

## Molecular Identification of Pathogenic Enterococci and Evaluation of Multi-drug Resistance in *Enterococcus* Species Isolated From Clinical Samples of Some Hospitals in Tehran, Iran

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### KEYWORDS

*Enterococcus faecalis*  
*Enterococcus faecium*  
Multi-drug Resistance  
Polymerase Chain Reaction

### ABSTRACT

**Background and Objectives:** Multidrug-resistant (MDR) enterococci cause many problems for physicians and infection control specialists in the recent years. Hence, by identification of antibiotic resistance patterns of enterococci in different geographical regions, an appropriate strategy can be developed to prevent bacterial antibiotic resistance and provide effective treatments. The current study aimed at identifying enterococci via molecular methods and evaluating multi-drug resistance patterns in *Enterococcus* species isolated from nosocomial samples of some hospitals in Tehran, Iran.

**Material and Methods:** The current study was conducted on 300 nosocomial samples from different hospitals in Tehran, Iran. The identified *Enterococcus* species of *E. faecalis* and *E. faecium* were isolated via biochemical testing and confirmed using polymerase chain reaction (PCR). The antibiotic resistance pattern was determined using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

**Results:** The highest antibiotic resistance was observed against quinupristin-dalfopristin, tetracycline, and erythromycin. Minimum inhibitory concentration (MIC) of vancomycin against the isolated antibiotic resistant *Enterococcus* spp. was  $\leq 256 \mu\text{g/mL}$ . According to the results of the current study, 69.6% of *E. faecalis* and 80% of *E. faecium* isolates showed multi-drug resistance.

**Conclusion:** Increase of antibiotic resistant bacteria, especially MDR species, is a severe health threatening problem worldwide. The increase of MDR bacteria limited the therapeutic solutions to the patients with enterococcal infection, increased treatment costs, and led to transmission of resistant genes among bacteria. It is highly important to find antibiotic resistant patterns to compile guidelines for infectious diseases.

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### Introduction

*Enterococcus* species are considered as one of the main nosocomial pathogens in the recent years. The urinary tract infection followed by pelvic and intra-abdominal infections, bacteremia, endocarditis, etc. are among the most common nosocomial infections caused by enterococci. Such bacteria are the second leading cause of urinary tract

infection and the third in nosocomial infections. *Enterococcus* spp. are Gram-positive cocci and the intestinal flora of human and some animals (1,2). *Enterococcus* species show poor pathogenicity due to lack of powerful toxins and remarkable virulence factors, but since they are intrinsically insensitive to penicillins, aminoglycosides, and glycopeptides,

and show high competence in receiving resistance genes; can transfer antibiotic resistance genes to other bacterial genera and species through mutations and/or receiving genetic elements via plasmids or transposons, *Enterococcus* spp. can cause severe problems in diagnosis, treatment, and control of the associated infections (3).

Nowadays, the multi-drug resistance and insensitivity of such bacteria to antibiotics is of great importance (4). Glycopeptides-resistant *Enterococcus* spp. are serious and alarming problem worldwide, which affect patients' general health. Glycopeptides such as vancomycin and teicoplanin are the selective and usually the last-resort choice for the hospital-acquired multidrug-resistant (MDR) Gram-positive infections (5). Inappropriate administration and indiscriminate use of broad-spectrum beta-lactams, aminoglycosides, and carbapenems as well as long-term hospitalization influence the spread of MDR bacteria (6).

Administration of proper antibiotics should always rely on the antibiotic resistance patterns because the treatment of nosocomial infections caused by such bacteria is very difficult; hence, periodic evaluation of the anti-microbial activity of different antibiotics and understanding the proper drug regimen to treat the infection and control its spread is of great importance because the antibiotic resistance pattern may change within short intervals (7). Transfer of the resistance genes among bacteria via transposons/plasmids, and genetic mutations are among the major causes of the prevalence and spread of antibiotic-resistant bacteria. Since a plasmid or transposon can be the carrier for several resistance elements, resistance to several antimicrobial agents can be acquired simultaneously; it is considered as the reason for the emergence of multidrug-resistant bacteria. Nowadays, increasing multidrug-resistant species and the associated complications are among the great concerns of medical community. Such infections are difficult-to-treat and even in some cases threaten the patients' lives. In addition, due to long-term hospitalization, the treatment is costly; hence, the infections can be significantly controlled and the prevalence can be remarkably limited by the application of cost-effective methods and hospital hygiene standards, as well as microbiologic

methods. Identification of Enterococci resistance patterns to commonly used antibiotics to determine therapeutic policy in the initial encounter and effectiveness of controlling programs are essential (8).

In the recent years, molecular techniques such as polymerase chain reaction (PCR) are developed; such techniques are used to identify enterococci as they are very sensitive and benefit from high speed and specificity, compared with the culturing methods (9). Employment of phenotypic methods parallel to genotypic PCR-based methods can provide reliable data. In the current study, both classic and molecular methods were used to identify genus and species. In addition, the frequency, antibiotic resistance pattern, and the prevalence of multidrug-resistance were also studied in *Enterococcus* species isolated from nosocomial infections in Tehran, Iran.

## Materials and methods

### Sampling

The current descriptive-cross sectional study was conducted on 300 samples including urinary tract, blood, wound infections, as well as ascites and bronchoalveolar lavage (BAL) collected from Baqiatallah, Milad, and some other hospitals in Tehran, 2015.

### Identification of *Enterococcus* species by phenotypic and genotypic methods

To identify bacteria isolated from clinical samples, Gram staining method as well as the evaluation of the biochemical characteristics such as catalase reaction, growth in 6.5% NaCl, and bile-esculin hydrolysis were performed. Besides, to identify the bacteria at the species level, the sugar fermentation test (lactose, arabinose, etc.) was conducted using a sugar-based media containing phenol red reagent and incubation at 37°C for 24 hours. All isolations were stored at -70°C in brain heart infusion (BHI) broth plus 50% glycerol until molecular analyses.

Bacterial genome was extracted using boiling methods; then, to confirm *E. faecalis* and *E. faecium*, PCR was amplified using specific primers. PCR reaction was performed in a total volume of 25 µL containing 1 µL DNA pattern (0.2 µg), 1 µL

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each of the primers (10 pmol), 12 µL 2X Master mix (purchased from Amplicon III, Denmark including 20 mM dNTP and 1.5 mM MgCl<sub>2</sub>), and 11 µL double distilled water. Then, the following PCR program was set using a Eppendorf thermocycler (Hamburg; Germany): early denaturation for 1

minute at 94°C, 35 cycles of denaturation for 1 minute at 94°C, and annealing for 1 minute at 55°C and early extension for 2 minutes at 72°C. The final extension was done for 5 minutes at 72°C. The PCR product was electrophoresed on 1.5% gel agarose in a Gel Documentation (Table 1).

**Table 1.** The Primer Sequences Used for PCR Amplification to Identify *Enterococcus faecalis* and *Enterococcus faecium*

Primer	Primer Sequence	Size of the Fragment	Reference
<b>ddl <i>E. faecalis</i></b>	F- 5'ATCAAGTACAGTTAGTCT 3' R- 5'ACGATTCAAAGCTAACTG 3'	941 bp	10
<b>ddl <i>E. faecium</i></b>	F- 5'TAGAGACATTGAATATGCC 3' R- 5'CTAACATCGTGTAAGCT 3'	550 bp	10

### Antibiotic profile

The disk diffusion method was used to determine the antibiotic sensitivity pattern of the isolates on Muller-Hinton Agar (Merck), according to the Clinical and Laboratory Standards Institute (CLSI) 2015 guidelines; results were reported as resistant, semi-sensitive, and sensitive for each antibiotic (11).

Vancomycin 30 µg, erythromycin 15 µg, quinupristin / dalfopristin 15 µg, tetracycline 30 µg, ciprofloxacin 5 µg, teicoplanin 30 µg, linzolaide 30 µg, fosfomycin 200 µg, chloramphenicol 30 µg, gentamicin 120 µg, ampicillin 10 µg, nitrofurantoin and 300 µg (Mast; UK), as well as *E. faecalis* ATCC29212 and *E. faecium* BM 4147 were used as the quality control species in the current study.

The minimum inhibitory concentration (MIC) for the isolates resistant to vancomycin was conducted based on the microdilution method. The isolation showed resistance to 3 or more antibiotic lines were considered as MDR species.

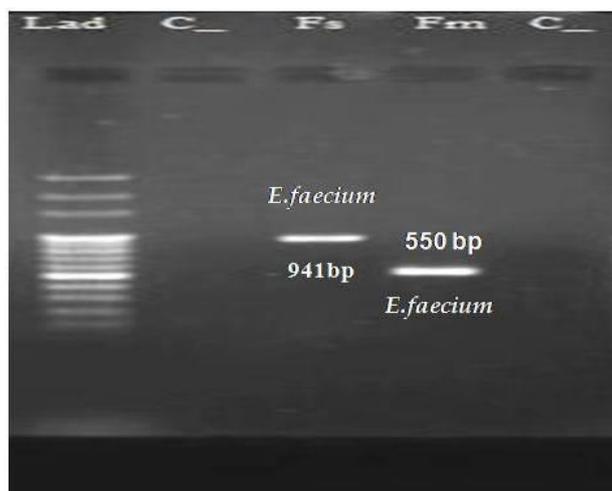
### Results

Out of 300 samples collected in the current study, 140 enterococcus species were identified, which constituted 68% of all urinary tract isolated species (Table 2).

Results of the phenotypic- based identification methods were confirmed by PCR amplification using specific primers; accordingly, out of 140 identified species 125 (89.28%) were *E. faecalis* and 15 (10.72%) *E. faecium* (Figure 1).

**Table 2.** The Frequency Percentage of *Enterococcus faecalis* and *Enterococcus faecium* Species Isolated from Nosocomial Samples in the Current Study

Sample Type	Isolated Enterococci , (N=140)	Isolated <i>E. faecalis</i> , N (%)	Isolated <i>E. faecium</i> , N (%)
<b>Urinary tract</b>	92	85 (68)	7 (46.7)
<b>Wound</b>	24	21 (16.8)	3 (20)
<b>Blood</b>	17	14 (11.2)	3 (20)
<b>BAL</b>	2	0	2 (13.3)
<b>Other</b>	5	5 (4)	0



**Figure 1.** PCR products; Lane 1) DNA marker 100 bp; Lane 2) The amplicon without the targeted gene in the

clinical isolations of *E. faecium*; Lane 3) The amplicon including *ddl* gene (550 bp) in the clinical isolation of *E. faecium*; Lane 4) The amplicon including *ddl* gene (941 bp) in the clinical isolations of *E. faecalis*; Lane 5) The amplicon without targeted gene in the clinical isolations of *E. faecalis*

### Antibiotic susceptibility testing

The highest antibiotic resistance in the studied isolates was observed against quinupristin / dalfopristin, followed by tetracycline and erythromycin. Results of antibiogram testing for the current study isolates, according to CLSI guidelines, are shown in Table 3.

**Table 3.** Results of Antibiotic Susceptibility Testing for the Study Isolates, Based on the Disc Diffusion Method

Antibiotic	<i>Enterococcus faecalis</i>			<i>Enterococcus faecium</i>		
	Sensitive, N (%)	Semi-sensitive, N (%)	Resistant, N (%)	Sensitive, N (%)	Semi-sensitive N (%)	Resistant, N (%)
Tetracyclin	23 (18.88)	0	102 (81.12)	4 (26.68)	0	11 (73.32)
Gentamicin	80 (64.51)	23 (18.29)	22 (17)	9 (60)	4 (26.8)	2 (13.2)
Ampicillin	115 (92)	0	10 (8)	9 (60)	0	6 (40)
Vancomycin	113(90.04)	9(6.8)	4 (3.16)	10 (66.8)	1 (6.52)	4 (26.68)
Fosfomycin		25 (20)	47(37.45)	53(42.55)	6 (40)	5 (33.32)
Linzeolid		116(92.8)	8 (6.27)	1 (0.08)	14(93.48)	0
Nitrofurantoin		120 (96)	3 (2.4)	2 (1.6)	10 (66.8)	2 (13.2)
Ciprofloxacin		38(30.04)	44(35.65)	43(34.4)	3 (20)	0
Erythromycin		19(15.2)	37(29.6)	69(55.2)	1(6.52)	1(6.52)
Teicoplanin		119(95.2)	4(3.51)	2(1.29)	10(66.8)	0
Chloramphenicol		85(68)	21(16.8)	19(15.2)	4(26.68)	3(20)
Quinpristin/dalfopristin		11(8.8)	5(4)	109(87.2)	4(26.68)	3(20)

**Table 4.** The Frequency of Multidrug-resistant *Enterococcus faecalis* and *Enterococcus faecium* Species Isolated in the Current Study

Species	Number of the Isolates Resistant to One or More Antibiotics				
	1	2	3	4	>4
<i>E. faecalis</i>	12	27	28	21	37
<i>E. faecium</i>	2	1	2	1	9

In the current study, 3.16% of *E. faecalis* and 26.68% of *E. faecium* species showed resistance to vancomycin. MIC of the isolated species to vancomycin was  $\geq 256$   $\mu\text{g/mL}$ . All vancomycin-resistant isolates showed MDR; in such a way that 1 vancomycin-resistant *E. faecalis* isolate showed resistance to 4 other antibiotics, 1 *E. faecium* isolate was resistant to 12 antibiotics, 1 isolates of *E. faecalis* was resistant to 7 antibiotics, 1 isolate of *E.*

*faecalis* was resistant to 9 antibiotics, 3 isolates of *E. faecium* were resistant to 8 antibiotics, and 2 *E. faecalis* isolates showed resistance to 6 antibiotics.

### Discussion

During the recent years, the growing prevalence of enterococci infections with antibiotic resistance caused severe and significant complications in the health care system of different countries (12). The

researchers showed that although the level of general health enhanced in the communities, the bacterial infections, particularly enterococcal infections, increase, which mainly results from intrinsic resistance of *Enterococcus* spp. to antibiotics and the linear transfer of resistant genes, transposons, and plasmids among enterococci and/or other bacterial genera. Besides, indiscriminate and improper administration of antibiotics is another reason for the spread of antibiotic resistance in different geographical regions. Uncontrolled consumption of broad-spectrum beta-lactams as well as long-term hospitalization influenced the spread of multidrug-resistant bacteria; the treatment of such infections is difficult and may lead to mortality (3, 5, 6).

*Enterococcus faecalis* is the most *Enterococcus* species isolated from clinical samples in the majority of the studies. In the current study, out of 140 isolated enterococci, 125 (89.28%) were *E. faecalis* and 15 (10.72%) *E. faecium*. In the studies by Dadfarma, Kafil, and Comerlato, most of the clinically isolated species were *E. faecalis*, consistent with the results of the current study (13-15). However, in another study by Bibalan and Oskuie, most of the isolated enterococci were *E. faecium*, inconsistent with the results of the current study; the difference between the results may be attributed to the type of samples and the type of commonly administered antibiotics (16, 17). In the current study, similar to those of Padmasini and Behnood, most of the enterococcal species were isolated from urinary tract samples. The dominance of enterococci among the species isolated from urinary tract samples is another reason for the prevalence of enterococci in the urinary tract infections. The bacterial multidrug-resistance has various frequencies in different countries, which may be attributed to genetic changes in bacterial species, different normal flora, and different antibiotic prescription patterns, as well as different rates of antibiotic consumption in the communities (18, 19).

In the current study, *E. faecalis* isolates showed the highest resistance to tetracycline, quinupristin / dalfopristin, erythromycin, and fosfomicin, respectively. In addition, *E. faecium* isolated species were mostly resistant to tetracycline, ciprofloxacin,

erythromycin, and quinupristin / dalfopristin. The results of the current study were similar to those of Seifi and Talebi (20, 21).

In the current study, out of 140 enterococci isolates, 2 isolates (*E. faecalis* and *E. faecium*) were resistant to linzolaide, similar to the results of Yaslani and Bhatt (22, 23). But, in the studies by Kafil Bibalan, Mahesh, and Wang, all isolated species were sensitive to linzolaide; the difference can be attributed to the type of study, geographical region, type of antibiotic, and the employed methods (14, 16, 24, 25). The resistance to gentamicin and ampicillin in the current study was similar to the results of Tabatabaei and in contradiction with the results of Yadegarynia (26, 27).

According to the results of the current study, resistance to vancomycin in *E. faecalis* and *E. faecium* was 3.16% and 26.68%, respectively. In the studies by Sanal and Tabatabaei, most of the species were sensitive to vancomycin (26, 28). In addition, studies by Hoseini Zadeh and Kafil reported higher levels of resistance to vancomycin, compared with the current study (14, 29).

The emergence of multidrug-resistant *Enterococcus* spp. is critically important and 69.6% of *E. faecalis* and 80% of *E. faecium* species isolated in the current study showed multidrug-resistance. In the studies by Bibalan and Aleyasin, the frequency of MDR species was lower than that of the current study (16 vs. 2 MDR species) (36, 16). Results of the studies by Wang et al. and Sharifi et al., showed that more than 80% and 79% of the enterococci isolates were MDR, respectively (15, 31). In addition, results of the studies by Dadfarma, Behnood, and Deshpande reported 45.7%, 49.59%, and 57% of the isolated enterococci as MDR, respectively (14, 19, 32).

Also, results of the studies by Telkar, Mira, and Mahesh reported the prevalence of MDR species (24, 33, 34).

### Conclusion

Results of the current study indicated that *E. faecalis* was the most common isolated species in the clinical samples. The majority of enterococcal species were isolated from urinary tract samples. In addition, results of the current study showed that the isolated species of *E. faecalis* were more resistant

against tetracycline, quinupristin / dalbapristin, erythromycin, and fosfomicin, but *E. faecium* showed more resistance against tetracycline, ciprofloxacin, erythromycin, and quinupristin / dalbapristin. Most of the isolated *Enterococcus* spp. were resistant against 3 or more antibiotics and accordingly were considered as MDR bacteria. Hence, administration of proper antibiotics based on the results of timely and meticulous antibiogram plays a significant role in the treatment and prevention of the MDR bacteria spread. Accordingly, it is recommended to compile more detailed and distinct instructions for more logical administration of antimicrobial agents and prevent infections, particularly nosocomial infections.

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