Molecular characterization of enterococci harboring genotype and phenotype incongruence related to glycopeptide resistance isolated in Brazilian hospitals

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Three Enterococcus faecalis *and one* Enterococcus faecium *strains were characterized by plasmid profile, pulsed-field gel electrophoresis (PFGE) and determination of antimicrobial minimal inhibitory concentrations. VanA elements were characterized by Long PCR, overlapping PCR and DNA sequencing. Enterococcal strains showed resistance to vancomycin and harbored the vanA gene, and three these were teicoplanin susceptible while one showed intermediate resistance to teicoplanin. Two* E. faecalis *strains showed indistinguishable PFGE profile while the third was unrelated.* E. faecalis *strains showed a deletion in the right terminal region of the Tn*1546*-like element. The* E. faecium *strain showed an insertion element in the* vanXY *intergenic region. Mutations in VanA elements were not found. Rearrangements in the VanA element could be responsible for incongruities in genotype and phenotype in these strains.*

Key words: vancomycin resistant enterococcci (VRE) - *vanA* genotype - VanB phenotype

Control of nosocomial infections caused by vancomycin-resistant enterococci (VRE) has emerged as a worldwide health problem (Courvalin 2005, Vilela et al. 2006). Until the 1990s, most enterococcal infections could be successfully treated by a combination of vancomycin and aminoglycosides antibiotics. As VREs have become more prevalent, treatment options have been greatly reduced (Schouten et al. 2000).

VanA and VanB are widespread globally and confer the most prevalent glycopeptide resistance phenotype (Courvalin 2005). The VanA phenotype is characterized by inducible, high-level resistance to vancomycin and teicoplanin. VanB-resistant strains are also inducible and resistant to vancomycin, but retain susceptibility to teicoplanin. Resistance phenotypes have been associated with the acquisition of two gene clusters, *vanA* and *vanB*. These gene clusters direct the synthesis of peptidoglycan precursors terminating with the depsipeptide D-alanyl-D-lactate, which bind glycopeptide with reduced affinity (Arthur et al. 1996).

The *vanA* gene cluster located on the Tn*1546* mobile genetic element is 10,851 bp in size and encodes nine polypeptides that can be assigned to different functional groups. The products of *vanA* gene cluster include transposase and resolvase (ORF 1 and ORF 2), VanS and VanR proteins, which are a response regulator and histidine kinase sensor, respectively. VanH and VanA synthesize the depsipeptide D-alanyl-D-lactacte, VanX hydrolyses D-ala-D-ala, and VanY hydrolyzes the terminal D-ala residue from the peptidoglycan precursor protein. Vancomycin or teicoplanin induce autophosphorylation of VanS, which in turn phosphorylates VanR. Phosphorylated VanR protein binds to the promoter region of *vanHAX* inducing transcription of genes encoding essential structural molecules (Arthur et al. 1992). The function of VanZ protein is unknown, but it has been related to teicoplanin resistance (Arthur et al. 1992, 1995).

Genetic heterogeneity in Tn*1546*-like element or VanA element has been documented (Hashimoto et al. 2000, Lee et al. 2004, Eom et al. 2004, Camargo et al. 2005). The polymorphisms described so far included mutations, insertion of insertions sequence (IS) and deletions.

In this study, the molecular characteristics of the Tn*1546*-like element in VanB phenotype-*vanA* genotype enterococccal strains isolated in Brazil are described in order to advance understanding of the resistance incongruities.

Bacterial strains - Two VRE strains studied, *Enterococcus faecalis* 28 and *Enterococcus faecium* 172 were isolated in 1998 during an outbreak (Casa de Saúde Santa Marcelina, São Paulo, Brazil). The other two strains included in this study, *E. faecalis* 211 and *E. faecalis* 217 were isolated in 2004 during a VRE surveillance program at the Hospital Paulistano (Zanella et al. 2006). The enterococcal strains that are described in this report were isolated from different patients.

Species identification and susceptibility profile - The isolates were identified as *Enterococcus* spp. at the Instituto Adolfo Lutz (IAL), São Paulo, by conventional biochemical tests (Teixeira & Facklam 2003). Minimal inhibitory concentrations (MIC) for vancomycin and

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teicoplanin were determined by agar dilution method according to the Clinical and Laboratory Standard Institute (CLSI 2005) and by Etest (AB BIODISK). The *vanA* and *vanB* genotype were determined by multiplex polymerase chain reaction (PCR) (Woodford et al. 1993). The strains were sent to the Laboratório Especial de Bacteriologia e Epidemiologia Molecular (LEBEM) at the Faculdade de Ciências Farmacêuticas of Ribeirão Preto – Universidade de São Paulo for confirmation of phenotype and genotype characterization. At LEBEM, the enterococcal species were confirmed using the protocol described by Dutka-Malen et al. (1995) followed by molecular characterization of the strains.

Pulsed-field gel electrophoresis (PFGE) profile - PFGE was performed after *SmaI* digestion of DNA (Campanile et al. 2003) in a Gene Navigator apparatus (Amersham, Uppsal, Sweden) at 180V for 25h at 7ºC with pulses of 20s for 10h, 8s for 10h and 3s for 5h. The DNA restriction patterns were interpreted as previously described (Tenover et al. 1995).

Plasmid profile - Enterococci plasmid DNAs were extracted by the alkaline lysis method (Birnboim & Doly 1979) and analyzed by electrophoresis on 1% agarose gels. Plasmid size was determined by comparison to plasmid standards from *Escherichia coli* V517 (NCTC 50193) and *E. coli* 39R861 (NCTC 50192) strains.

Location of VanA element - Southern blot hybridization analysis was performed using digoxigenin-labeled DNA probes following procedures described in the DIG System User Guide for filter hybridization (Boehringer, Mannheim, Germany). Southern blot hybridization was performed to determine molecular locations of the VanA element. Briefly, plasmid DNA was blotted onto nylon membranes (Hybond N+, Amersham) and hybridized with digoxigenin-labeled *vanA* probe (Woodford et al. 1993). Also, genomic DNA digested with I-*Ceu*I followed by PFGE was Southern blotted onto nylon membranes, and the blots were hybridized with digoxigenin- labeled *vanA* and 16S rRNA gene probes (Palazzo et al. 2006).

Characterization of Tn1546-like elements - The characterization of Tn*1546*- like elements was performed by Long PCR using the Expand Long Template PCR system (Boehringer, Mannheim, Germany) with a single prime target, the inverted repeat sequence of Tn*1546* (Woodford et al. 1997). VanA elements in enterococci strains were characterized by overlapping PCR using ten pairs of PCR primers (Woodford & Stigter 1998). The PCR products of VanA resistance element were purified using a GFX kit and were sequenced using the MegaBace^{TM} apparatus (Amersham Biosciences, Australia). *E. faecium* BM 4147, which carries a prototype of Tn*1546*, was used as a control.

Determination of promoter - The *vanXY* intergenic region was amplified by PCR, using *E. faecium* 4147 DNA as the template. It was cloned into a pKK232-8 vector (Amersham Biosciences, Australia) containing the promoterless choramphenicol acetyltransferase (*cat*) gene. The amplification primers were Pz1 GGATC-CGCTATTTTGATTTCCCCGTT and Pz2 GTCGAC-CCTAAGTATATTAAGAATAAC, which contained cut sites for the restriction enzymes *Sal*I and *BamH*I (underlined above). Restriction enzyme digestion is used to insure DNA fragment correct orientation upstream to the *cat* gene. The PCR product and pKK 232-8 were both digested with *Sal*I and *BamH*I enzymes and were ligated using T4 DNA ligase. The cloned product was transfected by electroporation into *E. coli* DH10B, which was cultured in Luria Bertani (LB) broth for 1 h at 37ºC with gentle agitation. Subsequently, the cells were spread on LB agar plates supplemented with 10 µg/ml of cloramphenicol. Expression of the *cat* gene was used to demonstrate presence of promoter.

Species identification and characterization of resistance profile - Three isolates identified by multiplex PCR as *E. faecalis* 28, 211 and 217 showed high resistance to vancomycin and susceptibility to teicoplanin which establishes the VanB phenotype. The other isolate, identified as *E. faecium* 172 showed resistance to vancomycin and intermediate resistance to teicoplanin. Vancomycin and teicoplanin MICs for isolates are shown in Table. The multiplex PCR assay indicated the presence of a 399 bp product corresponding to the *vanA* gene in all enterococci studied.

Analysis of clonality, plasmid profile and location of VanA element - *E. faecalis* 211 and 217, isolated during the 2004 surveillance showed indistinguishable PFGE profiles after *SmaI* digestion, indicating a single clone. *E. faecalis* 28 isolated in 1998 was genetically unrelated to these (Fig. 1). All strains showed the same plasmid 70 Kb plasmid (Fig. 2A). Southern blot hybridization with a *vanA* probe demonstrated presence of the *vanA* plasmid in *E. faecium* 172. The *vanA* probe hybridization with both fragments corresponding to chromosomal and plasmid DNA were shown by *E. faecalis* 211, *E. faecalis* 217 and *E. faecalis* 28 (Fig. 2B) but different results were obtained in subsequent assays. No co-hybridization of the 16SrRNA and *vanA* probes after *I-Ceu*I digestion of chromosomal DNA followed by PFGE indicated that those strains do not carry a copy of the *vanA* gene in their chromosomal DNA.

Molecular characterization of Tn1546-like elements - Characterization of Tn*1546*-like elements of the four VRE strains by Long PCR and overlapping PCR are summarized in Table. Three *E. faecalis* strains showed deletion of *vanYZ* genes and the right terminal repeat. *E. faecium* 172 showed insertion of an IS element, IS*Efa5*, between the *vanX* and *vanY* genes, located immediately after nucleotide 9,013 of Tn*1546* and flanked by 8 bp direct repeats, as previously reported by Camargo et al. (2005). According to the results obtained in this study, no promoter was detected between the *vanX* and *vanY* genes. DNA sequencing indicated the strains had no mutations in *vanS* gene.

The VREs in this study were isolated in hospitals located in the city of São Paulo, Brazil, and showed incongruities in genotype and phenotype such as the VanB phenotype and the *vanA* genotype (Palazzo et al. 2006, Zanella et al. 2006). The same incongruities have been described in studies conducted in Korea, Japan and

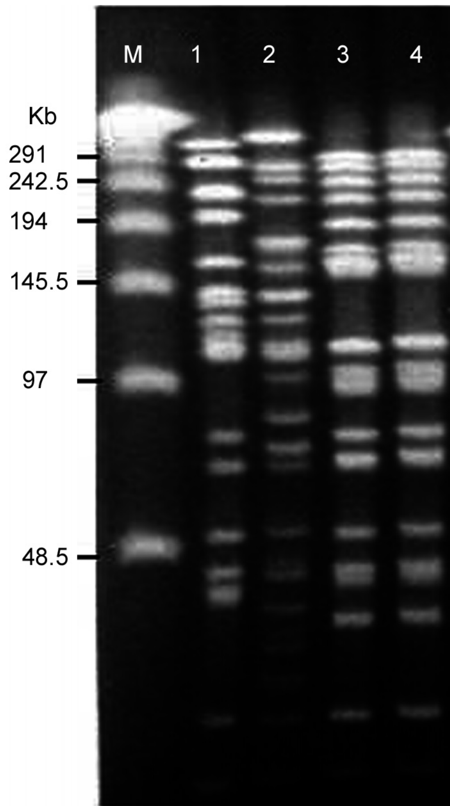

Fig. 1: pulsed-field gel electrophoresis (PFGE) patterns after *SmaI* digestion of total DNA extracted from the vancomycin resistant *Enterococcus faecalis* strains. Lanes - M: molecular size weight (Lambda Ladder PFG Marker, New England Biolabs); 1-4: *E. faecalis vanA* (control strain), *E. faecalis* 28, *E. faecalis* 211, *E. faecalis* 217, respectively.

Taiwan. This suggests that point mutations in the *vanS* regulatory gene or impairment of VanY and VanZ accessory proteins may serve as mechanism for loss of teicoplanin resistance (Hashimoto et al. 2000, Lauderdale et al. 2002, Lee et al. 2004, Ko et al. 2005, Tanimoto et al. 2005, Song et al. 2006). Although *E. faecalis* 28, *E. faecalis* 211 and *E. faecalis* 217 presented VanB phenotype-*vanA* genotype, no mutation was found in the *vanS* gene (Hashimoto et al. 2000).

The mechanism for VanZ protein associated teicoplanin resistance is unknown. Several authors (Arthur et al. 1994, Gold 2001, Courvalin 2006) reported that *vanRSHAX* genes promote cell wall alterations compatible with resistance to glycopeptides. These authors stated that the VanZ protein does not contribute to produce the pentadepsipeptide, the peptidoglycan precursor terminating in D-ala-D-lac, which is present in VRE strains (Arthur et al. 1995). Teicoplanin susceptibility in the strains in this study could be caused by genetic rearrangements in the right portion of Tn*1546*-like elements characterized by deletion of the *vanY* and *vanZ* genes which encode proteins important for the resistance mechanism in some situations. Thus, the denomination "accessory proteins" used in a majority of reports for products of *vanY* and *vanZ* genes are inadequate.

Interestingly, *E. faecium* 172 contained an insertion of IS*Efa5* in the intergenic region of *vanXY* genes, which could be the cause of the low-level resistance to teicoplanin. Other authors (Lee et al. 2004, Shin et al. 2006) have reported the presence of IS*1216V* in enterococci strains with the same phenotypic characteristics related to teicoplanin. The IS*Efa*5 insertion could interrupt a promoter preventing activation of *vanY* and *vanZ* genes

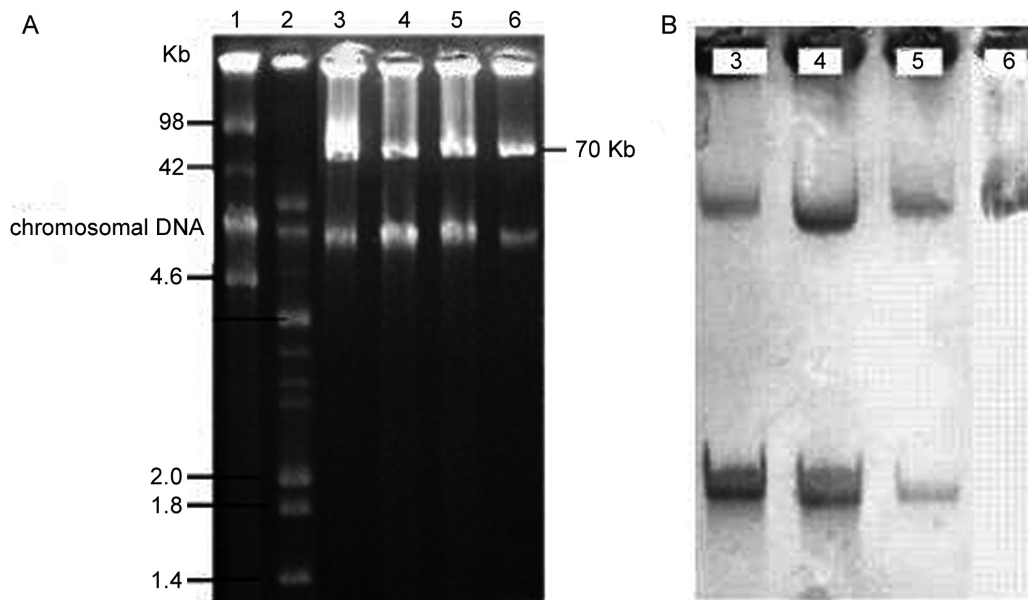

Fig. 2: agarose gel electrophoresis showing plasmid profiles and hybridization with the *vanA* probe. A: plasmid profiles of vancomycin resistant enterococcci (VRE) isolates after alkaline lyses extraction and agarose gel electrophoresis. Lines - 1-6: *Escherichia coli* 39R861; *E. coli* V517; *E. faecalis* 28; *E. faecalis* 211; *E. faecalis* 217; *E. faecium* 172, respectively; B: Southern blot corresponding to panel A and hybridization with *vanA* probe. Lines - 3-6: *E. faecalis* 28; *E. faecalis* 211; *E. faecalis* 217; *E. faecium* 172, respectively.

TABLE

Determination of minimal inhibitory concentrations (MIC) to glycopeptides and characterization of VanA elements by overlapping polymerase chain reaction (PCR)

Strains	MIC vancomycin	MIC teicoplanin	Overlapping PCR P1/P2[a] ORF-1[b] 1,309[c]	P3/P4 ORF-1 1,132	P5/P6 ORF-2 1,299	P7/P8 ORF-2 1,274	P9/P10 *VanR* 1,223	P11/P12 *vanS* 1,679	P13/P14 *vanHA* 1,793	P15/P16 *vanX* 1,941	P17/P18 *vanXY* 1,584	P19/P1 *vanZ* 423
E. faecium BM4147	> 256	> 256	+	+	+	+	+	+	+	+	+	+
E. faecalis 211	256	3	+	+	+	+	+	+	+	+	–	–
E. faecalis 217	256	6	+	+	+	+	+	+	+	+	–	–
E. faecalis 28	> 256	2	+	+	+	+	+	+	+	+	–	–
E. faecium 172	256	12	+	+	+	+	+	+	+	+	++	+

a: primers (Woodford et al 1998); *b*: genes amplified by PCR; *c*: size of amplified fragments in base pairs; (+): fragment is indistinguishable from prototype Tn*1546*; (++): fragment longer than prototype; (–): no fragment.

by phosphorylated VanR protein as occur in *vanH* promoter. Arguing against this is the fact that cloning analyses do not indicate the presence of a promoter between *vanX* and *vanY* genes. Another possibility is deletion of genes after the IS*Efa5* insertion. Refuting this is the fact that sequencing of the VanA element indicates that the *vanX* and *vanY* genes are intact. A more plausible explanation would be that the IS*Efa*5 insertion hinders activation of the transcription of *vanY* and *van*Z genes by promoter H.

Molecular characterization of Tn*1546*-like elements is important to better understand VRE epidemiology. *E. faecalis* 28 isolated in Hospital Santa Marcelina in 1998 shows the same Tn*1546*-like element and plasmid profile as strains *E. faecalis* 211 and *E. faecalis* 217 which were isolated much later, in 2004 in a different hospital (Hospital Paulistano).

In conclusion, the endemic nature of VRE occurrence is a serious medical problem. Exchange of genetic material among enterococcal strains is very likely and exacerbates the problem. Structural integrity of the VanA element and the presence of *vanY* and *vanZ* genes are important to maintain the glycopeptide resistant phenotype.

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