Prevalence of vancomycin resistance phenotypes among Enterococcus species isolated from clinical samples in a Romanian hospital

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ABSTRACT
Vancomycin resistant Enterococcus (VRE) has been increasingly reported since the 1980s. Although normally regarded as harmless commensals, enterococci can become the etiological agents of nosocomial infections causing urinary tract infections, peritonitis, bacteremia, wound infections and endocarditis. The acquisition of vancomycin resistance has seriously affected the treatment and infection control of these bacteria. VRE are frequently resistant to most antibiotics that are effective against vancomycin susceptible enterococci, the therapeutic options for VRE infections being thus very limited. The aim of this study was to assess the genetic support of vancomycin resistance among VRE strains isolated from patients admitted to intensive care units in one hospital unit from Bucharest, Romania. Out of 84 enterococcal strains isolated from urine prelevated from catheterized and non-catheterized patients with urinary tract infections, wound secretions, pus, blood, ascite liquid and stool, 28 strains had glycopeptide resistance. Out of these, in the 12 vancomycin-resistant Enterococcus faecalis strains, the VanA phenotype was detected in 9 strains and VanB in 3 strains. From the 16 E. faecium strains resistant to vancomycin, 10 strains showed the VanA phenotype and 6 strains had the VanB phenotype. According to the results of this study, the VanA phenotype, conferring high-level resistance to vancomycin and teicoplanin, was more prevalent than VanB in the Enterococcus sp. strains isolated from hospitalized patients.

Keywords: Enterococcus, VRE, vanA, vanB, PCR.

1. INTRODUCTION
Enterococci are Gram-positive bacteria that are a part of the normal microbiota of humans and animals [3]. These species can cause infection in hospital environment and in the community and can be the etiological agents of peritonitis, bacteremia, endocarditis and wound infections [5]. Vancomycin-resistant enterococci (VRE) were first reported in Europe in the late 1980, and since then they have become an important cause of nosocomial infections worldwide. In the clinical settings, the most common enterococci isolated are Enterococcus faecalis and E. faecium. Their intrinsic resistance to antibiotics is one of the main reasons that enterococci can survive common antibiotic treatments used in hospitals, but they have also the ability to acquire and transfer new resistance phenotypes [6]. There are nine types of vancomycin-resistance genotypes described in enterococci. Eight of them correspond to acquired resistance (vanA, vanB, vanD, vanE, vanG, vanL, vanM and vanN), while the VanC resistance phenotype is usually identified in strains of E. gallinarum, E. casseliflavus and E. flavescens; these strains exhibit an intrinsic resistance to low levels of vancomycin and susceptibility to teicoplanin [7, 8, 9]. Two phenotypes of vancomycin resistance have been mainly associated with nosocomial infections. The VanA phenotype confers high-level resistance to vancomycin and teicoplanin, while the VanB phenotype is characterized by vancomycin resistance and teicoplanin susceptibility. The genes that encode for these two phenotypes are located on a mobile genetic element represented by the transposon Tn1546 for the VanA phenotype and by the Tn1547 for the VanB phenotype [1, 2]. The aim of this study was to determine the prevalence of vancomycin resistance genotypes of Enterococcus sp. strains isolated from different clinical samples.

2. MATERIALS AND METHODS
2.1. Bacterial strains.
A total of 84 strains were selected from a collection of Enterococcus sp. strains isolated from different clinical specimens during May 2014 – March 2015 from patients admitted in the intensive care units of one hospital unit from Bucharest, Romania. The strains identification was done using the VITEK 2 system and E. faecalis ATCC 29212 was used as a reference strain.

2.2. Antimicrobial susceptibility testing.
The selected strains were tested for antibiotic susceptibility using the Kirby-Bauer disk-diffusion method on Mueller-Hinton agar. The antibiotics (disks from Oxoid Ltd, Basingstoke, Hampshire, England) used to determine the antibiotic susceptibility were: ampicillin (10 µg/mL), vancomycin (30 µg/mL), erythromycin (15 µg/mL), tetracycline (30 µg/mL), ciprofloxacin (5 µg/mL), nitrofurantoin (300 µg/mL), linezolid (30 µg/mL). The resulting inhibition zones were measured and interpreted according to the Clinical and Laboratory Standards Institute guidelines [10].
2.3. DNA extraction and PCR analysis.

Enterococcal DNA was extracted by suspending 1 - 5 overnight bacterial colonies in a 1.5 mL tube containing 20 µL solution of NaOH (sodium hydroxide) and SDS (sodium dodecyl sulphate). The following step was the addition of 180 µL of TE buffer (TRIS+EDTA) 1x followed by centrifugation at 13000 rpm for 3 minutes. The PCR reactions were performed using the Corbet Thermal Cycler. Resulting amplicons were visualized by electrophoresis in a 1% agarose gel stained with ethidium bromide (10 µg/mL). Amplicons were identified based on their size using specific molecular size markers (DNA Ladder).

2.4. Detection of antibiotic resistance genes (ARGs) by PCR.

Multiplex PCR was used for the characterization of the vancomycin-resistant genotypes of the selected strains. Six pairs of primers were used for detecting van genes. Primer sequences used for the vanA gene were the forward primer (5’-TTTGCYGYGCAGCTGCAA-3’) and the reverse primer (5’-TCTAAACCGCTGAGTTGGCGC-3’), for the vanB gene the forward primer (5’-GCGCGAGATGACCCGCGCA-3’) and the reverse primer (5’-CGCGTTCGTCCTCGGGCGTTGAAGGA-3’) and for the vanC gene the forward primer (5’-TCGAGGCGTAGATGCTCCTTGCGCA-3’) [21]. The PCR components for the amplification of the van genes were: 0.5 µM primers, 1.2 mM MgCl₂, 2 µM dNTPs, 0.2 U of Taq-pol, 1x buffer and 1 µL of bacterial DNA. The final volume was 20 µL. The PCR amplification program for vanA, vanB and vanC genes was as follows: denaturation at 94 °C for 2 minutes, annealing at 53 °C for 1 minute and the final extension was at 72 °C for 30 cycles.

3. RESULTS

A total of 84 VRE strains isolated from hospitalized patients during 2014-2015 was analyzed. Identification using VITEK 2 system revealed that 60 strains (71.42%) were E. faecalis and 24 (28.58%) belonged to E. faecium.

The strains were isolated from: urine (n=33, 39.3%), wound secretion (n=14, 16.7%), pus (n=6, 7.1%), blood culture (n=8, 9.5%), ascite liquid (n=8, 9.6%), stool culture (n=4, 4.8%) and catheter associated urinary tract infections (CAUTI) (n=11, 13.1%).

3.1. Antimicrobial susceptibility testing.

The antimicrobial susceptibility tests performed according to the CLSI guidelines have demonstrated that most of the strains were resistant to erythromycin (70.24%), tetracyclines (72.61%) and ciprofloxacin (58.33), while almost all strains were susceptible to nitrofurantoin (94.04%) and linezolid (95.23%). Out of the 84 strains tested, 28 (33.33%) strains were resistant to vancomycin (Table 1).

Table 1. Antibiotic resistance of the 84 enterococci strains isolated from clinical samples; N – number of strains, % - percentage.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>21</td>
<td>25</td>
<td>0</td>
<td>63</td>
<td>75</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>28</td>
<td>33.33</td>
<td>0</td>
<td>56</td>
<td>66.66</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>59</td>
<td>70.24</td>
<td>0</td>
<td>8</td>
<td>9.52</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>61</td>
<td>72.61</td>
<td>1</td>
<td>22</td>
<td>26.19</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>49</td>
<td>58.33</td>
<td>15</td>
<td>20</td>
<td>23.80</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>1</td>
<td>1.19</td>
<td>4</td>
<td>79</td>
<td>94.04</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1</td>
<td>1.19</td>
<td>3</td>
<td>80</td>
<td>95.23</td>
</tr>
</tbody>
</table>

Table 2. Antibiotic resistance profile of 60 E. faecalis strains; N-number of strains, % -percentage.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>15</td>
<td>25</td>
<td>0</td>
<td>45</td>
<td>75</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>12</td>
<td>20</td>
<td>0</td>
<td>48</td>
<td>80</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>42</td>
<td>70</td>
<td>10</td>
<td>16.66</td>
<td>8</td>
</tr>
<tr>
<td>Tetracycline</td>
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<td>73.33</td>
<td>1</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>26</td>
<td>43.33</td>
<td>6</td>
<td>28</td>
<td>46.66</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>1</td>
<td>1.66</td>
<td>4</td>
<td>55</td>
<td>91.66</td>
</tr>
<tr>
<td>Linezolid</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>57</td>
<td>95</td>
</tr>
</tbody>
</table>

Table 2 shows the results obtained for the 60 isolates of E. faecalis, in which 42 (70%) strains were resistant to erythromycin and 44 (73.33%) to tetracycline. Resistance to vancomycin was observed in 11 (18.33%) of the tested strains. Susceptibility to ampicillin (75%), vancomycin (81.66%), nitrofurantoin (91.66) and linezolid (95%) was observed.

For the E. faecium isolates, the most resistant isolates were to erythromycin (70.83%) and ciprofloxacin (62.50%). From the 24 (28.57%) strains identified as E. faecium, a high percentage (70.83%) were resistant to vancomycin. The E. faecium strains were susceptible to ampicillin (75%), nitrofurantoin and linezolid (91.66%) as shown in table 3.

3.2. Molecular detection of the ARGs.

The results showed that out of 84 isolates, 28 strains were VRE (Table 4).

Table 3. Antibiotic resistance profile of 24 E. faecium strains; N-number of strains.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Susceptible</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>6</td>
<td>25</td>
<td>0</td>
<td>18</td>
<td>75</td>
</tr>
<tr>
<td>Vancomycin</td>
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<td>66.66</td>
<td>0</td>
<td>8</td>
<td>33.34</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>17</td>
<td>70.83</td>
<td>2</td>
<td>5</td>
<td>20.83</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>11</td>
<td>45.83</td>
<td>1</td>
<td>4.16</td>
<td>12</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>15</td>
<td>62.50</td>
<td>3</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>8.33</td>
<td>22</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1</td>
<td>4.16</td>
<td>1</td>
<td>22</td>
<td>91.66</td>
</tr>
</tbody>
</table>

Table 4. Distribution of VRE isolates in clinical samples; N isolates – number isolates; N – number of strains; % - percentage; CAUTI – Catheter associated urinary tract infections.

The vanA genotype was present in 19 (67.86%) strains, out of which 9 (43.37%) strains were E. faecalis and 10 (52.63%) E.
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E. faecium. The vanB genotype was found in 9 (32.14%) strains, with 3 (33.33%) strains of E. faecalis and 7 (77.77%) strains of E. faecium.

E. faecalis was identified in 60 (71.42%) isolates from a total of 84 isolates. 12 (18.33%) strains had glycopeptide resistance. The vanA genotype was present in 9 (81.81%) strains which showed resistance to vancomycin and teicoplanin. The vanB genotype, known to be responsible for resistance to vancomycin, with preserved susceptibility to teicoplanin was found in 3 (18.19%) of the strains.

Even though only 24 (28.57%) strains from a total of 84 isolates were identified as E. faecium, 17 (79.83%) strains were VRE. 10 (58.82%) strains had the vanA genotype and were resistant to vancomycin and teicoplanin, while 7 (41.18%) strains contained the vanB genotype. The vanC genotype was not found in any of the selected strains.

The 12 (36.4%) strains of VRE isolated from urine culture showed that E. faecium had a higher prevalence than E. faecalis with 5 vanA and 2 vanB genotypes, while E. faecalis strains had 3 vanA and 2 vanB genotypes. Wound secretion isolates showed only 1 (7.1%) strain that was resistant to vancomycin. E. faecalis had the vanA genotype. Out of the 8 isolates of hemoculture (bacteremia), 2 (25%) E. faecium strains that had the vanA genotype were identified.

A study in the UK showed that the incidence of VRE in urinary isolates varied from 8% to 14% without a statistically significant trend across more than 9 years. They reported 15 (1.6%) strains out of 928 isolates were identified as vancomycin resistant E. faecalis and 107(51.2%) strains out of 209 isolates were identified as E. faecium strains resistant to vancomycin [22].

A German study that investigated the development of different nosocomial infections with VRE in German federal districts showed that between 2007 and 2016, the incidence of VRE in urinary tract infections increased from 2.9% to 9.9%, surgical site infections with VRE showed an increase from 0.9% to 5% and the incidence of bloodstream infections with VRE increased from 5.9% to 16.7% [23].

Six (45.6%) strains out of 11 strains isolated from CAUTI had glycopeptide resistance. While only 1 strain of E. faecalis showed the vanB genotype, 1 E. faecium strain had the vanA genotype and 4 isolates had vanB genotype. From the 8 ascites isolates, only 2 (25%) strains of E. faecalis presented the vanA genotype. Three (50%) out six strains isolated from pus were VRE. One E. faecalis strain had the vanA genotype and two E. faecium strains had the vanA genotype. Two (50%) E. faecalis out of 4 total isolates showed a vanA genotype. A survey in the UK reported high rates of asymptomatic carriage of VRE, similar to those reported in a study based in Ireland [16]. Asymptomatic colonization of patients, particularly those who are immunocompromised, presents a risk of transmission to other patients. VRE colonization has been shown to precede infection in hospital patients [17]. The authors of this survey were planning to analyze the whole genome to provide further insight into the population structure and transmission of VRE within the hospital setting [15].

The 28 enterococci strains that showed glycopeptide resistance were subjected to Multiplex PCR using 6 sets of primers for van genes described above.

Being opportunistic pathogens, enterococci can cause nosocomial and community-acquired infections. E. faecalis and E. faecium are the most commonly identified species and constitute about 85%–90% of all clinical isolates [12; 13], to which other species can be added, but with much lower frequency. A study in France reported an outbreak in a teaching hospital in the Paris area with 4 strains of E. raffinosus. These strains had the vanA genotype, and were also resistant to ampicillin and gentamicin. Clonal identity between all 4 isolates was confirmed by rep-PCR. E. raffinosus is rarely identified in France, the author assumes that the clonal strain was imported from Portugal, along with a patient native to Portugal [19].

An estimated percent of 14.3% of the patients admitted to ICU are infected with enterococci [20]. The vanA genotype confers resistance to vancomycin and teicoplanin and the vanB genotype confers resistance to vancomycin but not to teicoplanin. The vanC genotype usually confers only a partial resistance to vancomycin.

In this study, out of 84 strains of enterococci, 60 (71.43%) strains were identified as E. faecalis and 24 (28.57%) were E. faecium. 11 (18.33%) strains of E. faecalis were vancomycin resistant with 9 (81.81%) strains having the vanA genotype and 2 (18.18%) strains having the vanB genotype; out of 17 (70.83%) E. faecium strains, 10 (58.82%) strains had the vanA genotype and 7 (41.17%) strains had vanB genotype. vanC genotype was not found in any of the VRE isolates.

We found that the prevalence of vancomycin resistant E. faecium strains was higher than that of E. faecalis, which is similar with the results of another study [14] that reported that out of 23 VRE strains isolated from different clinical specimens at the same hospital in Bucharest, Romania, 18 strains were identified as E. faecium and 5 were E. faecalis. 16 E. faecium strains carried the vanA genotype and 2 strains had the vanB genotype. The E. faecalis strains had the vanA genotype in 4 strains and the vanB genotype in 1 strain. The high prevalence of vanA is worrying taking into account the localization of this gene on a mobile genetic element, that could be easily transferred horizontally to other strains. A study in Warsaw, Poland, showed a molecular analysis of vancomycin resistant E. faecium strains isolated from outbreaks at two hospitals in Warsaw. The results suggests that horizontal gene transfer of the whole vanA plasmids and Tn1546 transposons were responsible for the VanA phenotype among endemic populations of nosocomial E. faecium [18].

According to the ECDC surveillance report, vancomycin resistance in E. faecalis remained low in most countries, while E. faecium showed an increased trend in 8 countries (Bulgaria, Croatia, Denmark, Hungary, Ireland, Italy, Slovakia and United Kingdom) and a decreased trend in three (Belgium, France and Germany). In Romania, between 2014–2017, both E. faecalis and E. faecium strains that were resistant to vancomycin have been increasingly reported. E. faecalis showed an increase in the number of isolates, from 102 in 2014 to 128 in 2017, with about 29% of the isolates being reported from patients in ICU; E. faecium also showed an increase in the number of isolates, from
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56 in 2014 to 64 in 2017, with about 43% of the isolates being reported from patients in the ICU [11]. Figures 1 and 2 show the electrophoresis gel of the PCR amplification products for the *vanA*, *vanB* and *vanC* genes. *vanA* genotype was found in 8 (20.51%) strains, *vanB* genotype was encountered in 12 (30.79%) strains, while *vanC* genotype was not found in any of the enterococcal strains analyzed. Figure 3 shows the electrophoresis gel of the PCR amplicons for *vanA* and *vanB* genes. *vanA* genotype was found in 6 (40%) strains. The *vanB* genotype was found in 7 (46.66%) strains.

**Figure 1.** Electrophoresis gel for PCR amplification of *vanA*, *vanB* and *vanC* genes. L well – molecular size marker 3000 bp (Thermo Scientific); *vanA* positive wells: 1, 20, 21, 22, 23; *vanB* positive wells: 2, 7, 8, 10, 11, 12, 13, 16; *vanC* genes were not detected.

**Figure 2.** Electrophoresis gel for PCR amplification of *vanA*, *vanB* and *vanC* genes. L well – molecular size marker 3000 bp (Thermo Scientific); *vanA* positive wells: 4, 9, 10; *vanB* positive wells: 2, 8, 10, 13; *vanC* genes were not detected.

**Figure 3.** Electrophoresis gel for the PCR amplification of *vanA* and *vanB* genes. *vanA* positive wells: 2, 3, 4, 8, 15; *vanB* positive wells: 5, 7, 9, 10, 12, 13. Well L – molecular size marker (GeneRuler 1000 bp)

4. CONCLUSIONS

This study found that out of 84 enterococcal strains isolated from patients hospitalized in intensive care units, 28 were VRE. The *vanA* genotype was found in 9 strains of *E. faecalis* and in 10 strains of *E. faecium*, while the *vanB* genotype was found in 3 *E. faecalis* strains and in 7 strains of *E. faecium*. The *vanC* genotype was not found in any of the isolated enterococci strains. Vancomycin is an important antibiotic used in the treatment of serious infections. The prevalence of VRE in Romania is slowly increasing as reported by the ECDC and an outbreak of infections...
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with VRE could create many health problems for the patients and increase the costs associated with their treatment.

5. REFERENCES


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