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Changed epidemiology during intra and interhospital spread of high-risk clones of *vanA*-containing *Enterococcus* in Brazilian hospitals

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ABSTRACT

We report changes in the molecular epidemiology of *vanA*-containing *Enterococcus* during the intra and interhospital spread of high-risk clones, in Southeastern Brazil. While VRE *faecalis* predominated during 1998 to 2006, a reversal has been observed in the last years, where VRE *faecium* belonging to ST114, ST203, ST412, ST478 and ST858 have become endemic.

Vancomycin-resistant *Enterococcus* (VR*E*) are leading nosocomial pathogens for which there are limited treatment options. In this regard, among VRE species, *Enterococcus faecalis* (*Efl*) and *Enterococcus faecium* (*Efm*) have caused the vast majority of hospital-acquired infections worldwide (Miller et al., 2016). In Brazil, over the last 20 years, the incidence of VRE causing infection and colonization has increased markedly in clinical settings (Correa et al., 2015; Gales et al., 2009; Zanella et al., 2003). Indeed, VRE infections have become endemic in this region (Pereira et al., 2010; Tuon et al., 2011). In this study, we investigated the clonal relationships and genetic features of *vanA*-containing VRE isolates circulating in 26 hospitals, in Southeastern Brazil, during an 18-year period.

From January 1998 to December 2015, 2633 VRE isolates were collected by the Instituto Adolfo Lutz (IAL), a reference local center responsible for surveillance and epidemiology of multidrug-resistant bacteria in the city of São Paulo, the largest and most populous metropolitan area in Brazil. Of these isolates, 278 VRE strains were randomly selected, including 153 (55%) VRE*fl* and 125 (45%) VRE*fm*, from different patients hospitalized in 26 hospitals geographically dispersed, for further determination of the antimicrobial susceptibility profile and molecular characterization. Around twenty strains of VRE were selected per year (1998–2011), taking into account three criteria: i) clinical material, ii) hospitals in the city of São Paulo and, iii) exclusion of duplicates of the same species and even clinical material. These isolates selected were obtained from 107 urine samples, 81 blood cultures, 51 fluid cavities and 39 rectal swabs (Table S1).

All *Enterococcus* isolates were identified using conventional biochemical tests and PCR (Zanella et al., 2003). In all 2633 VRE isolates, the presence of *van*-type genes was examined by PCR amplification using specific primers and controls, as previously described (Zanella et al., 2003).

Minimal inhibitory concentrations (MICs) for vancomycin, teicoplanin, gentamicin, streptomycin, ampicillin, ciprofloxacin, tetracyclin, chloramphenicol, rifampicin, erythromycin, linezolid and quinupristin-dalfopristin were determined by a broth microdilution and agar dilution method (CLSI, 2016). Clonal relatedness of the 278 VRE isolates was investigated by pulsed-field gel electrophoresis (PFGE) (Zanella et al., 2003), and representative clonally unrelated *E. faecalis* and *E. faecium* strains were selected for MLST investigation (http://pubmlst.org/efaecalis/; http://pubmlst.org/efaecium/). Finally, whole genome sequence analysis of a representative *vanA*-containing *E. faecium* belonging to the PFGE-P6 pattern (strain VRE*fm* 320/07) was performed. In brief, the total genomic DNA was extracted, purified and sequenced using the Ion Torrent Personal Genome Machine TM Platform (Life Technologies, Carlsbad, CA, USA). A total of 636,605 reads were generated with 100X coverage. The sequence reads were de novo assembled using Mira v4.0.2 (Chevreux et al., 2004) resulting

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contigs were merged using Geneious software v7.9 (Biomatters). Finally, the draft genome sequence was automatically annotated using the NCBI PROKKA (https://github.com/tseemann/prokka). This whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NBVO00000000. The version described in this paper is version NBVO00000000.1. Multilocus sequence type (MLST), virulence and antimicrobial resistance genes were identified using MLST 1.8, ResFinder 2.1, PlasmidFinder 1.3, and VirulenceFinder 1.5 databases, respectively, available from the Center for Genomic Epidemiology (http://genomicepidemiology.org/).

Of the 2633 VRE isolates collected by the IAL, 1430 were identified as VR*Efl* and 1203 were identified as VR*Efm*. All strains showed high-level resistance to vancomycin (MIC ≥64 μg/mL) and teicoplanin (MIC ≥16 μg/mL). During the 18-year period, a reversal was observed in the epidemiology of VRE strains investigated. Fig. 1A represent the distribution of VRE isolates from 1998 to 2015, where is possible to observe that between 1998 and 2006 *E. faecalis* was the most frequently identified species, and since 2007 to 2015 a reversal occurred, where *E. faecium* was predominant. Fig. 1B graphs the transition between VRE species. In this regard, although the IAL performed the monitoring of VRE strains passively, after detection of the first VRE strain in the city of São Paulo

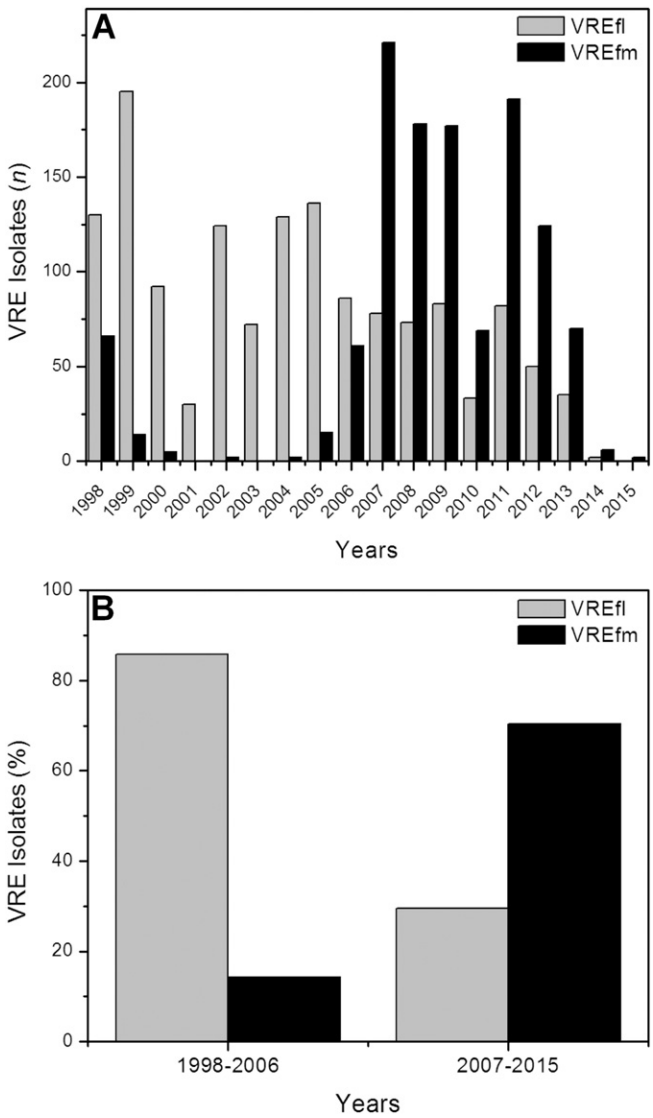

Fig. 1. Temporal distribution of 2633 *vanA*-containing VR*Efl* (light gray bars) and VR*Efm* (dark gray bars) clinical isolates collected from 26 hospitals, in Southeastern Brazil, during an 18-year study period.

(Zanella et al., 1999), the period studied represented the period of greatest collection of strains by the laboratory. Therefore, this data may reflect the epidemiological shift from VR*Efl* to VR*Efm*, in Sao Paulo city. In fact, other studies conducted in Brazil, with a lower number of isolates, have also confirmed this epidemiological change (Campos et al., 2014; d'Azevedo et al., 2008; Resende et al., 2014; Rossini et al., 2012; Ruzon et al., 2010), reinforcing this epidemiological landscape. Interestingly, in the last years, a gradual reduction of VRE has been observed in the hospitals studied. This seems to be a regional trend, since extended spectrum β-lactamases (ESBL)- and *Klebsiella pneumoniae* carbapenemase-producing Enterobacteriaceae have become widely disseminated in Brazil (Sampaio and Gales, 2016).

In this study, both VR*Efl* and VR*Efm* exhibited further resistance to ciprofloxacin (100/97%), quinupristin-dalfopristin (100/24%) [*E. faecalis* are naturally resistant to quinupristin/dalfopristin (Hollenbeck and Rice, 2012)], chloramphenicol (99/69%), rifampicin (98/93%), erythromycin (98/100%), gentamicin (97/22%), tetracycline (92/13%), streptomycin (28/85%) and ampicillin (9/100%), whereas for linezolid only VR*Efm* showed a resistance phenotype (5%).

PFGE performed on the 153 *vanA*-containing VR*Efl*, revealed four *Sma*I macrorestriction profiles named A ($n = 131$), B ($n = 7$), C ($n = 14$) and D ($n = 1$). Cluster A was further divided into 11 subtypes (A1-A11) (Fig. 2A). On the other hand, for the 125 *vanA*-containing VR*Efm* isolates, four PFGE clusters (P, Q, R and M) were identified, of which P-type was predominant ($n = 112$). The cluster P was further subdivided into 20 subtypes (P1-P20), with predominance of the P1 clone (39 isolates) (Fig. 2B). The PFGE designation adopted in this work was based on a previous study conducted to distinguish Brazilian VRE (Zanella et al., 2003). In this regard, the first *vanA*-containing *E. faecium* reported from Brazil was isolated in 1997 (Zanella et al., 1999). This strain was clustered into the PFGE M1-Brazilian clone, belonging to ST114. In this study, the first *vanA*-containing *E. faecium* was isolated in 1998, being also clustered into the PFGE M1-Brazilian clone and ST114. Only five representative VR*Efm* isolates clustered into 5 different PFGE groups (i.e., P1, P6, P9, P14 and M1) were selected for MLST analysis. In brief, the seven *vanA*-containing VR*Efm* (collected between 1998 to 2005), identified as belonging to the PFGE-M1 Brazilian lineage, could be clonally related to the nationwide disseminated ST114 (Camargo et al., 2006; Zanella et al., 2003).

Four representative VR*Efl* isolates clustered into PFGE A1, A7 and C1 were selected to MLST analysis. While, isolates clustered into PFGE-A1, —A5 and -A8 belonged to ST9 (clonal complex, CC9) or ST6 (CC2), the VR*Efl* strain grouped into PFGE-C1 belonged to ST4 (CC4). In this regard, this study confirms that the main VR*Efl* clones isolated in Brazilian hospitals have belonged to CC2, CC4 and CC9 (Ruiz-Garbajosa et al., 2006). MLST analysis of five representative VR*Efm*, clustered into PFGE patterns P1, P6, P9, P14 and M1, revealed the presence of ST858 (new ST), ST478, ST412, ST203 and ST114, respectively. The four former belonging to the previously designated CC17 (Willems and van Schaik, 2009), and the latter corresponding to a singleton. WGS analysis of the *E. faecium* strain VREfm 320/07 (PFGE-P6) identified 3452 coding sequences, 1080 pseudogenes, 62 tRNA, 6 rRNA, 4 ncRNAs and 37.7% GC. Furthermore, it was confirmed that this isolate belonged to high-risk hospital associated clone ST478 (*atp-15*, *ddl-43*, *gdh-1*, *purK-44*, *gyd-1*, *pstS-20*, *adk-1*). The *vanA* gene was harbored on a Tn*1546* element as previously reported (Merlo et al., 2015; Palazzo et al., 2006; Sacramento et al., 2015). Resistome analysis of VR*Efm* 320/07 identified the aminoglycoside resistance genes *aph(3′)-III* and *ant(6)-Ia*; glycopeptide resistance genes *vanX*, *vanH*, *vanZ*, *vanR*, *vanY*, *vanS*, *vanA*; the macrolide resistance gene *ermB*; the lincosamide resistance gene *lnuB*; and the gene *msrC* conferring resistance to macrolide, lincosamide, and streptogramin B. Four plasmids belonging to *rep*-families *rep2* (pRE25), *rep17* (pRUM), *rep18* (pEF418) and *repUS15*, were predicted by PlasmidFinder. Finally, virulence analysis identified functional collagen adhesin (*acm*) and cell wall adhesion (*efaAfm*) genes.