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ORIGINAL PAPER

Emergence of High-level Gentamicin Resistance among *Enterococci* Clinical Isolates from Burn Patients in South-west of Iran: Vancomycin Still Working

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Abstract

Enterococcus faecalis and *Enterococcus faecium* are among the main agents associated with nosocomial infections with high mortality in immunocompromised patients. Antibiotic resistance, especially against gentamicin and vancomycin among *Enterococci*, is a risk factor that could increase the morbidity and mortality rate. 179 *Enterococci* isolates from burn patients were included in this study. Antibiotic susceptibility testing was done using the disk diffusion test and minimum inhibitory concentration (MIC) was evaluated by agar microdilution. Vancomycin and gentamicin resistance associated genes including *vanA*, *vanB*, *vanC*, *aac (6')-Ie aph(2")*, *aph(3')-IIIa* and *ant(4')-Ia* were detected by PCR and their statistical relation with antibiotic resistance was evaluated. *E. faecalis* was the more prevalent strain among our local isolates and showed a higher antibiotic resistance in comparison to *E. faecium*. Vancomycin had a good antibacterial effect on the *Enterococcus* spp. isolates; however, resistance to this antibiotic and a high-level gentamicin resistance (HLGR) phenotype were observed. Among *van* operon genes, *vanA* was the most prevalent gene and among the gentamicin resistance genes, *aph (3')-IIIa* was more frequent. The HLGR *Enterococci* are a real challenge in nosocomial infections. Vancomycin is a key antibiotic to treat such infections but emergence of VRE in our region could be a real concern and, therefore, phenotypic and molecular surveillance must be considered.

Key words: *Enterococci*, gentamicin, burn, vancomycin, drug resistance

Introduction

Burn injury is a common cause of morbidity and mortality. In Iran, approximately 30 000 people with burns present to the emergency departments each year. Among these, 3 000 dies and others either have minor burn injuries that are treated primarily in the emergency department or sustain major burn injuries that require hospital admission. Both impose the health systems a burden of cost. These numbers are eight times larger than the world average and therefore are a source of concern (Karimi et al. 2015).

In most cases, the bacterial infection of burn wounds is an unquestionable phenomenon because of the skin destruction, which plays a role of the major barrier to bacterial access to the internal tissues. Gram-positive bacteria including *Staphylococcus aureus*, β-hemolytic *Streptococci* and *Enterococci*, and Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacteriaceae* are among the most frequent etiological agents of burn patients infections (Norbury et al. 2016).

The facultative anaerobic Gram-positive *Enterococcus* spp. normally colonize the gastrointestinal tract, oral cavity, and vaginal tract. *Enterococci* are among the major agents associated with nosocomial infections particularly in burn patients presenting with bacteremia, urinary tract infections and endocarditis (Hashem et al. 2017). The US National Nosocomial Infection Surveillance (NNIS) system, has ranked *Enterococci* among

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the top three most common pathogens of nosocomial infections and the leading cause of nosocomial infections in burn patients (Pan 2012).

Enterococci are intrinsically resistant to multiple antibiotic agents: cephalosporins, penicillinase-resistant penicillins, and low concentrations of aminoglycosides (Hollenbeck and Rice 2012). Vancomycin remains the drug of choice to treat enterococcal infections but nowadays vancomycin-resistant *Enterococci* (VRE) isolates are a global challenge. Such infections are treated by a combination of cell wall-active agents with aminoglycosides that achieve synergistic bactericidal activity. Aminoglycoside antibiotics are positively charged, carbohydrate-containing molecules that find a clinical use for the treatment of infections caused by both Gram-negative and Gram-positive bacteria (Miller et al. 2014). Two antibiotic resistance phenotypes among *Enterococci* including VRE and HLGR have emerged as important nosocomial causes throughout the world as well as in Iran (Sakoulas et al. 2013a; Emaneini et al. 2016; Osuka et al. 2016). Operon associated genes (*van* operon) are responsible for VRE emergence and are the best studied antibiotic resistance operon (Faron et al. 2016).

In contrast to the rest of aminoglycosides, gentamicin is not inactivated by target insensitivity and enzymatic modification and, therefore, remained for many years the aminoglycoside often used to achieve synergistic killing of *Enterococci*. However, there are some variants of aminoglycosides modifying enzymes (AMEs) that can affect gentamicin (Garneau-Tsodikova and Labby 2016).

There are limited studies regarding the simultaneous resistance to vancomycin and gentamicin in hospitalized burn patients in Iran and thereby, we aimed to follow up and monitor the antibiotic resistance pattern and the genes encoding resistance to these two antibiotics among *Enterococcus* spp. strains isolated from burn patients.

Experimental

Materials and Methods

Bacterial isolates. One hundred seventy-nine out of 628 bacterial isolates confirmed as *Enterococcus* spp. were isolated from different clinical specimens (wound biopsies and blood) of burned patients referred to Taleghani burn hospital (the only referral burn center in Ahvaz, Khuzestan province, Iran) during January 2015 to 2016. All isolates were primary identified by conventional microbiological methods and confirmed by specific tests such as bile esculin hydrolysis and PYR (Pyrrolidinyl Aminopeptidase) Test. Sugar fermentation (arabinose and sorbitol) were used for characterization of *Enterococcus* species (Emaneini et al. 2016). Molecular confirmation of the species was done by screening for the ddl_E (D-alanine-D-alanine ligase) gene (Dutka-Malen et al. 1995).

Antimicrobial susceptibility testing. Susceptibility to antimicrobial agents was determined by disk diffusion method according to CLSI criteria using commercially available disks (Mast, UK) including vancomycin, teicoplanin, gentamicin, chloramphenicol, linezolid, ciprofloxacin, and amoxicillin. The Minimum Inhibitory Concentration (MIC) against gentamicin and vancomycin were determined using two-fold serial agar dilution method with Mueller Hinton agar (Difco, USA) according to CLSI guidelines (CLSI 2015). *E. faecalis* ATCC 29212 and *E. faecium* IP 4107 (The Collection of Institut Pasteur, France) were used as quality control reference strains.

DNA extraction and PCR. DNA was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea). The oligonucleotide primers used in this study are listed in Table I. For each sample, the PCR assay was performed to identify *vanA, vanB, vanC* genes for vancomycin resistance and *aac(6')-Ie aph(2")*, *aph(3')-IIIa* and *ant(4')-Ia* genes for gentamicin

Table I
Primers used in this study.

Target gene	Oligonucleotide sequences (5'-3')	Size of product (bp)	Reference
van A	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	Dutka-Malen et al. 1995
vanB	ATG GGA AGC CGA TAG TC GAT TTC GTT CCT CGA CC	638	Dutka-Malen et al. 1995
vanC	AAT CGT CAA TTC CTG CAT GT TAA TCG TGG AAT ACG GGT TTG	299	Dutka-Malen et al. 1995
aac(6')-Ie aph(2")	AGGAATTTATCGAAAATGGTAGAAAAG CACAATCGACTAAAGAGTACCAATC	369	Vakulenko et al. 2003
aph(3')-IIIa	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	523	Vakulenko et al. 2003
ant(4')-Ia	CAAACTGCTAAATCGGTAGAAGCC GGAAAGTTGACCAGACATTACGAACT	294	Vakulenko et al. 2003

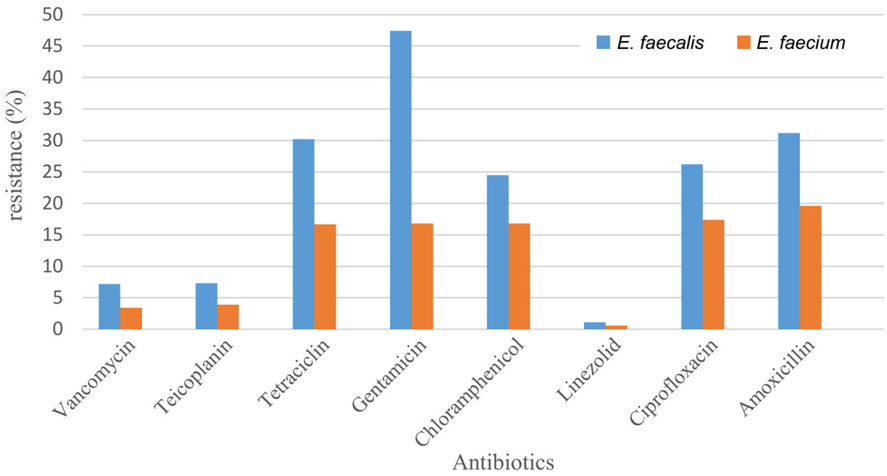

Fig. 1. Antibiotic resistance pattern of *E. faecalis* and *E. faecium* isolated in this study against eight antibacterial agents.

resistance. DNA amplification was carried out in a peqSTAR thermal cycler system using a defined protocol as described previously (Dutka-Malen et al. 1995; Vakulenko et al. 2003).

Statistical analysis. We used absolute and relative frequency to present descriptive statistics. Chi-Square test and also Fisher exact test (if it was necessary) used to explain analytical statistics. Data were analyzed by SPSS version 22.

Results

The patients referred to the hospital had different degrees of burn. From 628 bacterial isolates, isolated from patient referred to our hospital, 28.5% (179 isolates) were confirmed as *Enterococci* and among them 108 (60.3%) were *Enterococcus faecalis* and 71 (39.7%) were *Enterococcus faecium*. These species were isolated from blood (29.6%) and wound (70.4%) of burn patients. The drug resistance pattern of the isolates against eight antibiotics including chloramphenicol, linezolid, ciprofloxacin, amoxicillin, teicoplanin, vancomycin, and gentamicin is shown in Fig. 1. Overall, drug resistance of *E. faecalis* was significantly higher than that of *E. faecium* ($p < 0.03$). Among the aminoglycosides, the highest resistance rate was against gentamicin, and was observed in 45% of the *E. faecalis* and 15% of the *E. faecium* isolates, respectively. The prevalence of resistance to amoxicillin and tetracycline came in the second and third positions. However, a good sensitivity was observed toward vancomycin.

The frequency of vancomycin resistance associated genes: *vanA*, *vanB*, *vanC* and gentamicin resistance associated genes: *aph*(*3'*)-*IIIa*, *ant*(*4'*)-*Ia* and *aac*(*6'*)-*Ie aph*(*2'*) in the two *Enterococcus* species is reported in Table II.

The *vanA* gene was the most frequent vancomycin associated gene and it was detected in 23 positive cases (12.8%). As shown in Table II, the *vanA* and *vanB* genes were detected simultaneously in 6 isolates. The vancomycin MICs among VRE isolates ranged between 64 mg/l and 1024 mg/l. There was not a significant correlation between the presence of *van* operon genes and the vancomycin MICs.

Among the gentamicin resistance genes, the highest frequency was observed for *aph*(*3'*)-*IIIa* in 68% (n = 122) and *aac*(*6'*)-*Ie aph*(*2'*) in 61% (n = 109) of the cases.

The prevalence of *aph*(*3'*)-*IIIa, ant*(*4'*)-*Ia* and *aac*(*6'*)-*Ie aph*(*2'*) in HLGR isolates were 10.8%, 65.5% and 60.1% respectively.

The simultaneous presence of at least two gentamicin resistance associated genes also were observed

Table II
Frequency of vancomycin and gentamicin resistance genes among our local *Enterococci* isolates.

Genus	*van* gene				Gentamicin gene						
	vanA	*vanB*	*vanA, vanB*	*vanC*	*3'*	*4'*	*6'*	*3'+4'*	*3'+6'*	*4'+6'*	*3'+4'+6'*
E. faecalis	14	3	3	1	74	8	67	3	26	6	4
E. faecium	9	0	3	2	48	10	42	6	50	2	1
Total (*n*)	23	3	6	3	122	18	109	9	76	8	5

Abbreviations are as follows: *3: aph* (*3'*)-*III;, 4': ant* (*4'*)-*I;, 6': aac* (*6'*)-*Ie aph* (*2"*)

Table III
The association between presence of gentamicin resistance genes and MICs against gentamicin.

Gene target		MIC (μg/ml)					
		256		512		1024	
		E. faecalis	*E. faecium*	*E. faecalis*	*E. faecium*	*E. faecalis*	*E. faecium*
Gentamicin resistance gene	*aph(3')-IIIa*	0	0	3	1	2	3
	ant(4')-Ia	0	0	3	1	0	0
	aac(6')-Ie aph(2")	0	0	5	2	2	2

and the most frequently, the simultaneous presence of *aph*(*3'*)-*IIIa* and *aac*(*6'*)-*Ie aph*(*2'*) genes wad detected on 109 cases (60.8%). There was no significant correlation between aminoglycoside resistance emergence and the prevalence of these genes.

The relation between the aminoglycoside resistance genes and the MIC of aminoglycosides antibiotics is reported in Table III. Of 122 isolates that were harboring the *aph*(*3'*)-*IIIa* gene, 7.3% (n = 9) had the gentamicin MIC of ≥ 512 μg/ml. The gentamicin MICs for isolates harboring *ant*(*4'*)-*Ia* and *aac*(*6'*)-*Ie aph* (*2'*) were 3 μg/ml and 11 μg/ml respectively.

The HLGR phenotype was reported in 82.7% of the isolates in this study (57.4% of *E. faecalis* and 42.6% of *E. faecium* isolates, respectively). There was no significant correlation between HLGR rate and the species of *Enterococci* ($p = 0.2$).

Discussion

We studied 179 *Enterococci* isolates from patients, which were referred to a burn center in Ahvaz, southwest of Iran and this number is higher than other similar studies that could be a point for our study. The morbidity and mortality associated with nosocomial infections due to antimicrobial resistant *Enterococci* demonstrated a crude mortality rate of 17–100% in case of enterococcal bacteremia in different hospitals around the world (Edmond et al. 1996).

The clinical importance of *Enterococci* is directly related to their antibiotic resistance, which contributes to the risk of colonization and infection. The species of the greatest clinical importance are *E. faecalis* and *E. faecium* (Kajihara et al. 2015). In our study, *E. faecalis* was more prevalent, which is in concordance with other reports (Olawale et al. 2011; Komiyama et al. 2016).

The increase of antimicrobial resistance among *Enterococcus* spp. is a serious health problem globally and there are several reports of antimicrobial resistance among *Enterococci* isolated from hospitalized patients in Iran and other countries (Emaneini et al. 2016; Lan et al. 2016). In this study, antibiotic resistance rate in *E. faecalis* isolates was significantly higher than *E. faecium*. However, it has been reported that notwithstanding of its lower frequency, *E. faecium* has a more ability to develop antibiotic resistance (Werner et al. 2008).

Although there are an increasing number of reports on VRE emergence in other countries, in the present study most of the isolates were susceptible to vancomycin. This could be due to lower usage of vancomycin in the first place because treatment is done with other antibacterial agents. Moreover, the VRE phenotype is more often associated with *E. faecium* (Werner et al. 2008; Arias et al. 2010), which was not prevalent in our study. The *vanA* and *vanB* genes are the most frequently vancomycin resistance associated genes among *Enterococcus* spp. *Enterococci* which harbor the *vanA* gene, are resistant to vancomycin (MIC ≥ 64 μg/ml) and teicoplanin (MIC ≥ 8 μg/ml) at a high concentration. Resistance is induced by the presence of these drugs (Eliopoulos and Gold 2001). In our study the *vanA* gene was more prevalent but totally the vancomycin resistance was observed only in 10.6% of the isolates, which accordingly to the ability of the resistance induction in the presence of the antibiotic, could be associated with lack of exposure to vancomycin and teicoplanin, as it was mentioned previously. The *vanB* harboring *Enterococci* are resistant to a range of vancomycin concentrations: from 4 to over 1024 μg/ml. Such strains remain susceptible to teicoplanin. The *vanC* gene was reported in *E. gallinarum* and *E. casseliflavus*, which are intrinsically resistant to vancomycin at concentrations typically lower than or equal to 32 μg/ml (Eliopoulos and Gold 2001). In this study, we observed the *vanC* gene in three isolates and this gene could has been transmitted from these organisms to the clinically important *E. faecalis* and *E. faecium* spp.

In addition to the costs imposed to health systems, the importance of the VRE emergence is that these strains could serve as a *van* genes reservoir for other organisms, especially *Staphylococcus aureus*. This could be a real problem because vancomycin is the therapeutic agent of choice for methicillin-resistant *S. aureus* (Gardete and Tomasz 2014).

For the first time, the HLGR phenotype was reported in *E. faecalis* in 1979 in France, followed by some U.S. healthcare institutions, in which 25% of *E. faecium* iso-

lates displayed the HLGR phenotype and a decade later, more than 60% of *E. faecium* isolates demonstrated this phenotype (Kobayashi et al. 2003).

Nine genes that encode enzymes targeting eight different aminoglycosides have been identified. In most cases, the *aac(6')-Ie aph (2')* gene has been found to be associated with resistance to aminoglycosides (Ramirez and Tolmasky 2010).

Like in this study, the HLGR was reported high in other similar studies from Iran, (Emaneini et al. 2016; Heidari et al. 2017). The studies conducted in other countries have also been reported a high rate of the HLGR phenotype. Almost in all these studies, high rate of HLGR was more prevalent in *E. faecalis* which could be related to its higher prevalence in the clinic (Wendelbo et al. 2003; Adhikari, 2010). Thus, the prevalence of *aph*(*3'*)*-IIIa, ant*(*4'*)*-Ia* and *aac*(*6'*)*-Ie aph*(*2'*) was higher in *E. faecalis* isolates in comparison to *E. faecium* isolates.

We could not find any report that study the relation between the presence of aminoglycosides resistance genes and the HGLR rate. The prevalence of the HLGR phenotype and the aminoglycosides resistance genes varied in the studies from all over the world (Udo et al. 2004). Nowadays, there are several genes conferring aminoglycosides resistance among *Enterococci* including the *aph*(*2*")*-Ic, aph*(*2*")*-Id, aph*(*2*")*-Ib genes*, and the *aac*(*6'*)*-Ie-aph*(*2'*)*-Ia* gene is no longer the only gentamicin resistance gene (Chow et al. 1997; Tsai et al. 1998; Kao et al. 2000). Almost all these genes are located on transposable elements, which contribute to their easy dissemination and this poses a challenge for health systems. Treatment of HLGR *Enterococci* is a real problem. Recently, few reports suggested using an anti-peptidoglycan active agent in combination with a membrane active agent. Accordingly, a successful treatment of endocarditis caused by *E. faecalis* of HLGR phenotype was demonstrated after administration of daptomycin plus ceftaroline (Sakoulas et al. 2013b). Although the HLGR *Enterococci* are predominant in our region, vancomycin keeps its antimicrobial effect on such strains. However, we reported VRE isolates and this could be a "red alarm" to our health system, and thus a continuous surveillance using both genetically and phenotypically methods should be done for this type of resistance.

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Conflict of interest

The authors have no financial conflicts of interest.

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