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# Analysis of Linezolid Resistance Mechanisms and Epidemiology of Cfr-producing Strains in the United States

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SENTRY ANTIMICROBIAL SURVEILLANCE

## ABSTRACT

**Background:** The majority of linezolid-nonsusceptible strains possess G2576T mutations. Alterations in L3 and L4 ribosomal proteins and the plasmid-encoded Cfr have also been implicated in linezolid resistance. We report the genotypic characteristics of Cfr-producing staphylococci collected from USA hospitals.

**Methods:** Staphylococci (27,298) submitted as part of the SENTRY Program (2007 – 2010) were tested for susceptibility by reference CLSI methods. Isolates with linezolid MIC values at ≥4 µg/mL were screened for *cfr* and mutations in the 23S rRNA-, L3- and L4-encoding genes. *cfr* strains were selected and further evaluated. Clonality was accessed by PFGE, *spa* (*S. aureus* only) and MLST. Strains were identified by Vitek 2 and confirmed by 16S rRNA.

**Results:** Only 14 (0.05%) *cfr* strains were detected during 2007 – 2010. Linezolid MIC results in *S. aureus* were between 4 and 16 µg/mL, and these strains were wildtype for ribosomal mutations (23S, L3 and L4). Coagulase-negative staphylococci (CoNS), mostly *S. epidermidis*, showed higher linezolid MIC values (8 - >128 µg/mL) and these strains were often associated with ribosomal mutations. Two *S. aureus* isolates were sequence type (ST) 8 and t008 (USA300), while *S. epidermidis* were ST5 (clonal complex [CC] 2, cluster II-5), ST22 (CC2, cluster I) and ST186 (CC2, cluster I). *S. epidermidis* from Arizona were clonally-related.

**Conclusions:** *cfr* genes were rare in the studied population. *S. epidermidis* often possessed multiple linezolid resistance mechanisms, which can explain the higher MIC results compared with *S. aureus*. This study describes the second *cfr* report in a *S. aureus* USA300 strain in the USA. The *cfr* gene has mostly emerged in *S. epidermidis* CC2 cluster I or II-5, which are clones comonly associated with linezolid resistance in the USA.

## INTRODUCTION

Linezolid was introduced into clinical practice in 2000, and has been widely utilized for treating serious Gram-positive infections, including those caused by methicillin-resistant *Staphylococcus aureus* (MRSA). Since the early development and use of linezolid, the occurrence of resistance has been detected in laboratory-generated and clinical strains, and associated with target site mutations in rRNA and ribosomal proteins. These target site alterations are usually due to prolonged *in vitro* or clinical drug exposure.

The *cfr* gene has been recognized as an additional and mobile linezolid resistance mechanism, since it has been found almost exclusively on small plasmid DNAs (17- to 43-kb). Cfr causes post-transcriptional methylation of the 23S rRNA at position A2503, which affects the binding of several other drug classes. Recently, a mutation in the intrinsic *rlmN*, which encodes a ribosomal methyltransferase that modifies the 23S rRNA at position A2503 in *S. aureus,* was implicated in a slightly decreased susceptibility to linezolid.

The objective of this study was to evaluate the phenotypic and molecular characteristics of Cfr-producing staphylococcal clinical isolates collected in USA hospitals and submitted as part of the SENTRY Antimicrobial Surveillance Program (2007 – 2010).

## MATERIALS AND METHODS

**Clinical strains.** A total of 27,298 staphylococcal strains were submitted as part of the SENTRY Program (2007 – 2010). *cfr*-positive strains (six *S. aureus*, seven *S. epidermidis* and one *S. capitis*) were selected for further analysis and included in this study.

**Antimicrobial susceptibility testing.** Susceptibility testing was carried out by reference broth microdilution methods, according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (M07-A8, 2009). Minimum inhibitory concentration (MIC) interpretations were performed as described in the CLSI M100-S11 document (2011), when available. Retapamulin MIC results were interpreted according to the microbiological parameters reported by Traczewski et al. (2008). Susceptibility breakpoint interpretation for tigecycline was based on the FDA criteria for *S. aureus* (≤0.5 µg/mL for susceptibility). *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were concurrently tested for quality assurance purposes.

**Screening for ribosomal protein mutations.** Presence of mutations in the 23S rRNA, L3 and L4 ribosomal proteins were screened by PCR and sequencing. Amplicons were sequenced on both strands. Ribosomal proteins obtained were compared to those from wildtype ATCC strains using the Lasergene® software package (DNAStar; Madison, Wisconsin).

**Molecular typing.** Isolates recovered from the same hospital were subjected to pulsed-field gel electrophoresis (PFGE). SmaI-digested genomic DNA was resolved in CHEF-DR II (BioRad, Richmond, California). PFGE profiles were analyzed using the GelCompar II software (Applied Math, Kortrijk, Belgium). PFGE patterns of *S. aureus* strains were compared with those from representatives of USA100 – 1100 clones. The *cfr*-carrying strains were further characterized by single (*spa*; *S. aureus* only) and multilocus sequence typing (MLST). *spa* types were assigned through the Ridom web server (http://www.ridom.de/spaserver/). MLST alleles and sequence types (ST) were identified using the MLST database (http://www.mlst.net) and the eBURST program (http://eburst.mlst.net) was utilized to infer the evolutionary relatedness among STs.

## RESULTS

- Only 14 of 27,298 (0.05%) staphylococcal strains carrying *cfr* were detected during this four year period of the SENTRY Program. Within 2007 – 2008, three *cfr*-positive strains were observed, while six and five Cfr-producing staphylococci were detected during 2009 and 2010, respectively (Table 1).
- Table 2 shows the antimicrobial susceptibility profile of *cfr* strains included in this study. Except for one strain, *S. aureus* were methicillin-resistant, as were the *S. epidermidis* clinical strains.
- All included strains demonstrated elevated MIC results to those antimicrobial agents (phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A; $PhLOPS_A$) affected by the post-transcriptional methylation of 23S rRNA (A2503) caused by Cfr.
- Three *S. aureus* strains exhibited linezolid MIC results at the breakpoint for susceptibility (4 µg/mL), while the other *S. aureus* showed MIC values of 8 or 16 µg/mL. All *S. epidermidis* demonstrated linezolid MIC results (≥128 µg/mL) higher than *S. aureus*, except for one isolate (MIC, 16 µg/mL).
- Cfr-producing *S. aureus* exhibited wildtype L3 and L4 ribosomal protein sequences (Table 1). In contrast, alterations in the L3 and L4 proteins were commonly observed among *S. epidermidis*.
- *S. aureus* strains belonged to ST5 or ST8. In addition, isolate 22689 showed a PFGE profile similar to that of NRS382 (USA100), while *S. aureus* 1848 and 22631 displayed a PFGE pattern similar to that of NRS384 (USA300; Figure 1).
- All *cfr*-carrying *S. epidermidis* were associated with CC2 and 85.7% of the strains (four clonally related) belonged to cluster I, which included ST22 (a single-locus variant of ST2; two strains) and ST186 (a double-locus variant of ST2; four strains).
- PFGE results indicated the presence of *S. epidermidis* strains (2007 – 2010) with indistinguishable profiles in a medical center located in Arizona (site 426). In addition, isolate 12676 was genetically related to those responsible for a multi-city outbreak in Ohio hospitals (Bonilla et al., 2010).

**Figure 1.** PFGE pattern analysis of *S. aureus* included in this study. Percent similarities were identified on a dendrogram derived from the unweighted pair group method using arithmetic averages and based on Dice coefficients. Band position tolerance and optimization were set at 1.5% and 0.5%, respectively. Isolates showing similarity coefficient ≥80% were considered as genetically related. *S. aureus* NRS382 (USA100) and NRS384 (USA300) were included for comparison purposes. PFGE profiles of *S. aureus* strains 272, 1687 and 737 did not match any of those of representative isolates belonging to USA type scheme (USA100 – USA1100; data not shown).

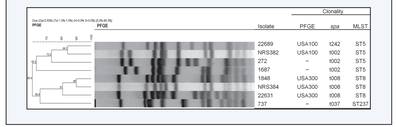


**Table 1.** Cfr-producing staphylococcal strains recovered from USA hospitals as part of the SENTRY Antimicrobial Surveillance Program (2007 – 2010).

| | | | | | Linezolid | Molecular typing | | | | Alteration in ribosomal proteins | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Organism | Isolate | State | Year | Site | MIC (µg/mL) | CC | MLST | *spa* | PFGE[a] | 23S | L3 | L4 |
| *S. aureus* | 737 | Ohio | 2007 | 004 | 8 | 8 | ST239 | t037 | – | WT | WT | WT |
| | 272 | Ohio | 2009 | 004 | 16 | 5 | ST5 | t002 | – | WT | WT | WT |
| | 1687 | Kentucky | 2009 | 027 | 16 | 5 | ST5 | t002 | – | WT | WT | WT |
| | 1848 | Maryland | 2009 | 401 | 4 | 8 | ST8 | t008 | USA300 | WT | WT | WT |
| | 22631 | Michigan | 2010 | 460 | 4 | 8 | ST8 | t008 | USA300 | WT | WT | WT |
| | 22689 | Utah | 2010 | 051 | 4 | 5 | ST5 | t242 | USA100 | WT | WT | WT |
| *S. epidermidis* | 3147 | Arizona | 2007 | 426 | >128 | 2-I | ST186 | NA | SEPI426A | C2534T | H146Q/V154L/A157R | 71_72insG |
| | 2104 | Arizona | 2008 | 426 | >128 | 2-I | ST186 | NA | SEPI426A | C2534T | H146Q/V154L/A157R | 71_72insG |
| | 2174 | Arizona | 2009 | 426 | >128 | 2-I | ST186 | NA | SEPI426A | C2534T | H146Q/V154L/A157R | 71_72insG |
| | 38449 | Arizona | 2009 | 426 | >128 | 2-I | ST186 | NA | SEPI426A | C2534T | H146Q/V154L/A157R | 71_72insG |
| | 4042 | Missouri | 2010 | 449 | 16 | 2-II-5 | ST5 | NA | NA | WT | A157R | WT |
| | 2907 | Kentucky | 2010 | 107 | 128 | 2-I | ST22 | NA | NA | WT | V154L/A157R | N158S |
| | 12676 | Ohio | 2010 | 004 | >128 | 2-I | ST22 | NA | NA | WT | H146Q/V154L/A157R | N158S/71_72insG |
| *S. capitis* | 4593 | Michigan | 2009 | 003 | 8 | NA | NA | NA | NA | WT | WT | WT |

NA, not applicable; WT, wildtype.
a. PFGE typing was performed for epidemiological purposes in isolates recovered from the same medical institution. PFGE profiles of *S. aureus* were evaluated according to the USA100-1100 scheme.

**Table 2.** Antimicrobial susceptibility profile of *cfr*-carrying staphylococcal strains recovered from USA hospitals and selected for this study.

| | | MIC (µg/mL) [susceptibility category][a] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Organism | Isolate | LZD | RET | TIA | CLI | CHL | Q/D | VIR | OXA | CIP | GEN | TET | TIG | DAP | VAN |
| *S. aureus* | 737 | 8 [R] | >8 [R] | 64 | >64 [R] | >128 [R] | 8 [R] | 16 | >2 [R] | >4 [R] | >8 [R] | >8 [R] | ≤0.03 [S] | 0.25 [S] | 0.5 [S] |
| | 272 | 16 [R] | >8 [R] | >64 | >64 [R] | >128 [R] | 2 [I] | 4 | >2 [R] | >4 [R] | ≤1 [S] | 0.5 [S] | 0.25 [S] | 0.25 [S] | 1 [S] |
| | 1848 | 4 [S] | >8 [R] | >64 | >64 [R] | >128 [R] | 2 [I] | 4 | >2 [R] | ≤0.5 [S] | ≤1 [S] | 0.25 [S] | 0.12 [S] | 0.25 [S] | 0.5 [S] |
| | 1687 | 16 [R] | >8 [R] | >64 | >64 [R] | >128 [R] | 2 [I] | 4 | >2 [R] | >4 [R] | ≤1 [S] | 0.25 [S] | 0.25 [S] | 0.5 [S] | 1 [S] |
| | 22631 | 4 [S] | >8 [R] | >64 | >64 [R] | >128 [R] | 2 [I] | 4 | 1 [S] | >4 [R] | ≤1 [S] | ≤0.12 [S] | 0.25 [S] | 0.25 [S] | 0.5 [S] |
| | 22689 | 4 [S] | >8 [R] | >64 | >64 [R] | 32 [R] | 2 [I] | 8 | >2 [R] | >4 [R] | >8 [R] | ≤0.12 [S] | 0.06 [S] | 0.5 [S] | 1 [S] |
| *S. epidermidis* | 3147 | >128 [R] | >8 [R] | >64 | >64 [R] | >128 [R] | 4 [R] | 8 | >2 [R] | >4 [R] | >8 [R] | 0.25 [S] | 0.12 [S] | 0.25 [S] | 2 [S] |
| | 2104 | >128 [R] | >8 [R] | >64 | >64 [R] | 64 [R] | 2 [I] | 8 | >2 [R] | >4 [R] | >8 [R] | 1 [S] | 0.25 [S] | 0.25 [S] | 2 [S] |
| | 2174 | >128 [R] | >8 [R] | >64 | >64 [R] | 128 [R] | 2 [I] | 8 | >2 [R] | >4 [R] | >8 [R] | 0.25 [S] | 0.12 [S] | 0.5 [S] | 2 [S] |
| | 38449 | >128 [R] | >8 [R] | >64 | >64 [R] | >128 [R] | 1 [S] | 8 | >2 [R] | >4 [R] | 8 [R] | 2 [S] | 0.12 [S] | 0.5 [S] | 2 [S] |
| | 4042 | 16 [R] | >8 [R] | >64 | >64 [R] | 32 [R] | 1 [S] | 4 | >2 [R] | >4 [R] | ≤1 [S] | ≤0.12 [S] | 0.06 [S] | 0.25 [S] | 1 [S] |
| | 2907 | 128 [R] | >8 [R] | >64 | >64 [R] | >128 [R] | 2 [I] | 8 | >2 [R] | >4 [R] | >8 [R] | ≤0.12 [S] | 0.12 [S] | 0.5 [S] | 2 [S] |
| | 12676 | >128 [R] | >8 [R] | >64 | >64 [R] | >128 [R] | 2 [I] | 16 | >2 [R] | >4 [R] | 8 [R] | 0.25 [S] | 0.12 [S] | 0.5 [S] | 2 [S] |
| *S. capitis* | 4593 | 8 [R] | >8 [R] | >64 | >64 [R] | >128 [R] | 1 [S] | 4 | ≤0.25 [S] | ≤0.5 [S] | ≤1 [S] | 0.25 [S] | 0.12 [S] | 0.5 [S] | 1 [S] |

a. MIC interpretive criteria as published by CLSI (2011), when available. Retapamulin MIC results were interpreted according to the microbiological parameters reported by Traczewski et al. (2008), whereas susceptibility breakpoint interpretations for tigecycline was based on FDA criteria for *S. aureus*. S, susceptible; I, intermediate; and R, resistant. LZD, linezolid; RET, retapamulin; TIA, tiamulin; CLI, clindamycin; CHL, chloramphenicol; Q/D; quinupristin/ dalfopristin; VIR, virginiamycin; OXA, oxacillin; CIP, ciprofloxacin; GEN, gentamicin; TET, tetracycline; TIG, tigecycline; DAP, daptomycin, VAN, vancomycin.

## CONCLUSIONS

- Cfr-producing staphylococcal strains remain rare (0.05%) among clinical isolates from USA hospitals participating in the SENTRY Antimicrobial Surveillance Program.
- An increase in the number of Cfr strains was observed in the last two years of the program (11 strains in 2009 – 2010) compared with previous years (three strains in 2007 – 2008). Clonal dissemination was observed in one medical center.
- Although all strains displayed an antimicrobial susceptibility profile compatible with Cfr production, three *S. aureus* were linezolid susceptible (MIC, 4 µg/mL). These results may hinder the detection of such strains and facilitate local strain/gene dissemination.
- Cfr-producing *S. epidermidis* were often associated with mutations in ribosomal proteins (L3 and/or L4), which may explain the higher linezolid MIC results when compared with *S. aureus* isolates.

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