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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

The phenotypic and genotypic characterization of vancomycin-resistant enterococci in outpatients' urine culture

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SUMMARY

Introduction/Objective In the era of emerging antibacterial resistance, the major burden of resistant strains is on hospitalized patients. Although community factors are also important in the spread of resistance, less attention has been paid to non-healthcare settings.
The aim of the study is to determine the prevalence of vancomycin-resistant enterococci (VRE) in the outpatient's urine culture and to perform phenotypic and genotypic characterization of VRE strains.

Methods During an 18-month period, a total of 5,164 *Enterococcus* spp. strains were isolated from urine and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Antimicrobial susceptibility testing was performed by disk diffusion method and by gradient test for glycopeptide-resistant strains. Genotypic characterization of VRE strains was done by multiplex polymerase chain reaction for the detection of the vancomycin resistance genes.

Results Among the isolated enterococci, 5,060 (98%) were *E. faecalis* and 104 (2%) were *E. faecium*. *E. faecalis* strains were susceptible to all tested antibiotics except norfloxacin (33% of strains were resistant), while *E. faecium* showed high level of resistance to most of the tested agents (91.3% to ampicillin, 77% to norfloxacin, and 75% to nitrofurantoin), and 26% of strains were resistant to vancomycin and teicoplanin. *VanA* gene was detected in all vancomycin resistant *E. faecium* (VRE*fm)* strains.

Conclusion A high proportion of VRE*fm* was noticed among outpatients in our country. All analyzed VRE*fm* strains belonged to *vanA* genotype. Future surveillance studies of VRE are needed to follow up on this baseline study to monitor any possible changes in abundance and genotype of VRE in this population group.

Keywords: VRE; urine; outpatients

INTRODUCTION

Enterococcus spp. are not generally regarded as highly virulent bacterial pathogens. These bacteria are part of normal intestinal flora of both humans and animals, and can cause vast majority of human infections, such as: urinary tract infections, bacteremia, endocarditis and, less frequently, infections of other sites (wounds, bones, meninges, etc.). *Enterococcus faecalis* is the most common isolated species of *Enterococcus* spp., but in the last couple of decades, *Enterococcus faecium* has caused a substantial proportion of enterococcal infections, especially in hospital settings [1, 2].

Enterococci have emerged as important nosocomial pathogens. The major reason for this is the trend of increasing antimicrobial resistance seen in these organisms [2]. One of the main reasons why these organisms have survived in the hospital environment is their intrinsic resistance to commonly used antibiotics and, perhaps more importantly, their ability to acquire resistance to all currently available antibiotics, either by mutation or by receipt of foreign genetic material through the transfer of plasmids and transposons [3]. In the past decade, antibiotic resistance has been increasingly identified in the community. Although community factors are also important in the spread of resistance, less attention has been paid to non-healthcare settings [4]. Community-acquired infections account for the majority of prescribed antibiotics, very often wide-spectrum antibiotic therapy, which increases the rate of multidrug-resistant bacteria, e.g. multidrug-resistant enterococci strains isolated from urine culture [5, 6, 7]. The emergence of vancomycin-resistant enterococci (VRE) in the community has emphasized the non-existence of boundaries between hospitals, between people and animals, between countries, and probably between continents [8].

The aim of the study is to determine the prevalence of vancomycin-resistant enterococci (VRE) in outpatients' urine culture and to perform phenotypic and genotypic characterization of VRE strains.

METHODS

Bacterial strains

From February 2014 to July 2015, a total of 53,348 urine samples were analyzed in our

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laboratory. In accordance with European guideline recommendations [9], 5,164 clinically significant enterococci strains were included in this study. If there was more than one sample per patient, only the first isolated strain was included. Identification was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Vitek MS®, bioMerieux, Marcy-l'Étoile, France).

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was done by Kirby–Bauer disk diffusion method using the following disks (Bio-Rad Laboratories, Inc., Hercules, CA, USA): ampicillin (2 μg), norfloxacin (10 μg), nitrofurantoin (100 μg), vancomycin (5 μg), and teicoplanin (30 μg). Minimum inhibitory concentration (MIC) in glycopeptides resistant strains was determined by gradient test (E-test, bioMerieux) for vancomycin and teicoplanin. The results were interpreted and quality control was done in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations from 2015 [10, 11].

Multiplex polymerase chain reaction

Genotypic identification and determination of glycopeptide resistance genotype was done by multiplex polymerase chain reaction (PCR). For identification, detection of the genes encoding D-alanine–D-alanine ligases specific for *E. faecium* ($ddl_{E.\ faecium}$) and for *E. faecalis* ($ddl_{E.\ faecalis}$) was performed. For detection of the vancomycin resistance genes, the attempt was made to identify the commonest ones, i.e., *vanA*, *vanB*, and *vanC* genotypes (*vanC1* gene or *vanC2/C3* gene) [12, 13]. *E. faecium* BM4147 (*vanA* positive strain) was used as a positive control strain.

The PCR conditions and the primers used for the genotypic characterization of vancomycin resistant strains were as previously described [14–17]. The following pairs of primers were used: for $ddl_{E.\ faecium}$F (5'-GCAAGGCTTCTTAGAGA-3'), $ddl_{E.\ faecium}$R (5'-CATCGTGTAAGCTAACTTC-3'), $ddl_{E.\ faecalis}$F (5'-ATCAAGTACAGTTAGTCTT-3'), $ddl_{E.\ faecalis}$R (5'-ACGATTCAAAGCTAACTG-3'), *vanA*F (5'-GGAAAACGACAATTGCTATT-3'), *vanA*R (5'-GTACAATGCGGCCGTTA-3'), *VanB*F (5'-ACTGGCCTACATTCTTACA-3'), *VanB*R (5'-AGCGTTTAGTTCTTCCGT-3'), *vanC1*F (5'-TCTCCAGAATACTCAGTGT-3'), *vanC1*R (5'-ACATGGCAACCAACATAAG-3'), *vanC2/C3*F (5'-CCTCAAAAGGGATCACTAA-3'), *vanC2/C3*R (5'-TCTTGATAGGATAAGCCGA-3').

Statistical analysis

The data obtained in this study were analyzed in the SPSS statistical program (PASW statistics for Windows, Version 18.0, SPSS Inc., Chicago, IL, USA) using methods of descriptive statistics and χ^2 test.

RESULTS

Among isolated enterococci, 5,060 (98%) strains were *E. faecalis* and 104 (2%) strains were *E. faecium*.

E. faecalis strains were susceptible to all tested antibacterial agents, except 33% of strains that were resistant to norfloxacin, which is used for fluoroquinolones resistance screening according to EUCAST recommendations (Table 1). Among tested *E. faecium* strains, 91.3% were resistant to ampicillin, 77% to norfloxacin, 75% to nitrofurantoin and 26% to vancomycin and teicoplanin, respectively (Table 1).

MIC for vancomycin among all detected vancomycin resistant *E. faecium* (VRE*fm*) strains was higher than 256 μg/ml and for teicoplanin it was in the 8–256 μg/ml range (Figure 1).

Out of 27 strains of VRE*fm* subjected to multiplex PCR for detecting vancomycin resistance genes, all strains were found to possess the *vanA* gene (Figure 2).

Table 1. Antibiotic susceptibility of enterococci isolated from the urine of outpatients

Antibiotics	*Enterococcus faecalis* (n = 5,060)		*Enterococcus faecium* (n = 104)	
	Susceptible	Resistant	Susceptible	Resistant
Ampicillin	5,060 (100%)	0 (0%)	9 (8.7%)	95 (91.3%)
Nitrofurantoin	5,060 (100%)	0 (0%)	26 (25%)	78 (75%)
Norfloxacin	3,390 (67%)	1,670 (33%)	24 (23%)	80 (77%)
Vancomycin	5,060 (100%)	0 (0%)	77 (74%)	27 (26%)
Teicoplanin	5,060 (100%)	0 (0%)	77 (74%)	27 (26%)

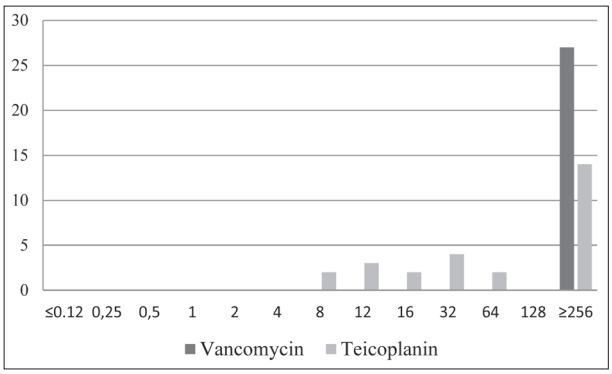

Figure 1. Glycopeptides MIC Distribution for VRE*fm* strains

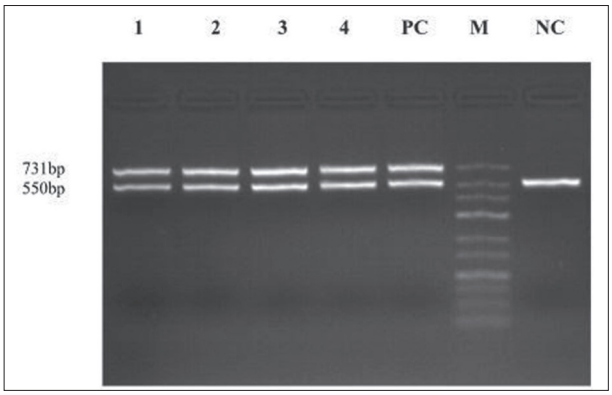

Figure 2. Gel electrophoresis of amplified products by PCR for vancomycin resistance genes; M – Gene Ruler Low range DNA Ladder (Thermo Scientific); PC – positive control for *vanA* gene; NC – negative control *VanA* gene; lines 1–4 positive for *vanA* gene (731 bp), $ddl_{E.\ faecium}$, D-alanine–D-alanine ligases *E.faecium* (550 bp)

Incidence of *vanA* gene was significantly higher in all strains of VRE*fm* compared to *vanB* and *vanC* genotypes ($p < 0.001$).

DISCUSSION

For accurate interpretation of antimicrobial resistance data, especially for glycopeptides, precise species identification is necessary. When interpreting the MIC/disk diffusion results, it is important to ensure that isolate is not *E. casselifalvus* or *E. gallinarum*, species that possess intrinsic resistance to glycopeptides. Furthermore, despite the number of studies on antibiotic-resistance in enterococci from Serbian clinical settings, there were no data about prevalence of VRE in the outpatients' settings in our country [18, 19, 20]. The results of the Central Asian and Eastern European Surveillance of Antimicrobial Resistance (CAESAR network) [7] showed high level of resistance to aminopenicillins among *E. faecalis* and *E. faecium* (41% and 94%, respectively). This result may reflect problems with species identification (comprising *E. faecium*, which is commonly resistant to aminopenicillins), rather than true high resistance in *E. faecalis*. On the other hand, high level of VRE*fm* among invasive isolates in Serbia (75%) may indicate difficulty in distinguishing *E. faecium* from *E. casseliflavus* and *E. gallinarum*. The application of the latest methods for identification such as MALDI-TOF MS or molecular methods, overcomes this problem [21]. Therefore, MALDI-TOF MS, as the most reliable phenotypic method for bacterial identification, was used in this study.

Antimicrobial resistance data in our study indicates overall significant level of multidrug-resistant *E. faecium* among enterococci in outpatients' urine culture, with 26% of VRE*fm* strains. Comparing the results with other studies [22, 23], where no VRE*fm* was detected, it can be concluded that increasing antibiotics resistance in community settings is a current trend.

Two principal phenotypes of acquired inducible vancomycin resistance have been described, VanA and VanB, encoded by two distinct gene clusters, the *vanA* and *vanB* clusters, respectively, which are carried on transposons Tn*1546* and Tn*1547*, respectively. The VanA phenotype confers high-level resistance to both vancomycin and teicoplanin, while the VanB phenotype confers only moderate to high-level resistance to vancomycin. A third type of vancomycin resistance, termed VanC, has been known for many years to be natural (intrinsic) vancomycin resistance found in the motile enterococci (*E. casseliflavus*, *E. gallinarum*, and *E. flavescens*). VanC confers only low-level resistance to vancomycin [24, 25]. Compared to other phenotypes, the VanA is the most common in European countries [26, 27]. Our results confirmed this fact: 100% of VRE*fm* strains belong toVanA phenotype.

To the best of our knowledge, this is the first molecular study on VRE strains among outpatients in our country. In accordance with phenotyping results, all strains were positive for *vanA* and negative for *vanB, vanC1*, and *vanC2/C3* genes as evidenced by PCR. Genotypic results in various studies show similar results. Libisch et al. [18] reported that the *vanA* gene was the dominant gene among invasive isolates in Serbia. Similar results were obtained for hospitalized patients in Turkey [27]. As per Werner et al. [28], the *vanA* and *vanB* resistance genotypes are by far the most prevalent in Europe. The reservoir for *vanA* and *vanB* type resistance in humans is *E. faecium*, which shows an enhanced capacity to disseminate in the nosocomial setting and are thus called epidemic or hospital-acquired. These clones of *E. faecium* are mostly ampicillin-resistant, partly high-level ciprofloxacin-resistant. In our study, *E. faecium* strains were 91.3% resistant to ampicillin and 77% resistant to quinolones. This may suggest that probably majority of our strains originated from nosocomial settings, but this requires further investigations.

CONCLUSION

A high proportion of VRE was noted among outpatients. All analyzed VRE strains belonged to *Enterococcus faecium* species associated with *vanA* genotype. Future surveillance studies of VRE are needed to follow up on this baseline study to monitor any possible changes in abundance and genotype of VRE in this population group.

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Фенотипска и генотипска карактеризација ентерокока резистентних на ванкомицин, изолованих из урина ванболничких болесника

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САЖЕТАК

Увод/Циљ У ери антимикробне резистенције највећи број резистентних сојева потиче од хоспитализованих болесника. Иако су ванболнички фактори важни у ширењу резистенције, мање пажње се поклања амбулантним болесницима. Циљ рада је одређивање учесталости енетерокока резистентних на ванкомицин (*VRE*) у уринокултури ванболничких болесника и њихова фенотипска и генотипска карактеризација.

Метода Током периода од 18 месеци укупно 5.164 ентерокока је изоловано из урина и идентификовано методом *MALDI-TOF MS*. Осетљивост на антимикробне агенсе је одређена диск-дифузионом методом и *E*-тестом за сојеве резистентне на гликопептидне антибиотике. Генотипизација *VRE* сојева је извршена мултиплекс *PCR* методом.

Резултати Међу изолованим ентерококама, *E. faecalis* чини 98% сојева, осетљивих на већину испитиваних антибиотика изузев норфлоксацина (33% сојева је било резистентно), док *E. faecium* чини 2% сојева, који показују висок ниво резистенције на већину тестираних антибиотика (91.3% сојева је било резистентно на ампицилин, 77% на норфлоксацин и 75% на нитрофурантоин), док је 26% сојева резистентно на ванкомицин и теикопланин. Код свих сојева *E. faecium* резистентних на ванкомицин (*VREfm*) утврђено је присуство *VanA* гена.

Закључак Међу ванболничким болесницима у нашој земљи утврђен је висок степен учесталости *VREfm*, што указује на неопходност сталног праћења антимикробне резистенције и у овој популационој групи.

Кључне речи: *VRE*; урин; ванболнички болесници