Identification of aminoglycoside resistance genes by Triplex PCR in Enterococcus spp. isolated from ICUs

Reza Mirnejad¹, Nikta Sajjadi², Sara Masoumi Zavaryani³, Vahhab Piranfar⁴, Maryam Hajihosseini³, Maliheh Roshanfekr²
¹Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
²Department of Microbiology, Science & Research Islamic Azad University, Damghan Branch, Damghan, Iran
³Department of Microbiology, Islamic Azad University Varamin-Pishva Branch, Tehran, Iran
⁴Department of Microbiology, Biology Faculty, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran.

SUMMARY

Early detection of antibiotic-resistant enterococci is an important part of patient treatment. Therefore, the aim of the present study was to evaluate the resistance patterns and simultaneously identify and characterise the resistance genes in Enterococcus spp. using a triplex polymerase chain reaction (PCR) method. In all, 150 consecutive Enterococcus spp were collected from several hospitals in Tehran (Iran) from January to December 2015. The Enterococcus species were identified by standard phenotypic/biochemical tests and PCR. The antimicrobial resistance patterns were determined using a disk diffusion method. The triplex PCR method was designed to identify gentamicin and other aminoglycoside resistance genes. Among the 150 Enterococcus specimens, 87 cases (58%) were Enterococcus faecalis, and 63 cases (42%) were Enterococcus faecium. The highest frequency of resistance was observed for tetracycline while the lowest was found for vancomycin. Among the identified samples, 56.9% contained the aac(6')-le-aph(2'')-Ia gene, 22.2% contained the aph(3')-IIIa gene, and 38.8% contained the ant(4')-Ia gene. Eight percent of the isolates contained the three aminoglycoside resistance genes. Data analysis showed that there was a significant correlation between the phenotypic gentamicin resistance and the presence of the aminoglycoside resistance genes (18.9%, p<0.05), while the correlation between the phenotypic streptomycin resistance and the corresponding genes was not significant (2.8%, p≥0.5). Nearly half of the identified Enterococcus strains had increased aminoglycoside resistance. The direct correlation between resistance genes, such as the aminoglycoside resistance factor, and phenotypic resistance was not significant (p>0.05).

Keywords: Enterococcus faecalis, Enterococcus faecium, resistance gene, Triplex PCR.

INTRODUCTION

Enterococci are fermentative gram-positive bacteria that are observed as single cocci or short chains [1]. These bacteria constitute the natural intestinal microflora humans and animals, and two species cause nosocomial infections, including Enterococcus faecalis, with a frequency of 90 to 95%, and Enterococcus faecium, with a frequency of 5 to 10%. Based on the nosocomial infection surveillance system developed in the U.S., these two species are reported as the fourth most frequent cause of nosocomial infections, the second most frequent cause of bacteremia, and the third most frequent cause of urinary tract infections [2, 3]. The presence of antibiotic resistance genes is a common characteristic of nosocomial infections caused by these species [4]. In accordance with the World Health Organization guidelines, some
Identification of aminoglycoside resistance genes by Triplex PCR in Enterococcus spp. isolated from ICUs

...frequency of resistance to aminoglycosides in Iran between January and December 2015.

MATERIALS AND METHODS

Clinical samples
This research was a cross-sectional study with simple random sampling. 150 consecutive strains isolated from patients affected by intra-abdominal infection and urinary tract infection from ICUs of three hospitals, including Milad, Imam Khomeini, and Baqiatallah (Tehran, Iran). Sampling was conducted between January and December 2015. The samples included blood, urine and wound swabs which were transferred under sterile conditions to the Baqiatallah molecular research laboratory. First, cultural, biochemical, and microbiological standard tests were used to identify the species (E. faecalis and E. faecium). In accordance with the Clinical Laboratory Standards Institute guidelines (CLSI 2015), HLGR and HLSR were confirmed using minimum inhibitory concentration (MIC) tests.

Antibiotic susceptibility tests
To determine antimicrobial susceptibility patterns, the Kirby–Bauer method was applied using antibiotic discs, including tetracycline (30 µg), erythromycin (15 mg), chloramphenicol (30 mg), ciprofloxacin (5 µg), streptomycin (30 mg), and gentamicin (120 mg). Based on CLSI 2015, the bacteria were reported as sensitive, semi-sensitive, and resistant. All discs were purchased from the MAST Company (UK). To confirm the results and evaluate reproducibility, the antibiotic susceptibility tests were performed three times for each sample. In addition, susceptibility to vancomycin was determined by macro gradient test on Brain Heart Infusion Agar (MIC ≥256 µg/mL) while HLGR (MIC ≥500 µg/mL) and HLSR (MIC ≥1,000 µg/mL) were determined using MIC Test Strip range on Mueller Hinton Agar according to the CLSI 2015 guidelines [22]. E. faecalis ATCC 2912 and ATCC 51299 were used as negative and positive controls, respectively.

Polymerase chain reaction
Identification of Enterococcus species
In order to confirm the results of the phenotypic and biochemical tests, the identification of species...
was performed by PCR using a GeneAmp PCR system (Eppendorf, Hamburg, Germany) and specific primers (ddl E. faecalis and ddl E. faecium) listed in Table 1.

To prepare DNA samples for PCR, five colonies of bacteria from positive cultures were suspended in 100 mL of distilled water and then boiled for 15 min. The boiled cell suspension was directly used as template DNA for PCR amplification. DNA amplification was performed in 25 μL of a reaction mixture containing 2.5 μL of 10× buffer, 0.75 μL of 50 mM magnesium chloride, 0.5 μL of 10 mM deoxynucleotide triphosphates (dNTPs), 20 pmol each primer, 0.2 μL of 5 U Taq polymerase, and 2 μL of a bacterial suspension, equal to 0.5 McFarland standard (1.5× 10⁸ cells/mL). The conditions for a PCR reaction were as follows: initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. All PCR reactions were performed in duplicate. The PCR products were analyzed by electrophoresis on 1.5% agarose gels, stained with SafeStain, and visualized under UV transilluminator. E. faecalis ATCC 2912, which carries the three genes, was used as a positive control sample in this study, and E. faecalis V583 was used as a negative control due to the absence of the aforementioned genes.

Purified PCR products of the three genes from the last step, aac(6')-le-aph(2'')-la, aph(3')-IIIa, and ant(4')-Ia, were sent to the MWG company for sequencing by the Sanger method.

Statistical analysis
The significance of the obtained results was determined by the chi-squared test at the significance level of p≤0.05 using SPSS v. 16.

Ethical approval of research
The study was approved by the Research Ethics Committee of Islamic Azad University of Damghan, Iran.

### RESULTS
The present study was conducted on 150 consecutive Enterococcus spp isolated from patients affected by intra-abdominal infection and urinary tract infection. The frequency of detection of the enterococci in urine, blood and other samples is
Identification of aminoglycoside resistance genes by Triplex PCR in Enterococcus spp. isolated from ICUs presented in Figure 1. The results revealed that at the level of $p<0.001$, significant infections were higher in urine than in the other samples.

In this study, various tests, including growth on bile esculin agar and in the presence of 6.5% NaCl, as well as arabinose fermentation, were performed on the strains isolated from the 150 clinical samples. Eighty-seven strains (58%) did not ferment arabinose and thus were identified as E. faecium, while the other strains that could ferment arabinose belonged to E. faecalis. The growth in BHI broth with 6.5% NaCl showed that all 150 enterococcal strains were able to tolerate high concentrations of sodium chloride in the environment.

**Confirmation of identified species by PCR**

The results of detection of two genes, $ddl$ of E. faecalis [941 base pairs (bp)] and $ddl$ of E. faecium (550 bp) confirmed the identification of the two species in the clinical samples (Figure 2).

**Antibiotic susceptibility tests**

In the antibiotic susceptibility tests performed by the disk diffusion method, the strains showed the highest resistance to tetracycline (57.6%) and
erythromycin (43.75%) and the lowest resistance to chloramphenicol and vancomycin (15.27 and 6.25%, respectively). In addition, 38% of the strains were resistant to a high level of gentamicin, and 25% of the strains showed resistance to a high level of streptomycin (Figure 3).

**Triplex PCR for resistance genes**

The PCR amplification of the three genes indicated that among the 150 identified enterococcal strains, 82 strains (56.9%) that showed a high level of resistance to gentamicin had the aac(6')-Ie-aph(2'')-Ia gene. Only 32 strains (22.2%) carried the aph(3')-IIIa gene and were resistant to a high level of streptomycin, while 56 samples (38.8%) contained the ant(4')-Ia gene.

The triplex PCR products of the three genes, aph(3')-IIIa, aac(6')-le-aph(2'')-Ia, and ant(4')-Ia, could be observed in the clinical sample isolates (Table 2). Furthermore, the strains that contained two resistance genes were also identified in this study. The frequency of these genes is shown in Table 2.

It was found by the PCR that 31 strains (21.5%) contained the resistance gene aac(6')-le-aph(2'')-Ia and showed resistance to a high level of gentamicin (MIC >500 µg/mL). Thirty-two strains (22.2%) contained aph(3')-IIIa and were resistant to a high level of streptomycin (MIC >1,000 µg/mL), and 56 strains (38%) contained ant(4')-Ia and showed resistance to other aminoglycosides. For all samples, there was no significant relationship among the frequencies of each gene (p=0.750).

**DISCUSSION**

In this study, 58% of the samples were found to contain E. faecalis, and 42% of the samples contained E. faecium. The difference in the occurrence of these species among the 150 Enterococcus-containing samples is not significant. This frequency is in line with the data reported by Nohi and Rahimi (2007), Dadfar (2013), and Padmasini (2014) [8, 15, 23]. Some studies, such as that of Mohammadi, indicated that the prevalence of E. faecalis was higher than that of E. faecium in clinical specimens in 2011, which is in contrast with the present study [24]. It is possible that the geographical differences and the sampling methods caused the differences in the frequency patterns for these two species.

The frequency of HLGR (56.9 %) reported in our study is the same as the frequency shown by Dadfar and Feyzabadi but higher than the percentages reported by Behnood, i.e (32.43%) and the studies were shown 46.15% in Italy, 45.5% in Brazil, 37.64% in Chicago and 46.06% in South Africa [8,9,25-29]. The lowest prevalence of HLGR was reported in Greece [30-32]. In this study, the prevalence of HLSR was 22.2%, similar to that reported in the Padmasini study [15]. These results are in contrast with those of Vakulenko’s study, suggesting a higher percentage [33].

The present study, however, confirms the resistance patterns revealed in the Mohammadi and Karmarkar’s studies [24, 34]. The increasing transfer of resistance genes of other aminoglycoside antibiotics, such as streptomycin, through plasmids and transposons, may explain the differences between the studies. High use of antibiotics causes the emergence of high-level-resistant strains. In contrast to the reports of Kobayashi in Korea and Vakulenko, showing that no strains contained all three resistance genes, aac(6')-le-aph(2'')-Ia, ant(4')-Ia, and aph(3')-IIIa, our study revealed that 8% of the strains contained all the three genes [35].

Presently, due to increased resistance of enterococci to antibiotic treatments (especially to gentamicin and streptomycin) in various countries, early detection of AME (Aminoglycoside modifying enzymes) resistance genes by molecular techniques is of utmost importance [36]. Among molecular genotypic methods, triplex PCR can simultaneously assess several resistance genes in a fast and efficient fashion, thus it saves time.

### Table 2 - Frequency of Enterococcus strains containing more than one resistance gene in the clinical samples.

<table>
<thead>
<tr>
<th>Certain Genes</th>
<th>Numbers</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>aac(6')-le-aph(2'')-Ia + aph(3')-IIIa</td>
<td>19</td>
<td>12.6%</td>
</tr>
<tr>
<td>aac(6')-le-aph(2'')-Ia + ant(4')-Ia</td>
<td>39</td>
<td>26%</td>
</tr>
<tr>
<td>ant(4')-Ia + aph(3')-IIIa</td>
<td>16</td>
<td>10.6%</td>
</tr>
<tr>
<td>'ant(4')-Ia + aph(3')-IIIa + aac(6')-le-aph(2'')-Ia</td>
<td>12</td>
<td>8%</td>
</tr>
</tbody>
</table>
Identification of aminoglycoside resistance genes by Triplex PCR in Enterococcus spp. isolated from ICUs and provides a precise diagnosis [37]. Thus, the detection of aac(6’)-Ie-aph(2’’)-Ia, aph(3’’)-IIIa, and ant(4’)-Ia genes is essential, considering the morbidity of the infections caused by Enterococcus species, particularly genitourinary tract infections and bacteremia in ICU wards around the world. In recent years, discovery of new aminoglycoside resistance genes made eradication of the infections more complicated [38]. Enterococcus species can easily acquire high resistance to aminoglycosides, particularly to gentamicin and streptomycin, by receiving these genes through mobile genetic elements, transposons and integrons [39]. Some of these genes, such as aac(6’)-Ie-aph(2’’)-Ia, are only responsible for gentamicin resistance, i.e., they act specifically [40]. High levels of aminoglycoside resistance cause a major problem in hospitals. The present study was conducted to monitor and rapidly detect Enterococcus species, for which an optimized triplex PCR method was designed. The results indicated a high frequency of resistance to high levels of aminoglycosides, particularly, gentamicin and streptomycin.

In the present study, 9.18% of the strains with genes of increased resistance to gentamicin did not show any elevated resistance to gentamicin in the disc diffusion test. It is likely that, even though there is no significant relationship between the differences, not all bacteria containing the aac(6’)-Ie-aph(2’’)-Ia gene are resistant to high concentrations of gentamicin. There was an inverse correlation between streptomycin resistance and aph(3’)-IIIa, and 8.2% of the strains that were resistant to the high concentration of streptomycin in the disk diffusion test lacked the aph(3’)-IIIa gene. This fact shows that bacteria develop different mechanisms of resistance to streptomycin. In addition, the presence of the aac(6’)-Ie-aph(2’’)-Ia gene not always causes increased resistance to gentamicin. This needs further investigation.

Increased resistance to antibiotics is detected in nearly 10% of the isolated Enterococcus strains, which was confirmed by the triplex PCR test. This technique is more accurate than the culture technique and can quickly reveal resistant species. In this study, the presence of resistance genes and the aminoglycoside resistance factor did not meaningfully correlate at the level of significance of p<0.5. Moreover, it was shown that a direct relationship between the presence of resistance genes and increased resistance does not always exist.

Conflict of interest: the authors declare that there is no conflict of interests.

Authors’ contributions: all authors read and approved the final manuscript.

ACKNOWLEDGMENT
This study obtained an MSc thesis in Microbiology from Islamic Azad University of Damghan.

REFERENCES
Identification of aminoglycoside resistance genes by Triplex PCR in Enterococcus spp. isolated from ICUs


