Linezolid Resistance Mechanisms among Staphylococci and Enterococci Collected from Global Resistance Surveillance Programs

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ABSTRACT

Background: Linezolid resistance in Gram-positive isolates has been mostly associated with 23S rRNA mutations. We assessed the molecular mechanisms associated with linezolid resistance in a worldwide collection of Gram-positive pathogens (2008-2009).

Methods: *S. aureus* (10,955), coagulase-negative staphylococci (CoNS; 2,958) and enterococci (4,061) were collected from 127 hospitals from North America (46.5%), Europe (29.1%), Latin America (13.0%) and Asia (14.4%). Isolates were tested for susceptibility by CLSI methods. Those with linezolid MIC values at ≥4 µg/mL were screened for *cfr* and mutations in the 23S rRNA, L3 and L4 proteins by PCR/sequencing. Sequences were compared with those from linezolid-susceptible ATCC strains. Clonality was assessed by PFGE.

Results: Five (0.2%) *E. faecalis*, eight (0.07%) *S. aureus*, 19 (1.5%) *E. faecium* and 37 (1.2%) CoNS met the screening criteria. G2576T was detected in 4 *S. aureus*, while 2 strains (2009) carried *cfr* and other strains showed L3 alterations. *E. faecalis* exhibited G2576T or L4 mutations. All *E. faecium* had G2576T, absence of L3 or L4 mutations and variable linezolid MICs. All enterococci were *cfr*-negative. Clonal dissemination among *E. faecium* was noted within institutions. 19 CoNS were 23S rRNA mutants, including one and two strains with T2504A and G2447T substitutions, respectively. Eight CoNS (2 strains from 2008 and 6 from 2009) were *cfr*-positive from unique sites in Mexico (3), Italy (2), Arizona (2), and Michigan (1); frequently (75.0%) representing clonal expansion within hospitals. These CoNS harboring 23S rRNA mutations or *cfr* also exhibited L3 and/or L4 alterations and linezolid MIC results at ≥32 µg/mL. The remaining CoNS had L3 and/or L4 mutations only and lower linezolid MICs (≤16 µg/mL). Co-presence of *cfr* and 23S rRNA mutations was not noted.

Conclusion: Linezolid resistance was rare and most commonly associated with ribosomal protein mutations (23S rRNA, L3 and L4). Strains with L3 and/or L4 alterations only, were frequently found among CoNS showing lower linezolid MIC values. *cfr* strains increased in 2009; however, mostly due to clonal dissemination.

INTRODUCTION

Linezolid is approved for the treatment of complicated skin and skin-structure infections (cSSSI) and nosocomial pneumonia caused by Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Linezolid inhibits protein synthesis by interfering with the formation of the 70S initiation complex. Although, linezolid resistance remains rare, sporadic staphylococci and enterococci non-susceptible isolates have been detected and usually associated 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 rRNA. Other mutations in this same region, such as T2500A, G2447T and T2504A have also been observed. Moreover, modifications in the conserved regions of L3 and L4 ribosomal proteins have been associated with decreased susceptibility to linezolid.

Recently, an oxazolidinone resistance mechanism was identified in staphylococci. This gene, named *cfr*, encodes a protein that causes post-transcriptional methylation of 23S rRNA (in the position A2503) affecting drugs belonging to several antimicrobial classes, including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A. We assessed the molecular mechanisms associated with elevated linezolid MIC values (≥4 µg/mL) in a collection of Gram-positive pathogens from worldwide surveillance programs (2008-2009).

MATERIALS AND METHODS

Bacterial isolates: *S. aureus* (10,955), coagulase-negative staphylococci (CoNS; 2,958) and enterococci (4,061) were collected from 127 hospitals from North America (46.5%), Europe (29.1%), Latin America (13.0%) and Asia (14.4%). These isolates were selected according to established protocols and submitted to a central monitoring laboratory (JMI Laboratories, North Liberty, Iowa, USA) as part of several surveillance programs. All processed isolates were identified by the submitting laboratory and confirmed by the central facility using the Vitek 2 System (bioMerieux, Hazelwood, Missouri, USA), or conventional reference manual methods.

Susceptibility testing: Isolates were susceptibility tested by broth microdilution procedure according to the Clinical and Laboratory Standards Institute (CLSI; M07-A8, 2009). Validation of the minimum inhibitory concentration (MIC) values was performed by concurrent testing of CLSI-recommended (M100-S20-U, 2010) quality control (QC) strains: *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213. Interpretation of MIC results was in accordance with published CLSI (M100-S20-U) guidelines.

Detection of linezolid-resistance mechanisms: Isolates with elevated linezolid MIC values at ≥4 µg/mL were screened for *cfr* and mutations in the 23S rRNA-, L3- and L4-encoding genes by PCR. Amplicons were sequenced on both strands and proteins compared with those from linezolid-susceptible *S. aureus* NCTC 8325, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 35667 and *Staphylococcus cohnii* ATCC 29974.

Molecular typing: Isolates with elevated linezolid MIC values (≥4 µg/mL) collected from the same medical site were subjected to pulsed-field gel electrophoresis (PFGE). Genomic DNA was prepared in agarose blocks and digested with specific restriction enzymes according to standard protocols. Electrophoresis was performed on the CHEF-DR II (BioRad, Richmond, California, USA).

RESULTS

- Among Gram-positive isolates selected for this study, five (0.2%) *E. faecalis*, eight (0.07%) *S. aureus*, 19 (1.5%) *E. faecium* and 37 (1.2%) CoNS met the screening criteria (linezolid MIC, ≥4 µg/mL).
- All selected *S. aureus* strains (MIC values, 8 – 16 µg/mL; Table 1) originated from USA medical centers and were susceptible to tigecycline (MIC_{50}, 0.25 µg/ml) and vancomycin (MIC_{50}, 1 µg/ml). Nevertheless, all isolates were resistant to oxacillin, ciprofloxacin and erythromycin.
- G2576T alterations were detected in four *S. aureus*, whereas one strain had a G2512T substitution. Two *S. aureus* isolates (both from 2009) carried *cfr* and one strain with a linezolid MIC value of 8 µg/mL had a L3 deletion at position 145 (Table 1).
- CoNS isolates displayed a broader range of linezolid resistance mechanisms and MIC values (4 – >128 µg/mL; Table 2).
- Mutations in the 23S rRNA were detected in 64.5% of CoNS, including T2504A (one strain) and G2447T (two strains). In addition, four 23S rRNA mutant strains also showed L3 and/or L4 alterations.
- Eight (21.6%) CoNS (two strains from 2008 and six from 2009) were *cfr*-positive. These isolates were from medical sites located in Mexico (three strains), Italy (two strains), Arizona (two strains), and Michigan (one strain). Eight of six (75.0%) Cfr-producing strains represented clonal expansion within hospitals (Table 2).
- *cfr*-harboring CoNS often exhibited L3 and/or L4 alterations and linezolid MIC results at ≥32 µg/mL. Isolates with L3 and/or L4 alteration but no 23S rRNA mutation had lower linezolid MICs (≤16 µg/mL). Co-presence of *cfr* and 23S rRNA mutations was not detected.
- Overall, *E. faecalis* and *E. faecium* exhibited G2576T, absence of L3 or L4 mutations and variable linezolid MIC values. No *cfr* gene was detected among linezolid-resistant enterococci. Clonal dissemination among linezolid-resistant *E. faecium* was noted within two institutions.

Table 1. Antimicrobial susceptibility profile and molecular findings among MRSA strain recovered from clinical specimens of hospitalized patients.

				Antimicrobial agent MIC (µg/mL)[a]												Resistance mechanisms			
Isolate	Year	State	Country	LZD	CLI	CHL	RET	TET	TIG	Q/D	CIP	ERY	GEN	T/S	VAN	*cfr*	23S rRNA	L3	L4
2265	2009	CT	USA	8	≤0.5	16	1	0.5	0.25	1	>4	>2	≤2	≤0.5	1	–	WT[b]	ΔS145	WT
3460	2008	MA	USA	16	>64	32	0.25	4	1	1	>4	>2	≤2	≤0.5	1	–	G2576T	WT	WT
3675	2008	NY	USA	16	≤0.5	8	0.25	0.25	0.12	1	>4	>2	≤2	≤0.5	2	–	C2512T	WT	WT
4303	2008	KY	USA	16	≤0.5	16	0.25	2	0.5	≤0.25	>4	>2	>8	>2	1	–	G2576T	WT	WT
2031	2009	CA	USA	16	≤0.5	16	0.5	≤0.12	0.06	0.5	>4	>2	≤2	≤0.5	2	–	G2576T	WT	WT
99	2009	KS	USA	16	>64	32	0.25	>16	0.25	1	>4	>2	>8	≤0.5	2	–	G2576T	WT	WT
272	2009	OH	USA	16	>64	>128	>8	0.5	0.25	2	>4	>2	≤2	≤0.5	1	+	WT	WT	WT
1687	2009	KY	USA	16	>64	>128	>8	0.25	0.25	2	>4	>2	≤2	≤0.5	1	+	WT	WT	WT

a. LZD, linezolid; CLI, clindamycin; CHL, chloramphenicol; RET, retapamulin; TET, tetracycline; TIG, tigecycline; Q/D, quinupristin/dalfopristin; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; T/S, trimethoprim/sulfamethoxazole; VAN, vancomycin.
b. WT, wildtype.

Table 2. Antimicrobial susceptibility profile and molecular findings among CoNS strains recovered from clinical specimens of hospitalized patients.

			Antimicrobial agent MIC (µg/mL)[a]											Resistance mechanisms			
Isolate	Year	Organism	LZD	CLI	CHL	RET	TET	TIG	Q/D	CIP	ERY	GEN	T/S	VAN	*cfr*	23S rRNA	L3	L4
2466	2009	*S. epidermidis*	4	≤0.5	4	0.06	>16	0.12	≤0.25	>4	>2	>8	>2	2	–	WT[b]	L101V/V154L/A157R	P171S
4593	2009	*S. capitis*	8	>64	>128	>8	0.25	0.12	1	≤0.5	≤0.25	≤2	≤0.5	1	+	WT	WT	WT
10725	2008	*S. epidermidis*	8	>64	16	8	1	0.12	2	>4	>2	>8	>2	2	–	G2576T	WT	WT
1708	2008	*S. epidermidis*	8	≤0.5	16	0.25	2	0.25	≤0.25	>4	≤0.25	>8	>2	4	–	G2576T	WT	WT
6546	2008	*S. epidermidis*	16	≤0.5	≤1	1	1	0.12	≤0.25	>4	>2	>8	>2	2	–	G2447T	WT	WT
586	2008	*S. epidermidis*	16	≤0.5	16	0.25	1	0.12	≤0.25	>4	≤0.25	>8	>2	2	–	G2576T	WT	WT
7715	2009	*S. epidermidis*	16	≤0.5	8	0.12	0.25	0.12	≤0.25	>4	2	4	2	2	–	WT	L101V/V154L/A157R	$_{21}G_{72}$
1590	2009	*S. epidermidis*	16	≤0.5	8	0.12	≤0.12	0.12	≤0.25	>4	>2	>8	>2	2	–	WT	H146Q	$_{21}G_{72}$
4596	2009	*S. epidermidis*	16	≤0.5	8	0.12	≤0.12	0.06	≤0.25	>4	>2	8	>2	2	–	WT	H146Q	WT
2563	2008	*S. epidermidis*	32	32	32	>8	2	0.25	0.5	>4	>2	4	>2	2	–	G2576T	WT	WT
2409	2009	*S. epidermidis*	32	1	128	0.5	1	0.25	≤0.25	>4	0.5	>8	>2	2	–	G2576T	WT	WT
12898[d]	2009	*S. epidermidis*	32	>64	16	8	2	0.25	2	>4	>2	>8	>2	2	+	WT	L101V/S158Y/D159Y	WT
5738[d]	2009	*S. epidermidis*	32	>64	16	>8	1	0.25	0.5	>4	>2	>8	>2	2	+	WT	L101V/S158Y/D159Y	WT
4615	2008	*S. epidermidis*	32	1	32	0.5	1	0.25	≤0.25	>4	≤0.25	>8	>2	2	–	G2576T	WT	WT
10842	2009	*S. cohnii*	32	>64	32	>8	≤0.12	0.06	4	>4	>2	>8	≤0.5	1	+	WT	S158F/D159Y	N20S
1440	2008	*S. epidermidis*	32	>64	32	0.5	2	0.25	≤0.25	>4	≤0.25	>8	>2	2	–	G2576T	WT	WT
2179	2009	*S. epidermidis*	32	16	32	8	2	0.5	0.5	>4	>2	>8	>2	4	–	G2576T	WT	WT
14078[d]	2009	*S. epidermidis*	32	>64	64	>8	1	0.12	8	>4	>2	>8	2	1	+	WT	F147L/A157R	WT
4303[d]	2008	*S. epidermidis*	64	>64	128	>8	2	0.5	8	>4	>2	>8	>2	2	+	WT	L101V/F147L/A157R	WT
6739	2008	*S. haemolyticus*	64	1	64	1	1	0.5	0.5	>4	>2	>8	>2	2	–	G2576T	WT	WT
5288[d]	2008	*S. epidermidis*	64	2	64	0.5	1	0.12	≤0.25	>4	>2	>8	>2	2	–	G2576T	WT	WT
2268	2008	*S. epidermidis*	64	≤0.5	≤1	0.5	1	0.25	≤0.25	>4	>2	>8	2	1	–	G2447T	WT	WT
2286	2009	*S. epidermidis*	128	2	128	1	2	0.5	0.5	>4	>2	>8	>2	2	–	G2576T	H146R/M156T	$_{21}G_{72}$
25	2009	*S. epidermidis*	128	1	64	1	1	0.25	0.5	>4	>2	>8	>2	2	–	G2576T	G137D/H146R/V154L/M156T	$_{21}G_{72}$
3417	2009	*S. epidermidis*	128	1	128	1	1	0.25	≤0.25	>4	>2	>8	>2	2	–	G2576T	H146P/M156T/G173S	$_{21}G_{72}$
1459[d]	2008	*S. epidermidis*	128	2	128	1	2	0.5	≤0.25	>4	>2	>8	>2	1	–	G2576T	WT	WT
1460[d]	2008	*S. epidermidis*	128	2	128	1	2	0.25	≤0.25	>4	2	>8	>2	1	–	G2576T	WT	WT
8676	2008	*S. epidermidis*	128	2	128	0.5	0.5	0.12	0.5	>4	>2	>8	>2	4	–	G2576T	WT	WT
5289[d]	2008	*S. epidermidis*	128	2	64	1	1	0.25	0.5	>4	>2	>8	>2	2	–	G2576T	WT	WT
9528[d]	2008	*S. epidermidis*	128	>64	64	>8	2	0.25	1	>4	>2	>8	>2	2	–	G2576T	WT	WT
13800	2009	*S. epidermidis*	128	1	64	1	4	0.5	≤0.25	>4	>2	8	>2	1	–	G2576T	H146R/M156T	$_{21}G_{72}$
3242	2008	*S. epidermidis*	>128	8	>128	>8	≤0.12	0.12	1	>4	≤0.25	8	>2	2	–	T2504A	WT	WT
9013[d]	2008	*S. epidermidis*	>128	4	>128	1	2	0.25	0.5	>4	>2	>8	>2	2	–	G2576T	WT	WT
2104[e]	2008	*S. epidermidis*	>128	>64	64	>8	1	0.25	2	>4	>2	>8	>2	2	+	WT	WT	WT
2174[e]	2009	*S. epidermidis*	>128	>64	128	>8	0.25	0.12	2	>4	>2	>8	>2	2	+	WT	H146Q/A157R	$_{21}G_{72}$
1188	2008	*S. epidermidis*	>128	2	128	1	2	0.5	≤0.25	>4	>2	>8	>2	2	–	G2576T	WT	WT
10728	2008	*S. hominis*	>128	4	128	1	4	0.5	0.5	>4	>2	4	>2	2	–	G2576T	WT	WT

a. LZD, linezolid; CLI, clindamycin; CHL, chloramphenicol; RET, retapamulin; TET, tetracycline; TIG, tigecycline; Q/D, quinupristin/dalfopristin; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; T/S, trimethoprim/sulfamethoxazole; VAN, vancomycin.
b. WT, wildtype.
c, d, e, f and g. Clonally-related strains.

Table 3. Antimicrobial susceptibility profile and molecular findings among enterococci strains recovered from clinical specimens of hospitalized patients.

					Antimicrobial agent MIC (µg/mL)[a]										Resistance mechanisms		
Isolate	Site	Year	Country	Organism	LZD	AMP	CHL	RET	TET	TIG	Q/D	CIP	DAP	VAN	TEC	*cfr*	23S rRNA	L3	L4
4071		232	2009	China	*E. faecalis*	4	≤1	64	>8	>16	0.12	8	2	1	1	≤2	–	WT[b]	WT	F101L
12978		089	2008	Sweden	*E. faecalis*	8	2	64	>8	>16	0.12	4	>4	2	4	≤2	–	G2576T	WT	WT
7904		133	2008	UK	*E. faecalis*	8	≤1	8	0.5	≤0.12	0.06	≤0.25	>4	1	1	≤2	–	G2576T	WT	WT
16		233	2008	China	*E. faecalis*	8	≤1	64	>8	>16	0.12	16	>4	1	2	≤2	–	G2576T	WT	WT
2978		420	2008	USA	*E. faecalis*	8	2	16	>8	>16	0.12	8	>4	0.5	2	≤2	–	G2576T	WT	WT
414		427	2009	USA	*E. faecium*	4	>16	16	0.12	0.25	0.06	0.5	>4	1	>16	>16	–	G2576T	WT	WT
939		021	2009	USA	*E. faecium*	8	>16	16	0.12	>16	0.06	0.5	>4	2	>16	>16	–	G2576T	WT	WT
6994		027	2008	USA	*E. faecium*	8	>16	16	8	0.25	≤0.12	≤0.03	≤0.25	>4	2	>16	–	G2576T	WT	WT
2539		030	2009	USA	*E. faecium*	8	>16	16	0.25	16	0.06	0.5	>4	2	>16	>16	–	G2576T	WT	WT
652		088	2009	Germany	*E. faecium*	8	>16	32	0.12	≤0.12	≤0.03	≤0.25	>4	2	>16	>16	–	G2576T	WT	WT
1686[c]		027	2009	USA	*E. faecium*	8	>16	16	0.25	0.25	0.06	1	>4	2	>16	>16	–	G2576T	WT	WT
870[c]		027	2009	USA	*E. faecium*	8	>16	16	0.25	≤0.12	0.06	≤0.25	>4	2	>16	>16	–	G2576T	WT	WT
5561[c]		027	2009	USA	*E. faecium*	16	>16	32	>8	4	0.25	4	>4	2	>16	>16	–	G2576T	WT	WT
5353		015	2008	USA	*E. faecium*	16	>16	32	0.12	>16	0.12	0.5	>4	1	>16	>16	–	G2576T	WT	WT
2997		015	2008	USA	*E. faecium*	16	>16	16	0.5	>16	0.25	2	>4	4	>16	>16	–	G2576T	WT	WT
2069		048	2008	Brazil	*E. faecium*	16	>16	16	0.25	0.5	0.12	2	>4	2	>16	>16	–	G2576T	WT	WT
6128		051	2009	USA	*E. faecium*	16	>16	16	0.5	0.5	0.12	0.5	>4	2	>16	>16	–	G2576T	WT	WT
6237		088	2008	Germany	*E. faecium*	16	>16	16	0.5	2	≤0.03	0.5	>4	4	>16	≤2	–	G2576T	WT	WT
12093[c]		027	2008	USA	*E. faecium*	32	>16	16	0.5	>16	0.12	1	>4	1	>16	>16	–	G2576T	WT	WT
14306[c]		027	2008	USA	*E. faecium*	32	>16	32	0.5	>16	0.12	1	>4	2	>16	>16	–	G2576T	WT	WT
6948[c]		027	2009	USA	*E. faecium*	32	>16	32	8	2	0.12	4	>4	2	>16	>16	–	G2576T	WT	WT
6803		088	2009	Germany	*E. faecium*	32	>16	32	0.5	0.25	0.06	1	>4	2	1	≤2	–	G2576T	WT	WT
30		219	2009	Korea	*E. faecium*	32	>16	64	1	0.25	≤0.03	1	>4	2	1	≤2	–	G2576T	WT	WT
243		406	2008	USA	*E. faecium*	64	>16	32	8	0.5	0.12	2	>4	2	>16	>16	–	G2576T	WT	WT

a. LZD, linezolid; AMP, ampicillin; CHL, chloramphenicol; RET, retapamulin; TET, tetracycline; TIG, tigecycline; Q/D, quinupristin/dalfopristin; CIP, ciprofloxacin; DAP, daptomycin; VAN, vancomycin; TEC, teicoplanin.
b. WT, wildtype.
c. Clonally-related strains

CONCLUSIONS

- Only 69 of 10,955 (0.4%) Gram-positive strains displayed elevated linezolid MIC values (≥4 µg/mL), where mutations within 23S rRNA were the main resistance mechanisms.
- Alterations in L3 among CoNS were often detected between amino acids 146 and 159. Substitutions within this region (i.e. His146, Gly155 and Ala157) have been associated with decreased susceptibility to linezolid in staphylococci.
- CoNS frequently possessed a glycine insertion at position 71 in L4. This position belongs to a conserved region, where mutations have been associated with cross resistance to linezolid, macrolides and chloramphenicol in *Streptococcus pneumoniae*, *S. aureus* and *Clostridium perfringens*.
- The vast majority (80.0%) of Cfr-producing staphylococci were recovered during the 2009 sampling period. However, this increase in *cfr* strains was likely due to clonal dissemination within medical sites.
- CoNS strains exhibited multiple linezolid resistance mechanisms, emphasizing the potential for accumulating mutations and/or resistance determinants.

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