Occurrence of high-level aminoglycoside resistance (HLAR) among Enterococcus species strains


Department of Microbiological Diagnostics and Infectious Immunology, Medical University of Białystok, Poland

ABSTRACT

Purpose: Today, Enterococcus species are one of the most frequent etiological agents in nosocomial infections. The aim of this study was to determine the susceptibility to antibiotics and the prevalence of high-level aminoglycoside resistance (HLAR) among Enterococcus strains.

Materials and methods: The susceptibility of 85 isolates of Enterococcus (47 E. faecalis and 38 E. faecium) was determined using the disk diffusion method. The results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. PASW Statistics 17.0 was used for statistical analysis.

Results: E. faecalis strains showed the highest susceptibility to ampicillin, tigecycline, vancomycin, imipenem, and linezolid and E. faecium to linezolid, tigecycline, and quinupristin/dalfopristin. Among all tested strains, high-level gentamicin resistance (HLGR) was found in 4% of E. faecalis and 8% of E. faecium strains, high-level streptomycin resistance (HLSR) in 45% and 42%, and HLAR in 50% and 32% of strains, respectively. HLGR was detected only in vancomycin-resistant Enterococcus (VRE)− strains (12%), while HLSR in 76.9% of VRE+ and 24% of VRE− strains, and HLAR in 23.1% of VRE+ and 64% of VRE− strains. The tested strains were also divided into two groups: HLSR+ and HLAR+. In both groups, statistically significant susceptibility differences (p<0.05) were found for ampicillin, imipenem and trimethoprim/sulfamethoxazole. The most frequent antibiotic resistance profile among E. faecalis strains was S\textsuperscript{R} (resistance phenotype to streptomycin), and among E. faecium, AMP\textsuperscript{R}, IMP\textsuperscript{R}, CN\textsuperscript{R}, S\textsuperscript{R}, SXT\textsuperscript{R} (ampicillin, imipenem, gentamicin, streptomycin, trimethoprim/sulfamethoxazole).

Conclusions: This study showed the slowly increasing prevalence of HLAR and resistance to newer antibiotics (linezolid and tigecycline) among Enterococcus strains. It is necessary to search for new directions in the treatment of enterococcal infections.

Key words: Enterococcus; aminoglycoside resistance; vancomycin-resistant Enterococcus (VRE).

*Corresponding author:
Department of Microbiological Diagnostics and Infectious Immunology
Medical University of Białystok
Waszyngtona Str., 15A, 15-269 Białystok, Poland
Tel.: 85 746 85 71
e-mail: piowie@umb.edu.pl

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INTRODUCTION

Enterococcus, especially E. faecalis and E. faecium, have in recent years become one of the most common etiological factors in nosocomial infections. Although these bacteria are part of the normal flora of the gastrointestinal and genitourinary tracts and they are characterized by low pathogenicity, they can lead to serious infections such as bacteremia, endocarditis, and infections of wounds and the urinary tract [1-3].

Enterococcus can survive in a hospital environment because of their resistance to a variety of antimicrobials. In addition to their intrinsic resistance to cephalosporins, lincosamides, low levels of aminoglycosides, and many β-lactams, Enterococcus are also able to acquire resistance to many antibiotics by means of mutations or as a result of the transfer of genes located in plasmids/transposons or due to the incorporation of integrons [4,5]. The largest threats are strains resistant to glycopeptides (vancomycin-resistant Enterococcus, VRE) and high-level aminoglycoside resistance (HLAR) [6].

In the treatment of enterococcal infections, the use of a cell wall active agent such as a penicillin or vancomycin with an aminoglycoside results in synergistic bactericidal activity [7,8]. The increasingly frequent occurrence of HLAR strains, caused by production of aminoglycoside-modifying enzymes (AMEs), makes standard therapy with aminoglycosides and β-lactams impossible. Two of the most prevalent AME genes, aac(6')-Ie and aph(2'')-Ia, are located on mobile genetic elements and are widespread among Enterococcus. These genes encode a bifunctional enzyme, AAC(6')-Ie-APH(2'')-Ia, that confers resistance to a broad spectrum of aminoglycosides. Recently, new AME genes such as aph(2'')-Ib, aph(2'')-Ic and aph(2'')-Id have been detected and they are responsible for gentamicin resistance; high-level streptomycin and kanamycin resistance are mediated by the aph(3')-IIIa gene. At present, over 70 such enzymes have been discovered. Therefore, we distinguish three different phenotypes: HLSR (high-level streptomycin resistance), which determines resistance only for streptomycin, HLGR (high-level gentamicin resistance), which determines resistance to all aminoglycosides except streptomycin, and HLAR (high-level aminoglycoside resistance), which means resistance to all aminoglycosides. Hereinafter, testing HLGR, HLSR, and HLAR in Enterococcus has required only the use of high concentrations of gentamicin and streptomycin [3-5,9].

Today, vancomycin resistance among Enterococcus strains (VRE), especially E. faecium, has emerged as a major problem in the healthcare system, and related infections cause serious therapeutic problems. Bacteria resistant to glycopeptides produce cell wall precursors with decreased affinity for the drug, which prevents the antibiotic from blocking cell wall synthesis [10-11]. Resistance to newer effective antibiotics, such as linezolid and tigecycline, is also slowly developing [12-13].

The increasing role of Enterococcus in infections and their increasing resistance to antibiotics call for constant monitoring of their susceptibility. The aim of this study was to determine the susceptibility to different groups of antibiotics and the frequency of occurrence of HLAR, HLGR, and HLSR phenotypes among E. faecalis and E. faecium strains.

MATERIALS AND METHODS

Strains

A total of 85 isolates of Enterococcus were investigated, including 47 E. faecalis and 38 E. faecium. The isolates belong to the collection of the Department of Microbiological Diagnostics and Infectious Immunology and were selected randomly (except the VRE strains).

Strains were isolated from August 2012 to March 2013 from various departments of the Medical University of Białystok Clinical Hospital and the Medical University of Białystok Children’s Clinical Hospital of L. Zamenhof.

Most of the E. faecalis strains were collected from the intensive care (21%), surgery (15%), gynecology (15%), and cardiology (12%) units, and were isolated mostly from wound swabs (21%) and urine (17%), whereas E. faecium strains were gathered from the hematology (45%) and intensive care units (31%) and were isolated from rectal swabs (24%), and urine (21%).

Identification

Each isolate was identified by using the automated VITEK 2 system (bioMérieux, USA) according to the manufacturer’s instructions. Gram-positive cocci cards (GP VITEK 2, bioMérieux) were used for identification.

Antimicrobial susceptibility testing

Susceptibility of Enterococcus strains was determined using the disk diffusion method. The test was performed for the following antibiotics and amounts: ampicillin, 10 µg, imipenem, 10 µg, gentamicin, high content, 30 µg, streptomycin, high content, 300 µg, vancomycin, 5 µg, linezolid, 10 µg, quinupristin/dalfopristin, 15 µg, trimethoprim/sulfamethoxazole, 25 µg, and tigecycline, 15 µg (disks were purchased from Oxoid, UK and Becton Dickinson, USA).

The inoculum was prepared as follows: 2–3 colonies were picked up from overnight growth on blood agar and suspended into 2 ml of a 0.9%
sodium chloride solution. The suspension was adjusted to a McFarland standard of 0.5 by using a DensiCheck (bioMérieux). Next, the bacteria were inoculated on Mueller-Hinton agar (Oxoid) using a sterile swab and, after placing the disks with antibiotics, incubated for 24 hours under aerobic conditions at 37°C. Inhibition zone diameters were measured in millimeters and interpreted according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines [8]. Staphylococcus aureus ATCC 29213 was used as a control strain.

**Statistical analysis**

PASW Statistics 17.0 (IBM SPSS, USA) was used for statistical analysis. Differences in the prevalence of antibiotic resistance between different groups of *Enterococcus* were assessed by the Chi-square test and Fisher’s exact test; results with *p*<0.05 were considered significant.

**RESULTS**

Antibiotic susceptibility of *E. faecalis* and *E. faecium* strains is presented in Figure 1. *E. faecalis* strains showed the highest susceptibility to ampicillin (100%), tigecycline (97.9%), vancomycin (96%), and linezolid (91.4%). The most active antibiotics against *E. faecium* strains were linezolid (100%), tigecycline (100%), and quinupristin/dalfopristin (92.1%). Both *E. faecalis* and *E. faecium* strains showed high-level resistance to aminoglycosides: resistance to gentamicin was detected in 36% of *E. faecalis* and 57.9% of *E. faecium* strains; resistance to streptomycin was detected in 76.6% of *E. faecalis* and 92.1% of *E. faecium* strains. Differences in the prevalence of resistance between groups of *E. faecalis* and *E. faecium* were statistically significant only in the case of gentamicin (*p*<0.046), ampicillin (*p*<0.001), imipenem (*p*<0.001), vancomycin (*p*<0.001), and trimethoprim/sulfamethoxazole (*p*<0.001).

![Fig. 1. Comparison of susceptibility to antibiotics among E. faecalis (n = 47) and E. faecium (n = 38) strains.](image)

Table 1 presents the occurrence of high-level aminoglycoside resistance (HLAR) among all *E. faecalis* and *E. faecium* strains and among vancomycin-resistant *E. faecium* (VRE+) and vancomycin-susceptible *E. faecium* (VRE-). High-level gentamicin resistance (HLGR) occurred in 4% of *E. faecalis* and 8% of *E. faecium* strains. High-level streptomycin resistance (HLSR) was detected in 45% and 42% of strains, respectively. Half of the tested *E. faecium* strains and 32% of *E. faecalis* strains had HLAR phenotype. Nineteen percent of *E. faecalis* strains showed susceptibility to all aminoglycosides, whereas *E. faecium* strains were not susceptible to both gentamicin and streptomycin. These differences were not statistically significant (for HLAR *p*=0.091, for HLSR *p*=0.122). Comparison of the amino-glycoside resistance between VRE+ and VRE− *E. faecium* showed that HLGR was detected only in VRE− strains (12%). HLSR occurred in 76.9% of VRE+ strains and in 24% of VRE− strains; this difference was statistically significant.
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(p=0.002). On the other hand, HLAR phenotype was detected in 23.1% of VRE+ strains and in 64% of VRE− strains (likewise significant, p=0.017).

Table 1. Occurrence of high-level aminoglycoside resistance (HLAR) among all E. faecalis and E. faecium strains and among E. faecium VRE+ and E. faecium VRE− strains.

<table>
<thead>
<tr>
<th></th>
<th>Percent of all E. faecalis strains</th>
<th>Percent of all E. faecium strains</th>
<th>Percent of E. faecium VRE+ strains</th>
<th>Percent of E. faecium VRE− strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-level gentamicin resistance (HLGR)</td>
<td>4%</td>
<td>8%</td>
<td>0%</td>
<td>12%</td>
</tr>
<tr>
<td>High-level streptomycin resistance (HLSR)</td>
<td>45%</td>
<td>42%</td>
<td>76.9%</td>
<td>24%</td>
</tr>
<tr>
<td>High-level aminoglycoside resistance (HLAR)</td>
<td>32%</td>
<td>50%</td>
<td>23.1%</td>
<td>64%</td>
</tr>
<tr>
<td>Aminoglycoside-susceptible strains</td>
<td>19%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>All</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

After analysing the prevalence of resistance to aminoglycosides, the tested strains were divided into two groups: one group with HLSR phenotype (HLSR+) and one group with HLAR phenotype (HLAR+). HLGR+ group was not created due to the low prevalence of this resistance. Figure 2 presents a comparison of susceptibility to antibiotics other than aminoglycosides among E. faecalis HLAR+ and E. faecium HLAR+ strains.

Fig. 2. Comparison of resistance to antibiotics other than aminoglycosides among E. faecalis HLAR+ (n = 15) and E. faecium HLAR+ (n = 19) strains. AMP – ampicillin, IMP – imipenem, VA – vancomycin, LZD – linezolid, QD – quinupristin/dalfopristin, SXT – trimethoprim/sulfamethoxazole, TGC – tigecycline

All of the E. faecalis strains were susceptible to ampicillin, whilst all E. faecium isolates were resistant to ampicillin and imipenem and susceptible to quinupristin/dalfopristin. About 7% of E. faecalis isolates were found to be susceptible to imipenem. In comparison with the E. faecium strains, the E. faecalis strains were characterized by a higher resistance to linezolid (0 versus 6.7%) and by slightly higher susceptibility to trimethoprim/sulfamethoxazole (26.7% were intermediate and the remainder were resistant, while all of the E. faecium isolates were resistant). For the tigecycline all of the strains from both groups showed susceptibility. Vancomycin resistance level was similar: 13.3% versus 15.8% between both strains. Interestingly, the comparison between HLSR+ groups (Fig. 3) showed higher resistance of E. faecalis strains to imipenem (9.5% resistant, 4.8% intermediate), linezolid (9.5% resistant), and tigecycline (4.8% intermediate); likewise, among E. faecium strains, 6.3% showed resistance to quinupristin/dalfopristin and 12.4% intermediate resistance to quinupristin/dalfopristin. In both groups (HLAR+ and HLSR+), statistically significant susceptibility differences (p<0.05) were found for ampicillin, imipenem, and trimethoprim/sulfamethoxazole. Results of the statistical analysis are summarized in Table 2.
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**Fig. 3.** Comparison of resistance to antibiotics other than aminoglycosides among *E. faecalis* HLSR+ (n = 21) and *E. faecium* HLSR+ (n = 16) strains. AMP – ampicillin, IMP – imipenem, VA – vancomycin, LZD – linezolid, QD – quinupristin/dalfopristin, SXT – trimethoprim/sulfamethoxazole, TGC – tigecycline.

**Table 2.** Statistical analysis of differences in the prevalence of antibiotic resistance between different groups of *Enterococcus* (all *E. faecalis* strains versus all *E. faecium* strains, *E. faecalis* HLAR+ strains versus *E. faecium* HLAR+ strains, *E. faecalis* HLSR+ strains versus *E. faecium* HLSR+ strains).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>All strains</th>
<th>HLR+</th>
<th>HLR+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. faecalis</em> (n=47)</td>
<td><em>E. faecium</em> (n=38)</td>
<td>p-value</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>23.4%</td>
<td>7.9%</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>76.6%</td>
<td>92.1%</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>36.2%</td>
<td>42.1%</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>76.6%</td>
<td>57.9%</td>
</tr>
<tr>
<td>AM</td>
<td>S</td>
<td>100%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>IMP</td>
<td>S</td>
<td>91.4%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>4.3%</td>
<td>100%</td>
</tr>
<tr>
<td>VA</td>
<td>S</td>
<td>96%</td>
<td>65.8%</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>4%</td>
<td>34.2%</td>
</tr>
<tr>
<td>LZD</td>
<td>S</td>
<td>91.4%</td>
<td>100%</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>8.6%</td>
<td>6.7%</td>
</tr>
<tr>
<td>QD</td>
<td>S</td>
<td>92.1%</td>
<td>x</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>5.3%</td>
<td>100%</td>
</tr>
<tr>
<td>R</td>
<td>2.6%</td>
<td>100%</td>
<td>x</td>
</tr>
<tr>
<td>SXT</td>
<td>S</td>
<td>&lt;0.001</td>
<td>0.017**</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>55.3%</td>
<td>26.7%</td>
</tr>
<tr>
<td>TGC</td>
<td>S</td>
<td>97.9%</td>
<td>100%</td>
</tr>
<tr>
<td>I</td>
<td>R</td>
<td>2.1%</td>
<td>100%</td>
</tr>
</tbody>
</table>

In the final stage of the research, the antibiotic resistance profiles of *E. faecalis* and *E. faecium* strains were characterized (Table 3). The most frequent antibiotic resistance profile among *E. faecalis* strains was S\(^R\) (resistance to streptomycin), which was detected in 10 strains. Eighteen strains of *E. faecium* had the following resistance profile: AMP\(^R\), IMP\(^R\), CN\(^R\), S\(^R\), SXT\(^R\) (resistance to ampicillin, imipenem, gentamicin, streptomycin, and trimethoprim/sulfamethoxazole, respectively).

**Table 3.** Characteristics of the antibiotic resistance profiles of *E. faecalis* and *E. faecium* strains.

<table>
<thead>
<tr>
<th>Number of inactive antibiotics</th>
<th>E. faecalis strains (n=47)</th>
<th>E. faecium strains (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RESISTANCE PHENOTYPE</td>
<td>Number of strains</td>
</tr>
<tr>
<td></td>
<td>AM</td>
<td>IPM</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>2</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>2</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>1</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>


**DISCUSSION**

In the last two decades, *Enterococcus* have received an increasing attention because of the antimicrobials and their common prevalence as development of resistance to multiple nosocomial pathogens [12].

In this study, the hospital departments and isolation sources were similar to those presented by other authors in different areas of Poland and Europe. *Enterococcus* strains were mostly collected from intensive care units and surgery wards. The most frequent sources of isolation were rectal swabs, urine, feces, and wound swabs [14-16].

This study investigated the prevalence of aminoglycoside resistance among *E. faecalis* and *E. faecium* strains; resistance to gentamicin was detected in 36% of *E. faecalis* and 57.9% of *E. faecium* strains; resistance to streptomycin was detected in 76.6% and 92.1% of strains. Studies by other authors showed different results; in Lodz and Warsaw, percentages of streptomycin-resistant strains were much lower, while resistance to gentamicin occurred more frequently [14,17]. Differences between these results indicate that the resistance to gentamicin does not always correlate with resistance to streptomycin.

In this study we detected that high-level gentamicin resistance (HLGR) occurred in 4% of *E. faecalis* and in 8% of *E. faecium* strains; high-level streptomycin resistance (HLSR) was detected in 45% and 42% of strains, respectively. Half of the tested *E. faecium* strains and 32% of *E. faecalis* strains had HLAR phenotype. Similar differences were observed by authors from Poland (Warsaw and Torun) and Spain: more HLAR+ *E. faecalis* than *E. faecium* were found; likewise, more HLSR+ *E. faecium* than *E. faecalis* strains were detected [15, 18-19].

Comparison of the aminoglycoside resistance between VRE+ and VRE− *E. faecium* strains showed that HLGR was detected only in VRE− strains (12%); HLSR was found in 76.9% of
VRE+ strains and in 24% of VRE− strains. HLAR phenotype was detected in 23.1% of VRE+ strains and in 64% of VRE− strains. These results did not coincide with the studies from other centers [20 - 21]. For example, in a Behnood et al. study, HLAR was found in 71.4% of VRE+ and 30.6% of VRE− strains [20].

Unfortunately, the high-level resistance to all aminoglycosides is now widespread across Europe, and the synergistic effect between β-lactams and aminoglycosides is lost [16,22]. According to a recent report [22], the prevalence of HLAR is stable, but high. The percentages of resistant E. faecalis isolates were between 0% in Iceland to 56% in Italy; in 7 countries this percentage varied between 10–25%; in 18 countries between 25–50%. Percentages of strains in Poland ranged between 41–56%, which gave a value similar to the results presented in this paper and those of other Polish researchers [14,15,17,19,23]. An alarming increase in the prevalence of HLAR strains, compared to previous years, was reported in Austria and Luxemburg; a downward trend was observed in Belgium, Greece, Portugal, Great Britain, and Cyprus [22]. There is no information about the downward trend in Poland; based on the results of this study and other Polish researchers [14-15,17,19, 23], we can speculate that in Poland the level is stable, but high. Likewise, in the case of E. faecium, the percentage of aminoglycoside-resistant strains was also high, and compared to previous years, had not decreased [22]. Despite this stable trend, HLAR among Enterococcus remains a major infection control challenge throughout Europe.

As reported in several publications, E. faecalis strains are largely susceptible to ampicillin and imipenem, and E. faecium are mostly resistant [14,17]. E. faecium resistance is associated with changes in penicillin-binding proteins. Similar results are presented in this paper. However, researchers from Spain obtained only 28.6% ampicillin-resistant E. faecium strains. On the other hand, in the Netherlands 24% of E. faecium strains were susceptible to imipenem [18,24]. Therefore, determination of β-lactam resistance among Enterococcus must always be performed.

This study showed that linezolid, quinupristin/dalfopristin and tigecycline, had excellent activity towards Enterococcus isolates, including those resistant to aminoglycosides and vancomycin. These results are confirmed by many researchers [17,25-27]. However, cases of resistance to these antibiotics have been recently reported [13-14,17,28] that may suggest that resistance to newer antimicrobials is slowly developing.

In conclusion, this study highlights that the increasing prevalence of HLAR among Enterococcus strains is a reason to strictly enforce antibiotic policies coupled with greater adherence to infection control measures to prevent the spread of antimicrobial-resistant bacteria. It is necessary to search in new directions for the treatment of enterococcal infections.

CONCLUSIONS

1. High-level streptomycin resistance (HLSR) occurred more frequently in E. faecalis strains than in E. faecium strains, but the difference was not statistically significant.
2. High-level gentamicin resistance (HLGR) and high-level aminoglycoside resistance (HLAR) occurred more frequently in E. faecium strains than E. faecalis strains, but the differences were not statistically significant.
3. Differences in the prevalence of resistance between groups of E. faecalis and E. faecium were statistically significant (p<0.05) in the case of gentamicin. Percentages of resistance to aminoglycosides were higher in E. faecium than E. faecalis strains.
4. Differences in the prevalence of aminoglycoside resistance among E. faecium VRE+ and VRE− strains were statistically significant in the case of HLAR and HLSR phenotypes; HLAR appeared more frequently in VRE− strains, while HLSR appeared more frequently in VRE+ strains.
5. Differences in the prevalence of resistance between groups of E. faecalis and E. faecium (E. faecalis HLAR+ versus E. faecium HLAR+ and E. faecalis HLSR+ versus E. faecium HLSR+) were statistically significant (p<0.05) for ampicillin, imipenem, and trimethoprim/sulfamethoxazole. Percentages of resistance to these antibiotics were higher in E. faecium than in E. faecalis strains.

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Conflicts of interest

The authors declare no conflicts of interest.

REFERENCES


