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Original paper

Description of vancomycin resistance genes in *Enterococcus* sp. clinical strains isolated from Bucharest, Romania

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Abstract

Vancomycin Resistant Enterococci (VRE) are a major cause of nosocomial infections. The purpose of this study was the investigation of the genetic background of vancomycin resistance in VRE strains isolated from various clinical sources in a major hospital in Bucharest. Identification of *Enterococcus faecium* and *E. faecalis* at the species level was performed using multiplex PCR for species specific ddl (D-alanine-D-alanine ligase) genes, as for 64% of the strains the VITEK system revealed only the genus of the strains. We used a multiplex PCR approach, using primers targeting the vanA, vanB, vanC, vanD, vanE, vanG genes, revealing the presence of vanA and vanB genes, To the best of our knowledge, this is the first description of vanA and vanB genes in Romania. This specific and sensitive technique allows detection of glycopeptide-resistant strains, that may escape phenotype-based automated rapid methods.

Keywords VRE, vanA, vanB, multiplex PCR, ddl

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Introduction

Enterococci naturally colonize the intestinal tract of human and animals (SILVA & al.[1]). There are two clinically common isolates, *Enterococcus faecalis* and *Enterococcus faecium*, causing nosocomial infections including bacteremia, endocarditis, intra-abdominal, pelvic, soft tissue infections, and urinary tract infections (GUZMAN & al. [2]; FISHER & al.[3] (AGUDELO & al. [8])). Since *Enterococcus* spp. are resistant to multiple antibacterial drugs, there are only limited options for effective therapy and prophylaxis of serious infections (KAWALEC & al. [4]). The emergence of vancomycin-resistant enterococci (VRE) followed a worst-case scenario for nosocomial pathogens. Vancomycin was the first glycopeptide antibiotic to be discovered as early as 1950 (MURRAY& al. [5]). VRE clinical isolates were first documented in Europe in the late 1980s (UTLEY& al. [6]; LECLERCQ &al.[7]).

There have been reported a phenotypic and genotypic heterogeneity regarding the resistance patterns of VRE isolates around the world (ARTHUR & al. [9]). There have been described nine types of glycopeptides resistance genes in enterococci that can be distinguished on the basis of the sequence of the structural gene for the resistance ligase (*van*A, *van*B, *van*C, *van*D, *van**E***, *van*G, *van*L, *van*M, and *van*N) (DEPARDIEU & al. [10], LEBRETON & al. [18]; BOYD & al. [19]; XU & al. [20]).

*Van*A resistance phenotype is characterized by high-level resistance to vancomycin and teicoplanin, whereas strains with *Van*B phenotype are resistant to variable levels of vancomycin, but susceptible to teicoplanin (ARTHUR & al. [11]). Strains of the *Van*D type are resistant to moderate level of vancomycin and low level of teicoplanin (DEPARDIEU & al. [12]). *Van*C (DUTKA-MALEN & al. [13]), *Van*E (FINES & al. [14]) and *Van*G (DEPARDIEU & al. [15]; MCKESSAR & al. [16]) resistance phenotype are characterized by constitutive low-level resistance to vancomycin and full susceptibility to teicoplanin.

Materials and Methods

1.1. Bacterial strains

A total number of 23 VREstrains were selected from a collection of *Enterococcus* sp. strains isolated during 21. June – 19 November 2017 from different clinical specimens at Fundeni Clinical Institute in Bucharest (Romania). The strains identification was performed in the Microbiology Laboratory using VITEK 2 system. Catalase activity was further tested.

1.2. Antimicrobial susceptibility testing

The antibiotic susceptibility testing was performed using the diffusion method (Kirby -Bauer), following the recommendations of CLSI editions 2017 and 2018. The antimicrobial disks used in this study were as follows: vancomycin (30 µg), vancomycin (5 µg), and teicoplanin (30 µg). *E. faecalis* ATCC 29212 and *E. faecium* ATCC 13590 were included as reference strains. The inhibition zones were scored after incubation at 37°C for 18-24 hour and interpreted according to the CLSI guidelines. Both the resistance and intermediate susceptibility were reported as resistance in this study.

The minimum inhibitory concentration (MIC) of *E. faecalis* and *E. faecium* isolates against a range of antibiotics was determined by agar dilution, using the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2018). Briefly, Mueller-Hinton Agar (Difco, USA) plates were prepared with vancomycin (30 µg), vancomycin (5 µg) and teicoplanin (30 µg) and inoculated with a bacterial suspension equivalent to 0.5 McFarland standard and incubated at 37°C for 18-24 hour. Isolates were considered susceptible or resistant according to breakpoint values recommended by the CLSI document M100-S27 (Wayne, PA, USA). *E. faecalis* ATCC 29212 and *E. faecium* ATCC 13590 were included a reference strain.

1.3. Molecular analysis

Genomic DNA was extracted using an adapted alkaline extraction method. In this purpose, one to five colonies of bacterial cultures were suspended in 1.5 ml tube containing 20 µL solution of 0,05M NaOH (sodium hydroxide) and 0.25% SDS (sodium dodecyl sulphate). For the permeabilization of the cell membrane the tubes were heated on a thermoblock at 95°C for 15 minutes. 180 µL TE buffer (Tris + EDTA) 1X was added and the tubes were centrifuged at 13000 rpm for 3 minutes. The supernatant was kept and stored at -20°C until further analyses (GHEORGHE & al., [17]). All PCR reactions were performed using a Thermal Cycler machine Corbet. The amplification products of each PCR reaction (multiplex / simplex) were visualized by electrophoresis on a 1% agarose gel, stained with ethidium bromide (10 µg / ml) and identified based on their size using specific molecular weight markers (100bp, Mid-Range DNA Ladder).

1.3.1. Molecular discrimination of *Enterococcus* sp. strains

In order to precisely identify the species, all *Enterococcus* sp. strains were analyzed by PCR (Table 1) using primers specific to *E. faecalis* (ddl *E. faecalis*) and *E. faecium* (ddl *E. faecium*) (Table 3).

1.3.2. Detection of antimicrobial resistance genes by PCR

The genotypic characterization of the vancomycin-resistance types present in the analyzed strains was performed using PCR methods (simplex and multiplex). Two reactions were performed using the multiplex PCR with six (for van genes) and two (for ddl genes) pairs of primers. The amplification program, reaction components and primer sequences are detailed in Table no. 1, 2 and 3.

Table 1. Condition of amplification in the PCR reactions

Genes	Condition of amplification			
	denaturation	annealing	final extension	cycles
vanA, vanB, vanC, vanD, vane, vanG, ddl	94° 2min	54° 1min	72° 1 min	30x

Table 2. Reaction components used in the PCR experiments

Concentration						Final volume
primer	$MgCl_2$	dNTP	DNATaq-pol	Reaction buffer	DNA	20µl
0,5µM	1,2mM	2µM	0,2U	1x	10x	

Table 3. Primer sequences and amplicon sizes used to amplify selected genes

Gene	Primer[a]	Sequence (5′→3′)	Size of PCR product (bp)	References
***van* A**	EA1 (+) EA2 (-)	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	Depardieu et al. (2004)
***van* B**	EB3 (+) EB4 (-)	ACGGAATGGGAAGCCGA TGCACCCGATTTCGTTC	647	Dutka-Malen et al. 1995
***van* C1/2**	EC5 (+) EC8 (-)	ATGGATTGGTAYTKGTAT[b] TAGCGGGAGTGMCYMGTAA[b]	815/827	Depardieu et al. 2004
***van* D**	ED1 (+) ED2 (-)	TGTGGGATGCGATATTCAA TGCAGCCAAGTATCCGGTAA	500	Depardieu at al. 2004
***van* E**	EE1 (+) EE2 (-)	TGTGGGATCGGAGCTGCAG ATAGTTTAGCTGGTAAC	430	Depardieu et al. 2004
***van* G**	EG1 (+) EG2 (-)	CGGCATCCGCTGTTTTTGA GAACGATAGACCAATGCCTT	941	Depardieu et al. 2004
ddl (*E. faecalis*)	DD13(+) DD3-2(-)	ATCAAGTACAGTTAGTCTTTATTAG ACGATTCAAAGCTAACTGAATCAGT	941	Sharifi et al. 2012
ddl (*E. faecium*)	FAC1-1(+) FAC2-1(-)	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	658	Sharifi et al. 2012

[a] +, forward primer; -, reverse primer.
[b] K - G or T; M - A or C; Y - C or T,

Statistical analysis (Fischer test) for assessing a correlation between *Enterococcus* species and a certain *van* gene was performed using GraphPad Quick Calcs (www.graphpad.com/quickcalcs/).

Results and discussion

This study was conducted on a total of 23VRE strains isolated during 2017 from patients hospitalized in the Fundeni Clinical Institute from Bucharest, Romania. The VITEK identification revealed the presence of Enterococcus sp. (n = 16); *Enterococcus faecium* (n = 6) and *Enterococcus faecalis* (n = 3). All strains proved to be catalase negative.

Subsequent identification using multiplex PCR for ddl genes revealed that, of the 16 VITEK identified strains as *Enterococcus* sp., 12 were assigned to *E. faecium* and the remaining 4 were assigned to *E. faecalis* (Figure 1) results that further support the importance of molecular diagnosis. Thus, the analyzed strains were comprised of 18 (83.33%) *E. faecium* and 5 (6.67%) *E. faecalis* strains.

1.2. Antimicrobial susceptibility testing

Regarding the susceptibility patterns of 18 *E. faecium* and 5 *E. faecalis* strains to vancomycin and teicoplanin, all strains were resistant to vancomycin. Among the 18 *E. faecium* strains, 15 (88.88%) were resistant to teicoplanin, while 4 (80%) out of the 5 *E. faecalis* strains were resistant to teicoplanin.

1.3. Molecular detection of ARGs (antibiotic resistance genes)

All the 25 isolates were subjected to Multiplex PCR using 6 sets of primers for van genes (Table 3). VanA gene was encountered in 16 of *E. faecium* strains (88.88%), while in *E. faecalis*was encountered in 4 (80%) of the strains. VanB gene was encountered in 2 (11.11%) of *E. faecium* and 1 (20%) of *E. faecalis*. These results are consistent with the phenotypical data, meaning that all teicoplanin resistant strains harbored vanA gene while all teicoplanin susceptible strains harbored vanB gene.

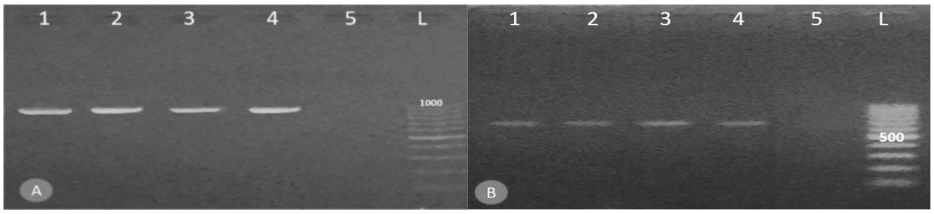

Figure 1. PCR amplification of *ddl E. faecalis*, *ddl E. faecium* genes. (A) PCR products *ddl E. faecalis* gene (941 bp). (B) PCR products *ddl E. faecium* genes (658 bp). L: molecular size marker 100 bp, 1: positive control, 2-4: samples, 5: negative control.

Statistical analysis revealed that there is no significant correlation between the presence of vanA or vanB in the *Enterococcus* species highlighted here, meaning that these genes are not associated with a particular species or clone. This result is limited however, by the low number of strains.

The presence of vanA gene results in high levels of vancomycin resistance, as well as resistance to teicoplanin and may be induced by both glycopeptides and nonglycopeptide antibiotics. Worldwide, VanA phenotype is linked to most of the human cases of vancomycin resistant enterococci and is mainly carried *by E. faecium*. Spread of vancomycin-resistant *E. faecium* is a major global issue due to its persistence in hospital environment, limited therapeutic alternatives, and plasmodial vanA transfer (CDC, [23]).

The MICs of vancomycin for enterococci positive for vanB gene may be from low to very high, but these enterococci remain susceptible to teicoplanin and is inducible by vancomycin alone. The genes encoding the VanB resistance phenotype are more commonly chromosomal but can also be transferred by conjugation. Both these genotypes of vancomycin resistance (VanA and VanB VRE) have been detected mainly in *E. faecium* and *E. faecalis*. These types of resistance are acquired and encoded by genetic elements, which are transferable.

Vancomycin-resistant *E. faecium* are the second most common cause of nosocomial infections in the USA (SIEVERT & al. [24]). In Europe, vancomycin resistant *E. faecium* prevalence is variable, ranging from less than 1% in France and Sweden to higher than 30% in Romania, Ireland and Cyprus (EARRS, [25]).

Conclusion

This is the first study regarding the genetic background of vancomycin resistance in VRE strains in Romania. Our study, although performed on a limited number of strains has shown that all teicoplanin resistant strains harbored vanA, while all teicoplanin susceptible strains harbored the vanB gene, irrespective to *Enterococcus* species.

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