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Detection of *cfr* rRNA Methyltransferases Among *Staphylococcus aureus* (SA) and Coagulase-Negative Staphylococci (CoNS) Recovered from Human Infections

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ABSTRACT

Background: *cfr,* a chloramphenicol resistance (R) gene in *S. sciuri*, also mediates R to lincosamides, streptogramin A, oxazolidinones and pleuromutilins, and has subsequently been found in *S. simulans* and SA, all of animal origin. Only one report of a *cfr*-positive human source SA (Colombia) has been described. This methyltransferase produces R by methylation of adenosine at position 2503 of the 23S rDNA. Here we describe identification of the *cfr* gene in SA and CoNS recovered from human infections.

Methods: Staphylococci recovered as part of international surveillance and research programs that demonstrated R to chloramphenicol, linezolid, clindamycin and quinupristin/dalfopristin when tested by the CLSI broth microdilution assay were further evaluated by PCR and amplicon sequencing for the linezolid-R G2576T mutation in the 23S rDNA and for the *cfr* gene (Kehrenberg et al. Antimicrobial Agents Chemother 2006;50:1156). Plasmid analysis followed by *cfr* amplification/sequencing was also performed.

Results: Four isolates meeting criteria were identified in 2006-2007, including two each SA (USA and Belgium) and CoNS (USA and Spain). All displayed a multidrug-resistant phenotype (see Methods) including R to oxacillin and ciprofloxacin. Two were R to erythromycin (Cfr-mediated methylation does not affect macrolide S), and concurrent presence of *ermA* was documented; all isolates were susceptible to vancomycin. While the G2576T mutation was not detected, all four were positive for *cfr*, confirmed by sequencing. Preliminary evaluation revealed presence of the *cfr* gene on plasmids in two strains. The genetic context of *cfr* differs from prior reports in some strains.

Conclusion: This is the first report of *cfr*-mediated linezolid-R among SA and CoNS in USA and European human isolates. The potential mobility of this gene via plasmidic spread, combined with the tendency towards clonal dissemination among *Staphylococcus* spp., is a serious threat to potent Gram-positive-active agents, including oxazolidinones. Active surveillance along with effective infection control should be utilized in minimizing spread of this worrisome R mechanism.

INTRODUCTION

Linezolid, the first oxazolidinone agent used in clinical practice, has demonstrated potent antimicrobial activity against Gram-positive pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci and *Streptococcus* spp. According to the surveillance program Linezolid Experience and Accurate Determination of Resistance (LEADER), nearly all *S. aureus* (>99.9%) and coagulase-negative staphylococci (CoNS; 98.4%) isolated in the United States (USA) were susceptible to linezolid in 2006.

Linezolid resistance has appeared only sporadically since its introduction in 2000, and it is usually mediated by the presence of mutations in one or more alleles of the target 23S rDNA gene. However, some linezolid-resistant isolates fail to display these mutations, indicating the presence of other resistance mechanisms.

The *cfr* gene was initially described as a chloramphenicol resistance mechanism in *S. sciuri* of animal origin. The product of this gene, a methyltransferase, provides post-transcriptional methylation of the 23S rRNA at position A2503. Further studies showed that *cfr* methylation affects the binding of at least five antimicrobial classes: phenicols, lincosamides, pleuromutilins, streptogramin A and oxazolidinones. This gene, that produces to a multidrug-resistant (MDR) phenotype, was recently reported in a *S. aureus* isolate recovered from the respiratory tract infection of a patient in Colombia.

In this study we detected four linezolid-resistant clinical isolates carrying *cfr.* These strains (two *S. aureus* and 2 CoNS) were collected in the USA and in Europe during international surveillance studies and clinical trial protocols.

MATERIALS AND METHODS

Bacterial isolates. *Staphylococcus* spp. isolates showing elevated linezolid MIC values (MIC, ≥4 µg/ml) were routinely confirmed and further evaluated at JMI Laboratories (North Liberty, Iowa, USA). These isolates were collected in different international surveillance studies and clinical trial protocols. Only clinically significant isolates were included in the studies; one per patient episode. Species identification was confirmed by standard biochemical tests and use of the Vitek System (bioMérieux; Hazelwood, Missouri, USA), when necessary.

Susceptibility testing. All *Staphylococcus* spp. isolates were susceptibility tested against more than 25 antimicrobials by the broth microdilution procedure as described by the Clinical and Laboratory Standards Institute (CLSI; 2006) using validated panels manufactured by TREK Diagnostics (Cleveland, Ohio, USA). Interpretations of susceptibility testing results were by CLSI (2008) criteria. *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were concurrently tested for quality assurance. MIC values to key antimicrobial agents were confirmed using broth microdilution or E-test® strips (AB BIODISK, Solna, Sweden) according to the manufacturer's instructions.

Detection of linezolid resistance mechanisms. Isolates with elevated linezolid MIC values were evaluated for the presence of a G2576T mutation in the 23S rDNA. Additionally, selected isolates were screened for mutations in ribosomal proteins L4 and L22 that have recently been linked to linezolid-resistance. The isolates lacking these resistance mechanisms were further tested for the presence of *cfr.* The nucleotide sequences and deduced amino acid sequences were analyzed using Lasergene software package (DNASTAR, Madison, WI) and compared with the sequences available through the internet using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

Genetic context of *cfr.* Plasmid DNA was extracted using the Plasmid DNA Midi Kit (Qiagen GmbH, Hilden, Germany), digested with XbaI and resolved on 1% agarose gel along with aliquots of uncut plasmids. DNA was transferred onto a nylon membrane by the Southern blot method. The *cfr* was amplified, labeled and used in hybridization reactions performed with nonradioactive DIG high Prime DNA labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). Plasmid sizes were determined by comparison with plasmids carried by *Escherichia coli* NCTC 50192 and NCTC 50193.

The DNA sequences surrounding *cfr* were elucidated with custom primers designed based on previous *cfr*-carrying elements by the primer walking strategy. Isolates showing negative amplification with these primers were characterized using degenerate random primers in combination with primers anchored in the *cfr* gene.

Nucleotide sequence accession number. The nucleotide sequences of the *cfr* gene from *S. aureus* 004-737X have been deposited in the GenBank database under the accession number EU598691.

RESULTS

- Four strains without mutations in G2576U 23S rDNA or in the L2 and L4 encoding regions, were positive for *cfr* (Table 1). Sequencing results confirmed the presence of this methylase gene.
- These four linezolid resistant strains included two *S. aureus* and two *S. epidermidis* (Table 1) that were recovered in Belgium, Spain and USA (Ohio and Arizona).

Table 1. Characterization of linezolid resistance mechanisms among *Staphylococcus* spp. isolates.

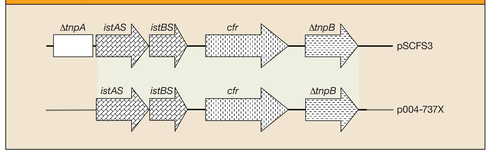
Isolate no.	Year	Organism	Mutations in 23S rDNA	*cfr* detection
30-2293A	2006	*S. epidermidis*	G2576T(-)	negative
86-5174A	2006	*S. epidermidis*	G2576T(-)	negative
86-15443A	2006	*S. epidermidis*	G2576T(-)	negative
409-512L	2006	*S. epidermidis*	G2576T(-)	negative
408-805L	2006	*S. epidermidis*	G2576T(-)	negative
25-1911X	2006	*S. aureus*	G2576T(-)	negative
129-5633X	2006	*S. epidermidis*	G2576T(-)	negative
131-6952X	2006	*S. aureus*	G2576T(-)	cfr(+)
116-7625X	2006	*S. epidermidis*	G2576T(-)	negative
4-737X	2007	*S. aureus*	G2576T(-)	cfr(+)
426-3147L	2007	*S. epidermidis*	G2576T(-)	cfr(+)
442-3204L	2007	*S. epidermidis*	G2576T(-)	negative
442-3207L	2007	*S. epidermidis*	G2576T(-)	negative
2363	2007	*S. epidermidis*	G2576T(-)	cfr(+)

Table 2. Antimicrobial susceptibility profiles of *cfr*-carrying *Staphylococcus* spp. isolates.

	MIC µg/ml			
Antimicrobial agent	004-737X *S. aureus*	131-6952X *S. aureus*	426-3147L *S. epidermidis*	2363 *S. epidermidis*
Linezolid	8	8	>256	>256
Chloramphenicol	>256	>256	>256	>256
Quinupristin/dalfopristin	8	4	1	8
Retapamulin	32	1	>32	>32
Clindamycin	>256	8	>256	>256
Erythromycin	>256	0.25	8	0.5
Oxacillin	>2	>2	>2	>2
Vancomycin	1	1	4	2
Teicoplanin	2	2	1	4
Daptomycin	0.25	0.25	0.25	0.25
Mupirocin	0.25	0.5	256	64
Ciprofloxacin	>32	>32	>32	>32

- The two *cfr* positive *S. aureus* isolates showed MIC values to linezolid lower than those for *S. epidermidis* isolates (MIC values of 8 and >256 µg/ml, respectively).
- All four isolates were highly resistant to chloramphenicol and retapamulin, although the resistance levels to clindamycin and quinupristin/dalfopristin were variable among the isolates (Table 2).
- The *cfr*-positive isolates were resistant to oxacillin and ciprofloxacin, but remained susceptible to vancomycin, teicoplanin and daptomycin (Table 2).
- PCR experiments used primers targeting structures previously associated with *cfr* in combination with primers anchored in this methylase gene, and showed that one *S. aureus* isolate (collected in Ohio, USA) was carried in a structure similar to the one found in the *S. aureus* isolate collected from the respiratory tract of swine (AM086211).
- In isolate 004-737X *istAS* and *istBS* genes were found upstream of the *cfr* and Δ*tnpB* was located downstream (Figure 1). However, Δ*tnpA* (located upstream of *istAS*/*istBS*/*cfr* in the isolate from animal origin) was not detected in isolate 004-737X evaluated in this study. The remaining three isolates showed negative results on PCR experiments for *cfr* associated structures previously described.
- The degenerated primer approach revealed a DNA fragment of 600 bp upstream of *cfr* from one *S. epidermidis* isolate. This sequence showed low homology with other known DNA sequences and is being investigated further.
- Analysis of plasmid content of the four isolates revealed the presence of different plasmid patterns, with bands varying from 55 to 555-kb.

Figure 1. Schematic representation of DNA sequences surrounding *cfr* in *S. aureus* (004-737X) and the genetic context of pSCFS3 shown for comparison purposes (AM086211). Genes are indicated by boxes and the arrows indicate their transcriptional orientations. Small arrows indicate primers targeting regions and their respective orientations. Background indicates identity to the pSCFS3 DNA sequence.



- Experiments showed that *cfr* was likely to be located in different plasmids having different sizes in each of the strains, suggesting that the element carrying *cfr* has been mobilized to various plasmid structures.

CONCLUSIONS

- Linezolid resistance is usually mediated by mutations in 23S rDNA or other mutations in constitutive genes, inferring limited dissemination of resistance.
- The detection of a plasmid-borne *cfr*-mediated linezolid resistance gene in staphylococci recovered from clinical specimens adds a new dimension to the threat of bacterial resistance to several antimicrobial classes, including the oxazolidinones.
- The dissemination of *cfr*-mediated resistance genes among staphylococcal clinical isolates is especially worrisome given the potential for rapid plasmid mobilization and spread.
- Continued surveillance of linezolid resistance mechanisms seems prudent to guide effective infection control and future therapeutic strategies.

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