In vitro Antimicrobial Activity of Linezolid Tested Against Vancomycin-Resistant Enterococci Isolated in Brazilian Hospitals

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The emergence of vancomycin-resistant enterococci (VRE) has been described recently in Brazil. This is in contrast to the USA and Europe, where the VRE appeared in the late 1980s. The progressive increase in VRE isolation poses important problems in the antimicrobial therapy of nosocomial infections. Treatment options and effective antimicrobial agents for VRE are often limited and the possibility of transfer of vancomycin genes to other Gram-positive microorganisms continues. In the search for antimicrobial agents for multiresistant Gram-positive cocci, compounds such as linezolid and quinupristin/dalfopristin have been evaluated. The present study was conducted to evaluate the *in vitro* activity of the oxazolidinone linezolid and 10 other antimicrobial agents, including quinupristin-dalfopristin, against multiresistant enterococci isolated in Brazilian hospitals. Thirty-three vancomycin resistant isolates (17 *Enterococcus faecium* and 16 *E. faecalis*), were analyzed. Strains were isolated from patients at São Paulo Hospital, Oswaldo Cruz Hospital, Hospital do Servidor Público Estadual, Santa Marcelina Hospital, Santa Casa de Misericórdia de São Paulo, and Hospital de Clínicas do Paraná. The samples were tested by a broth microdilution method following the National Committee for Clinical Laboratory Standards (NCCLS) recommendations. All isolates were molecular typed using pulsed-field gel electrophoresis (PFGE). Linezolid was the most active compound against these multiresistant enterococci, showing 100% inhibition at the susceptible breakpoints. Quinupristin/dalfopristin and teicoplanin showed poor activity against both species. The molecular typing results suggest that there has been interhospital spread of vancomycin resistant *E. faecium* and *E. faecalis* among Brazilian hospitals. The results of this study indicate that linezolid is an appropriate therapeutic option for the treatment of vancomycin-resistant enterococci infections in Brazil.
<u>**Key words**</u>**: Linezolid, vancomycin-resistant enterococci, quinupristin/dalfopristin-resistant *Enterococcus faecium*, clonal dissemination, nosocomial infection.**

Enterococci are considered opportunistic pathogens that frequently cause infections in patients hospitalized for a long period of time and/or receiving multiple courses

of antimicrobial therapy [1]. According to the Centers for Disease Control and Prevention, the percentage of nosocomial enterococal infections caused by strains resistant to vancomycin increased from 0.3% in 1989, to 15% in 1996 [2]. *E. faecalis* and *E. faecium* are the 2 most common species routinely isolated by clinical microbiology laboratories [3]. Both species are intrinsically resistant to cephalosporins. Ampicillin resistance among *E. faecalis* isolates is generally rare [3]. In contrast, *E. faecium* strains usually show high rates of resistance to most antimicrobial agents [3]. Vancomycin resistance has been observed predominantly in *E. faecium* and can be as high as 47% in the USA [4].

Acquired resistance to glycopeptides in enterococci is due to the production of peptidoglycan precursor ending in the depsipeptide D-alanyl-D-lactate (D-Ala-D-Lac) instead of the dipeptide D-Ala-D-ala produced by susceptible bacteria [5]. This substitution prevents the formation of the complexes between glycopeptides and peptidoglycan precursors at the cell surface, leading to inhibition of cell wall synthesis [5]. The genes encoding this mechanism of resistance are *van*A, *van*B, *van*D, and *van*E [5,6]. Species such as *E. gallinarum, E. casseliflavus* and *E. flavescens* show low level resistance to vancomycin due to the presence of chromosomally encoded *van*C genes [5,6].

In contrast to the USA and Europe, where VRE emerged in the 1980s, the emergence of VRE was only recently described in Brazil. The first isolation of a Brazilian VRE strain occurred in 1997, in Southern Brazil [7]. More recently, several cases of VRE were described in the Santa Marcelina Hospital in São Paulo city. Most cases were described as colonization by the pathogen, rather than clinical infection. Several isolates had identical or similar PFGE patterns, suggesting clonal dissemination [8]. In addition, VRE isolates showing the same PFGE were detected in several other hospitals located in São Paulo [8]. These findings indicate that this pathogen can easily disseminate within a hospital or even among different hospitals [5].

The emergence and dissemination of VRE is of great concern since treatment options for VRE infections are often limited. In the search for therapeutic agents for serious Gram-positive infections, compounds such as linezolid and quinupristin/dalfopristin have been recently approved for clinical use in several countries for serious and life threatening infections caused by strains of vancomycin-resistant *E. faecium* and for complicated skin and soft tissue infections [9,10]. In the United Kingdom, quinupristin/dalfopristin is licensed specifically for skin and soft tissue infections, nosocomial pneumonia and for *E. faecium* infections "where no other drug is appropriate" [11]. In addition, resistance to quinupristin/dalfopristin among *E. faecium* has been observed in Latin America, especially in São Paulo [8].

Linezolid (formally U-100766) is an oxazolidinone, an antimicrobial class unrelated to other known antimicrobial agents [11-13]. This compound inhibits the formation of the initiation complex constructed with 30S ribosomes, mRNA, initiation factors IF2 and IF3, and fMet-RNA [4,13,14]. *In vitro* and *in vivo* studies have demonstrated that linezolid has significant antimicrobial activity against multiresistant Gram-positive pathogens including VRE, methicillin-resistant strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*, penicillin-resistant pneumococci, *Corynebacterium* spp. and *Listeria monocytogenes*. Anaerobes such as *Clostridium* spp., *Peptostreptococcus* spp. and *Prevotella* spp. are also susceptible to linezolid [15].

Linezolid is bacteriostatic against most susceptible organisms, but displays bactericidal activity against some strains of pneumococci, *Bacteroides fragilis,* and *C. perfringens* [15]. In addition, due to its unique mechanism of action, cross-resistance with other classes of antimicrobial agents is not described, and bacterial rate of spontaneous mutation to resistance is very low. This may translate to a low incidence of resistance during therapy [4,15].

This study was conducted to evaluate the *in vitro* activity of the oxazolidinone linezolid against vancomycin-resistant enterococci isolated in Brazilian hospitals.

Materials and Methods

Bacterial strains

Thirty-three vancomycin resistant isolates were analyzed, 17 *Enterococcus faecium* and 16 *E. faecalis*. These strains were isolated from patients hospitalized at the following hospitals: São Paulo Hospital - Federal University of São Paulo, São Paulo (8 *E. faecalis*); Oswaldo Cruz Hospital, São Paulo (4 *E. faecalis*); Hospital do Servidor Público Estadual, São Paulo (2 *E. faecalis* and 6 *E. faecium*); Santa Marcelina Hospital, São Paulo (8 *E. faecium* and 1 *E. faecalis*); Santa Casa de

Table 1. Antimicrobial activities of linezolid and reference compounds against clinical isolates of vancomycin resistant enterococci

Organism (nº)	Antimicrobial Agents	MIC (μg/mL)			
		MIC_{50}	MIC_{90}	Range	% Susceptible
Enterococcus faecalis (16)	Linezolid	2.0	2.0	1–2	100.0
	Quinupristin/ Dalfopristin	>8	>8	4->8	0.0
	Teicoplanin	>16	>16	>16	0.0
	Vancomycin	>16	>16	>16	0.0
	Ampicillin	1.5	4.0	0.5–8	100.0
	Gentamicin	>1000	>1000	>1000	0.0
	Streptomycin	≤1000	≤1000	≤1000	100.0
	Doxycycline	2.0	4.0	≤0.5-4	100.0
	Erythromycin	>8	>8	>8	0.0
	Chloramphenicol	>16	>16	8->16	18.8
	Rifampin	2.0	>2.0	2->2	0.0
Enterococcus faecium (17)	Linezolid	2.0	2.0	1-2	100.0
	Quinupristin/ Dalfopristin	4.0	>8.0	1->8	11.8
	Teicoplanin	>16	>16	>16	0.0
	Vancomycin	>16	>16	>16	0.0
	Ampicillin	>16	>16	>16	0.0
	Gentamicin	>1000	>1000	>1000	0.0
	Streptomycin	≤1000	≤1000	≤1000	100.0
	Doxycycline	≤0.5	2.0	≤0.5->4	94.1
	Erythromycin	>8	>8	>8	0.0
	Chloramphenicol	8.0	>16	2->16	64.7
	Rifampin	>2	>2	2->8	0.0

Misericórdia de São Paulo, São Paulo (2 *E. faecium* and 1 *E. faecalis*); Hospital de Clínicas do Paraná – Federal University of Paraná, Curitiba (1 *E. faecium*). Only 1 isolate per patient was included in the study. The isolates were identified to the species level using the conventional biochemical tests described by Facklam, et al. [16].

Susceptibility tests

The samples were tested by broth microdilution method following the National Committee for Clinical Laboratory Standards (NCCLS) recommendations [17]. The minimum inhibitory concentration (MIC) was determined for linezolid, quinupristin-dalfopristin,

Table 2. Antimicrobial susceptibility of clinical isolates of vancomycin resistant enterococci

Hospitals [a]		Minimum inhibitory concentration in μg/mL (category) [b]						PFGE [c]
Organism (nº)		Linezolid [d]	Q/D [d]	Ampi [d]	Doxy [d]	Chlo [d]	Rif [d]	
E. faecalis	HSP	2 (S)	>8 (R)	0.5 (S)	2 (S)	8 (S)	2 (I)	I
(16)	HSP	1 (S)	>8 (R)	1 (S)	2 (S)	>16 (R)	>2 (R)	I
	HSP	1 (S)	8 (R)	2 (S)	2 (S)	>16 (R)	2 (I)	I
	HSP	2 (S)	>8 (R)	1 (S)	2 (S)	>16 (R)	2 (I)	I
	HSP	2 (S)	>8 (R)	1.5 (S)	4 (S)	>16 (R)	>2 (R)	I
	HOC	2 (S)	4 (R)	1.5 (S)	≤0.5 (S)	8 (S)	>2 (R)	II
	HOC	1 (S)	>8 (R)	1.5 (S)	2 (S)	>16 (R)	2 (I)	II
	HOC	1 (S)	>8 (R)	1.5 (S)	4 (S)	>16 (R)	>2 (R)	II
	HOC	2 (S)	>8 (R)	0.5 (S)	4 (S)	>16 (R)	>2 (R)	II
	SM	2 (S)	>8 (R)	3 (S)	2 (S)	>16 (R)	2 (I)	II
	HSP	2 (S)	>8 (R)	6 (S)	2 (S)	>16 (R)	>2 (R)	I
	HSP	1 (S)	8 (R)	8 (S)	1 (S)	8 (S)	2 (I)	I
	HSP	2 (S)	>8 (R)	1.5 (S)	4 (S)	>16 (R)	>2 (R)	II
	HSPE	2 (S)	8 (R)	1 (S)	2 (S)	>16 (R)	2 (I)	I
	HSPE	2 (S)	>8 (R)	1 (S)	4 (S)	>16 (R)	>2 (R)	I
	SC-SP	1 (S)	>8 (R)	4 (S)	1 (S)	>16 (R)	2 (I)	I
E. faecium	HSPE	1 (S)	4 (R)	>16 (R)	≤0.5 (S)	4 (S)	>2 (R)	E
(17)	SM	2 (S)	4 (R)	>16 (R)	≤0.5 (S)	8 (S)	>2 (R)	A2
	SC-SP	2 (S)	4 (R)	>16 (R)	≤0.5 (S)	8 (S)	>2 (R)	A1
	SM	2 (S)	4 (R)	>16 (R)	≤0.5 (S)	8 (S)	>2 (R)	A1
	SM	2 (S)	4 (R)	>16 (R)	≤0.5 (S)	8 (S)	>2 (R)	A3
	SM	2 (S)	4 (R)	>16 (R)	≤0.5 (S)	8 (S)	>2 (R)	A4
	HC-UFPR	2 (S)	1 (S)	>16 (R)	4 (S)	>16 (R)	>2 (R)	D
	SM	1 (S)	1 (S)	>16 (R)	2 (S)	>16 (R)	>2 (R)	H
	HSPE	2 (S)	4 (R)	>16 (R)	1 (S)	>16 (R)	2 (I)	A1
	HSPE	2 (S)	4 (R)	>16 (R)	≤0,5 (S)	>16 (R)	>2 (R)	A2
	HSPE	2 (S)	4 (R)	>16 (R)	≤0.5 (S)	2 (S)	>2 (R)	A1
	SC-SP	2 (S)	4 (R)	>16 (R)	≤0.5 (S)	8 (S)	>2 (R)	A1
	HSPE	2 (S)	4 (R)	>16 (R)	2 (S)	4 (S)	>2 (R)	A1
	SM	2 (S)	4 (R)	>16 (R)	≤0.5 (S)	8 (S)	>2 (R)	A1
	SM	2 (S)	4 (R)	>16 (R)	≤0.5 (S)	8 (S)	>2 (R)	G
	SM	2 (S)	>8 (R)	>16 (R)	1 (S)	>16 (R)	>2 (R)	L
	HSPE	1 (S)	>8 (R)	>16 (R)	2 (S)	>16 (R)	>2 (R)	A1

[a] HSP = São Paulo Hospital; HOC = Oswaldo Cruz Hospital; HSPE = Hospital do Servidor Público Estadual; SM = Santa Marcelina Hospital; SC-SP = Santa Casa de São Paulo; HC-PR – Hospital de Clínicas do Paraná; [b] S: susceptible; I: intermediate; R: resistant. All isolates showed resistance to vancomycin (MIC, ≥256μg/mL), teicoplanin (MIC, ≥256mg/mL) erythromycin (MIC, ≥16mg/mL) and high level resistance to gentamicin (MIC, > 500mg/mL); [c] Antimicrobial Agents: Q/D = quinupristin/dalfopristin; Ampi = ampicillin; Doxy = doxycycline; Chlo = chloramphenicol; Rif = rifampin; [d] Pulsed Field Gel Electrophoresis patterns.

Figure 1. PFGE patterns of *SmaI* digested chromossomal DNA showing examples of vancomycin-resistant *Enterococcus faecalis* with PFGE patterns I (lanes 1 - 6) and II (lanes 7 - 12) from different hospitals. Patterns I and II were considered similar since they differ from each other by only two bands. λ: 48.5 Kb lambda ladder.
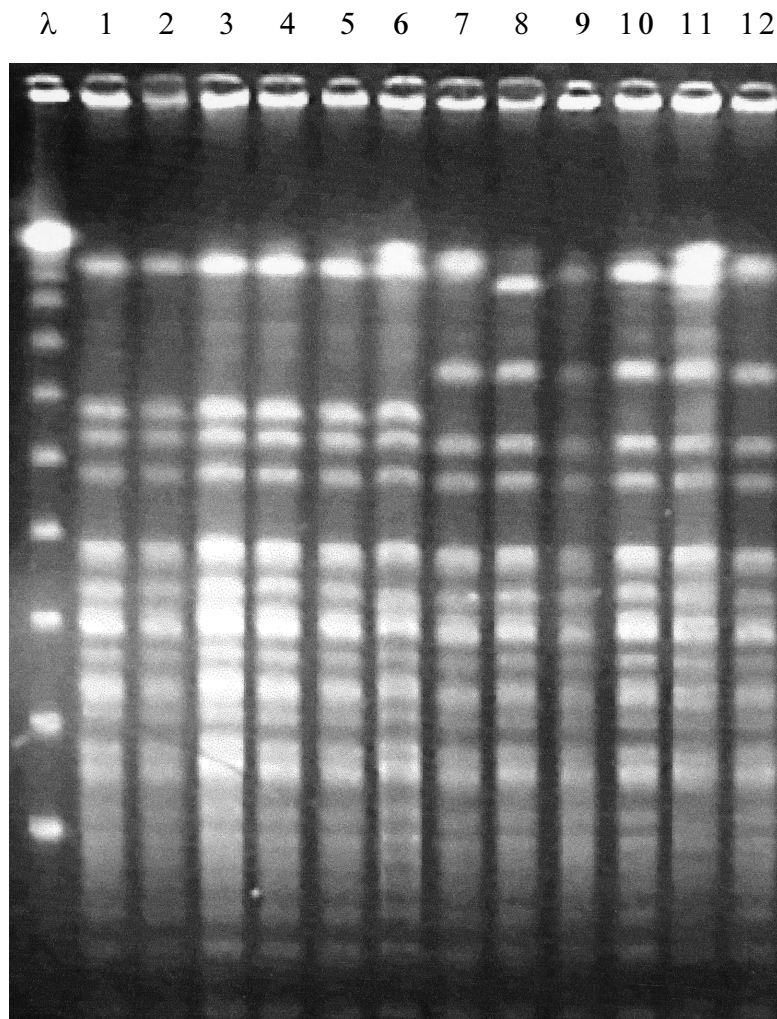
ampicillin, doxycycline, chloramphenicol, rifampin, gentamicin, streptomycin, erythromycin, vancomycin and teicoplanin [18]. Pure colonies were suspended in 5ml of Mueller-Hinton broth in order to match 0.5 Mc Farland turbidity standard. Then, 50µL were transferred to 10mL of Mueller Hinton and mixed well. Once the inoculum suspension was prepared, 100µL of this suspension were transferred to each plate well. The plates were incubated at 35°C in an ambient air incubator for 16 h to 20 h. The vancomycin results were interpreted after 24 h of incubation. The samples were categorized as susceptible, intermediate or resistant, according to susceptibility ranges established for dilution methods by the NCCLS [18].

The breakpoints for susceptibility and resistance are, respectively: quinupristin/dalfopristin at $\leq 1µg/mL$ and $\geq 4µg/mL$; rifampin at $\leq 1µg/mL$ and $\geq 4µg/mL$; chloramphenicol at $\leq 8µg/mL$ and $\geq 32µg/mL$; doxycycline at $\leq 4µg/mL$ and $\geq 16 µg/mL$; teicoplanin at $\leq 8µg/mL$ and $\geq 32µg/mL$; vancomycin at $\leq 4µg/mL$ and $\geq 32µg/mL$; ampicillin $\leq 8µg/mL$ and $\geq 16µg/mL$; erythromycin at $\leq 0.5µg/mL$ and $\geq 8µg/mL$, gentamicin at $\leq 500µg/mL$ and $> 500µg/mL$ (high level); streptomycin at $\leq 1000µg/mL$ and $>1000µg/mL$ (high level). The linezolid interpreting criteria was defined as $\leq 4µg/mL$ to susceptibility and $\geq 16µg/mL$ to resistance [19]. The quality control strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were tested along with the clinical isolates in each batch of tests.

Molecular typing

The strains were molecular typed using PFGE as described by Pfaller, et al. [20]. Restriction digestion of chromosomal DNA was performed with *Sma*I and the resultant restriction fragments were resolved in a 1% agarose gel with CHEF-DRII system (Bio-Rad Laboratories, Richmond, CA, USA). The pulsed time ramped from 5s to 30s over 23h at 13°C and 6V/cm. PFGE patterns were considered identical if they shared every band, similar if they differed from one to another by only 1 to 3 bands, and different if they differed by 4 or more bands.

Results

Table 1 shows MICs that inhibit 50% and 90% of the isolates tested (MIC50 and MIC90, respectively), MIC variation and the percentage of susceptible strains for each compound evaluated. Only linezolid inhibited all isolates of both species at the susceptible breakpoint ($\leq 4µg/mL$). The highest MIC detected for this compound was 2µg/mL. Table 2 presents the MIC results of linezolid and 10 other antimicrobial agents against all isolates evaluated.

As expected, quinupristin/dalfopristin showed poor *in vitro* activity against *E. faecalis* with 100% of strains showing resistance to this compound [3]. Among *E. faecium,* 15 of 17 isolates (88.2%) were resistant to quinupristin/dalfopristin.

High level resistance to streptomycin was not detected among the isolates evaluated and doxycycline showed excellent *in vitro* activity against both species (94.1% susceptibility among *E. faecium* and 100% susceptibility among *E. faecalis*). In contrast, high level resistance to gentamicin was observed in both species with 100% of resistance.

Chloramphenicol showed moderate activity against *E. faecium*, with 64.7% of susceptibility, but poor activity against *E. faecalis* (81.2% resistance) (Table 2).

Ampicillin was very active against *E. faecalis* with 100% susceptibility, but none of the *E. faecium* isolates were susceptible to ampicillin.

The molecular pattern of the strains are shown on Table 1. Of the 16 *Enterococcus faecalis* submitted to molecular typing, 10 strains presented a unique molecular pattern, which was called I. The 6 remaining isolates also showed a unique molecular pattern distinct from pattern I and was called II. Twelve of the 17 *Enterococcus faecium* (71%) strains showed a unique major pattern which was called A. This pattern presented 4 subtypes A1, A2, A3 and A4. The 5 remaining strains had distinct PFGE patterns, which were called D, G, H, E, and L.

Discussion

Linezolid was the most active compound against our collection of multiresistant enterococci. It was the only antimicrobial agent active against all isolates at the susceptible breakpoint (100% susceptibility) (Table 1). These data are in agreement with other published studies which show that the oxazolidinones represent a unique class of synthetic antimicrobials that maintain *in vitro* activity against multiresistant Gram-positive cocci, including vancomycin-resistant enterococci [15]. In addition to its excellent *in vitro* activity, *in vivo* studies with mice have shown results comparable to that of vancomycin for experimental systemic and soft tissue infections due to *Enterococcus* sp. [13]. Resistance and cross resistance with antimicrobial inhibitors of protein synthesis, such as chloramphenicol, macrolides, lincosamides, streptogramins, aminoglycosides, and tetracyclines, have not been demonstrated [4,21].

As expected, the streptogramin combination quinupristin/dalfopristin showed poor activity against *E. faecalis* [3]. However, the compromised activity of this compound (88.2% resistance) against *E. faecium* must be emphasized and has also been reported in Western Europe [22]. Resistance to quinupristin/dalfopristin has been associated with the presence of *sat*A and *sat*G genes, which encode acetyltransferases that hydrolyze streptogramin A. Both genes are related to the acetyltransferase genes *vat*, *vat*B, and *vat*C reported in staphylococci [10]. Resistance to streptogramin B has been related to the presence of *vgb*, *msr*A, and *erm*B genes. All three genes have been reported among staphylococci isolates, but only the *erm*B gene has been described among enterococci. The *erm*B gene is widespread among enterococci isolates and confers cross-resistance with macrolides and lincosamines due to modification in the ribosomal target [10].

Since most antimicrobial agents exhibit poor *in vitro* activity against enterococci, the options for the treatment of severe infections is generally restricted either to a glycopeptide or a β-lactam associated to an aminoglycoside. In our study, as shown in Table 1, among the aminoglycosides that can be used to treat systemic enterococcal infections, only streptomycin showed adequate *in vitro* activity against the vancomycin resistant strains. High level resistance to streptomycin was not detected among the isolates evaluated. However, all isolates showed high level resistance to gentamicin.

Until recently, the epidemiology of enterococcal infections was poorly understood due to a lack of discriminatory typing methods. However, the application of molecular methods to clusters of glycopeptide-resistant enterococci has provided a greater insight into this problem, and, recently, PFGE has been shown to be useful for epidemiological evaluations of nosocomial enterococcal infections [5,23]. Although there have been a number of sporadic cases of infection caused by glycopeptide-resistant enterococci, most reports have described clusters of cases involving either a single or multiple strains of *E. faecalis* or, in particular, *E. faecium* [5].

The spread of a single strain of VRE has been reported from a number of other centers and the majority of these clusters have been confined to a single hospital unit. However, interhospital spread of VRE has also been reported, and it was, in some instances, attributable to the transfer of patients [5,24].

The finding of several strains with identical or similar PFGE patterns in different medical centers in our study strongly suggests interhospital spread of vancomycin resistant *E. faecium* and *E. faecalis*. Further studies are needed to investigate the mode of dissemination of these multiresistant strains.

Until recently, the treatment options for patients infected with VRE were extremely limited [4,25]. Only small and/or uncontrolled clinical trials and anecdotal case reports evaluated therapeutic options for VRE infections, including doxycycline, rifampicin, or chloramphenicol, alone and in various combinations [3, 25].

In addition to the excellent *in vitro* activity against VRE isolates shown in the present study and in several other investigations, intravenous and oral linezolid has produced high rates of clinical sucess in clinical trials involving hospitalized patients with skin or soft tissue pneumonia [15]. Thus, this study presents *in vitro*

information that will be very helpful to determine the role of linezolid in the treatment of infections caused by multiresistant enterococci in Brazil.

Acknowlededgments

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