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Short Report

Anti-Microbial Resistance of Enterococci Isolated from Urinary Tract Infections in Iran

Mahnaz Saifi[1], Mohammad Reza Pourshafie[2], Mohammad Reaz Eshraghian[3] and Mohammad Mehdi Soltan Dallal[*1]

[1]Div. of Microbiology, Dept. of Pathobiology and [3]Dept. of Epidemiology and Biostatistic , School of Public Health, Medical Sciences Tehran University, Tehran; [2]Dept. of Bacteriology, Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT

Background: During the last decade, enterococci have become important nosocomial pathogens, representing the second leading cause of urinary tract infections. This increasing prevalence has been paralleled by the occurrence of multi-drug resistant (MDR) and high-level gentamicin resistant (HLGR) strains. **Methods:** From September 2005 to 2006, a total of 638 enterococcal isolates were collected from urine samples among 9 medical centers in Tehran (Iran). Confirmation of species and detection of gentamicin resistance genes were done by PCR method. Anti-microbial susceptibility test was determined with disk diffusion and minimal inhibitory concentration of gentamicin among HLGR isolates assayed by microdilution methods. **Results:** The isolates were found to consist of *Enterococcus faecalis* (77.8%) and *Enterococcus faecium* (22.2%). The results obtained from PCR showed a high rate of agreement with phenotypic assays for both species. MDR to most prevalent anti-microbials was present in 29% and 72% of the *E. faecalis* and *E. faecium* isolates, respectively. HLGR phenotype was detected in 64% of *E. faecalis* and 92% of *E. faecium* isolates. The *aac(6')-Ie-aph(2")-Ia* gene were identified in 83% of *E. faecalis* and 100% of *E. faecium* HLGR isolates. *E. faecalis* and *E. faecium* isolates differed in their susceptibilities to different antibiotics. **Conclusion:** Emergence of multi-resistant enterococci and high level resistance to gentamicin shown by enterococcal strains is of concern because of the decrease in the therapeutic options for treatment of infections caused by enterococci. *Iran. Biomed. J. 12 (3): 185-190, 2008*

Keywords: Anti-microbial resistance, Enterococci, Urinary tract infection (UTI)

INTRODUCTION

For years, Enterococci have been considered as harmless inhabitants of the gastrointestinal tracts of humans and animals. Of the strains belonging to the enterococcus genus *E. faecalis* and *E. faecium* are common human isolates. Recent studies have documented that enterococci are increasing as significant nosocomial pathogens, representing the second leading cause of urinary tract infections (UTI) in the USA [1, 2]. In addition, studies have shown increasing resistance of enterococci to anti-microbial agents such as ß-lactams, and high-level resistance to amino-glycosides and more recently to glycopeptides. This is possibly due to the use of broad-spectrum antibiotics or multi-antibiotic regimes, which allow for enterococcal overgrowth and superinfection [1]. ß-lactams and aminoglycosides are generally the antibiotics of choice for treating the serious infections caused by enterococci. High-level gentamicin-resistant (HLGR) enterococci frequently express additional resistance to multiple antibiotics, thereby causing therapeutic problems [2].

Enterococci exhibiting HLGR have been reported widely as a cause of nosocomial infections in Europe, the United States, and other geographic locations [3]. This study was performed to

*Corresponding Author; Tel. (+98-21) 6646 5404; E-mail: soltanda@tums.ac.ir

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determine the patterns of anti-microbial resistance of enterococci species', including high-level gentamicin resistance.

MATERIALS AND METHODS

Bacterial strains. From September 2005 to 2006, 638 enterococcal isolates were recovered from patients with UTI attending to 9 medical centers located in Tehran (Iran) .The urine samples were collected in sterile condition and all of them were cultured on sheep blood agar and incubated at 37°C. After 48 hours, each of the enterococcal positive cultures over than 10^5 CFU/ml was identified as UTI [4].

Identification of` enterococcal isolates. Identification of the strains to the genus level was performed by using the following characteristics: reaction of Gram-staining, growth and blackening of bile-esculin agar, growth in the presence of 6.5% NaCl, absence of catalase and presence of pyrrolidonyl arylamidase [4].

Susceptibility testing. All isolates were tested against vancomycin (30 µg), ciprofloxacin (5 µg), ampicillin (10 µg), teicoplanin (30 µg), trimethoprim/sulfametoxazole (1.25:22.75), tetracycline (30 µg), erythromycin (30 µg), nitrofurantoin (300 µg), linezolid (30 µg), quinupristin-dalfopristin (Synercid) (15µg) and high content gentamicin (120 µg) (BBL Microbiology System, Cockeysville, MD) by disk diffusion method according to the CLSI guidelines [5]. Isolates with intermediate levels of susceptibility were classified as sensitive [6]. *E. faecalis* ATCC 29212 and *E. faecium* IP 4107 (Microbial Collection of Pasteur Institute of Paris) were used as quality control reference strains.

Minimum inhibitory concentration measurements. The minimum inhibitory concentrations (MIC) for gentamicin (Sigma-Aldrich, Poole, UK) against enterococci were determined by microdilution technique. Serial two-fold dilutions of antibiotic were added to Mueller Hinton broth in order to obtain the following final concentrations of gentamicin ranged from 4 to 2048 mg/ml. The MIC was defined as the lowest antibiotic concentration resulting in complete inhibition of visible growth [5].

DNA extraction. Three milliliter of an overnight culture of the all enterococcal isolates was centrifuged in 5500 ×g for 15 minutes and the pellet was resuspended in Tris EDTA Sucrose (TES) buffer (Tris HCl, 10 mM; EDTA, 1 mM; sucrose, 50%, pH 7.5) containing lysozyme (20 mg/ml) and incubated at 37°C for 20 minutes. After adding 12 µl of 10% SDS, this mixture was placed in ice for 10 minutes and centrifuged in 7500 ×g for 15 minutes. The supernatant was removed in another tube and equal volume of phenol/ chloroform was added and after gently mixes, centrifuged in 13000 rpm for 10 minutes. Precipitation of DNA followed by adding 500 µl of cold ethanol and finally DNA was dissolved in 50 µl of TE buffer containing RNase [7].

Detection of genus and species by PCR. Enterococcal genus and species primers were as previously published [8]. The base master mix consisted of 0.8 mM dNTP, 0.5 U of Taq DNA polymerase, 2.5 µl of 10× PCR buffer, 1.5 mM $MgCl_2$ (Roche Diagnostic, Manheim, Germany) and 2.5 pM of each primer. PCR were performed in a final volume of 25 µl containing 20 µl of master mix and 5 µl of DNA template. following an initial denaturation at 94ºC for 5 min, products were amplified by 30 cycles of denaturation at 94ºC for 1 min, annealing at 54ºC for 1 min and elongation at 72ºC for 1 min. Amplification was followed by a final extension at 72ºC for 7 min [8]. Ten microliters of product was electrophoresed on a 1.5% 1X ris-acetate-EDTA agarose gel and stained with ethidium bromide.

Detection of aac(6')-Ie-aph(2")-Ia gene by PCR. PCR experiments were performed in a volume of 25 µl with the following content: 2 µl of DNA template, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 2.5 µl of 10X PCR buffer, 2.5 U Taq DNA polymerase ((Roche Diagnostic, Manheim, Germany) and primers with a 2 pM final concentration. PCR was performed with an initial lysing step of 3 min at 94ºC, 35 cycles of 40 s at 94ºC, 40 s at 55ºC, and 40 s at 72ºC, and a final extension step of 2 min at 72ºC [9]. PCR products were analyzed by electrophoresis in 1.5% agarose gel and stained with ethidium bromide.

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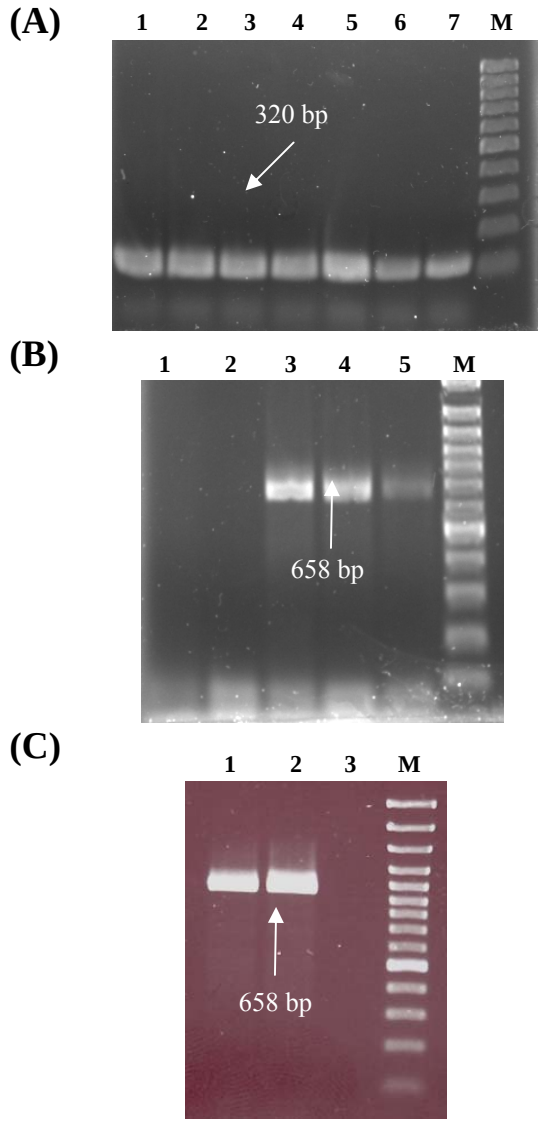

Fig. 1. **(A)** PCR product of genus specific (lanes 1-7, *Enterococcus* spp.; lane 8, molecular weight marker), **(B)** PCR product of *E. faecium* species (lanes 1 and 2, negative control; lanes 3-5, *E. faecium* and lane 6, molecular weight marker, **(C)** PCR product of *E. faecalis* species (lane 1 and 2, *E. faecalis*; lane 3, negative control; lane 4, molecular weight marker).

RESULTS

A total of 638 enterococci isolates from UTI were collected. The distribution of species according to biochemical and PCR tests was 77.8% (527) *E. faecalis* and 22.2% (149) *E. faecium*. Amplification of genus, *E. faecals*-specific and *E. faecium* -specific targets produced 320 bp, 941 bp and 658 bp bands, respectively (Fig. 1).

The susceptibility patterns of all isolates are shown in Table 1. Among *E. faecalis* isolates with the exception of intrinsic resistance to quinupristin-dalfopristin, tetracycline, erythromycin and co-trimoxazole resistant strains were isolated at relatively high frequencies of about 48-88% when compared to other drug resistances. Drug resistance among the isolates showed many resistant patterns.

E. faecium isolates had a higher rate of combined resistance than *E. faecalis*. According to the different antibiotic groups, the frequency of multi-drug resistant (MDR) strain was between 30-72% and 0.3-29% among *E. faecium* and *E. faecalis*, respectively (Table 2). Among MDR isolates, HLGR was detected by the high content disk in 92% and 30% of *E. faecium* and *E. faecalis* isolates, respectively. Excellent correlation was observed between the high-level disk tests and the MIC test in detection of HLGR resistance. The MIC of gentamicin was ≥1024 mg/l for all HLGR isolates.

Resistance to all of the anti-microbials except quinopristin-dalfopristin, tetracycline and linezolid was higher in *E. faecium* than *E. faecalis.* Vancomycin resistance was detected in 2.7% of *E. faecalis* and 63% of *E. faecium* isolates.

None of the *E. faecium* isolates tested demonstrated any *in vitro* resistance to linezolid while 3.8% of *E. faecalis* isolates were resistant. Among *E. faecium* isolates, resistance frequency against quinupristin-dalfopristin was 2.4%.

Table 1. Anti-microbial resistance profile among *E. faecalis* and *E. faecium* isolates.

E. faecalis		*E. faecium*	
Antibiotics	**Resistance (%)**	**Antibiotics**	**Resistance (%)**
Synercid	100.0	Erythromycin	89.0
Tetracycline	88.0	Ciprofloxacin	85.0
Erythromycin	53.0	Ampicillin	82.0
Co-trimoxazole	48.0	Gentamicin	82.0
Ciprofloxacin	39.0	Co-trimoxazole	72.0
Gentamicin	30.0	Vancomycin	63.0
Linezolid	3.8	Tetracycline	58.5
Vancomycin	2.7	Teicoplanin	52.0
Teicoplanin	2.5	Nitrofurantoin	7.3
Ampicillin	1.4	Synercid	2.4

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Table 2. Multi-resistance patterns of enterococcal species.

Antibiotic groups	*E. faecium* resistance (%)	*E. faecalis* resistance (%)
Am, TE, E	46	0.3
Am, TE, Cip	46	0.6
Am, TE, Gm	42	0.3
Am, TE, V	31	0.3
Am, TE, SXT	43	0.3
Am, E, Cip	72	0.3
Am, E, Gm	69	0.3
Am, E, SXT	67	0.3
Am, E, V	59	0.3
Am, Cip, SXT	65	0.3
Cip, Gm, V	59	2.0
Cip,Gm, SXT	63	23.0
Cip, SXT, E	65	29.0
Cip, SXT, V	53	2.3
TE, Cip, V	30	2.3
TE, Cip, SXT	40	29.0
TE, Cip, Gm	40	25.0
E, Cip, Gm	65	24.0

V, vancomycin; Te, tetracycline; Am, ampicillin; E, erythromycin; Cip, ciprofloxacin; Tei, teicoplanin; Fm, nitrofurantoin; Syn, quinupristin-dalfopristin (Synercid); Lin, linezolid; SXT, trimethoprime-sulfamethoxazole.

PCR results showed that 83% and 100% of the HLGR *E. faecalis* and *E. faecium* isolates contained the *aac(6')-Ie-aph(2'')-Ia* gene, respectively (Fig. 2).

DISCUSSION

MDR bacteria in both the hospital and community environment are important concern to the clinician. During the last few decades, the frequency and spectrum of antibiotic resistant infections have increased within the United States, Europe and the developing countries [10]. The epidemiology of enterococci is not fully understood since striking differences among resistant isolates from different species and resistant isolates from different geographic locations have been reported [11].

More than a dozen species of enterococci are currently recognized, but 85-95% of enterococcal infections are caused by *E. faecalis*, with another 5-10% caused by *E. faecium* [1]. This study showed *E. faecalis* is the most common species (77.8%) recovered more frequently in culture and the less prevalent type included *E. faecium* (22.2%). We had a clear dominance of *E. faecalis* as expected but the frequency of *E. faecium* was more than other reports [12-14]. Diversity in the species distribution might be obtained when enterococci isolated in different geographic regions are involved. The results obtained from PCR and phenotypic assays showed a high rate of agreement for both species.

Several multi-resistant enterococci strains obtained in this study are a cause of concern due to limitation in clinical uses of anti-microbial agents especially by loss of synergistic combination which is often needed for treatment of enterococcal infections.

Our study signals the emergence of the high frequency of HLGR isolates in Iran. Disks of high content to detect high-level gentamicin resistance and MIC tests did not present incompatible results. Thus, since it is easily applicable, routine use of disks with high content of gentamicin is considered more appropriate. Earlier studies in Iran have reported the prevalence of HLGR strains in clinical samples about 52% [15] and in other reports ranging from 14 to 49% [16]. In this study, the prevalence of HLGR enterococci was significantly higher (30% of *E. faecalis* and 82% of *E. faecium* strains). In the case of HLGR enterococcal isolates, the synergistic activity of the combination of penicillin with gentamicin is totally abolished. In such instances, controlling the spread of these organisms becomes of paramount importance.

HLGR enterococci are due to the synthesis of modifying enzymes. Ribosomal resistance and aminoglycoside modifying enzymes coding on self-transferable plasmids cause high-level resistance [1]. The *aac(6')-Ie-aph(2'')-Ia* gene is the most clinically important gene among HLGR isolates [7, 10]. The high frequency of this gene in our study indicates widespread dissemination of this resistance determinant.

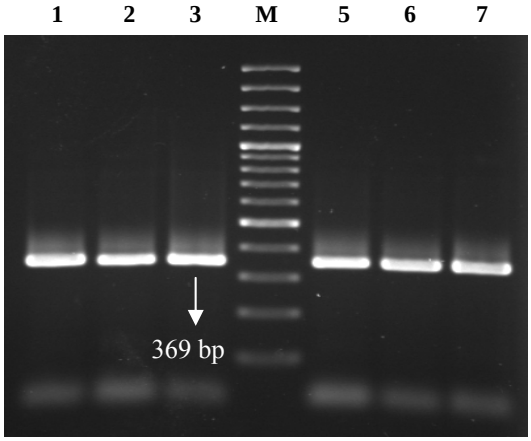

Fig. 2. PCR product of *aac(6')-Ie-aph(2'')-Ia* gene (lane 1-3 and 5-7, HLGR strains and lane 4, molecular weight marker).

There are some reports about increasing resistance of enterococci to ciprofloxacin [7, 17]. High resistance to ciprofloxacin and co-trimoxazole in the present study may be due to a widespread usage of these antibiotics for UTI first line treatment in Iran.

The prevalence of resistance to vancomycin in *E. faecium* isolated in the present study was higher than *E. faecalis* and this was in concordance to teicoplanin resistance frequency. The alarming point about the spreading potential of resistance is that vancomycin resistance genes could be transferred among enterococci and from enterococci to staphylococci species [1].

Resistance rate to ampicillin among *E. faecium* isolates was higher than *E. faecalis* (82% versus 1.4%) and this finding is similar to Jureen's report [18]. The decreased affinity of penicillin-binding proteins (PBP) with low molecular weights (particularly PBP5) and some strains with plasmid-mediated ß-lactamases have been held responsible for this resistance [1].

Quinupristin/dalfopristin and linezolid are new antibiotics, have a spectrum of *in vitro* activity against enterococci [18-20]. In our study, both species showed low resistance only to linezolid and the most of *E. faecium* isolates were sensitive to quinupristin- dalfopristin. According to our results, it seems that nitrofurantoin because of the lower resistance especially in *E. faecalis* strains, can be considered as a good alternative therapy in enterococcal UTI.

Characterization and identification of enterococci by using the traditional phenotypic differentiation can be a tedious process requiring numerous tests. Other methods for identification of enterococci have utilized molecular techniques such as PCR [11]. Indeed, correct definition of species enabled us to assess species-specific antibiotic susceptibility patterns in our area. Species identification can also play a role in the treatment of patients with infection due to these organisms. Periodic evaluation of antibiotic susceptibility of enterococci and early detection of microorganisms are required for empirical treatment planning.

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