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Virulence determinants and biofilm formation in clinical isolates of *Enterococcus*: A cross-sectional study

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ABSTRACT

Objective: To evaluate the relationship between biofilm formation and incidence of virulence determinants in clinical isolates of *Enterococcus*.

Methods: In this cross-sectional study, the clinical isolates of *Enterococcus* strains were collected from the university teaching hospitals in Ahvaz, Iran from June 2017 to June 2018. Then, the prevalence of *Enterococcus* species, antibiotic resistance, virulence factors, and biofilm-producing ability were determined.

Results: Of the 119 tested isolates, 17 (14.3%) were *Enterococcus faecalis*, 72 (60.5%) were *Enterococcus faecium* and 30 (25.2%) were other *Enterococcus* spp. Gelatinase was detected in 97 (81.5%) isolates, enterococcal surface protein in 41 (34.5%) isolates, serine protease in 39 (32.8%) cases, accessory colonization factor in 111 (93.3%) cases and pathogenicity islands in 17 (14.3%) cases. The biofilm formation ability was observed in 75 (63.0%) of all isolates and the association between the presence of enterococcal surface protein gene and biofilm formation was statistically significant. Higher resistance to vancomycin, gentamycin, and teicoplanin was indicated in *Enterococcus faecium* with 81.8%, 58.4%, and 85.7% resistance rate, respectively. All *Enterococcus faecalis* isolates were sensitive to teicoplanin and vancomycin.

Conclusions: The presence of antibiotic-resistance with several virulence factors in *Enterococcus* spp has become a concern. High prevalence of enterococcal surface protein gene among biofilm-producing isolates suggests a potential relation between biofilm formation and the enterococcal surface protein gene, and further studies are needed to identify the mechanism of biofilm inhibition.

KEYWORDS: *Enterococcus* spp; Biofilm formation; Antibiotic resistance; Virulence genes

1. Introduction

Enterococci are commensal bacteria in the gastrointestinal flora of animals and humans. In recent years, enterococci have evolved into the main causes of nosocomial infections, and they are one of the most frequent opportunistic pathogens isolated from urinary tract infections, infected surgical sites, and septicemia[1,2].

Enterococcus has two common species *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) which are involved in nosocomial infections with the prevalence of about 90% and 10%, respectively. The most infections caused by these bacteria are endogenous but cross-infection usually happens in hospitalized patients[3]. Also, the treatment of these infections has been clinically challenging because of the increasing resistance to different types of antibiotics, including β lactams, glycopeptides, aminoglycosides, macrolides and fluroquinolones[4,5]. The capability of *Enterococcus* to acquire antibiotic resistance through the chromosomal exchange, transfer of transposons and plasmids, or mutation makes it difficult to implement appropriate therapeutic measures[6].

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Virulence factors involve in the pathogenesis through the mediation of adherence and colonization, invasion into the host tissues, modulation of the host immunity, secretion of toxins and enzymes, which can enhance the infection intensity. Several virulence factors including the capsule formation and gelatinase [encoded by the chromosomal gelatinase (*gelE*)], aggregation substance, enterococcal surface protein [encoded by the chromosomal enterococcal surface protein (*esp*)] are involved in bacterial adherence and/or in biofilm production in the environment of hospitals[7].

Several enterococcal virulence factors have been identified to date, of which pathogenicity islands (*pai*), accessory colonization factor (*ace*), *esp*, serine protease (*sprE*) and *gelE* have been studied most intensively. Gelatinase, an extracellular zinc-containing metalloprotease, hydrolyzed collagen, and gelatin, has been recognized in dairy strains of *E. faecium* and has been shown to aggravate endocarditis in an animal model. Pathogenicity islands represent genetic elements that encode virulence factors related to bacterial pathogenesis[8,9].

In addition, one of the virulence factors that play a significant role in the pathogenesis of enterococcal infections is biofilm formation, which also helps the survival of the disease by preventing the penetration of antimicrobial agents[7]. The clinical impact of *E. faecium* may be increased by biofilm production, and these bacteria are frequently found in conditions where biofilm is necessary, including periodontitis, catheter-associated urinary tract infections, endocarditis, and other device-related infections, thereby making treatment of *E. faecium* with antibiotics more difficult. According to the study of Almohamad *et al.*, biofilm formation occurs less commonly in *E. faecium* compared with *E. faecalis*[10].

The aim of our study is to identify virulence genes, evaluate biofilm production and the antibiotic resistance in clinical isolates of *Enterococcus* obtained from the teaching hospital of Ahvaz and find the relationship between virulence genes and biofilm formation ability.

2. Materials and methods

2.1. Ethics statement

This study was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1396.1047).

2.2. Strains collection

In this cross-sectional study, clinical isolates that were suspected to be *Enterococcus* strains were collected from teaching hospitals in Ahvaz, Iran, from June 2017 to June 2018.

All isolates were cultured on MacConkey agar, blood agar, and bile esculin agar (Himedia, India). Culture characteristics and colony morphology were observed macroscopically. The genus *Enterococcus* was identified using gram staining, cultural characteristics, and biochemical tests, including *L*-pyrrolidinyl-β-naphthalyamide hydrolysis, bile esculin hydrolysis, and growth on 6.5% NaCl media at pH 9.6[11].

2.3. Identification of Enterococcus spp. strains by PCR assay

The DNA was extracted by the boiling method. Specific primers for *E. faecalis* and *E. faecium* were used (Table 1). Species were identified by PCR assay as follows: 1.5 mM $MgCl_2$, 1.5 pmol of each primer, 0.2 mM of each dNTP, and 0.625 U of Taq DNA polymerase[12]. The PCR conditions were as follows: 94℃ for 4 min, followed by 30 cycles of 94℃ for 40 s, 55℃ or 56℃ for 40 s, and 72℃ for 40 s and an extension at 72℃ for 5 min. The PCR amplicons were electrophoresed on 1% agarose gel. *E. faecium* ATCC 19434 and *E. faecalis* ATCC 29212 were used as control strains.

2.4. Susceptibility testing

Susceptibility of enterococcal isolates against teicoplanin (30 μg), vancomycin (30 μg), linezolid (30 μg), gentamycin (10 μg), fosfomycin

Table 1. The primers used in PCR.

Targets	Primer sequence(5'-3')	Product size	Temperature (℃)	Reference
Enterococcus faecalis	F-TCAAGTACAGTTAGTCTTTATTAG	940 bp	56	[12]
	R-ACGATTCAAAGCTAACTGAATCAGT			
Enterococcus faecium	F-TTGAGGCAGACCAGATTGACG	658 bp	55	[12]
	R-TATGACAGCGACTCCGATTCC			
gelE	F-CGAAGTTGGAAAAGGAGGC	372 bp	54	[8]
	R-GGTGAAGAAGTTACTCTGA			
pai	F-GACGCTCCCTTCTTTTGAC	387 bp	54	[8]
	R-CCAGAGAAATTACTACCAT			
esp	F-TTTGGGGCAACTGGAATAGT	407 bp	56	[8]
	R-CCCAGCAAATAGTCCATCAT			
ace	F-CAGGCCAACATCAAGCAACA	125 bp	58	[8]
	R-GCTTGCCTCGCCTTCTACAA			
sprE	F-GGTAAACCAACCAAGTGAATC	300 bp	56	[8]
	R-TTCTTCCGATTGACGCAAAA			

(200 μg), nitrofurantoin (200 μg), ampicillin (10 μg), ciprofloxacin (5 μg), erythromycin (15 μg), tetracycline (30 μg), chloramphenicol (30 μg) disks (Mast, United Kingdom) was determined using the Kirby-Bauer disk diffusion method on muller-Hinton agar, according to CLSI (2017) guidelines[13]. *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as quality control organisms.

2.5. Detection of virulence genes

The DNA was extracted by the boiling method. Genes including *ace*, *gelE*, *sprE* , *esp*, and *pai* were detected by PCR using primers listed in Table 1[8]. The PCR conditions were as follows: initial denaturation at 94℃ for 4 min; 30 cycles at 94℃ for 30 s, annealing for 30 s at the TA of the primer pairs, and extension at 72℃ for 30 s; followed by an extension at 72℃ for 5 min. PCR products were analyzed in 1.5% agarose gel (Invitrogen, Carlsbad, CA, USA) prepared in TBE buffer at 95 V for 60 min. After staining with ethidium bromide, it was observed under ultraviolet light.

2.6. Detection of biofilm production

For the detection of biofilm production, a 1: 10 dilution of overnight cultures in tryptone soy broth was inoculated in a microtitre polystyrene plate. After growth for 18 h at 37℃, the plates were washed thrice with phosphate-buffered saline. The adherent bacterial film was fixed by air drying at 60℃ for 1 h and stained with crystal violet; excess stain was washed with tap water. Then, the biofilm optical density was measured at 570 nm by a spectrophotometer. Biofilm formation ability was recorded as follows: OD<0.120, nonproducers, 0.120<OD<0.240, weak producers, OD>0.240, strong producers. Biofilm measurements were repeated at least thrice for each isolate[14].

2.7. Statistical analysis

SPSS v.22.0 statistics software (IBM Corporation, Armonk, NY, USA) was used for statistical analysis. Data were expressed as percentages, and analyzed by *Chi*-square test. The significance level was set as α=0.05.

3. Results

3.1. Bacterial isolates

A total of 119 clinical strains of *Enterococcus* spp. were obtained from different wards of Ahvaz teaching hospitals. A total of 43 (36.1%) isolates were from female patients and 76 (63.9%) were from male patients. A total of 92.5% of *Enterococcus* spp. were isolated from urine, 3.4%, 3.4% and 0.8% from blood wound secretion and ascites fluid, respectively. In addition, 72 (60.5%) isolates were identified as *E. faecium*, 17 (14.3%) as *E. faecalis* and 30 (25.2%) as other *Enterococcus* spp.

3.2. Antibiotic resistance pattern

The antibiotic-resistance of *Enterococcus* spp in different samples is shown in Table 2. More *Enterococcus* isolates were resistant to erythromycin (80.7%), followed by gentamicin (74.8%) and ampicillin (69.7%), tetracycline (59.7%). Resistance to these antibiotics was higher in *E. faecium* than other species. In this study, high sensitivity was observed to linezolid, fosfomycin, and nitrofurantoin.

3.3. Virulence factors

According to PCR results, 41 (34.5%) had *esp* gene, 97 (81.5%) had *gelE* gene, 39 (32.8%) had *sprE* gene, 111 (93.3%) had *ace* gene and 17 (14.3%) had *pai* gene (Table 3). One hundred twelve (94.1%) of the enterococci isolates carried 2-5 tested virulence genes. Three of *E. faecium*, 2 of *E. faecalis* and 1 of other *Enterococcus* spp harbored all tested virulence genes. In contrast, only one of the other *Enterococcus* spp was negative for all virulence genes.

3.4. Biofilm formation

The biofilm formation ability was observed in 75 (63.0%) of all isolates; 24 (20.2%), 33 (27.7%) and 18 (15.1%) were classified as weekly, moderately and strongly adherent, respectively. The relationship between virulence genes and biofilm formation is shown in Table 4. Association between *esp* positive and biofilm positive strains was statistically significant (P=0.030). No significant differences were found when comparing *gelE*, *sprE*, *ace*, *pai* positive and biofilm positive isolates (P>0.05) (Table 4).

Among the biofilm formers isolates, the highest resistance rate was 73% to gentamicin. Although, the resistance was higher in biofilm positive isolates there were no statistically significant differences between biofilm formation and antimicrobial resistance (P>0.05).

Table 2. Prevalence of antibiotic resistance among *Enterococcus* spp. isolates.

Antibiotics	Resistant [*n* (%)]	Intermediate [*n* (%)]	Susceptible [*n* (%)]
Vancomycin	33 (27.7)	21 (17.6)	65 (54.6)
Teicoplanin	21 (17.6)	8 (6.7)	90 (75.6)
Nitrofurantoin	12 (10.1)	9 (7.6)	98 (82.4)
Linezolid	9 (7.6)	4 (3.4)	106 (89.1)
Fosfomycin	3 (2.5)	9 (7.6)	107 (89.9)
Gentamicin	89 (74.8)	8 (6.7)	22 (18.5)
Erythromycin	96 (80.7)	15 (12.6)	8 (6.7)
Chloramphenicol	31 (26.1)	20 (16.8)	68 (57.1)
Tetracycline	71 (59.7)	5 (4.2)	43 (36.1)
Ampicillin	83 (69.7)	8 (6.7)	28 (23.5)
Ciprofloxacin	60 (50.4)	15 (12.6)	44 (37.0)

Table 3. Prevalence of virulence genes and biofilm among *Enterococcus* spp.

Species	*esp* positive [*n* (%)]	*gelE* positive [*n* (%)]	*sprE* positive [*n* (%)]	*ace* positive [*n* (%)]	*pai* positive [*n* (%)]	Biofilm positive [*n* (%)]
Enterococcus faecalis	8 (19.5)	12 (12.4)	8 (20.5)	16 (14.4)	5 (29.4)	10 (13.3)
Enterococcus faecium	29 (70.7)	57 (58.8)	26 (66.5)	66 (59.5)	10 (58.8)	46 (61.3)
Other spp.	4 (9.8)	28 (28.8)	5 (13.0)	29 (26.1)	2 (11.8)	19 (28.3)

Table 4. Relation between the presence of virulence gene and biofilm formation.

Genes	Biofilm positive isolates (*n*=75)		Biofilm negative isolates (*n*=44)		χ^2	*P*-value
	P^1 [*n* (%)]	N^2 [*n* (%)]	P^1 [*n* (%)]	N^2 [*n* (%)]		
esp	31 (41.33)	44 (58.66)	10 (22.73)	34 (77.27)	4.251	0.030
gelE	63 (84.00)	12 (16.00)	34 (77.27)	10 (22.73)	0.832	0.250
sprE	28 (37.33)	47 (62.67)	11 (25.00)	33 (75.00)	1.914	0.118
ace	70 (93.33)	5 (6.67)	41 (93.18)	3 (6.82)	0.001	0.624
pai	11 (14.67)	64 (85.33)	6 (13.64)	38 (86.36)	0.024	0.553

P^1: Positive, N^2: Negative.

4. Discussion

Over the past decades, enterococci have emerged as important nosocomial pathogens[15]. Because of its intrinsic resistance to harsh environments and antibacterial drugs, it can survive and spread in hospitals.

Biofilm plays a critical role in enterococcal infections and produces a context to increase bacterial survival in the host[16]. Due to the controversial status of enterococci, this research assessed biofilm formation, virulence genes and antibiotic resistance in 119 clinical enterococci isolates. Based on the results, the incidence of *E. faecium* was higher than *E. faecalis* isolates; however, *E. faecalis* is the main cause of enterococcal infections. This is in accordance with Arshadi *et al*.[17] and Moosavian *et al*.[18] who isolated enterococci from clinical samples in Ahvaz in the southwest of Iran. But it was different from the results of Shokoohizadeh *et al*.[19] and Emaneini *et al*.[11] in Tehran and Hashem *et al*.[20] in Egypt. They showed that the prevalence of *E. faecalis* (62.5%, 64.4%, and 72.2%) was higher than *E. faecium* isolates (37.5%, 35.6%, and 24.4%). In current years, an increase in *E. faecium* nosocomial infections can be seen in hospitals due to the emergence of vancomycin-resistant enterococci strains[17]. On the other hand, 92.5% of *Enterococcus* spp. were isolated from urine which is similar to previous studies[17,21,22].

Antibiotic resistance is a factor contributing to the pathogenesis of enterococci that can be acquired or found internally[23]. The highest resistance among all isolates was to erythromycin, gentamycin, and ampicillin. A similar study by Khani *et al*.[24] in Kermanshah indicated that most isolates of enterococci were resistant to ampicillin and erythromycin.

Also, the high prevalence of resistance to gentamycin has been previously reported[25,26]. In this study, according to drug susceptibility testing, 7.6% of our isolates showed resistance to linezolid and 3.4% of them had intermediate resistance. Previous studies conducted by Arabestani *et al*.[27] in 2017 and Feizabadi *et al*.[28] in 2008 in the west of Iran and Tehran shows that no resistance was reported for linezolid. In the Yasliani *et al*., a study in 2009, 17 (8.5%), 6 (3%) and 4 (2%) of the isolates were resistant to vancomycin, teicoplanin, and linezolid, respectively[29]. Also, Labibzadeh in 2018 reported the same resistance to these antibiotics. Our study showed a higher resistance of linezolid, teicoplanin, and vancomycin among clinical enterococcal isolates in Ahvaz. In this study, antibiotic resistance rate in *E. faecalis* isolates was higher than *E. faecium*, and all the linezolid-resistant isolates were vancomycin-resistant enterococci and teicoplanin-resistant, which is a major therapeutic concern.

In this study, the *gelE* gene was the most important virulence factor. The *gelE* gene is responsible for gelatinase production which can hydrolyze fibrinogen, insulin, casein, collagen, gelatin, and hemoglobulin[30].

Biofilm formation in enterococci is a multifactorial property and the role of various virulence genes in this process is controversial[12]. Several studies were performed to report the main virulence genes of enterococci that are related to biofilm formation in these bacteria[12,20,31-33]. Previous studies investigated the relation of virulence genes and biofilm formation, especially the presence of *esp* and *gelE*. *Esp* has been implicated as a contributing factor in the colonization and persistence of the infection[31]. In this study, the prevalence of all 5 virulence factors was significantly high in *E. faecium* than *E. faecalis*. Our study demonstrates a high frequency (63%) of biofilm formation, which is consistent with other studies[34-36]. In our study, 41.33% of biofilm positive isolates carried *esp* gene, which is in agreement with the incidence reported by other researchers[16,34].

Also, the prevalence of the *esp* gene in biofilm positive isolated from different sources showed a significant trend (P=0.03), which was similar to the Soares *et al*.[12] and Zheng *et al*.[37] studies (P<0.001) while others found no significant correlation between biofilm formation and the presence of *esp*.

The *esp* gene encodes an extracellular surface protein that helps adhesion, colonization, and evasion of the immune system. Also, this protein contributes to biofilm formation and persistence of *E. faecalis* in the urinary tract[16,38]. Our results showed that there was no significant correlation between the presence of *gelE*, *sprE*, and *ace* and the ability of isolates to biofilm formation.

According to our findings, the presence of antibiotic-resistant *Enterococcus* with several virulence factors can be a concern. Also,

the high prevalence of the *esp* gene among biofilm-producing clinical isolates suggests a potential link between biofilm formation and the *esp* gene but further studies should be needed to identify the mechanism of biofilm inhibition.

Conflict of interest statement

The authors report no conflict of interest.

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Authors' contribution

F.S. and H.H. conceived and designed the experiments; performed the experiments, contributed reagents, materials, analysis tools or data and interpreted. S.K., G.M. and A.A.D. performed the experiments, analyzed and interpreted the data. A.F.S. contributed reagents, materials and wrote the paper.

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