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Distribution of Gentamicin Resistant Genes of Nosocomial *Enterococcus spp* from Intensive Care Unit of Shahid Beheshty Hospital in Kashan, Iran

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The aim of this study was to determine, the rate and molecular characterization of aminoglycoside resistance genes (*aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(23)-Ic*, and *aph(23)-Id*) among high level gentamicin resistance (HLGR) *enterococcus* isolates in Kashan, Iran. A total of 180 *enterococcus species* were tested for high level gentamicin resistance by using disk diffusion method and minimum inhibitory concentration (MIC>500 ¼g/mL) confirmatory test. High level gentamicin resistance strains were further assessed for aminoglycoside resistance genes. Antibiotic susceptibility pattern revealed that 43 isolates (23.9%) were high level gentamicin resistance (HLGR) (MIC>500¼g/ml), 24 isolates (55.8%) of HLGR isolates were resistant to Chloramphenicol, 13 isolates (30.2%) to Quinupristin-dalfopristin, 7 isolates (16.3%) to Linezolid and 9 isolates (20.9%) of HLGR isolates were multi-drug resistant. The PCR method revealed that 76.7% of high level gentamicin resistance isolates carried *aac(62)Ie-aph(23)Ia* gene; but *aph(23)Ib*, *aph(23)Ic*, and *aph(23)Id* genes were not detected among our isolates. The *aac (6')-Ie-aph (2'')-Ia* was detected in (71.9%) and (28.1%), of *Enterococcus faecalis* and *E. faecium*, respectively. These results point to that high level aminoglycoside resistance genes are extensively disseminated among ICU isolates of *enterococci*.

Keywords: *Enterococcus*, aminoglycoside, Intensive Care Unit.

Enterococcus is one of the most important causes of nosocomial infections among patients in intensive care unit (ICU). Due its importance it was recently ranked as second common agents of bacteremia in ICU[1]. Although at first it was considered as an endogenous colon[2, 3], by acquisition resistance genes with horizontal transformation mechanism and conjugation changed to hazardous pathogen[4]. The *aac (62) Ie-aph (23) Ia* is one of the main genes that carried by high-level gentamicin resistance (HLGR) *enterococcus*[5]. The importance of this gene is elimination of synergistic effects between penicillin and glycopeptide or aminoglycoside antibiotics by encoding a bi functional enzyme[6, 7]. The *aph (23) Ib*, *aph (23) Ic* and *aph (23) Id* are the other genes that are coded by phosphotransferases which cause HLGR strains[6]. The abuse of antibiotics especially in patients with acute diseases at this ward poses enormous nosocomial infection with multi-drug resistant (MDR) bacteria[3]. Multi-drug resistant *enterococcus* can be a serious problem for treating human and causes increased rates of failure treatment[8]. Since then, the high level aminoglycoside resistance has become a serious problem in most of hospitals; so identification of clinical isolates of HLGR *enterococcus* strains is

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essential for an appropriate management for curing the infections. Little is known about the prevalence of aminoglycoside resistance genes in HLGR *enterococcus* strains recovered from rectal swabs of patients in ICU in Iran. So the aim of this study was determination of high level gentamicin resistance and distribution of aminoglycoside resistant genes of *Enterococcus species* from ICU. And also this study evaluates the rate of resistance to linezolid, Quinupristin-dalfopristin and Chloramphenicol among high level gentamicin resistance of *enterococcus species*. The other criterion which is determined is distribution of MDR isolates among high level gentamicin resistance of *enterococcus species*.

MATERIALS AND METHODS

Sample collection

A cross-sectional study was organized between October 1, 2013, and October 15, 2014. One hundred eighty non-repetitive *enterococcus* isolates were recovered from two hundred ten cotton rectal swabs of patients after 48 hours of their hospitalization at ICU (74 isolates of surgical ICU, 59 isolates of Neurosurgical ICU and 47 medical ICU) in Shahid Beheshti Hospital of Kashan, Iran. This is a general teaching hospital with different wards and 516 beds. There was no age and sex restriction for preparing samples. Any complication or underlying disease such as diabetes didn't cause disturbance in this study. The study protocol was approved by the Ethics Committee of Kashan University of Medical Sciences. And also written informed consent was obtain from all study participants or their parents/ guardian.

Bacterial isolates

A cotton rectal swab which was acquired from each patient at ICU immediately transferred to the 6.5% Nacl broth medium (Merk, catalogue number: 105459). It was incubated in 37ÚÙC within 2 hr and cultured on Bile-Esculin agar medium (Merk, catalogue number: 105459). Cultured plates were incubated at 37ÚÙC and were examined after overnight incubation. Phonotypical test for identification of *enterococcus spp* performed based on the conventional microbiological tests. Just one *enterococcus* isolate was analyzed from each patient[9, 10].

Antimicrobial susceptibility test

The disk diffusion method and minimum inhibitory concentration (MIC) test were performed by using Mueller Hinton agar and Brain Heart Infusion agar for detection of HLGR isolates among 180 *enterococcus species* according to the Clinical and Laboratory Standards Institute (CLSI 2014) recommendation[11]. And also susceptibility of HLGR strains was determined to Chloramphenicol (S: e" 18mm, I: 13–17mm, R: d" 12), Quinupristin-dalfopristin (S: e" 19mm, I: 16–18mm, R: d" 15mm), and Linezolid (S: e" 23mm, I: 21–22mm, R: d" 20mm) (MAST, UK). The reference strain *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 was used as a control. Results were clarified as susceptible, intermediate or resistant according to the criteria recommended by the CLSI and the manufacturer protocols (Mast, UK)[11].

DNA extraction

The crude DNA was extracted from 108 *E.faecalis* and 72 *E.faecium* by boiling method for confirming the species and identifies the genes of interest among HLGR isolates. The template DNA stored at "20¨C until polymerase chain reaction (PCR) amplification was performed[12].

Genus identification of enterococcus by PCR

The identified genus of *enterococcus species* were confirmed by distinguishing *ddl* genes with PCR method using specific primers (Table1). Amplification of *ddl* genes were performed under the conditions that were used in similar study[13]. 25 5ØßL Final reaction mixtures was prepared with 10 pmol of each primer, 200mM of dNTP, 1 unit of Taq polymerase, 2.5 5ØßL of 10x reaction buffer, 1.5mM MgCl2 in final concentration, and100 ng DNA template. Amplification reactions were carried out in a thermocycler (Eppendorf master cycler, MA) under the following conditions: initial denaturation at 94ÚÙC for 3 min, followed by 30 cycles of amplification at 94! for 1min, 54ÚÙC for 1 min and 72ÚÙC for 1 min with final extension at 72! for 7 min[13]. The amplified products were electrophoresed on 2% agarose gels. The gels were stained in ethidium bromide (0.5mg/mL) visualized in gel document system (Biorad, UK).

Characterization of aminoglycoside resistance genes among HLGR strains

Aminoglycoside modifying enzymes (AMEs) which includes *aac(62)Ie-aph(23)Ia*,

aph(23)Ib, *aph(23)Ic* and *aph(23)Id* genes identified by polymerase chain reaction. Amplification reactions were carried out in a thermocycler (Eppendorf master cycler, MA) under conditions that were experiment in similar studies[14, 15, 16]. Amplification for *aac (62) Ie-aph (23) Ia* gene was performed under underneath conditions: denaturation at 94ÚÙC for 3 min, followed by 32 cycles, annealing at 60ÚÙC for 45 sec, extension at 72! for 1 min and final extension at 72! for 2 min [14]. PCR conditions for *aph (23) Ib* and *aph (23) Id* were as follows: denaturation at 94ÚÙC for 1 min, followed by 30 cycles, annealing at 55ÚÙC for 1 min and extension at 72! for 2 min [15]. And also the Amplification conditions that used for *aph (23) Ic* gene were as follows: denaturation at 94ÚÙC for 40 sec, followed by 30 cycles, annealing at 56ÚÙC for 30 sec, extension at 72! for 50 sec[16]. A total volume of 50µl containing 100 ng genomic DNA from *enterococcus species* culture, 200 mM each of dNTP, 1 × PCR buffer (20mM Tris-HCl, pH 8.4), 50mM KCl, 1.5mM MgCl2, 0.5 mM of each primer (Table 1) and 1.5U of Taq polymerase used for performing PCR. 10µl of Amplified samples were electrophoresed on 2% agarose gel in TBE buffer. The gel was stained with ethidium bromide 0.5 mg/ml. The amplified bands were observed under gel document system (Biorad, UK). As negative control reaction mixture without a DNA template was used. The positive amplicons were sequenced to confirm the result of PCR.

DNA Sequencing and Sequence Analysis

Sequencing performed for positive favorable gene (*aac (62) Ie-aph (23) Ia*) using the ABI Capillary System (Macrogen Research, Seoul, Republic of Korea). The sequence was analyzed using Chromas Pro version 1.7.5 Technelysium (http://technelysium.com.au/) and performed online by using the BLAST program of the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/).

Statistical analysis

The statistical analysis of data was conducted using SPSS software version 15 (SPSS, Inc.). The Chi-square test or the Fisher's exact test was used to compare proportions. *P-Values* < 0.05 were considered statistically significant. Prevalence data is presented with 95% confidence intervals (CI).

RESULTS

180 isolates of *enterococcus species* obtained from 210 hospitalized patients in ICU. These isolates were collected from patients who had been hospitalized for two days or more in ICU of Shahid Beheshti Hospital of Kashan. The prevalence rate of *enterococcus* was 85.7% (108 *E. faecalis* and 72 *E.faecium*). The majority of patients who carried *enterococcus* isolates were males (67%). More than fifty seven percent of these patients who carried *enterococcus species* were 57e" years.

The prevalence of HLGR *enterococcus* was 23.9% (43/180) (MIC >500 ¼g/mL). Of these HLGR *enterococcus* isolates, 26 isolates (60.5%) were *E.faecalis* (Table 2). The majority of patients with HLGR strains were males (74.4%). More than

Table 1. Primers used for polymerase chain reaction and sequencing

Gene	Primer Sequences (5'-3')	PCR product (bp)	References
ddl (*E. faecalis*)	CACCTGAAGAAACAGGC ATGGCTACTTCAATTTCACG	475	[13]
ddl (*E. faecium*)	GAGTAAATCACTGAACGA CGCTGATGGTATCGATTCAT	1091	[13]
aac(62)-Ie-aph(23)a	CCAAGAGCAATAAGGGCATA CACTATCATAACCACTACCG	220	[14]
aph(23)Ib	ACTCCGTTATTTATCGTCCG TCATCATATGCAAGGGCATC	279	[15]
aph(23)-Ic	GAGGGCTTTAGGAATTACGC ACACAACCGACCAACAGAGG	125	[16]
aph(23)-Id	GGTGGTTTTTACAGGAATGCCATC CCCTCTTCATACCAATCCATATAACC	642	[15]

sixty five percent of patients with HLGR strains were 60e" years. The mean duration of hospitalization was 12.56 ±13.927 day. In this study 20 isolates of HLGR strains (46.5%) were collected from surgery ICU (70% *E.faecalis* and 30% *E.faecium*), 5 isolates (11.6%) from neurosurgery ICU (60% *E.faecalis* and 40% *E.faecium*) and 18 isolates (41.9%) from medical ICU (72.2% *E.faecalis* and 27.8% *E.faecium*).

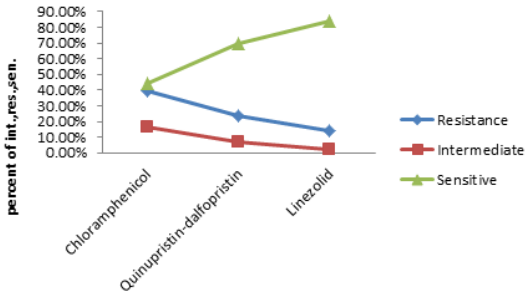

Fig. 1. Antimicrobial resistance of high level gentamicin resistance (HLGR) *enterococcus* isolates was measured by disk diffusion method

Antibiotic susceptibility pattern revealed that among 43 HLGR isolates, 55.8% (16 *E.faecalis* and 8 *E.faecium*) were resistant to Chloramphenicol, 30.2% (11 *E.faecalis* and 2 *E.faecium*) to Quinupristin-dalfopristin and 16.3% (6 *E.faecalis* and 1 *E.faecium*) to Linezolid (figure 1).

Among HLGR isolates of *enterococcus*, 20.9% (9/43) indicated multidrug-resistant (MDR) pattern. This pattern demonstrated resistant to at least one agent in three or more antimicrobial categories. Of isolates that were supposed HLGR bacteria by disk diffusion method and MIC test, 76.7% (33/43) comprising *aac (62) Ie-aph (23) Ia*. PCR assays and sequencing revealed that 60.6% (n=20) of *E.faecalis* and 39.4% (n=13) of *E.faecium* carried this gene. None of the *aph (23) Ib*, *aph (23) Ic*, and *aph (23) Id* genes were found at HLGR isolates. The nucleotide sequence of the PCR products of *ddl* genes and aminoglycoside resistant gene were identical to *ddl* (*E.faecalis*), *ddl* (*E.faecium)* and *aac (62) Ie-aph (23) Ia* in the GenBank nucleotide database (http://

Table 2. The prevalence of 43 HLGR isolates of *enterococcus species* that confirmed with minimum inhibitory concentration (MIC) method in different ICU.

Species	Surgery ICUNO. (%)	Neurosurgery ICUNO. (%)	Medical ICUNO. (%)	MIC>500µg/mL NO. (%)
E. faecalis	14(70)	3(60)	13(72.2)	26(60.5)
E. faecium	6(30)	2(40)	5(27.8)	17(39.5)
Total	20(100)	5(100)	18(100)	43(100)

Table 3. Association between patient characterizations and the rate of high level gentamicin resistance *enterococcus species* that may carry aminoglycoside resistance genes.

Risk factor	HLGR positive No. (%)	HLGR negativeNo. (%)	P- Value	Odds ratio (95% CI)
Diabetes				
Yes (28)	11 (39.3%)	17 (60.7%)	0.038	2.426
No (152)	32 (21.1%)	120 (78.9%)		(1.03-5.69)
Using ciprofloxacin				
Yes (56)	23 (41.1%)	33 (58.9%)	0.001	3.624
No (124)	20 (16.1%)	104 (83.9%)		(1.771-7.415)
Using meropenem				
Yes (70)	24 (34.3%)	46 (65.7%)	0.009	2.499
No (110)	19 (17.3%)	91 (82.7%)		(1.243-5.025)
Using amikacin				
Yes (9)	6 (66.7%)	3 (33.3%)	0.002	7.243
No (171)	37 (21.6%)	134 (78.4%)		(1.728-30.355)

www.ncbi.nlm.nih.gov/blast/) and accession numbers obtained for them in current study are KP793143, KP793142, and KP793141. The statistical analysis confirmed proved that diabetes and using some antibiotics by patients such as ciprofloxacin, meropenem, and amikacin were clinical factors that significantly associated with the presence of HLGR *enterococcus species* that would result isolates that include aminoglycoside resistance genes (Table 3).

DISCUSSION

Gentamicin is one of the most commonly used aminoglycosides against *enterococcus*, since its discovery in 1963. High-level gentamicin resistance (HLGR) has been widely investigated and different frequencies have been reported depending on various regions due to the variety in climate of hetero geographical regions and origin of isolates[17]. So, the high prevalence of resistance to high level gentamicin is forecast. In this study the rate of *E.faecalis* and *E.faecium* were 60% and 40%, respectively, indicates high rates of *E.faecalis* which was identical to the other results of study conducted in Tehran, 64.4% *E.faecalis* and 35.6% *E.faecium*[18]. *E.faecalis* was dominant in this study and similar to other studies from Iran, USA and UK and some of European countries and in contrast *E.faecium* is more prevalent in some countries such India and Japan[17]. Our result showed high frequency of high-level gentamicin resistance (23.9%) in ICU. Versus our result a study in turkey showed a low frequency of HLGR, approximately 9.9% among fecal samples of patients[19]. It could be due to inappropriate use of gentamicin among patients in Kashan. A higher frequency was mentioned in northwestern of Iran (60.4%), Kuwait (47%), China (64.15%) and Thailand (55.6%)[20, 21, 6, 16]. So according to the different studies in most part of the world high level gentamicin resistance in *enterococcus species* is increasing, however low frequency was noted in some countries such India (2%) and Saudi Arabia (20.9%)[22, 23]. At this study *E.faecalis* (60.5%) was the most prevalent species among HLGR isolates, as well as the prevalence of *E.faecalis* among non HLGR *enterococcus species*. In northwestern of Iran the frequencies of HLGR between *E.faecalis* and *E.faecium* were 59.4% and 40.6% and in northern Tehran was 61.3% and 33.9%, respectively, which is in agreement with our findings[20, 17]. And also in Sweden the prevalence of HLGR isolates between *E.faecalis* and *E.faecium* were 20% and 0%[24]. It sounds low administration of extended spectrum antibiotic in this country reduced the rate of HLGR isolates in this country. Although the prevalence of HLGR isolates in *E.faecalis* is more than *E.faecium* in our findings and some countries, these results are in contrast to studies conducted in china and turkey that shown 51.5% and 88% HLGR in *E.faecium*[6, 25]. To date, *aac (62) Ie-aph (23) Ia* gene is the most prevalent aminoglycoside-modifying enzymes genes .The present study demonstrated high prevalence (76.7%) of *aac (62) Ie-aph (23) Ia* gene among 43 (23.9%) HLGR isolates. This result is alarming due to the ability of *enterococcus species* for being reservoir and transporting antibiotic resistance genes among different bacteria. Although our result is more prevalent than Chile (14.8%), but a higher prevalence was shown in Iran (100%) and china (86.8%)[26, 27, 6]. In this study high frequency of this gene is more prevalent among *E.faecalis* 60.6% than *E.faecium* 39.4%. In addition, a study in Japan showed the frequency of this gene increased to 28% in *E.faecium*[28]. The present study was shown a low frequency of this gene in gentamicin susceptible isolates that may be due to the presence of non-functional gene at these isolates[16]. The result of this study revealed that none of the isolates possess *aph(23)Ib*, *aph(23)Ic* and *aph(23)Id* genes which are comparable to the results of the studies in northwestern of Iran, China, Thailand, Kuwait and India[20,6,16,21,29]. The high rate of *aac (62) Ie-aph (23) Ia* among *enterococcus species* isolates in our region may be associated to clonal spread of a single clone, although further studies using molecular typing methods such as pulsed field gel electrophoresis (PFGE) are needed for approval this statement. Whereas a low frequency of *aph(23)Ib* gene in Cuba 5%, America 5% in clinical specimens and 3.4% in *enterococcus* blood strains in Cuba demonstrated[30, 31]. And also there were evidences in the presence of *aph (23) Ic* gene in Cuba 1.1% in *enterococcus* blood strains and 1.6% on clinical specimens and in America 2.5% in human specimens[30, 32]. In disagreement of our findings a low frequency of *aph (23) Id* gene in America 2.5% was demonstrated[32]. However, the frequency

about 20.9% of multi-drug resistant (MDR) in our findings among 9 HLGR isolates (8 *E.faecalis* and 1 *E.faecium*) is comparably lower than the rate of MDR isolate in northern Tehran which was about 31.7%[17]. One of the reasons on this disagreement may due to the origin of the specimens. In our findings 5 *E.faecalis* MDR isolates are 100% resistant to Chloramphenicol, Linezolid, Quinupristin-dalfopristin and gentamicin. Two MDR isolates (1 *E.faecalis* and 1 *E.faecium*) are resistant to Linezolid, Chloramphenicol and gentamicin and also two *E.faecalis* that are MDR, are resistant to Quinupristin-dalfopristin, Chloramphenicol and gentamicin. There is warning because infection caused by such resistant isolates can be difficult to treat. The rate of Chloramphenicol resistant among HLGR isolates 55.8% in our findings (66.7% *E.faecalis* and 33.3% *E.faecium*) is considerably more than the rate of Chloramphenicol resistant among multi-drug resistant *enterococcus* (26.3% *E.faecalis* and 4.8% *E.faecium*) in china[7]. It reveals that *E.faecalis* isolates are more resistant to this antibiotic which is in agreement to the result of study in china[7]. Although in China none of the isolates were resistant to linezolid but in our findings 16.3% of isolates were resistant to the linezolid[6]. An important step in controlling the dissemination of this microorganism is to identify the risk factors that associate with it. Finally, in term of relation between patients characterizations and acquisition HLGR *enterococcus*, we found that using extended-spectrum antibiotics such ciprofloxacin, meropenem, amikacin, and chronic disease such as diabetes in hospitalized patients in ICU tend them to acquisition HLGR *enterococcus* ($p<0.05$) (according table 3). In contrast, in study conducted in turkey with these risk factors no significant differences observed[33].

CONCLUSION

All of these data indicates that *aph (2") Ib*, *aph (2") Ic* and *aph (2")Id* genes don't play an important role in producing HLGR isolates in this region, but *aac(6')Ie-aph(2")Ia* gene which is more prevalent is a main gene that produces HLGR isolates by enzymatic mechanisms.

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