Molecular analysis and distribution of multidrug-resistant *Enterococcus faecium* isolates belonging to clonal complex 17 in a tertiary care center in Mexico City

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**Abstract**

**Background:** *Enterococcus faecium* has recently emerged as a multidrug-resistant nosocomial pathogen involved in outbreaks worldwide. A high rate of resistance to different antibiotics has been associated with virulent clonal complex 17 isolates carrying the *esp* and *hyl* genes and the purK1 allele.

**Results:** Twelve clinical vancomycin-resistant *Enterococcus faecium* (VREF) isolates were obtained from pediatric patients at the Hospital Infantil de México Federico Gómez (HIMFG). Among these VREF isolates, 58.3% (7/12) were recovered from urine, while 41.7% (5/12) were recovered from the bloodstream. The VREF isolates showed a 100% rate of resistance to ampicillin, amoxicillin-clavulanate, ciprofloxacin, clindamycin, chloramphenicol, streptomycin, gentamicin, rifampicin, erythromycin and teicoplanin. In addition, 16.7% (2/12) of the isolates were resistant to linezolid, and 66.7% (8/12) were resistant to tetracycline and doxycycline. PCR analysis revealed the presence of the *vanA* gene in all 12 VREF isolates, *esp* in 83.3% (10/12) of the isolates and *hyl* in 50% (6/12) of the isolates. Phylogenetic analysis via molecular typing was performed using pulsed-field gel electrophoresis (PFGE) and demonstrated 44% similarity among the VREF isolates. MLST analysis identified four different sequence types (ST412, ST757, ST203 and ST612).

**Conclusion:** This study provides the first report of multidrug-resistant VREF isolates belonging to clonal complex 17 from a tertiary care center in Mexico City. Multidrug resistance and genetic determinants of virulence confer advantages among VREF in the colonization of their host. Therefore, the prevention and control of the spread of nosocomial infections caused by VREF is crucial for identifying new emergent subclones that could be challenging to treat in subsequent years.

**Keywords:** *Enterococcus faecium*, Multidrug-resistant, Clonal complex, Pulsotypes, Virulence
Background

Enterococci are opportunistic pathogens of the normal intestinal microbiota of humans and animals [1,2]. The most common species of Enterococcus involved in nosocomial infections is Enterococcus faecium (E. faecium) [1,2]. This pathogen is associated with hospital-acquired infections such as UTIs (urinary tract infections), wounds, bacteremia, endocarditis and meningitis [1,2].

In recent years, the emergence of multidrug-resistant E. faecium has increased [3-5]. The recommended treatment for Enterococcus infections has been penicillin alone or combined with aminoglycosides. However, due to increased resistance to aminoglycosides, vancomycin is currently the antibiotic employed to treat these infections. In the last several decades, the number of vancomycin-resistant enterococci (VRE) has increased. The first VRE isolates were reported in the United Kingdom in the late 1980s [6]. In the United States, more than 80% of E. faecium isolates from hospitals are now resistant to vancomycin, and virtually all of them (>90%) exhibit ampicillin resistance [7]. Vancomycin-resistant Enterococcus faecium (VREF) has been associated with outbreaks in hospitals worldwide [2]. The rates of VREF colonization and infection have risen steadily, with most cases being caused by strains displaying glycopeptide resistance to VanA and VanB [8-11].

In addition to multidrug resistance, E. faecium produces diverse factors that contribute to its pathogenesis, including virulence molecules such as secreted antigen SagA [12], cell wall-anchored collagen adhesin (Acm) [13], hyaluronidase (Hyl) [14] and enterococcal surface protein (Esp) [15]. However, the traits that contribute to the transition of E. faecium from a commensal to a nosocomial pathogen have not been identified [16].

Molecular typing methods are essential for identifying hospital-associated outbreaks of E. faecium. Multilocus sequence typing (MLST) has revealed the existence of host-specific genogroups, including a specific genetic lineage designated clonal complex 17, associated with hospital-related isolates [1,17]. MLST of E. faecium is based on identifying alleles from DNA sequences in internal fragments of housekeeping genes (atpA, ddl, gdh, purK, gyd, pstS and adk), resulting in a numeric allelic profile, with each profile then being assigned a sequence type (ST) [17].

Complex 17 most likely evolved from the primary E. faecium ancestor ST-22, while ST-17 represents an important secondary founder with additional lineages designated to complex 17 [18]. Clonal complex 17 is characterized by ampicillin and quinolone resistance and the presence of a putative pathogenicity island that includes the esp and/or hyl genes in the majority of isolates [1,18-20]. Various STs belonging to clonal complex 17, such as ST16, ST17, ST18, ST203 and ST412, are currently being disseminated worldwide [21,22]. Interestingly, half of the STs within the clonal complex 17 polyclonal subpopulation have also been identified in samples obtained from healthy humans, swine, poultry and pets [16].

In Mexico, there is little available information about the prevalence of VREF isolates, and no study related to clonal complex 17 has been performed in pediatric patients. The aim of this study was to genotypically and phenotypically characterize VREF clinical isolates from 12 immunocompromised pediatric patients at the Hospital Infantil de México Federico Gómez (HIMFG). This study involved amplification of the resistance genes vanA and vanB and two virulence genes (esp and hyl) and molecular typing via pulsed-field gel electrophoresis (PFGE) and MLST.

Methods

Bacterial isolates

Twelve E. faecium isolates of clinical importance were obtained from 12 patients with nosocomial infections in the PICU (Pediatric Intensive Care Unit), oncology, gastroenterology and transplant wards of HIMFG during the period from July 2009 to April 2011. The isolates were maintained at −70°C in skim milk (Becton Dickinson, New Jersey, USA) and cultured on 5% sheep blood agar plates (Becton Dickinson, New Jersey, USA) at 37°C under 5% CO2 for 24 h. The E. faecalis ATCC® 29212, E. faecalis ATCC® 51299 and E. faecium ATCC® 51559 strains (American Type Culture Collection Manassas, VA, USA) were used as controls.

Biochemical tests

Bacteria were grown on blood agar, and identification was performed using manual methods. All colonies were grown in brain heart infusion broth (BHI) (Becton Dickinson, New Jersey, USA) with 6.5% NaCl and on bile esculin agar (Oxoid Sunnyvale, California, USA) to determine their hydrolysis grade. Disks impregnated with the substrate L-putidamidyl-beta-naphthylamide were used to perform pyrrolidonase tests (Oxoid Biochemical Identification System, Oxoid LTD., Basingstoke, Hampshire, England). Reduction of tellurite (Merck, Darmstadt, Germany) was evaluated via growing the bacteria on 0.04% potassium tellurite.

Antibiotic susceptibility

The antibiotic susceptibility profiles of the 12 VREF isolates were determined via the minimum inhibitory concentration (MIC) technique by means of the micro-dilution method using Mueller-Hinton broth (MHB), as recommended by the Clinical and Laboratory Standards Institute. MIC tests were performed for vancomycin (MP Biomedicals, Solon, Ohio, USA),
teicoplanin (Sigma-Aldrich, St. Louis, Missouri, USA), chloramphenicol (MP Biomedicals, Solon, Ohio, USA), ciprofloxacin (MP Biomedicals, Solon, Ohio, USA), streptomycin (Alexis Biochemical, San Diego California, USA), linezolid (Sigma-Aldrich, St. Louis, Missouri, USA), rifampicin (MP, Biomedicals, Ohio, USA), nitrofurantoin (MP Biomedicals, Solon, Ohio, USA), tetracycline (MP Biomedicals, Solon, Ohio, USA), doxycycline (Sigma-Aldrich, St. Louis, Missouri, USA), erythromycin (MP Biomedicals, Solon, Ohio, USA), tigecycline (Sigma-Aldrich, St. Louis, Missouri, USA), gentamicin (MP Biomedicals, Solon, Ohio, USA) and amoxicillin-clavulinate (Glaxo-Smith-Kline, Philadelphia, Pennsylvania, USA). Several concentrations (256–0.625 μg/ml) of the antibiotics were tested in Mueller Hinton broth, with 100 μl of those dilutions being loaded into each well of a microplate. For each dilution, 100 μl of a bacterial suspension (1.5×10⁸ CFU/ml) was inoculated and grown overnight at 37°C under a CO₂ atmosphere. After bacterial growth was detected, the MIC for each isolate of E. faecium was reported as the highest concentration in which no growth was observed. The E. faecalis ATCC® 29212 strain (American Type Culture Collection Manassas, VA, USA) was used as a control. These isolates were also evaluated for high-level aminoglycoside resistance (HLAR) to streptomycin (Alexis Biochemical, San Diego California, USA), ciprofloxacin (MP Biomedicals, Solon, Ohio, USA), tigecycline (Sigma-Aldrich, St. Louis, Missouri, USA), erythromycin (MP Biomedicals, Solon, Ohio, USA), doxycycline (Sigma-Aldrich, St. Louis, Missouri, USA), rifampicin (MP, Biomedicals, Ohio, USA), nitrofurantoin (MP Biomedicals, Solon, Ohio, USA), tetracycline (MP Biomedicals, Solon, Ohio, USA), amoxicillin-clavulinate (Glaxo-Smith-Kline, Philadelphia, Pennsylvania, USA), and gentamicin (MP Biomedicals, Solon, Ohio, USA). Several concentrations (256–0.625 μg/ml) and gentamicin (500 μg/ml).

Detection of the glycopeptide resistance genes vanA and vanB

PCR was performed to detect the glycopeptide resistance genes vanA and vanB in the 12 E. faecium clinical isolates using specific primers (Table 1) [23]. Briefly, genomic DNA was purified using the Wizard Genomic DNA Purification Kit (Promega Madison, Wisconsin, USA) from a bacterial culture grown in BHI broth incubated at 37°C for 24 h. The amplification reactions were prepared in a final volume of 50 μl, as follows: 25 μl of amplification mix (22 mM Tris/HCl, pH 8.4; 55 mM KCl; 1.65 mM MgCl₂; 25 μM each dNTP; 0.6 U recombinant Taq DNA polymerase/ml), 100 ng/μl of bacterial DNA, 10 μl of H₂O and 5 μl of primer solution (10 pg/μl). A Perkin Elmer 9600 thermocycler was programmed to run for 30 cycles with the following parameters: denaturing at 94°C for 3 s, annealing at 55°C for 45 s and extension at 72°C for 1 m, with a final extension at 72°C for 2 m. The samples were analyzed via electrophoresis in 1% agarose gels (Agarose LE, Promega) using a 100 bp DNA ladder (Gibco/BRL Life Technologies, Breda, The Netherlands). E. faecium strain ATCC 51559 (vanA⁺) and E. faecalis strain ATCC* 51299 (vanB⁺) were used as controls in the PCR experiments [24].

Table 1 Primers sequences used in this study

| Gene | Primer | Sequence (5' to 3') | Size (bp) | Reference |
|------|--------|---------------------|-----------|-----------|
| vanA | vanA-F | CATGAATAGAATAAAAGTTGCAATA | 1,030 | (Clark et al., 1993) [23] |
|      | vanA-R | CCCCCTTAAAGCTTATACGATCAA | 433 | (Clark et al., 1993) [23] |
| vanB | vanB-F | GTCACAAACCGGAGGCGAGGA | 945 | (Shankar et al., 1999) [25] |
|      | vanB-R | CCGCCATCCTCCTGCAAAAA | 433 | (Clark et al., 1993) [23] |
| espEfm | esp-F | TTGCTAATGCTAGTCCACGACC | 945 | (Shankar et al., 1999) [25] |
|      | esp-R | GCCTCAACACCTTGCAATGGCCGA | 661 | (Rice et al., 2003) [14] |

PCR screening for the esp and hyl genes

DNA from bacterial cultures was extracted and amplified via PCR using primers for the espEfm and hylEfm genes (Table 1), generating bands of 954 bp and 661 bp, respectively [14,25].

Molecular typing of VREF

PFGE of the 12 VREF clinical isolates was carried out following the protocols of Morrison et al. [26,27]. Briefly, the samples were digested with 50 U of SmaI (New England Biolab, Ipswich, MA, USA) for 4 h at 25°C. The digested plugs were separated via electrophoresis in 1% agarose gels (BioRad Laboratories, Hercules, California, USA) using ultra-pure DNA agarose (BioRad, Hercules, California, USA), with 0.5X TBE as the running buffer in the CHEF MAPPER system (BioRad Laboratories, Hercules, California, USA), run at 6 V/cm at 14°C under two different linear ramped pulse times: 1 to 10 s for 16 h and 10 to 40 s for 22 h. A PFGE lambda ladder (New England Biolabs, Hertfordshire, England, UK) was used as a molecular weight marker, and the gels were stained for 40 m with 0.5 mg/ml of ethidium bromide for visualization under UV light. The obtained banding patterns were initially interpreted via visual inspection according to the criteria specified by Tenover et al. [28]. Cluster analysis was performed with BioNumerics (Applied Maths, Inc., Austin, TX, USA) using the DICE correlation coefficient and the unweighted pair group
The mathematical average algorithm (UPGMA) as the grouping method [29]. The PFGE pulstypes of the 12 VREF clinical isolates were also genotyped through multilocus sequence typing (MLST) according to a standard protocol described by Homan et al. [17]. Fragments of seven housekeeping genes (atpA, ddl, gdh, purK, ygd, pstS and adk) were sequenced using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA), thus obtaining their allelic profiles, and the STs for each unique allelic profile were designated on the basis of information from the MLST website (http://efaecium.mlst.net).

Results

Origin of the strains

A total of 12 VREF clinical isolates obtained during the period from July 2009 to April 2011 were included in this study. The risk factors of the 12 patients were characterized by a minimum hospital stay of 4 days, assistance in the PICU and treatment with vancomycin. During their stay, the 12 patients were subjected to surgical procedures and received a central venous catheter, steroids and immunosuppressive treatment. Among the VREF isolates, 58.3% (7/12) were obtained from urine, while 41.6% (5/12) were obtained from the bloodstream. The VREF isolates were obtained from patients with different pathologies (Table 2).

Detection of susceptibility patterns and glycopeptide resistance in the VREF isolates

The results obtained for the 12 VREF clinical isolates showed a 100% rate of resistance to ampicillin, amoxicillin-clavulanate, ciprofloxacin, clindamycin, chloramphenicol, streptomycin, gentamicin, rifampicin, erythromycin and teicoplanin. The MIC values for each VREF isolate are presented in Table 3. In addition, 16.7% (2/12) of the VREF clinical isolates were resistant to linezolid, and 67% (8/12) were resistant to tetracycline and doxycycline (Table 3). However, all of the VREF isolates were susceptible to nitrofurantoin and tigecycline (Table 3). The HLR values for gentamicin (500 μg/ml), streptomycin (1,000 μg/ml) and gentamicin/streptomycin (500/1,000 μg/ml) were determined with 50% (6/12), 25% (3/12) and 25% (3/12), respectively.

The vanA and vanB genes of the 12 VREF clinical isolates were amplified via PCR. Interestingly, only the vanA gene was detected in all the VREF clinical isolates, as a 1,030 bp amplicon (data not shown), whereas the vanB gene, with a length of 433 bp, was not identified in the isolates (data not shown). The E. faecium ATCC® 51559 (vanA⁺) and E. faecalis ATCC® 51299 (vanB⁺) strains were used as positive controls in the PCR assays [24].

Prevalence of the esp and hyl virulence genes in the VREF isolates

The esp and hyl virulence genes, which are associated with a clonal subcluster known as clonal complex 17 in VREF clinical isolates, were detected via PCR. The esp and hyl genes were highly prevalent in the isolates. The esp virulence gene was detected in 83.3% (10/12) of the isolates, and the hyl virulence gene was present in 50% (6/12) of them. Therefore, three genotypes were determined for the VREF clinical isolates: esp+/hyl−, esp+/hyl⁺ and esp−/hyl⁺, at prevalence rates of 50% (6/12), 33.3% (4/12) and 16.7% (2/12), respectively.

Table 2 Characteristics of the 12 VREF isolates related to the patients’ clinical diagnosis, source of clinical samples, ward, PFGE, sequence type and clonal complex

| Clinical isolate | Clinical diagnosis | Sources of clinical samples | Wards | PFGE | MLST/STs | CC  |
|-----------------|-------------------|-----------------------------|-------|------|----------|-----|
| 133H            | Acute lymphocytic leukemia L1, fever, and neutropenia | Bloodstream | ONC  | A    | 757      |     |
| 926U            | Aplastic anemia, neutropenic colitis, septic shock | Urine | ONC  | A    | 203      | 17  |
| 821U            | Lupus erythematosus, septic Shock | Urine | TRPU | A    | 412      | 17  |
| 851H            | Anaplastic lymphoma, tumor lysis syndrome, sepsis | Bloodstream | PICU | B    | 757      |     |
| 215H            | Venous catheter infection, Down syndrome | Bloodstream | PICU | B    | 612      | 17  |
| 222U            | Acute myeloid leukemia M2, tumor lysis syndrome, Septic shock | Urine | ONC  | B    | 412      | 17  |
| 127U            | Acute lymphocytic leukemia L1, fever, and neutropenia. | Urine | PICU | B1   | 412      | 17  |
| 30H             | Wilms tumor | Bloodstream | PICU | B1   | 412      | 17  |
| 634U            | Septic shock, hemophagocytic lymphohistiocytosis | Urine | ONC  | C    | 757      |     |
| 459U            | Lupus erythematosus, sacroiliac ulcers | Urine | PICU | C    | 412      | 17  |
| 422H            | Acute myeloid leukemia M4, fever, and neutropenia | Bloodstream | SS   | D    | 412      | 17  |
| 155U            | Cholestatic syndrome, choledochal cyst. | Urine | GST  | D    | 203      | 17  |

Multilocus sequence typing (MLST), sequence types (STs), clonal complex (CC). ONC (Oncology Ward), TRPU (Transplant Unit), PICU (Pediatric Intensive Care Unit), SS (Short Stay Ward) and GST (Gastroenterology Ward).
Molecular typing analysis of the *E. faecium* isolates via PFGE and MLST

The VREF isolates were analyzed via PFGE following SmaI digestion of genomic DNA. Data obtained through PFGE were analyzed using a dendrogram profile, which included the PFGE pulsotypes obtained from VREF (Figure 1). A total of four clusters (I-IV) with five DNA pulsotypes were identified, showing patterns consisting of 12 to 20 DNA fragments ranging in size from 48.5 to 339.5 Kb (Figure 1). Interestingly, 25% (3/12) of the VREF clinical isolates observed via PFGE were categorized as pulsotype B and 16.7% (2/12) as pulsotype B1, with 92% genetic similarity being observed among these isolates (Figure 1). Meanwhile, 25% (3/12) of the VREF isolates were classified as pulsotype A, showing a different pattern from pulsotypes B, C and D (Figure 1). However, 16.7% (2/12) of the VREF isolates were classified as pulsotypes C and D, which displayed 50% genetic similarity. In addition, a maximum of 44% similarity was observed among all clusters of VREF isolates.

In this study, 12 VREF clinical isolates were subjected to MLST genotyping. Six of the 12 VREF isolates (50%) belonged to ST412, three to ST757, two to ST203 and one to ST612 (Table 2). eBURST analysis of the VREF isolates revealed four different STs (ST412, ST612, ST757 and ST203), three of which belonged to clonal complex 17; ST757 was not related to this clonal complex (Figure 2).

### Discussion

*E. faecium* is a highly resistant nosocomial pathogen and has recently emerged as an important threat in hospitals worldwide [2]. In this study, the 12 examined VREF isolates exhibited multidrug resistance to ampicillin, amoxicillin-clavulanate, ciprofloxacin, clindamyacin, chloramphenicol, streptomycin (S), rifampin (RA), erythromycin (E), vancomycin (Va), teicoplanin (TEI), tetracycline (Te), doxycycline (D), linezolid (LZN), nitrofurantoin (F/M), and tigecycline (TGC). *Cut-off values for resistance to MIC (μg/ml), Percentage of resistant (%R).*

### Table 3 Minimum Inhibitory Concentration (MIC) to 12 clinical isolates of multidrug-resistant *Enterococcus faecium*

| Clinical isolate | Am | Amc | CIP | CC | C | GM | S | RA | E | Va | TEI | Te | D | LZN | F/M | TGC |
|------------------|----|-----|-----|----|---|----|---|----|---|----|-----|---|---|----|-----|-----|
| 133H             | 128| 128 | 512 | 128| 64| 32 | 512| 4  | 32 | 512| 16  | 1  | 1  | 8  | 2   |
| 926U             | 256| 256 | 4   | 128| 32| 32 | 64 | 4  | 32 | 512| 32  | 64 | 8  | 2  | 2   |
| 821U             | 128| 128 | 256 | 256| 32| 32 | 32 | 32 | 32 | 256| 128 | 64 | 64 | 2  | 16  |
| 851H             | 128| 128 | 512 | 256| 32| 32 | 252| 4  | 32 | 512| 16  | 2  | 1  | 4  | 16  |
| 215H             | 128| 128 | 512 | 256| 32| 32 | 252| 4  | 32 | 512| 16  | 2  | 0.25 | 1 | 4   |
| 222U             | 64 | 128 | 256 | 256| 32| 32 | 32 | 16 | 32 | 256| 32  | 64 | 16 | 2  | 32  |
| 127U             | 128| 128 | 256 | 256| 32| 32 | 32 | 32 | 32 | 256| 64  | 64 | 8  | 32 | 2   |
| 30H              | 128| 128 | 256 | 256| 32| 32 | 32 | 16 | 32 | 256| 256 | 64 | 16 | 1  | 16  |
| 634U             | 64 | 64  | 256 | 256| 32| 32 | 32 | 4  | 32 | 256| 16  | 4  | 0.5 | 2  | 8   |
| 459U             | 256| 256 | 256 | 64 | 64| 32 | 32 | 32 | 32 | 32 | 256 | 32 | 64 | 16 | 2   |
| 422H             | 64 | 128 | 256 | 256| 32| 32 | 32 | 8  | 32 | 256| 64  | 64 | 8  | 2  | 16  |
| 155U             | 128| 128 | 256 | 256| 32| 32 | 32 | 8  | 32 | 256| 64  | 64 | 16 | 2  | 16  |
| CVR*             | ≥16| ≥8  | ≥4  | ≥4 | ≥32| ≥16| ≥16| ≥4 | ≥8 | ≥32| ≥16 | ≥8 | ≥4 | ≥128| ≥16 |
| % R              | 100| 100 | 100 | 100| 100| 100| 100| 100| 100| 100| 100 | 100| 100| 100| 0   |

Ampicillin (Am), amoxicillin-clavulanate (Amc), ciprofloxacin (CIP), clindamyacin (CC), chloramphenicol (C), gentamicin (GM), streptomycin (S), rifampin (RA), erythromycin (E), vancomycin (Va), teicoplanin (TEI), tetracycline (Te), doxycycline (D), linezolid (LZN), nitrofurantoin (F/M), and tigecycline (TGC).
HLAR to gentamicin (500 μg/ml), streptomycin (1,000 μg/ml) and gentamicin/streptomycin (500/1,000 μg/ml), displaying resistance values of 50%, 25% and 25%, respectively. Treatment of severe enterococcal infection requires combined therapy to achieve a synergistic bactericidal effect [35]. However, the results obtained in cases of severe infections associated with enterococci have shown that HLAR should not be treated with combined therapy (gentamicin/ampicillin) [35]. Therefore, the treatment of HLAR E. faecium is restricted [36].
The enterococcal surface protein Esp, which is encoded by genes that appear to have been acquired and localized within a pathogenicity island, is commonly found in clinical isolates and anchors to the cell wall. This protein also affects biofilm formation and plays a role in experimental UTI and/or endocarditis models [2]. The presence of the esp gene has been associated with hospital outbreaks, although this gene is not exclusively found in epidemic strains [19,30,37,38]. The esp gene was detected in 83.3% of our VREF clinical isolates. In addition, the majority of esp+ strains of E. faecium isolates were multidrug-resistant to more than three antibiotics, in accord with data reported by other researchers [39-41].

On the other hand, the hyl gene was found in 50% of the VREF clinical isolates and displayed a higher prevalence compared to the prevalences of 29.8% (29/131) reported in isolates of E. faecium in the Picardy Region of France, 38% (83/220) in isolates from the US and 3% in European clinical isolates. However, in the United Kingdom, a hyl gene prevalence of 71% (20/28) was observed in E. faecium isolates [14,42,43]. We believe that the differences observed in the detection rates of the hyl gene are due to the region in which the samples were isolated. The rates of the occurrence of esp+/hyl+, esp+/hyl+ and esp-/hyl- isolates were found to be 50% (6/12), 33.3% (4/12) and 16.7% (2/12), respectively, which is in accord with the findings of Vankerckhoven et al. and Rice et al. [14,42,44]. The VREF clinical isolates of Mexican origin in which the esp and/or hyl gene was amplified (alone or together), were resistant to more than three antibiotics; in contrast, other studies have shown a significant correlation between the presence of the esp gene and resistance to ampicillin, imipenem and ciprofloxacin [40,41].

![Figure 2 Clustering of MLST profiles using the eBURST database algorithm. Our profiles showed that ST412, ST612 and ST203, but not ST757, belong to clonal complex 17.](http://www.biomedcentral.com/1471-2180/13/291)
PFGE and MLST analyses have been proposed as alternative methods for the molecular characterization of clinical isolates of *E. faecium* [45]. According to our PFGE analysis, the 12 VREF isolates showed a heterogeneous pattern associated with a profile of multidrug resistance to different antibiotics and the presence of the vanA gene. The data obtained through PFGE revealed four clusters (I-IV), with a low similarity of 44% being detected among the VREF isolates and therefore high diversity. Furthermore, the VREF isolates within clusters I, II-B and III showed an identical banding profile in the PFGE analysis. However, the MLST data indicated different STs due to changes in the nucleotide sequences of the analyzed housekeeping genes; these data are consistent with the findings of Poh et al. [46]. In addition, the VREF isolates within clusters II-B1 and IV displayed identical PFGE and MLST profiles, in agreement with other authors [22,33]. Nevertheless, pulsotypes from different wards showed similar multidrug resistance profiles, possibly due to horizontal genetic transference between these isolates.

MLST is an important tool for studying the molecular epidemiology of outbreaks of *E. faecium* and microbial population biology [44]. MLST analysis of VREF clinical isolates revealed four STs: ST203, ST412, ST612 and ST757. As previously reported, clonal complex 17 harbors various STs that have been involved in hospital outbreaks. Our results revealed two allelic profiles, ST203 and ST412, belonging to clonal complex 17 STs involved in hospital outbreaks. However, clonal complex 17 has been resolved into two different subgroups, one of which harbors ST17 and ST18, while the second harbors ST78 [47]. ST17, ST18 and ST203 are the major groups in the genetic lineage of *E. faecium*; they are distributed worldwide and have been associated with outbreaks [18,48]. ST412 was the most frequent sequence type found in the VREF isolates from HIMFG and was genetically linked to the ST78 lineage. Interestingly, ST412 has been identified worldwide and associated with outbreaks [49]. According to the eBURST analysis, ST612 showed characteristics of the STs belonging to the 18 lineage. ST757 has not been characterized within clonal complex 17. In addition, ST757 displayed resistance markers (ampicillin and quinolones), virulence genes (*esp* and/or *hyl*) and the *purK1* allele; however, it has not been associated with outbreaks. Nevertheless, this community of multidrug-resistant strains is able to infect humans and might contribute to the spreading of these bacteria in the hospital, highlighting the importance of molecular typing via MLST to identify STs involved in nosocomial outbreaks.

Recently, it was shown that MLST analysis of typified *E. faecium* based on selected alleles may generate misleading results due to the recombination of five alleles (*atpA*, *ddl*, *gdh*, *gyd* and *pstS*). As only the *purk* and *adk* alleles are located in regions where there is no predicted recombination, the results must be interpreted with care [50]. The genome of *E. faecium* is highly plastic due to the few existing barriers to the acquisition of foreign genetic elements [51,52]. Recent studies have provided evidence of high levels of recombination through comparative genomics analyses [51-54]. Whole-genome sequencing platforms are superior to conventional typing methods, providing an excellent tool for determining phylogenies and regions of recombination and for accurately discriminating between outbreak- and non-outbreak-causing VREF isolates [50,55]. Thus, whole-genome sequence information, rather than data on just one or a few genes, could be used to distinguish between closely related strains.

In this study, MLST and PFGE analysis were applied for the molecular characterization of clinical VREF isolates to identify different clonal complexes with different pulsotypes that were not related to outbreaks. However, according to the results obtained through PFGE, four multidrug-resistant clones of VREF were identified at HIMFG; in addition, these VREF isolates were identified at different periods. Therefore, these data suggest that these clones have circulated endemically at HIMFG.

In the case of cluster II, the clones have evolved from cluster II-B to cluster II-B1 due to the high similarity (> 90%) observed via PFGE analysis and based on the acquisition of three bands for B1, suggesting a mechanism of horizontal gene transfer. The results obtained in this study highlight the importance of monitoring circulating VREF isolates in different wards of this institution to efficiently control multidrug resistance and prevent outbreaks of these clones.

**Conclusion**

Little is known about VREF isolates in Mexican hospitals. In this study, the detected virulence genes (*esp* and *hyl*), multidrug profiles and allelic patterns were associated with clonal complex 17 VREF clinical isolates obtained from pediatric patients at HIMFG. To our knowledge, this is the first report describing clonal complex 17 VREF isolates in a tertiary care center in Mexico City.

Multidrug resistance and genetic determinants of virulence confer advantages in VREF in the colonization of their hosts. The genome of *E. faecium* is highly plastic, showing an ability to readily acquire genes involved in environmental persistence, colonization and virulence, favoring the selection of specific clonal complexes in a hospital environment. Therefore, the prevention and control of the propagation of nosocomial infections caused by VREF is crucial for identifying new emergent subclones that could be challenging to treat in subsequent years.
Ethics statement
The study was reviewed and approved by the Research (Dr. Onofre Muñoz Hernández), Ethics (Dr. Amparo Faure Fontenla) and Biosecurity (Dr. Herlinda Vera Hermosillo) Committee of HIMFG, under permit numbers HIM/2011/019. After looking at the medical history of each patient, E. faecium isolates were recovered from clinical samples, and the patients were asked by the physicians in the Infectology Department of HIMFG for their permission for their samples be used in this study. Analyses of E. faecium isolates obtained from clinical samples are not considered routine studies. Informed consent was obtained from the patient for the publication of this report and any accompanying images.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
SAO, LBD and GE performed the susceptibility pattern analysis, molecular genetics experiments and PFGE and MLST assays. SAO, ZZ and ACC participated in editing the manuscript and the data analysis. VCD, CAE, BLM, RHC and GAJ conducted the diagnoses of the patients, interpreted data, collaborated in the collection of samples and revised the manuscript. JXC is the principal investigator and conceived the study, designed the experiments, performed data analysis and wrote the manuscript. All authors read and approved the final version.

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