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Next Generation Sequencing for Genomic Analysis of Cfr(B)-producing *Enterococcus faecium* Causing Infections in a New Orleans Medical Center

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Abstract

Background: CC17 is a major group of genetic lineage of *E. faecium* that has spread worldwide and is associated with hospital outbreaks. We previously reported *cfr*(B) among two VRE linezolid-resistant clinical *E. faecium* index isolates (18203 and 18961) belonging to CC17 from a medical center in New Orleans. In the last year, four additional isolates (three in 2015 and one in 2016) harboring *cfr*(B) were isolated in this hospital system. This study presents the characterization of these isolates in comparison to the index strains using next generation sequencing (NGS).

Methods: *E. faecium* isolates were tested for susceptibility by reference methods (M07-A10) and categorized according to CLSI (M100-S26). Screening for oxazolidinone resistance mechanisms including *cfr*, *optr*A and mutations in 23S rRNA, L3 and L4 proteins was performed by PCR and sequencing techniques. Isolates were subjected to NGS and epidemiological and *cfr*(B) genetic context information extracted.

Results: Four additional VRE and linezolid-resistant isolates were recovered (three from blood and one from bone culture; May 2015-Jan. 2016) from immunocompromised/suppressed patients with severe underlying diseases. Index isolates and one of the four additional *E. faecium* were recovered from patients with previous linezolid treatment. All isolates (these four plus two previously reported) were multidrug resistant, and tedizolid MIC values (1 – 2 µg/mL) were eight-fold lower than linezolid (8 - 16 µg/mL). G2576T in 23S rRNA was present along with *cfr*(B) in all *E. faecium*. L3 and L4 proteins showed wildtype sequences, and *optr*A was not detected. The two index isolates belonged to ST794 (CC17), while the additional four *E. faecium* were categorized as ST794 or a close variant, ST78. *cfr*(B) was located on a Tn*6218* structure and embedded in chromosomal DNA in all isolates.

Conclusion: NGS and analysis demonstrate that *E. faecium* isolates originated from a common ancestor; however, alterations in conserved MLST housekeeping gene suggest distant temporal relationships indicating prolonged persistency within the Ochsner system. Both G2576T and *cfr*(B) were chromosomally located; therefore, infection control measures should be effective in minimizing the spread of these MDR *E. faecium* isolates.

Introduction

Enterococcus faecalis and *Enterococcus faecium* have become established as human nosocomial pathogens in the last decades, emerging as the second most frequently reported cause of surgical wound and nosocomial urinary tract infections and the third most frequently reported cause of bacteremia, respectively. Clonal complex 17 (CC17), a major group of genetic lineage of *E. faecium,* has spread worldwide and has been associated with hospital outbreaks. This clonal complex is characterized by multilocus sequence typing (MLST), resistance to ampicillin, and quinolones and presence of a putative pathogenicity island, including the *esp* (enterococcal surface protein) gene in a majority of the isolates.

Linezolid is a key antimicrobial agent used to treat pneumonia and complicated and uncomplicated skin and skin structure infections (SSSIs) caused by Gram-positive pathogens, including vancomycin-resistant *E. faecium* (VRE). Linezolid resistance can occur after prolonged administration. Local investigations have reported sporadic outbreaks and dissemination of linezolid-dependent isolates. Alterations in linezolid binding sites (23S rRNA and L3 and L4 ribosomal proteins) remain the most common mechanisms of oxazolidinone resistance among staphylococci and enterococci.

A transferrable gene, *cfr,* has emerged as a mechanism of linezolid resistance. This gene encodes a methyltransferase that catalyzes the posttranscriptional methylation of adenosine at nucleotide position 2503 (*Escherichia coli* numbering) in 23S rRNA, thus interfering with the binding of linezolid. However, due to overlapping binding sites, *cfr* methylation also affects the binding of four other classes of antimicrobial agents and results in the multiresistance $PhLOPS_A$ phenotype, i.e., resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptograminA compounds. Recently, a homologue of Cfr named Cfr(B) has been identified. Although Cfr(B) shares only 75% amino acid identity with Cfr, it functions as a radical S-adenosylmethionine (SAM) family of methylases and catalyzes the same reaction as Cfr, leading to a documented oxazolidinone resistance phenotype in *Staphylococcus aureus*. Since it was first reported in *Peptoclostridium difficile* this gene has already successfully crossed several species barriers.

We previously reported *cfr*(B) in two vancomycin- and linezolid-resistant clinical *E. faecium* index isolates (18203R and 18961R) belonging to CC17 and recovered from patients in a medical center in New Orleans (2012—2013). In the last year, four additional isolates harboring *cfr*(B) were isolated in this hospital system. This study uses next generation sequencing (NGS) to present the characterization of these isolates compared to the index strains.

Methods

Bacterial Isolates: Two *E. faecium* isolates harboring *cfr*(B) have previously been detected and reported from Ochsner Health System (2012—2013). Four additional isolates recovered during 2015—2016 were submitted to JMI Laboratories (North Liberty, Iowa, USA), where bacterial identifications were confirmed by MALDI-TOF-MS (Bruker Daltonics, Bremen, Germany). The isolates were studied for antimicrobial susceptibility and molecular characterization.

Antimicrobial Susceptibility Testing: Susceptibility testing was performed by broth microdilution methods, according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (M07-A10, 2015). In addition, the inoculum density was monitored by colony counts to ensure an adequate inoculum size for each testing event. MIC values were validated by performing concurrent testing of CLSI-recommended quality control reference strains (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212). MIC interpretations were based on the CLSI M100-S26 and European Committee on Antimicrobial Susceptibility Testing (EUCAST) documents, when available.

Screening for Mechanisms of Resistance: Isolates were screened for oxazolidinone resistance mechanisms including *cfr*, *optr*A, and mutations in 23S rRNA, L3 and L4-encoding genes by PCR and sequencing techniques. G2576T mutation was identified by NheI digests of the V domain amplicon of 23S gene and sequencing. *cfr*, *rpl*C (L3) and *rpl*D (L4) amplicons were sequenced on both strands. L3 and L4 deduced amino acid sequences were compared to sequences from known wildtype *E. faecium* strain.

Epidemiological typing: Pulsed-field gel electrophoresis (PFGE) was performed. Genetic relatedness among *E. faecium* isolates also was assessed by MLST scheme described at http://efaecium.mlst.net/.

Next Generation Sequencing: One index strain (ST794) and one *E. faecium* isolate in 2016 (ST78) were subjected to NGS. Briefly, total genomic DNA was extracted using the fully automated ThermoScientific™ KingFisher™ Flex Magnetic Particle Processor (Cleveland, OH, USA). DNA extracts were quantified using the Qubit™ High Sensitivity DS-DNA assay (Invitrogen, ThermoFIsher Inc.) and normalized to 0.2 ng/ µL. A total of 1 ng high quality genomic DNA was used as input material for library construction using the Nextera XT™ DNA library preparation kit (Illumina, San Diego, CA, USA). Libraries were normalized using the bead based normalization procedure (Illumina) and sequenced on MiSeq using the V2- 500 cycle reagent kit. Fastq files generated at the end of the run were assembled and information regarding *cfr*(B) genetic context, virulence, and resistance genes was extracted using a proprietary algorithm (JMI Laboratories, North Liberty, Iowa).

Results

- Four additional vancomycin-resistant (VanA), linezolid-resistant *E. faecium* were isolated between May 2015 and Jan 2016. Three isolates were recovered from blood cultures and one from bone culture. All patients could be categorized as immunocompromised or immunosuppressed with severe underlying diseases. Case histories of patients are described, as follows:

  - **Case 1-JMI #13471J:** In April 2015, an 80-year-old female with malignant carcinoid metastatic to liver post radiation, heart failure, and chronic kidney disease was treated for *Escherichia coli* and *Clostridium perfringens* bacteremia with ceftriaxone, azithromycin, and ciprofloxacin followed by a course of cephalexin and metronidazole. Two weeks later she was treated for sepsis and a hepatic abscess. Blood and hepatic abscess cultures yielded linezolid- and vancomycin-resistant *E. faecium.* Patient received daptomycin for one month. One month later she was readmitted due to recurrent hepatic abscess and sepsis progressing to shock and expired.
  - **Case 2-JMI#13472J:** In July 2015, a 91-year-old female nursing home resident with type 2 diabetes, congestive heart failure, and osteomyelitis of the foot, treated with cefepime and metronidazole, presented with altered mental status and fever. Blood cultures resulted in linezolid- and vancomycin-resistant *E. faecium* and vancomycin-susceptible *Staphylococcus epidermidis*. Urine culture yielded linezolid-susceptible vancomycin-resistant *E. faecium*. She was initially treated with vancomycin, cefepime, and metronidazole. On hospital day 3, patient was started on linezolid for four days. Blood cleared on day 5 despite the linezolid resistance phenotype observed. She expired at a home-hospice facility.
  - **Case 3-JMI#13575J:** A 66-year-old female with type 2 diabetes was admitted with *Streptococcus gallolyticus* bacteremia and urinary tract infection caused by *E. coli* in September 2015. She was treated in ICU with cefepime, moxifloxacin, metronidazole, vancomycin, azithromycin, and ceftriaxone and developed a decubitus ulcer. In November 2015, patient underwent surgical debridement for a sacral ulcer, and bone culture resulted in *E. coli* and linezolid- and vancomycin-resistant *E. faecium*. She responded to eight weeks of daptomycin and cefazolin treatment.
  - **Case 4-JMI#13578J:** A 26-year-old male underwent his fifth liver transplant in September 2015. Abdominal culture grew linezolid-susceptible and vancomycin-resistant *E. faecium*. Patient received two weeks of linezolid treatment, but cultures remained positive. In January 2016, blood cultures yielded linezolid- and vancomycin-resistant *E. faecium*. Bacteremia persisted despite multiple courses of antibiotics, and the patient expired a few weeks later.

- Both index isolates and one of the four additional *E. faecium* were recovered from patients with previous exposure to linezolid.
- All isolates (6) demonstrated a multidrug-resistant (MDR) phenotype, including VanA. MIC results for several antimicrobial agents are listed in **Table 1**.
- G2576T mutations were present in 23S rRNA, along with Cfr(B)-encoding gene in all *E. faecium*. L3 and L4 proteins showed wildtype sequences and *optr*A was not detected (**Table 2**).
- All isolates showed similar PFGE profiles. The two index isolates belonged to ST794, while the additional four *E. faecium* were categorized as ST794 or a double-locus variant, ST78 (**Table 1**). All MLST types belong to the CC17.
- *cfr*(B) was located on a Tn*6218* structure and embedded in the chromosomal DNA of all isolates. Transposon structures from the index and the recent strains were identical and virtually identical to the *cfr*(B) transposon first described in *Peptoclostridium difficile* (**Figure 1**).
- The *esp* virulence gene implicated as a signature of CC17 was present in all isolates. Other resistance genes detected via NGS analysis are listed in **Table 2**. Minor variations in gene content were observed.

Table 1. Antimicrobial susceptibility profiles of Cfr(B)-producing *E. faecium* isolated in the Ochsner New Orleans hospital systems[a].

	MIC in µg/mL (susceptibility interpretation)[b] by isolate					
	2012	2013	2015	2015	2015	2016
Antimicrobial agent	18203R	18961R[c]	13471J	13472J	13575J	13578J[c]
Linezolid	8 (R)	8 (R)	16 (R)	16 (R)	8 (R)	8 (R)
Tedizolid	1	1	2	2	1	1
Clindamycin[d]	>64	≤0.5	>64	2	>64	≤0.5
Erythromycin[d]	>16 (R)	2 (I)	>16 (R)	8 (R)	8 (R)	1 (I)
Chloramphenicol	16 (I)	4 (S)	16 (I)	32 (R)	16 (I)	8 (S)
Retapamulin	0.5	0.5	0.5	2	0.25	0.5
Quinupristin/dalfopristin	1 (S)	1 (S)	0.5 (S)	0.5 (S)	1 (S)	0.5 (S)
Ampicillin	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)
Tetracycline	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)
Doxycycline	4 (S)	4 (S)	2 (S)	8 (I)	4 (S)	8 (I)
Tigecycline	0.06 (S)	0.06 (S)	0.06 (S)	0.12 (S)	0.06 (S)	1 (R)
Vancomycin	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Teicoplanin	>16 (R)	>16 (R)	>16 (R)	64 (R)	>64 (R)	>16 (R)
Gentamicin	>512	>512	8	>512	8	8
Trimethoprim-sulfamethoxazole	>4 (R)	>4 (R)	≤0.5 (I)	>4 (R)	≤0.5 (I)	>4 (R)
Ciprofloxacin	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)	>4 (R)
MLST	ST794	ST794	ST794	ST78	ST794	ST78

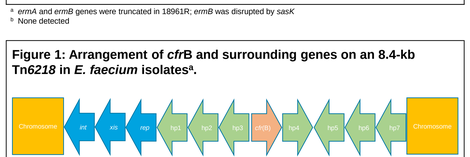
[a] Index strains are highlighted in grey
[b] MIC interpretations per CLSI (M100-S26) where available; tigecycline, gentamicin (≤128 µg/mL for low-level intrinsic resistance and ≥256 µg/mL for high-level resistance), and trimethoprim-sulfamethoxazole interpretations per EUCAST criteria; R= resistant, S= susceptible, I= intermediate
[c] Isolates subjected to NGS
[d] *ermB* gene was disrupted in 18961R resulting in a low erythromycin MIC result; *erm*B was not detected in 13578J

Table 2: Virulence and resistance genes detected in Cfr(B)-producing *E. faecium* isolates by NGS.

	Isolate genotypes	
Resistance/ phenotype	18961R	13578J
Linezolid resistance	*cfr*(B), 23S_G2576T	*cfr*(B), 23S_G2576T
Macrolide resistance[a]	*ΔermA, ΔermB*;*sasK, msrC*	*msrC*
Tetracycline resistance	*tetL, tetM, tet*(38)	*tetL, tetM*
Trimethoprim resistance	*dfrG*	-[b]
Vancomycin resistance	*vanA* operon	*vanA* operon
Efflux pumps	*emrB-qacA, lmrA, relA*	*emrB-qacA, lmrA, relA*
Aminoglycoside resistance	*aac*(6´)-*aph*(2´'), *aph*(3´)-III	*aph*(3´)-III
Virulence	*esp*, no *hyl*	*esp*, no *hyl*

[a] *ermA* and *ermB* genes were truncated in 18961R; *ermB* was disrupted by *sasK*
[b] None detected

Figure 1: Arrangement of *cfr*B and surrounding genes on an 8.4-kb Tn*6218* in *E. faecium* isolates[a].



[a] Figure reproduced from Deshpande et. al (2015).

Conclusions

- Next generation sequencing and analysis demonstrate that *E. faecium* isolates included in this study originated from a common ancestor. However, alterations in conserved MLST housekeeping genes suggest distant temporal relationships indicating prolonged persistency within the Ochsner system.
- Both G2576T and *cfr*(B) linezolid resistance mechanisms were chromosomally located and persisted in these isolates via clonal dissemination; therefore, infection control measures and active surveillance could be effective in minimizing the spread of these MDR isolates.
- Presence of *cfr*(B) carrying transposon in multiple species (*P. difficile*, *E. faecium*) on multiple continents (Europe, USA) and over a period of time highlights its potential to disseminate and persist in the environment. This also implicates that the transposon structure may be widespread in natural habitats of these species.

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