Article/Artigo

Trends in antimicrobial resistance among clinical isolates of enterococci in a Brazilian tertiary hospital: a 4-year study

Evolução da resistência aos antimicrobianos entre isolados clínicos de enterococos em um hospital terciário brasileiro: um estudo de 4 anos

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ABSTRACT

Introduction: In the past two decades members of the genus *Enterococcus* have emerged as important nosocomial pathogens worldwide. This study prospectively analyzed the distribution of species and trends in antimicrobial resistance among clinical isolates of enterococci in a Brazilian tertiary hospital from 2006-2009. **Methods:** Enterococcal species were identified by conventional biochemical tests. The antimicrobial susceptibility profile was performed by disk diffusion in accordance with the Clinical and Laboratory Standards Institute (CLSI). A screening test for vancomycin was also performed. Minimal inhibitory concentration (MIC) for vancomycin was determined using the broth dilution method. Molecular assays were used to confirm speciation and genotype of vancomycin-resistant enterococci (VRE). **Results:** A total of 324 non-repetitive enterococcal isolates were recovered, of which 87% were *E. faecalis* and 10.8% *E. faecium*. The incidence of *E. faecium* per 1,000 admissions increased significantly ($p < 0.001$) from 0.3 in 2006 to 2.3 in 2009. The VRE rate also increased over time from 2.5% to 15.5% ($p < 0.001$). All VRE expressed high-level resistance to vancomycin (MIC ≥256µg/mL) and harbored *vanA* genes. The majority (89.5%) of VRE belonged to *E. faecium* species, which were characteristically resistant to ampicillin and quinolones. Overall, ampicillin resistance rate increased significantly from 2.5% to 21.4% from 2006-2009. Resistance rates for gentamicin, chloramphenicol, tetracycline, and erythromycin significantly decreased over time, although they remained high. Quinolones resistance rates were high and did not change significantly over time. **Conclusions**: The data obtained show a significant increasing trend in the incidence of *E. faecium* resistant to ampicillin and vancomycin.

Keywords: Antimicrobial resistance profile. Enterococci. Vancomycin-resistant enterococci. *Enterococcus faecium*.

RESUMO

Introdução: Nas últimas duas décadas, os enterococos emergiram como importantes patógenos nosocomiais no mundo inteiro. Neste estudo, foi analisada a distribuição das espécies e a evolução da resistência aos antimicrobianos entre isolados clínicos de enterococos obtidos em um hospital terciário, no período de 2006 a 2009. **Métodos:** As espécies foram identificadas por testes bioquímicos convencionais e o perfil de sensibilidade foi determinado pelo método de disco difusão. A sensibilidade à vancomicina foi também determinada pela triagem em agar e pela concentração inibitória mínima (CIM). Testes moleculares foram utilizados para confirmar as espécies e determinar os genótipos dos enterococos resistentes à vancomicina (VRE). **Resultados:** Foram analisadas 324 amostras de enterococos, sendo 87% *E. faecalis* e 10,8% *E. faecium*. A incidência de *E. faecium* por 1.000 pacientes internados aumentou significativamente ($p < 0,001$) de 0,3 em 2006 para 2,3 em 2009. A taxa de VRE também aumentou significativamente de 2,5% para 15,5% ($p < 0,001$). Todos os VRE apresentaram genótipo VanA e CIM ≥256µg/mL para vancomicina. A maioria (89,5%) dos VRE pertencia à espécie *E. faecium* e foram resistentes à ampicilina e quinolonas. Foi observado um aumento significativo na taxa de resistência à ampicilina, de 2,5% (2006) para 21,4% (2009). As taxas de resistência para gentamicina, cloranfenicol, tetraciclina e eritromicina diminuíram significativamente no período do estudo. Para as quinolonas, as taxas de resistência foram elevadas não alteraram significativamente, no período do estudo. **Conclusões:** Os resultados do presente estudo mostram um aumento significativo na incidência de *E. faecium* resistentes à ampicilina e vancomicina.

Palavras-chaves: Perfil de resistência a antimicrobianos. Enterococos. Enterococos resistentes à vancomicina. *Enterococcus faecium.*

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INTRODUCTION

Enterococci are widespread in nature and are normal constituents of the human gastrointestinal tract, but nowadays they have been recognized as important pathogens, especially among hospitalized patients. Enterococci may cause a range of different disorders, such as urinary tract infections, intraabdominal abscesses, wound infections, endocarditis and bacteraemia[1]. According to the SENTRY Antimicrobial Surveillance Program, enterococci are the fourth most common pathogen of bacteremia in North America and the fifth in Europe[2]. In Brazil, they are the eighth agent of bacteremia overall and the third among the Gram-positive cocci[3].

Intrinsic or acquired resistance to various commonly used antimicrobial agents is a remarkable characteristic of enterococci[4]. Acquired resistance to glycopeptides (vancomycin and teicoplanin), penicillins and aminoglycosides (high-level resistance) are the most clinically important, because therapeutic options in these cases are limited.

Six types of acquired vancomycin resistance have been reported in enterococci; however, the most prevalent are VanA and VanB, in which the genes encoding resistance are associated with mobile genetic elements that allow resistance to spread clonally and laterally[5]. The VanC type confers an intrinsic nontransferable low-level resistance to vancomycin that has been observed primarily in *Enterococcus gallinarum* and *Enterococcus casseliflavus*.

Clinical vancomycin-resistant enterococci (VRE) isolates were first recognized in the 1980s in Europe and USA[4]. Approximately ten years later, the first VRE were isolated in Brazil, in the States of Paraná and São Paulo, located in the southern and southeastern regions of the country, respectively[6,7]. Although these VRE isolates belong to *Enterococcus faecium* species, until recently in Brazil, *Enterococcus faecalis* was the predominant VRE commonly reported in hospitals in the State of São Paulo[8-13].

Despite the increasing number of reports regarding the evolution of antimicrobial resistance of enterococci in different countries, published data on this subject are still sporadic in Brazil. In this study, our group analyzed the distribution of species and trends in antimicrobial resistance among enterococci recovered from clinical specimens in a Brazilian tertiary hospital over a four-year period. The genotypes of VRE isolates were also determined.

METHODS

Study design

A prospective study was conducted from 2006 to 2009 in the hospital of the Triangulo Mineiro University Hospital of the Federal University (*Universidade Federal do Triângulo Mineiro*, UFTM). This hospital, located in the State of Minas Gerais in southeastern Brazil, is a 294-bed tertiary-care teaching hospital with a 40-bed intensive care unit (ICU) and a full range of medical specialties. Over the study period, an average of 11,000 patients were admitted annually. All enterococci isolates recovered from hospitalized patients were included in the study, but only the first isolate from each patient was used.

Phenotypic identification of *Enterococcus* species

Isolates were identified at genus level by Gram staining, cellular morphology, absence of catalase production, hydrolysis of L-pyrrolidonyl-β-naphthylamide (PYR test), hydrolysis of esculin in presence of bile salts (bile-esculin test) and tolerance to 6.5% NaCl. Species identification was determined based on tests of carbohydrate fermentation, arginine hydrolysis, mobility, yellow pigment production and growth in 0.04% tellurite[14].

Susceptibility testing

The antimicrobial susceptibility profile was performed using the disk diffusion method. The antimicrobials agents tested were: vancomycin (30μg), teicoplanin (30μg), ampicillin (10μg), penicillin (10U), streptomycin (300μg), gentamicin (120μg), norfloxacin (10μg), ciprofloxacin (5μg), chloramphenicol (30μg), tetracycline (30μg), and erythromycin (15μg). Beta-lactamase production was tested with chromogenic nitrocefin disk (Becton, Dickinson and Company, Cefinase™, USA), in accordance with the manufacturer's instructions. A screening test for vancomycin was performed on BHI agar supplemented with 6μg/mL of this drug for all enterococcal isolates. For isolates resistant according to the screening test, the minimal inhibitory concentration (MIC) for vancomycin was determined using the broth dilution method. All susceptibility tests were performed and interpreted according to guidelines established by the Clinical and Laboratory Standards Institute (CLSI)[15]. *Staphylococcus aureus* ATCC (American Type Culture Collection) 25923, *S. aureus* ATCC 29213, and *E. faecalis* ATCC 29212 were used for quality control.

Molecular testing

Bacterial DNA was extracted from the enterococcal isolates that were phenotypically resistant to vancomycin using the QIAamp® DNA Mini kit (**Qiagen,** Hilden, Germany), in accordance with the manufacturer's guidelines. Detection of vancomycin resistance genes was performed by a multiplex polymerase chain reaction (PCR) assay, in accordance with procedures described[16] by Woodford et al. Species identification of VRE isolates was confirmed by PCR, as described previously[17]. PCR products were analyzed by electrophoresis on 1.5% agarose gels and stained by ethidium bromide.

Statistical analysis

To evaluate the trend in antimicrobial resistance among enterococci over time, the χ^2-test for trend was performed using Epi Info (CDC, Atlanta, GA) statistical software (version 3.5.1). The significance level was set at $p \leq 0.05$.

Ethical considerations

The present study was approved by Research Ethics Committee of the UFTM.

RESULTS

A total of 324 non-repetitive enterococcal isolates were consecutively recovered during the study period. These isolates were recovered from different clinical specimens, but they were more frequent in wounds (38.9%) and in urine (29.9%). **Table 1** shows the distribution of enterococcal species according to clinical specimens. The species identified were *E. faecalis* (87%), *E. faecium* (10.8%), *E. casseliflavus* (1.2%), *E. gallinarum* (0.3%), *E. hirae* (0.3%) and *E. pseudoavium* (0.3%).

Figure 1 shows the incidence of *E. faecalis*, *E. faecium* and other enterococcal species per 1,000 patient admissions from 2006 to 2009. A significant increasing trend in the incidence of *E. faecium* ($p < 0.001$) from 0.3 in 2006 to 2.3 in 2009 was observed, but not of *E. faecalis* or other enterococcal species over time.

The rate of VRE also increased significantly over time ($p < 0.001$), from 2.5% in 2006 to 15.5% in 2009 in our institution, although in 2007 there were no VRE and in 2008 the rate was only 1.4% **(Table 2)**. Among the 19 VRE isolates, 2 (10.5%) were identified phenotypically and by PCR as *E. faecalis* (VREfs) and 17 (89.5%)

TABLE 1 - Distribution of *Enterococcus* species isolated from hospitalized patients from 2006 to 2009, according to clinical specimens.

	Clinical specimens													
	wounds		urine		secretions		blood		catheter		other		Total	
Species	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Enterococcus faecalis	117	41.5	80	28.4	45	16.0	19	6.7	14	5.0	7	2.5	282	87.0
Enterococcus faecium	6	17.1	16	45.7	9	25.7	4	11.4	-	-	-	-	35	10.8
Enterococcus casseliflavus	2	50.0	-	-	2	50.0	-	-	-	-	-	-	4	1.2
Enterococcus gallinarum	-	-	-	-	-	-	-	-	-	-	1	100.0	1	0.3
Enterococcus hirae	-	-	1	100.0	-	-	-	-	-	-	-	-	1	0.3
Enterococcus pseudoavium	1	100.0	-	-	-	-	-	-	-	-	-	-	1	0.3
Total (%)	**126**	**38.9**	**97**	**29.9**	**56**	**17.3**	**23**	**7.1**	**14**	**4.3**	**8**	**2.5**	**324**	**100.0**

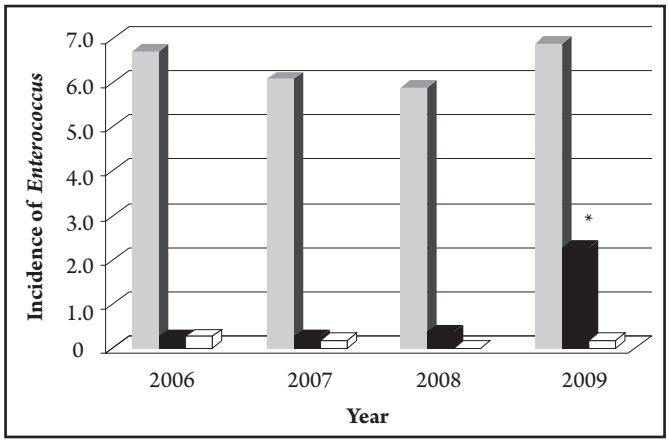

FIGURE 1 - Incidence of *Enterococcus faecalis* (gray bars), *Enterococcus faecium* (black bars) and others species of *Enterococcus* (white bars) per 1,000 patient admissions from 2006 to 2009. *χ^2 for trend $p < 0.001$.

as *E. faecium* (VREfm). The first two VREfs were isolated in 2006. Thereafter, no other VREfs were isolated during the study period. The first VREfm appeared at the end of 2008, since then an increasing number of VREfm were noted in 2009.

All VRE isolates uniformly harbored *vanA* genes, as demonstrated by PCR. They were resistant to teicoplanin and expressed high-level resistance to vancomycin (MIC ≥256μg/mL). The two VREfs showed the same antimicrobial susceptibility profile characterized by resistance to norfloxacin, ciprofloxacin, chloramphenicol, tetracycline, and erythromycin, and susceptibility to ampicillin, penicillin, streptomycin, and gentamicin. All VREfm were resistant to ampicillin, penicillin, norfloxacin, ciprofloxacin, and erythromycin and susceptible to streptomycin, gentamicin, chloramphenicol, and tetracycline.

Enterococcus gallinarum (n = 1) and *E. casseliflavus* (n = 4) isolates were susceptible to vancomycin by the disk diffusion method, but grew on agar screening with 6μg/mL of vancomycin, while the MIC observed for this drug was 8μg/mL. These five isolates were not considered as VRE in this study.

As demonstrated in **Table 2**, ampicillin and penicillin resistance rates increased from 2006 to 2009 from 2.5% to 21.4% and 23.8% to 35.9%, respectively, but only the increasing rate of ampicillin was statistically significant ($p < 0.001$). Among the 282 *E. faecalis* and 35 *E. faecium* isolates, 4 (1.4%) and 25 (77.4%) of them, respectively, were resistant to ampicillin and to penicillin. However, 63 (22.3%) *E. faecalis* isolates were resistant to penicillin, but remained susceptible to ampicillin. Beta-lactamase producing isolates were not detected.

Rates of resistance to gentamicin (41.2% to 25.2%), chloramphenicol (55% to 29.1%), tetracycline (75% to 57.3%), and erythromycin (95% to 80.6%) significantly decreased from 2006 to 2009, as demonstrated in **Table 2**. Regarding streptomycin, norfloxacin and ciprofloxacin, the resistance rates did not change significantly over the four-year study period.

TABLE 2 - Trends in antimicrobial resistance among clinical isolates of enterococci recovered from hospitalized patients from 2006 to 2009.

	Resistant isolates per year (number/%)								
	2006 (n = 80)		2007 (n = 72)		2008 (n = 69)		2009 (n = 103)		
Antimicrobial agent	n	%	n	%	n	%	n	%	p-value
Vancomycin	2	2.5	-	-	1	1.4	16	15.5	< 0.001
Teicoplanin	2	2.5	-	-	1	1.4	16	15.5	< 0.001
Ampicillin	2	2.5	2	2.8	3	4.3	22	21.4	< 0.001
Penicillin	19	23.8	18	25.0	19	27.5	37	35.9	0.059
Streptomycin	24	30.0	13	18.1	22	31.9	28	27.9	0.861
Gentamicin	33	41.2	21	29.2	22	31.9	26	25.2	0.037
Norfloxacin	60	75.0	39	54.2	45	65.2	66	64.1	0.351
Ciprofloxacin	62	77.5	37	51.4	45	65.2	63	61.2	0.124
Chloramphenicol	44	55.0	33	45.8	25	36.2	30	29.1	< 0.001
Tetracycline	60	75.0	49	68.1	39	56.5	59	57.3	0.006
Erythromycin	76	95.0	65	90.3	59	85.5	83	80.6	0.002

DISCUSSION

Although there are at least 30 species of the genus *Enterococcus*, both *E. faecalis* and *E. faecium* are the most common species causing human infections[1,14]. Similarly, in this study, *E. faecalis* was the most prevalent species followed by *E. faecium*, while the other species were rarely recovered from clinical specimens. Nevertheless, a significant increasing trend in the incidence of *E. faecium* per 1,000 patient admissions from 0.3 in 2006 to 2.3 in 2009 was verified, approximately an eight-fold increase. In a study conducted in a hospital in Greece, the authors reported a similar increased incidence of *E. faecium* infections (0.7 in 2002 to 2.4 in 2007), although that hospital is larger than ours, with more than 60,000 admissions annually[18].

The vancomycin-resistant enterococci rate also increased in our institution, by almost the same proportion as the incidence of *E. faecium* incidence. Most (89.5%) of the VRE isolates were *E. faecium* exhibiting the *vanA* genotype. In a retrospective study conducted at a tertiary Brazilian hospital located in the State of São Paulo, Furtado *et al.* also showed an increase in the rate of VRE over time from 9.5% in 2000 to 14.7% in 2001 and to 15.8% in 2002. However, the authors did not identify the VRE species or genotypes[19]. According to more recent data from the SENTRY Program, the percentage of VRE in Brazil increased from 6.9% in 2003 to 31.1% in 2008 and the majority (68.5%) of these isolates were *E. faecium*[20]. These VRE rates are much higher than that observed in other Latin America countries and in our hospital.

In the last two decades, the importance of *E. faecium* as a nosocomial pathogen has increased throughout the world due to

the greater ability of this species to acquire resistance to multiple drugs than *E. faecalis.* Molecular epidemiological studies have reported the spread of a hospital-adapted complex of *E. faecium* designated as clonal complex-17 (CC-17), which is associated with the majority of hospitals outbreaks and clinical infections on all continents[21,22]. This complex is characterized by ampicillin and quinolones resistance and by the presence of a putative pathogenicity island. Currently, the increase in *E. faecium* resistant to ampicillin usually precedes increasing rates of VREfm in various locations around the world, especially in certain European countries, where until recently, VRE rates were low[4,23-26]. In the USA, since the 1990s, *E. faecium* isolates account for more than 95% of all VRE recovered and most of them are also resistant to ampicillin[24].

Likewise, our group observed a significant increasing trend in resistance to ampicillin among clinical isolates of enterococci due to increased incidence of *E. faecium* in our institution, since resistance rates to this drug among *E. faecium* (71.4%) were much higher than among *E. faecalis* (1.4%) isolates. Of note, all VREfm isolated in this study were resistant to both ampicillin and quinolones, showing the same antimicrobial resistance profile observed for *E. faecium* of CC-17. Nevertheless, molecular epidemiological studies conducted with Brazilian *E. faecium* isolates from several hospitals showed that this complex is not common in our country[27,28].

In contrast to trends in ampicillin resistance, a significant decrease in resistance rates to chloramphenicol and tetracycline occurred from 2006 to 2009 and it is probably related to the increasing incidence of *E. faecium* in the present study. Decreasing rates of chloramphenicol resistance among enterococci has previously been observed from 1997 to 1999 in the USA (19% to 12%) and Latin America (34% to 27%), according to the SENTRY Program[29]. In addition, all VREfm isolates during this study were uniformly susceptible to both drugs. A similar chloramphenicol resistance rate among VREfm of 0.5% was observed in North America[21]; however, chloramphenicol use for the treatment of VRE infections is known to result in the development of resistance[30].

Regarding tetracycline, although the enterococci resistance rate decreased over time, it remained high in 2009 (57%), but was slightly lower than that reported by the SENTRY Program for Latin America (67.2%)[31] and by other Brazilian studies performed with clinical enterococci strains isolated in 1996 and 1997 (62%)[32] and in 2006 and 2007 (66.5%)[8]. Likewise, the resistance rates to erythromycin were very high, despite the significant decrease observed (95% to 80.6%), confirming that resistance to this drug is very common among enterococci[1,8,31,33].

Overall, moderate rates of high-level resistance to aminoglycosides were observed over the four-year, similar to those observed recently in Brazil of 32.1% and 26.7% and in the USA of 30.6% and 25.2% for streptomycin and gentamicin, respectively[3,34]. Regarding quinolones, resistance rates did not change significantly during the study period, whereas the overall resistance rates to ciprofloxacin were slightly higher compared to Latin America (50%) and the USA (58%)[29,31].

In conclusion, the present data show an increasing incidence of *E. faecium* resistant to ampicillin and vancomycin, corroborating the worldwide trends. Considering that all the VREfm identified in our institution expressed resistance to ampicillin and quinolone, it is quite probable that they belong to the hospital-adapted CC-17. However, future molecular characterization is necessary to verify this, since there are no studies demonstrating the spread of *E. faecium* CC-17 in Brazil to date. Therefore, more rigorous strategies should be developed in order to prevent further spread of *E. faecium* in Brazil.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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