



High level of gentamicin resistance (HLGR) among enterococcus strains isolated from clinical specimens

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KEYWORDS

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Summary

Background: Enterococci are pathogens that can cause nosocomial infections and acquire resistance properties via several molecular mechanisms. The *aac(6')Ie-aph(2'')Ia* gene plays a significant role in the emergence of high-level gentamicin-resistant (HLGR) strains. The screening of resistant strains and the provision of appropriate antibiotic therapy can decide the outcome of serious nosocomial infections.

Methods: In the present study, 142 enterococci were isolated from patients, and the species were identified using standard methods. An antimicrobial susceptibility test was performed using the disc diffusion method, and the minimum inhibition concentration (MIC) of gentamicin was determined according to the broth micro-dilution method. Additionally, PCR was utilized to detect the *aac(6')Ie-aph(2'')Ia* gene, the presence of which was confirmed by digestion with Sca1 and sequencing.

Results: Of the 142 isolates, 62 (43.7%) were found to exhibit the HLGR phenotype. All except one of the HLGR isolates contained the *aac(6')Ie-aph(2'')Ia* gene. The prevalence of resistance to other antibiotics and multi-drug resistance (MDR) was higher among the HLGR isolates compared to the non-HLGR isolates.

Conclusions: Our results indicate that high prevalence rates of MDR and HLGR enterococci are an important problem associated with medical treatment. Furthermore,

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the presence of the *aac(6')Ie-aph(2'')Ia* gene was shown to correspond to the presence of the HLGR phenotype among enterococci.

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Introduction

Enterococci are Gram-positive microorganisms found in the mammalian gut flora, soil, water, plants, vegetables and foods and are known to be potent pathogens responsible for nosocomial infections [1,2]. This genus contains more than 20 species, of which *Enterococcus faecium* and *Enterococcus faecalis* are isolated at a high frequency [3] from hospital-acquired infections, particularly bacteremia and endocarditis, as well as the genitourinary and gastrointestinal tracts. Hence, the proper treatment of these cases is an important area of focus [2]. Extensive administration and misuse of antimicrobial agents lead to the emergence of *Enterococcus* species with acquired resistance to antibiotics, including high concentrations of aminoglycosides, β -lactams and glycopeptides. These resistance events can include the loss of the synergistic effects of aminoglycoside antibiotics on cell-wall active components. Furthermore, some *Enterococcus* species intrinsically possess genes that encode aminoglycoside-modifying enzymes (AME). These genes can be contained within DNA located on plasmids or chromosomes as well as transposons [4]. In 1979, high-level gentamicin resistance (HLGR) was reported in France for the first time [2]. Until recently, the expression of only one bi-functional AME with both 6'-acetyltransferase and 2''-phosphotransferase activities, encoded by the structural gene *aac(6')Ie-aph(2'')Ia*, had been described as resulting in the emergence of HLGR in enterococci. However, three new corresponding genes, termed *aph(2'')-Ib*, *aph(2'')-Ic* and *aph(2'')-Id*, have also been shown to contribute to gentamicin resistance in enterococci [5]. The spread of resistant species via chromosomal exchange as well as plasmid and transposon transfer from one patient to another can lead to an increase in dangerous nosocomial infections that are difficult to treat [1]. Therefore, the identification of such resistant strains can be helpful in limiting serious nosocomial infections. Methods such as biotyping, antimicrobial susceptibility testing

and molecular techniques are effective for this purpose [6].

In this study, our aim was to identify antibiotic resistant profiles in HLGR and non-HLGR enterococci isolated from clinical samples in an Iranian hospital. Furthermore, we assessed the frequency of the *aac(6')Ie-aph(2'')Ia* gene in the HLGR group.

Materials and methods

Isolation and identification of bacterial strains

To isolate enterococcus strains, samples of urine, blood, wound secretions, body fluids, pulmonary secretions and abscess fluids were collected from patients hospitalized in the Educational Hospital in northern Tehran during a 6 month period (March–September 2009). Pure strains obtained from the growth of each sample on blood-agar plates at 37 °C were examined using conventional methods to identify the genus and species. To determine the genus, Gram staining, catalase testing, growth in the presence of 6.5% NaCl and bile and hydrolysis of esculin and PYR (L-pyrrolidonyl- β -naphthylamidase) were evaluated. In addition, the species type of each isolate was identified using biochemical tests, such as the motility test and arginine hydrolysis. In addition, the sugar fermentation patterns of arabinose, sorbitol, mannitol, sorbose and sucrose were assessed [7].

Antimicrobial susceptibility testing

The antimicrobial susceptibility patterns to seven antibiotics, including ampicillin (10 μ g), gentamicin (120 μ g), vancomycin (30 μ g), chloramphenicol (30 μ g), ciprofloxacin (5 μ g), erythromycin (15 μ g) and tetracycline (30 μ g), were investigated for all isolates using the disc diffusion method (Kirby–Bauer) according to CSLI guidelines (Booth, Mast Mersey Side, UK). In addition, the microdilution method was used to screen for HLGR strains (MIC \geq 500 μ g/ml). Interpretation of the obtained

results and the MIC determination was performed according to CSLI guidelines (8–1024 µg/ml) [8].

DNA extraction and PCR amplification of the resistance gene

The presence of the *aac(6')Ie-aph(2'')Ia* gene in the genome of HLGR isolates was established using PCR. DNA was extracted according to the method described by Belanger et al. [9] and was then amplified in 20 µl reaction mixtures containing 10× reaction buffer, 0.2 mM of the deoxynucleoside triphosphates (dNTPs), 2.5 mM MgCl₂, 10 pmol of each of the primers, 100 ng of genomic DNA as the template and 1.5 U of Taq DNA polymerase (Fermentas). The primers used in this study were designed by Qu et al. [10]. PCR was performed in an Eppendorf thermal cycler with an initial denaturation step of 4 min at 95 °C; 32 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C; and a final extension step of 5 min at 72 °C. Then, the presence of products with a size of 505 bp was assessed by electrophoresis on a 1% agarose gel stained with 0.5 mg/ml ethidium bromide and visualized under UV illumination. *E. faecalis* JH22 was used as a negative control strain. Finally, 3 µg of each PCR product was digested with the endonuclease Sca1 (Roche, Germany) to confirm the presence of this gene. Sca1 is able to cut a position within the amplified *aac(6')Ie-aph(2'')Ia* gene. The sizes of the two created fragments were 242 bp and 263 bp (VEB Cutter2 software). For further corroboration, the DNA was sequenced (Macrogen Research, Seoul, Korea), and the sequenced genes were subjected to BLAST comparison with the M13771 standard strain; the sequence homology was 99% as determined using the MEGA4 software.

Results

Strain isolation

Enterococcal strains were isolated from 142 specimens collected during the study period at frequencies of 63% (90) for *E. faecalis*, 33% (47) for *E. faecium*, 0.7% (1) for *Enterococcus gallinarum*, 1.4% (2) for *Enterococcus casseliflavus* and 1.4% (2) for *Enterococcus sulitarius*. Furthermore, these strains were isolated from urine, wounds, blood, body fluid, pulmonary secretions, abscess fluid and catheter specimens at frequencies of 74% (105), 14% (20), 2.8% (4), 3.5% (5), 3.5% (5), 1.4% (2) and 0.7% (1), respectively.

The HLGR phenotype was detected in 62 (43.7%) patients. The majority of the HLGR strains were *E.*

Table 1 Number and percent of HLGR isolates according to MIC.

Species	Concentration (µg/ml)	
	512	≥1024
<i>E. faecalis</i>	9 (28.1%)	23 (71.9%)
<i>E. faecium</i>	2 (9.5%)	19 (90.5%)
<i>E. sulitarius</i>	0	1 (100%)
<i>E. casseliflavus</i>	1 (100%)	0

faecalis (38) (61.3%) and *E. faecium* (21) (33.9%). The frequency pattern of the HLGR phenotype in these specimens is shown in Table 1. The MIC values for the HLGR isolates were measured in the range from 512 to >1024 µg/ml using the micro-dilution method. MIC levels greater than 500 µg/ml were demonstrated in 55 (89%) of the HLGR isolates.

Antimicrobial susceptibility testing

To evaluate the antibiotic resistance status of each strain, we used an antimicrobial susceptibility test. As illustrated in Fig. 1, the frequencies of resistance to tetracycline and vancomycin were found to be the highest and lowest, respectively. Furthermore, more than 50% of the isolates showed resistance to chloramphenicol and ciprofloxacin. The most frequent type of antibiotic resistance among the *E. faecalis*, *E. faecium* and *E. gallinarum* isolates was against tetracycline (Fig. 1). All of the *E. casseliflavus* isolates were resistant to chloramphenicol and ciprofloxacin. In addition, with the exceptions of erythromycin and vancomycin, the *E. sulitarius* isolates were resistant to all of antibiotics examined in this study.

Of the strains with the HLGR phenotype, more than 70% of the *E. faecalis* and *E. faecium* isolates were resistant to erythromycin, tetracycline and ciprofloxacin (Table 2). Ampicillin resistance was detected in 76% of the *E. faecium* isolates. All of the *E. casseliflavus* and *E. sulitarius* isolates with the HLGR phenotype were resistant to all antibiotics except vancomycin.

In addition, 45.7% of the isolates that displayed resistance to more than three antibiotics were categorized as multi-drug resistant (MDR). The majority of these isolates also belonged to the HLGR category; 31.7% of the HLGR strains and 14% of the non-HLGR strains were MDR.

In the HLGR group, MDR was observed in 57.8% of *E. faecalis* isolates and 70% of *E. faecium* isolates. Table 3 shows the MDR patterns of the HLGR *E. faecalis* and *E. faecium* isolates.

The frequencies of both antibiotic resistance and MDR were higher in HLGR isolates as compared to

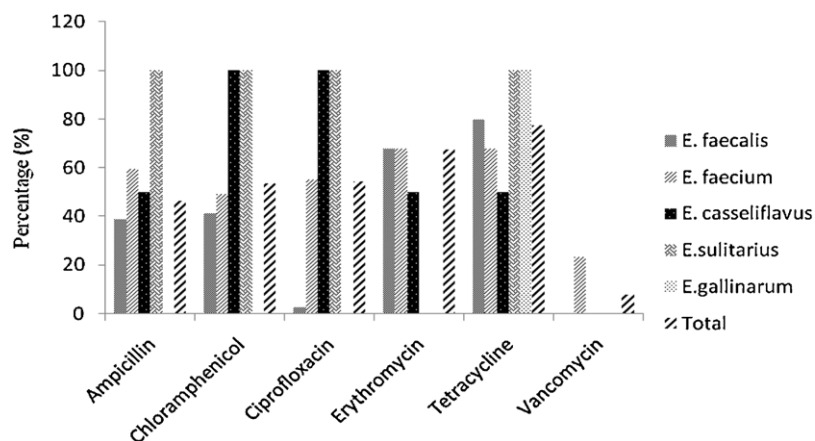


Figure 1 Percentage of enterococcal resistance to several antibiotics. The pattern of antibiotic resistance for each species is illustrated in this figure. The total data indicate the percentage of resistance among all of the isolates.

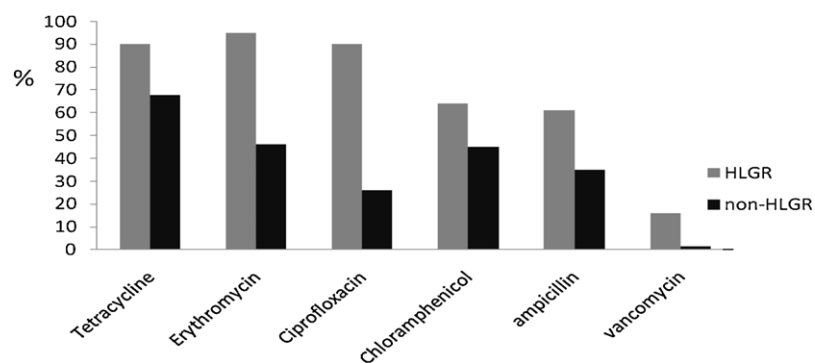


Figure 2 Percentage of enterococcal resistance to several antibiotics among HLGR and non-HLGR strains according to the disc diffusion method.

non-HLGR isolates (Fig. 2) as well among *E. faecium* isolates as compared to *E. faecalis* isolates.

All of the 11 (7.7%) *E. faecium* vancomycin-resistant isolates were also resistant to ampicillin, erythromycin and ciprofloxacin.

PCR and sequencing results

Only 1 of 62 HLGR isolates lacked the *aac(6')-aph(2'')* gene. Figs. 3 and 4 show an example of a PCR product obtained from an isolate containing this gene and the BLAST comparison of

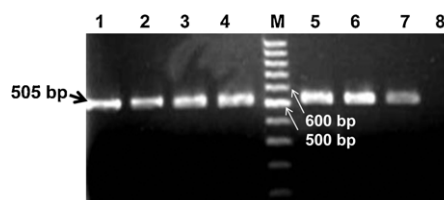


Figure 3 Gel electrophoresis of *aac(6')/le-aph(2'')* gene PCR products from HLGR isolates. The size of the positive band was 505 bp. Lanes numbered 1–7 show the presence of this gene, whereas lane 8 represents a negative control strain. M indicates the DNA ladder.

Antibiotics	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. casseliflavus</i>	<i>E. sulitarius</i>
Ampicillin	19 (50%)	16 (76%)	1 (100%)	2 (100%)
Chloramphenicol	18 (47.4%)	12 (57%)	1 (100%)	2 (100%)
Ciprofloxacin	33 (86.5%)	20 (95.2%)	1 (100%)	2 (100%)
Erythromycin	36 (94.5%)	20 (95.2%)	1 (100%)	2 (100%)
Tetracycline	37 (94%)	15 (71.4%)	1 (100%)	2 (100%)
Vancomycin	0	10 (47.6%)	0	0

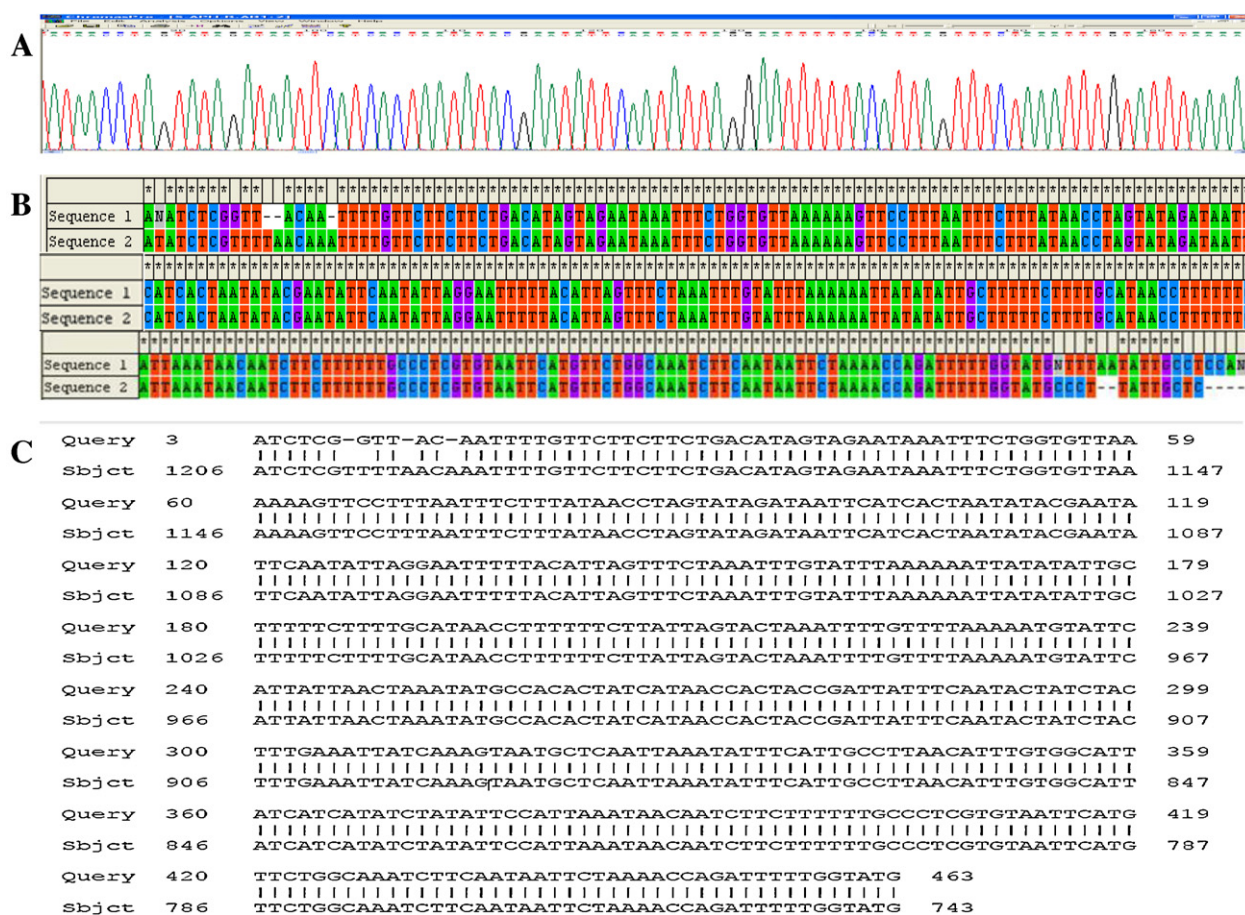


Figure 4 Data from the sequencing and aligning of the PCR products containing the *aac(6')Ie-aph(2'')Ia* gene. Section A shows a part of a chromatograph obtained from the above-mentioned gene sequencing. Sections B and C show the alignment of the PCR product sequence with the M13771 standard strain. Sequence 1 and the query indicate the sequence of our product. In addition, sequence 2 and the subject indicate the result from the M13771 standard strain.

this PCR product to the M13771 standard strain, respectively. Seven isolates with MICs ranging from 64 to <256 µg/ml for gentamicin also carried the *aac(6')Ie-aph(2'')Ia* gene. These data support the

hypothesis that the presence of this gene is associated with moderate-level gentamicin resistance in enterococci.

Discussion

The increasing number of nosocomial infections due to resistant Enterococcus species in healthy Iranians and their often ineffective treatments represent major health problems. The inherent resistance of many pathogens to several antibiotics that are commonly used at hospitals and the ability of the organisms to acquire antibiotic resistance via mutation or conjugation have led to increasing rates of resistance [11,12]. To combat this issue, the collection of sufficient information about the types and frequencies of antimicrobial-resistant enterococci, including an understanding of their glycopeptides and aminoglycosides, is essential.

Table 3 MDR patterns in of *E. faecalis* and *E. faecium* belonged to HLRG group. CIP, TE, E, C and AP indicate ciprofloxacin, tetracycline, erythromycin, chloramphenicol and ampicillin, respectively.

Antibiotics	<i>E. faecalis</i>	<i>E. faecium</i>
CIP, TE, E, C, AP	8 (20.7%)	5 (23.8%)
CIP, TE, E, AP	5 (13%)	6 (28.5%)
CIP, TE, E, C	9 (23.6%)	2 (9.5%)
CIP, E, C, AP	—	4 (19%)
TE, E, C	6 (15.7%)	2 (9.5%)
CIP, TE, AP	—	1 (4.7%)
CIP, C, AP	—	1 (4.7%)
CIP, TE, E	5 (13)	—
TE, E	5 (13)	—

This study assessed the prevalence of Enterococcus species in an Iranian hospital. The frequencies of *E. faecium* and *E. faecalis* isolates in our study were 36% and 63%, respectively. Because of differences in climate and bacterial prevalence, the distributions of Enterococcus species differ between regions. Unlike countries such as India and Japan, where *E. faecium* is dominant, in Iran, as in the USA, UK and some European countries, *E. faecalis* is the most common species [13]. Although *E. faecalis* plays an important role in generating nosocomial infections because of its high binding potency and its proliferation in the intestine, *E. faecium* can more easily acquire antibiotic resistance [14]. Hence, even a low frequency of *E. faecium* can have dangerous consequences. In agreement with previous studies, we isolated a high number of vancomycin-resistant *E. faecium* strains [15]; in fact, all of the vancomycin-resistant strains we isolated were *E. faecium*. This result indicates the importance of this species in distributing the genetic ability to resist vancomycin after colonization in the intestinal tracts of hospitalized patients and spreading resistance across health care centers and the wider community via patients and staff.

We investigated the presence of a gene responsible for HLGR in the current study and observed that 43.7% of the examined isolates were positive for this gene. Data from other studies in Iran conducted by Emaneini et al. (52%) [16] and Feaizabadi et al. (52%) [14] and in Kuwait (47%) [17] produced similar results, although the frequency of HLGR isolates was found to be lower in a study conducted in Turkey (24%) [18]. The frequency of HLGR strains in countries located in eastern Asia is also higher, as frequencies reported from Thailand and China were 56% and 64.2%, respectively [4,10].

Numerous studies have confirmed that the majority of HLGR phenotypes are correlated with the expression of the *aac(6')Ie-aph(2'')Ia* gene and that genes such as *aph(2'')-Ib*, *aph(2'')-Ic* and *aph(2'')-Id* are also involved in HLGR phenotypes [10]. Previous studies in Iran have also reported that a high percentage of HLGR enterococcus strains with a gentamicin MIC >500 µg/ml contain the *aph(2'')-Ib*, *aph(2'')-Ic* and *aph(2'')-Id* genes [19,20]. In our study, only one of the HLGR isolates lacked the *aac(6')Ie-aph(2'')Ia* gene, and in all but 7 isolates, the presence of the gene was accompanied by a gentamicin MIC >500 µg/ml. These results indicate that the *aac(6')Ie-aph(2'')Ia* gene has a major role in creating the HLGR phenotype, both in isolates from our country as well as others [14,16–18].

Irregular administration of antibiotics targeting sensitive strains promotes the emergence of resistant strains, especially MDR strains, with the ability

to colonize the gut lumens of patients, which leads to an increase in the direct and indirect transfer of the genetic material of resistant strains. Recently, significant increases in the prevalence of resistant enterococci with the MDR phenotype have been reported in the USA, Europe and other developed countries. Our results show that the majority of our MDR strains were also HLGR and contained the *aac(6')Ie-aph(2'')Ia* gene.

The high frequency of antibiotic resistance in our HLGR isolates as well as the high percentage of MDR (resistance to more than three antibiotics) can be viewed as a warning to the community because the eradication and treatment of infections resulting from these resistant species is difficult. Therefore, control and prevention methods to limit these infections are essential. Because the *aac(6')Ie-aph(2'')Ia* gene frequently exists in nosocomial infections associated with HLGR enterococci, screening for this gene with molecular techniques as well as high-dose aminoglycoside disc tests in the laboratory may help to efficiently select an appropriate protocol for antibiotic therapy and confine dangerous infections.

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