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ORIGINAL PAPER

Characterization of Glycopeptides, Aminoglycosides and Macrolide Resistance among *Enterococcus faecalis* and *Enterococcus faecium* Isolates from Hospitals in Tehran

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Abstract

The prevalence of glycopeptides, aminoglycosides and erythromycin resistance among *Enterococcus faecalis* and *Enterococcus faecium* was investigated. The susceptibility of 326 enterococcal hospital isolates to amikacin, kanamycin, netilmicin and tobramycin were determined using disk diffusion method. The minimum inhibitory concentration (MIC) of vancomycin, teicoplanin, gentamicin, streptomycin, and erythromycin were determined by microbroth dilution method. The genes encoding aminoglycoside modifying enzymes described as AMEs genes, erythromycin-resistant methylase (*erm*) and vancomycin-resistant were targeted by multiplex-PCR reaction. High level resistance (HLR) to gentamicin and streptomycin among enterococci isolates were 52% and 72% respectively. The most prevalent of AMEs genes were *aac (6')-Ie aph (2")* (63%) followed by *aph (3')-IIIa* (37%). The erythromycin resistance was 45% and 41% of isolates were positive for *ermB* gene. The *ermA* gene was found in 5% of isolates whereas the *erm*C gene was not detected in any isolates. The prevalence of vancomycin resistant enterococci (VRE) was 12% consisting of *E. faecalis* (6%) and *E. faecium* (22%) and all of them were VanA Phenotype. The results demonstrated that AMEs, *erm* and *van* genes are common in enterococci isolated in Tehran. Furthermore our results show an increase in the rate of vancomycin resistance among enterococci isolates in Iran.

Key words: *Enterococcus faecalis*, *Enterococcus faecium*, vancomycin resistant enterococci, VRE, *erm*, AMEs

Introduction

During the last decade, enterococci, particularly vancomycin resistant enterococci (VRE) have emerged as important nosocomial pathogen in Iran as well as throughout the world (Feizabadi *et al*., 2006; Witte *et al*., 1999; Cetinkaya *et al*., 2000). Knowing the prevalence of antibiotic resistance genes is important for treatment and controlling of enterococci infections. Studies have shown increasing resistance of enterococci to antimicrobial agents such as β-lactams, high-level resistance (HLR) to aminoglycosides and more recently to glycopeptides (Cetinkaya *et al*., 2000, Kaçmaz and Aksoy, 2005). Various different antibiotic resistance genes have been identified in enterococci, especially in *Enterococcus faecium* (Takeuchi *et al*., 2005). Resistance to high concentrations of aminoglycoside antibiotics, usually due to aminoglycoside-modifying enzymes (AMEs) encoded within mobile genetic elements and is widespread among enterococci (Shaw *et al*., 1993). One of the most prevalent AMEs genes among gram positive bacteria such as enterococci is *aac (6')-Ie aph (2")* encodes a bifunctional enzyme, AAC (6')-APH (2'') that confers resistance to a broad spectrum of aminoglycosides (Chow *et al*., 2001).

Resistance to macrolides such as erythromycin is prevalent among enterococci (Portillo *et al*., 2000). One of common resistance mechanisms is ribosomal target modification by 23S rRNA methylases encoded by the erythromycin-resistant methylase (*erm*) genes (De Leener *et al*., 2004). High-level resistance to vancomycin encoded by the *vanA* and *vanB* gene clusters which are carried on transposon (Cetinkaya *et al*., 2000).

The aim of present study was to determine the prevalence of AMEs genes, *erm* genes and *van* genes among a clinical collection of enterococci isolates from Tehran.

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Experimental

Material and Methods

Patient specimens and bacterial strains. Three hundred and twenty six (326) enterococcal isolates were recovered from different clinical samples of patients with suspected of clinical infections from four hospitals in Tehran, namely, Ahari Children Medical Center (ACMC) and shariaty, which are referral tertiary care centers and Tehran University of Medical Sciences teaching hospitals, Mehrad Hospital and Pars Hospital. The last two hospitals are tertiary care and secondary care facilities, respectively. Only one enterococcal isolate was analyzed from each patient.

Identification of the *Enterococus* genus was performed based on the following microbiological tests: Gram reaction, catalase reaction, presence of pyrrolidonyl arylamidase (PYR), growth on bile-aesculin agar and 6.5% NaCl media. A previously published scheme was used in this study to identify the enterococcal species (Facklam and Collins, 1989; Forbes *et al.*, 1998). This scheme utilized a motility test, arginine decarboxylation in Moeller decarboxylase media, pyruvate utilization, and fermentation of carbohydrates (Arabinose, Raffinose, Mannitol, Ribose).

Susceptibility testing procedures. Susceptibility to antimicrobial agents was tested by a disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (NCCLS 2004). The antibacterial agents tested were amikacin (30 µg), erythromycin (15 µg), gentamicin (120 µg), kanamycin (30 µg), netilmicin (30 µg), tobramycin (10 µg), streptomycin (300 µg), teicoplanin (30 µg) and vancomycin (30 µg) per milliliter. The minimum inhibitory concentration (MIC) of gentamicin, streptomycin, erythromycin, vancomycin and teicoplanin were determined by the agar dilution method on Mueller-Hinton agar. *E. faecalis* ATCC 29212 was used as control.

Amplification of AMEs, *erm* and *van* genes. Genes encoding the aminoglycoside modifying enzymes, 6'-N-acetyltransferase-2''-O-phosphotransferase (AAC(6')-APH(2'')), 3'-O-phosphotransferase (APH (3')), 4'-O-adenyltransferase (ANT (4')), *erm* genes (*ermA*, *ermB*, *ermC*) and the vancomycin-resistance determinants *vanA* and *vanB* were detected by Multiplex-PCR using specific primers listed in Table I.

Amplification was performed in a final volume of 25 µl containing 300 nM of each primer, reaction buffer 1x, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 µl of template DNA, 1.5 U of Taq polymerase (Fermentas, UAB, Lithuania). The PCR conditions consisted of a pre-denaturation step at 94°C for 5 min, followed by 30 cycles of 45 sec at 94°C, 45 sec at 55°C (50°C for *erm* genes) and 45 sec at 72°C. A final extension step was performed at 72°C for 5 min. Amplified products were analyzed by electrophoresis on 1.5% agarose gel. DNA bands were visualized by staining with ethidium bromide and photographed under UV illumination. The *E. faecalis* strain JH2-2, *E. faecalis* HH22 (Kindly provided by Dr. Feizabadi), *E. faecalis* E206 and *E. faecium* E2781 (courtesy of Dr. Edet Udo) were used as controls in the experiments.

Statistical analyses. SPSS software, version 11.5, was used for statistical analysis. Differences in resistance patterns between *E. faecalis* and *E. faecium* isolates were calculated using the chi-square test for each antimicrobial agent. A *P* value of ≤0.05 was considered statistically significant.

Table I
The oligonucleotide primers used in this study

Gene	Primers (oligonucleotide sequence 5'-3')	Size of amplified product (bp)	Reference
aac(6')-Ie aph(2'')	AGGAATTTATCGAAAATGGTAGAAAAG CACAATCGACTAAAGAGTACCAATC	369	Vakulenko *et al*., 2003
aph(3')-IIIa	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG-3	523	Vakulenko *et al*., 2003
ant(4')-Ia	CAAACTGCTAAATCGGTAGAAGCC GGAAAGTTGACCAGACATTACGAACT	294	Vakulenko *et al*., 2003
ermA	TAT CTT ATC GTT GAG AAG GGA TT CTA CAC TTG GCT GAT GAA A	139	Martineau *et al*., 2000
ermB	CTA TCT GAT TGT TGA AGA AGG ATT GTT TAC TCT TGG TTT AGG ATG AAA	142	Martineau *et al*., 2000
ermC	AAT CGT CAA TTC CTG CAT GT TAA TCG TGG AAT ACG GGT TTG	299	Strommenger *et al*., 2003
vanA	GGG AAA ACG ACA ATT GC GTA CAA TGC GGC CGT TA	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

Results

Of 326 enterococcal isolates, 210 and 116 were *E. faecalis* and *E. faecium*, respectively. They were cultured from urine (n = 172), blood (n = 60), wound (n = 68) and other clinical samples (n = 26).

Antimicrobial susceptibility. The MIC breakpoint and the MIC_{50} and MIC_{90} value of enterococcal isolates which were examined in this study are shown in Table II. The HLR to gentamicin (MIC > 500 µg/ml), and streptomycin (MIC > 2000 µg/ml) was detected in 52 and 72% of the isolates respectively. In addition 45% of isolates were resistant to erythromycin. Vancomycin and teicoplanin resistance were detected in 12% of isolates consisting of 4% (8/210) *E. faecalis* and 26% (30/116) *E. faecium* (Table II).

Detection of AMEs genes, *erm* genes and *van* genes. Distribution of genes encoding aminoglycoside modifying enzymes among the strains of *E. faecalis* and *E. faecium* are summarized in Table III. All isolates with gentamicin MIC>500 µg/ml in both species of *E. faecalis* (n = 106) and *E. faecium* (n = 65) gave positive results for the presence of the *aac (6')-Ie aph (2")* gene with a 369-bp fragment in size. This gene was detected only in the 21% (33/155) of isolates with gentamicin MIC≤500 µg/ml. The *aph (3')-IIIa* gene encoding the APH (3') enzyme were detected in 37% (122/326) of isolates. None of the isolates contained *ant (4')-Ia* gene. Coexistence of *aac (6')-Ie aph (2")* and *aph (3')-IIIa* among *E. faecalis* and *E. faecium* isolates were 28% (60/210) and 49% (57/116) respectively. There was correlation between resistance to tested aminoglycosides and existence of the *aac (6')-Ie aph (2")* and *aph (3')-IIIa* genes (Table IV).

The results of amplification of *erm* and *van* genes encoding resistance to erythromycin and vancomycin respectively, among enterococci isolated from different sources by multiplex-PCR are shown in Table V. The most prevalent *erm* genes were *ermB* which was detected in 41% of the isolates. The *ermA* gene was

Table II
The minimum inhibitory concentration of tested antibiotic among *E. faecalis* and *E. faecium* isolates

Organism	Antibiotic	Break point	MIC (µg/ml) 50%	MIC (µg/ml) 90%	% R	*P* value
Total (n = 326)	Gentamicin	> 500	1000	> 4000	52	
	Streptomycin	> 2000	> 4000	> 4000	72	
	Erythromycin	≥ 8	1	512	45	
	Teicoplanin	≥ 32	2	32	12	
	Vancomycin	≥ 32	2	128	12	
E. faecalis (n = 210)	Gentamicin	> 500	1000	> 4000	50	
	Streptomycin	> 2000	4000	> 4000	69	
	Erythromycin	≥ 8	≤ 0.5	512	39	
	Teicoplanin	≥ 32	< 0.25	8	6	
	Vancomycin	≥ 32	< 0.25	8	6	
E. faecium (n = 116)	Gentamicin	> 500	1000	> 4000	56	0.3558
	Streptomycin	> 2000	> 4000	> 4000	78	0.1973
	Erythromycin	≥ 8	64	512	56	0.0026
	Teicoplanin	≥ 32	2	64	22	0.0001
	Vancomycin	≥ 32	8	512	22	0.0001

Table III
Distribution of genes encoding aminoglycoside-modifying enzyme (AMEs) among *E. faecalis* and *E. faecium* isolates with source of infection

	N (%) of isolates								
	MIC > 500		MIC ≤500		Total (n = 326)	Urine (n = 172)	Blood (n = 60)	Wound (n = 68)	Others (n = 26)
	E. faecalis (n = 104)	*E. faecium* (n = 51)	*E. faecalis* (n = 106)	*E. faecium* (n = 65)					
aac(6')-Ie aph(2")	14 (13)	19 (37)	106 (100)	65 (100)	204 (63)	93 (54)	46 (77)	52 (76)	13 (50)
aph(3')-IIIa	11 (11)	17 (33)	53 (50)	41 (63)	122 (37)	52 (31)	31 (52)	36 (53)	3 (11)
ant(4')-Ia	0	0	0	0	0	0	0	0	0
aac(6')-Ie aph(2") + *aph(3')-IIIa*	10 (10)	15 (29)	50 (47)	42 (65)	117 (36)	47 (27)	31 (52)	35 (51)	4 (15)

Table IV
Phenotypic and genotypic resistance to aminoglycoside antibiotics among enterococcal isolates

Phenotype of resistance	N (%) of isolates	*aac(6')-Ie aph(2")*	*aph(3')-IIIa*
AMK*, GEN, KAN, NET, TOB	96	+	+
AMK, GEN, KAN, NET	10	+	+
GEN, KAN, NET, TOB	47	+	–
AMK, GEN, KAN	11	+	+
GEN, KAN, NET	16	+	–
GEN, KAN, TOB	11	+	–
GEN, KAN	3	+	–
KAN, TOB	2	+	–
KAN	5	+	–
TOB	3	+	–
AMK	5	–	+
–	117	–	–

* AMK; amikacin, GEN; gentamicin, KAN; kanamycin, NET; netilmicin, TOB; tobramycin

found in 5% of isolates whereas the *ermC* gene was not detected in any isolates. Coexistence of *ermA* and *ermB* was detected in three of isolates consisting of one *E. faecalis* (from blood) and two *E. faecium* (from urine and wound).

All of the *E. faecalis* and *E. faecium* isolates that were resistant to vancomycin were positive for *vanA* gene. The *vanB* gene was not detected in any isolates. The rates of isolation of VRE from different sources were urine (n = 20), blood (n = 5) and wound (n = 13).

Discussion

The spread of antimicrobial resistance among enterococcal species in Iran has presented a serious challenge for the Iranian medical community (Feizabadi *et al.*, 2006). Unfortunately, treatment failures in enterococcal infections are on the rise because of the lack of adequate information regarding antimicrobial resistance especially glycopeptide resistance among endemic enterococci. Such information is required for appropriate treatment of patients with enterococcal infections, which rank among the third common cause of bacteremia and the second most frequent cause of UTI (Cetinkaya *et al.*, 2000). Comprehensive data concerning the endemic prevalence and susceptibility patterns of enterococci in various health institutions is also necessary to prevent spread of antimicrobial resistance in Iran.

This study investigated the prevalence of genes encoding aminoglycoside resistant, erythromycin resistant and vancomycin resistant in enterococci isolated from clinical samples in Iranian hospitals. Of 326 enterococcal isolates tested, the prevalence of high-level resistance to gentamicin and streptomycin were 52 and 72% respectively. These results are similar to those from Kuwait and another report from Iran and higher than Turkey, 24 and 36% respectively (Feizabadi *et al.*, 2006; Kaçmaz and Aksoy, 2005; Udo *et al.*, 2004). Compared with our isolates, in the Spanish enterococcal isolates HLR to gentamicin (65%) was more than to streptomycin (42%) (del Campo *et al.*, 2000). In the present study, no significance difference was observed between *E. faecalis* and *E. faecium* in HLR to gentamicin and streptomycin (*P* value; 0.3558 and 0.1973). This result was in contrast to report from Europe where high level aminoglycoside resistance has been more frequently found in *E. faecium* than in *E. faecalis* and was similar to report from USA where HLR to gentamicin rates were equal between the two species (Zarrilli *et al.*, 2005; Gordon *et al.*, 1992).

The HLR to aminoglycoside in enterococci is usually coded by *aac (6')-Ie aph (2")* gene (Feizabadi *et al.*, 2006). The results of PCR of isolates with gentamicin MIC > 500 µg/ml confirmed that all isolates contained *aac (6')-Ie aph (2")* gene. However isolates with gentamicin MIC ≤ 500 µg/ml also contained this gene. Detection of the *aac (6')-Ie aph (2")* gene in isolates with gentamicin MIC ≤ 500 µg/ml has also reported in other studies which supporting the presence of the *aac (6')-Ie aph (2")* gene in low-level gentamicin-resistant enterococci (Feizabadi *et al.*, 2006; Udo

Table V
Distribution of erythromycin-resistant methylase (*erm*) and vancomycin-resistant genes (*van*) among *E. faecalis* and *E. faecium* isolates with source of infections

N (%) of isolates	*E. faecalis*				*E. faecium*				Total (n = 326)
	Urine n = 130	Blood n = 36	Wound n = 33	Others n = 11	Urine n = 42	Blood n = 24	Wound n = 35	Others n = 15	
ermA	3 (2)	1 (3)	1 (3)	0	5 (12)	2 (8)	3 (9)	1 (7)	16 (5)
ermB	35 (27)	18 (50)	20 (61)	1 (9)	25 (59)	16 (67)	17 (49)	2 (13)	134 (41)
ermC	0	0	0	0	0	0	0	0	0
ermA + *ermB*	0	1 (3)	0	0	1 (2)	0	1 (3)	0	3 (1)
vanA	7 (5)	1 (3)	4 (12)	0	13 (31)	4 (17)	9 (26)	0	38 (12)
vanB	0	0	0	0	0	0	0	0	0

et al., 2004). Like many reports, in our study the *aac (6')-Ie aph (2")* gene was the most common AME gene in gentamicin-resistant enterococci (Feizabadi *et al*., 2006; Udo *et al*., 2004; Kaçmaz and Aksoy, 2005). The second AMEs gene that was detected in this study was *aph (3')-IIIa* which confers resistance to amikacin and kanamycin, but not gentamicin (Feizabadi *et al*., 2006; Takeuchi *et al*., 2005). Importantly, it was found among 33% of *E. faecium* isolates with gentamicin MIC≤500 μg/ml. However, the types and distribution of aminoglycoside-modifying genes in enterococci vary in different geographical regions (Udo *et al*., 2004; del Campo *et al*., 2000; Zarrilli *et al*., 2005; Gordon *et al*., 1992).

In our study, coexistence of *aac (6')-Ie aph (2")* and *aph (*3')*-IIIa* among *E. faecalis* and *E. faecium* isolates were 28% (60/210) and 49% (57/116) respectively. Isolation of multiple AMEs gene in enterococci have also observed in other reports (Feizabadi *et al*., 2006; Udo *et al*., 2004; del Campo *et al*., 2000). The presence of these multiple genes for aminoglycoside-modifying enzymes in the isolates implies that gentamicin can not be used to obtain synergy with a glycopeptide or β-lactam for the treatment of enterococcal infections (Udo *et al*., 2004).

In the present study the *ermB* gene was the most prevalent gene in erythromycin resistant enterococci, it was detected in 52% (60/116) of the *E. faecium* isolates and with *ermA* in 2% (2/116). This is in agreement with results from Korea, Belgium, 24 European university hospitals and Colombian hospitals (De Leener *et al*., 2004; Lim *et al*., 2002; Schmitz *et al*., 2000; Reyes *et al*., 2007). The frequency of isolates that displayed erythromycin MIC≥8 μg/ml and gave negative results by PCR for *erm* genes were 4%. This implies that other mechanisms contribute to erythromycin resistance in tested enterococcal isolates. The 12% rate of VRE prevalence in the present study is higher than other report of VRE prevalence (7%) in Tehran (Feizabadi *et al.*, 2004). In addition, finding of alarmingly high rate of vancomycin resistance in Iran is in sharp contrast with studies from other countries in the Middle East, where low incidence (0–1%) of VRE has been reported (Zouain and Araj, 2001; Udo *et al.*, 2002).

Despite the recent isolation of a single *vanB* genotype enterococcal strain from a Tehran hospital (Emaneini *et al*., 2005), the finding that all VRE's isolated in this investigation were *vanA* genotype illustrates that, *vanA* genotype is the predominant type of enterococcal vancomycin resistance in Iran, as reported in other countries (Udo *et al.,* 2002; Kholy *et al*., 2003).

In conclusion, our study demonstrated that high level resistance to gentamicin, erythromycin and vancomycin in enterococcal isolates in Tehran, Iran Hospitals mainly related to the presence of *aac (6')-Ie aph (2")*, *ermB* and *vanA* gene respectively. Increasing in resistance to vancomycin in Iran contributes to the challenges confronted by the infection control specialists in hospitals throughout the Middle East region, as well as causing problems in the treatment of patients with enterococcal infections.

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