Identification and characterization of cfr-positive Staphylococcus aureus isolates from community-onset infectious patients in a county hospital in China

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The cfr gene was detected in 14 meticillin-sensitive Staphylococcus aureus isolates recovered from outpatients with community-onset infections in a county hospital in China. The MIC of linezolid was 4 μg ml⁻¹ in eight isolates and 2 μg ml⁻¹ in six isolates. All isolates were susceptible to vancomycin and teicoplanin, but had elevated MICs for penicillin (0.5–128 μg ml⁻¹), chloramphenicol (2–32 μg ml⁻¹), clindamycin (0.5–128 μg ml⁻¹) and erythromycin (4–128 μg ml⁻¹). Nine isolates had mutations on domain V of 23S rRNA and/or the ribosomal L proteins that were not located close to the linezolid-binding pocket. Southern blotting experiments demonstrated that the cfr genes in all 14 isolates resided on plasmids. Sequence analysis of the 5.6 kb cfr-carrying plasmid segment revealed 99 % identity to the corresponding sequences in plasmid pSS-01 from animal staphylococci and plasmid pRM-01 from human staphylococci. Five isolates belonged to sequence type (ST)188 and three to ST965; the two ST types were previously reported in isolates of animal origin in some areas of China. These results indicate that the cfr-carrying plasmids in this study are likely of animal origin. The present study shows that cfr-harbouring S. aureus isolates have emerged in some areas of China and that cfr-carrying isolates may be transmitted between animals and humans.

INTRODUCTION

Linezolid, a synthetic oxazolidinone antibiotic, was approved by the Food and Drug Administration in 2000, and was introduced in China in 2007. Linezolid is widely prescribed to treat respiratory tract and skin infections caused by meticillin-resistant Staphylococcus aureus (MRSA), penicillin-resistant Streptococcus pneumoniae and vancomycin-resistant enterococci (Brickner et al., 2008). This drug works as a protein synthesis inhibitor by binding to the ribosomal peptidyltransferase centre (Shinabarger et al., 1997). Although it was expected initially that linezolid would have low potential for resistance owing to its unique mechanism of action, linezolid resistance emerged shortly after the drug entered into clinical use. The first linezolid-resistant clinical isolate was reported in an MRSA isolate in the USA (Tsiodras et al., 2001). Since then, the occurrence of linezolid-resistant staphylococci has been increasingly reported in the United States (Mendes et al., 2008; Scheetz et al., 2008), Mexico (Mendes et al., 2010a), Japan (Ikeda-Dantsuji et al., 2011), Italy (Bongiorno et al., 2010; Mendes et al., 2010b), Spain (Sánchez García et al., 2010; Seral et al., 2011) and Ireland (Kelly et al., 2008).

Linezolid resistance is mainly due to mutations in domain V of the 23S rRNA gene, among which G2567U occurs most frequently (Bonilla et al., 2010; Marshall et al., 2002). In addition, the mutations T2500A (Meka et al., 2004), C2534T (LaMarre et al., 2013), G2215A (Kosowska-Shick et al., 2010), T2504A and G2447T (Wong et al., 2010) have been reported among clinical isolates. Mutations of the ribosomal proteins L3 (Locke et al., 2009) and L4 (LaMarre et al., 2013; Wong et al., 2010) in the regions close to the ribosomal peptidyltransferase centre were also reported to reduce the efficacy of linezolid in the control of infections.

The transferrable multidrug resistance gene cfr, which encodes a methyltransferase that targets A2503 in the 23S...
RESULTS

Identification of the cfr gene in S. aureus

The screening results revealed that, of the 476 S. aureus tested, 14 isolates carried the cfr gene. Detailed information on the 14 cfr-positive isolates is given in Table 1. The isolates were all recovered from outpatients in the same county hospital in Fujian Province, China. The patients’ ages ranged from 1 to 86 years, and 10 patients were male. The strains were isolated from secretions, sputum or abscesses, or by fine needle aspiration. The cfr genes of the 14 isolates were all identical to previously reported cfr sequences (accession nos AJ579365, JF834910, JQ041372 and FR675942).

Antimicrobial susceptibility and MLST

Susceptibility testing results are shown in Table 1. All isolates were identified as MSSA and susceptible to teicoplanin and vancomycin. Fourteen isolates showed linezolid MIC values of either 2 (six isolates) or 4 μg ml⁻¹ (eight isolates). For the other antimicrobial agents tested, similar multidrug-resistant profiles were observed in all 14 isolates, which displayed resistance or elevated MICs to penicillin (0.5–128 μg ml⁻¹), chloramphenicol (2–32 μg ml⁻¹), clindamycin (0.5–128 μg ml⁻¹) and erythromycin (4–128 μg ml⁻¹). Eight sequence types (STs) were identified, comprising ST1, ST6, ST15, ST30, ST59, ST121, ST188 and ST965. Among the 14 isolates, five were ST188 and three were ST965; the remaining six ST types were sporadic.

Molecular detection of mutations

Mutations in domain V of the 23S rRNA gene were detected in isolates sklx4267 (C2165T) (Escherichia coli numbering), sklx4281 (G2371A) and sklx4283 and sklx4284 (G2389A). The isolates sklx4268 and sklx4277 had mutations on L3 (Val28Leu and Ile23Thr, respectively). A mutation on Ala118 of L4 was found in sklx4263, sklx4264, sklx4266 and sklx4281, among which Ala was mutated to Thr in three isolates and to Val in one isolate. The sklx4283 isolate was found to contain an Ala83Thr mutation in L3 and an Ala98Glu mutation in L4 (Table 1).

Location and genetic environment of the cfr gene

Hybridization experiments with a probe specific for the cfr gene indicated that all isolates harboured the cfr gene in plasmids (data not shown). To gain insight into the structure and genetic environment of the cfr gene in the plasmids, ~5.6 kb fragments of the plasmids carried by isolates sklx004266, sklx004267 and sklx004274 were sequenced by PCR mapping. The genetic environments of cfr in these isolates were identical and showed 99 % identity to the sequence of the pSS-01 plasmid found in Staphylococcus cohnii (accession no. JQ041372) (Fig. 1). The resistance gene cfr was flanked by two copies of the IS256-like insertion sequence, with a downstream orf1 gene (Fig. 1).

METHODS

Bacterial isolates. A total of 476 clinical S. aureus isolates from community-onset infections were collected from 34 county hospitals in 12 provinces of China from August 2010 to December 2011. Samples obtained from outpatients accounted for 94.8 % of the isolates. Samples isolated from abscesses, secretion and sputum accounted for 32.3, 30.4 and 17.4 % of the samples, respectively.

Antimicrobial susceptibility testing. The MICs for penicillin, linezolid, chloramphenicol, clindamycin and erythromycin were determined by the broth microdilution method, and susceptibility to oxacillin, vancomycin and teicoplanin was tested by the agar dilution method. The results of antimicrobial susceptibility tests were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute. Isolates that had oxacillin MICs of ≤ 2 were identified as MSSA. S. aureus subspecies ATCC 25923 and ATCC 29213 were used for quality control.

Multilocus sequence typing (MLST). The S. aureus isolates were characterized by MLST as described previously (Enright et al., 2000). Briefly, ~500 bp fragments of seven housekeeping genes (arc, aro, glp, gmk, pta, tpi and yqi) were amplified by PCR and sequenced (Biosune). The sequences were compared with those in the S. aureus database (http://saureus.mlst.net).

Molecular detection of cfr genes and mutations. The isolates were treated with 4 μl 2 mg ml⁻¹ lysostaphin (Sigma) for 2 h at 37 °C before DNA extraction. Total DNA was extracted using the Total DNA Isolation kit (Omega Bio-Tek) according to the manufacturer’s instructions and used as templates for PCR amplification. The cfr gene, domain V of the 23S rRNA gene, and the rplC, rplD and rplV genes, which encode the ribosomal proteins L3, L4 and L22, respectively, were amplified as described previously (Schwarz et al., 2000; Pillai et al., 2002; Gentry et al., 2007). The PCR products were sequenced on both strands and the sequences were analysed using DNASTAR Lasergene v7.1 software. The BLAST (Basic Local Alignment Search Tool) program was used to compare DNA sequences against those in the National Center for Biological Information database. A cfr-positive Staphylococcus capitis (stored in our laboratory) was used as a positive control.

PCR walking. Plasmid DNA was extracted using the Plasmid Midi kit (Qiagen) according to the manufacturer’s instructions. The partial sequence of the cfr-carrying plasmid was determined by PCR walking (TsingKe Biological Technology). The sequences were compared with those deposited in GenBank using the BLAST program.
### Table 1. Characteristics of the cfr-positive S. aureus strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sample type</th>
<th>Date of isolate</th>
<th>Sex</th>
<th>Age</th>
<th>ST</th>
<th>MIC (µg ml⁻¹)</th>
<th>Mutations on L proteins and 23S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PEN</td>
<td>OXA</td>
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<tr>
<td>ATCC 29213</td>
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<td>m</td>
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<td>m</td>
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<td>0.5</td>
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<tr>
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</table>

se, Secretion; sp, sputum; fn, fine needle aspiration; as, abscess; PEN, penicillin; OXA, oxacillin; LNZ, linezolid; TEC, teicoplanin; VAN, vancomycin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin.
DISCUSSION

Linezolid is an important alternative in the treatment of infections caused by MRSA and meticillin-resistant coagulate-negative staphylococci, and is the last line of defence against heterogeneous vancomycin-intermediate S. aureus and vancomycin-resistant S. aureus. However, the first linezolid-resistant isolate emerged shortly after its introduction into the market. Since then, resistance has spread to many countries. So far, linezolid-resistant staphylococci and enterococci isolates from tertiary hospitals in China have been described (Cai et al., 2012; Tian et al., 2014; Chen et al., 2013; Cui et al., 2013). In China, the cfr gene is widely distributed in isolates of human and animal origin, particularly coagulate-negative staphylococcus isolates (Cai et al., 2012; Tian et al., 2014; Chen et al., 2013; Cui et al., 2013; Kehrenberg & Schwarz, 2006; Wang et al., 2013). This study identified 14 S. aureus isolates that harbour the cfr gene; this is, we believe, the first time that the cfr gene has been reported in community-onset S. aureus in China.

The rRNA methyltransferase coded by the cfr gene modifies the adenine residue at position 2503 of the 23S rRNA, resulting in resistance to antibiotics including lincomamides, phenicols, pleuromutilins and streptogramin A (Long et al., 2006). Among the 14 cfr-positive isolates, 12, 11 and 10 isolates exhibited altered resistance to erythromycin, chloramphenicol and clindamycin, respectively, whereas all isolates were susceptible to vancomycin and teicoplanin. All 14 isolates showed linezolid MIC values ranging from 2 to 4 µg ml⁻¹, which is a twofold or fourfold increase compared with the reference strain ATCC 29213. To further evaluate the contribution of the cfr gene to the elevated linezolid MICs in these isolates, mutations in the 23S rRNA domain V and in the ribosomal L proteins were analysed. Nine isolates had mutations in the 23S rRNA domain V and/or the L proteins. However, the nine isolates with the cfr gene and these additional mutations exhibited the same linezolid MIC values as the other five isolates, which only had the cfr gene, indicating that there was no synergistic effect on linezolid MIC values. It is possible that the mutations were located away from the linezolid-binding site on the rRNA and therefore might have no effect on the interaction between linezolid and 23S rRNA. Thus, the cfr gene itself is primarily responsible for the elevated linezolid MICs of the cfr-positive isolates in this study. The presence of the cfr gene therefore increases the likelihood of high-level resistance to linezolid in isolates with additional significant mutations (Flamm et al., 2012, 2013; Baos et al., 2013).

The cfr gene was first identified in chloramphenicol-resistant or florfenicol-resistant isolates, and a high occurrence of this gene was reported in isolates of animal origin, ranging from 3.2 to 18.6 % (Kehrenberg & Schwarz, 2006; Wang et al., 2013; Zeng et al., 2014). It is believed that this high occurrence is related to the widespread use of chloramphenicol in animals. Recently, plasmids that harbour the cfr gene, such as pSS-01, pSS-02, pSS-03 (Wang et al., 2012a) and pMSA16 (Wang et al., 2012b), were isolated from staphylococci of animal origin in China. In these plasmids, the cfr gene was flanked by mobile genetic elements such as IS21558, IS256 and premob, which are involved in the recombination and mobilization of the plasmid (He et al., 2014). The genetic environment of cfr in this study showed 99 % identity to that of the plasmid pSS-01 (accession no. JQ041372), which was isolated from a swine-origin S. cohnii. The cfr gene in plasmid pSS-01 was flanked upstream by a Tn4001-like transposon and downstream by a Tn558. The IS256-like mobile genetic element around cfr may account for its spread and maintenance in pathogens (LaMarre et al., 2013). In addition, the plasmids pRM01 (accession no. KC820815) and pHS, which were detected in clinical S cohnii and S. aureus isolates from tertiary hospitals in China, were also similar to plasmid pSS-01 with respect to the genetic
environment of the cfr gene (Tian et al., 2014; Chen et al., 2013). In 2012, a clinical Staphylococcus epidermidis from the USA was reported to have a similar plasmid, p7LC (accession no. JX910899) (LaMarre et al., 2013). This cfr-carrying plasmid was first detected in animal pathogens, and a similar genetic environment was identified in the clinical staphylococci in this study. The similarity in the genetic environments of the cfr gene in isolates from human and animal origin indicates that this gene originated in staphylococci from animal settings.

MLST analysis showed that 5 of the 14 isolates belonged to ST188 and 3 belonged to ST965. ST188 was identified by MLST analysis showed that 5 of the 14 isolates belonged to ST188 and 3 belonged to ST965. ST188 was identified in China in recent years (He et al., 2013). In 2012, a study in Shanghai found that ST188 accounted for 55.2% of S. aureus of food origin (Li & Shi, 2012). The outpatient isolates in this study were from county hospitals in rural areas and have a greater chance of coming into contact with food animals and farms. Thus, the possibility that ST188 and ST956 might have been transmitted from animals to humans cannot be excluded. However, further studies are needed to investigate the molecular epidemiology of animal-derived S. aureus and to examine the differences between S. aureus of human and animal origin.

In conclusion, this is the first report to our knowledge of cfr-positive plasmids in community-onset S. aureus in China. The cfr-positive isolates showed elevated MICs to linezolid, which might increase their chances of high linezolid resistance when additional mutations occur. Based on the results of the MLST analysis and the genetic environment of the cfr gene, this cfr-carrying plasmid could be transmitted between staphylococci of human and animal origin. Thus, further work is needed to study the molecular epidemiology of the S. aureus strains on local farms, in the environment, and in animals, and to examine the presence of the cfr gene among these pathogens to identify the underlying mechanism(s) by which this gene is transmitted from animals to humans.

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