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Original Article

Local spread of Tn*1546*-like element among three species of vancomycin resistant enterococci in an intensive care unit

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Abstract

Introduction: In an intensive care unit (ICU) of the Emergency Center in the Clinical Center of Serbia, four species of vancomycin resistant enterococci (VRE) were isolated in a 17-month period mostly from blood cultures, including *E. faecalis, E. faecium, E. raffinosus* and *E.gallinarum.*

Methodology: The relationship between isolates from each species was investigated by PFGE, and PCR experiments for detection of pathogenicity factor genes and *van* genes to determine the nature of each clone. A PCR-based method, using 10 primer pairs (p1/2-p19/20), was used to investigate the presence of the Tn*1546*-like structure.

Results: PFGE indicated the presence of two different *E. faecium* clones, while the three other enterococcal species belonged to one clone each. Transposon typing revealed that isolates of *E. raffinosus* (4), *E. gallinarum* (4) and *E. faecalis* (3) yielded gene sequences identical to 10 primer pairs (p1/2-p19/20), suggesting the possibility of identical transposon-like structure in these species.

Conclusions: The results of the study indicate probable horizontal spread of Tn*1546*-like structure in three species of VRE obtained from the same ICU.

Key words: vancomycin resistant enterococci; PFGE; Tn*1546*.

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Introduction

VRE displaying the VanA and VanB resistance phenotype have been reported widely as a cause of nosocomial infections in the United States and Europe [1-3]. The genes encoding the VanA and VanB types of vancomycin resistance are often located on mobile DNA elements and are therefore easily transmitted between members of the same species or genus or even different genera [4-6]. For that reason, the horizontal transfer of resistance genes among enterococci may have a more significant impact on the dissemination of vancomycin resistance than the clonal spread of resistant enterococci. The *vanA* gene is the most frequently encountered gene among isolates causing VRE infections in humans [6]. This gene is part of the transposable element Tn*1546,* cluster consisting of genes: *orf1 and orf2,* associated with transposition functions; *vanR* and *vanS* (regulatory genes); *vanH, vanA* and *vanX* (critical for resistance determination) and *vanY* and *van Z* (accessory genes) [7]. Previous authors developed a set of 10 primers (p1p2-p19p20), encoding overlapping fragments of prototype *vanA* element, to investigate the content of the element. These investigations documented genetic heterogeneity in Tn*1546*-related elements [8,9].

In this study, in addition to investigation of resistance elements, we therefore investigated the content of virulence factor genes. Virulence factors of interest include those that promote binding to extracellular matrix proteins, like collagen binding protein Ace and cell wall adhesins expressed in serum by *E. faecalis* and *E. faecium*, encoded by *ace*, *EfaAfs* and *EfaAfm* genes, respectively; adaptation and survival in different environments, such as *Enterococcus* surface

protein Esp and aggregation substance AS, encoded by *esp* and *asa1* genes; factors that enable secretion of toxins (*e.g.* cytolysin - production regulated by an operon which includes *cylA* and *cyl B* genes) and enzymes that damage and degrade host tissue (gelatinase and hyaluronidase, encoded by *gelE* and *hyl* genes). Sex pheromone genes (*cpd, cob*) are part of a complex system of gene transfer by conjugation controlled by intercellular signalling via peptide mating pheromones.

E. faecium and *E. faecalis* are well known and frequent human pathogens, which account for 12% of healthcare-associated infections [1]. Outbreaks of other enterococci, *E. raffinosus* and *E. gallinarum* [2,10,11], have been reported less frequently than the two most frequently encountered species. This study therefore also investigated the presence of Tn*1546* in four species of enterococci, taking into account that they were isolated at the same ICU of the Emergency Center at the Clinical Center of Serbia (ICUEC) in a relatively short period. There is very little information from earlier studies on molecular epidemiology specific to the transposon itself, or its association with specific antimicrobial resistance or virulence traits. Our hypothesis was that the same genetic element carrying *vanA* resistance gene could have been shared among these different species of enterococci. Having an understanding of epidemiology is critical in designing control measures in intensive care units.

Methodology

The study was carried out between May 2005 and October 2006. It was focused on patients hospitalized at ICUEC, also called "intensive care unit for septic patients". It is intended for the patients already hospitalized in other intensive care units, either in the Clinical Center of Serbia or other healthcare institutions, whose status became septic and critical. In most of the patients abdominal operations had been performed, but some were suffering from traffic injuries. Blood cultures were incubated in semi-automatic blood culture system BACTEC 9240 (Becton Dickinson Diagnostic Instrument Systems, Sparks, USA) and subsequently cultivated on blood agar. Isolate of vancomycin resistant enterococcus from vaginal swabs, provided from colleagues from Bacteriology laboratory at Emergency Center, Department of Microbiology, was included in the study. VRE were identified to the species level by conventional tests [12].

Susceptibility testing

MICs for vancomycin, teicoplanin, tetracycline, ciprofloxacin, linezolid, ampicillin, penicillin, chloramphenicol, nitrofurantoin, erythromycin, gentamicin, and streptomycin were determined by the agar dilution method on Mueller-Hinton II agar plates (Torlak, Belgrade, Serbia) according to CLSI criteria [13].

PFGE

Genomic DNA for PFGE was prepared in agarose plugs and digested with *Sma*I (New England Biolabs, Beverly, USA) enzyme [14]. PFGE was performed on a CHEF-DRIII (Bio-Rad, Hercules, USA). *Sma*I-digested *Staphylococcus aureus* NCTC 8325 was a molecular mass standard on all PFGE gels. Identical fragment patterns were considered the same type while those with two or three band differences resulting from a single genetic event [15] were considered closely related and assigned as subtype. The different types or subtypes were identified by numbers and small-letter codes for *E. faecium* or capital-letters with number added (for subtype) for other enterococci.

Vancomycin resistance and pathogenicity factor genes

Bacterial DNA in this study was extracted by use of InstaGene Matrix (Bio-Rad Laboratories, Hercules, USA), as suggested by manufacturer. Mutiplex PCR investigation of *vanA* and *vanB* genes was performed according to the method of Dutka-Malen [16]. PCR experiments for detection of pathogenicity factor genes: *esp, cylA, cylB, efaAfs, efaAfm, cpd* and *cob* were performed as described previously [17]. Briefly, PCR was performed in a GeneAmp PCR System 9600 (PerkinElmer Life and Analytical Sciences, Wellesley, USA). PCR amplifications were performed in 50-µl reaction mixtures using 5 µg of DNA, 15 mM $MgCl_2$, 20 pmol of each primer, and 1 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, USA). Samples were subjected to an initial cycle of denaturation (94°C for 2 minutes), annealing (at an appropriate temperature for 2 min and elongation (72°C for 2 minutes), followed by 29 cycles of denaturation (92°C for 15 seconds), annealing (at an appropriate temperature for 15 seconds), and elongation (72°C for 15 seconds). A multiplex PCR for the pathogenicity factor genes: *asa1, gelE,* and *hyl* was carried out as previously described [18]. In brief, Each 50-µl PCR mixture consisted of 5 µl of bacterial suspension; a 0.1 µM concentration (each) of primers specific for *asa1,gelE,* and *hyl*; 25 µl of Master mixture, which consisted of 2.5 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, and 200 µM

deoxynucleoside triphosphates; and an additional 1.0 mM $MgCl_2$. An initial activation step at 95°C for 15 minutes was followed by 30 cycles of denaturation (94°C for 1 minute), annealing (56°C for 1 minute), and extension (72°C for 1 minute), followed by one cycle consisting of 10 minutes at 72°C. PCR products were run on 2% agarose gels at 60V for 1.5 hours. A 100-bp DNA ladder (Invitrogen, Carlsbad, USA) was used as a molecular size marker.

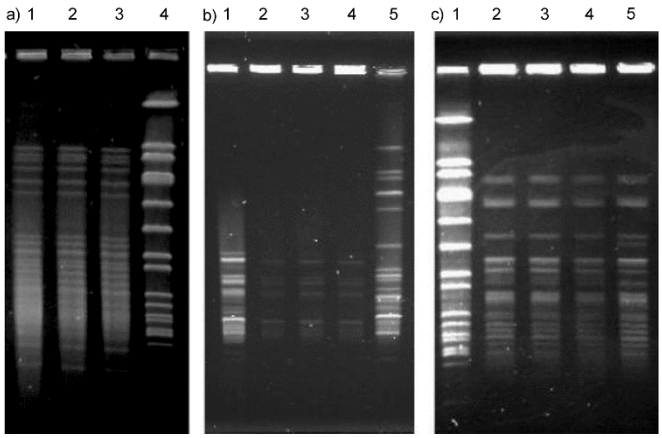
Primers for detection of virulence and *van* genes are listed in Table 1.

Amplification of VanA resistance elements

Each pair of primers (p1p2, p3p4, p5p6, p7p8, p9p10, p11p12, p13p14, p15p16, p17p18, p19p20) was used in a separate assay.

Briefly, the primers (Table 2) were adjusted, and a touchdown PCR strategy was employed using Master mix. The initial denaturation step of 15 minutes at 95°C was followed by 10 cycles consisting of 30 seconds at 94°C, 30 seconds at 61°C, down to 51° C and 1 minute at 72°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 51°C and 1 minute at 72°C, with a final extension step of 7 minutes at 72°C. BM4147 was included as a positive control. The result was visualized by running the PCR products on a 1% agarose gel with 0.01% ethidium bromide.

Figure 1.PFGE patterns of *E. faecalis, E. gallinarum, and E. raffinosus.*



a) Lanes 1,2,3: *E. faecalis* gel type C, lane 4: 8325 QC; b) Lanes 1,2,3,4: *E. gallinarum* gel type G1, lane 5: 8325 QC; **c)** Lane 1: 8325 QC; lanes 2,3,4: *E. raffinosus* R4, lane 5: *E. raffinosus* R3

The VanA element in each isolate was scored for the presence or absence of each of the 10 overlapping fragments of Tn*1546* [7,8].

Results

In the study period 1156 blood cultures from ICUEC were processed. Bacterial isolates were

Table 1. The primers encoding for virulence and *van* genes.

Gene	Phenotype	Sequences (5' to 3')	Size of product (bp)
asa1	aggregation substance AS	F GCA CGC TAT TAC GAA CTA TGA R TAA GAA AGA ACA TCA CCA CGA	375
cylA	cytolysin A	F TGG ATG ATA GTG ATA GGA AGT R TCT ACA GTA AAT CTT TCG TCA	688
efaAfs	*E. faecalis* A antigen	F GAC AGA CCC TCA CGA ATA R AGT TCA TCA TGC TGT AGT A	735
ace	collagen binding adhesin	F GAG CAA AAG TTC AAT CGT TGA C R GTC TGT CTT TTC ACT TGT TTC T	1,003
hyl	hyaluronidase	F ACA GAA GAG CTG CAG GAA ATG R GAC TGA CGT CCA AGT TTC CAA	276
gelE	gelatinase	F ACC CCG TAT CAT TGG TTT R ACG CAT TGC TTT TCC ATC	419
cylB	cytolysin B	F ATT CCT ACC TAT GTT CTG TTA R AAT AAA CTC TTC TTT TTC AAC	843
esp	enterococcal surface protein, esp	F TTG CTA ATG CTA GTC CAC GAC C R GCG TCA ACA CTT GCA TTG CCG AA	933
efaAfm	*E. faecium* A antigen	F AAC AGA TCC GCA TGA ATA R CAT TTC ATC ATC TGA TAG TA	735
cpd	sex pheromone	F TGG TGG GTT ATT TTT CAA TTC R TAC GGC TCT GGC TTA CTA	782
cob	sex pheromone	F AAC ATT CAG CAA ACA AAG C R TTG CCA TAA AGA GTG GTC AT	1,400
vanA	vanA type of resistance	F CAAT GAA TAG AAT AAA AGT TGC AAT A R CCC CCT TAA CGC TAA TAC GAT CAA	1,030
van B	vanB type of resistance	F GTG ACA AAC CGG AGG CGA GGA R CCG CCA TCC TCC TGC AAA AAA	433

Table 2. The 10 primer pairs used for Tn1546 transposon typing.

Primer pair	sequences 5'-3'	
	Forward primer	Reverse primer
p1/p2	GGA TTT ACA ACG CTA AGC C	GCC TTT ATC AGA TGC TAC C
p3/p4	GGT TTT CGA TTA TTG GAA G	TAA AAA TAA TAG AAC GCA TCG AAT AC
p5/p6	CTT GAA AGT CAC GGA ATG	GGT TAA CAC CAG CCA TTA C
p7/p8	GGA TGG ACT AAC ACC AAT C	GTA TAA TTC AAC CAA ATC GG
p9/p10	GTG AAG GGA TTG AAT TGG	CCA ATC CCC AAG TTT CC
p11/p12	CGA CTA TTC CAA ACT AGA ACG A	CAT AGT ATA ATC GGC AAC GC
p13/p14	CTT CTT GCG CTG AAG AG	CTA TTT CCA TGC TTA TCA CC
p15/p16	CAG GAG CAT GAA TAG AAT AAA AG	GGA TTT ACT ATT ATC ACC AAT GTA G
p17/p18	CAC TTA TGA AAA TTC ATC TAC ATT G	CCA AGA AAG CCT CCA ACA
p19/p20	GCT ATT GGA GCG ACA GAC A	GCG GAT TTA CAA CGT TAA G

obtained from 856 of them. In 129 blood cultures enterococci have been confirmed, and 20 of them were resistant to vancomycin: 3 *E.faecalis*, 4 *E. raffinosus*, 4 *E. gallinarum* and 9 *E. faecium.* PFGE for chromosomal relatedness identified one pattern of *E.faecalis* - clone C (Figure 1a), one pattern of *E. gallinarum* - clone G (Figure 1b), and two closely related patterns of *E. raffinosus* - clones R3 and R4 (Figure 1c), while members of *E. faecium* were placed in two different groups named 3b and 29, which included subgroup 29a. Multiplex PCR proved *vanA* gene in all VRE, whereas results for pathogenicity factor genes are displayed in Table 3.

Amplification experiments showed that 4 *E. gallinarum*, 4 *E. raffinosus* and *3 E. faecalis* carried a Tn*1546*-like element, given the amplification of all primers (p1p2-p19p20), constituents of Tn*1546* (Table 4). The same table contains antimicrobial susceptibility patterns of the investigated strains. The isolates were found to be multidrug resistant, uniformly demonstrating resistance to penicillins, tetracycline and high concentrations of aminoglycosides. The antimicrobial resistance pattern was identical within each clone, with characteristic susceptibility of *E. faecalis* to ampicillin; clear susceptibility of *E. gallinarum* and *E. raffinosus* to ciprofloxacin and resistance of *E. raffinosus* to chloramphenicol. The order of appearance of the clones of enterococci containing the entire and defective Tn*1546*-like structure is presented on Figure 2. The study commenced in May 2005 and the first isolate containing *vanA* gene was *E. raffinosus*, obtained in July 2005. It was also the first clone to disappear, four months after. *E. faecalis* was detected in August 2005, a month after the appearance of the first *E. raffinosus*, and the last member of that clone was isolated in April 2006, eight months later. *E. gallinarum* persisted for seven months. Clones 29a and 3b persisted for four and five months. If Tn*1546*-like structure was common to three species

Figure 2. The order of appearance of *E. raffinosus*, *E. gallinarum*, *E. faecalis* and *E. faecium* in 2005 and 2006.

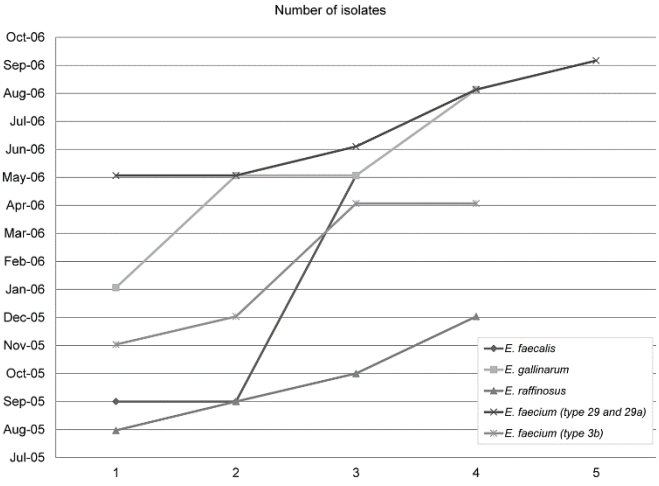


Table 3. Pathogenicity factor genes of *E. faecalis, E. raffinosus*, *E. gallinarum*, and *E. faecium.*

Type and subtype[1)]	Species	*esp*	*gelE*	*cylB*	*cpd*	*cob*	*ace*	*efaAfs* or *efaAfm*	*cylA*	*asa1*	*hyl*
3b (4)	*E. faecium*	-	-	-	-	-	-	+	-	-	-
29 (4)	*E. faecium*	-	-	-	-	-	-	+	-	-	+
29a (1)	*E. faecium*	-	-	-	-	-	-	+	-	-	-
C (3)	*E. faecalis*	+	+	-	+	+	+	+	-	+	-
R3 (1)	*E. raffinosus*	-	-	-	-	-	-	NA	-	-	-
R4 (3)	*E. raffinosus*	-	-	-	-	-	-	NA	-	-	-
G (4)	*E. gallinarum*	-	-	-	-	-	-	NA	-	-	-

[1)]: Results for one representative strain per PFGE pattern. Numbers in parenthesis are numbers of the member strains; NA, not applicable.

of enterococci it seems that they persisted for 4-8 months.

Discussion

Glycopeptide resistant enterococci are important causative agents in bacteremias. According to Brown [19], most clinical isolates of *Enterococcus spp*., whether glycopeptide susceptible or resistant, represent colonization rather than infection, which is typically endogenous. Since the isolates from patients in this study were from blood cultures in critically ill patients in intensive care units, we considered the infections to be clinically significant [20].

Investigation of patients' blood cultures revealed four species, clonally spread, of enterococci carrying *vanA* gene. Although the most frequent causative agent in human infections is *E. faecalis*, outbreaks with different types of enterococci carrying *vanA* genotype have been previously reported [10,21]. One isolate of *E. raffinosus*, part of R4 clone, was obtained from vaginal swab, suggesting spread of that clone to other body parts, or that the other body parts were the source of vancomycin resistant *E. raffinosus*.

As *vanA* gene is the most frequently located on *Tn1546*, as a part of a plasmid, we investigated the presence of genes encoding ten fragments of *Tn1546* in all members of enterococci from the outbreak (Table 3). Indeed, a total set of genes was present in all *E. faecalis, E. raffinosus* and *E. gallinarum*. Clonal relatedness of enterococci from the study was congruent with identical antimicrobial resistance within the clone. The entireTn*1546*-like element was likely identical or closely related to the original Tn*1546* transposon detected by Woodford [8] and transmitted probably *en bloc* with other genes encoding high level resistance to aminoglycosides, erythromycin, and tetracycline in three species of enterococci. It remains unknown in which strain the Tn*1546*-like element appeared originally, since Tn*1546* has a wide range of hosts, encompassing *Enterococcus, Staphylococcus aureus, Staphylococcus epidermidis, Bacillus circulans, Oerskovia turbata, Arcanobacterium haemolyticum, Paenibacillus, Rhodococcus* [7,22,23]. Generally,

Table 4. Amplicons obtained and resistance patterns of each strain of *E. raffinosus*, *E. gallinarum*, *E. faecium* and *E. faecalis.*

No	Strain	p1/2	p3/4	p5/6	p7/8	p9/10	p11/12	p13/14	p15/16	p17/18	p19/20	Strains resistant to:	Source	Gel type
1	1242	+	+	+	+	+	+	+	+	+	+	van, tei, tet, cip, pen, er, gm, stre	blood	Efs C
2	x248	+	+	+	+	+	+	+	+	+	+	van, tei, tet, cip, pen, er, gm, stre	blood	Efs C
3	x236	+	+	+	+	+	+	+	+	+	+	van, tei, tet, cip, pen, er, gm, stre	blood	Efs C
4	x230	+	+	+	+	+	+	+	+	+	+	van, tei, tet, pen, amp, caf, er, gm, stre	blood	R3
5	x250	+	+	+	+	+	+	+	+	+	+	van, tei, tet, pen, amp, caf, er, gm, stre	vagina	R4
6	x298	+	+	+	+	+	+	+	+	+	+	van, tei, tet, pen, amp, caf, er, gm, stre	blood	R4
7	x256	+	+	+	+	+	+	+	+	+	+	van, tei, tet, pen, amp, caf, er, gm, stre	blood	R4
8	1476	+	+	+	+	+	+	+	+	+	+	van, tei, tet, pen, amp, er, gm, stre	blood	G
9	1286	+	+	+	+	+	+	+	+	+	+	van, tei, tet, pen, amp, er, gm, stre	blood	G
10	1970	+	+	+	+	+	+	+	+	+	+	van, tei, tet, pen, amp, er, gm, stre	blood	G
11	x315	+	+	+	+	+	+	+	+	+	+	van, tei, tet, pen, amp, er, gm, stre	blood	G
12	x266	-	-	-	-	+	+	+	+	+	+	van, tei, tet, cip, pen, amp, er, gm, stre	blood	Efm 3b
13	x292	-	-	-	-	+	+	+	+	+	+	van, tei, tet, cip, pen, amp, er, gm, stre	blood	Efm 3b
14	984	-	-	-	-	+	+	+	+	+	+	van, tei, tet, cip, pen, amp, er, gm, stre	blood	Efm 3b
15	981	-	-	-	-	+	+	+	+	+	+	van, tei, tet, cip, pen, amp, er, gm, stre	blood	Efm 3b
16	2429	-	-	-	-	-	-	-	-	-	+	van, tei, tet, cip, pen, amp, gm, stre, caf[1)]	blood	Efm 29
17	1911	-	-	-	-	-	-	-	-	-	+	van, tei, tet, cip, pen, amp, gm, stre, caf[1)]	blood	Efm 29
18	1124	-	-	-	-	-	-	-	-	-	+	van, tei, tet, cip, pen, amp, gm, stre, caf[1)]	blood	Efm 29
19	1052	-	-	-	-	-	-	-	-	-	+	van, tei, tet, cip, pen, amp, gm, stre, caf[1)]	blood	Efm 29
20	1474	-	-	-	-	-	-	-	-	-	+	van, tei, tet, cip, pen, amp, gm, stre, caf[1)]	blood	Efm 29a

R3 and R4, E. raffinosus; G, *E. gallinarum*; Efm*, E. faecium*; Efs C, *E. faecalis*; van, vancomycin; tei, teicoplanin; tet, tetracycline; cip, ciprofloxacin; pen, penicillin; amp, ampicillin; caf, chloramphenicol; er, erythromycin; gm, gentamicin; stre, streptomycin; [1)]: intermediate resistance to chloramphenicol.

intra-species transfer of enterococcal *vanA* plasmids is far more frequent than transfer across species or genus barriers and transconjugants could also serve as additional donors, being capable of transferring resistance plasmids to further recipients [6].

Vancomycin resistance genes are sometimes physically linked with virulence determinants on the same plasmid [24]. Investigation of virulence determinants was previously shown to be important for *E. faecalis* and *E. faecium*; our study detected none of them in *E. raffinosus* and *E. gallinarum*. *E. faecium* contained only *EfaAfm* gene inherent to that species and *hyl* gene in clone 29. Instead, in the C clone of *E. faecalis* the presence of *asa1* gene has been detected, encoding aggregation substance (AS), associated with *vanA*-containing pheromone-responsive plasmids in *E. faecalis* [25]. The clone contained pheromone genes (*cpd, cob*) as well. Previous mating experiments on transfer of *vanA* genes across species barriers revealed higher rates from *E. faecalis* donors to *E. faecium* recipients than vice versa [5,6], especially in presence of AS genes to facilitate conjugative exchange [5]. Pheromone induction results in increased transcription of numerous structural genes required for conjugation and consecutive transfer of resistance genes. According to this, it seems more likely that the source of *vanA* gene in our study was *E. faecalis* than *E. raffinosus*. *E. faecalis* clone persisted in the time when *E. gallinarum* clone emerged, which leaves the possibility for transfer of resistance genes to *E. gallinarum* as well.

In the study of Dahl [4] exconjugants containing a plasmid harbouring the *vanA* transposon Tn*1546* persisted stably in vivo in the absence of glycopeptides for more than 20 days. Transposon stability would have been enhanced under the glycopeptide pressure, exerted to the isolates from our study, considering that the patients were in intensive care unit, receiving the high dozes of glycopeptide and carbapenem antibiotics. A period of 4-8 months when clones of enterococci from our study persisted seems to be the period of transposon stability.

Although results suggest horizontal spread of Tn*1546*-like element, which is usually located on the plasmid, only the complete sequencing of the plasmid genome would confirm the existence of entire Tn*1546*. It is necessary to determine and analyze the whole sequence of the plasmid to reveal the dynamics of the genes. This study was unable to make a direct impact on infection control measures to prevent the transmission of the strains or modify the antibiotic usage in that ICU. Finally, this study was performed in an eighteen month period and historical isolates were not tested. Further study is required to understand if there is ongoing transmission and how long the horizontal spread of the Tn*1546*-like element has been occurring.

Conclusion

In this study a case of polyclonal outbreak of at least three species of enterococci, *E. faecalis, E. raffinosus* and *E. gallinarum*, was described, in which horizontal transmission of entire Tn*1546*-like elementwasprobable. The event occurred in a 17-month period, after which the investigation was ceased. This event is an example of dissemination of single or related resistance determinants among related, but clearly different organisms in a single Intensive Care Unit.

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