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Molecular epidemiology of vancomycin-resistant *Enterococcus faecium* in Argentina☆

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Summary
Objective: To characterize the mechanism of glycopeptide resistance and to determine the genetic relatedness among strains by pulsed-field gel electrophoresis (PFGE) in vancomycin-resistant *Enterococcus faecium* from Argentina.
Materials and methods: A total of 189 vancomycin-resistant single-patient isolates of *Enterococcus faecium* recovered between January 1997 and December 2000 from 30 hospitals in Argentina were studied. Minimum inhibitory concentrations were determined by the agar dilution method and *van* genes were detected by PCR. PFGE was used for molecular typing.
Results: All isolates except three (*van*B) were of genotype *van*A. For 189 vancomycin-resistant *Enterococcus faecium*, *Sma*I-PFGE indicated 35 clonal types. Most of the isolates (56%) belonged to the same clonal type 1, which was present in 19 hospitals and dominant in 17.
Conclusions: The emergence of vancomycin-resistant *Enterococcus faecium* in Argentina seems to be related to the intra- and inter-hospital dissemination of an epidemic clone carrying the *van*A element.

Introduction

Enterococci as a cause of nosocomial infection have become more prevalent over the last 20 years, both in the USA and in Western European countries. Strains of enterococci have acquired resistance to almost all antimicrobial agents, including vancomycin.

Vancomycin-resistant *Enterococcus* (VRE) was first isolated in the UK and France in 1986,[1] and one year later,

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the first cases of VRE were documented in the USA.[2] Important differences in the epidemiology of VRE in the USA and Europe were observed. In the USA, the major factor contributing to the dissemination of VRE was the excessive use of glycopeptides and other antibiotics in the healthcare environment. In contrast, in Europe the emergence of VRE took place outside hospitals. A large reservoir of transferable *van*A gene cluster was identified in animal husbandry and has been associated with the use of avoparcin as a growth promoter in animal feed.[3–5]

Nowadays, VRE are distributed worldwide. The incidence of VRE infection in the USA has greatly increased over the past 15 years. Within 10 years VRE represented more than 25% of enterococci associated with bloodstream infections in hospitalized patients in the USA.[6] In Latin America, VRE have been reported in Brazil[7] and Colombia.[8] In Argentina, the first reported VRE, an *Enterococcus faecium* isolate carrying the *van*A gene, was isolated in 1997 from a blood culture.[9] After that, many hospitals in our country implemented a survey of stool or rectal swab cultures in order to detect VRE colonization and to prevent and control nosocomial transmission.

Between January 1997 and December 2000, at the Antimicrobial Division of the National Institute of Infectious Diseases (INEI) ''Dr. C.G. Malbrán'', we received a total of 189 vancomycin-resistant *E. faecium* from 30 hospitals. Almost all Argentinean hospitals that had identified vancomycin-resistant *E. faecium* up to December 2000 contributed their isolates to this study. The main objectives of the present study were to characterize the mechanisms of glycopeptide resistance and to evaluate the mode of dissemination of vancomycin-resistant *E. faecium* isolates in Argentina.

Materials and methods

Clinical isolates

From January 1997 to December 2000, 189 vancomycin-resistant *E. faecium* from 30 hospitals were received at the INEI to confirm the genotype. Eleven enterococci (5.8%) were recovered during 1997, 49 (25.9%) in 1998, 67 (35.5%) in 1999, and 62 (32.8%) in 2000. Isolates were identified in each hospital to the species level with conventional biochemical tests as described by Carvalho et al.[10] One hundred and twenty-five (66.1%) isolates were collected from 20 hospitals in Buenos Aires City, 52 (27.5%) from six hospitals in the Province of Buenos Aires, three (1.6%) from two hospitals in the Province of Córdoba (700 km from Buenos Aires City), seven (3.7%) from one hospital in the Province of Chaco (970 km from Buenos Aires City), and two (1.1%) from one hospital in the Province of Santa Fe (480 km from Buenos Aires City). Names, locations, and the number of isolates recovered from each hospital are listed in Table 1.

Vancomycin-resistant *E. faecium* strains were isolated from rectal swabs in 145 (76.7%), urine in 17 (9%), blood in nine (4.8%), and from other sources in 18 (9.5%). Only the initial isolate of each patient was included in the study. Most of the strains were isolated in the intensive care units (ICU) (46.5%) and general medicine wards (36%). One hundred and fifty-two patients (80.4%) were colonized with VRE, 28 (14.8%) exhibited signs of clinical infection, and in nine (4.8%) cases an assessment of the clinical significance was not possible.

Antimicrobial susceptibility

Minimum inhibitory concentrations (MICs) to ampicillin (Bagó Argentina), vancomycin (Lilly), teicoplanin (Aventis Pharma), gentamicin (Schering—Plough), streptomycin (Rontag), tetracycline (Phoenix), chloramphenicol (Parke Davis), erythromycin (Lilly), and ciprofloxacin (Roemmers Argentina) were determined by the agar dilution procedure according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) recommendations.[11,12] Quality control strains used were *Staphylococcus aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *E. faecalis* ATCC 51299.

PCR amplification of glycopeptide resistance genes

The presence of *van* genes was detected by PCR using specific primers for *van*A and *van*B and conditions already described.[13] DNA template was prepared by the boiling method. Reactions were performed with a Biometra thermal cycler (Whatman Biometra GmbH, Göttingen, Germany). The PCR amplification products were analyzed in 1% agarose gel. *E. faecalis van*A Tx2403 and *E. faecium van*A WHO-3 used as positive controls, were kindly provided by Barbara Murray (University of Texas at Houston, USA) and Fred Tenover (CDC, Atlanta, GA, USA), respectively. Specific primers for the 16S ribosomal RNA gene were used as controls of DNA extraction.[14] Amplification of the intergenic *van*S—*van*H region was performed using the specific primers, *van*S-f (forward) and *van*H-r (reverse), as described by Brown et al.[15] PCR-RFLP was performed on *van*SH amplicons, using *Hind*III and *Eco*RI enzymes as recommended by the manufacturer (New England Biolabs, Beverly, MA, USA).

PFGE typing

Isolates were grown overnight in brain—heart infusion broth. Chromosomal DNA was prepared in agarose plugs and subjected to endonuclease digestion with *Sma*I (New England Biolabs, Beverly, MA, USA) as previously described.[16] DNA fragments were separated in 0.8% agarose using a CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA, USA), with running conditions of 6 V/cm and pulses ranging from 5 to 35 seconds during 26 h at 7 °C. Lambda ladder (New England Biolabs) was used as molecular size standard. Gels were stained with 1 μg/mL ethidium bromide and photographed under UV illumination. The similarity between isolates was determined by visual comparison of isolate banding patterns. The interpretation of the band patterns was carried out according to previously published guidelines.[17] Isolates were defined as distinct strain types, or unrelated, if their PFGE patterns differed by more than six bands. Types were named using a consecutive Arabic number (e.g. type 1, 2, 3). Subtypes were defined as strains that differed by 2—6 bands, which were considered closely or possibly related, and were named using a letter following the Arabic number (e.g. subtype 1a, 1b, 1c). Those isolates whose restriction patterns had the same number and size of bands were considered genetically indistinguishable and were assigned to the same strain type and subtype.

Table 1 Origin and clonal type of 189 clinical isolates of vancomycin-resistant *Enterococcus faecium* from Argentina

Hospital[a]	City[b]	Type of hospital	Resistance gene	Total no. of isolates	No. of isolates by clonal type	PFGE type and subtype[c] (No. of isolates)
AER	BAC	Community	*van*A	4	4	5 (4)
ANT	BAC	Community	*van*A	3	2	8a (2)
					1	34 (1)
BAN	BAC	Community	*van*B	2	2	10 (2)
BAZ	BAC	Community	*van*A	3	3	**1d** (3)
CML	BAP	Community	*van*A	1	1	**1l** (1)
COS	BAC	Community	*van*A	18	13	**1a** (1); **1e** (1); **1g** (1); **1i** (1); **1j** (1); **1k** (3); **1l** (1); **1m** (1); **1v** (1); **1x** (1); **1y** (1)
			*van*A		2	3a (2)
					1	8b (1)
			*van*A		1	11 (1)
			*van*A		1	32 (1)
DUR	BAC	Community	*van*A	20	8	**1a** (1); **1c** (3); **1d** (1); **1h** (1); **1i** (1); **1l** (1)
			*van*A		5	4a (5)
			*van*A		3	2b (3)
			*van*A		2	15 (2)
			*van*A		1	30 (1)
			*van*A		1	31 (1)
EVI	BAC	Community	*van*A	18	17	**1d** (5); **1l** (1); **1s** (8); **1t** (1); **1u** (2)
			*van*A		1	27 (1)
FAV	BAC	Cardiology	*van*A	5	3	3a (3)
			*van*A		2	6a (2)
FER	BAC	Community	*van*A	18	14	**1a** (8); **1c** (2); **1d** (1); **1l** (2); **1n** (1)
			*van*A		1	2a (1)
			*van*A		1	11 (1)
			*van*A		1	12 (1)
			*van*A		1	26 (1)
FLE	BAC	Neurology	*van*A	2	2	**1a** (1); **1o** (1)
GAR	BAC	Pediatrics	*van*A	1	1	33 (1)
HCC	COR	Community	*van*A	2	2	2c (1); 2d (1)
HIE	BAP	Obstetrics	*van*B	1	1	16 (1)
IPA	SFE	Community	*van*A	2	2	**1a** (2)
LAE	BAC	Community	*van*A	1	1	**1b** (1)
MIT	BAC	Community	*van*A	1	1	18 (1)
MUN	BAC	Infectious Disease	*van*A	9	8	**1a** (7); **1c** (1)
			*van*A		1	24 (1)
PER	CHA	Community	*van*A	7	6	**1a** (1); **1p** (1); **1q** (3); **1r** (1)
			*van*A		1	17 (1)
PIN	BAC	Community	*van*A	15	10	7a (6); 7b (2); 7c (1); 7d (1)
			*van*A		5	**1a** (2); **1c** (2); **1f** (1)
PIR	BAC	Community	*van*A	6	2	13 (2)
			*van*A		2	**1a** (1); **1b** (1)
			*van*A		1	2e (1)
			*van*A		1	28 (1)
POS	BAP	Community	*van*A	11	2	3b (2)
			*van*A		2	9 (2)
			*van*A		2	14a (1); 14b (1)
			*van*A		1	19 (1)
			*van*A		1	20 (1)
			*van*A		1	21 (1)
			*van*A		1	22 (1)
			*van*A		1	23 (1)
QUE	BAC	Burn Center	*van*A	12	7	2a (7)

Table 1 (*Continued*)

Hospital[a]	City[b]	Type of hospital	Resistance gene	Total no. of isolates	No. of isolates by clonal type	PFGE type and subtype[c] (No. of isolates)
			*van*A		2	**1a** (2)
			*van*A		2	6b (2)
			*van*A		1	25 (1)
REI	COR	Community	*van*A	1	1	**1a** (1)
RIV	BAC	Community	*van*A	1	1	**1a** (1)
SMP	BAP	Community	*van*A	20	19	**1a** (14); **1d** (1); **1e** (3); **1g** (1)
			*van*A		1	9 (1)
STJ	BAC	Community	*van*A	2	1	4 (1)
			*van*A		1	35 (1)
TOR	BAC	Community	*van*A	1	1	**1a** (1)
VLP	BAP	Community	*van*A	1	1	**1m** (1)
ZUB	BAC	Community	*van*A	1	1	29 (1)

[a] Hospital codes: AER, Htal. Aeronáutico; ANT, Sanatorio Antártida; BAN, Policlínico Bancario; BAZ, Clínica Bazterrica; CML, Clínica Modelo de Lanús; COS, Htal. Argerich; DUR, Htal. Durand; EVI, Htal. Evita; FAV, Fundación Favaloro; FER, Htal. Fernandez; FLE, FLENI; GAR, Htal. Garrahan; HCC, Htal. Córdoba; HIE, Htal. Tetamanti; IPA, Centro Médico IPAM; LAE, Laboratorio Especializado; MIT, Sanatorio Mitre; MUN, Htal. Muñiz; PER, Htal. Perrando; PIN, Htal. Piñero; PIR, Htal. Pirovano; POS, Htal. Posadas; QUE, Htal de Quemados; REI, Htal. Reina Fabiola; RIV, Htal. Rivadavia; SMP, Htal. ''San Martín'' La Plata; STJ, Htal. Santojanni; TOR, Htal. Tornú; VLP, Htal Vicente López y Planes; ZUB, Htal. Zubizarreta.

[b] City codes: BAC, Buenos Aires City; BAP, Province of Buenos Aires; COR, Province of Córdoba; SFE, Province of Santa Fe; CHA, Province of Chaco.

[c] Isolates of clonal type 1 are shown in bold.

Results and discussion

The emergence of VRE is a serious nosocomial problem with important implications for hospital infection control. Among the 189 isolates characterized in this study, 186 (98%) carried the *van*A gene and only three the *van*B gene (Table 1). All *van*A *E. faecium* showed high-level resistance to vancomycin (MICs 32—512 mg/L) and teicoplanin (MICs 8—64 mg/L), which is characteristic of the *van*A phenotype. The three isolates with the *van*B phenotype showed MICs of 16—32 mg/L for vancomycin and 0.12—1 mg/L for teicoplanin as we described in a previous study.[18] A predominance of *E. faecium* with the *van*A genotype, as well as the predominance of *van*A over *van*B observed in our study have been previously described in VRE[6,7,19,20] and may be related to the less efficient mobilization of the *van*B complex.[16] However, we cannot dismiss the possibility that the ratio between *van*A and *van*B genes could be biased by the detection methods used in the primary laboratories.

Enterococci are intrinsically resistant to many antimicrobial agents, including cephalosporins, low concentrations of aminoglycosides, and trimethoprim—sulfamethoxazole. Furthermore, the ability of enterococci to acquire resistance to other agents like erythromycin, rifampin, chloramphenicol, ciprofloxacin, high concentrations of aminoglycosides, and vancomycin is well recognized. Consequently, treatment of VRE blood stream infections is a clinical challenge of great concern. In our collection the percentage of resistance was, as expected, high for vancomycin (100%), teicoplanin (97.9%), ampicillin (98.4%), erythromycin (100%), ciprofloxacin (98.9%), gentamicin (77.2%), and streptomycin (95.8%), but relatively low for tetracycline (6.3%) and chloramphenicol (3.7%). Antimicrobial resistance profiles with MIC_{50} and MIC_{90} are shown in Table 2. These results are similar to those previously reported by Sader et al. in Brazil.[19]

The analysis of molecular typing demonstrated 35 PFGE patterns among the 189 vancomycin-resistant *E. faecium* isolates, as shown in Figure 1. One hundred and six isolates (56%) belonged to the most frequent clone 1, which was

Table 2 Antibiotic susceptibilities among 189 vancomycin-resistant *Enterococcus faecium* isolates from Argentina

Antibiotic	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range	% Resistance
Vancomycin	256	512	16—512	100
Teicoplanin	16	32	0.12—64	97.9
Ampicillin	64	128	16—512	98.4
Erythromycin	>2048	>2048	1—>2048	100
Ciprofloxacin	64	>128	1—>128	98.9
Chloramphenicol	4	8	0.5—64	3.7
Tetracycline	0.25	0.5	≤0.03—128	6.3
Gentamicin	2048	>2048	2—>2048	77.2
Streptomycin	>2048	>2048	16—>2048	95.8

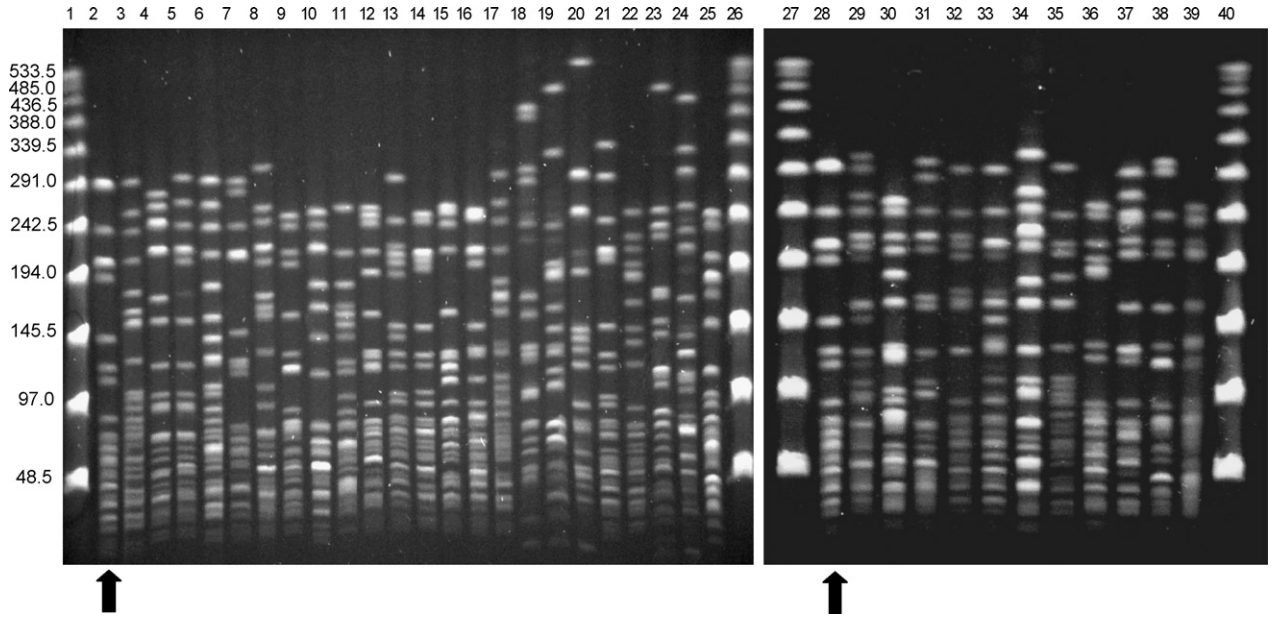

Figure 1 PFGE banding patterns of *Sma*I-digested chromosomal DNAs of vancomycin-resistant *Enterococcus faecium* strains. Lanes 1, 26, 27 and 40, lambda ladder; lane 2 and 28, epidemic clone 1a; lane 3, clone 2a; lane 4, clone 3a; lane 5, clone 4a; lane 6, clone 5; lane 7, clone 6a; lane 8, clone 7b; lane 9, clone 8a; lane 10, clone 9; lane 11, clone 10; lane 12, clone 12; lane 13, clone 14b; lane 14, clone 15; lane 15, clone 16; lane 16, clone 17; lane 17, clone 18; lane 18, clone 19; lane19, clone 20; lane 20, clone 21; lane 21, clone 22; lane 22, clone 23; lane 23, clone 24; lane 24, clone 25; lane 25, clone 26; lane 29, clone 13; lane 30, clone 28; lane 31, clone 29; lane 32, clone 30; lane 33, clone 31; lane 34, clone 32; lane 35, clone 33; lane 36, clone 34; lane 37, clone 35; lane 38, clone 11; lane 39, clone 27. The sizes of the fragments (in Kb) are shown to the left. PFGE pattern of the epidemic vancomycin-resistant *Enterococcus faecium* clone 1 is indicated by arrows.

present in 19 of 30 hospitals (Table 1). The vancomycin-resistant *E. faecium* clone 1 presented 24 subtypes (1a to 1y), out of which the most abundant was subtype 1a, shared by 43/189 (22.8%) of the isolates. Clone 1 was present in all the cities involved in the survey, representing 52% of the isolates from Buenos Aires City, 73% from the Province of Buenos Aires, and 75% from other cities. Clone 1 isolates were resistant to all of the antibiotics tested with the exception of tetracycline and chloramphenicol. Clone 2 was represented by 14 isolates (7.4%) and was detected in four hospitals from Buenos Aires City (DUR, FER, PIR, and QUE) and one from Córdoba (HCC). Seven isolates representing clone 3 were found in three hospitals, two from Buenos Aires City (COS and FAV) and one from the Province of Buenos Aires (POS). Six isolates of clone 4 were detected in two hospitals from Buenos Aires City (DUR and STJ). Thus, most of the isolates (70.4%) belonged to one of these four major clonal types. The remaining 56 isolates (29.6%) were highly diverse, belonging to 31 clonal types. Dominant clones distinct from clone 1 were detected in some hospitals. For example, in hospital QUE, 7/12 isolates were of clonal type 2, in hospital AER 4/4 isolates were of clonal type 5, and in hospital FAV 3/3 were of clonal type 3. In contrast, in some hospitals, high genetic diversity was observed. In hospital POS, there were eight clonal types among the 11 vancomycin-resistant *E. faecium* isolates.

The presence of a dominant vancomycin-resistant *E. faecium* clone (clone 1) in 17 of 30 hospitals shows that their spread has occurred not only within individual hospitals but also between hospitals of various geographic locations. Other studies have documented the spread of vancomycin-resistant *E. faecium*[19,21–23] and *E. faecalis*[24–26] clones among hospitals. In the present study, molecular typing results indicate the clonal dissemination of vancomycin-resistant *E. faecium* clone 1 in different wards of the same hospital, in different hospitals, and in different cities. The absence of an ongoing alert system for patients infected or colonized with vancomycin-resistant enterococci upon hospital readmission in our country may have contributed to this dissemination.

In some hospitals we observed a high number of clone 1 subtypes. In hospital COS there were 11 subtypes of the clone 1 and in hospital DUR six subtypes. The number of subtypes of a particular clone may reflect the evolution of the clone in a region and its relative age. Mutations, chromosomal rearrangements, as well as loss and acquisition of plasmids, transposons, or insertion sequences could be responsible for observed changes in PFGE profiles, resulting in different clonal subtypes. Probably, an epidemic clone that has persisted for a long time in a hospital subjected to an intensive selective pressure had a higher chance of incorporating more rearrangements in its genetic background than a non-epidemic clone.

Transposon Tn*1546* was the first element described to carry the *van*A cluster.[27] Tn*1546* is highly heterogeneous, because of the occurrence of deletions, insertions, and point mutations.[16,28] Although these events originated different *van*A elements, they could derive from a unique ancestral Tn*1546*.[29] De Lencastre et al. reported that the occurrence of IS*1251* is indicative of the presence of a larger transposon (~26 kb) named Tn*5482*.[16] The insertion sequence IS*1251* was found in the intergenic *van*S–*van*H region, mainly in isolates from the USA.[16,28] We selected one representative isolate

from each of the 33 *van*A clonal types identified in this study, and determined the presence of IS*1251* by PCR of the intergenic region *van*S—*van*H. All 33 isolates carrying the *van*A gene yielded an 1871 bp amplimer when *van*S-f and *van*H-r primers were used, suggesting the presence of an IS*1251*-like element. The analysis of the 1871 bp amplimer by endonuclease digestion with *Hind*III and *Eco*RI resulted in the same RFLP profile as expected from the reported sequence (Genbank accession numbers: Tn*1546*, **M97297** and IS*1251*, **L34675**) (data not shown). These results indicate the presence of IS*1251* in the intergenic region *van*S—*van*H and suggest the possible horizontal dissemination of *van*A through Tn*5482*. Recently we described the transfer of the *van*A element by conjugation from highly resistant *E. gallinarum* isolates, carrying Tn*5482*, to vancomycin-susceptible *E. faecium*.[30]

The most common clinical impact of VRE is intestinal colonization, which may persist for long periods.[31] Colonized individuals are potential reservoirs for transmission of VRE and should be identified and included in infection control interventions, because they constitute a major route of exposure.[32] Transmission is mediated by factors such as patient characteristics, antimicrobial use, and the prevalence of VRE within the hospital.[20] A large number of clinical studies have described the association of VRE colonization or infection with the use of vancomycin, antibiotics with activity against anaerobes, and extended-spectrum cephalosporins.[20,31,33] Recent reports of three vancomycin-resistant *S. aureus*[34,35] and the already demonstrated horizontal transfer of the *van*A gene from vancomycin-resistant *E. faecalis* to *Staphylococcus aureus*, underscores the importance of understanding VRE epidemiology.[36]

The emergence of predominant clonal types among multiple strains in several institutions of the survey, may suggest that some strains contain bacterial factors that enhance their spread within hospitals. Recently, Shankar et al. and Willems et al. have identified the *esp* gene encoding a surface protein associated with virulence for *E. faecalis*[37] and *E. faecium*,[38] residing on a pathogenicity island.[37,39] Several authors have reported that the *esp* gene is not associated with vancomycin resistance.[40,41] On the other hand, Harrington et al. support the hypothesis that in a clinical setting in which vancomycin-resistant *E. faecium* is endemic, the combination of vancomycin resistance and the *esp* gene could lead to dissemination of particular clones. Recently, Willems et al. have identified a genetic lineage of *E. faecium*, named complex-17, associated with hospital outbreaks in five continents, representing the first globally dispersed nosocomial clonal lineage.[42] The subsequent acquisition of *van*A or *van*B genes for the hospital-adapted complex-17 resulted in vancomycin-resistant *E. faecium* with pandemic potential. In this way, it is probable that in Argentina a particular *E. faecium* clone has been selected and has adapted well to the hospital environment with the ability to spread. Although the presence of the *esp* gene was not assessed in the present study, we cannot dismiss the role of this factor in the spread of dominant clones. Further studies are necessary in order to investigate the presence of *esp* or other genes belonging to pathogenicity islands. Moreover, additional studies on the relation between the epidemic clone 1 and complex-17 lineage would be valuable in elucidating the epidemicity of this clone.

In summary, our results strongly suggest that intra- and inter-hospital spread of an epidemic vancomycin-resistant *E. faecium* clone carrying the *van*A element seems to be the main mechanism of vancomycin-resistance dissemination in Argentina.

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