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Genotypic Evaluation of a Collection of Cfr-producing Staphylococcal Clinical Isolates from the SENTRY Antimicrobial Surveillance Program

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ABSTRACT

Objectives: To evaluate the genotypic and phenotypic characteristics of Cfr-producing staphylococcal clinical isolates submitted as part of the SENTRY Antimicrobial Surveillance Program. Cfr-encoding gene is often plasmid-located and therefore this recent linezolid resistance mechanism has the potential for mobilization.

Methods: Staphylococcal strains (76,303) submitted to the SENTRY Program were tested for susceptibility by reference CLSI methods (M07-A8) and interpretations by EUCAST (2011). Isolates with elevated linezolid MIC results (≥4 mg/L) were screened for resistance mechanisms, including *cfr* and mutations in the 23S rRNA-, L3- and L4-encoding genes. *cfr*-positive strains were selected for this study and further evaluated. Clonality was assessed by PFGE, *spa* (*S. aureus* only) and MLST. Strains were identified by Vitek 2 and confirmed by 16S rRNA sequencing.

Results: Within 2006 – 2008, five *cfr*-positive strains were detected, while nine and five Cfr-producing staphylococci were detected during 2009 and 2010, respectively. Most (73.7%) *cfr* strains were identified in coagulase-negative staphylococci (CoNS) and the linezolid MIC results in *S. aureus* (4 – 16 mg/L) were usually lower than those noted in CoNS (8 - >128 mg/L). All *S. aureus* were methicillin-resistant (MRSA) and associated to internationally disseminated MRSA lineages, including USA300 (isolate 1848). Among *S. aureus*, no sequence type (ST) prevailed, whereas ST23 was the commonest ST among CoNS. PFGE results indicated the presence of CoNS strains with indistinguishable profiles in medical centers in Guadalajara (Mexico), Tempe (USA) and Rome (Italy). Overall, mutations in the 23S rRNA were not detected, except in isolates 3147 (C2534T) and 27805 (G2576T), while alterations in the L3 and L4 proteins were commonly observed, especially among CoNS.

Conclusions: Cfr-producing *S. aureus* strains usually exhibited lower linezolid MIC values when compared with CoNS. This finding may be associated with additional linezolid resistance mechanisms in CoNS, such as mutations in 23S rRNA, L3 and L4 proteins. Presence of *cfr* and 23S rRNA alterations seems to be rare. The prevalence of *cfr* strains apprears to be increasing slightly over the years in staphylococcal strains submitted to this program; however, clonal dissemination was observed in several medical sites, including the presence of a persistent clone (SEPI426A; 2007 – 2010) in a USA hospital.

INTRODUCTION

Linezolid was the first member of the oxazolidinone class to be introduced into clinical practice (2000). Emergence of resistance is usually associated with prolonged therapy and mutation(s) in the active binding site region, the peptidyl transferase center (PTC) of the 50S bacterial ribosome. The vast majority of mutations occur in the domain V of 23S ribosomal RNA (usually G2576T). Other alterations in this region, such as T2500A, G2447T and T2504A have also been observed. Furthermore, modifications in the L3 and L4 ribosomal proteins close to the PTC have been associated with decreased susceptibility to linezolid.

Resistance mechanisms related to target site modifications develop slowly due to the redundancy of rRNA genes in bacteria. In addition, these are not transferable among clinical species. Recently, however, 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 ribosomal RNA at position A2503, which affects the binding of several drugs. The objective of this study was to evaluate the molecular epidemiology of Cfr-producing staphylococcal clinical isolates submitted as part of the SENTRY Antimicrobial Surveillance Program (2006 – 2010).

MATERIALS AND METHODS

Clinical strains. A total of 76,303 staphylococcal strains were submitted as part of the SENTRY Program (2006 – 2010). *cfr*-positive strains (five *Staphylococcus aureus*, 12 *S. epidermidis,* one *S. capitis* and one *S. cohnii* 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 by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2011), when available. Retapamulin MIC results were interpreted according to the microbiological parameters reported by Traczewski et al. *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 19 of 76,303 (0.02%) staphylococcal strains carrying *cfr* were detected during a five year period of the SENTRY Program. Within 2006 – 2008, five *cfr*-positive strains were detected, while nine 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. All *S. aureus* were methicillin-resistant (MRSA), as were the coagulase-negative staphylococci (CoNS). Overall, *S. aureus* exhibited linezolid MIC results (4 – 16 mg/L), lower than CoNS (8 - >128 mg/L).
- Overall, mutations in the 23S rRNA were not detected among CoNS, except in isolate 3147 (C2534T) and clonally related strains, and 27805 (G2576T). In contrast, alterations in the L3 and L4 proteins were commonly observed in CoNS. Cfr-producing *S. aureus* demonstrated wildtype L3 and L4 ribosomal protein sequences (**Table 1**).
- All *S. aureus* strains were recovered from hospitalized patients in the United States (USA), except for one isolate from Antwerp (Belgium; **Table 2**). Moreover, these strains were associated with internationally disseminated MRSA lineages.
- No MLST type prevailed among *S. aureus*. Strains 272 and 1687 were *spa* type t002 and associated with ST5. Isolate 1687 showed a PFGE profile identical to that of NRS382 (USA100), while *S. aureus* 272 displayed a PFGE pattern other than those of the USA typing scheme. Strain 1848 was *spa* type t008, ST8 and exhibited a PFGE pattern indistinguishable from that of NRS384 (USA300).
- All *cfr*-carrying *S. epidermidis* were associated with CC2 and 50.0% of the strains belonged to cluster I, which included ST2 (one strain), 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 CoNS strains with indistinguishable profiles in medical centers in Guadalajara (site 115; Mexico), Tempe (site 426; USA) and Rome (site 086; Italy).

Table 1. Cfr-producing staphylococcal strains submitted as part of the SENTRY Antimicrobial Surveillance Program (2006 – 2010).

						Molecular typing				Alteration in ribosomal proteins		
Organism	Isolate	Country	Year	Site	Linezolid MIC (mg/L)	CC	MLST	*spa*	PFGE[a]	23S	L3	L4
S. aureus	6952	Belgium	2006	131	8	45	ST45	t740	–	WT	WT	WT
S. aureus	737	USA	2007	004	8	8	ST239	t037	–	WT	WT	WT
S. aureus	1848	USA	2009	401	4	8	ST8	t008	USA300	WT	WT	WT
S. aureus	272	USA	2009	004	16	5	ST5	t002	–	WT	WT	WT
S. aureus	1687	USA	2009	027	16	5	ST5	t002	USA100	WT	WT	WT
S. capitis	4593	USA	2009	003	8	NA	NA	NA	NA	WT	WT	WT
S. cohnii	10842	Mexico	2009	115	32	NA	NA	NA	NA	WT	S158F/D159Y	N20S/A133T/V155I
S. epidermidis	5738	Mexico	2009	115	32	2-II-89	ST23	NA	SEPI115A	WT	S158Y/D159Y	WT
S. epidermidis	12898	Mexico	2009	115	32	2-II-89	ST23	NA	SEPI115A	WT	S158F/D159Y	WT
S. epidermidis	14078	Italy	2009	086	32	2-II-89	ST23	NA	SEPI86A	WT	F147L/A157R	N158S
S. epidermidis	4303	Italy	2008	086	64	2-II-89	ST23	NA	SEPI86A	WT	F147L/A157R	N158S
S. epidermidis	3147	USA	2007	426	>128	2-I	ST186	NA	SEPI426A	C2534T	H146Q/V154L/A157R	71_72insG
S. epidermidis	2104	USA	2008	426	>128	2-I	ST186	NA	SEPI426A	C2534T	H146Q/V154L/A157R	71_72insG
S. epidermidis	2174	USA	2009	426	>128	2-I	ST186	NA	SEPI426A	C2534T	H146Q/V154L/A157R	71_72insG
S. epidermidis	38449	USA	2010	426	>128	2-I	ST186	NA	SEPI426A	C2534T	H146Q/V154L/A157R	71_72insG
S. epidermidis	4042	USA	2010	449	16	2-II-5	ST5	NA	NA	WT	A157R	WT
S. epidermidis	27805	Spain	2010	066	64	2-I	ST2	NA	NA	G2576T	WT	WT
S. epidermidis	2907	USA	2010	107	128	2-I	ST22	NA	NA	WT	V154L/A157R	N158S
S. epidermidis	12676	USA	2010	004	>128	2-I	ST22	NA	NA	WT	H146Q/V154L/A157R	N158S/71_72insG

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

Table 2. Antimicrobial susceptibility profile of *cfr*-carrying staphylococcal strains selected for this study.

		MIC (mg/L) [susceptibility category][a]													
Organism	Isolate	LZD	RET	TIA	CLI	CHL	Q/D	VIR	OXA	CIP	GEN	TET	TIG	DAP	VAN
S. aureus	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]
S. aureus	737	8 [R]	>8 [R]	64	>64 [R]	>128 [R]	8 [R]	16	>2 [R]	>4 [R]	>8 [R]	>8	≤0.03 [S]	0.25 [S]	0.5 [S]
S. aureus	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]
S. aureus	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]
S. aureus	6952	8 [R]	>8 [R]	>64	16 [R]	128 [R]	2 [I]	8	>2 [R]	>4 [R]	≤1 [S]	≤0.12 [S]	0.06 [S]	0.25 [S]	1 [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]
S. cohnii	10842	32 [R]	>8 [R]	>64	>64 [R]	32 [R]	4 [R]	16	>2 [R]	>4 [R]	>8 [R]	≤0.12 [S]	0.06 [S]	0.25 [S]	1 [S]
S. epidermidis	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]
S. epidermidis	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]
S. epidermidis	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]
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]
S. epidermidis	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]
S. epidermidis	4303	64 [R]	>8 [R]	>64	>64 [R]	128 [R]	8 [R]	64	>2 [R]	>4 [R]	>8 [R]	2 [I]	0.5 [S]	0.5 [S]	2 [S]
S. epidermidis	5738	32 [R]	>8 [R]	>64	>64 [R]	16 [R]	0.5 [S]	2	>2 [R]	>4 [R]	>8 [R]	1 [S]	0.25 [S]	0.5 [S]	2 [S]
S. epidermidis	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. epidermidis	12898	32 [R]	8 [R]	64	>64 [R]	16 [R]	2 [I]	8	>2 [R]	>4 [R]	>8 [R]	2 [I]	0.25 [S]	0.5 [S]	2 [S]
S. epidermidis	14078	32 [R]	>8 [R]	>64	>64 [R]	64 [R]	8 [R]	32	>2 [R]	>4 [R]	>8 [R]	1 [S]	0.12 [S]	0.25 [S]	1 [S]
S. epidermidis	38449	>128 [R]	>8 [R]	>64	>64 [R]	>128 [R]	1 [S]	8	>2 [R]	>4 [R]	8 [R]	2 [S]	0.25 [S]	0.5 [S]	2 [S]
S. epidermidis	27805	64 [R]	>8 [R]	>64	>64 [R]	>128 [R]	≤0.25 [S]	2	>2 [R]	>4 [R]	>8 [R]	1 [S]	0.12 [S]	0.25 [S]	2 [S]

a. MIC interpretive criteria as published by EUCAST (2011), when available. Retapamulin MIC results were interpreted according to the microbiological parameters reported by Traczewski et al. 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 staphylococci still remain rare among clinical isolates submitted as part of the SENTRY Program. An increase in the number of *cfr*-carrying strains was observed in the last two years of the program (14 strains in 2009 – 2010) compared with previous years (five strains in 2006 – 2008). However, clonal dissemination was observed in three medical sites.
- *S. aureus* clinical isolates demonstrated linezolid MIC results lower than CoNS isolates. The latter often harboured mutations in ribosomal proteins, previously associated with decreased susceptibility to linezolid.
- *S. aureus* strains carrying *cfr* were associated with different MLST types (four STs among five strains) suggesting sporadic emergence of resistance. The *S. epidermidis* population appears to be more homogeneous (five STs among seven unique strains) and all isolates belonged to the same CC2 (three clusters).
- The *S. epidermidis* population structure has not been well characterized as that of *S. aureus* and needs further studies. In addition, these *cfr* strains may represent the usual population of *S. epidermidis* circulating in the hospital environment; therefore, the molecular epidemiology of these strains needs to be further assessed.

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