Original Article

Emergence of *vanA* vancomycin-resistant *Enterococcus faecium* in a hospital in Porto Alegre, South Brazil

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Abstract

Introduction: In Porto Alegre (South Brazil), since the first VRE isolation in 2000 until the middle of the last decade, the epidemiology of enterococcal infections presented the peculiarity that, as opposed to other regions of the country, almost all VRE were *E. faecalis*. The aim of this study was to investigate the microbiological and epidemiological characteristics of a VRE outbreak that occurred between August 2010 and September 2011 in Porto Alegre, South Brazil.

Methodology: Twenty-nine isolates from inpatients of Mãe de Deus Hospital that were identified and characterized for their susceptibility profile, vancomycin genotype, presence of *esp* gene, biofilm production, and clonal relationship were collected. Patients' records were reviewed for clinical information.

Results: All isolates were identified as vancomycin/ampicillin resistant *E. faecium* carrying the *vanA* gene. Almost all were susceptible to gentamicin and streptomycin. Most patients died and were associated with a hemodialysis unit stay. All but the first isolate were clustered in a main clone.

Conclusions: An important change in vancomycin-resistant enterococci was observed. Studies like this are necessary to monitor the dissemination of VRE, especially of some individual clones.

Key words: vancomycin-resistant *Enterococcus faecium*; PFGE; VRE outbreak

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Introduction

Vancomycin-resistant enterococci (VRE) emerged in the 1970s and are now one of the leading problems in many hospitals because of their ability to colonize and cause disease in high-risk patients [1]. The capacity of enterococci to survive outside the human body for prolonged periods of time contributes to cross-contamination, through either the hands of healthcare workers, equipment, or surfaces, leading to outbreaks [2].

Over the last two decades, vancomycin-resistant *Enterococcus faecium* (VRE_{fm}) has emerged worldwide as an important cause of nosocomial infection. Molecular epidemiological studies have revealed the existence of host-specific genogroups, including a particular genetic lineage designated clonal complex 17 (CC17), associated with hospital isolates. These strains have been characterized by ampicillin and quinolone resistance. In addition, the majority of these CC17 isolates contain over one hundred hospital-clade-specific genes, including mobile elements, phage genes and plasmid sequences, hypothetical and membrane proteins, antibiotic and regulatory genes, and a putative pathogenicity island including the enterococcal surface associate protein (*esp*) gene. Although controversial, the *esp* gene seems to have an important role in biofilm production and in conjugation [3].

In Porto Alegre, a city of 1.4 million inhabitants in South Brazil, the first VRE was isolated in 2000 and characterized as an *E. faecalis* carrying the *vanA* gene [4]. While around the world, and even in other regions of Brazil, VRE outbreaks have been especially associated with *E. faecium* [5,6,7], in our city, outbreaks have been almost exclusively related to *E. faecalis*. Some non-published data from Porto Alegre demonstrated the sporadic occurrence of VRE infections/colonization from 2000 to 2009 in different health institutions of the city, but all were associated with *E. faecalis*. From 2009, an increase of VRE_{fm} was

observed. Nevertheless, until 2010, these cases were sporadic, not associated with an outbreak.

The aim of this study was to investigate the microbiological and epidemiological characteristics of a VRE_{fm} outbreak that occurred between August 2010 and September 2011, in Porto Alegre, South Brazil.

Methodology

This cross-sectional study included isolates from inpatients of Mãe de Deus Hospital (a tertiary, non-teaching hospital) obtained between August 2010 and September 2011. The MicroScan Walkaway System (Siemens, Sacramento, California) was used to identify isolates and to determine the susceptibility profiles as part of the routine of Mãe de Deus Hospital Microbiology Laboratory. Only one isolate per patient (case) was considered. For confirmatory and additional tests, all isolates were sent to the Microbiology Laboratory of the Federal University of Health Science of Porto Alegre, where they were suspended in a skim milk solution (10%) with glycerol (10%) and maintained at -20°C. Conventional phenotypic tests [8] were used to confirm previous results; genus was confirmed by morphological characteristics on Gram stain, catalase production, esculin hydrolysis in the presence of 40% of biliary salts, growth in 6.5% sodium chloride, hydrolysis of L-pyrrolidonyl-β-arylamidase (PYR), and L-Leucin-β-naftilamide. Species were determined by the observation of pigment production, motility, arginine hydrolysis, pyruvate utilization, and sugar fermentation (arabinose, mannitol, methyl-α-D-Glucopyranoside [MGP], raffinose, sucrose, sorbitol, and sorbose). E-test (bioMérieux, La-Balme-les-Grottes, France) (ampicillin, vancomycin, gentamicin and streptomycin) was used to confirm susceptibility profiles. Linezolid resistance was confirmed by determining minimum inhibitory concentration (MIC) using broth microdilution [9]. MICs were interpreted according to CLSI (2011) [9]. *Enterococcus faecalis* ATCC51299 and *Enterococcus faecium* ATCC51559 were used as control strains.

Surveillance culture, which is not routinely done in Mãe de Deus Hospital, was performed in patients in whom VRE_{fm} was recovered from clinical sites. Some patients, however, were not submitted to surveillance cultures because they died or had been discharged. To recover VRE_{fm}, rectal swabs were collected and plated onto chromID VRE (bioMérieux, La-Balme-les-Grottes, France), following the manufacturer's instructions.

The presence of vancomycin-resistant genes was evaluated by multiplex PCR[10], in which genes *vanA*, *vanB*, *van*C1, *vanC2/3*, *vanD*, *vanE*, and *vanG* were searched in a reaction containing 1.5 mM of $MgCl_2$, 0,5 µM of each primer, and 2.5 U of *Taq* DNA polymerase. Amplification occurred according to the following parameters: 94°C for three minutes and 30 cycles of 94°C for one minute, 54°C for one minute, and 72°C for one minute. Reaction ended up in a final extension step at 72°C for seven minutes. The *esp* gene was detected by conventional simplex PCR [11], and biofilm formation on polystyrene microplate was determined using the crystal violet staining method [12]. The clonal relationship among isolates was evaluated by comparing PFGE profiles of genomic DNA digested with *SmaI* [13]. Electrophoresis was performed in a CHEF DR III apparatus (Bio-Rad, Berkeley, USA), according to the following parameters: initial and final pulse of 5 and 35 seconds, respectively, with 6V/cm at 12°C for 22 hours.

Patients' records were reviewed for clinical information. Those asymptomatic patients who had a positive culture for VRE in the investigation of an infectious process were considered to be colonized, based on the criteria of the National Healthcare Safety Network (NHSN) and ANVISA [14,15].

Statistical analysis was done with SPSS version 16.0 (SPSS Inc., Chicago, USA) for Windows 7. Categorical variables were compared using the chi-squared test and Fisher's exact test when appropriate. A p-value of 0.05 was considered statistically significant. Similarity matrices and dendrograms were obtained by the unweighted pair group method using arithmetic averages (UPGMA). Similarity coefficients were calculated according to the Dice method (tolerance of 2.0% and 0% of optimization). All isolates presenting 80% or higher similarity in the dendrogram were considered clones (three or fewer differences in band patterns). Within a clone, isolates could be considered indistinguishable (identical patterns) or closely related (one to three bands of difference). Isolates closely related were considered subclones.

Results

A total of 29 isolates with vancomycin MICs higher than 256 µg/mL (all carrying the *vanA* gene) were identified as *E. faecium*. Microbiological characteristics of isolates are presented in Tables 1a and 1b. Ampicillin resistance was observed for all VRE_{fm}, as well as non-susceptibility to quinolones.

Table 1a. General microbiological characteristics of VRE_{fm} isolated during the outbreak (MICs were interpreted according to CLSI 211)

n°	Site	Antimicrobials[a]								*esp* gene	Biofilm production
		AMP	CIP	HLG	LEV	LIN	HLS	Q/D	TET		
1	Urine	R	I	S	R	S	S	I	S	+	No
2	Blood	R	R	S	R	S	S	I	S	-	No
3	Blood	R	R	S	R	S	S	S	S	+	No
4	Urine	R	I	S	R	S	S	I	S	-	No
5	Blood	R	R	S	R	S	S	S	S	+	Yes
6	Urine	R	I	S	R	S	S	S	S	+	Yes
7	Urine	R	R	S	R	R	R	R	R	+	Yes
8	Blood	R	R	S	R	S	S	S	S	+	No
9	Urine	R	R	S	R	I	S	S	S	+	Yes
10	Blood	R	I	S	R	S	S	S	S	+	No
11	Urine	R	I	S	R	S	S	S	S	+	Yes
12	Urine	R	I	S	R	S	S	S	R	+	No
13	Urine	R	I	S	R	S	S	S	S	+	Yes
14	Urine	R	I	S	R	S	S	S	S	+	No
15	Urine	R	R	S	R	S	-	R	R	+	Yes
16	Urine	R	R	S	R	S	S	S	S	+	No
17	Urine	R	I	S	S	S	S	I	S	-	No
18	Blood	R	R	S	R	S	S	I	S	-	No
19	Urine	R	I	S	R	S	S	S	S	+	Yes
20	Urine	R	R	S	R	S	S	I	S	-	No
21	Blood	R	R	S	R	S	S	S	S	+	No
22	Blood	R	R	S	R	S	S	S	S	-	No
23	Blood	R	I	S	R	S	S	I	S	-	No
24	Ascitic fluid	R	R	R	R	S	R	S	S	+	Yes
25	Blood	R	R	S	R	S	S	S	S	+	No
26	Urine	R	R	S	R	S	S	S	S	+	Yes
27	Urine	R	R	S	R	S	S	S	S	-	Yes
28	Blood	R	R	S	R	S	S	S	S	+	No
29	Urine	R	R	S	R	S	S	S	S	+	No

[a]AMP: ampicillin; CIP: ciprofloxacin; HLG: high-level gentamicin; levofloxacin; LIN: linezolid; HLS: high-level streptomycin; Q/D: quinupristin-dalfopristin; TET: tetracycline

Table 1b. Biofilm formation among VRE_{fm} isolated during the outbreak and the relationship with clinical interpretation of isolates

Site (n)	Clinical interpretation (n)	Number of biofilm producers / non producers
Urine (17)	Infection (9)	3*/6
	Colonization (8)	6*/2
Blood (11)	Infection (8)	0/8
	Colonization (3)	1/2
Ascitic fluid (1)	Infection (1)	1/0

*$p < 0.05$

The rate of high-level resistance to aminoglycoside was low; one (3.4%) and two (6.8%) isolates were resistant to gentamicin and streptomycin, respectively. Quinupristin/dalfopristin non-susceptibility occurred in nine (33.3%) isolates (seven intermediate and two fully resistant). The dominant susceptible profile (17/29; 58.6%) included resistance to ampicillin and quinolones, and susceptibility to all other antimicrobials (aminoglycosides, linezolid, quinupristin/dalfopristin and tetracycline). One VRE_{fm} (isolate 7) presented linezolid resistance, which was confirmed by broth microdilution (MIC = 32 µg/mL). The *esp* gene was detected in 72.4% (21/29) of *E. faecium* (Table 1a). Among biofilm producers (11/29; 38%), all but one were positive for the *esp* gene. However, there was no significant association between biofilm production and the presence of the *esp* gene (Table 1b). Also, no association was observed between *esp* and infection or death.

Clinical characteristics of patients are summarized in Table 2. The average age of patients was 75.2 years (ranging from 32 to 94 years; median, 78 years). Virtually all patients presented underlying diseases: heart (7/29), pulmonary (4/29), liver (4/29), and infectious (4/29) diseases were the most common (65.5%). The remaining underlying diseases included kidney, gastric, hematological, oncological, and psychiatric conditions. Seventeen (58.6%) *E. faecium* were isolated from urine, eleven (37.9%) from blood, and one from ascitic fluid. Among VRE_{fm} from blood, 72.7% (8/11) were from clinically defined bloodstream infections. Urinary tract infection was clinically characterized in 47% (8/17) of patients with VRE_{fm} grown in urine cultures. The death rate was higher among patients with bloodstream infections compared to patients with urinary tract infections. Most patients (18/29; 62%) died. Death, although not statistically significant (p = 0.2481), was more

Table 2. Clinical characteristics of patients infected with VRE_{fm} during the outbreak and clonal relationship among strains

Case	Isolation date (m/d/y)	Site	Clinical interpretation	Surveillance culture	Hemodialysis	Clonal type	Treatment	Outcome
1	08/26/2010	Urine	Colonization	None	Yes	B	Fosfomycin	Death
2	02/26/2011	Blood	Infection	Negative	No	A_2	Linezolid + nitrofurantoin	Discharged
3	02/27/2011	Blood	Infection	None	Yes	A_1	Linezolid	Death
4	03/22/2011	Urine	Infection	Negative	Yes	A_2	Linezolid	Discharged
5	03/22/2011	Blood	Colonization	Negative	Yes	A_1	Not treated	Discharged
6	03/25/2011	Urine	Colonization	Negative	No	A_1	Linezolid	Death
7	04/25/2011	Urine	Colonization	Positive	No	A_1	Fosfomycin + nitrofurantoin	Death
8	05/01/2011	Blood	Infection	Negative	Yes	A_1	Not treated	Death
9	05/02/2011	Urine	Infection	Negative	No	A_1	Not treated	Discharged
10	05/05/2011	Blood	Infection	Positive	Yes	A_1	Not treated	Death
11	05/16/2011	Urine	Infection	Negative	Yes	A_1	Linezolid	Discharged
12	05/24/2011	Urine	Infection	Positive	Yes	A_1	Linezolid	Death
13	06/06/2011	Urine	Infection	Positive	Yes	A_1	Fosfomycin	Discharged
14	06/11/2011	Urine	Infection	Positive	Yes	A_1	Linezolid	Death
15	06/19/2011	Urine	Colonization	Positive	Yes	A_5	Fosfomycin	Discharged
16	06/21/2011	Urine	Infection	Negative	Yes	A_1	Linezolid	Death
17	07/04/2011	Urine	Colonization	Positive	Yes	A_2	Not treated	Death
18	07/11/2011	Blood	Infection	None	Yes	A_2	Not treated	Death
19	07/29/2011	Urine	Infection	None	Yes	A_1	Not treated	Death
20	08/01/2011	Urine	Infection	Positive	Yes	A_2	Fosfomycin	Death
21	08/09/2011	Blood	Infection	Positive	No	A_4	Daptomycin	Death
22	08/22/2011	Blood	Colonization	Positive	Yes	A_2	Daptomycin	Hospitalized
23	08/31/2011	Blood	Infection	Positive	Yes	A_2	Daptomycin	Death
24	09/01/2011	Ascitic fluid	Infection	Positive	Yes	A_1	Daptomycin	Death
25	09/09/2011	Blood	Infection	None	Yes	A_1	Not treated	Death
26	09/23/2011	Urine	Colonization	Positive	No	A_1	Not treated	Hospitalized
27	09/23/2011	Urine	Colonization	Positive	No	A_2	Not treated	Death
28	09/25/2011	Blood	Colonization	Positive	No	A_3	Not treated	transferred
29	09/26/2011	Urine	Colonization	Positive	No	A_1	Not treated	Hospitalized

frequent among infected patients than colonized patients: 13/18 (72.2%) in infected versus 5/11 (45.4%) in colonized patients. Among all patients, surveillance culture was performed on 82.7% (24/29), which yielded positive results in 66.7% (16/24) of cases. However, association between positive surveillance culture and infection with VRE was not observed.

The average length of hospitalization was 83.6 days, ranging from 12 to 291 days. All patients had received antimicrobial therapy previously to VRE_{fm} isolation. Seventeen (58.6%) had received VRE_{fm}-specific treatment/decolonization with the following antimicrobials: fosfomycin (4/17), linezolid (7/17), daptomycin (4/17), nitrofurantoin plus fosfomycin (1/17), and nitrofurantoin plus linezolid (1/17). There was no significant association between death and any of the treatments or decolonization, not even between treated/decolonized and untreated/not decolonized patients and death. Most patients were from the hemodialysis unit (20/29; 68.9%), and this feature was significantly associated with death ($p = 0.001$). The remaining patients were from the special care (3/29), intensive care (3/29), and inpatient units (3/29). During the outbreak, patients were transferred from one unit to another (all of them cited above).

PFGE profile analysis characterized a main clone (A), which clustered all but one (first case) VRE_{fm}. Two major subclones were identified (A1 and A2). The absence of the *esp* gene was exclusively associated with subclone A2. On the other hand, aminoglycoside and linezolid resistance (isolates 7 and 24) were related to subclone A1, while most (77.8%; 7/9) quinupristin/dalfopristin non-susceptible isolates were grouped in subclone A2. Table 2 shows VRE_{fm} characteristics according to their clonal type and subtype and their relationship with the hemodialysis sector.

Discussion

We described the microbiological and epidemiological characteristics of the first (to our knowledge) VRE *vanA* outbreak associated with *E. faecium* that occurred in our city. This study characterizes an important shift in terms of epidemiological features of VRE outbreaks in Porto Alegre.

Our data, which showed that all patients had used antimicrobial therapy prior to VRE isolation and that most VRE surveillance cultures were positive, are in accordance with other studies, demonstrating that VRE colonization occurs with certain frequency after the continued use of antimicrobials [16-18]. Some studies have demonstrated that VRE infection is preceded by intestinal colonization [16,17,19]. Nevertheless, we did not find this correlation, which could be explained by the clonal characteristic of the referred outbreak, corroborating the idea of cross-spread and exogenous infection [16].

In our study, although not statistically significant, death was more frequently observed among infected patients compared to colonized ones. The relationship between VRE infection and mortality remains controversial because studies drawn to evaluate associated VRE mortality frequently present confusion bias, represented by the severe underlying conditions of patients. In this context, some authors suggest that the treatment for underlying conditions, as well as its severity, are more relevant for survival [20,21]. Indeed, we demonstrated that the VRE treatment/decolonization (occurrence and type) did not change the outcome.

However, a meta-analysis comparing mortality associated to VRE and vancomycin-susceptible enterococci demonstrated that the effect of vancomycin resistance is consistent, even after controlling for severity of illness [22]. There are several biologically plausible explanations for this fact. One explanation includes the suboptimal activity against VRE among the antimicrobials used to treat the infection. In this context, Diazgranado *et al.* (2005) [22] demonstrated that the effect of vancomycin resistance on mortality was dependent on the duration of bacteremia.

Patients undergoing hemodialysis are at high risk of VRE acquisition because of several factors such as the close contact between patients in the dialysis unit, the presence of co-morbid conditions, repeated exposure to antibiotics, and frequent hospitalization [23,24]. In our study, 68.9% of patients had been admitted to the hemodialysis unit. We also found an association between hemodialysis and death, reinforcing the fact that characteristics of the underlying disease associated with the infection itself further increase the risk of death.

Although *E. faecium* is considered an important healthcare-associated pathogen, little is known about its virulence. The *esp* gene is a common feature of hospital-associated VRE_{fm}, especially some specific well-adapted lineages [3,25-27]. Although some authors [26] found a low prevalence of *esp* (33%), it is important to point out that, in this specific study, VRE_{fm} population was characterized as being highly heterogeneous, considering PFGE profiles. This data

support the idea that presence of the *esp* gene is associated with well-established clones.

The majority of our isolates were *esp*-positive, consistent with most studies [3,26,27]. However, although most biofilm producers (10/11) were *esp* carriers, no correlation between the presence of the gene and biofilm formation was observed. Experimental studies showed that, although *esp* could mediate the initial interaction between bacteria and the surface where the biofilm is formed, it is not essential. In the absence of this protein during biofilm formation, its function may be fulfilled by other adhesion proteins [29,30]. In fact, numerous genes associated with biofilm formation besides *esp* have been recently described in *E. faecium* [31-33], so its relevance alone is questionable.

Esp protein is also important for the initial adherence without biofilm formation; strains harboring this gene have significantly higher conjugation rates than strains without it [23]. As most VRE_{fm} were esp producers, one could assume these isolates have greater possibilities of exchanging mobile genetic elements, giving them evolutionary advantages since adaptation to a hospital environment is facilitated.

The first *vanA E. faecium* in Brazil was isolated from a meningitis patient in São Paulo in June 1997 [34]. After that, this pathogen has been periodically isolated from clinical samples, including outbreaks, in different states of Brazil [27,35-38].

Around the world and even in Brazil, most recent VRE_{fm} outbreaks have been found to be related to monoclonal spread [36,39-41]. Our data describes a monoclonal outbreak, with two major subclones, during a period of almost a year. The pulsotype of the prevalent clone/subclone (A1) in this study seems to be the same, by optical evaluation, as the one found by d'Azevedo *et al.* (2008) circulating in a hospital in São Paulo, Brazil [36]. This finding could suggest that there is a well-established common clone in Brazilian hospitals. However, a large epidemiological study of isolates around the country is required to confirm this suspicion.

The isolate not clustered in A clone was the first VRE detected, six months before other isolations. The participation of this isolate as part of the outbreak is debatable. One could assume that for microorganisms with an extraordinary genetic plasticity, such as Enterococci, six months may represent enough time for the occurrence of diverse random genetic events, justifying clonal diversity between the first VRE and all others. However, there is not enough published data to support this hypothesis.

Molecular epidemiological studies have reported the spread, around the world, of a hospital-adapted complex of *E. faecium* designated as clonal complex 17 (CC17), which is associated with the majority of hospital outbreaks and clinical infections on all continents. Recently, phylogenetic analysis confirmed the existence of three separate hospital lineages within CC17: ST17, ST18, and ST78, highlighting different evolutionary trajectory. All of them are strongly associated with a hospital environment [42]. The global success of CC17 seems to have been facilitated by the cumulative acquisition of antibiotic resistance, putative virulence traits, and the ability to acquire different mobile genetic elements [3,38,43,44]. Our VRE_{fm} presented some characteristics of CC17, such as resistance to ampicillin and non-susceptibility to quinolones [3]. Moreover, 72% harbored *esp* gene, another common feature of CC17 [3].

Molecular epidemiological studies have shown that the incidence of outbreaks caused by VRE_{fm} strains in Brazil is not as high as in European countries and in the United States [25,35,45]. Palazzo *et al.* (2011) [35] revealed that VRE_{fm} outbreaks in Brazil are caused by strains that do not share a common evolutionary history. In this context, molecular epidemiological studies are of great importance to the knowledge of circulating strains in our country. Further studies will be necessary to establish if VRE_{fm} strains pertaining to CC17 are now predominant in Brazil as in other countries.

The number of isolates in our study was a major limitation; it may have compromised the analysis of the relationship among some risk factors and the occurrence of VRE colonization/infection.

In conclusion, our study describes a *vanA*-type vancomycin-resistant *E. faecium* outbreak and contributes to the local knowledge about epidemiological and microbiological characteristics of VRE infections.

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