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JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
319.665.3370, fax 319.665.3371
rodrigo-mendes@jmilabs.com

Detection of Plasmid-encoded CFR in Clinical Isolates of *Enterococcus faecalis* from Panamá City: Report from the SENTRY Antimicrobial Surveillance Programme

L. M. DESHPANDE[1], S. VEGA[2], J. E. ROSS[1], R. N. JONES[1], R. E. MENDES[1]
[1]JMI Laboratories, North Liberty, Iowa, USA; [2]Social Security Hospital, Panamá City, Panamá

SENTRY ANTIMICROBIAL SURVEILLANCE

ABSTRACT

Objectives: To report the detection of two clonally-related Cfr-producing *E. faecalis* strains from Panamá, Central America. Linezolid has been in clinical use for a decade and surveillance studies have reported low and stable resistance rates among Gram-positive pathogens. Cfr alters the 23S rRNA (A2503) conferring a multidrug-resistance phenotype. Most commonly detected in *Staphylococcus* spp., *cfr* was recently found in an *E. faecalis* strain (MLST16; Clonal Clomplex [CC]16) of human origin from Thailand (2010).

Methods: *E. faecalis* strains (32789X and 32791X) with linezolid MIC values of 4 mg/L were detected during the SENTRY Programme (2011) and selected for further studies. Susceptibility testing was performed by CLSI methods. Strains were screened for *cfr* and mutations in 23S rRNA, L3 and L4. Location of *cfr* was determined by S1 digestion, followed by PFGE, Southern blot and hybridization. Plasmid conjugation was attempted using EF 32789X and OG1RF as donor and recipient strains, respectively. Strains were subjected to PFGE and multilocus sequence typing (MLST). Clonal complexes (CCs) were assigned based on MLST.

Results: *E. faecalis* 32789X was isolated (Aug, 2011) from a 41 year-old male, while 32891X was recovered (Aug, 2011) from a 58 year-old male patient, both associated with nosocomial infections. Subjects had previous hospital admissions (12-21 days), when clindamycin was prescribed. Subjects also received clindamycin prior to the culture of *E. faecalis* and both patients survived. *E. faecalis* strains were susceptible to ampicillin (MIC, 1 mg/L), but showed elevated MIC values to clindamycin (>64 mg/L), chloramphenicol (16 mg/L), tiamulin (>64 mg/L), quinupristin/dalfopristin (>16 mg/L) and tetracycline (MIC, >16 mg/L). Lower MIC results were obtained for tigecycline (0.06 mg/L), daptomycin (0.5-1 mg/L) and vancomycin (2 mg/L). PCR assays were positive for *cfr*, and wildtype sequences were noted for 23S rRNA, L3 and L4. *E. faecalis* strains had indistinguishable PFGE patterns and belonged to ST103 (CC388). *cfr* was plasmid-located (ca. 97-kb), but conjugation experiment attempts did not yield transconjugant strains.

Conclusion: This is the first report of *cfr*-carrying *E. faecalis* in the Americas and emergence seems associated to clindamycin use. *E. faecalis* strains clustered within CC338 and related STs were associated with nosocomial strains recovered in France, Portugal and the USA. These findings warrant further monitoring of *cfr*-carrying Gram-positive isolates.

INTRODUCTION

Linezolid has been widely prescribed to treat serious infections caused by multidrug-resistant (MDR) Gram-positive pathogens since its clinical introduction as the first oxazolidinone in 2000. Linezolid is currently approved by the United States Food and Drug Administration (USA-FDA) for the treatment of complicated and uncomplicated skin and skin structure infections, and nosocomial and community-acquired pneumonia caused by susceptible organisms. Linezolid also has an FDA indication for the treatment of vancomycin-resistant *Enterococcus faecium* (VRE) infections (including bacteremia).

This oxazolidinone alters protein synthesis by interacting with the 50S ribosomal subunit, and recent data suggests that this drug binds to the A site of the peptidyl-transferase center (PTC) of bacterial ribosome interfering with the positioning of aminoacyl-tRNA, resulting in protein synthesis inhibition. The mechanisms responsible for linezolid resistance have been mostly comprised of mutations in the 23S rRNA, but alterations in the ribosomal proteins L3 and L4 have also been associated with decreased susceptibility. Moreover, *cfr* encodes a methyltransferase that catalyzes the post-transcriptional methylation of nucleotide A2503 in the 23S rRNA causing decreased susceptibility to phenicol, lincosamide, oxazolidinone, pleuromutilin, and streptogramin A ($PhLOPS_A$) compounds.

Up-to-date, there has been only one report in the scientific literature characterizing a human clinical case of linezolid-resistant *E. faecalis* carrying a plasmid-located *cfr.* This clinical isolate was recovered from a skin specimen of a 72 year-old diabetic woman in Bangkok, Thailand (July, 2010), who presented with multiple skin abscesses due to *Mycobacterium abscessus* and pulmonary tuberculosis. The patient received multiple courses of antimicrobial therapy, including linezolid for at least three months prior to the isolation of the linezolid-resistant organism. This study reports the detection and characterization of two clonally-related Cfr-producing *E. faecalis* strains collected from hospitalized patients in Panamá, Central America.

MATERIALS AND METHODS

Bacterial strains. *E. faecalis* clinical isolates 32789X and 32791X were submitted to a central monitoring laboratory (JMI Laboratories, North Liberty, Iowa), as part of the SENTRY Antimicrobial Surveillance Programme (2011), according to defined protocols. These isolates exhibited elevated MIC results for linezolid (4 mg/L) and were selected for further studies. Species identification was performed by the Vitek® 2 System (bioMérieux; Hazelwood, Missouri).

Antimicrobial susceptibility testing. Susceptibility testing was performed by the local site using the automated Vitek® 2 System (bioMérieux) for identification and antibiotic susceptibility testing of Gram-positive cocci, according to the manufacturer's instructions. Reference broth microdilution methods was conducted by the monitoring laboratory according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (M07-A9, 2012). Validation of the MIC values was performed by concurrent testing of CLSI-recommended quality control reference strains (*Staphylococcus aureus* ATCC 29213 and *E. faecalis* ATCC 29212). MIC interpretations were based on the CLSI M100-S23 (2013) and European Committee on Antimicrobial Susceptibility Testing (EUCAST; 2013) breakpoint criteria, as available. In addition, the inoculum density was monitored by colony counts to assure an adequate number of cells for each testing event.

Screening for linezolid resistance mechanisms. Isolates were screened for the presence of *cfr* and mutations in the 23S rRNA and ribosomal proteins (L3 and L4) by PCR and sequencing. Amplicons were sequenced on both strands. Ribosomal proteins obtained were compared to those from wildtype linezolid-susceptible *E. faecalis* ATCC 29212 using the Lasergene® software package (DNAStar; Madison, Wisconsin).

Plasmid analysis. Whole genomic DNA was prepared in 1% agarose blocks and partially digested with S1 endonuclease. DNA fragments were resolved by pulsed-field gel electrophoresis (PFGE) using CHEF-DR II (BioRad, Richmond, California) and transferred to a nylon membrane. Membrane was hybridized using a digoxigenin-labeled *cfr*-specific probe (Roche Diagnostics GmbH, Mannheim, Germany).

Transfer of *cfr*-carrying plasmid. Plasmid conjugation was attempted by filter matting using *E. faecalis* 32789X and OG1RF as donor and recipient strains, respectively. Selection of transconjugants was performed using chloramphenicol (20 mg/L).

Molecular typing. Isolates were subjected to PFGE and profiles obtained were compared visually and by the GelCompar II software (Applied Math, Kortrijk, Belgium). As the *E. faecalis* isolates demonstrated undistinguishable PFGE patterns (**Figure 1**), one representative strain was selected for multilocus sequence typing (MLST) using the established methodologies for *E. faecalis* (http://efaecalis.mlst.net).

RESULTS

- *E. faecalis* 32789X was isolated (Aug, 2011) from a 41 year-old male, while isolate 32891X was recovered (Aug, 2011) from a 58 year-old male patient, both associated with nosocomial infections.
- Both subjects had previous hospital admissions, which varied between 12 and 21 hospital-days, when clindamycin was prescribed. Subjects also received clindamycin prior to the culture of *E. faecalis* and both patients survived.
- *E. faecalis* isolates were categorized as susceptible to linezolid when tested for susceptibility by the Vitek® 2 System; whereas the MIC result obtained by frozen-form broth microdilution panels (MIC, 4 mg/L for both strains; **Table 1**) was categorized as susceptible and intermediate based on EUCAST and CLSI criteria, respectively.
- The *E. faecalis* exhibited low MIC values for ampicillin (MIC, 1 mg/L), tigecycline (0.06 mg/L), daptomycin (0.5-1 mg/L) and vancomycin (2 mg/L), while elevated MIC results were observed for quinupristin/dalfopristin (>16 mg/L), virginiamycin (32 mg/L) chloramphenicol (16 mg/L), tiamulin (>64 mg/L), and tetracycline (MIC, >16 mg/L; **Table 1**).
- PCR assays yielded positive results for *cfr*, and wildtype sequences were noted for 23S rRNA, L3 and L4 (**Table 1**). *E. faecalis* strains had indistinguishable PFGE patterns and belonged to ST103, clonal complex 338 (CC; **Figure 1** and **Table 1**).
- *cfr* was located in a large plasmid (ca. 97-kb), but conjugation experiment attempts did not yield any transconjugant strains (data not shown).

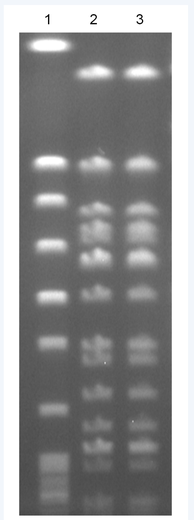
Table 1. Antimicrobial susceptibility profiles and molecular findings for *cfr*-carrying *E. faecalis* clinical isolates included in this study.

Parameters	MIC (mg/L) [susceptibility category; CLSI/EUCAST][a]	
	E. faecalis (32789X)	*E. faecalis* (32791X)
Antimicrobial agent		
Linezolid	4 [I/S]	4 [I/S]
Ampicillin	2 [S/S]	1 [S/S]
Chloramphenicol	16 [R/-[b]]	16 [R/-]
Clindamycin	>64 [-/-]	>64 [-/-]
Virginiamycin	32 [-/-]	32 [-/-]
Quinupristin/dalfopristin	>16 [R/R]	>16 [R/R]
Tiamulin	>64 [-/-]	>64 [-/-]
Tigecycline	0.06 [-/S]	0.06 [-/S]
Tetracycline	>16 [-/-]	>16 [-/-]
Doxycycline	8 [I/-]	8 [I/-]
Vancomycin	2 [S/S]	2 [S/S]
Daptomycin	1 [S/-]	0.5 [S/-]
Molecular findings		
cfr	positive	positive
23S rRNA	WT	WT
L3	WT	WT
L4	WT	WT
Epidemiological findings		
PFGE	EF346A	EF346A
MLST	103	103
Clonal complex	338	338

S, susceptible; I, intermediate; R, resistant; WT, wildtype; PFGE, pulsed-field gel electrophoresis.
a. MIC interpretive criteria as published by CLSI M100-S23 (2013) and EUCAST (2013), as available.
b. Not available.

Figure 1.

PFGE profiles of *S. aureus* NCTC 8325 (ladder; lane 1) and *cfr*-carrying *E. faecalis* 32789X (lane 2) and 32791X (lane 3).



CONCLUSIONS

- This is the first report of human clinical isolates of *E. faecalis* carrying the *cfr*-gene in the Americas and the emergence of these clinical cases of linezolid-resistant strains seem to be associated with previous therapeutic courses of clindamycin.
- Both strains demonstrated modestly elevated MIC results for linezolid (4 mg/L) and were considered as susceptible by the Vitek® 2 System and when applying the EUCAST criteria.
- In addition, although the results suggested that the *cfr* gene was located in a non-transferable plasmid DNA, these findings warrant monitoring for resistance, including Cfr-producing Gram-positive isolates.

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