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Evolving Linezolid Resistance Mechanisms in a Worldwide Collection of Enterococcal Clinical Isolates: Results from the SENTRY Antimicrobial Surveillance Program

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Abstract

Background: Linezolid has been in clinical use for several years and large surveillance studies have reported low and stable resistance rates among Gram-positive clinical pathogens. The linezolid resistance mechanisms are well known and mostly comprised of G2576T alterations. However, a new transferable determinant (*optrA*) was recently reported in enterococcal isolates from human and animal origin in China. This study evaluated the linezolid resistance mechanisms among a global collection of enterococcal clinical isolates.

Methods: Isolates from the SENTRY Antimicrobial Surveillance Program (2008-2015) were selected. Identification and MIC testing were performed by MALDI-TOF and broth microdilution (CLSI), respectively. Isolates with linezolid MIC at ≥4 μg/ml were screened for *cfr*, *optrA* and mutations in the 23S rRNA-, L3 and L4-encoding genes. *optrA*-carrying isolates were submitted to whole genome sequencing for characterization of genetic context.

Results: A total of 26 *E. faecalis* and 60 *E. faecium* had LZD MIC at ≥4 μg/ml (0.35% of surveillance isolates received). *E. faecalis* had a linezolid MIC range of 2 - 16 μg/ml ($MIC_{50/90}$, 8/16 μg/ml), while *E. faecium* displayed higher values (4 - 64 μg/ml; $MIC_{50/90}$, 8/32 μg/ml). *optrA* was detected in 14 (53.5%) *E. faecalis* and 10 (38.5%) isolates had G2576T mutations in the 23S rRNA. The remaining *E. faecalis* had *cfr* (1 isolate) or L4 alterations (1) alone. Most (53.8%) *optrA*-carrying *E. faecalis* were from the Asia-Pacific region (China [5], Malaysia [1], Taiwan [1] and Thailand [1]), but other isolates originated from the USA (2), Ireland (2), Panama (1) and Ecuador (1). Two *optrA*-carrying *E. faecalis* (Thailand and Panama) also produced Cfr. All *E. faecium* had G2576T mutations, while two isolates each had concomitant presence *cfr*(B) (USA). *optrA*-carrying enterococci (14 *E. faecalis*) exhibited high MICs for chloramphenicol, retapamulin and tiamulin. *optrA* was mostly plasmid-located and genetic context varied greatly.

Conclusions: Linezolid resistance mechanisms differed between *E. faecalis* and *E. faecium*. 23S rRNA alterations remained the main resistance mechanism in *E. faecium*, while *optrA* prevailed in *E. faecalis*. Plasmid-born *optrA* was detected in isolates from countries other than China and showed a diverse genetic context.

Introduction

Linezolid is an anti-gram-positive agent approved for the treatment of acute bacterial skin and skin structure infections (ABSSSIs), with potent activity against clinical pathogens causing infections worldwide in large surveillance studies. Linezolid resistance occurs especially after prolonged administration, and local investigations have reported occurrences of 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.

More recently, additional and transferrable resistance determinants, such as *cfr*, *cfr*(B) and *optrA* have been detected as newer mechanisms responsible for decreased susceptibility to linezolid. These plasmid-borne resistance genes have been documented in numerous species of human clinical isolates in several regions worldwide. In addition, studies have reported on the concomitant detection of *cfr* and *optrA* genes in *Staphylococcus sciuri* and *Enterococcus faecium.* This study was conducted to evaluate the linezolid resistance mechanisms among a global collection of enterococcal clinical isolates.

Methods

Bacterial strain. A total of 16,176 *E. faecalis* and 8,354 *E. faecium* clinical isolates were submitted to the coordinating monitoring laboratory (JMI Laboratories, North Liberty, Iowa, USA), where bacterial identifications were confirmed by standard algorithms and Vitek® 2 (bioMérieux, Hazelwood, Missouri, USA) or MALDI-TOF-MS (Bruker Daltonics, Bremen, Germany). These isolates were recovered from hospitalized patients and selected by the participating centers following specific protocols, as part of the SENTRY Antimicrobial Surveillance Program for 2008 through 2015. Among these isolates 86 displayed linezolid MIC results at ≥4 μg/ml and were selected for this study.

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 assure an adequate number of cells for each testing event. Validation of the MIC values was performed by concurrent testing of CLSI-recommended quality control reference strains (*Staphylococcus aureus* ATCC 29213 and *Enterococcus 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 linezolid resistance mechanisms. Isolates were screened for the presence of *cfr*, *cfr*(B) and *optrA* and mutations in the 23S rRNA-, L3 and L4-encoding genes by PCR and sequencing. Amplicons were sequenced on both strands and amino acid sequences compared with those from *E. faecalis* and *E. faecium* reference strains.

Characterization of *optrA* genetic context by next generation sequencing (NGS). Isolates carrying *optrA* gene were subjected to NGS. Total genomic DNA of selected isolates was extracted using the fully-automated Thermo Scientific™ KingFisher™ Flex Magnetic Particle Processor (Cleveland, OH, USA). Total genomic DNA was used as input material for library construction. DNA libraries were prepared using the NexteraXT™ library construction protocol (Illumina, San Diego, CA, USA) following the manufacturer's instructions and sequenced on a MiSeq Sequencer (JMI Laboratories, North Liberty, IA, USA). Assembled genomes were subjected to a proprietary software (JMI Laboratories, North Liberty, Iowa) for screening of *optrA* and surrounding regions.

Results

- A total of 26 *E. faecalis* and 60 *E. faecium* had LZD MIC at ≥4 μg/ml (0.35% of surveillance isolates received). *E. faecalis* had a linezolid MIC range of 2 - 16 μg/ml ($MIC_{50/90}$, 8/16 μg/ml), while *E. faecium* displayed higher values (4 - 64 μg/ml; $MIC_{50/90}$, 8/32 μg/ml; **Table 1**).
- Most *E. faecalis* exhibited a susceptible phenotype to ampicillin (96.3 - 100.0% susceptible), as they did for tigecycline (100.0% susceptible) and daptomycin (100.0% susceptible). *optrA*-carrying *E. faecalis* were susceptible to ampicillin, tigecycline, daptomycin and the glycopeptides (**Table 1**).
- *E. faecium* showed a multidrug resistance (MDR) phenotype and high susceptibility rates were observed only for tigecycline (96.7% susceptible) and daptomycin (100.0% susceptible; **Table 1**).
- *optrA* was detected in 14 (53.8%) *E. faecalis* with two isolates (Thailand and Panama) having concomitant presence of *cfr* (**Table 2** and **Figure 1**). Ten (38.5%) isolates had G2576T mutations in the 23S rRNA and remaining *E. faecalis* had *cfr* (1 isolate) or L4 alteration (1) alone (**Table 2**).
- Most (57.1%) *optrA*-carrying *E. faecalis* were from the Asia-Pacific region (China [5], Malaysia [1], Taiwan [1] and Thailand [1]), but other isolates originated from the USA (2), Ireland (2), Panama (1) and Ecuador (1) (**Figure 1**).
- All *E. faecium* had G2576T mutations, while two isolates each had concomitant presence *cfr*(B) or *cfr* (USA) (**Table 2**).
- *E. faecalis* carrying *optrA* and belonging to the same clonal complex (CC) were observed in distinct regions, such as isolates belonging to CC16 and CC116 that were detected in USA/Thailand and China/Ireland, respectively (**Table 3**). In contrast, two *E. faecalis* isolates from medical site 127 (Ireland) exhibited distinct genetic backgrounds.
- NGS analysis revealed that *optrA* was plasmid-located in the majority of isolates (8/14). Chromosomal location was confirmed in five strains (**Table 3**). Transposon structures and phenicol resistance encoding gene *fexA* were associated with *optrA* in plasmid backgrounds. An *ermA*-like gene was present in the vicinity of *optrA* in chromosomal backgrounds (**Figure 2**).

Table 1. Activity of antimicrobial agents tested against the enterococcal collection included in this study.

Organism (number tested)/ Antimicrobial agent[a]	MIC (μg/ml) Range	50%	90%	% Susceptible/%Intermediate/%Resistant[a] CLSI			EUCAST		
***E. faecalis* (26)**									
Ampicillin	≤1 — 8	≤1	2	100.0	-	0.0	96.3	3.7	0.0
Linezolid	2 — 16	8	16	3.7	40.7	55.6	44.4	-	55.6
Clindamycin	8 — >64	>64	>64	-	-	-	-	-	-
Chloramphenicol	8 — 128	32	128	14.8	22.2	63.0	-	-	-
Retapamulin	0.5 — >8	>8	>8	-	-	-	-	-	-
Virginiamycin	≤1 — 32	8	32	-	-	-	-	-	-
Quinupristin-dalfopristin	≤0.25 — >16	16	>16	-	-	-	4.3	8.7	87.0
Levofloxacin[b]	1 — >4	>4	>4	18.5	0.0	81.5	18.5	-	81.5
Doxycycline	≤0.06 — >8	8	>8	22.2	38.9	38.9	-	-	-
Tetracycline	≤0.25 — >8	>8	>8	7.4	0.0	92.6	-	-	-
Tigecycline	≤0.03 — 0.25	0.06	0.25	-	-	-	100.0	0.0	0.0
Daptomycin	0.12 — 2	1	2	100.0	-	-	-	-	-
Teicoplanin	≤2 — >8	≤2	>8	88.5	0.0	11.5	85.2	-	14.8
Vancomycin	1 — >16	2	>16	85.2	0.0	14.8	85.2	-	14.8
***optrA*-positive *E. faecalis* (14)**									
Ampicillin	≤1 — 4	≤1	2	100.0	-	0.0	100.0	0.0	0.0
Linezolid	2 — 16	4	16	7.1	42.9	50.0	50.0	-	50.0
Clindamycin	32 — >64	>64	>64	-	-	-	-	-	-
Chloramphenicol	16 — 128	64	128	0.0	7.1	92.9	-	-	-
Retapamulin	>8	>8	>8	-	-	-	-	-	-
Virginiamycin	4 — 32	8	32	-	-	-	-	-	-
Quinupristin-dalfopristin	4 — >16	16	>16	-	-	-	0.0	9.1	90.9
Levofloxacin[b]	1 — >4	>4	>4	21.4	0.0	78.6	21.4	-	78.6[b]
Doxycycline	8 — >8	>8	>8	0.0	33.3	66.7	-	-	-
Tetracycline	>8	>8	>8	0.0	0.0	100.0	-	-	-
Tigecycline	0.03 — 0.25	0.06	0.25	-	-	-	100.0	0.0	0.0
Daptomycin	0.12 — 2	1	2	100.0	-	-	-	-	-
Teicoplanin	≤2	≤2	≤2	100.0	0.0	0.0	100.0	-	0.0
Vancomycin	1 — 2	1	2	100.0	0.0	0.0	100.0	-	0.0
***E. faecium* (60)**									
Ampicillin	>8	>8	>8	0.0	-	100.0	0.0	0.0	100.0
Linezolid	4 — 64	8	32	0.0	11.7	88.3	11.7	-	88.3
Clindamycin	≤0.5 — >64	>64	>64	-	-	-	-	-	-
Chloramphenicol	4 — 64	16	32	39.0	35.6	25.4	-	-	-
Retapamulin	≤0.06 — >8	0.25	1	-	-	-	-	-	-
Virginiamycin	≤1 — 8	≤1	2	-	-	-	-	-	-
Quinupristin-dalfopristin	≤0.25 — 4	1	2	77.8	15.6	6.7[b]	78.2	21.8	0.0
Levofloxacin[b]	>4	>4	>4	0.0	0.0	100.0	0.0	-	100.0[c]
Doxycycline	≤0.12 — >8	1	>8	63.8	14.9	21.3	-	-	-
Tetracycline	≤2 — >8	≤2	>8	53.3	0.0	46.7	-	-	-
Tigecycline	≤0.03 — 2	0.06	0.25	-	-	-	96.7	1.7	1.7
Daptomycin	0.5 — 4	2	4	100.0	-	-	-	-	-
Teicoplanin	≤2 — >8	>8	>8	24.5	3.8	71.7	21.7	-	78.3
Vancomycin	1 — >16	>16	>16	16.7	1.7	81.7	16.7	-	83.3

a. Breakpoint criteria for telavancin according to CLSI (M100-S26, 2016) and EUCAST breakpoint criteria for telavancin comparator agents, as available.
b. Uncomplicated UTI only.

Table 2. Summary of resistance mechanisms detected among isolates included in this study.

Year	Organism	No. isolates	Resistance mechanism AA alterations[a]	Efflux-pump *optrA*	Methyltransferase *cfr*
2008	***E. faecalis***	1	–	+	–
2008		3	G2576T	–	–
2009		2	–	+	–
2009		1	L4 (F101L)	–	–
2010		1	–	+	–
2010		1	–	+	+
2010		2	G2576T	–	–
2011		1	–	+	+
2011		2	G2576T	–	–
2011		1	–	–	+
2012		3	–	+	–
2012		2	G2576T	–	–
2013		1	–	+	–
2013		1	G2576T	–	–
2014		3	–	+	–
2015		1	–	+	–
2008	***E. faecium***	8	G2576T	–	–
2009		13	G2576T	–	–
2009		1	G2576T	–	–
2010		12	G2576T	–	–
2011		9	G2576T	–	–
2012		1	G2576T	–	–
2012		3	G2576T	–	–
2012		1	G2576T	–	+[b]
2013		5	G2576T	–	–
2013		1	G2576T	–	+[b]
2014		4	G2576T	–	–
2014		1	G2576T	–	+
2015		1	G2576T	–	–

a. Amino acid alterations were investigated in the 23S rRNA and the L3 and L4 ribosomal proteins.
b. Represents isolates with the *cfr*(B) variant.

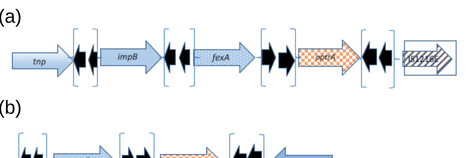
Table 3. Molecular information obtained from *optrA*-carrying *E. faecalis* clinical isolates included in this study.

Year	Isolate No	Country	Site Code	*optrA* gene location	Epidemiology[a] MLST	CC	PFGE
2010	50427	Thailand	603	Chromosome	ST16	16	
2015	35178	USA	013	Plasmid	ST179	16	
2014	4664	USA	122	Chromosome	ST585	585	
2009	7922	China	232	Chromosome	ST69	96	
2009	3279	China	234	Plasmid	ST632	96	
2012	53344	China	234	Plasmid	ST585	585	
2008	16	China	233	Plasmid	ST116	116	
2010	20618	China	237	Plasmid	ST116	116	
2012	33526	Ireland	127	Plasmid	ST116	116	EF127A
2014	19570	Ireland	127	Plasmid	ST41	41	EF127B
2012	55372	Taiwan	215	Chromosome	ST1154	1154	
2014	18844	Malaysia	601	ND[b]	ST59	59	
2011	32791	Panama	346	Plasmid	ST103	103	
2013	9235	Ecuador	364	Chromosome	ST86	86	

a. MLST - multilocus sequence typing; CC - clonal complex; PFGE - pulsed-field gel electrophoresis. Species recovered from the same site were subjected to PFGE and isolates from center 127 showed distinct profiles.
b. ND - gene location could not be determined.

Figure 1. Schematic distribution of *optrA*-carrying enterococcal isolates detected during the SENTRY Antimicrobial Surveillance Program for 2008-2015.

Figure 2. Schematic representation of *optrA* genetic context. (a) Common array of genes found in plasmid context. (b) Most common gene array found in chromosomal context. Arrows indicate direction of transcription; black arrows in parenthesis indicate miscellaneous genes with no known functions. These were variable in number and arrangement in different strains.



Conclusions

- Isolates included in this study had decreased susceptibility to linezolid and several other antimicrobial agents had compromised *in vitro* activity, emphasizing the MDR nature of these enterococcal clinical isolates.
- Resistance mechanisms to oxazolidinone differed between *E. faecalis* and *E. faecium*. 23S rRNA alterations (G2576T) remained the main resistance mechanism in *E. faecium*, while *optrA* prevailed in *E. faecalis*.
- Overall, *optrA*-carrying *E. faecalis* showed a very diverse genetic background, likely due to the dissemination of this gene via plasmid transfer. However, isolates belonging to the same CC were detected in distinct geographic regions.
- Finally, this study reports the global dissemination of *optrA*-carrying enterococci in isolates recovered from patients in countries other than those of Asia-Pacific region. The MDR nature of such isolates in combination with the possible dissemination warrants constant surveillance for monitoring.

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