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Multilocus sequence typing and analysis of putative virulence factors in vancomycin-resistant and vancomycin-sensitive *Enterococcus faecium* isolates from Brazil

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ABSTRACT

Enterococci are leading causes of hospital-acquired infections that are often difficult to treat because of high-level aminoglycoside and glycopeptide resistance. Vancomycin-resistant enterococci are a global problem, and have been isolated with increasing frequency in hospitals in Brazil. The objective of this study was to determine the genetic relatedness of vancomycin-resistant *Enterococcus faecium* (VREFM) and vancomycin-sensitive *E. faecium* (VSEFM) isolated from human infections and faecal sources in Brazil, and to compare these isolates with those from domesticated animals. Isolates (n = 56) were classified by multilocus sequence typing (MLST) and assessed for putative virulence traits. The *acm* gene was detected in 98% of all isolates. The 56 isolates studied comprised 26 different MLST types. VSEFM isolates from the faeces of pigs were found to be distinct from all human isolates characterised previously by MLST, and were assigned new sequence type (ST) numbers. VREFM isolates were represented by four different STs (ST-114, ST-17, ST-281, ST-50). Among the 26 STs identified in this study, eBURST detected three groups of STs with related allelic profiles, and 19 unrelated STs. Among *E. faecium* isolates from Brazil, the *esp* gene was restricted to vancomycin-resistant isolates. Furthermore, isolates classified as ST-17 by MLST, an epidemic strain type isolated internationally with the *purK-1* gene, were found among VREFM isolates from Brazil that also harboured the *esp* and *hyl* genes.

Keywords *Enterococcus faecium*, epidemiology, *esp* gene, *hyl* gene, multilocus sequence typing, vancomycin resistance

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INTRODUCTION

Enterococcus faecium causes a large proportion of human enterococcal infections, which can be extremely difficult to treat because of resistance to glycopeptides and ampicillin, and high-level resistance to aminoglycosides [1]. Six different phenotypes for vancomycin-resistant enterococci (VRE) have been described: VanA, VanB, VanC, VanD, VanE and VanG [2–4]. In Brazil, VRE have recently become endemic in hospitals. The first vancomycin-resistant enterococcal strain identified was a divergent VanD strain of *E. faecium* isolated in 1996 from a patient with aplastic anaemia [3,5]. The first documented case of VanA *E. faecium* infection occurred in Brazil during 1997. The strain was isolated from a case of meningitis in São Paulo [6]. One year later, several vancomycin-resistant *E. faecium* and *Enterococcus faecalis* strains were isolated from the same hospital in São Paulo, providing evidence of an outbreak caused by VanA enterococci [7]. A study of these isolates identified several different clones of *E. faecium*. VRE have also been isolated from different hospitals in São Paulo, indicating inter-hospital dissemination [8–10].

In addition to antibiotic resistance, putative virulence factors described in vancomycin-resistant *E. faecium* (VREFM) include a collagen-binding adhesin (*acm*) and hyaluronidase (*hyl*) [11,12]. Rice *et al*. [12] described an open reading frame, designated hyl_{Efm}, which had a deduced amino-acid sequence with significant identity to known

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hyaluronidase genes. hyl_{Efm} has been detected mostly in multiresistant *E. faecium* isolates from non-stool cultures from patients in hospitals in the USA [12], but has also been found in an endemic VREFM strain in a survey in Taiwan [13]. A collagen-binding adhesin of *E. faecium* (Acm), expressed on the surface of the bacterium, was characterised as a new member of the MSCRAMMs (Microbial Surface Components Recognising Adhesin Matrix Molecules) family. Despite the detection of the *acm* gene in all 32 isolates studied, the Acm phenotype is identifiable only in certain clinical isolates [11]. The attachment of Acm-positive *E. faecium* to collagen type I has been studied, but a role for Acm in *E. faecium* pathogenesis has not yet been demonstrated [11].

Gelatinase, aggregation substance and cytolysin are virulence factors found in *E. faecalis*, although they have also been sought among *E. faecium* isolates [14–16]. Gelatinase (GelE) is an extra-cellular zinc metallo-endopeptidase that hydrolyses collagen, gelatin and small peptides [17] and plays a role in biofilm development [18–20]. Aggregation substance, encoded by *asc10*, *asa1* and other related genes, represents a family of pheromone-inducible surface proteins that promote aggregation during bacterial conjugation [21]. Cytolysin has bacteriocin activity and is also a haemolytic toxin for human, horse and rabbit erythrocytes [22]. Cytolysin and aggregation substance appears to act synergically to enhance the severity of endocarditis [23].

A virulence factor found in both *E. faecalis* and *E. faecium* is enterococcal surface protein (Esp), encoded by the *esp* gene [15,24,25]. Esp is a surface protein that enhances biofilm formation by *E. faecalis* in the presence of glucose [26], and is one of the virulence factors encoded by the pathogenicity island that is present in both *E. faecalis* [27] and *E. faecium* [28]. Some studies have observed an association between the occurrence of *esp* and specific variants of the housekeeping gene *purK* (phosphoribosylaminoimidazole carboxylase ATPase subunit), which is a gene that has been used for typing VREFM [24,25,29,30]. Willems *et al*. [25] suggested that the *purK* allele may be useful as a marker for epidemic VREFM genogroups in infection control.

Homan *et al*. [30] have proposed a scheme for characterisation of *E. faecium* by multilocus sequence typing (MLST). An advantage of MLST for epidemiological investigations and surveillance is that it allows easy comparison of local isolates with all strains contained in an electronic database (http://efaecium.mlst.net/). The eBURST program permits analysis of the MLST dataset into non-overlapping groups of related sequence types (STs) or clonal complexes, and then indicates the predicted progenitor and the evolutionary relationship of isolates [31].

Recent studies have shown that VanA *E. faecium* isolates in Brazil have a number of different pulsed-field gel electrophoresis profiles, indicating that several strains are involved in infection and colonisation of patients [7,8]. However, the carriage of virulence factors and the MLST profiles of *E. faecium* isolates from Brazil have not been investigated previously. The present study aimed to determine the sequence-based relatedness of isolates from Brazil by MLST, and then to relate these findings to the presence of known virulence traits.

MATERIALS AND METHODS

Bacteria

Fifty-six isolates of *E. faecium* were investigated in this study: ten vancomycin-sensitive *E. faecium* isolates that colonised hospitalised patients (VSERM chp) and one infection-derived vancomycin-sensitive isolate from a hospitalised patient (VSEFM ihp) were chosen randomly from the collection of Maschieto *et al*. [32]; 12 VSEFM isolates that colonised healthy humans (chh), unrelated to the hospital environment, were collected from undergraduate students of the University of São Paulo; ten VSEFM isolates from the faeces of pigs (fp) were collected randomly from two different farms, 300 km apart (Braganca Paulista and Ribeirao Preto); ten vancomycin-resistant infection-derived isolates from hospitalised patients (VREFM ihp), and 13 vancomycin-resistant isolates that colonised hospitalised patients (VREFM chp) were obtained from the southern part of Brazil [5,7,8,10]. More details concerning the source of isolates are shown in Table 1.

All isolates were tested for high-level resistance to aminoglycosides on plates of brain–heart infusion agar (Difco, Detroit, MI, USA) containing gentamicin (500 mg/L) or streptomycin (2000 mg/L). MICs of vancomycin, quinupristin–dalfopristin and penicillin were determined using the CLSI (NCCLS) agar dilution method [33]. PCR was used to determine the genotype (*vanA*, *vanB*, *vanD* or *vanC-1/vanC-2*), as described previously [34].

MLST scheme

Internal fragments of seven housekeeping genes were amplified with specific primers, using reaction conditions described previously [30]. The genes amplified were *adk*, *atpA*, *ddl*, *gyd*, *gdh*, *purK* and *pstS*. All PCR-amplified fragments were sequenced, using both forward and reverse primers and the