Linezolid-resistant clinical isolates of meticillin-resistant coagulase-negative staphylococci and Enterococcus faecium from China

Jia Chang Cai, Yan Yan Hu, Rong Zhang, Hong Wei Zhou and Gong-Xiang Chen

2nd Affiliated Hospital of Zhejiang University, Zhejiang University, 88 Jiefang Road, Hangzhou 310009, PR China

Seventeen meticillin-resistant coagulase-negative staphylococci (MRCoNS), including ten Staphylococcus capitis, four Staphylococcus cohnii, two Staphylococcus haemolyticus and one Staphylococcus sciuri, and an Enterococcus faecium isolate with various levels of linezolid resistance were isolated from intensive care units in a Chinese hospital. PFGE indicated that the four S. cohnii isolates belonged to a clonal strain, and that nine of the S. capitis isolates were indistinguishable (clone A1) and the other one was closely related (clone A2). A G2576T mutation was identified in domain V of the 23S rRNA gene in the E. faecium isolate. Besides the G2576T mutation, a novel C2104T mutation was detected in the nine clone A1 S. capitis isolates. The cfr gene was detected in all the staphylococci except an S. sciuri isolate, whose 23S rRNA gene contained the G2576T mutation. There was a clonal dissemination of linezolid-resistant MRCoNS in intensive care units of our hospital, and this is the first report, to our knowledge, of linezolid-resistant staphylococci and enterococci in China.

INTRODUCTION

Linezolid is an oxazolidinone antimicrobial approved for clinical use in 2000. Linezolid inhibits initiation of bacterial protein synthesis by preventing the formation of the tRNA–ribosome–fMet–mRNA ternary complex (Wilson et al., 2008) and is active against the majority of clinically relevant Gram-positive cocci, including meticillin-resistant staphylococci, vancomycin-resistant enterococci and penicillin-resistant streptococci (Diekema & Jones, 2001).

The first clinical isolates of linezolid-resistant enterococci and staphylococci were reported in 2001 (Gonzales et al., 2001; Tsiodras et al., 2001). Linezolid resistance in the vancomycin-resistant enterococci and meticillin-resistant Staphylococcus aureus was caused by G2576T mutation in the domain V region of the 23S rRNA gene, which was the most common mechanism. Besides the most frequent G2576T mutation, other mutations, including T2500A, G2603T, C2534T, T2504A, G2447T and G2631T, have been reported (Meka et al., 2004; Wong et al., 2010) among clinical staphylococcal isolates. Two other mechanisms involved in linezolid resistance of staphylococci have been reported. One is the presence of the cfr gene, which encodes an rRNA methyltransferase (Kehrenberg et al., 2005; Long et al., 2006; Schwarz et al., 2000), and the second is mutations in the ribosomal proteins L3 and L4 (Locke et al., 2009; Mendes et al., 2010a).

Recently, linezolid-resistant staphylococci have become an increasing problem, with several outbreaks in European countries and the USA (Bonilla et al., 2010; Kelly et al., 2008; Sánchez García et al., 2010). To date, clinical isolates of linezolid-resistant staphylococci and enterococci have not been reported in China. In the current study, we characterized 17 meticillin-resistant coagulase-negative staphylococci (MRCoNS) and an Enterococcus faecium isolate with linezolid resistance that were recovered from intensive care units in a Chinese hospital.

METHODS

Bacterial strains. Seventeen linezolid-resistant MRCoNS isolates, including ten Staphylococcus capitis, four Staphylococcus cohnii, two Staphylococcus haemolyticus and one Staphylococcus sciuri, were collected from March 2011 to August 2011. All isolates were recovered from blood culture, except one which was from cerebrospinal fluid culture. Eight patients were hospitalized in the neurological intensive care unit, eight in the central intensive care unit and one in the emergent intensive care unit. Nine patients were treated with linezolid before linezolid-resistant staphylococci were isolated (Table 1).

The linezolid-resistant E. faecium LRE1 and S. capitis LRS8 were isolated from the same patient, a 35-year-old man who was admitted to the neurological intensive care unit due to cerebral haemorrhage in June 22 and received left lateral cerebral ventricular external drainage the next day. Cefoperazone/sulbactam [3.0 g three times a day
Table 1. Clinical characteristics of patients with linezolid-resistant MRCoNS and *E. faecium*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Collection date (dd/mm/yy)</th>
<th>Source*</th>
<th>Ward†</th>
<th>Mechanisms of resistance</th>
<th>Linezolid MIC (µg ml⁻¹)</th>
<th>Antibiotic exposure‡</th>
<th>Sex</th>
<th>Age</th>
<th>Underlying condition§</th>
<th>Intensive care unit outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. capitis</em> LRS1</td>
<td>05/04/11</td>
<td>CSF</td>
<td>NICU</td>
<td>G2576T, C2104T, cfr</td>
<td>&gt;256</td>
<td>MEM, VA, LZD</td>
<td>Male</td>
<td>48</td>
<td>Cerebral haemorrhage</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. capitis</em> LRS2</td>
<td>23/06/11</td>
<td>Blood</td>
<td>CICU</td>
<td>G2576T, C2104T, cfr</td>
<td>&gt;256</td>
<td>TZP</td>
<td>Male</td>
<td>51</td>
<td>Pneumonia</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. capitis</em> LRS3</td>
<td>24/06/11</td>
<td>Blood</td>
<td>CICU</td>
<td>G2576T, C2104T, cfr</td>
<td>&gt;256</td>
<td>TZP, LZD</td>
<td>Male</td>
<td>65</td>
<td>COPD</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. capitis</em> LRS4</td>
<td>29/06/11</td>
<td>Blood</td>
<td>NICU</td>
<td>cfr</td>
<td>6</td>
<td>MEM, SCF, VA</td>
<td>Male</td>
<td>44</td>
<td>Craniocerebral injury</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. capitis</em> LRS5</td>
<td>30/06/11</td>
<td>Blood</td>
<td>NICU</td>
<td>G2576T, C2104T, cfr</td>
<td>&gt;256</td>
<td>TZP, LZD</td>
<td>Male</td>
<td>40</td>
<td>Brainstem haemorrhage</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. capitis</em> LRS6</td>
<td>03/07/11</td>
<td>Blood</td>
<td>EICU</td>
<td>G2576T, C2104T, cfr</td>
<td>&gt;256</td>
<td>LZD, TZP</td>
<td>Male</td>
<td>67</td>
<td>Gastric cancer</td>
<td>Died</td>
</tr>
<tr>
<td><em>S. capitis</em> LRS7</td>
<td>04/07/11</td>
<td>Blood</td>
<td>NICU</td>
<td>G2576T, C2104T, cfr</td>
<td>&gt;256</td>
<td>MEM</td>
<td>Female</td>
<td>74</td>
<td>Cerebral infarction</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. capitis</em> LRS8</td>
<td>27/07/11</td>
<td>Blood</td>
<td>NICU</td>
<td>G2576T, C2104T, cfr</td>
<td>&gt;256</td>
<td>MEM, SCF, LZD</td>
<td>Male</td>
<td>35</td>
<td>Cerebral haemorrhage</td>
<td>Died</td>
</tr>
<tr>
<td><em>S. capitis</em> LRS9</td>
<td>09/08/11</td>
<td>Blood</td>
<td>CICU</td>
<td>G2576T, C2104T, cfr</td>
<td>&gt;256</td>
<td>SCF</td>
<td>Male</td>
<td>76</td>
<td>Coronary heart disease</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. capitis</em> LRS10</td>
<td>19/08/11</td>
<td>Blood</td>
<td>CICU</td>
<td>G2576T, C2104T, cfr</td>
<td>&gt;256</td>
<td>MEM</td>
<td>Female</td>
<td>87</td>
<td>Coronary heart disease</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. cohnii</em> LRS11</td>
<td>16/03/11</td>
<td>Blood</td>
<td>CICU</td>
<td>cfr</td>
<td>&gt;256</td>
<td>MEM, TZP, TEC, LZD</td>
<td>Male</td>
<td>84</td>
<td>COPD</td>
<td>Died</td>
</tr>
<tr>
<td><em>S. cohnii</em> LRS12</td>
<td>13/05/11</td>
<td>Blood</td>
<td>NICU</td>
<td>cfr</td>
<td>&gt;256</td>
<td>SCF, LZD</td>
<td>Male</td>
<td>46</td>
<td>Multiple injury</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. cohnii</em> LRS13</td>
<td>03/07/11</td>
<td>Blood</td>
<td>NICU</td>
<td>cfr</td>
<td>&gt;256</td>
<td>SCF, LZD</td>
<td>Male</td>
<td>67</td>
<td>Conscious disturbance</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. cohnii</em> LRS14</td>
<td>27/08/11</td>
<td>Blood</td>
<td>CICU</td>
<td>cfr</td>
<td>&gt;256</td>
<td>SCF, LZD</td>
<td>Male</td>
<td>67</td>
<td>Diabetes mellitus</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. haemolyticus</em> LRS15</td>
<td>08/07/11</td>
<td>Blood</td>
<td>CICU</td>
<td>cfr</td>
<td>6</td>
<td>MOX</td>
<td>Male</td>
<td>73</td>
<td>Cervical cord injury</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. haemolyticus</em> LRS16</td>
<td>21/08/11</td>
<td>Blood</td>
<td>NICU</td>
<td>cfr</td>
<td>6</td>
<td>SCF, MEM</td>
<td>Male</td>
<td>67</td>
<td>Respiratory failure</td>
<td>Survived</td>
</tr>
<tr>
<td><em>S. sciuri</em> LRS17</td>
<td>08/08/11</td>
<td>Blood</td>
<td>CICU</td>
<td>G2576T</td>
<td>64</td>
<td>MOX</td>
<td>Male</td>
<td>74</td>
<td>COPD</td>
<td>Survived</td>
</tr>
<tr>
<td><em>E. faecium</em> LRE1</td>
<td>29/07/11</td>
<td>Blood</td>
<td>NICU</td>
<td>G2576T</td>
<td>8</td>
<td>MEM, SCF, LZD</td>
<td>Male</td>
<td>35</td>
<td>Cerebral haemorrhage</td>
<td>Died</td>
</tr>
</tbody>
</table>

*CSF, Cerebrospinal fluid.
†NICU, Neurological intensive care unit; CICU, central intensive care unit; EICU, emergent intensive care unit.
‡MEM, Meropenem; VA, vancomycin; LZD, linezolid; TZP, piperacillin/tazobactam; SCF, cefoperazone/sulbactam; TEC, teicoplanin; MOX, moxifloxacin.
§COPD, Chronic obstructive pulmonary disease.
intravenously (i.v.) and meropenem (1.0 g three times a day i.v.) were given for the treatment of hyperpyrexia after the operation. Blood culture yielded linezolid-susceptible *Staphylococcus hominis* on day 6 and the patient was treated with linezolid (600 mg twice a day i.v.) for 24 days. Subsequently linezolid-resistant *S. capitis* LRS8 and *E. faecium* LRE1 were isolated from blood culture on day 30 and day 32, respectively, and vancomycin (500 mg twice a day i.v.) was given. Two days later, the patient died of multiple organ failure. Species identification was performed with the Vitek 2 compact system (bioMérieux), and the identities of the isolates were confirmed by 16S rRNA gene sequencing. Two other patients died of multiple organ failure 1 week (*S. cohnii* LRS11) and 40 days (*S. capitis* LRS6), respectively, after the pathogen was isolated.

**Antimicrobial susceptibility testing.** The MICs of antimicrobial agents were determined by Etest (AB Biodisk) according to the manufacturer's instructions and confirmed by the disc diffusion method. The Clinical and Laboratory Standards Institute recommendations were used for antimicrobial susceptibility testing interpretation (CLSI, 2011). *S. aureus* ATCC 25923 was used as a quality control strain.

**PFGE.** Linezolid-resistant MRCoNS isolates were genotyped by PFGE following the PulseNet protocol from the website of the US Centers for Disease Control and Prevention (http://www.cdc.gov/pulsenet/protocols.htm) with some modifications. The bacterial cells were treated with proteinase K/lysozophin (Sigma) overnight and digested with *Smal* restriction enzyme overnight. PFGE was performed in a Rotaphor System 6.0 instrument (Whatman Biotracka). The restriction patterns of the genomic DNA from the isolates were analysed and interpreted according to the criteria of Tenover et al. (1995).

**PCR amplification of domain V of the 23S rRNA gene and cfr gene.** Genomic DNA was extracted by the Axyprep Bacterial Genomic DNA Miniprep kit (Axygen Scientific) and used as templates in PCR amplification. Staphylococci were treated with nsylastaphin (50 µg; Sigma) for 2 h at 37 °C before DNA extraction. Domain V of the 23S rRNA gene and cfr gene were amplified using conditions described by Kohenberg & Schwarz (2006) and Toh et al. (2007). The PCR products were sequenced on both strands. Four linezolid-susceptible MRCoNS isolates (*S. capitis*, *S. cohnii*, *S. haemolyticus* and *S. sciuri*) and one linezolid-susceptible *E. faecium* isolate from our hospital during the same period were used as negative control strains in DNA sequence analysis of the domain V region of the 23S rRNA gene.

**RESULTS AND DISCUSSION**

**Antimicrobial susceptibility**

Susceptibility profiles of nine of the ten *S. capitis* strains were similar. The four *S. cohnii* isolates also had similar susceptibility profiles (Table 2). The 17 coagulase-negative staphylococci were all resistant to penicillin G, oxacillin and cefoxitin. These MRCoNS showed various levels of linezolid resistance with MIC values of >256 µg ml\(^{-1}\) for nine *S. capitis* and four *S. cohnii*, MIC of 64 µg ml\(^{-1}\) for *S. sciuri* and MICs of 6 µg ml\(^{-1}\) for one *S. capitis* and two *S. haemolyticus*. The 17 MRCoNS were resistant to chloramphenicol, clindamycin, ciprofloxacin and gentamicin (except one *S. sciuri*), but were susceptible to tetracycline, trimethoprim–sulfamethoxazole, rifampicin and vancomycin. For teicoplanin, however, one *S. haemolyticus* isolate showed high-level resistance, and the MIC value for the other isolate was 8 µg ml\(^{-1}\). Previous studies (Howe et al., 2002; Wilson et al., 2003) found a loss of erythromycin resistance in linezolid-resistant metillin-resistant *Staphylococcus aureus*. The majority of linezolid-resistant MRCoNS in our study remained resistant to erythromycin. The *E. faecium* LRE1 was resistant to linezolid with an MIC of 8 µg ml\(^{-1}\) and exhibited high-level resistance to most of the antibiotics tested, except for tetracycline, vancomycin and teicoplanin.

**PFGE typing**

As shown in Fig. 1, nine *S. capitis* isolates with linezolid MIC of >256 µg ml\(^{-1}\) were indistinguishable (designated clone A1) and the other *S. capitis* isolate with a linezolid MIC of 6 µg ml\(^{-1}\) was closely related to them with a difference of two bands (clone A2). The four *S. cohnii* isolates showed identical band patterns and belonged to the same clonal strain. The two *S. haemolyticus* isolates showed more than seven band differences and were considered to be distinguishable. These data indicated that the majority of staphylococci were genetically related, and suggested the transmission of resistant clones from patient to patient.

**Mechanisms of linezolid resistance in MRCoNS and *E. faecium***

The most common mutation in domain V of the 23S rRNA gene, G2576T, and a novel mutation, C2104T, were identified in the nine *S. capitis* A1 clones. No mutation was detected in *S. capitis* LRS4 (clone A2), whose linezolid MIC was 6 µg ml\(^{-1}\). The G2576T mutation was also present in *S. sciuri* LRS17 but was absent in the four *S. cohnii* and two *S. haemolyticus* isolates. The sequence chromatogram of the domain V region of the 23S rRNA gene containing the G2576T mutation revealed a single peak. The G-to-T point mutation generates a *Nhel* site in the mutant domain V region. Five microlitre PCR products of the domain V region of the 23S rRNA gene were digested with 10 U *Nhel* (MBI Fermentas) for 4 h at 37 °C. Separation of the fragments by electrophoresis showed complete digestion (data not shown), suggesting that all copies of the 23S rRNA gene were mutated.

The *cfr* gene was detected in all linezolid-resistant MRCoNS except for *S. sciuri* LRS17. For *cfr*-positive isolates, the linezolid MIC for the four *S. cohnii* isolates was rather higher than that for *S. capitis* LRS4 and *S. haemolyticus* isolates LRS15 and LRS16 (>256 µg ml\(^{-1}\) vs 6 µg ml\(^{-1}\)), implying the possibility of other mechanisms involved in linezolid resistance in *S. cohnii*. Genes encoding ribosomal proteins L3 (*rplC*) and L4 (*rplD*) from linezolid-resistant and linezolid-susceptible *S. cohnii* were amplified as described by Locke et al. (2009). The result demonstrated the presence of six amino acid mutations (Asp108Glu, Ser158Phe, Asp159Tyr, Thr190Ala, Asn193Lys and Tyr208Phe) in L3, RplC and RplD from linezolid-resistant and linezolid-susceptible *S. cohnii* were amplified as described by Locke et al. (2009). The result demonstrated the presence of six amino acid mutations (Asp108Glu, Ser158Phe, Asp159Tyr, Thr190Ala, Asn193Lys and Tyr208Phe) in L3, RplC and RplD from linezolid-resistant and linezolid-susceptible *S. cohnii* were amplified.
Ala133Thr and Val156Ile) in L4 in linezolid-resistant S. cohnii when compared with the sensitive strain. None of these amino acid alterations has been previously reported. However, it still remains to be determined whether these modifications result in elevated resistance to linezolid.

Though cfr-positive S. capitis LRS4 and S. haemolyticus LRS15 and LRS16 were not resistant to linezolid according to Clinical and Laboratory Standards Institute criteria (resistant, ≥ 8 μg ml⁻¹), the MIC values for the three cfr-positive isolates were significantly higher than those for linezolid-susceptible S. capitis and S. aureus ATCC 25923 (6 μg ml⁻¹ vs 0.19–0.25 μg ml⁻¹). This suggests that Cfr confers low-level resistance to linezolid.

NheI restriction enzyme digestion of the PCR-amplified product of the domain V region of E. faecium LRE1 showed the presence of both NheI-digested bands and an undigested band, and the amount of undigested fragment was much more than that of digested fragment (data not shown). Careful examination of the sequencing traces found double peaks at position 2576 (Escherichia coli numbering), and the signal intensity of the mutated thymine (T) was about twice as strong as that of the wild guanine (G). Therefore, we estimated that only one or two of six copies of the 23S rRNA gene contained the G2576T mutation. It is important to note that such an isolate with two copies or a single copy of the 23S rRNA gene mutation, NheI restriction enzyme digestion of the PCR-amplified product of the domain V region of E. faecium LRE1 showed the presence of both NheI-digested bands and an undigested band, and the amount of undigested fragment was much more than that of digested fragment (data not shown). Careful examination of the sequencing traces found double peaks at position 2576 (Escherichia coli numbering), and the signal intensity of the mutated thymine (T) was about twice as strong as that of the wild guanine (G). Therefore, we estimated that only one or two of six copies of the 23S rRNA gene contained the G2576T mutation. It is important to note that such an isolate with two copies or a single copy of the 23S rRNA gene mutation,
which is intermediate in resistance or has low-level resistance, respectively, could be omitted by standard susceptibility testing in a clinical microbiology laboratory. Therefore, linezolid resistance should be carefully monitored, especially for patients who have received prolonged linezolid therapy. Previous studies have demonstrated that the MIC of linezolid increases in proportion to the number of copies of mutant 23S rRNA genes (Marshall et al., 2002), and isolates may increase their resistance if re-exposed to linezolid (Boumghar-Bourchaï et al., 2009).

Southern blot analysis (DIG High Prime DNA Labelling and Detection Starter kit I; Roche Applied Science) of plasmid DNA (extracted using an AxyPrep Plasmid Miniprep kit; Axygen Scientific) and genomic DNA (extracted using an AxyPrep Bacterial Genomic DNA Miniprep kit; Axygen Scientific) from the representative cfr-positive isolates S. capitis LRS1, S. capitis LRS4, S. cohnii LRS1 and S. haemolyticus LRS15 and LRS16 hybridized with the cfr probe showed that no positive signal was detected in plasmid DNA or genomic DNA in any isolates (data not shown). It is presumed, therefore, that the cfr gene may be located on the plasmid with low-copy number, which was unable to be detected by Southern blot hybridization.

Though linezolid resistance in staphylococci and enterococci has been reported in many European countries (Sánchez Garcia et al., 2010; Seedat et al., 2006; Witte & Cuny, 2011), the USA (Bonilla et al., 2010; Scheetz et al., 2008), Brazil (Gales et al., 2006), Mexico (Mendes et al., 2010b), Japan (Ikeda-Dantsuji et al., 2011) and Korea (An et al., 2011), to our knowledge, clinical isolates of linezolid-resistant staphylococci, either S. aureus or coagulase-negative staphylococci, or enterococci have never been described in China. This first emergence of linezolid-resistant MRCoNS and E. faecium in intensive care units in our hospital during a short period is very worrying, as many isolates were clonally related, suggesting the intrahospital dissemination of resistant clones.

REFERENCES


