Emergence of *cfr*-harbouring coagulase-negative staphylococci among patients receiving linezolid therapy in two hospitals in China

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This study reports on the emergence of *cfr*-harbouring coagulase-negative staphylococci (CoNS) among patients who received linezolid therapy in two hospitals in Hangzhou, China. The mechanisms of resistance and transmission were analysed for these resistant isolates. Eight *Staphylococcus capitis* isolates, one *Staphylococcus epidermidis* isolate and one *Staphylococcus hominis* isolate, obtained from patients who had received linezolid therapy in two hospitals in Hangzhou, China, were confirmed as linezolid resistant, with MICs ranging from 8 to >256 mg l⁻¹. The linezolid usage data of the ten patients before isolation of the linezolid-resistant CoNS were collected. PFGE analysis showed that the eight *S. capitis* isolates from the two hospitals belonged to the same clone. Nine of the linezolid-resistant CoNS isolates carried the *cfr* gene, which was located on plasmids of a similar size. A 5.3 kb fragment containing the *cfr* gene, revealing 99% identity to the sequence of the *cfr*-harbouring plasmid pSS-01 reported previously, was determined by PCR mapping for all *cfr*-positive isolates, and the *cfr* gene was flanked by two copies of IS256-like elements. Thus, these results document the emergence of linezolid-resistant CoNS isolates carrying the *cfr* gene in Hangzhou, China. Effective nosocomial infection control strategies and the judicious use of antibiotics will be required to prevent further spread of this resistance mechanism.

INTRODUCTION

Linezolid, the first oxazolidinone with antimicrobial activity against resistant Gram-positive bacteria, has been used to treat infections caused by meticillin-resistant *Staphylococcus aureus*, meticillin-resistant coagulase-negative staphylococci (CoNS), vancomycin-resistant enterococci and *Streptococcus* spp. (Meka & Gold, 2004). Linezolid binds to rRNA, specifically to domain V of the 23S rRNA of the 50S ribosomal subunit, thus inhibiting protein synthesis (Meka & Gold, 2004; Toh et al., 2007). A frequently reported mechanism of linezolid resistance is the presence of point mutation at the drug target site, such as G2576T (Bonilla et al., 2010; Marshall et al., 2002; Witte & Cuny, 2011). Resistance caused by mutation develops slowly, as all bacteria possess multiple copies of the 23S rRNA and this mechanism is not transmissible (Witte & Cuny, 2011). Meanwhile, another mechanism of linezolid resistance, mediated by a methyltransferase that catalyses methylation of A2503 in the 23S rRNA gene of the large ribosomal subunit (Morales et al., 2010), was described initially in a bovine *Staphylococcus sciuri* isolate. The Cfr enzyme confers resistance to several other antibiotic classes besides oxazolidinones, such as phenicols, lincosamides, pleuromutilins and streptogramin A.

The emergence of *cfr* in nosocomial staphylococci has been reported in several countries, including Italy, Spain, the USA and Mexico (Witte & Cuny, 2011). Recently, veterinary staphylococcal isolates harbouring the *cfr* gene were reported in China and dissemination of the *cfr* gene was studied (Wang et al., 2012). However, *cfr*-positive human isolates have rarely been reported in China.

Abbreviations: CoNS, coagulase-negative staphylococci; DDD, defined daily dose; ICU, intensive care unit.

The GenBank/EMBL/DDBJ accession number for the *cfr* gene sequence of *Staphylococcus capitis* MHZ is JX232067.
In the present study, we report on ten Staphylococcus isolates, nine of which harboured the cfr gene, from two hospitals in Hangzhou, China.

METHODS

Patients and bacterial strains. Ten linezolid-resistant clinical Staphylococcus isolates were studied, three of which were isolated from the First Affiliated Hospital of Zhejiang Chinese Medicine University (1500-bed tertiary care hospital) and seven from the First Affiliated Hospital of Zhejiang University (2500-bed tertiary care hospital). The ten isolates were obtained between November 2011 and March 2012, and all were isolated from blood culture. Species identification was performed using the API Staph system.

The linezolid usage data of the ten patients before isolation of the linezolid-resistant CoNS were collected. The defined daily dose (DDD) of linezolid was 1200 mg.

Antimicrobial susceptibility testing. The MICs of linezolid and vancomycin were determined using Etest, and susceptibilities to quinupristin/dalfopristin, rifampicin, chloramphenicol, clindamycin, teicoplanin, levofloxacin, trimethoprim/sulfamethoxazole, erythromycin, penicillin, tetracycline and gentamicin were tested by agar disc diffusion method. Staphylococcus aureus ATCC 25923 and S. aureus ATCC 29213 were used for quality control in the antimicrobial susceptibility testing. The results of susceptibility testing were interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2012).

Molecular detection of resistance genes and mutations. The cfr gene and the G2576T mutation were confirmed by PCR and sequencing using specific primers described previously (Toh et al., 2007).

PFGE. PFGE was performed according to the method described by Bannerman et al. (1995) with some modifications. Genomic DNA was prepared in agarose blocks and digested with Smul. The DNA fragments were separated using a CHEF-Mapper XA PFGE System (Bio-Rad) for 22 h at 6 V cm\(^{-1}\) and 14 °C, with a pulse angle of 120° and pulse times of 3–40 s. The PFGE banding patterns were analysed visually.

Plasmid analysis and PCR mapping. Plasmid DNA was extracted using a Plasmid DNA Midi kit (Qiagen). Plasmid DNA was digested with HindIII and EcoRI according to the instructions of the manufacturer (Takara). The fragments were separated by electrophoresis in 1% agarose gels in TBE buffer and stained with GelRed (Biotium). HindIII-digested λ DNA (Takara) was used as a molecular mass marker.

Genomic DNA was digested with S1 nuclease and separated by PFGE as above, but with a switch time from 2.16 to 63.8 s for a 17 h runtime. The DNA fragments were transferred to nylon membranes (Millipore), hybridized with DIG-labelled cfr-specific probes and detected using a nitro-blue tetrazolium/5-bromo-4-chloro-3′-indolyl phosphate colour detection kit (Roche Applied Sciences).

The partial nucleotide sequences of the cfr-harbouring plasmids were determined by primer mapping using primers specifically designed based on the sequence of the cfr-harbouring plasmid pSS-01 (GenBank accession no. JQ041372), as described previously (Wang et al., 2012).

RESULTS

Patients and bacterial strains

Ten linezolid-resistant CoNS isolates were recovered from blood samples of ten male patients from two hospitals in Hangzhou. Eight of these isolates were identified as Staphylococcus capitis, one as Staphylococcus epidermidis and one as Staphylococcus hominis.

All patients had received at least one course of linezolid before isolation of the linezolid-resistant CoNS, and the total number of days of linezolid DDD ranged from 5 to 40.5. Eight of the patients were hospitalized in an intensive care unit (ICU). All ten isolates were recovered from blood cultures (Table 1).

Antimicrobial susceptibility

All isolates had linezolid MICs >256 mg l\(^{-1}\) except for S. epidermidis NGG (8 mg l\(^{-1}\)) and S. capitis YQ45 (16 mg l\(^{-1}\)). For the other antimicrobial agents tested, similar multidrug-resistant phenotypes were observed in the ten isolates, which displayed resistance to chloramphenicol, clindamycin, erythromycin, levofloxacin, penicillin and gentamicin. However, all the isolates remained susceptible to vancomycin, teicoplanin and tetracycline, and most were susceptible to rifampicin and trimethoprim/sulfamethoxazole. The ten isolates were susceptible or intermediate to quinupristin/dalfopristin (Table 1).

Resistance genes and mutations

Except for S. capitis YQ45, all isolates were positive for the cfr gene. A G2576T mutation was detected in all eight S. capitis isolates, and the sequence chromatograms of three isolates showed mixed signals (G and T) existing at these loci. Both S. epidermidis NGG and S. hominis YQ39 isolates were negative for this mutation (Table 1).

Molecular typing and plasmid analysis of the cfr-positive staphylococci

PFGE analysis showed that the eight S. capitis isolates exhibited indistinguishable band patterns, whilst the S. epidermidis NGG and S. hominis YQ39 isolates had different profiles (Fig. 1). Southern blot analysis with a probe specific for the cfr gene showed that the nine cfr-positive isolates had the cfr gene on a plasmid with a similar size, ranging from ~20.5 to ~54.7 kb (Fig. 1), and no hybridization signal was observed for bands of isolate YQ45. The plasmids extracted from the nine cfr-positive isolates were analysed further by restriction enzyme digestion, which showed that the HindIII- and EcoRI-digested restriction patterns for the plasmids of the seven cfr-carrying S. capitis were identical but were different from the patterns obtained from the S. epidermidis and S. hominis isolates (data not shown).

The genetic environment surrounding the cfr gene was accessed by PCR mapping. A 5.3 kb fragment containing the cfr gene was determined by sequencing in S. capitis MHZ (GenBank accession no. JX232067) and showed 99% identity to the sequence reported for the cfr-harbouring plasmid pSS-01 (GenBank accession no. JQ041372) (Fig. 2).
### Table 1. Characteristics, antibiotic susceptibilities and resistance mechanism of the ten linezolid-resistant CoNS isolates

| Strain | Species      | Date of isolate | Days of linezolid DDDs | Patient age (years) | Underlying disease                                                   | Ward* | Outcome | Origin† | MIC (µg ml⁻¹)‡ | Resistance pattern§ | cfr|| nt |
|--------|--------------|-----------------|------------------------|---------------------|---------------------------------------------------------------------|-------|---------|---------|---------------|---------------------|-------|
| YQ2028 | S. capitis   | 6/11/2011       | 40.5                   | 97                  | Coronary artery disease                                             | ICU   | Discharge | FHZJU  | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | T     |
| YQ2236 | S. capitis   | 12/12/2011      | 9                      | 52                  | Brain trauma, pulmonary infection                                   | SICU  | Death    | FHZJU  | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | G, T   |
| YQ2238 | S. capitis   | 14/12/2011      | 26                     | 87                  | Septicaemia, chronic bronchitis                                      | ICU   | Discharge | FHZJU  | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | T     |
| YQ2324 | S. capitis   | 31/12/2011      | 15                     | 51                  | Hepatic cirrhosis                                                   | Liver transplant | Discharge | FHZJU  | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | G, T   |
| YQ23   | S. capitis   | 3/1/2012        | 14                     | 69                  | Infectious shock                                                    | ICU   | Discharge | FHZJU  | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | T     |
| YQ45   | S. capitis   | 8/1/2012        | 5                      | 76                  | Septicaemia                                                         | SICU  | Discharge | FHZJU  | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | T     |
| YQ39   | S. hominis   | 19/1/2012       | 20                     | 55                  | Septicaemia, skin infections                                        | Infectious diseases | Discharge | FHZJU  | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | G     |
| MHZ    | S. capitis   | 31/1/2012       | 40                     | 78                  | Brain infarction, pulmonary infection, septicaemia                  | ICU   | Death    | FHZCMU | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | G, T   |
| XWZ    | S. capitis   | 4/2/2012        | 8                      | 84                  | Brain infarction, severe pneumonia, respiratory failure             | ICU   | Death    | FHZCMU | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | G, T   |
| NGG    | S. epidermidis| 15/3/2012      | 12                     | 86                  | Brain infarction                                                    | ICU   | Death    | FHZCMU | >256 1        | CHL, CLI, LVX, ERY, PEN, GEN | Pos | G     |

* SICU, Surgical ICU.
†FHZCMU, First Affiliated Hospital of Zhejiang Chinese Medicine University; FHZJU, First Affiliated Hospital of Zhejiang University.
‡LZD, Linezolid; VAN, vancomycin.
§Antimicrobial agents tested: RIF, rifampicin; CHL, chloramphenicol; CLI, clindamycin; LVX, levofloxacin; SXT, trimethoprim/sulfamethoxazole; ERY, erythromycin; PEN, penicillin; GEN, gentamicin.
||Pos, positive; Neg, negative.
¶Mixed nucleotide signal in the 23S rRNA 2576 position.
The \( cfr \) gene was flanked by two copies of an IS256-like element, with a downstream \( orf1 \) gene. These 5.3 kb fragments were also obtained from the other eight \( cfr \)-positive isolates, indicating that similar genetic structures surrounding the \( cfr \) gene existed in these isolates.

**DISCUSSION**

Resistance to linezolid is rare in China. Here, we reported ten linezolid-resistant CoNS isolates with linezolid MICs ranging from 8 to >256 mg l\(^{-1}\) from ten patients who had received linezolid therapy in two hospitals in Hangzhou between November 2011 and March 2012. Although these ten CoNS isolates were isolated from a single set of blood cultures, all patients had received short-term or prolonged linezolid treatment before isolation of these resistant isolates, indicating that the emergence of these linezolid-resistant isolates was associated with exposure to this agent. Therefore, concerns need to be raised about these resistant isolates, as they could threaten the clinical benefits of linezolid use.

The PFGE pattern indicated that the linezolid-resistant \( S.\) capitis strains disseminated in the two hospitals belonged to one clone, and eight of the ten patients had passed through the same ICUs in the two hospitals, suggesting that this nosocomial outbreak was associated with intra- and interhospital clonal spread. The clonal spread of closely related meticillin-resistant CoNS strains within hospitals and even between hospitals has been reported in several studies, which could be explained by colonized patients.
moving between hospitals (Piette & Verschraegen, 2009; Widerström et al., 2006). Although the epidemiological relationship should be studied further for these patients from whom linezolid-resistant CoNS were isolated to prove this interhospital spread, we assumed that this clone of linezolid-resistant CoNS had persisted in certain wards such as the ICU of the two hospitals for >3 months, and ongoing monitoring of environmental samples in these wards may show the capacity of these resistant CoNS isolates to survive in this environment and disseminate among patients.

Mutations in the central loop of the V domain of the 23S rRNA and acquisition of the cfr gene are the most common resistance mechanisms of staphylococci to linezolid. Of the ten linezolid-resistant isolates, all the S. capitis isolates had the G2576T mutation. Considering the history of linezolid use for all the patients, the emergence of these mutation-carrying S. capitis was most likely due to the selective pressure of linezolid exposure. In an earlier report, the level of linezolid resistance increased when copies of the rRNA operon with the G2576T mutation increased (Wilson et al., 2003). For the three mutation-carrying S. capitis isolates, nucleotide mixtures were observed in their sequence chromatograms, indicating that not all copies of the rRNA operon had the G2576T mutation in these isolates but that this mutation mechanism had evolved during linezolid therapy. However, the contribution of the G2576T mutation was difficult to determine because of the presence of the cfr gene, which was detected in nine isolates. The cfr gene, encoding an rRNA methyltransferase, confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics (Long et al., 2006). Isolates carrying the cfr gene displayed higher levels of linezolid resistance than isolate YQ45 without the cfr gene, except for the one S. epidermidis isolate. This may be explained by varied expression of the cfr gene in this host. However, it also should be noted that other, less common, linezolid-resistance mutations (e.g. L3, L4 and T2500A) were not investigated in this study.

Unlike the mechanism of mutation, which develops slowly and is not transmissible, the cfr gene has been found in plasmids and appears to be capable of horizontal transfer between staphylococci (Kehrenberg et al., 2007; Morales et al., 2010). Wang et al. (2012) confirmed the wide dissemination of the cfr gene in five different species of staphylococci from swine farms, and four different types of plasmid were involved in the spread of this gene. In agreement with this finding, the cfr gene in all the cfr-carrying isolates we collected was located on plasmids of similar sizes ranging from 20.5 to 54.7 kb. Wang et al. (2012) also described a structure with two copies of an IS256-like element that played an important role in transfer of the cfr gene. To access the DNA sequences surrounding the cfr gene in this study, PCR mapping was performed, and we found that similar structures with the cfr gene flanked by two copies of IS256-like elements were present in all cfr-carrying isolates, indicating that this 5.3 kb region surrounding the cfr gene was conserved and that transfer of the cfr gene was possibly mediated by the IS256-like elements for these clinical isolates. Further sequencing of these cfr-carrying plasmids is ongoing to complete the genetic characterization and determine the similarity and relationships between the cfr-carrying plasmids from clinical isolates and animal isolates in China.

Mutational resistance to linezolid is troublesome in clinical practice, but acquisition of the cfr gene is a more worrying threat because of its rapid spread. In this study, we described the emergence of linezolid-resistant staphylococci in two hospitals in Hangzhou, China, and confirmed that cfr-carrying isolates were spreading among the patients. We believe that patients colonized with cfr-carrying staphylococci in the two hospitals were a reservoir of the cfr gene and that selective pressure imposed by using linezolid led to the outbreak of these resistant isolates. Dissemination of the cfr gene would reduce the efficacy of linezolid in treating infections caused by resistant Gram-positive bacteria, currently an effective nosocomial infection control strategy, and reinforcement of hand hygiene and monitoring of colonization of these resistant isolates in the wards, as well as a principle of judicious use of antibiotics, should be established in case of further spread of this resistance mechanism in China.

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