Molecular Epidemiology and Antimicrobial Susceptibility of *Enterococci* Recovered from Brazilian Intensive Care Units

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We studied the antimicrobial resistance and the molecular epidemiology of 99 enterococcal surveillance isolates from two hospitals of Brasília, Brazil. Conventional biochemical tests were used to identify the enterococcal species and the disk diffusion method was used to determine their resistance profiles. *Enterococcus faecalis* (76%) and *E. faecium* (9%) were the most prevalent species. No enterococci showed the vanA or vanB vancomycin resistance phenotypes or genotypes. Only the intrinsically resistant species *E. gallinarum* (n=2) and *E. casseliflavus* (n=3) harbored the vancomycin-resistance genes vanC1 and vanC2/3, respectively. We found *E. faecalis* isolates with high-level resistance to gentamicin (22%) and streptomycin (8%) and both *E. faecalis* and *E. faecium* isolates with resistance to more than two antimicrobials (84% and 67%, respectively). Nine *E. faecalis* isolates (12%) were resistant to ampicillin; the minimal inhibitory concentration (MIC) values were 16μg/mL (n=6) and 32μg/mL (n=3). Among these ampicillin-resistant *E. faecalis*, seven were also resistant to gentamicin, ciprofloxacin, rifampin, penicillin, chloramphenicol, tetracycline and erythromycin. Pulsed-field gel electrophoresis classified those isolates in three different genotypes, suggesting dissemination of genetically related ampicillin-resistant *E. faecalis* strains among different patients.

Key Words: Enterococci, antimicrobial resistance, genotyping.

Enterococci are opportunistic pathogens; they have been recognized as an important cause of infective endocarditis for almost a century. In addition, these bacteria have also been recovered from urinary, wound, and bloodstream infections [1]. The intrinsic (e.g., against cephalosporins and semisynthetic penicillinase-resistant penicillins) and acquired (e.g., against aminopenicillins and glycopeptides) resistance to antibiotics is a subject of considerable concern [2,3]. First reported in Europe in 1987, vancomycin-resistant enterococci (VREs) have received increasing attention since the late 1980s, when a rapid rise in the numbers of nosocomial infections was reported by the Center for Disease Control and Prevention – CDC [4]. The bacteria spread throughout the world, causing hospital outbreaks of enterococcal infection and colonization [5]. Several studies demonstrated that elevated prevalences of VRE in health-care settings can compromise the control of VRE dissemination, reinforcing the importance of surveillance and early

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detection [3]. Since vancomycin-resistant *E. faecium* and *E. faecalis* have already been isolated in other regions of Brazil [6-13], we conducted a surveillance study in Brasília city to determine the prevalence of vancomycin resistance, the general antimicrobial resistance profiles and the molecular epidemiology of ampicillin-resistant *E. faecalis* isolates.

Materials and Methods

Bacterial isolates

A total of 99 enterococci were isolated from rectal swabs from patients of intensive care units (ICU) of two hospitals of Brasília (University Hospital of Brasília and Santa Luzia Hospital), Brazil, during a two-year period (2000-2001).

Identification of bacterial isolates

Enterococci were identified on the basis of the following criteria: Gram-positive staining, growth on bile-aesculin agar and in 6.5% NaCl broth, absence of catalase and presence of pyrrolidonyl arylamidase (PYR Test Probac, Brazil). Species-level identification was performed by standard biochemical tests: formation of acid in mannitol, sorbitol, sucrose, arabinose, raffinose, pyruvate and sorbose broth, pigmentation, motility, growth on tellurite agar, and arginine hydrolysis [14].

Susceptibility testing

The isolates were subjected to disk diffusion antimicrobial susceptibility testing (Kirby-Bauer) according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines [15]. The antibiotics (Oxoid, Basingstoke, UK) tested were ampicillin, vancomycin, teicoplanin, ciprofloxacin, tetracycline, chloramphenicol, penicillin, erythromycin, rifampin; high-level resistance to gentamicin and streptomycin was also assessed [16]. Isolates with intermediate levels of susceptibility were classified as resistant. The ampicillin-resistant isolates were subjected to broth microdilution susceptibility testing to determine the minimal inhibitory concentration (ampicillin, Sigma, St. Louis, Mo., USA), based on NCCLS guidelines [16,17]. The density of the inoculum was first adjusted by suspending colonies in 0.9% saline to the 0.5 McFarland turbidity standard. The bacterial suspension was diluted 1:100 in cation-adjusted Mueller-Hinton broth to obtain the desired final inoculum concentration of colony-forming units (5 x 10^5 CFU/mL) in each well of the microdilution trays. Colony-forming units on inoculum suspensions were determined for each batch of tests. The plates were incubated at 35°C for 16 to 20 hours.

Quality control

Staphylococcus aureus ATCC 25923 and *E. faecalis* ATCC 29212 were used as quality control reference strains according to NCCLS guidelines. We performed quality control tests for the disks, disk diffusion media and microdilution trays, using the reference strains. In addition, the quality control strains were tested along with the bacterial isolates in each antimicrobial susceptibility test batch [15-17].

Pulsed-field gel electrophoresis (PFGE)

The bacterial genomic DNA was digested with *Sma I* and the PFGE assay was performed according to previous publications [18]. The samples were electrophoresed on a BioRad CHEF mapper (block 1: run-time 10 h, switch time: 0.5 - 15 s and block 2: run-time 8 h, switch time: 15-30 s, 6 V/cm, temperature 14ºC). Gels were stained with ethidium bromide and photographed under UV irradiation. Isolates were differentiated by visual inspection, and they were classified according to generally accepted criteria [19].

Multiplex PCR

The multiplex PCR scheme that we developed allows the simultaneous identification of enterococcal species and vancomycin-resistance genes. The

multiplex PCR assays were performed on material from colonies touched with a sterile tip after overnight growth on a blood agar plate. For each reaction, five to ten colonies were suspended in 25 μL of a PCR mixture containing the six pairs of primers described below: 10 mM Tris-HCl (pH 8.3); 50 mM KCl, 3.0 mM $MgCl_2$; 0.25 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP); and 2 U of *Taq* DNA polymerase. The oligonucleotide primers used in the PCR reaction, named according to the gene detected, were: $ddl_{E.\ faecalis}$, $ddl_{E.\ faecium}$, vanC1, vanC2/3 (18.0 pmol of each primer), vanA (3.0 pmol of each primer) [20], and vanB (1.5 pmol of each primer) [8]. The tubes were overlaid with two drops of mineral oil. PCR amplification was carried out with the following program: initial denaturation step at 94ºC for 5 min, 30 cycles of amplification (denaturation at 94ºC for 1 min, annealing at 52ºC for 1 min, extension at 72ºC for 2 min), and a final extension at 72ºC for 5min in a DNA thermocycler PTC-100 (MJ Research, Inc, Waltham, MA, USA). Fifteen-microliter samples of the PCR products were electrophoresed through a 1% agarose gel stained with ethidium bromide for 1 hour at 60 V. The gel was photographed under UV light.

Results

Amongst the 99 isolates of *Enterococcus spp*., 75 were identified by biochemical tests as *E. faecalis* (76%), 9 as *E. faecium* (9%), and 15% as other species (five *E. hirae*, four *E. raffinosus*, two *E. gallinarum*, three *E. casseliflavus*, and one *E. avium*) (Table 1).

In vitro susceptibility patterns of the isolates were determined (Table 1). None of the enterococcal isolates were resistant to vancomycin or teicoplanin. Ampicillin-resistance was detected in *E. faecalis* (12%) and absent in *E. faecium*. In total, 28% of the *E. faecalis* and 11% of the *E. faecium* were resistant to penicillin. The high-level aminoglycoside resistance phenotype (HLAR) and chloramphenicol resistance levels differed between *E. faecalis* and *E. faecium* strains. Although none of the *E. faecium* isolates expressed HLAR, 22% and 8% of the *E. faecalis* were highly resistant to gentamicin and streptomycin, respectively. A total of 37% of the *E. faecalis* strains were resistant to chloramphenicol, as opposed to none of the *E. faecium* strains.

The number of *E. faecalis* and *E. faecium* isolates resistant to ciprofloxacin (79% and 56%, respectively), rifampin (91% and 67%, respectively), tetracycline (71% and 78%, respectively), and erythromycin (92% and 89%, respectively) were high. Also, high prevalences of resistance to two or more antimicrobials were found in the *E. faecalis* and *E. faecium* isolates (84% and 67%, respectively). In addition, seven ampicillin-resistant *E. faecalis* isolates were also resistant to gentamicin, ciprofloxacin, rifampin, penicillin, chloramphenicol, tetracycline and erythromycin.

Among the other enterococcal species, none exhibited resistance to vancomycin, teicoplanin, gentamicin, streptomycin or chloramphenicol. Resistance to ampicillin (7%), ciprofloxacin (43%), rifampin (29%), penicillin (21%), tetracycline (43%) and erythromycin (29%) was found.

To evaluate the ability of the multiplex PCR reaction to amplify the DNA targets, control strains *E. faecium*, *E. faecium* (vanA), *E. faecalis*, *E. faecalis* (vanA), *E. faecalis* (vanB), *E. gallinarum* (vanC1), and *E. casseliflavus* (vanC2) were first tested. The expected PCR products were observed for all the control strains (Figure 1). In a second step, multiplex PCR was performed on every isolate (Figure 1). The results obtained from PCR and from phenotypic assays showed a high rate of agreement for all the species, as follows: *E. faecalis* (95%), *E. faecium* (95%), *E. gallinarum* (100%) and *E. casseliflavus* (100%). As none of the enterococcal isolates were resistant to the glycopeptide antibiotics, vancomycin and teicoplanin, PCR products for vanA and vanB genes were not found. As expected, no PCR products were obtained for *E. hirae*, *E. raffinosus*, and *E. avium* because of the absence of specific primers for these species.

The nine ampicillin-resistant *E. faecalis*, detected by the disk diffusion method, showed minimal inhibitory concentrations (MIC) for ampicillin ranging from 16

Table 1. Antimicrobial resistance profile among *Enterococcus* spp.

Species (no. of isolates; %)	Antimicrobial agent	Nº of resistant enterococci isolates[a]	%
E. faecalis	Vancomycin	0.0	
(75; 75,8)	Teicoplanin	0	0.0
	Ampicillin	9	11.8
	Gentamicin[b]	17	22.4
	Streptomycin[b]	6	7.9
	Ciprofloxacin	60	78.9
	Rifampin	69	90.8
	Penicillin	21	27.6
	Chloramphenicol	28	36.8
	Tetracycline	54	71.1
	Erythromycin	70	92.1
E. faecium	Vancomycin	0	0.0
(9; 9,1)	Teicoplanin	0	0.0
	Ampicillin	0	0.0
	Gentamicin[b]	0	0.0
	Streptomycin[b]	0	0.0
	Ciprofloxacin	5	55.6
	Rifampin	6	66.7
	Penicillin	1	11.1
	Chloramphenicol	0	0.0
	Tetracycline	7	77.8
	Erythromycin	8	88.9
Enterococcus spp.[c]	Vancomycin	0	0.0
(15; 15,1)	Teicoplanin	0	0.0
	Ampicillin	1	7.1
	Gentamicin[b]	0	0.0
	Streptomycin[b]	0	0.0
	Ciprofloxacin	6	42.9
	Rifampin	4	28.6
	Penicillin	3	21.4
	Chloramphenicol	0	0.0
	Tetracycline	6	42.9
	Erythromycin	4	28.6

[a]Isolates with intermediate levels of susceptibility were classified as resistant.
[b]High-level resistance to gentamicin, streptomycin.
[c]Includes five *E. hirae*, four *E. raffinosus*, three *E. casseliflavus*, two *E. gallinarum*, and one *E. avium*.

Figure 1. Agarose gel electrophoresis of multiplex PCR from enterococci isolates. The PCR products of the control strains are shown in gel A, lanes: (1) *Enterococcus faecalis* ATCC 29212 (941 bp); (2) *E. faecalis* van-B ATCC 51299 (941bp e 300bp); (3) *E. faecalis* van-A A256 (941bp e 732bp); (4) *E. faecium* (550bp); (5) *E. faecium* van-A (550bp e 732bp); (6) *E. casseliflavus* (439bp); (7) *E. gallinarum* ATCC 12359 (822bp). The PCR products of the isolates recovered from the individuals are shown in gel B, lanes: (1) *E. faecium* (550bp); (2) *E. faecalis* (941 bp); (4) *E. casseliflavus* (439bp); (5) *E. gallinarum* (822 bp). Lane (3) molecular size standard 100-bp DNA Ladder (Gibco/BRL Life Technology).

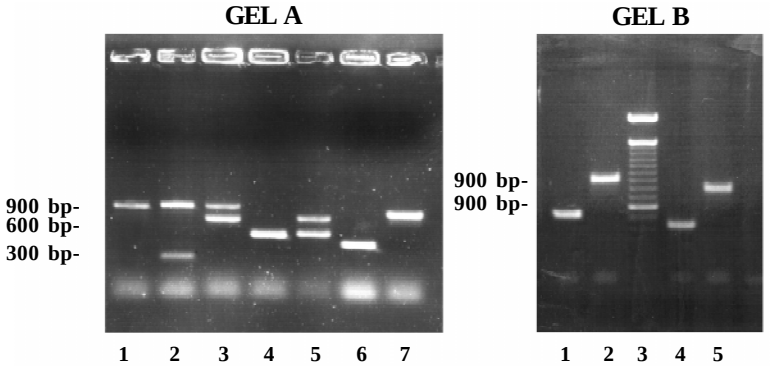
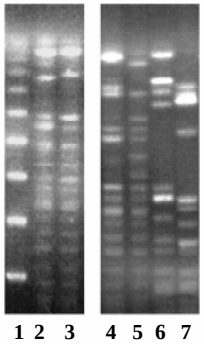


Figure 2. PFGE results of *SmaI*-digested chromosomal DNA from two ampicillin-susceptible *Enterococcus faecalis* (lanes 6-7) and four ampicillin-resistant *E. faecalis* isolates resistant to gentamicin, ciprofloxacin, rifampin, penicillin, chloramphenicol, tetracycline and erythromycin, representatives of the genotypes A, B, and C (lanes 2-5). Lanes: **1** CHEF ladder standard (Biorad, Hercules, Calif., USA); **2** and **3** genotype A; **4** genotype B; **5** genotype C; **6** genotype D; **7**, genotype E.



μg/mL (n=6) up to 32 μg/mL (n=3). Furthermore, seven isolates with resistance to ampicillin were also resistant to gentamicin, ciprofloxacin, rifampin, penicillin, chloramphenicol, tetracycline and erythromycin. These seven isolates, showing similar resistance phenotypes, were typed by PFGE and classified into genotypes named A (n=4), B (n=2), and C (n=1). Four representative strains from these genotypes and also two examples of ampicillin-susceptible *E. faecalis* with divergent genotypes (D and E) are shown in Figure 2.

Discussion

Enterococci are part of the microbial communities that colonize mammals, composing 1% of the human intestinal microbiota. Among members of the genus *Enterococcus*, *E. faecalis* and *E. faecium* are the most common species isolated from human feces, and they are the most common agents recovered from enterococcal infectious diseases [3]. In our investigation, *E. faecalis* (76%) and *E. faecium* (9%)

were the most prevalent species colonizing the gastrointestinal tract of patients from ICUs of two hospitals of Brasília, Brazil. Similar results were obtained for clinical isolates in other studies from Brazil [10,21-23] and the United States [24]. These two species were also the most prevalent enterococcal isolates when considering only ICU isolations in studies in Sweden and Lebanon [25, 26].

Enterococcal infections have received much attention after the emergence of isolates resistant to glycopeptide antibiotics [3,4]. VREs are uncommon nosocomial pathogens in Europe [27], but they have been isolated from livestock, small animals and healthy people [3,18]. In contrast, the community reservoir seems to be absent in the USA, where VREs pose an alarming problem in hospitals [5]. The first Brazilian VREs were isolated in Paraná (1996) and São Paulo (1997), with vanD and vanA phenotypes, respectively [6,7]. After that, VREs were detected in hospitals from various cities, including São Paulo, Marília, Rio de Janeiro, Uberlândia, and Porto Alegre [8-13]. Due to the geographic dimensions of Brazil, it was expected that each state would isolate VRE at different times. We found no VREs in the hospitals investigated in Brasília.

Several PCR protocols have been developed in order to identify enterococcal species and to detect glycopeptide resistance genotypes [20,28]. Woodford et al. (1997) tested a multiplex PCR method on bacterial colonies, with the same set of primers for species identification used in our study [29]. They found 95% agreement between genotypic and phenotypic methods, and they established the use of PCR to identify enterococci submitted to the Laboratory of Hospital Infection of the Central Public Health Laboratory, London, United Kingdom [29]. In our investigation, the primer concentration, annealing temperature, and amplification cycles, were carefully adjusted in order to develop a multiplex PCR assay that allows the direct suspension of bacterial colonies in the PCR mixture. The rate of agreement observed between biochemical and PCR results (95% for *E. faecalis and E. faecium*; 100% for *E. gallinarum* and *E. casseliflavus*) showed that our multiplex PCR protocol for bacterial colonies can be used for rapid and reliable identification of enterococcal species (*E. faecalis*, *E. faecium*, *E. casseliflavus* and *E. gallinarum*).

Ampicillin, in association with gentamicin or streptomycin, are the first choice drugs to treat severe enterococcal infections [3]. Therefore, in the case of HLAR, there is no synergism between the aminoglycosides and b-lactams, which compromises antibiotic therapy [1]. In our investigation, none of the *E. faecium* isolates expressed HLAR, a result that contrasts with previous publications [8,9]. However, 22% of the *E. faecalis* isolates were highly resistant to gentamicin. Investigations conducted in other countries found prevalences of gentamicin resistance in *E. faecalis* ranging from 14% to 41% [26,30,31]. In Brazil, previous publications have described resistance to gentamicin in all *E. faecalis* strains found resistant to vancomycin [8, 9]. In addition, we found that 8% of the *E. faecalis* isolates had high-level resistance to streptomycin. The streptomycin resistance prevalence among the *E. faecalis* isolated during the first Brazilian outbreak of vancomycin-resistant enterococci was higher (22%) [8]. In contrast, Reis et al. (2001) found absence of streptomycin resistance among the vancomycin-resistant enterococci isolates that they studied [9]. Stern et al. (1994) found a clear tendency towards increasing enterococcal HLAR over the years (e.g., 29%, 1985-1986; 55%, 1989-1990), which could explain the differences in prevalence described earlier [21]. Finally, previous investigations have shown that endemic and high-level aminoglycoside resistant isolates have gained vancomycin resistance genes, which has been a subject of great concern [8,10].

Resistance to ciprofloxacin, rifampin, tetracycline, and erythromycin was prevalent among *E. faecalis* and *E. faecium* isolates. Zanella et al. (2003) have described resistance to tetracycline (98%) and ciprofloxacin (96%) in Brazilian vancomycin-resistant *E. faecalis* isolates [8]. Studies conducted in several countries (e.g. Poland, South Africa, Lebanon, Kuwait, and Italy) have also found prevalence of ciprofloxacin and tetracycline resistance phenotypes among enterococci isolates [26,32-35]. In addition, the presence of resistance genes within transferable genetic structures, such as plasmids, enables

the horizontal spreading of resistance among isolates [2]. In a recent study, it was observed that mobile genetic elements account for more than a quarter of the complete *E. faecalis V583* genome and, consequently they play important roles in the acquisition and dissemination of drug resistance among enterococci [36].

Resistance to ampicillin, the drug of choice for enterococcal infections, was observed in 12% of the *E. faecalis* isolates in our investigation. The prevalence rate was near that observed during the first Brazilian vancomycin-resistant outbreak (9.4%), which occurred in São Paulo [8]. D'Azevedo et al. (2001) have also found a similar rate of resistance to ampicillin (10%) among vancomycin-resistant *E. faecalis* recovered from Porto Alegre city, in southern Brazil [37]. In contrast, ampicillin-resistance was absent among vancomycin-resistant *E. faecalis* isolates from another study [9]. A remarkable variation in the *E. faecalis* ampicillin resistance prevalence has been found in various countries, as follows: Poland (0%), Lebanon (0.9%), South Africa (1.4%), Brazil, Argentina, Chile, and Ecuador (1-7%), Croatia (5.2%), and Italy (17%) [26,32-34,38,39].

The ampicillin-resistant *E. faecalis* isolates in our investigation had MIC values ranging from 16 μg/mL to 32 μg/mL, being classified as resistant (MIC ≥ 16 μg/mL) [16]. However, some authors consider that ampicillin would be useful to treat infections (e.g. lower urinary tract infection) caused by enterococci with MIC for ampicillin ≤ 64 μg/mL [1]. This antibiotic therapy would avoid the use of glycopeptides and newer drugs (e.g. linezolid), thus reducing the selective pressure for resistant isolates [3].

Interestingly, no *E. faecium* isolates showed resistance to ampicillin in our investigation. This resistance phenotype occurred in 100% of the vancomycin-resistant *E. faecium* of the first Brazilian outbreak and among isolates recovered from different hospitals of the cities of São Paulo and Curitiba [8,9]. Data gathered from a recent investigation, as part of an international surveillance program, called GSMART (Global Synercid® Microbiologic Assessment of Resistance Trends), described the ampicillin resistance prevalence among Latin America countries, including Brazil, Argentina, Chile, Ecuador and Venezuela [38]. We considered this a representative study of the Brazilian enterococcal resistance profiles, because it included isolates from four different cities (São Paulo, Rio de Janeiro, Florianópolis and Porto Alegre). In addition, 81% of the vancomycin-resistant *E. faecium* isolates were from Brazil. In that investigation, Sader et al. (2001) found resistance to ampicillin in 100% of the vancomycin-resistant *E. faecium*, which corroborated previous publications [8,9,38]. However, most of the vancomycin-susceptible *E. faecium* were susceptible to ampicillin (72% to 84%). We also found a high prevalence of susceptibility to ampicillin (100%) among vancomycin-susceptible *E. faecium* isolates, corroborating the GSMART results.

In our study, seven ampicillin-resistant *E. faecalis* isolates showing resistance to gentamicin, ciprofloxacin, rifampin, penicillin, chloramphenicol, tetracycline and erythromycin were isolated from different UTI patients from the University Hospital of Brasília. The PFGE analysis of the seven ampicillin resistant *E faecalis* isolates with multiple resistance phenotypes revealed three different genotypes, named A, B, and C. Genotype C was represented by only one isolate. However, four and two isolates from different patients shared genotypes A and B, respectively. These endemic clones (types A and B) spread among patients during different periods of time. Previous investigations performed in several countries, including Brazil, have described intra and inter-hospital spread, as well as persistence of genetically related VREs [9,40]. In addition, vanA and vanB genes have been incorporated into endemic ampicillin-resistant vancomycin-sensitive *E. faecium* [41,42]. The long-term presence of genetically related enterococcal lineages in nosocomial wards, and the relationship between resistance and virulence are factors that drive local epidemiology and bacterial evolution [3,43]. A previous publication found both ampicillin resistance and presence of the esp gene as associated events among vancomycin-sensitive *E. faecium* isolates [44].

In conclusion, none of the enterococcal isolates from the Brasília ICUs were resistant to vancomycin or teicoplanin, and most of them remain susceptible to

ampicillin. In contrast, the prevalence of HLAR, the resistance patterns found, and the clonal dissemination of endemic ampicillin-resistant *E. faecalis* are subjects of concern. We suggest actions promoting the rational use of antibiotics in health-care settings, the execution of surveillance studies in order to monitor changes in enterococcal resistance patterns and the adoption of measures to prevent the spreading of genetically-related resistance isolates.

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