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Original article

Phenotypic and genotypic characterization of vancomycin-resistant *Enterococcus faecium* clinical isolates from two hospitals in Mexico: First detection of VanB phenotype-*vanA* genotype

Paola Bocanegra-Ibarias[a,◊], Samantha Flores-Treviño[a,◊], Adrián Camacho-Ortiz[b], Rayo Morfin-Otero[c], Licet Villarreal-Treviño[d], Jorge Llaca-Díaz[e], Erik Alan Martínez-Landeros[b], Eduardo Rodríguez-Noriega[c], Andrés Calzada-Güereca[d], Héctor Jesús Maldonado-Garza[a], Elvira Garza-González[a,e,*]

[a] *Servicio de Gastroenterología, Hospital Universitario Dr. José Eleuterio González, Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico*
[b] *Servicio de Infectología, Hospital Universitario Dr. José Eleuterio González, Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico*
[c] *Hospital Civil de Guadalajara Fray Antonio Alcalde e Instituto de Patología Infecciosa y Experimental, Universidad de Guadalajara, Guadalajara, Jalisco, Mexico*
[d] *Departamento de Microbiología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, Mexico*
[e] *Departamento de Patología Clínica, Hospital Universitario Dr. José Eleuterio González, Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico*

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ABSTRACT

Introduction: *Enterococcus faecium* has emerged as a multidrug-resistant nosocomial pathogen involved in outbreaks worldwide. Our aim was to determine the antimicrobial susceptibility, biofilm production, and clonal relatedness of vancomycin-resistant *E. faecium* (VREF) clinical isolates from two hospitals in Mexico.
Methods: Consecutive clinical isolates ($n = 56$) were collected in two tertiary care hospitals in Mexico from 2011 to 2014. VREF isolates were characterized by phenotypic and molecular methods including pulsed-field gel electrophoresis (PFGE).
Results: VREF isolates were highly resistant to vancomycin, erythromycin, norfloxacin, high-level streptomycin, and teicoplanin, and showed lower resistance to tetracycline, nitrofurantoin and quinupristin-dalfopristin. None of the isolates were resistant to linezolid. The *vanA* gene was detected in all isolates. Two VanB phenotype-*vanA* genotype isolates, highly resistant to vancomycin and susceptible to teicoplanin, were detected. Furthermore, 17.9% of the isolates were classified as biofilm producers, and the *espfm* gene was found in 98.2% of the isolates. A total of 37 distinct PFGE patterns and 6 clones (25% of the isolates as clone A, 5.4% as clone B, and 3.6% each as clone C, D, E, and F) were detected. Clone A was detected in 5 different wards of the same hospital during 14 months of surveillance.
Conclusion: The high resistance to most antimicrobial agents and the moderate cross-transmission of VREF detected accentuates the need for continuous surveillance of *E. faecium* in the hospital setting. This is also the first reported incidence of the *E. faecium* VanB phenotype-*vanA* genotype in the Americas.

Caracterización fenotípica y genotípica de aislamientos clínicos de *Enterococcus faecium* resistente a vancomicina de 2 hospitales en México: primera detección del fenotipo VanB-genotipo *vanA*

Palabras clave:
Resistencia a vancomicina
Enterococcus faecium
México

RESUMEN

Introducción: *Enterococcus faecium* multifarmacorresistente es un importante patógeno intrahospitalario que a nivel mundial se ha asociado con brotes hospitalarios. El objetivo de este trabajo fue determinar la susceptibilidad a los antimicrobianos, la formación de biopelícula y la relación clonal de los aislamientos clínicos de *Enterococcus faecium* resistentes a vancomicina (EFRV) en México.

* Corresponding author.
E-mail address: elvira_garza_gzz@yahoo.com (E. Garza-González).
◊ PBI and SFT contributed equally to this work.

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Diversidad clonal
Fenotipo VanB-genotipo *vanA*

Métodos: Se recolectaron 56 aislamientos clínicos en 2 hospitales mexicanos de 2011 a 2014. Los aislamientos de EFRV fueron caracterizados por métodos fenotípicos y moleculares.
Resultados: Los aislamientos de EFRV fueron resistentes a vancomicina, eritromicina, norfloxacina, estreptomicina de alto nivel y teicoplanina. Presentaron baja resistencia a tetraciclina, nitrofurantoína y quinupristina-dalfopristina. Ningún aislamiento presentó resistencia a linezolid. El gen *vanA* se detectó en todos los aislamientos. Dos aislamientos presentaron un fenotipo VanB-genotipo *vanA*, que se caracteriza por la resistencia a vancomicina y la susceptibilidad a teicoplanina. El 17,9% de los aislamientos fueron productores de biopelícula y el 98,2% presentaron el gen *espfm.* Se obtuvieron 37 patrones de bandas diferentes y 6 clonas (25% de la clona A, 5,4% de la clona B y 3,6% de las clonas C, D, E y F, respectivamente). La clona A se detectó en 5 diferentes salas hospitalarias en el mismo hospital durante 14 meses.
Conclusión: La alta resistencia a los antimicrobianos, junto con la moderada transmisión cruzada de EFRV encontradas en este estudio, acentúan, la necesidad de una vigilancia continua de este microorganismo en el ambiente hospitalario. Además, este es el primer reporte de *E. faecium* con un fenotipo VanB-genotipo *vanA* en América.

Introduction

Over the past few decades, *Enterococcus* species have become one of the most challenging nosocomial problems worldwide due to, in part, the increased use of vancomycin and broad-spectrum antibiotics in hospital. Enterococci can cause a range of nosocomial infections, including urinary tract infections, endocarditis, intra-abdominal and pelvic infections, catheter-related infections, surgical wound infections, and central nervous system infections.[1] One of the most common species of *Enterococcus* involved in nosocomial infections is *Enterococcus faecium* which is much more frequently resistant to vancomycin and ampicillin than *Enterococcus faecalis*. Consequently, vancomycin-resistant *E. faecium* (VREF) represents a growing threat in hospital-acquired infections.[2]

Enterococci are intrinsically resistant to several antibiotics, and they also readily accumulate mutations and exogenous genes that confer additional resistance. The acquisition of resistance genes often occurs via conjugation with plasmids or conjugative transposons that can potentially carry multiple antibiotic resistance genes.[1]

Acquired resistance to vancomycin in *E. faecium* is an increasing problem particularly in hospital-acquired infections. Different genotypes of vancomycin resistance, *vanA–G*, have been described in enterococci. The *vanA* and *vanB* genotypes in *E. faecium* are most commonly found worldwide, with *vanA* predominating.[3] These genotypes are associated with different levels of antibiotic resistance. The *vanA* genotype is associated with a high level of vancomycin and teicoplanin resistance, the *vanB* genotype is associated with a moderate to high level of vancomycin resistance and teicoplanin susceptibility, and the *vanC* genotype is associated with an intrinsically low level of vancomycin resistance.[4]

In addition to antibiotic resistance, biofilm formation may be an important factor in the pathogenesis of enterococcal infection. *E. faecium* is capable of producing biofilms, which consist of a population of cells encased in a hydrated matrix of exopolymeric substances that attach irreversibly to various biotic and abiotic surfaces.[5] In fact, enterococci can survive on environmental surfaces, including medical equipment, for long time periods.[1] It has been proposed that many environmental and genetic factors are associated with biofilm production, including the enterococcal surface protein, Esp (*fm*).[5,6] Few studies have included an analysis of VREF clinical isolates from Mexican hospitals[7,8] and even fewer have reported a molecular analysis of such isolates.[7,9–12] Therefore, the aim of this study was to examine the antimicrobial susceptibility, biofilm production, and clonal relatedness of VREF clinical isolates obtained from two tertiary care hospitals in Mexico.

Methods

Clinical isolates and study population

From 2011 to 2014, consecutive *E. faecium* isolates were collected at two tertiary care hospitals in Mexico: Hospital Universitario "Dr. José Eleuterio González" in Nuevo Leon and Hospital Civil de Guadalajara and Instituto de Patología Infecciosa y Experimental "Dr. Francisco Ruiz Sánchez" in Jalisco. The Hospital Universitario "Dr. José Eleuterio González" is a 450 bed tertiary-care teaching hospital located in the city of Monterrey, the third largest city in Northeastern Mexico. The hospital provides care to adult and pediatric patients in 20 wards located in a main building and additional services in 3 connected buildings. This hospital serves a population that includes the Monterrey metropolitan area, including 51 municipalities (approximate 5 million Hab.) and surroundings states. During 2013 there were 24,572 discharges, 7667 surgical procedures and a 96.3% daily occupancy rate with patients having a mean length of stay of 4.94 days. The Hospital Civil de Guadalajara Fray Antonio Alcalde is an 899-bed tertiary-care teaching hospital located in the city of Guadalajara, the second largest city in western Mexico. The hospital provides care to adult and pediatric patients in 31 wards situated among 3 connected buildings. This hospital serves a population that includes the greater Guadalajara metropolitan area, including 7 municipalities (approximately 4.0 million Hab) and surroundings states. During 2013 there were 45,982 discharges, 25,415 surgical procedures, and a 94.7% daily occupancy rate with patients having a mean length of stay of 6.2 days.

During the 4-year study period, 151 isolates of *E. faecium* were collected, and 56 isolates (37.1%) were vancomycin-resistant, of which 48 (85.7%) isolates were from Monterrey and 8 (14.3%) were from Guadalajara. All isolates were considered as presumptive causative agents of infection. One isolate per patient was assayed. The majority of the isolates were recovered from urine (33.9%), followed by blood (23.2%), and soft tissue (14.3%). Most isolates (58.9%) were obtained from intense care unit (ICU) patients. All isolates were cultured on 5% sheep blood agar plates at 37 °C for 24 h and identified using biochemical tests (i.e., 6.5% NaCl growth, bile esculin hydrolysis, and potassium tellurite reduction). All *E. faecium* isolates were stored at −80 °C until further use. We retrieved

demographic data from the patients' charts as well as information about common risk factors, such as prior antibiotic exposure, hospitalization, and invasive device usage.

Ethics statement

The local ethics committee (Comité de Ética y Comité de Investigación, Facultad de Medicina y Hospital "Dr José Eleuterio González", Universidad Autónoma de Nuevo León, Mexico) approved this study with the following reference number GA14-015. Informed consent was waived by the Ethics Committee because no intervention was involved and no patient identifying information was included.

Antimicrobial susceptibility and presence of vancomycin resistance-associated genes

Broth microdilution susceptibility testing of *E. faecium* was performed in Sensititre plates (Thermo Scientific-TREK Diagnostic Systems, Ohio, USA) according to the manufacturer's instructions. The antibiotics tested included erythromycin, ampicillin, high-level streptomycin, high-level gentamicin, linezolid, nitrofurantoin, norfloxacin, quinupristin/dalfopristin, tetracycline, teicoplanin, and vancomycin. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria.[13] All *E. faecium* isolates were screened for the presence of *vanA*, *vanB*, and *vanC* as previously described.[14]

Biofilm production and presence of biofilm production-associated genes

Biofilm formation was determined using crystal violet staining as previously described with some modifications.[15] All isolates were tested in two different experiments conducted on different days. Cultures diluted 1:40 in 200 μl of tryptic soy broth containing 1% glucose were inoculated into 96-well polystyrene plates (Falcon, Franklin Lakes, NJ, USA). After 24 h of incubation at 37 °C, planktonic cells were removed for determination of cell density at 600 nm, and plates were washed 3 times with sterile phosphate buffered saline (PBS), pH 7.3. The adherent biofilms were stained with 0.1% crystal violet for 15 min at room temperature, washed 5 times with sterile PBS, and allowed to dry. Finally, an ethanol:acetone (80:20) solution was added to the biofilm samples, and the optical density at 595 nm (OD_{595}) was read. The American Type Culture Collection (ATCC) strains, *Staphylococcus aureus* 29213 (high biofilm producer) and *Escherichia coli* 25922 (low biofilm producer) were used as controls. Isolates were classified as non-adherent (OD ≤ 0.120), weakly adherent (OD > 0.120 to 0.240), or strongly adherent (OD > 0.240) according to the criteria described by Christensen et al.[16] Determination of the biofilm index was performed using the method previously described by Kristich et al.[15] Presence of the *espfm* gene was determined by PCR using the designed primer, espfm-F (5′-TTGCTAATGCAAGTCCACGTCC-3′) and primer TE105 (5′-GCATCAACACTTGCATTACCGAA-3′), the sequence of which has been previously reported.[17] Each 25-μl reaction mixture consisted of 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 200 nM of each primer, 1 U of AmpliTaq polymerase (Bioline USA Inc., Randolph, MA, USA) and 2 μl of DNA. PCR was initiated by denaturation for 1 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C with final extension for 1 min at 72 °C. The PCR products were separated on a 2% agarose gel with a molecular weight standard ladder, and the ethidium bromide-stained bands were visualized by UV transillumination.

Clonal diversity by pulsed-field gel electrophoresis (PFGE)

The genetic relatedness of all *E. faecium* isolates was analyzed by PFGE as previously described with modifications.[18] The plugs were incubated overnight in lysis buffer supplemented with RNase (5 mg/ml) and lysozyme (1 mg/ml), followed by incubation in fresh lysis buffer with proteinase K (0.5 mg/ml) at 55 °C for 24 h. DNA was digested with the restriction enzyme, *Sma*I, and electrophoresis was performed on the CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA) at 6 V/cm, with linear switching interval ramps from 3.5 s to 25 s for 12 h at 14 °C for the first block, followed by 1 s to 5 s for 8 h for the second block. Band patterns were visually compared after ethidium bromide staining, and the interpretation was based on the criteria of Tenover et al.[19]

Statistical analysis

Statistical analysis was performed in the SPSS statistics 20.0 software (IBM Corporation, Somers, NY, USA). Demographic and clinical characteristics were compared using Fisher's exact test for percentages and Wilcoxon rank test for continuous variables; a P value of <0.05 was considered as statistically significant. PFGE band patterns were generated by Labworks 4.5 software (Ultra-Violet Products, Upland, CA, USA) with 1% tolerance. The similarity coefficients were generated from a similarity matrix calculated using the Jaccard's coefficient.

Results

Demographic and clinical characteristics

We retrieved demographic data from 49 of 56 patients (41 from Monterrey and 8 from Guadalajara). Patients from Monterrey had a mean age of 49.8 years and were hospitalized mainly for intra-abdominal or lower respiratory tract infections. The majority of patients had been in the ICU with a median stay of 19 days, and 60.9% ($n = 25$) of the patients had received one to three antibiotics before *E. faecium* was detected. Most patients were treated with carbapenems and cephalosporins. Patients from Guadalajara had a mean age of 44.6 years and were hospitalized mainly for lower respiratory tract or other infections. The majority of patients had been in the ICU with a median stay of 26.2 days, and 87.5% ($n = 7$) of the patients had received at least four antibiotics before *E. faecium* was detected. Most patients were treated with carbapenems and cephalosporins. There were significant differences in the percentage of isolates from blood, patients with neutropenia and a higher number of patients with prior use of 4 or more antibiotics (especially aminoglycosides) in the group from Guadalajara compared to the group from Monterrey ($p < 0.05$ for all, Table 1).

Antimicrobial susceptibility

The minimum inhibitory concentration (MIC) range, MIC_{50}, and MIC_{90}, as well as the percentage of isolates resistant, intermediately resistant, and susceptible to each of the antimicrobial agents tested are shown in Table 2. Based on the CLSI interpretive criteria, the *E. faecium* isolates from Monterrey were highly resistant to vancomycin (100%), ampicillin (100%), erythromycin (100%), norfloxacin (100%), high-level streptomycin (85.4%) and teicoplanin (81.3%). The isolates exhibited lower resistance to high-level gentamicin (68.8%), tetracycline (54.2%), nitrofurantoin (45.8%), and quinupristin/dalfopristin (6.25%). The *E. faecium* isolates from Guadalajara were highly resistant to vancomycin (100%), teicoplanin (100%), ampicillin (100%),

Table 1
Demographic and clinical characteristics of patients.

Characteristic	No. (% of patients or range) Monterrey	No. (% of patients or range) Guadalajara	*P* value
No. of patients	41	8	
Mean age (range)	49.8 (16–86)	18.9 (0–74)	0.007
Male	23 (56)	6 (75)	0.444
Specimens			
Urine	19 (46.3)	–	–
Blood	6 (14.6)	6 (75)	0.001
Soft tissue	8 (19.5)	–	–
Ascities	4 (9.7)	–	–
Respiratory secretions	2 (4.8)	–	–
Other	1 (2.4)	2 (25)	0.065
Primary diagnosis			
Lower respiratory tract infection	5 (12.1)	3 (37.5)	0.110
Intra-abdominal infection	12 (29.2)	–	–
Urinary tract infection	4 (9.7)	–	–
Bacteremia	0	2 (25)	–
Other	20 (48.7)	3 (37.5)	0.706
Comorbidities			
End stage renal disease			
With RRT	9 (21.9)	–	–
Without RRT	18 (43.9)	–	–
Cancer	5 (12.1)	–	–
Neutropenia	3 (7.3)	4 (50)	0.009
Previous hospitalization			
>72 h (%)	13 (31.7)	1 (12.5)	0.410
<72 h (%)	3 (7.3)	–	–
Median ICU LOS (days)	19 (4–59)	26.2 (0–66)	0.663
Median time of mechanical ventilation (range)	18 (4–50)	18 (11–25)	0.984
Median duration of central line use (range)	23 (4–54)	15	0.705
Median duration of urinary catheter use (range)	23.5 (4–80)	20	0.914
LOS prior to positive culture (range)	16 (0–66)	21.7 (10–33)	0.372
LOS after positive culture (range)	9 (0–116)	11.8 (0–36)	0.498
Number of antibiotics used prior to E. faecium isolation			
1–3	25 (60.9)	1 (12.5)	0.018
4 or more	16 (39)	7 (87.5)	0.018
Class of antibiotic used prior to E. faecium isolation			
Carbapenems	23 (56.0)	6 (75)	0.4440
Cephalosporins	22 (53.6)	6 (75)	0.438
Fluoroquinolones	9 (21.9)	4 (50)	0.183
Vancomycin	12 (29.2)	1 (12.5)	0.663
Metronidazole	10 (24.3)	2 (25)	0.970
Aminoglycosides	5 (12.1)	4 (50)	0.028
Penicillins	3 (7.3)	2 (25)	0.182
Other	6 (14.6)	3 (37.5)	0.151

RRT = renal replacement therapy; APACHE = acute physiology and chronic health evaluation; LOS = length of stay.

erythromycin (100%), norfloxacin (100%), high-level streptomycin (100%) and high-level gentamicin (100%). The isolates exhibited lower resistance to tetracycline (87.5%), nitrofurantoin (37.5%), and quinupristin/dalfopristin (12.5%). None of the isolates were resistant to linezolid. The *vanA* gene was detected in all isolates; however, neither *vanB* nor *vanC* was detected in any isolate. We also detected two VanB phenotype-*vanA* genotype isolates (MX13-1542 and MX13-0631), which were highly resistant to vancomycin

Table 2
Antimicrobial susceptibility of *E. faecium* isolates.

Antimicrobial agent	MIC ($\mu g\,ml^{-1}$)			No. (%) of isolates[a]		
	Range	50%	90%	Resistant	Intermediate	Susceptible
Ampicillin	64 to >64	>64	>64	56 (100.0)	0 (0.0)	0 (0.0)
Erythromycin	>4 to >4	>4	>4	56 (100.0)	0 (0.0)	0 (0.0)
High-level streptomycin	≤1000 to >1000	>1000	>1000	49 (87.3)	0 (0.0)	7 (12.5)
High-level gentamicin	≤500 to >500	>500	>500	41 (73.2)	0 (0.0)	15 (26.8)
Linezolid	≤2 to ≤2	≤2	≤2	0 (0.0)	0 (0.0)	56 (100.0)
Nitrofurantoin	64 to >64	64	>64	25 (44.6)	31 (55.5)	0 (0.0)
Norfloxacin	>8 to >8	>8	>8	56 (100.0)	0 (0.0)	0 (0.0)
Quinupristin/dalfopristin	≤1 to >2	≤1	>2	3 (5.4)	7 (12.5)	46 (82.1)
Tetracycline	≤2 to >8	8	>8	27 (48.2)	1 (1.8)	28 (50.0)
Teicoplanin	≤8 to >16	>16	>16	47 (83.9)	7 (12.5)	2 (3.6)
Vancomycin	>16 to >16	>16	>16	56 (100.0)	0 (0.0)	0 (0.0)

[a] Classification of isolates as resistant, intermediate, or susceptible based on CLSI interpretive criteria.

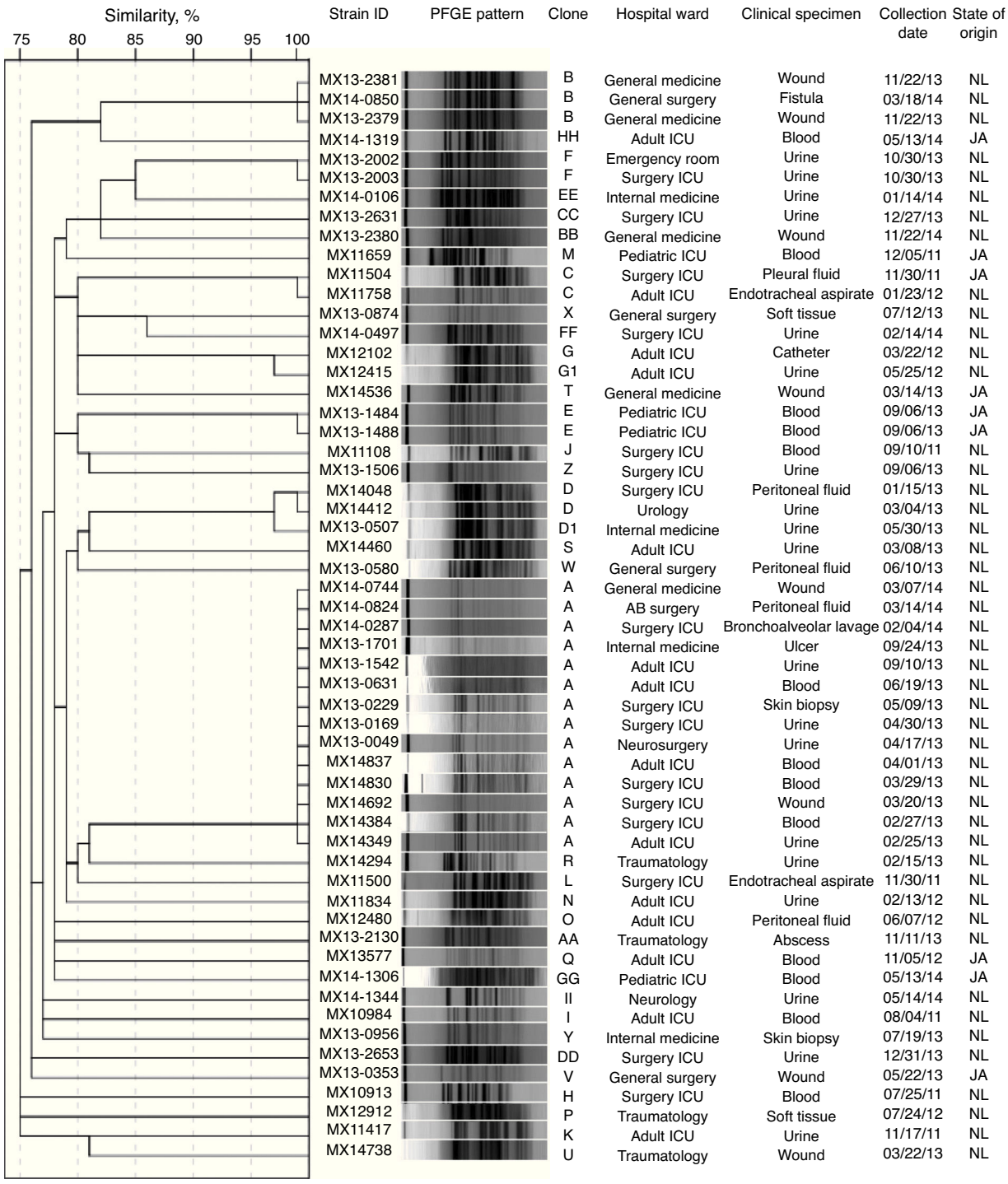

Fig. 1. Dendogram and PFGE patterns of VREF clinical isolates. The clinical characteristics of the patients are also included. NL corresponds to Nuevo Leon and JA corresponds to Jalisco. ICU corresponds to intensive care unit.

and susceptible to teicoplanin; these isolates were collected in Monterrey.

Biofilm production

Ten of the 56 isolates (17.9%) were able to form biofilms. These biofilm-producing isolates were classified as weak biofilm producers (90%) and strong biofilm producers (10%). The *espfm* gene was found in 98.2% of the isolates. Nine of the isolates (18.8%) obtained in Monterrey were biofilm producers and one of the isolates (12.5%) obtained in Guadalajara was biofilm producer.

Clonal diversity

A total of 37 distinct PFGE patterns were identified. The percentage of similarity among the analyzed isolates ranged from 75% to 100%, and the isolates exhibited restriction patterns consisting of 11–20 bands. Isolates that showed 100% similarity in their restriction patterns were classified as a clone. As well, 44.6% (25/56) of the isolates were distributed in 6 clones (A to F), as shown in Fig. 1. Clones A (14/56), B (3/56), C (2/56), D (2/56), and F (2/56) were clusters of isolates obtained from Nuevo Leon. Clone E (2/56) was the only clone obtained from Jalisco. Clone

A was detected in 5 different wards of the same hospital during 14 months of surveillance. Among the clone A isolates, 78.6% (11/14) of them were susceptible to high-level gentamicin and resistant to high-level streptomycin (data not shown). We also detected two subtypes (D1 and G1) with restriction patterns that were 97% similar to the D and G patterns, respectively.

Discussion

In this study, we examined the antimicrobial susceptibility, biofilm production, and clonal relatedness of 56 VREF clinical isolates obtained from two tertiary care hospitals in Mexico during a 4-year study period. Our results demonstrate the presence of VREF isolates that are strongly resistant to vancomycin, erythromycin, norfloxacin, high-level streptomycin, and teicoplanin. Compared to the few previous studies that analyzed VREF clinical isolates from Mexican hospitals[7–12] this study included the largest number of VREF clinical isolates to date. Therefore, this work represents a contribution to the national surveillance of emerging nosocomial pathogens.

All the VREF isolates we examined were of the *vanA* genotype. Similarly, previous studies in Mexico showed that VREF isolates carried the *vanA* gene,[7,9–12] although the *vanB* gene was also detected.[9,11] Interestingly, we detected two VanB phenotype-*vanA* genotype isolates (MX13-1542 and MX13-0631), which were highly resistant to vancomycin and susceptible to teicoplanin. The VanB phenotype-*vanA* genotype in *E. faecium* has been rarely reported worldwide.[20–23] In fact, to our knowledge, this is the first detection of this particular *E. faecium* isolate in the Americas.

As of yet, we do not know the mechanism for the emergence of these VanB phenotype-*vanA* genotype VREF isolates. The *vanA* gene cluster is a widely studied vancomycin/teicoplanin resistance determinant which includes 7 *van* genes (*vanA*, *vanH*, *vanR*, *vanS*, *vanX*, *vanY*, and *vanZ*) in a Tn1546-type transposon.[3,24] It has been reported that the deletion of the intergenic region between *vanY* and *vanZ* can partly explain the difference between phenotype and genotype in the VanB phenotype-*vanA* genotype *E. faecium* isolate.[21] Therefore, further studies should be performed to fully characterize the Tn1546 structure of the two isolates we detected in this study. Tn1546 is generally carried on plasmids and thus is effectively disseminated by horizontal gene transfer. An acquisition of *vanA* plasmid by a strain of nosocomial *E. faecium* may result in a spread of VREF infection. Therefore, both characterization of the Tn1546 structure and its linkage to particular plasmid groups is crucial for understanding of VREF dissemination in hospital environments.

There are several risk factors for developing a nosocomial VRE infection, including close physical proximity to patients infected or colonized with VRE, a long period of hospitalization, multiple courses of antimicrobials, hospitalization in intensive-care units and co-morbidities such as diabetes, renal failure or hemodialysis.[1] Certainly, the majority of our patients had been in the ICU with a prolonged stay. In addition, half of our patients had received at least four antibiotics before *E. faecium* was detected, most of them being carbapenems and cephalosporins.

We detected no resistance to linezolid in any of the clinical isolates. Linezolid is one of the two antibiotics that are approved by the Food and Drug Administration (FDA) to treat VREF, and it is used worldwide.[1] It has been reported that linezolid resistance in VREF is dependent on prior exposure and duration of therapy.[25] Enterococcal infections are commonly treated with ampicillin as the first line treatment, while vancomycin is recommended for cases of ampicillin resistance. Linezolid, daptomycin, and tigecycline are used as alternatives in cases of vancomycin-resistance. Despite linezolid resistance still being rare worldwide[1] it has been documented in enterococcal outbreaks[26] and even sporadically in patients who have never received the antibiotic.[1] Taking these findings and recent reports of linezolid resistant VREF in Mexico[11,12] into account, the need for continuous surveillance of *E. faecium* antimicrobial resistance in hospitals throughout our country is mandatory.

Biofilm formation reportedly occurs less commonly among *E. faecium* isolates than among *E. faecalis* isolates, the latter which exhibit a much higher frequency of biofilm formation (95–100%), according to previous reports.[27,28] Our result (17.9%) also indicates low biofilm production in *E. faecium*, and this frequency was even lower than that (53.75%) in a previous report.[28] This finding indicates that the clinical impact of these particular VREF isolates is not enhanced by biofilm formation. Furthermore, most of these isolates in our hospitals were obtained from blood and urine specimens, whereas it is thought that biofilm formation is important in conditions such as endocarditis, periodontitis, and a variety of device-related infections[5] that were not evident among the patients in our study. Conflicting outcomes regarding the role of the *espfm* gene in biofilm formation have been published.[5] While this gene reportedly plays a role in *E. faecalis* biofilm formation,[29] it is apparently neither essential nor sufficient for biofilm production in infectious *E. faecium* isolates.[27,28] Indeed, we detected a high frequency of the *espfm* gene in our isolates despite the low level of biofilm production.

Molecular typing methods are essential for identifying hospital-associated outbreaks of *E. faecium*. Using PFGE, we detected a moderate presence of clones as well as the predominance of clone A, which was detected in 5 different wards of the same hospital during 14 months of surveillance. In addition, the two VanB phenotype-*vanA* genotype isolates (MX13-1542 and MX13-0631) were detected within the clone A. These two isolates were obtained with a 3-month span from different patients at the ICU. This finding indicates cross-transmission of VREF in the hospital setting. Enterococci can survive for long periods on environmental surfaces, including medical equipment, which may help explain why these organisms were disseminated in the nosocomial setting. In addition, the fact that the two VanB phenotype-*vanA* genotype isolates were part of clone A underlines the need for continuous monitoring of currently circulating VREF strains to efficiently prevent and control the propagation of nosocomial VREF infections.

One limitation of this study is the lack of using multilocus sequence typing (MLST) for the molecular characterization of *E. faecium* isolates.[30] MLST has revealed the existence of host-specific genogroups, including a specific genetic lineage designated as clonal complex 17 (CC17), which is associated with hospital-related isolates. These strains are characterized by ampicillin and quinolone resistance. The first report of multidrug-resistant VREF isolates in Mexico City that belong to CC17 has been recently published.[12] Thus, further studies are needed to analyze our VREF isolates using MLST.

E. faecium isolates resistant to vancomycin, erythromycin, norfloxacin, high-level streptomycin, and teicoplanin exist in Mexican hospitals. The moderate clonal diversity and high frequency of clone A detected among these isolates indicate cross-transmission of VREF in the hospital setting. In addition, this is the first reported incidence of the *E. faecium* VanB phenotype-*vanA* genotype in the Americas. This study therefore represents a contribution to the national surveillance of emerging nosocomial pathogens.

Conflict of interest

The authors declare no conflict of interest.

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References

1. Arias CA, Murray BE. The rise of the *Enterococcus*: beyond vancomycin resistance. Nat Rev Microbiol. 2012;10:266–78.
2. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, et al. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. Infect Control Hosp Epidemiol. 2008;29:996–1011.
3. Werner G, Strommenger B, Witte W. Acquired vancomycin resistance in clinically relevant pathogens. Futur Microbiol. 2008;3:547–62.
4. Hegstad K, Mikalsen T, Coque TM, Werner G, Sundsfjord A. Mobile genetic elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. Clin Microbiol Infect. 2010;16:541–54.
5. Mohamed JA, Huang DB. Biofilm formation by enterococci. J Med Microbiol. 2007;56 Pt 12:1581–8.
6. Eaton TJ, Gasson MJ. A variant enterococcal surface protein Esp(fm) in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. FEMS Microbiol Lett. 2002;216:269–75.
7. Llaca-Diaz JM, Mendoza-Olazaran S, Camacho-Ortiz A, Flores S, Garza-Gonzalez E. One-year surveillance of ESKAPE pathogens in an intensive care unit of Monterrey, Mexico. Chemotherapy. 2012;58:475–81.
8. Garcia GM, Leo-Amador GE, Avila-Morales J, Zaldivar-Lelo de Larrea G, Sanchez-Corona J. Vancomycin-resistant *Enterococcus* spp isolated from community acquired infections and colonizations in Queretaro City, Mexico. Am J Infect Control. 2014;42:578–80.
9. Calderon-Jaimes E, Arredondo-Garcia JL, Aguilar-Ituarte F, Garcia-Roca P. In vitro antimicrobial susceptibility in clinical isolates of *Enterococcus* species. Salud Publica Mex. 2003;45:96–101.
10. Cuellar-Rodriguez J, Galindo-Fraga A, Guevara V, Perez-Jimenez C, Espinosa-Aguilar L, Rolon AL, et al. Vancomycin-resistant enterococci, Mexico City. Emerg Infect Dis. 2007;13:798–9.
11. Leo-Amador GE, Borbolla-Ramos A, Morales-Lara JA, Perez-Gonzalez HA, Hernandez-Montiel HL, Solis SJ. Infection or colonization and antimicrobial susceptibility of *Enterococcus* spp at a regional hospital in Queretaro, Mexico. Am J Infect Control. 2011;39:615–6.
12. Ochoa SA, Escalona G, Cruz-Cordova A, Davila LB, Saldana Z, Cazares-Domimguez V, et al. Molecular analysis and distribution of multidrug-resistant *Enterococcus faecium* isolates belonging to clonal complex 17 in a tertiary care center in Mexico City. BMC Microbiol. 2013;13:291.
13. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. In: Twenty-fourth informational supplement M100-S24. 2014.
14. Clark NC, Cooksey RC, Hill BC, Swenson JM, Tenover FC. Characterization of glycopeptide-resistant enterococci from U.S. hospitals. Antimicrob Agents Chemother. 1993;37:2311–7.
15. Kristich CJ, Li YH, Cvitkovitch DG, Dunny GM. Esp-independent biofilm formation by *Enterococcus faecalis*. J Bacteriol. 2004;186:154–63.
16. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol. 1985;22:996–1006.
17. Togay SO, Keskin AC, Acik L, Temiz A. Virulence genes, antibiotic resistance and plasmid profiles of *Enterococcus faecalis* and *Enterococcus faecium* from naturally fermented Turkish foods. J Appl Microbiol. 2010;109:1084–92.
18. Weng PL, Ramli R, Shamsudin MN, Cheah YK, Hamat RA. High genetic diversity of *Enterococcus faecium* and *Enterococcus faecalis* clinical isolates by pulsed-field gel electrophoresis and multilocus sequence typing from a hospital in Malaysia. Biomed Res Int. 2013;2013:938937.
19. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33:2233–9.
20. Jung MK, Ahn SH, Lee WG, Lee EH. Molecular epidemiology of vancomycin-resistant enterococci isolated from non-tertiary-care and tertiary-care hospitals in Korea. Epidemiol Infect. 2014;142:2372–7.
21. Gu L, Cao B, Liu Y, Guo P, Song S, Li R, et al. A new Tn1546 type of VanB phenotype-*vanA* genotype vancomycin-resistant *Enterococcus faecium* isolates in mainland China. Diagn Microbiol Infect Dis. 2009;63:70–5.
22. Strateva T, Atanasova D, Mitov I, Sirakov I, Katrandjieva A. Emergence of VanB phenotype-*vanA* genotype *Enterococcus faecium* clinical isolate in Bulgaria. Braz J Infect Dis. 2014;18:693–5.
23. Al-Ahdal MN, Abozaid SM, Al-Shammary HF, Bohol MF, Al-Thawadi SI, Al-Jaberi AA, et al. Characterization of *Enterococcus faecium* isolates and first report of vanB phenotype-*vanA* genotype incongruence in the Middle East. Eur J Clin Microbiol Infect Dis. 2012;31:3223–9.
24. Depardieu F, Podglajen I, Leclercq R, Collatz E, Courvalin P. Modes and modulations of antibiotic resistance gene expression. Clin Microbiol Rev. 2007;20:79–114.
25. Pai MP, Rodvold KA, Schreckenberger PC, Gonzales RD, Petrolatti JM, Quinn JP. Risk factors associated with the development of infection with linezolid- and vancomycin-resistant *Enterococcus faecium*. Clin Infect Dis. 2002;35:1269–72.
26. de Almeida LM, de Araujo MR, Iwasaki MF, Sacramento AG, Rocha D, da Silva LP, et al. Linezolid resistance in vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolates in a Brazilian hospital. Antimicrob Agents Chemother. 2014;58:2993–4.
27. Di Rosa R, Creti R, Venditti M, D'Amelio R, Arciola CR, Montanaro L, et al. Relationship between biofilm formation, the enterococcal surface protein (Esp) and gelatinase in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. FEMS Microbiol Lett. 2006;256:145–50.
28. Almohamad S, Somarajan SR, Singh KV, Nallapareddy SR, Murray BE. Influence of isolate origin and presence of various genes on biofilm formation by *Enterococcus faecium*. FEMS Microbiol Lett. 2014;353:151–6.
29. Toledo-Arana A, Valle J, Solano C, Arrizubieta MJ, Cucarella C, Lamata M, et al. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. Appl Environ Microbiol. 2001;67:4538–45.
30. Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, et al. Multilocus sequence typing scheme for *Enterococcus faecium*. J Clin Microbiol. 2002;40:1963–71.

[a] Facultad de Química, Universidad Autónoma de Yucatán, Mérida, Yucatán, Mexico.
[b] Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, Mexico.