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Low CEM-102 (Fusidic Acid) Resistance Rates and High Prevalence of Acquired Genes among *Staphylococcus* spp. from North America and Australia

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Amended Abstract

Background: Target mutations have been considered the primary *Staphylococcus* spp. fusidic acid resistance (R) mechanism; however, acquired fusidic acid genes have been recently shown to have a role on fusidic acid R. We evaluated fusidic acid R mechanisms in *Staphylococcus* spp. collected from the USA, Canada and Australia (2007-2008).

Methods: 4,167 *S. aureus* and 790 coagulase-negative *Staphylococcus* spp. (CoNS not *S. saprophyticus*) were consecutively collected from North American and Australian hospitals. Strains displaying a fusidic acid MIC at ≥2 µg/ml were tested by multiplex PCR for *fusB*, *fusC* and *fusD*. *fusA* was sequenced for all multiplex negative *S. aureus*.

Results: *S. aureus* fusidic acid R rates were very low in the USA (0.3%) being higher in Canada (6.0%) and Australia (7.0%). CoNS fusidic acid R was considerably elevated (7.2-20.0%; highest in Canada). All 52 (41 CoNS) USA strains showed low levels of fusidic acid R (MIC, ≤64 µg/ml). 7 of 11 USA *S. aureus* carried *fusC* and 39 of 41 CoNS carried *fusB* or *fusC*. Many fusidic acid resistant strains were from New York (18/52). 3 (27.3%) strains did not carry acquired fusidic acid R genes and *fusA* mutations were detected in only one strain (M453I). In Canada, *fusB* and *fusC* were similarly found among *S. aureus* and CoNS and low level of fusidic acid R was observed. *fusA* mutations were detected in one Canadian *S. aureus* (H457Q). *Staphylococcus* spp. strains from Australia showed low R levels (MIC at ≥32 µg/ml) and *S. aureus* were predominantly *fusC*-positive.

Conclusions: Low levels of fusidic acid R were observed in these three countries, two with distinct patterns of fusidic acid usage. Moreover, acquired fusidic acid genes were most prevalent among fusidic acid resistant strains (>90%) with few *fusA* mutations observed. CEM-102 would be a valuable treatment for *Staphylococcus* spp. infections particularly in the USA.

Locations (% of FA-R SA/CoNS)	Strains at MIC (µg/ml)			% of (SA/CoNS)		
	≤8	16	≥32	*fusB*	*fusC*	multiplex negative
Australia (7.0/10.8)	7/4	-/2	-/5	-/54.5	85.7/45.5	-/-
Canada (6.0/20.0)	5/2	-/3	1/5	50.0/70.0	33.3/30.0	16.6/-
USA (0.3/7.2)	9/4	2/26	-/11	9.1/70.7	63.6/24.4	27.3/-

Introduction

Fusidic acid interacts with elongation factor G (EF-G), preventing its release from the ribosome and thereby inhibiting bacterial protein synthesis. Resistance to fusidic acid was considered to be primarily caused by spontaneous mutations on the EF-G-encoding gene, *fusA*, which recently was experimentally documented to elevate *Staphylococcus aureus* MIC values.

Acquired fusidic acid resistance mechanisms have also been described, and *fusB* and *fusC* were characterized in various clinical strains. These genes can be chromosomal- or plasmid-mediated and *fusB* was reported as being carried in pUB101, a ubiquitous plasmid. FusB was shown to protect EF-G from binding with fusidic acid molecules and strains harboring the gene encoding this protein were associated with outbreaks in Scandinavian countries. *fusD* is responsible for intrinsic fusidic acid resistance among *Staphylococcus saprophyticus.*

Alterations in the L6 portion of *rplF* were recently observed to encode fusidic acid non-susceptibility among small colony-variants (SCV) of *S. aureus*, indicating that fusidic acid could have a secondary site of action in this ribosomal region, named *fusE*.

In this study, we analyzed the prevalence of fusidic acid elevated MIC results among large collections of *Staphylococcus* spp. from countries with different usage patterns of fusidic acid: the United States (USA), Canada and Australia. Fusidic acid specific resistance mechanisms, including acquired genes and mutations on *fusA* and *fusE* were evaluated in the 86 strains showing elevated MIC values to this established compound.

Materials and Methods

Bacterial strains. *Staphylococcus* spp. strains from established surveillance initiatives were included in this study and data from those were used to generate the prevalence of fusidic acid resistance. A total of 4,605 staphylococcal strains collected from 26 USA hospitals in 2008 as part of the SENTRY Antimicrobial Surveillance Program were analyzed. Additionally, among 277 *Staphylococcus* spp. strains collected in 2007-2008 from two Canadian hospitals, 150 strains were screened by disk diffusion for detection of fusidic acid resistance. These strains were submitted to a surveillance program that evaluated consecutively collected Gram-positive strains recovered from various infection sites. Strains from Australia were collected from patients hospitalized in the Women's and Children's Hospital during 2008. These strains were selected according to fusidic acid disk diffusion results among 202 staphylococcal infection isolates.

Only one isolate per patient from documented infections was included in these surveys. Species identification was confirmed by standard biochemical tests, the Vitek System (bioMerieux, Hazelwood, MO) or 16S rRNA sequencing, when necessary.

Antimicrobial susceptibility testing. Initial screening of fusidic acid resistance was performed by disk diffusion for strains from Canada and Australia. All strains from USA were tested by broth microdilution method. Strains displaying disk zone diameters ≤17 mm or MIC results at ≥2 µg/ml were tested by broth microdilution with an extended dilution range of fusidic acid (0.5-1024 µg/ml) and comparator agents as described by the Clinical and Laboratory Standards Institute (CLSI). Categorical interpretations for all antimicrobials were those found in M100-S19 and quality control (QC) was performed using *Escherichia coli* ATCC 25922, *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212. All QC results were within specified ranges as published in CLSI documents.

Detection of fusidic acid acquired resistance. All strains displaying fusidic acid MIC at ≥2 µg/ml were tested for the presence of *fusB*, *fusC* and *fusD* in a multiplex PCR approach. The reaction included one set of primers for each of the fusidic acid resistance encoding genes (Table 1) and internal control primers (16S rRNA) to exclude extraction and/or amplification failures. Bacterial DNA was prepared using DNAzol Direct (Molecular Research Center, Ohio, USA) or QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. Reactions used Multiplex PCR kit (Qiagen), 0.5 µM of each primer for detection of fusidic acid resistance and 0.4 µM for internal control primers. Cycling conditions were as previously described. Amplification products of 496, 128 and 525 bp were expected for *fusB*, *fusC* and *fusD*, respectively, which were resolved on 1.5% agarose gel electrophoresis. Detection of *fusD* was included in this reaction with two purposes: (i) to detect *S. saprophyticus* strains that were incorrectly identified and (ii) to detect possible mobilization of *fusD* to other staphylococcal species. At least one amplicon of each type was sequenced and used as a control for the following experiments.

Mutations on *fusA* and *fusE*. Constitutive genes *fusA* and *fusE* were amplified and sequenced using Extensor Hi-fidelity Master Mix (ABGene, Sussex, UK) and custom and previously described oligonucleotides (Table 1). Sequencing was performed in five and two reactions, respectively. The nucleotide sequences and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI) and compared with sequences available through the internet using BLAST (http://www.ncbi.nlm.nih.gov/blast/).

Results

- Among 4,957 staphylococcal strains analyzed, 86 (1.7%) showed MIC results at ≥2 µg/ml for fusidic acid. Strains were collected from blood cultures (75.4%), skin and skin structure infections (11.6%), respiratory tract infections (5.8%) and other sources (7.2%).
- Overall, strains had low-level fusidic acid resistance (all but one strain with MIC values at ≤64 µg/ml; Table 2).
- Oxacillin resistance was detected in 33.3 and 85.1% of *S. aureus* and CoNS that were fusidic acid non-susceptible, respectively (data not shown).
- Of the fusidic acid resistant staphylococci, acquired genes were present in 79 of 86 (91.8%) strains: *fusB* was detected in 46 (53.5%) strains and 33 (38.8%) strains carried *fusC* (Table 2).
- *fusB* was more prevalent among the CoNS (65.0% of resistant CoNS), whereas *fusC* was similarly detected in both staphylococcal groups (29.0% of resistant CoNS and 32.6% of resistant *S. aureus*; Table 2).
- Only one *S. aureus* from Canada and one from the USA showed mutations on *fusA*, generating amino acid substitutions of H457Q and M453I, respectively (Table 3). Mutations on *fusE* were not detected.
- In the USA, fusidic acid-resistant strains were primarily observed among CoNS (41 of 52 strains); 18 were from New York, including 12 from the same hospital (Table 3).
- Canadian strains also showed low fusidic acid MIC values and *fusB* and *fusC* were detected in most of the strains from both monitored staphylococcal groups (Table 3). Only one *S. aureus* was negative for acquired resistance genes.
- Resistant *S. aureus* from Australia were predominantly positive for *fusC* (no *fusB*-carrying strain detected) and CoNS carried *fusB* or *fusC* (6 and 5 strains, respectively; Tables 2 and 3).

Table 1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')	Reference
Multiplex PCR		
fusB-1F	TCA TAT AGA TGA CGA TAT TG	This study
fusB-1R	ACA ATG AAT GCT ATC TCG AC	This study
fusC-1F	GAT ATT GAT ATC TCG GAC TT	This study
fusC-1R	AGT TGA CTT GAT GAA GGT AT	This study
fusD-1F	TGC TTA TAA TTC GGT CAA CG	This study
fusD-1R	TGG TTA CAT AAT GTG CTA TC	This study
16S-8F	AGA GTT TGA TCC TGG CTC AG	Mendes et a.
16S-1493R	ACG GCT ACC TTG TTA CGA CTT	Mendes et al.
Amplifications and sequencing		
fusA-F2	CTC GTA AYA TCG GTA TCA TG	This study
fusA-R2	GCA TAG TGA TCG AAG TAC	This study
fusA_seq1	TAA GGG TCA GTC ATAT ACT TT	McLaws et al.
fusA_seq2	TTC AAA AAC AAA GGT GTT CA	McLaws et al.
fusA_seq3	ATG TAT TCA CGA GGA AC	McLaws et al.
fusE(rplF)-1F	CCT AGT GAC GTA ACA GTA AC	This study
fusE(rplF)-1R	CGG CGW ACR TAT TCA CCT TG	This study
fusC-2F	GTA CAA ACG ATA TGA ATT CC	This study
fusC-2R	ATC ATC TAG GTT CTG ATT AC	This study

Table 3. Summary of the prevalence of the fusidic acid resistance mechanisms.

Location (no. overall SA/CoNS)	Strains tested (no. FA[b] ≥ 2 µg/ml SA/CoNS)	*S. aureus*		CoNS		Strains negative by multiplex	Mutations (no. tested)	
		fusB	*fusC*	*fusB*	*fusC*		*fusA*	*fusE*
Australia (100/102)	18 (7/11)	-	6	6	5	1	-	-
Canada (100/50)[c]	16 (6/10)	3	2	7	3	1	1 H457Q (2)	0 (1)
USA (3967/638)	52 (12/46)	1	7	29	10	5	1 M453I (5)	0 (5)
Arkansas	2	-	1	1	-	-	-	-
Hawaii	3	-	3	-	-	-	-	-
Iowa	2	-	2	-	-	-	-	-
Kentucky	5	-	-	4	-	1	1 M453I (1)	0 (1)
Massachusetts	4	-	-	4	-	-	-	-
Michigan	4	-	1	-	1	2	0 (2)	0 (2)
Nebraska	4	-	-	1	2	1	0 (1)	0 (1)
New Jersey	5	-	-	5	-	-	-	-
New York	18	-	-	12	6	-	-	-
Ohio	1	1	-	-	-	-	-	-
Texas	1	-	-	1	-	-	-	-
Virginia	3	-	-	1	1	1	0 (1)	0 (1)

a. SA= *S. aureus*.
b. FA= Fusidic Acid.
c. Strains collected in 2007-2008, two medical sites.

Table 2. Fusidic acid (FA) MIC frequency distributions when tested against 86 *Staphylococcus* spp. strains and the occurrences of acquired resistance genes among these tested strains.

Organism group (no. tested)	Overall prevalence of FA MIC at ≥ 2 µg/ml	Number (cumulative %) of strains inhibited at FA MIC (µg/ml):										Acquired resistance genes (% of occurrence)	
		2	4	8	16	32	64	128	256	512	≥1024	*fusB*	*fusC*
S. aureus													
USA (11)	0.3%	-	1 (9.1)	8 (81.8)	2 (100.0)	-	-	-	-	-	-	1 (9.1)	7 (63.6)
Canada (6)	6.0%	-	1 (16.7)	4 (83.3)	-	-	-	-	1 (100.0)	-	-	3 (50.0)	2 (33.3)
Australia (7)	7.0%	1 (14.3)	-	6 (100.0)	-	-	-	-	-	-	-	-	6 (85.7)
CoNS													
USA (41)	7.2%	1 (2.4)	1 (4.9)	2 (9.8)	26 (73.2)	10 (97.6)	1 (100.0)	-	-	-	-	29 (70.7)	10 (24.4)
Canada (10)	20.0%	-	-	2 (20.0)	3 (50.0)	4 (90.0)	1 (100.0)	-	-	-	-	3 (70.0)	3 (30.0)
Australia (11)	10.8%	-	-	4 (36.3)	2 (54.5)	5 (100.0)	-	-	-	-	-	6 (54.5)	5 (45.5)

Conclusions

- Of the 86 strains (1.7%) from the USA, Canada and Australia that were non-susceptible to fusidic acid (MIC ≥2 µg/ml), acquired resistance genes, *fusB* and *fusC*, were very prevalent (91.8%).
- Our data demonstrated that these genes are not restricted to certain geographic areas and that their distribution is wider than previously appreciated and not directly related to clinical use, since fusidic acid is not used in the USA.
- Mutations on the primary (*fusA*) and secondary putative (*fusE*) fusidic acid binding sites were rare. Furthermore, *fusE* mutations seem to be uncommon among non-SCV staphylococci.
- Fusidic acid continues to provide a potentially useful treatment option for infections caused by multi-drug resistant staphylococci, including methicillin-resistant *S. aureus* (MRSA).

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