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Characterization of Enterococcal Isolates with a Linezolid MIC at 4 µg/ml Collected as Part of the ZAAPS Oxazolidinone Surveillance Program (2002-2004)

HS SADER, JE ROSS, LM DESHPANDE, TR FRITSCHE, RN JONES
JMI Laboratories, North Liberty, IA, USA

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

ABSTRACT

Background: Acquired resistance (R) to linezolid (LZD) remains very uncommon among Gram-positive (GP) clinical isolates in geographic regions of high LZD use. We evaluated the occurrence of the G2576T mutation in enterococcal (ENT) isolates with LZD MIC values in the intermediate CLSI/NCCLS category (4 µg/ml) using phenotypic and molecular methods.

Methods: The Zyvox Annual Appraisal of Potency and Spectrum (ZAAPS) Program monitors for LZD activity in >60 medical centers worldwide. A total of 20,471 GP clinical strains, including 3,827 ENT isolates, were collected in 2002-2004. The majority of ENT (1,979 strains; 51.7%) were collected in the USA. The strains were tested for susceptibility (S) by CLSI broth microdilution method in a central reference laboratory and isolates with LZD MIC ≥ 4 µg/ml were also tested by the disk diffusion method and Etest (AB BIODISK, Solna, Sweden). Isolates with reproducible LZD MIC values at 4 µg/ml were evaluated for the presence of the G2576T mutation in domain V of the 23S rRNA by PCR and restriction endonuclease digestion of the PCR fragments.

Results: Only 4 ENT isolates (0.10%) had a LZD MIC at 4 µg/ml and the G2576T mutation was detected in 3 of those strains. The strains were *E. faecalis* (EF, 2) and *E. faecium* (2). The strains were collected in 2002 (2) and 2003 (2) in the USA (3) and Sweden (1 EF with negative result for the G2576T mutation). Seven ENT strains (0.18%) had LZD MIC values at ≥ 8 µg/ml and all contained a G2576T mutation by the described molecular methods. A LZD MIC value of 4 µg/ml was also extremely rare among 7,889 *S. aureus* (0.03%) and 4,354 *S. pneumoniae* (0.02%) strains tested, and was not observed among CoNS (2,671 strains tested), ß-haemolytic (1,097) or viridans group streptococci (633).

Conclusion: Increased LZD MIC values (> 2 µg/ml) remain very rare among clinical strains of GP and the G2576T mutation may be present among ENT isolates with an intermediate (4 µg/ml) LZD MIC value.

INTRODUCTION

Linezolid was the first clinically applied oxazolidinone and has become a significant addition to those agents used to treat infections caused by resistant Gram-positive cocci. Although rare, oxazolidinone (linezolid) resistance has occurred, particularly among *Staphylococcus aureus* and enterococci, following mutations in domain V of the 23S rRNA (primarily the G2576U). Most bacterial species possess multiple copies of 23S rRNA genes (alleles) and an increased percentage of genes with this mutation is associated with an increased level of resistance expressed (gene dosage effect).

Linezolid resistance is usually associated with prolonged therapy and indwelling device infections. Some oxazolidinone-resistant strains have emerged in patients without prior drug exposure, each event attributed to clonal spread of strains from other patients in the hospital environment. The Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) has established linezolid breakpoints of ≤ 2 µg/ml (susceptible) and ≥ 8 µg/ml (resistant) for enterococci, while only susceptible breakpoints have been established for staphylococci (≤ 4 µg/ml) and streptococci (≤ 2 µg/ml).

The Zyvox® Annual Appraisal of Potency and Spectrum (ZAAPS) Program is a post-marketing risk management tool designed to monitor the evolution of linezolid resistance among Gram-positive cocci from over 50 medical centers located in North America, South America, Europe and the Western Pacific. In the 2002-2004 period of the ZAAPS Program, a total of 20,471 Gram-positive clinical strains were reference tested against linezolid and numerous comparator agents. In the present study, we evaluated the occurrence of the G2576U mutation in enterococcal isolates with linezolid minimum inhibitor concentration (MIC) values in the intermediate CLSI category (4 µg/ml) using phenotypic and molecular methods.

MATERIALS AND METHODS

The central monitoring site (JMI Laboratories, North Liberty, IA, USA) collected a total of 20,471 Gram-positive clinical strains for the ZAAPS Program from sites in Latin America (four nations/10 sites), Europe (six nations/16 sites), the Western Pacific (five nations/11 sites) and North America (Canada/five sites and the USA [2002 and 2003]/25 sites) in the 2002-2004 period. Each participating site or country forwarded a target total of 200 consecutive, non-duplicate patient specimens originating from patients having infections of the bloodstream, respiratory tract, urinary tract or wounds/skin and soft tissue.

A total of 3,827 enterococcal isolates were collected during this period, and the majority of them (1,979 strains; 51.7%) were collected in the USA. The collection also included 7,889 *S. aureus*, 2,671 coagulase-negative staphylococcal (CoNS), 4,354 *Streptococcus pneumoniae,* 1,097 ß-haemolytic and 633 viridans group streptococcal strains.

The strains were tested for susceptibility by CLSI broth microdilution methods in a central reference laboratory. The validated, dry-form microdilution panels and cation-adjusted Mueller-Hinton broth (with 2 - 5% lysed horse blood added for testing of streptococci) were purchased from TREK Diagnostics (Cleveland, OH, USA). Interpretations of quantitative MIC results and quality control strains (*S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *S. pneumoniae* ATCC 49619) were in accordance with the CLSI tables. Isolates with linezolid MIC values ≥ 4 µg/ml were also tested by the disk diffusion method and Etest (AB BIODISK, Solna, Sweden).

Isolates with reproducible linezolid MIC values at 4 µg/ml were evaluated for the presence of the G2576T mutation in domain V of the 23S rRNA gene by PCR and restriction endonuclease digestion of the PCR fragments as described previously. The primers used to amplify the 389 bp region were: 5'-GCA GAA GGG AGC TTG ACT GCG AG-3' (forward) and 5'-ACC CAG CAA TGC CCT TGG CAG-3' (reverse). The PCR products obtained were cleaned and digested with NheI, which cuts at the G2576T mutation site. The restriction endonuclease digests were submitted to electrophoresis on 1.5% agarose and stained with ethidium bromide. Isolates with no G2576T mutation show a single uncut 389 bp band whereas isolates with a G2576T mutation exhibit two other bands with molecular sizes adding to 389 bp. The original 389 bp band will not be present if the strain has the mutation in all 23S rRNA genes (homozygous pattern, high level resistance). If the mutation is not present in all genes (heterozygous pattern), the 389 bp band will still be present and the strain will express low to moderate levels of resistance, depending on the proportion of 23S rRNA genes effected.

RESULTS

- Linezolid MIC distributions for all organism groups evaluated in the ZAAPS Program are shown in Table 1 and Figure 1. Among 20,471 strains tested, only nine strains (0.04%) exhibited linezolid MIC values of 4 µg/ml: four enterococci, four *S. aureus* and one *S. pneumoniae*.
- Linezolid MIC values ≥ 8 µg/ml were detected in nine strains (0.04%), including seven enterococci, one *S. aureus*, one CoNS and one viridans group *Streptococcus*.
- Among enterococcal strains (3,827 tested), isolates with linezolid MIC value of 4 µg/ml represented only 0.10% of isolates tested, while isolates with linezolid MIC values at ≥ 8 µg/ml numbered 0.18%.
- The G2576T gene mutation was detected in three of four (75%) enterococcal strains with a reproducible linezolid MIC value of 4 µg/ml (Table 2). All three strains showed a heterozygous pattern (three bands; Figure 2).

Table 1. Linezolid MIC distributions among Gram-positive isolates evaluated by the ZAAPS Program (2002 - 2004).

	No. of isolates at linezolid MIC (µg/ml) of:							
Organisms (no. tested)	≤0.12	0.25	0.5	1	2	4	8	>8
Enterococcus spp. (3,827)	1	4	56	1,621	2,134	4	3	4
S. aureus (7,889)	2	10	49	2,332	5,492	3	0	1
CoNS (2,671)	5	11	388	2,045	221	0	0	1
S. pneumoniae (4,354)	25	142	1,355	2,666	165	1	0	0
ß-haemolytic streptococci (1,097)	5	3	118	953	18	0	0	0
viridans group streptococci (633)	6	13	169	425	19	0	1	0
Total (20,471)	44	138	2,135	10,042	8,049	8	4	6

Table 2. Microbiologic and demographic results of enterococcal isolates with linezolid MIC values of 4 µg/ml identified by the ZAAPS Program worldwide.

				Linezolid MIC (µg/ml)		Disk diffusion	Presence of
Isolate no.	Species	Location	Year	Broth	Etest	(mm-category)	G2576T mutation
2-681V	*faecium*	Indiana, USA	2003	4	8	18-R	Positive
30-97V	*faecium*	Virginia, USA	2003	4	6	20-R	Positive
27-12964A	*faecalis*	Kentucky, USA	2002	4	6	21-I	Positive
89-8106A	*faecalis*	Stockholm, Sweden	2002	4	4	21-I	Negative

- Two of three isolates with the G2576T gene mutation were considered resistant to linezolid by both disk diffusion and Etest (MIC, 8 µg/ml) methods, while the third isolate (27-12974A) showed an intermediate disk diffusion inhibition zone diameter and a MIC of 6 µg/ml by Etest (Table 2).
- The enterococcal strains with linezolid MIC values at 4 µg/ml were *E. faecalis* (two) and *E. faecium* (two), and they were collected in 2002 (two) and 2003 (two) in the USA (three) and Sweden (one *E. faecalis* with negative results for the G2576T gene mutation; Table 2).

Table 3. Antimicrobial susceptibility pattern of enterococcal isolates with linezolid MIC values of 4 µg/ml.

	MIC (µg/ml)			
Antimicrobial agent	2-681V	30-97V	27-12964A	89-8106A
Linezolid	4	4	4	4
Gentamicin (HL)	≤500	>1000	1000	≤500
Streptomycin (HL)	≤1000	>2000	>2000	≤1000
Erythromycin	>8	>8	>8	2
Tetracycline	>8	≤4	≤4	>8
Chloramphenicol	16	8	16	8
Quinupristin/dalfopristin	1	0.5	8	8
Rifampin	>2	>2	1	2
Ampicillin	>16	>16	≤2	≤2
Vancomycin	>16	>16	1	2
Teicoplanin	>16	>16	≤0.12	0.25

- A linezolid MIC value of 4 µg/ml was also extremely rare among 7,889 *S. aureus* (0.07%) and 4,354 *S. pneumoniae* (0.02%) strains tested, and was not observed among CoNS (2,671 strains tested), ß-haemolytic (1,097) or viridans group streptococci (633; Table 1).
- The *E. faecium* strains with a linezolid MIC value of 4 µg/ml also had resistances to most antimicrobial agents tested, including vancomycin and teicoplanin; while the *E. faecalis* strains were usually susceptible to glycopeptides and ampicillin (Table 3).

Figure 1. Linezolid MIC distributions among Gram-positive isolates evaluated by the ZAAPS Program (2002 - 2004).

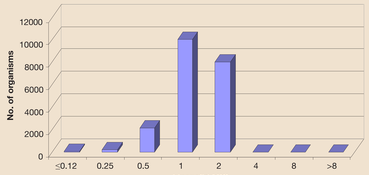
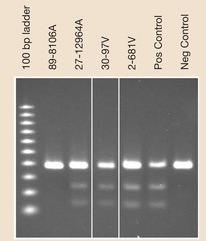


Figure 2. Electrophoresis of PCR products after digestion with Nhe I, which cuts at the G2576T mutation site.



CONCLUSIONS

- Increased linezolid MIC values (> 2 µg/ml) remain extremely rare among clinical strains of Gram-positive monitored by the ZAAPS Program.
- The G2576T gene mutation may be present on enterococcal isolates with an intermediate (4 µg/ml) linezolid MIC value.
- Continued surveillance appears prudent, especially in nations where linezolid use has escalated due to emerging or endemic multidrug-resistant Gram-positive pathogens.

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