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

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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′)-Ie-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%, $p\geq0.5$). 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

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antibiotics are used for the treatment of enterococcal infections, such as glycopeptide antibiotics, beta-lactams, and aminoglycosides [5]. In some cases, they are recommended in the form of synergistic antibiotic treatment (penicillin and gentamicin) [6]. Gentamicin and streptomycin are the most important aminoglycosides which are still commonly used for the treatment of enterococcal infections in Iran [7, 8]. Due to the unstable conditions of the patients and an invasive or semi-invasive nature of these microorganisms, resistance to the high doses of antibiotics creates the major problem in hospitals worldwide, especially in intensive care unit (ICU) wards [9, 10].

Resistance to antibiotics in these species is classified as inherent and acquired resistance [11]. The inherent resistance includes resistance to low levels of penicillin, cephalosporin, and aminoglycosides [12]. Obtaining genetic components through plasmids or mutations can lead to acquired resistance to glycopeptides and high concentrations of aminoglycosides. Acquired resistance genes can encode aminoglycoside-modifying enzymes (AMEs) [13] and cause high-level gentamicin resistance (HLGR) and high-level streptomycin resistance (HLSR) [14, 15].

In enterococci, the *aac(6′)-Ie-aph(2″)-Ia* gene, which is located on the Tn5281 transposon, plays a major role in the emergence of resistance to gentamicin [16]. This gene encodes an AME that has two functions, one as acetyltransferase and the other as phosphotransferase; and consequently confers gentamicin resistance to the host [17, 18]. In recent years, new genes have been detected as the determinants of gentamicin resistance in *Enterococcus* species, including *aph(2″)-Ib*, *aph(2″)-Ic*, and *aph(2″)-Id*, which encode the APH(3′) and ANT enzymes [19,20]. Moreover, *aph(3′)-IIIa* and *ant(6)-Ia* have been introduced as streptomycin and kanamycin resistance genes. The *ant(4′)-Ia* gene, which has an adenylyltransferase function, has been identified as a resistance gene responsible for the resistance to other aminoglycoside antibiotics [21]. Because the frequency of aminoglycoside resistance genes is not certain in Iran, the aim of this study was identify three resistance genes which have a key role to resist to aminoglycoside antibiotics among *Enterococcus spp*. A triplex polymerase chain reaction (PCR) was designed to detect three aminoglycoside resistance genes in one reaction PCR. This study describes the latest 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

Table 1 - PCR primers used for the amplification of and resistance genes.

Gene	*Primer Sequence*	*Size*	*Reference of AMES gene sequence*
acc(6′)-le-aph(2′′)-la	5′CAGGAATTTATCGAAAATGGTAGAAAAG3′ 5′-CACAATCGACTAAAGAGTACCAATC-3′	369	23
aph(3′)-IIIa	5′-GGCTAAAATGAGAATATCACCGG-3′ 5′-CTTTAAAAAATCATACAGCTCGCG-3′	523	23
ant(4′)-Ia	5′-CAAACTGCTAAATCGGTAGAAGCC-3′ 5′-GGAAAGTTGACCAGACATTACGAACT-3′	296	7
ddl E. feacalis	5′-ATCAAGTACAGTTAGTCTT-3′ 3′-ACGATTCAAAGCTAACCTG-5′	941	22
ddl E. faecium	5′-GCAAGGCTTCTTAGAGA-3′ 3′-CATCGTGTAAGCTAACTTC-5′	550	22

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 (1.5×10^8 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. The PCR products were analyzed by electrophoresis on 1.5% agarose gels, stained with SafeStain, and visualized under an UV transilluminator. The PCR products were confirmed by sequencing.

Triplex PCR method to detect resistance genes

After DNA extraction by the boiling method as described in the previous section, triplex PCR was performed on samples resistant to streptomycin and gentamicin to identify the *aph(3′)-IIIa*, *aac(6′)-Ie-aph(2′′)-Ia*, and *ant(4′)-Ia* genes using the specific primers shown in Table 1. Each PCR reaction mixture contained 15 µL of 2× Master Mix (Ampliqon III Co., Denmark), 1 µL of template DNA (0.5 µg), 20 pmol each forward and reverse primer, and sterile distilled water up to 30 µL. The PCR cycling conditions were as follows: initial denaturation at 95°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 run for 1 h at 85 V and 25 mA, 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′)-Ie-aph(2′′)-Ia*, *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 $p\leq 0.05$ 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

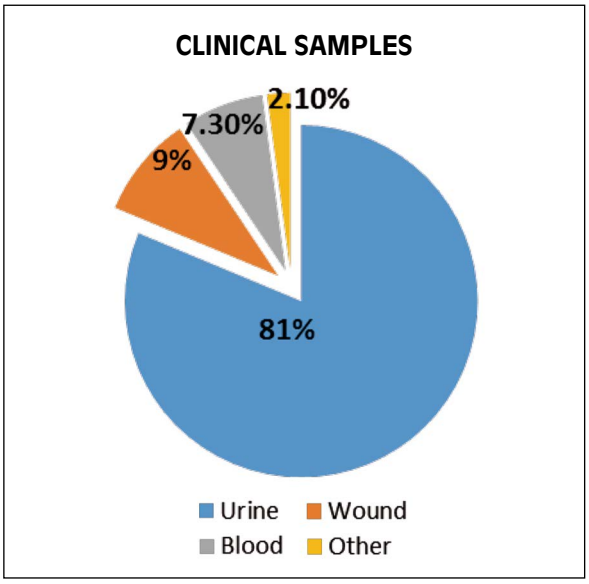


Figure 1 - Frequency of isolation of enterococci from clinical specimens.

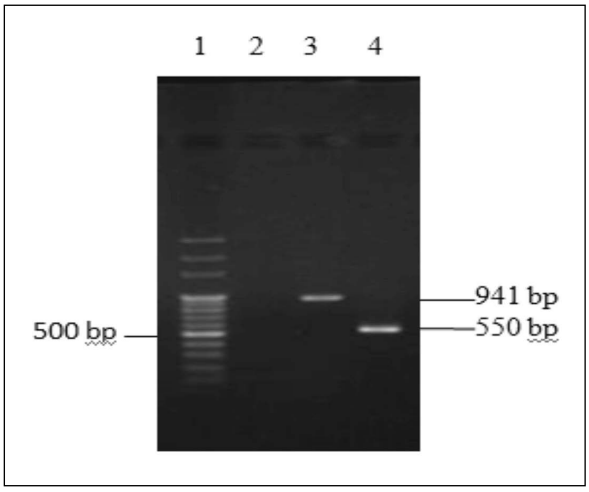


Figure 2 - PCR products. Lane 1, 100 bp DNA Marker; Lane 2, amplified products from clinical specimens lacking the target genes; Lane 3, amplified products from clinical specimens with *ddl* of E. *faecalis* (941 bp); Lane 4, amplified products from clinical specimens with *ddl* of *E. faecium* (550 bp).

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.

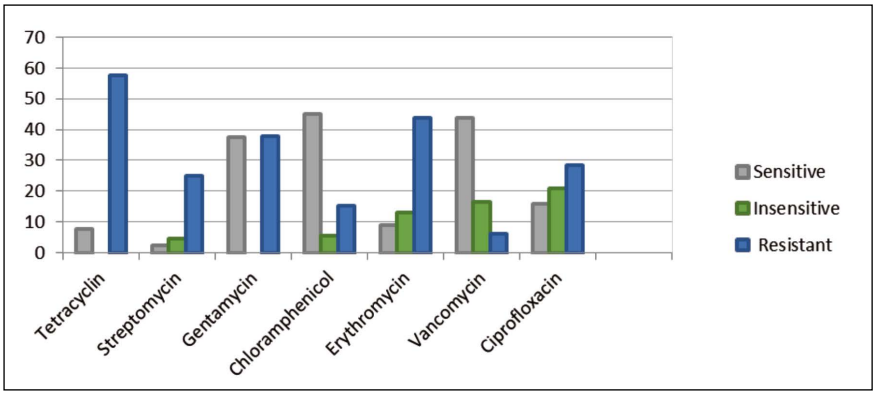
Confirmation of identified species by PCR

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

Figure 3 - Results of the disk diffusion test for all isolated *Enterococcus* strains from clinical samples.



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′)-Ie-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′)-Ie-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 Dadfarma 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′)-Ie-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.

Certain Genes	*Numbers*	*Frequency*
aac(6′)-Ie-aph(2″)-Ia + aph(3′)-IIIa	19	12.6%
aac(6′)-Ie-aph(2″)-Ia + ant(4′)-Ia	39	26%
ant(4′)-Ia + aph(3′)-IIIa	16	10.6%
'ant(4′)-Ia + aph(3′)-IIIa + aac(6′)- Ie-aph(2″)-Ia	12	8%

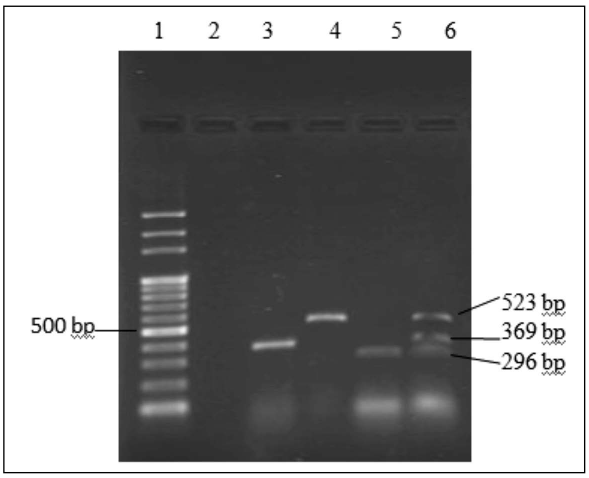


Figure 4 - Triplex PCR products. Lane 1, 100 bp DNA Marker; Lane 2, amplified products from clinical samples lacking the target genes; Lane 3, amplified products of the *aac(6′)-Ie-aph(2″)-Ia* gene (369 bp) from clinical samples; Lane 4, amplified products of the *aph(3′)-IIIa* gene (523 bp) from clinical samples; Lane 5, amplified products of the *ant(4′)-Ia* gene (296 bp) from clinical samples; Lane 6, amplified products of the three genes, *aac(6′)-Ie-aph(2″)-Ia*, *aph(3′)-IIIa*, and *ant(4′)-Ia*, from clinical samples.

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.

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REFERENCES

[1] Buchan B.W., Ginocchio C.C., Manii R., et al. Multiplex identification of gram-positive bacteria and resistance determinants directly from positive blood culture broths: evaluation of an automated microarray-based nucleic acid test. *PLoS Med*. 10, e1001478, 2013.

[2] Arias C.A., Murray B.E. The rise of the Enterococcus: beyond vancomycin resistance. *Nat. Rev. Microbiol*. 10, 266-278, 2012.

[3] Djahmi N., Boutet-Dubois A., Nedjai S., et al. Molecular epidemiology of Enterococcus sp. isolated in a university hospital in Algeria. *Scand. J. Infect. Dis*. 44, 656-662, 2012.

[4] Davies J., Davies D. Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev*. 74, 417-433, 2010.
[5] Kristich C.J., Rice L.B., Arias C.A., Enterococcal Infection-Treatment and Antibiotic Resistance, In: M.S. Gilmore, D.B. Clewell, Y. Ike, N. Shankar (Eds.) Enterococci: From Commensals to Leading Causes of Drug Resistant Infection, Boston, 2014.
[6] Leekha S., Terrell C.L., Edson R.S. General principles of antimicrobial therapy. *Mayo Clin. Proc*. 86, 156-167, 2011.
[7] Agudelo Higuita N.I., Huycke M.M., Enterococcal Disease, Epidemiology, and Implications for Treatment, In: M.S. Gilmore, D.B. Clewell, Y. Ike, N. Shankar (Eds.) Enterococci: From Commensals to Leading Causes of Drug Resistant Infection, Boston, 2014.
[8] Dadfarma N., Imani Fooladi A.A., Oskoui M., Mahmoodzadeh Hosseini H. High level of gentamicin resistance (HLGR) among enterococcus strains isolated from clinical specimens. *J. Infect. Public Health.* 6, 202-208, 2013.
[9] Behnood A., Farajnia S., Moaddab S.R., Ahdi-Khosroshahi S., Katayounzadeh A. Prevalence of aac(6')-Ie-aph(2'')-Ia resistance gene and its linkage to Tn5281 in Enterococcus faecalis and Enterococcus faecium isolates from Tabriz hospitals. *Iran J. Microbiol*. 5, 203-208, 2013.
[10] Dellinger R.P., Levy M.M., Carlet J.M., et al. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Intensive Care Med*. 34, 17-60, 2008.
[11] Miller W.R., Munita J.M., Arias C.A. Mechanisms of antibiotic resistance in enterococci. *Expert Rev. Anti Infect. Ther*. 12, 1221-1236, 2014.
[12] Hollenbeck B.L., Rice L.B. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence.* 3, 421-433, 2012.
[13] Soleimani N., Aganj M., Ali L., Shokoohizadeh L., Sakinc T. Frequency distribution of genes encoding aminoglycoside modifying enzymes in uropathogenic *E. coli* isolated from Iranian hospital. *BMC Res. Notes.* 7, 842, 2014.
[14] Fair R.J., Tor Y. Antibiotics and bacterial resistance in the 21st century. *Perspect. Medicin. Chem*. 6, 25-64, 2014.
[15] Padmasini E., Padmaraj R., Ramesh S.S. High level aminoglycoside resistance and distribution of aminoglycoside resistant genes among clinical isolates of *Enterococcus* species in Chennai, India. *Scientific World Journal.* 2014, 329157, 2014.
[16] Lebreton F., van Schaik W., McGuire A.M., et al. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. *MBio*. 4, 4, e00534-13, 2013.
[17] Ramirez M.S., Tolmasky M.E. Aminoglycoside modifying enzymes. *Drug Resist. Updat*. 13, 1 151-171, 2010.
[18] del Campo R., Tenorio C., Rubio C., et al. Aminoglycoside-modifying enzymes in high-level streptomycin and gentamicin resistant *Enterococcus* spp. in Spain. *Int. J. Antimicrob. Agents*. 15, 221-226, 2000.
[19] Shokravi Z., Mehrad L., Ramazani A. Detecting the frequency of aminoglycoside modifying enzyme encoding genes among clinical isolates of methicillin-resistant *Staphylococcus aureus. Bioimpacts.* 5, 87-91, 2015.
[20] Krzyminska S., Szczuka E., Dudzinska K., Kaznowski A. Virulence and the presence of aminoglycoside resistance genes of *Staphylococcus haemolyticus* strains isolated from clinical specimens. *Antonie Van Leeuwenhoek.* 107, 857-868, 2015.
[21] Li W., Li J., Wei Q., et al. Characterization of aminoglycoside resistance and virulence genes among Enterococcus spp. isolated from a hospital in China. *Int. J. Environ. Res. Public Health.* 12, 3014-3025, 2015.
[22] Ross J.E., Scangarella-Oman N.E., Flamm R.K., Jones R.N. Determination of disk diffusion and MIC quality control guidelines for GSK2140944, a novel bacterial type II topoisomerase inhibitor antimicrobial agent. *J. Clin. Microbiol*. 52, 2629-2632, 2014.
[23] Rahimi F., Talebi M., Saifi M., Pourshafie M.R. Distribution of enterococcal species and detection of vancomycin resistance genes by multiplex PCR in Tehran sewage. *Iran Biomed. J*. 11, 161-167, 2007.
[24] Mohammadi F., Tabaraie B., Davudian E., et al. Evaluation of drug resistance frequency among *Enterococcus faecium* and *Enterococcus faecalis* strains and detection of vanA/B genes in vancomycin resistance isolated by PCR method in Ilam and Kermanshah hospitals. *Iran. J. Med. Microbiol*. 5, 14-18, 2011.
[25] Feizabadi M.M., Maleknejad P., Asgharzadeh A., et al. Prevalence of aminoglycoside-modifying enzymes genes among isolates of *Enterococcus faecalis* and *Enterococcus faecium* in Iran. *Microb. Drug Resist*. 12, 265-268, 2006.
[26] Zarrilli R., Tripodi M.F., Di Popolo A., et al. Molecular epidemiology of high-level aminoglycoside-resistant enterococci isolated from patients in a university hospital in southern Italy. *J. Antimicrob. Chemother*. 56, 827-835, 2005.
[27] Vigani A.G., Oliveira A.M., Bratfich O.J., Stucchi R.S., Moretti M.L. Clinical, epidemiological, and microbiological characteristics of bacteremia caused by high-level gentamicin-resistant *Enterococcus faecalis. Braz J Med Biol Res*. 41, 890-895, 2008.
[28] Sahm D.F., Gilmore M.S. Transferability and genetic relatedness of high-level gentamicin resistance among enterococci. *Antimicrob. Agents Chemother*. 38, 1194-1196, 1994.
[29] Keddy K.H., Klugman K.P., Liebowitz L.D. Incidence of high-level gentamicin resistance in enterococci at Johannesburg Hospital. *S. Afr. Med. J*. 86, 1273-1276, 1996.
[30] Tsakris A., Pournaras S., Maniatis A.N., Douboyas

J., Antoniadis A. Increasing prevalence of high-level gentamicin resistance among Enterococci isolated in Greece. *Chemotherapy.* 47, 86-89, 2001.
[31] Daikos G.L., Bamias G., Kattamis C., et al. Structures, locations, and transfer frequencies of genetic elements conferring high-level gentamicin resistance in *Enterococcus faecalis* isolates in Greece. *Antimicrob. Agents Chemother*. 47, 3950-3953, 2003.
[32] Papaparaskevas J., Vatopoulos A., Tassios P.T., et al. Diversity among high-level aminoglycoside-resistant enterococci. *J. Antimicrob. Chemother*. 45, 277-283, 2000.
[33] Vakulenko S.B., Donabedian S.M., Voskresenskiy A.M., et al. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. *Antimicrob. Agents Chemother*. 47, 1423-1426, 2003.
[34] Karmarkar M.G., Gershom E.S., Mehta P.R. Enterococcal infections with special reference to phenotypic characterization & drug resistance. *Indian J. Med. Res*. 119 Suppl, 22-25, 2004.
[35] Kafil H.S., Asgharzadeh M. Vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* isolated from education hospital of iran. *Maedica (Buchar)*. 9, 323-327, 2014.
[36] Schroder U.C., Beleites C., Assmann C., et al. Detection of vancomycin resistances in enterococci within 3 (1/2) hours. *Sci. Rep*. 5, 8217, 2015.
[37] Murugesan S., Perumal N., Mahalingam S.P., Dilliappan S.K., Krishnan P. Analysis of antibiotic resistance genes and its associated SCCmec types among nasal carriage of methicillin resistant coagulase negative staphylococci from community settings, Chennai, Southern India. *J Clin. Diagn. Res*. 9, DC01-05, 2015.
[38] Samadi N., Pakzad I., Monadi Sefidan A., Hosainzadegan H., Tanomand A. Study of aminoglycoside resistance genes in enterococcus and salmonella strains isolated from ilam and milad hospitals, Iran. *Jundishapur J. Microbiol*. 8, e18102, 2015.
[39] Hidano A., Yamamoto T., Hayama Y., et al. Unraveling antimicrobial resistance genes and phenotype patterns among *Enterococcus faecalis* isolated from retail chicken products in Japan. *PLoS One*. 10, e0121189, 2015.
[40] Yu J., Shi J., Zhao R., et al. Molecular characterization and resistant spectrum of enterococci isolated from a Haematology Unit in China. *J. Clin. Diagn. Res*. 9, DC04-07, 2015.