Original Article

Identification of Enterococci faecalis & E. faeacium pathogens viaTehran hospitals clinical samples by phenotypic and genotypic methods and Evaluation of Antimicrobial Susceptibility in 2015

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Abstract

Introduction:
Epidemiology of Enterococcus infections has attracted much attention. Precise identification of pathogenic strains can be effective in the control process of microorganism antibiotic resistance. This study aimed to identify the Enterococcus species isolated from hospitals in Tehran and examine their antibiotic resistance pattern by phenotypic and genotypic methods.

Methods & Materials:
This study was performed on 400 clinical samples from different hospitals in Tehran during 2015-2016. Specific cultures and biochemical tests were used to identify Enterococcus, distinguish E. faecalis and E. faeacium species and PCR method was used to identify Enterococcus species. Antibiotic susceptibility was examined using Kirby-Bauer disc diffusion, and CLSI and vancomycin MIC were measured using broth dilution.

Results:
Of the 400 samples, 278 Enterococcus species were recognized. Phenotypic methods recognized 70.86% E. faecalis, 15.46% E. faeacium and 13.68% other species. PCR identified 72.3% E. faecalis, 10.43% E. faeacium and 17.27% other Enterococcus species. Results of the antibiograms showed the highest resistance (83.34%) to quinupristin/dalfopristin, and the lowest (1.41%) to linezolid. Also, resistance to vancomycin was observed in 5.95% with MIC ≥ 512 µg/ml in 9 cases.

Conclusion:
Rapid diagnosis can prevent massive outbreaks of Enterococcus. Given the prevalence of vancomycin resistance in Enterococcus, preventive measures are imperative. The right antibiotics should be prescribed according to the resistance patterns after susceptibility test is performed for each patient.

Keywords: Enterococcus faecalis, Enterococcus faecium, Antibiotic Resistance, PCR

Introduction

Enterococci are the most important normal flora of the digestive system of human and many animals (1). These bacteria have little virulence due to lack of toxins and

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strong pathogenic agents, however, they can cause important diseases such as urogenital tract infections, endocarditis, bacteremia, wound infections, intra-abdominal infections, pelvic infections and meningitis in infants (2-4). Typically, Enterococci are Gram-positive fermentative cocci that often occur in pairs or short chains (5). The catalase-negative bacteria lack spores and are anaerobic. They grow in salt 6.5% (sodium chloride), bile salts 40% and pH=9.6. Enterococcus can grow in 10-45 °C (6). Enterococcus faecalis and Enterococcus faecium can withstand a temperature of 60 °C for 30 minutes which distinguish them from other Enterococcus species (7, 8). The pathogenesis of Enterococcus was reported by Hastings and McCallum in the late nineteenth century (8).

These bacteria are presently known as the major infectious agents, especially nosocomial infections. The epidemiology of enterococcal infections has attracted a lot of attention recently and have led to dramatic changes in this field. According to National Nosocomial Infections Surveillance (NNIS) System of the US, Enterococci are considered as one of the most important hospital-acquired pathogens. These bacteria are the fourth leading cause of nosocomial infections and the third leading cause of bacteremia and the second leading cause of urinary tract infections. Various studies blame the increasing resistance to antibiotics around the world for this situation (9). Accordingly, and since bacteria can survive in a wide range of environments, detection of pathogenic strains is particularly important for control and prevention of infections (10). The accurate detection of pathogenic strains is effective in controlling the process of antibiotic-resistance of microorganisms. Today, increasing resistance to all antibiotics is a major global problem. Enterococci have intrinsically moderate resistance to cephalosporins, penicillins, and aminoglycosides. Since 1986, there have been increasing reports of resistance to vancomycin which was first used in 1972 for the treatment of Enterococcus (11-13). Hence, the accurate and rapid detection of pathogenic strains of Enterococcus improves detection methods and creates new detection methods (14-16).

Previously, conventional phenotypic methods were used to detect enterococcal infections. Although these methods are sometimes associated with useful information, they are not enough for distinguishing species and are limited (17). Since the development of molecular methods in 1990, especially PCR, rapid and accurate differentiation of bacteria species including Enterococci have become possible for investigating the prevalence of diseases (18-21). Furthermore, phenotypic methods combined with genotypic methods based on PCR molecular techniques can provide valuable information. The present study was conducted to identify Enterococcus species through phenotypic and PCR molecular methods. The antibiotic resistance of the isolated samples was studied, too.

Materials and Methods

Samples collection

This cross-sectional descriptive study was conducted on 400 clinical samples of urine, wounds, blood, ascites, etc. suspected to Enterococcus infection by simple random sampling from Baqiyatallah Hospital and Milad Hospital in Tehran, Iran, from March 2014 to January 2014. The samples were transferred from hospitals' diagnostic laboratory to the research laboratory in sterile conditions at 4 °C.

The identification of Enterococcus species by phenotypic method

After preparing 24-hour pure cultures of clinical samples on the blood agar media, positive cultures underwent Gram staining, catalase test, bile salt hydrolysis (bile salts 40%) and growth in BHI containing salt

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6.5% (sodium chloride) in order to distinguish Enterococcus species by phenotypic method (16).
The fermentation of arabinose, mannitol, sorbitol, sorbose, lactose and other sugars was performed in tubes containing the Phenol Red Broth medium and one percent of the sugars and incubation at 37 °C for 24 hours in order to differentiate Enterococcus species. The Phenol Red Broth medium was sterilized by autoclave and its pH was set at 7.4-7.5 by sterile NaOH. The yellow color of the broth indicated a positive reaction (16).

Antibiotic test clinical specimens

Antibiotic test was conducted using gentamicin 10 µg, vancomycin 30 µg, teicoplanin 30 µg, linezolid 30 µg, phosphomycin 50 µg and quinu-/dalfo-pristin 15 µg discs (Mast company) by disk diffusion method on Mueller-Hinton agar medium (Merck) and 0.5 McFarland bacterial suspension. Resistance phenotype was determined according to the CLSI instructions. After 18 hours of incubation at 37 °C, the minimum inhibitory concentration (MIC) was read. The Broth Dilution method was used to determine MIC of Vancomycin-resistant Enterococci. The standard strains of E. faecalis ATCC 29212 was used as a positive control in this study.

The identification of Enterococcus species by PCR method

After extracting bacterial genomes of positive culture samples by boiling, specific primers of $ddl_{E.\ faecalis}$ and $ddl_{E.\ Faecium}$ species were used in order to identify Enterococcus species (17) (Table 1).
PCR was performed with a final volume of 25 µl including 1 µl of template DNA (5.0 mcg/L), 1 µl of each primer (10 pmol), 12 µl 2X Master mix (Ampliqon III, Denmark, containing 20 mM dNTP, 1.5 mM MgCl2) and 11 µl of double distilled water. PCR was set in a thermocycler device (Humburg, Germany, Eppendorf) with primary denaturation of one minute at 94 °C and 35 cycles at 94 °C for one minute, denaturation temperature of 55 °C for one minute, and the temperature of initial elongation of 72 °C for two minutes. The final elongation was at 72 °C for 5 minutes. The PCR product was observed on Agarose gel 1.5% containing Safe Stain Electrophoresis by Gel Documentation device (Cambridge, England, Uvitec).
All ethical issues were observed in this study under the 93-10 code.

Table 1: The sequence of primers used in gene amplification of $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$

Reference	Size	(5'→ 3') Sequence	Primer's name
(17)	941 bp	F:ATCAAGTACAGTTAGTCT R:ACGATTCAAAGCTAACTG	$ddl_{E.\ faecalis}$
(17)	550 bp	F:TAGAGACATTGAATATGCC R:CTAACATCGTGTAAGCT	$ddl_{E.\ faecium}$

Statistical Analysis

The collected data was analyzed by the SPSS software version 16 using Fisher and Mann-Whitney tests at a confidence level of 95%. The statistical significance level was considered as $P<0.05$.

Results

In this study, 278 Enterococcus samples were identified from 400 clinical isolates, and 80% of identified species were isolated from urine. Figure 1 shows the frequency of sample sources from urine, wounds, blood, vagina, BAL, etc. Age and gender were not considered in collecting samples.

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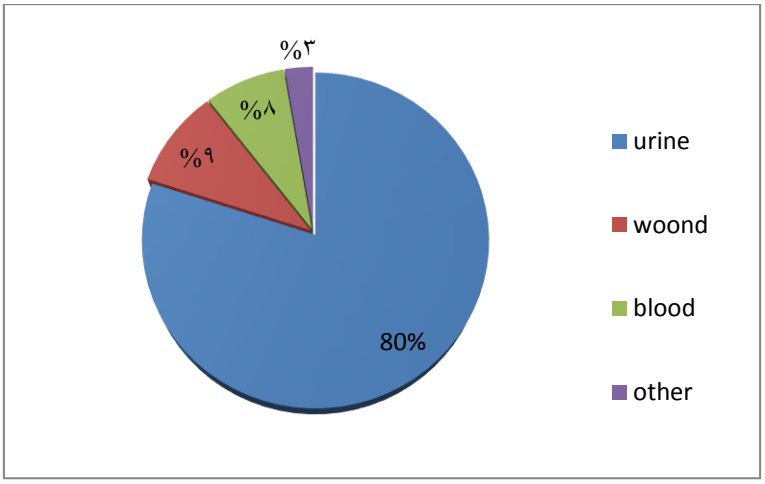

Figure 1: The frequency of Enterococci isolated from clinical samples

The confirmation of Enterococcus species by phenotypic methods

The results of species identification using biochemical methods showed that genus Enterococcus was detected in 278 samples, and proprietary tests detected 197 cases of E. faecalis (70.86%), 43 cases of E. faecium (15.46%) and 38 isolates of other Enterococcus species (13.68%). Among the 208 samples from Baqiyatallah hospital, 137 strains were E. faecalis (65.86%) and 32 strains were E. faecium (15.38%); and among the 70 samples of Milad Hospital, 50 strains were E. faecalis (71.42%) and 11 strains were E. faecalis (15.71%).

Antibiotic resistance test results

Based on antibiotic resistance pattern, the Enterococcus species showed the highest resistance to quinu-/dalfo-pristin (83.34%) and phosphomycin (37.84) and the lowest resistance to linezolid (1.41%) (Table 2 and Figure 2).

The strains with a halo diameter of ≤14 mm for vancomycin were selected for determining MIC. The studied isolates that well grew around the 30 µg vancomycin disc were highly resistant to vancomycin (MIC≥512g/mL). These samples contained 6 E. faecalis and 3 E. faecium strains.

From the 6 vancomycin-resistant E. faecalis strains, 2 were obtained from the Baqiyatallah Hospital and 4 from the Milad Hospital. Also, 1 vancomycin-resistant E. faecium strain was from the Baqiyatallah Hospital and 2 were from the Milad Hospital.

Table 2: Various antibiotic resistance in Enterococci

Antibiotics	Total resistance	Baqiyatallah Hospital	Milad Hospital
Gentamicin	20.87%	15.18%	26.56%
Vancomycin	5.95%	2.53%	9.37%
Ticoplanin	5.32%	1.26%	9.38%
Phosphomycin	37.84%	30.37%	45.31%
Linezolide	1.41%	1.26%	1.56%
Quinu-/dalfo-pristin	83.34%	81.01%	85.67%

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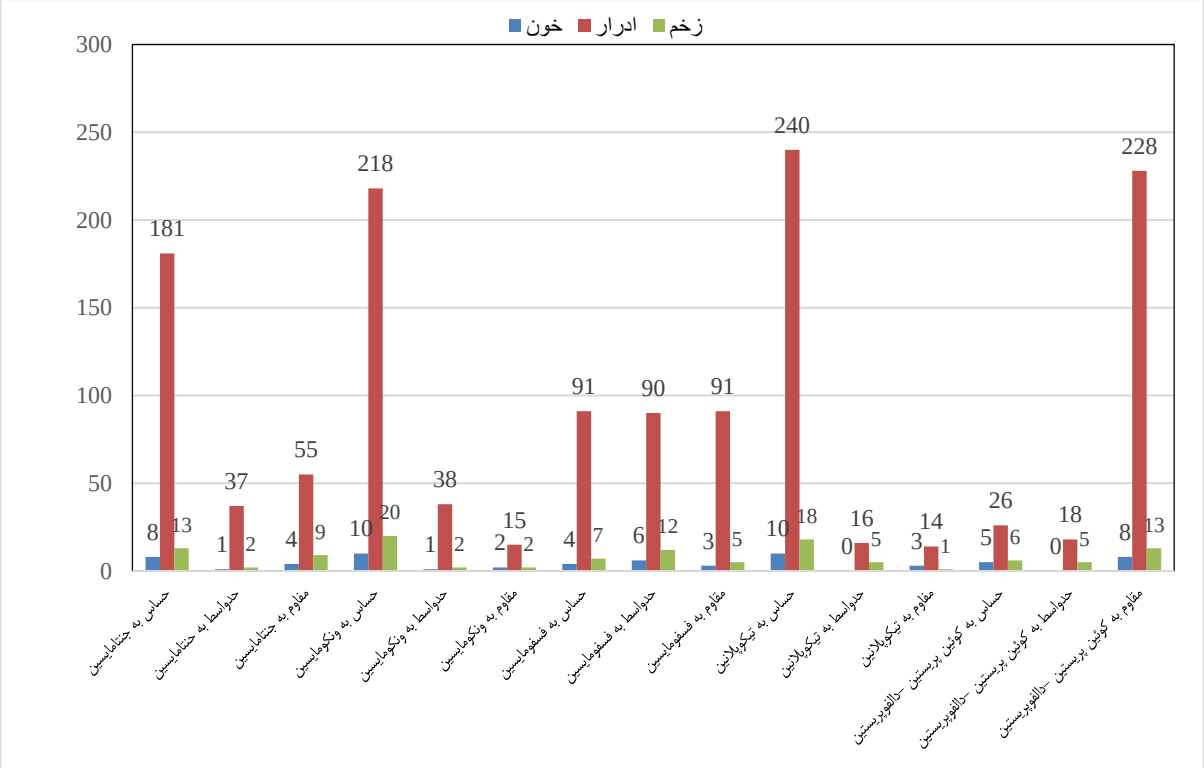

Figure 2: Antibiotic resistance of Enterococcus samples based on hospital

The confirmation of Enterococcus species by PCR methods

The results of PCR using specific primers showed (Figure 1) that of the 278 samples detected as genus Enterococcus by phenotypic testing, the specific primers of E. faecalis and E. faecium detected 201 strains of E. faecalis (72.3%) and 29 strains of E. faecium (10.43%) and 49 strains of other Enterococcus species (17.27%). Among the 208 samples from Baqiyatallah Hospital, 154 strains were E. faecalis (70.3%) and 21 strains were E. faecium (10.09%); and among the 70 samples of Milad Hospital, 50 strains were E. faecalis (80%) and 8 strains were E. faecalis (11.42%) (Table 3).

Statistical analysis results of the samples

The results of data analysis with Mann-Whitney test (Table 3) showed that the prevalence of Enterococcus in Milad Hospital was significantly higher than Baqiyatallah Hospital ($P<0.05$).

Fisher's test in R software showed that based on the origin of samples, resistance to quinu-/dalfo-pristin in urine samples was significantly prevalent compared to the sum of all other antibiotics ($P=0.048$). Vancomycin had the highest and gentamicin had the lowest resistance among wound samples ($P=0.007$). The resistance to quinu-/dalfo-pristin in blood samples was significantly prevalent as in urine samples.

The analysis of all antibiotic resistance patterns, regardless of the origin of the isolates, showed that resistance to vancomycin had no significant relationship with simultaneous resistance to phosphomycin ($P=0.488$) and teicoplanin ($P=0.310$).

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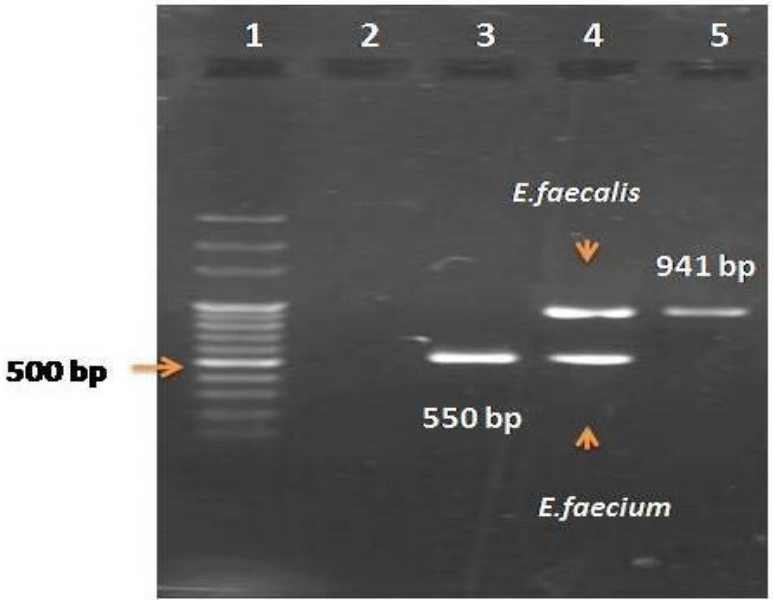

Figure 1: PCR products; Well (1) marker (DNA 100bp); Well (2) unamplified product of clinical samples without genes; Well (3) amplified product of ddl E. faecium gene in the studied clinical samples (550 bp); Well (4) amplified product of both genes; Well (5) amplified product of ddl E. faecalis gene (941 bp) in the studied clinical samples.

Table 3: The prevalence of strains isolated from clinical samples in Milad and Baqiyatallah hospitals

	Bacterial species	Total prevalence	Prevalence at Baqiyatallah Hospital	Prevalence at Milad Hospital
Phenotypic methods	E. faecalis	70.86%	65.68%	71.42%
	E. faecium	15.46%	38.38%	15.71%
	Other Species	13.68%	18.94%	12.87%
Genotypic methods	E. faecalis	72.3%	70.03%	80%
	E. faecium	10.43%	10.09%	11.42%
	Other Species	17.27%	19.88%	8.58%

Discussion

In this study, phenotyping methods reported 70.86% E. faecalis, 15.46% E. faecium and 13.68% other Enterococcus species; and molecular methods reported 72.3% E. faecalis, 10.43% E. faecium and 17.27% other Enterococcus species. The prevalence in this study was consistent with a study by Valenzuela et al. (2014) to identify Enterococcus species using biochemical and molecular tests on 153 samples isolated from milk and cheese (22). The results are also similar to those of Schouten et al. (23). In that study in 27 European countries, the prevalence of Enterococcus strains was more diverse. The strains were identified using biochemical tests, the results of which were similar to the present study (23). Accordingly, E. faecalis and E. faecium accounted for the highest prevalence percentage of enterococcal infections, respectively.

Furthermore, according to the studies mentioned above, it is important to correctly identify Enterococcus at species level. Thus, PCR method is used as a quick and easy method for rapid detection of Enterococcus species to prevent its massive outbreaks. Besides, phenotypic methods used for primary identification of genus Enterococcus can be problematic because of its similarity with the Streptococcus Group in appearance and structure.

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There are studies that unlike the results of this study have reported a lower prevalence of E. faecalis. Labib et al. (2013) identified Enterococcus species using phenotypic and molecular methods and reported statistical differences between the prevalence of E. faecalis and E. faecium (24). They also demonstrated that there are statistical differences between detected strains that might be due to the materials and conditions of culture media preparation in phenotypic methods.
According to the latest studies, the prevalence and antimicrobial resistance have increased in Iran since 2011. Mohammadi et al. (2011) conducted a study in Kermanshah where 5.5% of 128 Enterococcus samples were resistant to vancomycin (25). Shokoohizadeh (2014) reported 19 resistant strains of 144 Enterococcus strains (26). In the same year, Moaddab et al. reported 22 resistant strains of 193 samples in Zanjan hospitals (27). In 2015, Abbasi et al. reported 10 strain with resistance genotype to vancomycin in Shahr-e Kord (28). All studies in Iran have reported more prevalence of E. faecalis compared to E. faecium.

Conclusion

In the present study, the frequency of detected E. faecalis and E. faecium isolates were different in phenotypic and genotypic methods. Due to the phenotypic similarity of Enterococci to Streptococcus of Group D and according to the results of this study, genotypic methods can be used to identify the genus Enterococcus. However, it is better to use a combination of phenotypic and genotypic methods as complementary methods for rapid and definitive detection. Rapid detection can be useful to prevent a massive outbreak of the Enterococci. Furthermore, due to the diversity of vancomycin-resistant enterococci strains, it is necessary to take preventive measures. Due to resistance patterns, antibiogram is recommended for each patient before treatment in order to prescribe the right antibiotics.

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Conflict of interest

There is no conflict of interest between the authors and the journal.

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