Molecular epidemiology and antibiotic resistance patterns of *Enterococcus faecalis* isolates from hospitals in Tehran

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SUMMARY

Enterococcus faecalis is one of the most important causative agents of nosocomial infections. Control and prevention of these infections require appropriate epidemiological knowledge. This study investigated the correlation between molecular characteristics and drug resistance of *E. faecalis* isolates from local hospitals. A total of 125 isolates of *Enterococcus faecalis* from two hospitals in Tehran were identified by using culture and biochemical method. An antibiotic resistance assay was carried out by a disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines. The genetic diversity of the strains was determined using a repetitive sequence-based polymerase chain reaction (REP-PCR) method. All isolates were typed by REP-PCR, and different PCR amplification products (from 9 to 17 amplified DNA fragments) were detected by gel electrophoresis. There were eight different PCR patterns (A-H), and a significant correlation was detected between the resistance to antibiotics and the obtained PCR patterns. The most relevant cases (28%) belonged to the fourth group (D). All group D isolates were resistant to tetracycline and quinupristin/dalfopristin. A high resistance to certain common antibiotics and clonal propagation were detected among isolates from patients in different wards of the hospitals. This study was the first to investigate *E. faecalis* isolates from Iranian patients and to describe patterns that showed correlation between infection prevalence and genetic origin/similarity of the isolates.

Keywords: *Enterococcus faecalis*, antibiotic resistance, nosocomial infections, molecular epidemiology, REP-PCR.

INTRODUCTION

Enterococci are known as an important cause of nosocomial infections [1]. This genus of bacteria is considered as the second most common cause of bacteremia and the third cause of urinary tract infections [2]. In hospitalized patients, Enterococci colonization, especially by resistant strains to different antibiotics, provides conditions for patients that with interventions such as surgery, shunt and catheter, may cause disease [3]. Enterococcus species is involved in urinary tract infections, pelvic and intra-abdominal infections, bacteremia, endocarditis, neonatal sepsis and meningitis [4]. The most important problem of Enterococci is their relative and absolute resistance against the antibiotics. Most of enterococci are resistant to glycopeptide and beta-lactam antibiotics, making necessary their simultaneous use with an aminoglycoside for treatment of the most serious enterococcal infections such as endocarditis, meningitis. The efficacy of such drug combination is disturbed with the appearance of re-

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sistant strains to some antibiotics, including high resistance to aminoglycosides and glycopeptides. For this reason, it is important to control and prevent the outbreak of these microorganisms [5-8].
In order to control the dissemination of these microorganisms, it is essential to identify the sources and genetic diversity of strains, which is known as typing. In the past, phenotypic characteristics such as serotypes, biotypes, antibiotic sensitivity profiles, and phage typing were used for bacterial typing, but today molecular typing methods have replaced the classical methods, which were time consuming and expensive. Besides, the classical methods were not capable of distinguishing closely related strains. The molecular techniques currently used for bacterial typing include pulsed-field gel electrophoresis, ribotyping, methods based on enzymatic digestion, plasmid analysis, and typing techniques based on polymerase chain reaction (PCR) [9-11]. One of the PCR-based typing methods is a repetitive sequence-based technique (REP-PCR). Genetic patterns of repetitive sequences can be reproduced by the REP-PCR method [12, 13]. Then, strain relationships can be determined by analyzing their dendrograms. Hence, the knowledge of genetic diversity of bacterial strains associated with a region is important for finding the source of infection in the cases of epidemics and nosocomial infections. There have been no studies yet regarding the typing of *E. faecalis* strains in Iran. Therefore, this study was performed to evaluate the epidemiology of molecular and genotypic diversity of *E. faecalis* isolates from patients in Tehran by the REP-PCR method. In addition, this study attempted to evaluate the efficiency of the molecular method for typing these strains.

MATERIALS AND METHODS

Isolation and identification of strains
In this cross-sectional study, 125 clinical samples of *Enterococcus faecalis* were collected from Milad and Baghiyatallah hospitals in 2015 from the following: urine, blood, wound samples, ascites, and bronchoalveolar lavage (BAL) fluid. All the isolates were kept at -70°C in brain-heart infusion broth containing 50% glycerol.
The identification of the isolates was carried out by colony morphology, Gram staining, and conventional biochemical characteristics such as a catalase reaction, growth at 5.6% salt, and esculin hydrolysis in the presence of bile. Furthermore, a medium containing the phenol red indicator was used to identify the species by fermentation of sugars (lactose, arabinose, etc.) [14]. A PCR with specific primers was used to confirm the isolates as *E. faecalis* [15].

Antibiotic susceptibility profiles
An antibiotic susceptibility test was performed using the disk diffusion method on Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The results for each antibiotic were recorded as sensitive, intermediate, and resistant based on a validated protocol. The antibiotic disks selected for the current study included vancomycin (30 µg), erythromycin (15 µg), quinupristin/dalfopristin (15 µg), ciprofloxacin (5 µg), tetracycline (30 µg), linezolid (30 µg), teicoplanin (30 µg), chloramphenicol (30 µg), phosphomycin (200 µg), nitrofurantoin (300 µg), gentamicin (120 µg), and ampicillin (10 µg) (Mast, England). In this study, *E. faecalis* ATCC 29212 was used as a reference control. Minimum inhibitory concentrations (MICs) of vancomycin for resistant isolates were determined using a broth microdilution method. Isolates resistant to three or more antibiotic classes were defined as multidrug resistant.

REP-PCR test
DNA was extracted by the boiling method, and REP-PCR was performed using appropriate primers to identify repeated sequences (Table 1) [16]. Then, a PCR reaction was performed in a final volume of 25 µL, which contained 2 µL of template DNA, 1 µL of each primer, 12 µL of 2× master mix (Ampliqon III Co., Denmark), and 9 µL of distilled water. The cycling conditions were as follows: initial denaturation at 95°C for 7 min, followed by 35 cycles of denaturation at 90°C for 30 s, annealing at 40°C for 1 min, and extension at 65°C for 8 min, and a final extension at 65 °C for 16 min.

Table 1 - Oligonucleotide sequences for primers used in study.

Primer Sequence	*Primers*
5-IIINCGNCGNCATCNGGC–3	F
5-NCGNCTTATCNGGCCTAC –3	R

Table 2 - The results of antibiotic susceptibility by disk diffusion method.

Antibiotic	*Resistant Number (percentage)*	*Semi-sensitive Number (percentage)*	*Sensitive Number (percentage)*
Tetracycline	109(87)	0	16 (12)
Gentamycin	23 (18)	22 (17)	80 (64)
Ampicillin	9 (7)	0	116 (92.8)
Vancomycin	5 (4)	8 (6.4)	112 (89.6)
Fosfomycin	52 (41)	44 (35.2)	29 (23.2)
Linezolid	2 (1.6)	3 (2.4)	121 (96.8)
Nitrofurantoin	2 (1.6)	3 (2.4)	120 (96)
Ciprofloxacin	43 (34)	41 (32.8)	41 (32.8)
Erythromycin	71 (56)	34 (27.2)	20 (16)
Teicoplanin	2 (1.6)	2 (1.6)	121 (96.8)
Chloramphenicol	21 (16.8)	21 (16.8)	83 (66.4)
Quinupristin-dalfopristin	109 (87)	5 (4)	11 (8.8)

Statistical analysis

The analysis of DNA profiles based on PCR products was performed using the GelClust software with the Dice algorithm for clustering. In the distance matrix, the presence or absence of a band was marked with 1 or 0, respectively.

RESULT

In this study, 125 isolates of *Enterococcus faecalis* from urine samples (100), ulcers [12], blood (9) and other samples (4) were isolated. Also the strains were collected respectively from the ICU, surgery, internal medicine, emergency, obstetrics and gynecology ward and outpatient unit.

The most frequent antibiotic resistance patterns observed were for tetracycline, quinupristin/dalfopristin, and erythromycin. The antibiotic susceptibility based on the CLSI guidelines are shown in Table 2.

Vancomycin resistance was observed in 4% of the *E. faecalis* isolates. Among the *E. faecalis* strains, 69.6% were resistant to three or more classes of antibiotics (Table 3).

The 125 strains of *E. faecalis* were further analyzed by REP-PCR. According to the cluster analysis plot shown in Figure 1, the REP-PCR results revealed eight different genetic patterns in the clinical isolates. The number of PCR fragments varied among the strains from 9 to 17.

After obtaining the fingerprint patterns of *E. faecalis* strains, a similarity coefficient (% similarity) was calculated using the Dice coefficient. The patterns were named A to H, and based on the patterns, 20% of the strains were in Group A, 9.6%

Table 3 - The frequency of multidrug resistant E. faecalis.

The number of resistant samples to one or more antibiotic					
The number of antibiotic	1	2	3	4	4>
E. faecalis	12	27	28	21	37

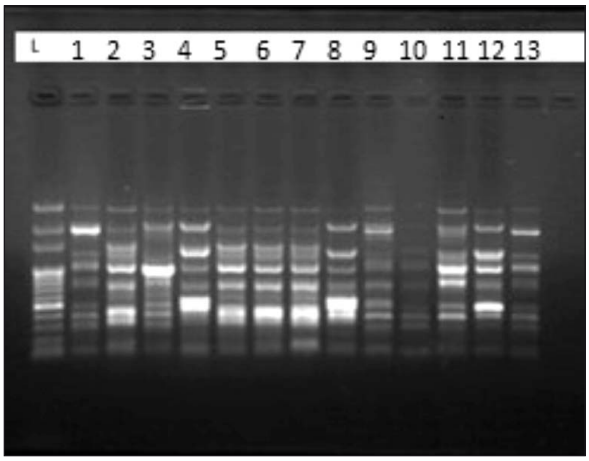

Figure 1 - The patterns of the REP-PCR – Lane 1: Ladder–100bp, Lane 2–14: isolated E. faecalis strains from patients

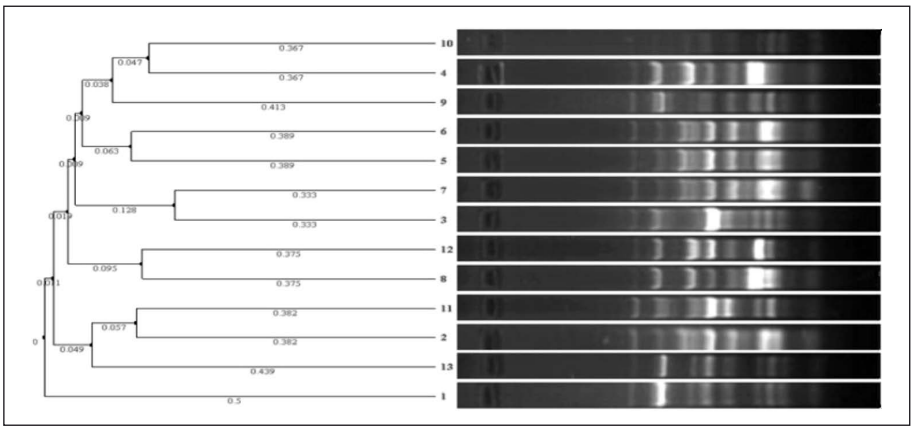

Chart 1 - *E. faecalis* isolates were divided into eight groups according to the PCR products of REP-PCR based on their similarity coefficient (Dice coefficient).

were in Group B, 9.6% were in Group C, 28% were in Group D, 4% were in Group E, 10.4% were in Group F, 8.8% were in Group G, and 8.8% were in Group H.

To determine the genotypic groups, the lengths of the fragments and their numbers (the difference in more than two PCR products) were compared (Table 4). Most of the isolates from different wards of the Milad hospital had C, D, and H genetic patterns, and the majority of the isolates from different wards of the Baqiyatallah hospital had E, A, B, F, and G genetic patterns.

The groups containing F, C, and A genetic patterns had the most important role in the spread of infection in the intensive care units (ICUs). The group containing genetic pattern D, B, H had the greatest role in the spread of infection in emergency units, internal surgery and gynecology ward. The most frequent genetic pattern that observed in outpatient unit was reported in G, E groups.

The closest genetic relationship was observed between groups A and B, which may indicate that these two groups have the same genetic origin.

The comparison of the antibiograms and REP-PCR results revealed that all samples isolated from the ICUs were resistant to quinupristin/dalfopristin and were sensitive to linezolid. On the other hand, the results indicated that all group H strains were resistant to ampicillin and sensitive to phosphomycin.

All group F strains were resistant to six or more antibiotic classes. All tetracycline-resistant strains belonged to Group E. The complete results of genotyping and resistance to antibiotics are shown in Table 5.

Table 4 - Distribution of genotyping patterns obtained from 125 strains.

Number of PCR amplifications of each pattern	*Number of strains*	*Genotyping patterns*
9	25	A
9	12	B
17	12	C
17	36	D
13	5	E
17	13	F
16	11	G
14	11	H
Total 112	125	total

Table 5 - Correlation between antibiotic resistance and genotyping patterns.

Genoyping patterns	*Antibiotic- resistance*
A,B,C,D,E,F,G,H	Tetracycline
A,C,D,G,F	Gentamycin
A,D,G	Ampicillin
A,C,F	Vancomycin
A,B,C,D,E,F,G,H	Fosfomycin
B,G	Linezolid
D,F	Nitrofurantoin
A,B,C,D,E,F,G,H	Ciprofloxacin
A,B,C,D,E,F,G,H	Erythromycin
F	Teicoplanin
A,B,C,D,E,F,G,H	Chloramphenicol
A,B,C,D,E,F,G,H	Quinupristin- dalfopristin

DISCUSSION

Among *Enterococcus* strains, *E. faecalis* is responsible for the majority of nosocomial infections [17]. The numbers of enterococcal infections have increased in the past two decades. Inherent increasing resistance to antibiotics, such as cephalosporin, has caused major complications in enterococcal infection treatments. Enterococci have a high ability to acquire dissemination factors, which are responsible for antibiotic resistance. Moreover, there is a wide range of health issues since resistance genes can be transferred between these microorganisms or to other species [18, 19].

Determining different types of bacteria causing nosocomial infections and genetic connections between them is very important to assess the origin of infection. A better insight about the means of microbial transmission among patients, personnel, and medical devices in a hospital environment can be gained by bacterial typing [20]. At present, the outbreaks of nosocomial infections can be prevented using various typing techniques in hospitals, which are of tremendous help to the economy and health communities [10, 21].

In this study, most of the strains were isolated from urine, and the rest were isolated from wound samples, blood, BAL fluid, and ascites. The results of this study are similar to those of Behnood's study. The high number of strains isolated from urine can be explained by the fact that enterococci are frequently responsible for urinary tracts infections [22].

In the current study, antibiotic resistance patterns to vancomycin, ciprofloxacin, erythromycin, tetracycline, chloramphenicol, and teicoplanin were the same as those in Ydo report (2003) from Kuwait [23].

The resistance patterns to vancomycin, observed in this study, corroborate those observed by Samadi et al. and the results are also similar to those revealed by Panesso from South America [24, 25].

In contrast to the results of Behnood's study, which showed that the resistance to linezolid was 18.9%, only 1.6% of our isolates were resistant to linezolid, which may be due to the difference in clinical specimens (all strains in Behnood's study were isolated from stool). Unlike the current study, Loza and Mihajlović-Ukropina showed that all isolated enterococci were sensitive to linezolid in Europe. Probably the differences in geographical regions, types of antibiotics, and methods used can be responsible for the discrepancies in prevalence [22, 26, 27].

The results showing that the majority of the 125 isolates of *E. faecalis* were resistant to tetracycline and quinupristin/ dalfopristin are similar to the data from Li study and those obtained by Jia in China [28, 29].

In the present study, the REP-PCR results demonstrated that the clinical isolates have eight different genetic patterns (A to H), which is in contrast to Malathum's study, in which isolates were classified into 26 clonal groups. This difference may be due to the geographical difference and the variety of clinical specimens (samples in the latter study were collected from the United States to Chile and Argentina) [16].

Our study showed that all isolates from the ICUs were resistant to quinupristin/dalfopristin and sensitive to linezolid. All group H strains were resistant to phosphomycin and sensitive to ampicillin. Five strains were resistant to tetracycline in group E, while teicoplanin resistance was observed only in group F, with the other groups sensitive. All strains of Group F were resistant to six or more antibiotic classes. This was in contrast with the results of Djahmi and Sherer's study, showing no relationship between genotyping data and antibiotic resistance patterns. It should be noted, however, that the above studies were conducted with limited numbers of clinical specimens, which could explain the lack of relationship between the patterns and their resistance to antibiotics [30, 31].

In this study, strains with identical patterns were found in both hospitals; this signifies a massive transfer of bacteria between the hospitals. Bacteria can be transferred via medical devices, hospital personnel, and other people who have been in contact with patients. In Singh and Dunne's studies, the same shared patterns were observed among hospitals as in this study [32, 33]. According to the Singh's report, isolates collected from patients and environmental samples generated similar PCR products and were classified in the same group [32]. In this study, the transfer of bacteria via medical tools and personnel was confirmed. Our study also detected identical patterns within the hospitals, which indicates the spread of bacteria among various sectors. These results are in line with the studies of Djahmi and colleagues

performed in Algeria, where they obtained patters of typing indicated that the infection prevailing in a hospital had the same origin [30].
In conclusions, the results of the present study show a great resistance of *E. faecalis* isolates to some commonly used antibiotics and their clonal propagation in different wards of the hospitals. Furthermore, this study showed that the strains responsible for infection in the hospital wards have a shared genetic origin and are genetically related. The similarity between patterns from different hospitals can be explained by the transfer of patients between hospitals in Iran. The results also indicate that the REP-PCR technique can be the best approach to distinguishing *E. faecalis* strains and an appropriate way of control and prevention of bacterial spread. Hopefully, this molecular method would be essential for hospitals in the future.

Conflict of interest. The authors have no conflicts of interest to disclose.

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