strains

Five linezolid-resistant S. capitis strains (I-0553, I-0676, I-1184, I-2648 and I-0507) were characterized, with a linezolid-susceptible strain (I-0428) as control. All strains were isolated from inpatients during the period 2012–2014 at a hospital in Chiba, Japan. The characteristics of the organisms and associated clinical data are listed in Table 1.

Antimicrobial susceptibility

Isolates were tested for antimicrobial susceptibility by the reference broth microdilution method using Mueller–Hinton broth in validated panels (DPI3, Dryplate, Eiken Chemical), according to the CLSI method. The MICs of linezolid were determined by serial 2-fold dilution using Mueller–Hinton agar plates. The MIC results were interpreted on the basis of published CLSI criteria. Linezolid was purchased from Sigma-Aldrich Co.

Table 1. Demographic data and antimicrobial susceptibility profile of linezolid-resistant S. capitis isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Collection date</th>
<th>Clinical sample</th>
<th>Lzd</th>
<th>Van</th>
<th>Tec</th>
<th>Oxa</th>
<th>IpM</th>
<th>Abk</th>
<th>Ery</th>
<th>ClI</th>
<th>Lvx</th>
<th>MIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-0553</td>
<td>May 2012</td>
<td>vascular catheter</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>&gt;4</td>
<td>&gt;8</td>
<td>0.5</td>
<td>&gt;4</td>
<td>2</td>
<td>&gt;4</td>
<td>8</td>
</tr>
<tr>
<td>I-0676</td>
<td>Jun 2012</td>
<td>digestive organ</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>&gt;4</td>
<td>&gt;8</td>
<td>0.5</td>
<td>&gt;4</td>
<td>2</td>
<td>&gt;4</td>
<td>8</td>
</tr>
<tr>
<td>I-1184</td>
<td>Sep 2012</td>
<td>vascular catheter</td>
<td>16</td>
<td>2</td>
<td>1</td>
<td>&gt;4</td>
<td>&gt;8</td>
<td>0.5</td>
<td>&gt;4</td>
<td>2</td>
<td>&gt;4</td>
<td>8</td>
</tr>
<tr>
<td>I-2648</td>
<td>Dec 2013</td>
<td>blood culture</td>
<td>32</td>
<td>2</td>
<td>2</td>
<td>&gt;4</td>
<td>&gt;8</td>
<td>0.5</td>
<td>&gt;4</td>
<td>2</td>
<td>&gt;4</td>
<td>8</td>
</tr>
<tr>
<td>I-0507</td>
<td>Apr 2014</td>
<td>vascular catheter</td>
<td>32</td>
<td>2</td>
<td>4</td>
<td>&gt;4</td>
<td>&gt;8</td>
<td>0.5</td>
<td>&gt;4</td>
<td>2</td>
<td>&gt;4</td>
<td>8</td>
</tr>
<tr>
<td>I-0428</td>
<td>Apr 2014</td>
<td>blood culture</td>
<td>0.5</td>
<td>2</td>
<td>4</td>
<td>&gt;4</td>
<td>&gt;8</td>
<td>1</td>
<td>&gt;4</td>
<td>2</td>
<td>&gt;4</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

Lzd, linezolid; Van, vancomycin; Tec, teicoplanin; Oxa, oxacillin; IpM, imipenem; Abk, arbekacin; Ery, erythromycin; ClI, clindamycin; Lvx, levofloxacin; MIN, minocycline.

aA linezolid-susceptible S. capitis strain, isolated in the same hospital, was included as the comparative control.

PCR amplification and DNA sequencing

To test for mutations in individual copies of 23S rRNA by PCR amplification, the six specific primer sets described in Table S1 (available as Supplementary data at JAC Online) were used to amplify the copies of 23S rRNA from linezolid-resistant isolates individually. The resulting DNA fragments were sequenced using the same primers. For analysis of ribosomal protein genes by PCR amplification, followed by DNA sequencing, a specific primer set to amplify genes for L3 and L4, presented in Table S1, was used. The presence of cfr was investigated by PCR amplification using the forward primer 5′TGAAGTATAAACGAGGTGGAGTC and the reverse primer 5′ACCATAATTGACCAAAGGC.

PFGE

PFGE was performed using the method described by Schnellmann et al. with some modifications. Genomic DNA was prepared in agarose blocks and digested with SmaI (TaKaRa Bio, Japan). The DNA fragments were separated in a 1% agarose slab gel using a CHEF-Mapper system (Bio-Rad Laboratories Inc., CA, USA) for 27 h at 6 V/cm and 14°C, with a pulse angle of 120° and a ramped pulse time of 2.98–35.38 s. A CHEF DNA size standards lambda DNA ladder (Bio-Rad) was used as a reference marker.

Results

Characteristics of clinically isolated linezolid-resistant S. capitis strains

This study included five linezolid-resistant S. capitis strains recovered from different inpatients in a hospital at Chiba, Japan, during the interval May 2012 to April 2014. The susceptibility profiles of the linezolid-resistant S. capitis isolates I-0553, I-0676, I-1184, I-2648 and I-0507 are presented in Table 1. The MICs of linezolid ranged from 8 to 32 mg/L for these strains. These linezolid-resistant strains were resistant to oxacillin, imipenem, erythromycin, clindamycin, levofloxacin and minocycline. All isolates were susceptible to vancomycin, teicoplanin and arbekacin.

The clonal relatedness of the isolates was examined by PFGE of SmaI-digested genomic DNA (Figure 1). The five linezolid-resistant isolates were indistinguishable from each other, suggesting that they were derived from a similar clone. However, they were unrelated to the susceptible strain, I-0428, isolated in the same hospital. The linezolid resistance mechanisms in the five isolates were characterized.
Analysis of individual copies of 23S rRNA and ribosomal protein genes in linezolid-resistant S. capitis isolates

Determination of the number of 23S rRNA alleles in S. capitis

Resistance to linezolid has been associated with mutations in the central loop of the domain V region of 23S rRNA. Prior to testing for 23S rRNA mutations in the linezolid-resistant isolates, the copy numbers of 23S rRNA genes in *S. capitis* were determined because strains of *Staphylococcus* are known to have five or six 23S rRNA operons. For this purpose, the read sequences of the whole *S. capitis* SK14 genome deposited in NCBI by next-generation sequencing (ftp://ftp-trace.ncbi.nlm.nih.gov/sra/sra-instant/reads/ByRun/sra/SRR/SRR005/SRR005141/) were assembled on to the sequences of six copies of 23S rRNA in the genome of *S. epidermidis* RP62A (GenBank accession no. NC_002976) as a reference. The sequences used as reference included the region from 500 bp upstream to 1000 bp downstream of each gene *rrlA*, *rrlB*, *rrlC*, *rrlD*, *rrlE* and *rrlF*. GS Reference Mapper Software (Roche) was used for assembly. All six copies of 23S rRNA in *S. capitis* were successfully amplified by PCR using primer sets designed on the basis of the assembled data for *S. capitis* 23S rRNAs. The primer sequences are presented in Table S1. Nucleotide sequences for the six different copies were confirmed by sequencing the amplified products.

Mutations in 23S rRNA alleles

The isolates were first tested for the most common mechanism, mutations in 23S rRNA. We designed PCR primers specific for each copy (Table S1), used them to amplify each copy individually and subsequently sequenced the copies. The G2576U mutation in domain V of 23S rRNA was identified in all isolates except the linezolid-susceptible one (Table 2). This mutation arose in different numbers of copies of the 23S rRNA gene: (i) three copies in strains I-0553 (MIC = 8 mg/L) and I-0676 (MIC = 16 mg/L); (ii) four copies in strain I-2648 (MIC = 32 mg/L); and (iii) five copies in strains I-1184 (MIC = 16 mg/L) and I-0507 (MIC = 32 mg/L).

G2576U is the most frequently reported mutation in staphylococci, although other mutations such as U2500A, U2504A and G2447U have also been identified in 23S rRNA in clinical isolates. In the present isolates we found no mutations except G2576U, which has previously been associated with linezolid resistance.

Loss of a single copy of the 23S rRNA gene in isolates with high-level resistance to linezolid

Whereas PCR analysis detected six copies of 23S rRNA with the predicted size (4908 bp) in the linezolid-resistant strains I-0553, I-0676, I-1184, I-2648 and I-0507, respectively.

**Table 2.** Characteristics of linezolid-resistant *S. capitis* isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutations in 23S rRNA domain V allele sequence</th>
<th>Mutations in ribosomal protein</th>
<th>Mutation in RlmN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>rrlA</em></td>
<td><em>rrlB</em></td>
<td><em>rrlC</em></td>
</tr>
<tr>
<td>I-0553</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>I-0676</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>I-1184</td>
<td>G2576U</td>
<td>—</td>
<td>G2576U</td>
</tr>
<tr>
<td>I-2648</td>
<td>deletion</td>
<td>—</td>
<td>G2576U</td>
</tr>
<tr>
<td>I-0507</td>
<td>deletion</td>
<td>—</td>
<td>G2576U</td>
</tr>
<tr>
<td>I-0428</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*a* A long dash indicates no mutation.

*b* A long dash indicates that *cfr* was not detected.

*c* A 3018 bp deletion was found in the region covering the 3′-terminus of 16S rRNA and the 5′-terminus of 23S rRNA as represented in Figure 2.

*d* Linezolid-susceptible strain as the comparative control.
I-0676 and I-1184 and the linezolid-susceptible strain I-0428, analysis revealed that the rrlA gene was smaller (1885 bp) in one product, with five amplification products of the predicted size in strains I-2648 and I-0507 exhibiting increased resistance to linezolid (MIC = 32 mg/L). Figure S1 (available as Supplementary data at JAC Online) illustrates the agarose gel electrophoretic pattern of PCR-amplified products using the primer set for rrlA, indicating a DNA deletion had occurred in rrlA in the strains I-2648 and I-0507. Comparing the DNA sequence of the amplified products encapsulating rrlA with the corresponding region of S. capitis standard strain SK14 revealed that the 3018 bp sequence extending from the 3’-end of DNA for 16S rRNA to the 5’-end of DNA for 23S rRNA was lost in both I-2648 and I-0507 (Figure 2). In the standard strain SK14, we identified a 10 bp direct repeat sequence, GACGGGTGAG; one is in the region encoding 16S rRNA and the other is in that encoding 23S rRNA. The 3018 bp deletion could possibly have arisen by recombination between the two repeated sequences to generate a defective rrlA in I-2648 and I-0507.

Mutations in ribosomal protein genes

Linezolid resistance has also been associated with mutations in the ribosomal proteins L3 and L4, although both are located further from the bound drug. All isolates in the present study had the mutation Thr-83→Ile in the L3 protein, which was not present in the control S. capitis SK14. Since Thr-83→Ile was also identified in the linezolid-susceptible isolate I-0428, it could not be responsible for linezolid resistance. An additional mutation, Ala-142→Thr, was found in the isolate I-1184 with an MIC of 16 mg/L. No mutation was identified in L4 in any isolate. Previous studies have reported mutations including Phe-147→Ile, Gly-139→Arg, Met-156→Thr and Ala-157→Arg in the L3 proteins of other species. None of these mutations were found in any isolate in the present study.

**Mutation of the endogenous RlmN methyltransferase in linezolid-resistant S. capitis isolates**

In addition to the ribosomal gene mutations, acquisition of the 23S rRNA methyltransferase gene cfr is known to render staphylococci linezolid-resistant through modification of the C-8 position of A2503 in the PTC, thus preventing the drug from binding to the target site. The cfr gene is found on plasmids and appears to be capable of horizontal transfer between staphylococcal species. PCR demonstrated that no cfr is harboured by the linezolid-resistant S. capitis isolates characterized in the present study.

In addition to being the target for the Cfr methyltransferase, A2503 is also modified at the C-2 position by the endogenous RlmN methyltransferase, which is widespread and is found in the genomes of most bacteria. A recently described rlmN mutation in an S. aureus isolate from a MRSA-infected patient treated with linezolid was thought to decrease susceptibility to the drug. Furthermore, it was reported that a mutant lacking RlmN activity because of rlmN knockout outcompeted those with active RlmN under selective pressure from linezolid, suggesting that loss of RlmN activity decreases susceptibility to linezolid. We therefore looked for mutations in rlmN on the genome of the linezolid-resistant S. capitis. The rlmN gene was individually amplified in all isolates using the primer set described in Table S1 and then subjected to DNA sequencing. Sequence analysis identified mutations in rlmN leading to Thr-62→Met and Gly-148→Ser in all linezolid-resistant isolates, but the gene was not altered in the linezolid-susceptible isolate I-0428 (Table 2).

**Discussion**

S. capitis commonly inhabits human skin and mucosa, but is now recognized as an important opportunistic pathogen causing nosocomial bloodstream infections and indwelling catheter-related bacteremia. Recent studies have reported the emergence of methicillin-resistant S. capitis with reduced vancomycin susceptibility as an important cause of late-onset sepsis in neonatal intensive care units. The incidence of linezolid resistance remains exceedingly low for staphylococci. In this study, the resistance mechanisms in five S. capitis strains with various levels of linezolid resistance isolated from different inpatients between 2012 and 2014 were characterized.

Linezolid resistance has been associated with mutations in domain V of 23S rRNA and ribosomal proteins L3 and L4, and the acquisition of a transmissible Cfr ribosomal methyltransferase gene. In the present linezolid-resistant isolates, we detected the mutation G2576U. This mutation was present in different numbers of copies of the 23S rRNA gene. Previously, S. aureus isolates were reported to demonstrate an increase in linezolid MIC as the number of mutant 23S rRNA genes increased. In a series of S. aureus linezolid-resistant mutants, this gene dose effect has been described for mutations arising in the 23S rRNA after serial passages with linezolid, the number of mutated rRNA copies being directly related to the linezolid MIC. Gene dosage effects have also been demonstrated in clinical Enterococcus faecium and E. faecalis isolates. In contrast, the correlation is imperfect in the present series of S. capitis isolates, as seen by the discrepancy between I-0553 and I-0676, both of which had mutations in three operons, rrlD, rrlE and rrlF, yet had linezolid MICs of 8 and 16 mg/L, respectively. The difference in MIC could reflect other mutations, drug efflux or another unidentified mechanism. There was also a discrepancy between strains I-0676 and I-1184, both of which had the same linezolid MIC (16 mg/L), but had mutations in different numbers of 23S rRNA copies. On the other hand, there was a large deletion of 3018 bp extending
from the 3’ end of 16S rRNA to the 5’ end of 23S rRNA in the operon rrlA (Figure 2) in isolates I-2648 and I-0507, which exhibited the highest linezolid MIC (32 mg/L). The loss of a WT copy of the 23S rRNA gene, in the setting of existing mutant copies with G2576U, would increase the overall ratio of mutant to WT 23S rRNA copies. This would be expected to enhance the effect of the number of mutant copies present, resulting in an increased linezolid MIC.

The common mechanisms for linezolid resistance are the acquisition of a transmissible cfr methyltransferase gene and the ribosomal mutations. While mutational resistance to linezolid is troublesome in clinical practice, the acquisition of the cfr gene is more worrisome because of its rapid spread. A very recent study reported the presence of cfr in almost half (48%) of linezolid-resistant S. epidermidis isolated in California between 2007 and 2012. In contrast, no cfr was detected in any of the linezolid-resistant S. capitis included in the present study. On the other hand, the present results revealed that all S. capitis isolates except the linezolid-susceptible strain have uniform mutations, Thr-62 → Met and Gly-148 → Ser, in the endogenous RlmN methyltransferase that modifies the C-2 position of A2503, the C-8 position being methylated by the Cfr methyltransferase. In view of a previous report that an rlmN mutation in strains emerging from the parental MRSA in a patient after linezolid treatment increased the linezolid MIC from 0.74 mg/L (in the original MRSA) to 2 mg/L, it can be speculated that inactivation of the endogenous RlmN methyltransferase in S. aureus possibly increases linezolid resistance.

According to a recent report, an S. aureus strain lacking RlmN outcompeted those with active RlmN in mixed cultures in the presence of linezolid, suggesting that inactivation of RlmN in S. aureus increases linezolid resistance. Although inactivation of rlmN seems to have little effect on linezolid resistance in staphylococci, the lack of RlmN-mediated modification might contribute to higher resistance in combination with other resistance factors, e.g. mutation of the 23S rRNA domain V.

We characterized the linezolid resistance in five linezolid-resistant isolates of S. capitis and revealed that multiple mechanisms are responsible: a G2576U mutation in different numbers of copies of the 23S rRNA gene, loss of a single copy of 23S rRNA and a mutation in the ribosomal protein L3. The results suggest the accumulation of independent mutational events rather than a single mutation in CoNS strains, presumably following exposure to linezolid, and subsequent spread throughout our health system. These findings underscore the need to develop strategies to prevent the emergence of linezolid resistance.

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Transparency declarations
None to declare.

Supplementary data
Table S1 and Figure S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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Multiple mechanisms of linezolid resistance in S. capitis isolates


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