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IS*21-558* Insertion Sequences Associated with *cfr* Dissemination in *Staphylococcus epidermidis* and *Staphylococcus aureus* Recovered from Two Medical Facilities in Ohio

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SENTRY ANTIMICROBIAL SURVEILLANCE

Amended Abstract

Background: During Oct/06 – May/08, 19 clonally-related *cfr*-carrying *S. epidermidis* caused a nosocomial outbreak in two centers in Northeast Ohio. Concurrently, in one of these centers, two Cfr-producing *S. aureus* were isolated. We assessed the *cfr* location and determined if this gene was mobilized via plasmid transfer or other elements.

Methods: 22 staphylococci were included: 19 *S. epidermidis* (13 hospital A; 6 hospital B), two *S. aureus* (1609 and 1626; hospital B) and the *S. aureus* Ohio *cfr* index strain from SENTRY (737 [2007]; same hospital A complex; ST-239 and *spa* t037). Strains 1609 and 1626 were subjected to PFGE, MLST and *spa* typing. Cells were embedded in agarose blocks, digested (S1) and DNA resolved by PFGE. Extracted plasmid DNA was digested and separated in agarose gels. DNA was blotted and hybridized with a *cfr* probe. Genetic context was mapped by PCR with primers targeting the *cfr* adjacencies (*istAS*, *istBS*, *ΔtnpB*) of 737.

Results: *S. aureus* 1609 and 1626 were ST-5 and t002, but clustered within different PFGE types. All *S. epidermidis* showed three plasmid bands of ca. 50-, 165- and 300-kb, while 1609 and 737 displayed bands of 50- and 480-kb. *S. aureus* 1626 had bands of 35-, 250- and 480-kb. All *S. epidermidis* and *S. aureus* 1609 and 737 exhibited hybridization signals from 50-kb bands, whereas signals from the 35- and 250-kb bands were noted in 1626. All *S. epidermidis* displayed identical plasmid restriction profiles. *S. aureus* strains showed distinct patterns, which were different from *S. epidermidis*. *S. aureus* 1609 and 737, and *S. epidermidis* had similar hybridization signal profiles, as well as adjacent *cfr* sequences (*istAS*, *istBS*, *ΔtnpB*). *S. aureus* 1626 showed a unique context and multiple *cfr* copies.

Conclusions: *cfr* was located in distinct plasmids for all *S. aureus*, which were different from that noted among *S. epidermidis*. But, similar *cfr* hybridization profiles among *S. epidermidis*, and *S. aureus* 1609 and 737 suggested related genetic context, as shown by PCR mapping. These data indicate common *cfr* origins and that IS*21-558* (*istAS* and *istBS*) may be involved in inter-species gene mobilization.

Introduction

Linezolid was the first member of the oxazolidinone class to be introduced into clinical practice (2000). Isolates resistant to linezolid remain uncommon, but emergence of resistance has been reported in patients with prolonged linezolid therapy. The vast majority of linezolid-resistant organisms detected in the nosocomial environment possess G2576T mutation(s) in the domain V of 23S ribosomal RNA. Other mutations in this same region, such as T2500A, G2447T and T2504A have also been observed. Moreover, modifications in the conserved regions of ribosomal proteins L3 and L4 have been associated with decreased susceptibility to linezolid.

Resistance mechanisms related to ribosomal mutations develop slowly due to the redundancy of rRNA genes in bacteria, and are not transferable between pathogenic species. However, *cfr* 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 drugs belonging to at least five antimicrobial classes (phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A compounds).

Although, *cfr* was initially recovered from animal sources, this gene has been lately reported in *Staphylococcus* spp. recovered from human clinical specimens in the USA, Colombia, Spain and Mexico. During Oct/06 – May/08, 19 clonally-related *cfr*-carrying *Staphylococcus epidermidis* caused a nosocomial outbreak in two medical centers in Northeast Ohio. Concurrently, two Cfr-producing *Staphylococcus aureus* were isolated in one of these centers. The main objective of this study was to assess the *cfr* location and determine the genetic elements responsible for gene mobilization among these clinical strains. In addition, a *cfr*-carrying *S. aureus* (737) recovered from one of these facilities in Ohio during the 2007 SENTRY Program was included for comparison purposes.

Methods

Clinical strains. A total of 22 staphylococci were included in this investigation. Nineteen *S. epidermidis* (13 strains from hospital A and six from hospital B), two *S. aureus* (isolates 1609 and 1626 from hospital B) and the *cfr* index strain (737; ST-239 and *spa* t037) recovered from the same hospital A complex facilities during the 2007 SENTRY Program.

Molecular typing. *S. aureus* 1609 and 1626 were subjected to pulsed-field gel electrophoresis (PFGE). Smal-digested genomic DNA was resolved in CHEF-DR II (BioRad, Richmond, California) and PFGE profiles obtained were compared to that from the index *cfr* strain 737 and representatives of USA clones using the GelCompar II software (Applied Math, Kortrijk, Belgium). 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 ≥95% were considered as genetically identical (subtype), while those with similarity coefficient between 80.0 and 94.9% were classified as genetically related (type). The *cfr*-carrying *S. aureus* 1609 and 1626 were further characterized by single (*spa*) and multilocus sequence typing (MLST). *spa* types were assigned through the Ridom web server (http://www.ridom.de/spaserver/) and MLST alleles and sequence types (ST) were identified using the MLST database (http://www.mlst.net).

Antimicrobial susceptibility testing. Susceptibility testing was performed by reference broth microdilution methods, according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (M07-A8, 2009). Minimum inhibitory concentrations (MIC) interpretations were applied as described in M100-S20-U (CLSI, 2010). Retapamulin MIC results were interpreted according to microbiological parameters reported by Traczewski et al. (2008). Tigecycline MIC values were interpreted based on the *S. aureus* breakpoint for susceptibility approved by the US Food and Drug Administration (FDA; Tygacil Product Package Insert, 2005). *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 *S. aureus* RN4220 and *S. epidermidis* ATCC 12228 using the Lasergene® software package (DNAStar; Madison, Wisconsin).

***cfr* plasmid analysis.** Whole genomic DNA was prepared in 1% agarose blocks and partially digested with S1 endonuclease. DNA fragments were resolved by PFGE using CHEF-DR II (BioRad), gel was photographed and plasmid band sizes were determined by comparison with bacteriophage λ concatemers (New England Biolabs; Ipswich, Massachusetts). Plasmid extraction was performed by using the Plasmid DNA MIDI Kit (Qiagen GmbH, Hilden, Germany). Plasmids were digested with restriction enzymes (Ear I, Nsi I and Asi I; New England Biolabs) and separated in 1% agarose gel. DNA fragments from agarose gels were transferred to a nylon membrane by Southern blot. Membranes were hybridized using a digoxigenin-labeled *cfr*-specific probe (Roche Diagnostics GmbH, Mannheim, Germany).

PCR mapping of *cfr* genetic context. The *cfr* gene in the *S. aureus* index strain 737 was previously reported to harbor an IS*21*-like (i.e. IS*21-558*) element upstream containing two overlapping reading frames, *ist*AS and *ist*BS. In addition, *ΔtnpB* was located downstream. Primers targeting *istAS*, *ΔtnpB* and *cfr* were utilized to PCR map the *cfr* genetic context. PCR products were digested with Bgl II, Dra I and Xba I (New England Biolabs), and separated in 1% agarose gel.

Results

- PFGE analysis revealed that *S. aureus* 1626, 1609 and 737 clustered within three PFGE types. PFGE pattern of strain 1626 (subtype A1) was similar to that of NRS382 (subtype A), a USA100 clone representative (similarity coefficient of 94.7%; Figure 1). *S. aureus* 1609 and index strain 737 belonged to PFGE B and C, respectively.
- Further typing analysis demonstrated that *S. aureus* strains 1626 and 1609 were ST-5 and *spa* t002 (Figure1). All *S. epidermidis* included in this investigation displayed identical PFGE profiles (data not shown).
- *S. aureus* strains exhibited linezolid MIC results between 8 and 16 μg/mL, while *S. epidermidis* showed MIC values of ≥128 μg/mL (Table 1). Elevated MIC results were noted for chloramphenicol (≥64 μg/mL), clindamycin (>64 μg/mL), virginiamycin (8 – 32 μg/mL), quinupristin/dalfopristin (2 – 8 μg/mL), retapamulin (>8 μg/mL), and tiamulin (>64 μg/mL) among staphylococcal isolates (Table 1).
- *S. aureus* demonstrated wildtype sequences for all ribosomal proteins investigated. L3 amino acid alterations (H146Q/V154L/A157R) were observed in one representative *S. epidermidis* from each hospital (A and B), as well as a L4 $_{71}G_{72}$ insertion (Table 1).
- All *S. epidermidis* showed similar plasmid band profiles (Figure 2A). Three plasmid bands of ca. 50-, 165- and 300-kb were observed among *S. epidermidis* during S1 endonuclease experiments. Hybridization signals were observed from the 50-kb plasmid bands (Figure 2B).
- *S. aureus* 1609 and index strain 737 displayed bands of ca. 50- and 480-kb, while *S. aureus* 1626 had bands of 35-, 250- and 480-kb (Figure 2A). *S. aureus* 1609 and 737 exhibited hybridization signals from plasmid bands of 50-kb, whereas signals from the 35- and 250-kb bands were noted in 1626 (Figure 2B).
- Representative *S. epidermidis* from each hospital (A and B) displayed similar plasmid restriction profiles, while selected *S. aureus* strains showed distinct patterns. Plasmid restriction profiles from *S. aureus* were different from that noted among *S. epidermidis* clinical isolates (Figure 3A).
- *S. aureus* 1609 and 737, and *S. epidermidis* strains had similar hybridization signal profiles (Figure 3B). In addition, PCR mapping suggested that these isolates possessed equivalent sequences upstream of *cfr* (*istAS* and *istBS*; Figure 4B), as well as downstream (*ΔtnpB*; data not shown).
- PCR mapping using primers targeting the *cfr* adjacencies of index strain 737 failed to generate amplicons in the *S. aureus* 1626, indicating a distinct genetic context (Figure 4B).

Table 1. Antimicrobial susceptibility profile and molecular findings for one representative *S. epidermidis* each from hospital A (1243) and B (1519), and *S. aureus* from hospital B (1609 and 1626).

	MIC (μg/mL) [susceptibility category][a]			
	S. epidermidis		*S. aureus*	
Parameters	1243	1519	1609	1626
Antimicrobial agent				
Linezolid	128 [R]	>128 [R]	16 [R]	8 [R]
Chloramphenicol	>128 [R]	>128 [R]	>128 [R]	64 [R]
Clindamycin	>64 [R]	>64 [R]	>64 [R]	>64 [R]
Virginiamycin	8	16	32	16
Q/D	2 [I]	2 [I]	8 [R]	4 [R]
Retapamulin	>8 [R]	>8 [R]	>8 [R]	>8 [R]
Tiamulin	>64	>64	>64	>64
Tigecycline	0.25 [S]	0.12 [S]	0.25 [S]	0.12 [S]
Tetracycline	0.25 [S]	0.5 [S]	0.5 [S]	0.25 [S]
Vancomycin	2 [S]	2 [S]	1 [S]	1 [S]
Daptomycin	0.25 [S]	0.25 [S]	0.25 [S]	0.25 [S]
Oxacillin	>2 [R]	>2 [R]	>2 [R]	>2 [R]
Ciprofloxacin	>4 [R]	>4 [R]	>4 [R]	>4 [R]
Erythromycin	4 [I]	4 [I]	>4 [R]	>4 [R]
Gentamicin	8 [I]	8 [I]	>8 [R]	≤1 [S]
SXT	4 [R]	4 [R]	≤0.5 [S]	≤0.5 [S]
Molecular findings				
cfr	Positive	Positive	Positive	Positive
23S rRNA	WT	WT	WT	WT
L3	H146Q/V154L/A157R	H146Q/V154L/A157R	WT	WT
L4	$_{71}G_{72}$	$_{71}G_{72}$	WT	WT

Q/D, quinupristin/dalfopristin; SXT, trimethoprim/sulfamethoxazole; S, susceptible; I, intermediate; R, resistant; WT, wildtype.

a. MIC interpretive criteria as published by CLSI M100-S20-U, when available. Retapamulin MIC results were interpreted according to the microbiological breakpoints proposed by Traczewski et al. (2008; i.e ≤0.5 μg/mL for susceptibility; 1 μg/mL for intermediate; and ≥2 μg/mL for resistant). Tigecycline breakpoint for susceptibility was that for *S. aureus* (≤0.5 μg/mL) approved by the US FDA.

Figure 1. PFGE profiles of Cfr-producing *S. aureus* 1626 and 1609 (hospital B) compared with 737 (hospital A complex facilities) and NRS382 (USA100).

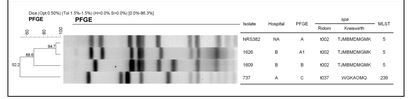


Figure 2. A. S1 partial-digested genomic DNA of *cfr*-carrying *S. epidermidis* (lanes 1 through 19) and *S. aureus* strains 1609 and 1626 (lanes 20 and 21, respectively; from hospital B). Lane 22 represents the *cfr*-carrying *S. aureus* index strain 737 (from hospital A complex facilities). λ represents Lambda ladder PFGE marker also used as negative control (New England Biolabs). **B.** Hybridization signals (horizontal arrow) obtained with a *cfr*-specific probe.

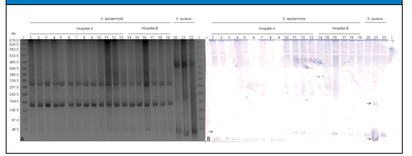


Figure 3. A. Lanes 1 and 2 represent plasmid DNA restriction profile analysis of representative *S. epidermidis* strains from each hospital (A and B). Lanes 3, 4 and 5 correspond to *S. aureus* 1609, 1626 (hospital B) and 737 (hospital A complex facilities), respectively. L represents 1 kb DNA Ladder (New England Biolabs). **B.** Hybridization signals (horizontal arrow) obtained with a *cfr*-specific probe. Hybridization signals for one of the *S. epidermidis* strains are not visible.

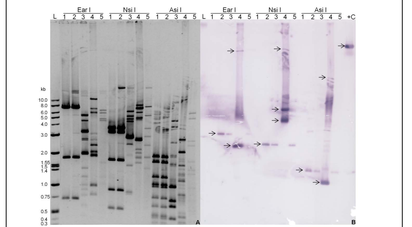
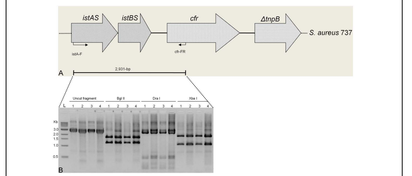


Figure 4. A. Schematic representation of surrounding *cfr* DNA sequences detected in *S. aureus* index strain 737. **B.** Analysis of *cfr* genetic context among investigated strains was performed by PCR mapping using primers istA-F and cfr-FR. Band patterns of uncut, and Bgl II-, Dra I- and Xba I-digested amplicons. L represents 1 kb DNA Ladder (New England Biolabs). Lanes 1, 2, 3 and 4 correspond to representative *S. epidermidis* strains from each hospital (A and B), *S. aureus* 1609 (hospital B) and 737 (hospital A complex facilities), respectively. *S. aureus* 1626 (hospital B) failed to produce amplicons.



Conclusions

- *S. aureus* strain 1626 showed a PFGE profile similar to that of a USA100 clone representative, which is the predominant clone responsible for healthcare-associated infections in the United States
- *S. aureus* 1626 and 1609 clustered within different PFGE types; however, they showed the same MLST profile and *spa* type as USA100. These findings suggest these isolates derived from a common ancestor.
- Representative *S. epidermidis* strains from hospital A and B exhibited linezolid MIC values higher than *S. aureus* 1626 and 1609. These differences in MIC results could be explained by additional L3 and L4 mutations observed in *S. epidermidis*.
- *cfr* was plasmid located in all strains included in this investigation. Similar *cfr*-carrying plasmids were noted among all *S. epidermidis*, while *S. aureus* strains 1626, 1609 and 737 possessed distinct *cfr* plasmids, which were different from that noted among *S. epidermidis*.
- Similar *cfr* hybridization profiles among *S. epidermidis*, and *S. aureus* 1609 and 737 strains suggest related genetic context. This finding was in agreement with PCR mapping results demonstrating similar surrounding *cfr* DNA sequences, which included *istAS* and *istBS* (IS*21-558*) upstream of *cfr* gene.
- IS*21-558* has been previously suggested as the genetic element responsible for *cfr* mobilization. The results obtained in this study strengthen the association between IS*21-558* and *cfr*.
- Notably, *S. aureus* strain 1626 showed a different genetic context and multiple *cfr* copies. The latter has not been previously reported.

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