

Virulence Genes and Antibiotic Susceptibility of *Enterococcus* spp. in Bandar Abbas City, Iran

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ABSTRACT

Backgrounds: In recent years, *Enterococcus* species have emerged as a leading cause of nosocomial infections worldwide. The aim of this study was to determine the virulence biomarkers and antibiotic resistance profiles of *Enterococcus* spp. collected from a main tertiary teaching hospital in Bandar Abbas, Iran.

Materials & Methods: A total of 71 *Enterococcus* were isolated from clinical specimens of patients in different wards of a hospital. *Enterococcus* spp. were verified by detecting *ddl* gene using PCR-based method. Virulence-encoding genes including *gelE* and *cylA* were detected using PCR. Antibiotic resistance was assessed using the disk diffusion assay, and vancomycin resistance was identified using the E-test method.

Findings: Among *Enterococcus* isolates, 50 and 21 isolates were identified as *E. faecalis* and *E. faecium*, respectively. Most of the *Enterococcus* species were isolated from urine, followed by wound samples. The most prevalent virulence genes among *E. faecalis* isolates were *cylA* (60%) and *gelE* (30%); also, 19 and 14% of *E. faecium* isolates were positive for *cylA* and *gelE* genes, respectively. Many isolates of *E. faecalis* (84%) and *E. faecium* (76%) were resistant to one or more antibiotics and showed high resistance to gentamicin and ciprofloxacin.

Conclusion: This study revealed a high prevalence of ciprofloxacin and gentamicin resistance and a high frequency of virulence genes among *E. faecalis* isolates. Due to the high prevalence of MDR *Enterococcus* strains, control measures are necessary to prevent the emergence and transmission of these strains in different hospital wards.

Keywords: *Enterococcus faecalis*, *Enterococcus faecium*, Antibiotic resistance, Virulence factors

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Introduction

Enterococcus species are considered as a major part of the gastrointestinal tract normal flora, the third leading cause of bacterial infections, the fourth leading cause of nosocomial infections, and the second leading cause of urinary tract infections worldwide [1]. *Enterococcus* spp. are important causes of nosocomial infections, especially in patients with prolonged hospital stays, immunocompromised patients, or those previously treated with broad-spectrum antibiotics. These isolates are the causative agents of multiple infections such as bacteremia, surgical site infections, urinary tract infections, and endocarditis [2]. *Enterococcus faecium* (*E. faecium*) and *Enterococcus faecalis* (*E. faecalis*) species are the most common causes of healthcare-associated and nosocomial infections. *E. faecalis* is responsible for 80% of all *Enterococcus* infections. However, a recent study reported that the prevalence of *E. faecium* increased during 2012 to 2019, while the prevalence of *E. faecalis* remained stable for 10 years [3, 4]. The traditional method used to detect *Enterococcus* spp. is bacterial growth on a culture medium, while this method takes more than 24-48 hrs. Moreover, after antimicrobial therapy, the number of bacteria is significantly reduced. Polymerase chain reaction (PCR)-based techniques are applied to detect microorganisms because these methods are sensitive and specific [5]. The pathogenesis of *E. faecalis* and *E. faecium* species isolated from hospitalized patients is attributed to an array of genes encoding virulence biomarkers, including hyaluronidase (*hyl*), gelatinase (*gelE*), aggregation substance (*asa1*), enterococcal surface protein (*esp*), cytolysin (*cylA*), and collagen-binding-protein (*ace*) [6]. Gelatinase hydrolyzes gelatin and collagen, causing damages to host tissues and facilitating bacterial spread, colonization, and biofilm

formation. Cytolysin production by hemolytic strains significantly contributes to the exacerbation of enterococcal infections. Cytolysin-encoding genes (*cyl*) are integrated into a chromosome or carried on a plasmid [7, 8].

Enterococcus spp. are increasingly resistant to two or three groups of antimicrobial agents, known as multiple-drug resistant (MDR) strains. These strains show resistance to aminoglycosides, fluoroquinolones, penicillin, and glycopeptides [9, 10]. Also, the emergence of vancomycin-resistant enterococci (VRE) with high levels of resistance to aminoglycoside and vancomycin poses great challenges for controlling enterococci infections [11]. Teicoplanin and vancomycin-resistant strains are of great concern due to the extensive therapeutic use of these antibiotics against MDR enterococci infections. *Enterococcus* spp. are intrinsically resistant to many antibiotics. Intrinsic resistance of *E. faecium* to many antimicrobial agents, especially glycopeptides, as well as *E. faecalis* to quinupristin/dalfopristin and clindamycin has been reported [12].

Objectives: This study was designed to determine the prevalence, virulence genes, and antibiotic resistance profiles of *E. faecalis* and *E. faecium* isolates collected from a main tertiary teaching hospital in Bandar Abbas, southern Iran.

Methods and materials

Clinical samples: In this study, 71 *Enterococcus* isolates were collected from different wards of a main tertiary teaching hospital in Bandar Abbas located in the south of Iran (Payambar-e-Azam therapeutic center) during 2017-2018, including outpatient department (OPD), internal, neurology, cardiac care unit (CCU), intensive care unit (ICU), ear-nose and throat (ENT), gastroenterology, burn, urology, and surgery rooms. *Enterococcus* isolates were

retrieved from various clinical samples, including urine (n=51), wound (n=9), blood (n=3), abdominal drainage aspirate (n=3), bronchoalveolar lavage (n=2), abscess (n= 2), and central venous catheter (n=1). Clinical samples were collected from 41 females and 30 males. They belonged to different age groups, including ≤15 years (n=4), 15 to 30 years (n=14), 30 to 45 years (n=24), and 45 to 85 years (n=29). All specimens were cultured on blood agar (Merck, Germany). Then in order to confirm *Enterococcus* isolates, standard biochemical and bacteriological tests were used according to the standard protocols [13].

DNA extraction: *Enterococcus* isolates genomic DNA was extracted by Cinnapure™ DNA extraction kit (Cinnagen, Iran).

***Enterococcus* spp. isolation:** To verify *E. faecium* and *E. faecalis* isolates, the *ddl* gene was detected by PCR-based method as described previously [11] (Table 1). Confirmed *E. faecium* and *E. faecalis* isolates were stored at -70 °C for further study.

Virulence genes: Multiplex PCR was performed to determine the presence of enterococcal virulence genes (*cylA*, and *gelE*) as described previously [14] (Table 1).

Antimicrobial susceptibility testing: Antibiotic susceptibility testing of *Enterococcus* isolates was performed by disk diffusion method following the Clinical Laboratory Standards Institute guidelines using commercial antimicrobial disks

(Mast. Co., UK), including ciprofloxacin (5 µg), ampicillin (10 µg), vancomycin (30 µg), gentamicin (10 µg), teicoplanin (30 µg), linezolid (30 µg), and tigecycline (15 µg). The minimum inhibitory concentration (MICs) of vancomycin was determined using the E-test method based on the CLSI guidelines (2016). MIC breakpoints to determine vancomycin susceptibility were as follows: MIC values ≤4 were considered as sensitive, between 4-32 as intermediate resistant, and ≥32 as resistant [15].

Findings

Bacteria: A total of 71 *Enterococcus* isolates, comprising 50 (70%) *E. faecalis* and 21 (30%) *E. faecium*, were isolated. Most *Enterococcus* species were isolated from patients in the age range of 30-45 and 45-85 years. *E. faecalis* strains were mostly isolated from urine (n=35; 70%), followed by wound (n=6; 12%), blood (n=3; 6%), abdominal drainage aspirate, bronchoalveolar lavage, and abscess (n=2; 4% for each of them) samples; also, 21 *E. faecium* strains were isolated from urine (n=16; 76%), wound (n=3; 14%), abdominal drainage aspirate, and central venous catheter (n=1; 4% for each of them) samples (Table 2). The clinical departments from which *Enterococcus* spp. were isolated (Table 2) included: OPD (n=26), internal (n=18), surgery (n=9), ICU (n=6), burn (n=4), urology (n=3), CCU (n=2), neurology (n=1), ENT (n=1), and gastroenterology (n=1).

Table 1) Primers used for identification of *Enterococcus* species and virulence genes in this study

Target	Sequence (5' to 3')	Ref
<i>ddl</i> (<i>E. faecalis</i>)	ATCAAGTACAGT TAGTCTTTATTAG ACGATTCAAAGCTAACTGAATCAGT	8
<i>ddl</i> (<i>E. faecium</i>)	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC	8
<i>gelE</i>	CGAAGTTGAAAAGGAGGC GGTGAAGAAGTTACTCTGA	11
<i>cylA</i>	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	11

Table 2) Characteristics of *E. faecium* and *E. faecalis* isolates

Ward*	Species	Sample**	Resistance Pattern***	MIC (µg/mL)	Virulence Genes
OPD	<i>faecium</i>	Urine	VA, CIP, GM, TEC	-	-
	<i>faecium</i>	Urine	VA, CIP, GM, TEC	0.75	-
	<i>faecium</i>	Urine	VA, CIP, AP, TEC	1.5	-
	<i>faecalis</i>	Urine	VA, CIP, GM, TEC	0.5	-
	<i>faecium</i>	Urine	CIP, AP, GM	-	-
	<i>faecalis</i>	Urine	CIP, GM	-	<i>gelE</i>
	<i>faecalis</i>	Urine	CIP, AP	-	-
	<i>faecalis</i>	BAL	CIP, GM	-	<i>cylA</i>
	<i>faecalis</i>	Urine	CIP, GM	-	<i>cylA</i>
	<i>faecalis</i>	Urine	CIP, GM	-	<i>cylA</i>
	<i>faecalis</i>	Urine	CIP, GM	-	<i>cylA, gelE</i>
	<i>faecalis</i>	Urine	GM	-	-
	<i>faecalis</i>	Urine	GM	-	<i>cylA, gelE</i>
	<i>faecalis</i>	Urine	GM	-	<i>cylA, gelE</i>
	<i>faecalis</i>	Urine	GM	-	<i>cylA</i>
	<i>faecalis</i>	Urine	GM	-	<i>cylA</i>
	<i>faecalis</i>	Urine	GM	-	<i>cylA</i>
	<i>faecalis</i>	Urine	-	-	<i>cylA, gelE</i>
	<i>faecalis</i>	Urine	-	-	<i>gelE</i>
	<i>faecium</i>	Urine	-	-	-
	<i>faecalis</i>	Urine	-	-	<i>cylA</i>
	<i>faecalis</i>	Urine	-	-	<i>cylA</i>
	<i>faecalis</i>	Urine	-	-	<i>cylA</i>
	<i>faecalis</i>	Urine	-	-	<i>cylA</i>
	<i>faecium</i>	Urine	-	-	<i>cylA, gelE</i>
	<i>faecium</i>	Urine	-	-	-
	Internal	<i>faecium</i>	Abdominal	VA, CIP, AP, GM, TEC	0.5
<i>faecalis</i>		Urine	VA, CIP, AP, GM, TEC	2	-
<i>faecalis</i>		Urine	VA, CIP, AP, GM, TEC	-	<i>gelE</i>
<i>faecium</i>		Urine	VA, CIP, AP, GM, TEC	1.5	-
<i>faecium</i>		Urine	VA, CIP, AP, GM	1.5	-
<i>faecium</i>		Urine	VA, CIP, AP, GM	0.75	<i>cylA</i>
<i>faecalis</i>		Blood	CIP, GM, LZD	-	-
<i>faecium</i>		Urine	GM, TGC	-	<i>gelE</i>
<i>faecalis</i>		Abscess	CIP, GM	-	-
<i>faecalis</i>		Urine	CIP, GM	-	<i>cylA</i>
<i>faecalis</i>		Urine	GM	-	<i>gelE</i>
<i>faecium</i>		Urine	GM	-	-
<i>faecalis</i>		Wound	CIP	-	<i>cylA, gelE</i>
<i>faecalis</i>		Urine	GM	-	<i>gelE</i>
<i>faecium</i>		Wound	GM	-	<i>cylA</i>
<i>faecalis</i>		Urine	GM	-	<i>cylA</i>
<i>faecium</i>		Urine	-	-	<i>gelE</i>
<i>faecalis</i>	Urine	-	-	<i>cylA</i>	

Table 2) Characteristics of *E. faecium* and *E. faecalis* isolates

Ward*	Species	Sample**	Resistance Pattern***	MIC ($\mu\text{g/mL}$)	Virulence Genes
Surgery	<i>faecalis</i>	Urine	VA, CIP, AP, GM, TEC	0.75	<i>cylA</i>
	<i>faecalis</i>	Wound	VA, CIP, GM, TEC	0.5	-
	<i>faecium</i>	Wound	VA, GM, TEC	0.75	-
	<i>faecalis</i>	Wound	VA, CIP, GM	1.5	<i>cylA</i>
	<i>faecalis</i>	Abdominal	CIP, AP	-	<i>cylA</i>
	<i>faecalis</i>	Abdominal	GM	-	<i>gelE</i>
	<i>faecalis</i>	Abscess	GM	-	<i>gelE</i>
	<i>faecium</i>	Urine	GM	-	-
	<i>faecium</i>	Urine	GM	-	-
ICU	<i>faecalis</i>	Urine	GM, LZD	-	-
	<i>faecium</i>	Catheter	VA, TEC	-	-
	<i>faecalis</i>	Blood	GM, TGC	-	<i>cylA</i>
	<i>faecalis</i>	Urine	CIP, GM	-	-
	<i>faecalis</i>	Urine	CIP, GM	-	-
	<i>faecalis</i>	Urine	CIP, GM	-	<i>cylA</i>
	<i>faecalis</i>	Urine	CIP, GM	-	<i>cylA</i>
Burn	<i>faecalis</i>	BAL	AP, GM	-	<i>cylA</i>
	<i>faecalis</i>	Wound	GM	-	<i>cylA</i>
	<i>faecium</i>	Wound	GM	-	<i>cylA</i>
	<i>faecalis</i>	Wound	-	-	<i>cylA</i>
Urology	<i>faecalis</i>	Urine	CIP, GM	-	<i>cylA</i>
	<i>faecalis</i>	Wound	GM	-	<i>cylA</i>
	<i>faecalis</i>	Urine	GM	-	<i>cylA, gelE</i>
CCU	<i>faecalis</i>	Urine	CIP, GM	-	-
	<i>faecium</i>	Urine	-	-	-
Digestive	<i>faecalis</i>	Blood	CIP, GM	-	<i>gelE</i>
Neurology	<i>faecalis</i>	Urine	GM	-	<i>cylA</i>
ENT	<i>faecalis</i>	Urine	GM	-	<i>cylA, gelE</i>

*OPD: outpatient department

**BAL: bronchoalveolar lavage, abdominal: abdominal drainage aspirate, catheter: central venous catheter

***CIP: ciprofloxacin, AP: ampicillin, VA: vancomycin, GM: gentamicin, TEC: teicoplanin, LZD: linezolid, TGC: tigecycline

PCR detection of virulence genes among *Enterococcus* isolates: Among *E. faecalis* isolates, *cylA* was the most prevalent gene (n=30; 60%), followed by *gelE* (n=15; 30%). Seven *E. faecalis* isolates were positive for both *gelE* and *cylA* genes. Among *E. faecium* isolates, four (19%) isolates possessed *cylA* gene, whereas three (14%) isolates showed the genetic marker *gelE*. Only one *E. faecium* isolate possessed both *gelE* and *cylA* genes (Table 2).

Antibiotic resistance profiles: In the disk

diffusion assay, 42 (84%) *E. faecalis* isolates were resistant to one or more antibiotics, and the highest resistance was shown against gentamicin (n=39; 78%), ciprofloxacin (n=23; 46%), vancomycin and ampicillin (n=6; 12% for each of them), teicoplanin (n=5; 10%), linezolid (n=2; 4%), and tigecycline (n=1; 2%). Out of 21 *E. faecium* strains isolated, 16 isolates (76%) showed resistance to at least one antibiotic. The highest resistance was observed against gentamicin (n=14; 67%), followed by vancomycin (n=9; 43%),

ciprofloxacin (n=8; 38%), teicoplanin (n=7; 33%), ampicillin (n=6; 28%), and tigecycline (n=1; 5%). In addition, eight *E. faecalis* and five *E. faecium* isolates were sensitive to all of the antibiotics surveyed in this study. The antimicrobial resistance profile of *Enterococcus* isolates is presented in Table 2. Designation of MIC levels showed that 11 *Enterococcus* isolates were susceptible to vancomycin with MIC values in the range of 0.5 to 2 µg/mL (Table 2).

Discussion

In the current study, virulence genes and antibiotic susceptibility profiles of *Enterococcus* spp. isolated from clinical samples were investigated. The isolation rate of *E. faecalis* (70%) was higher than that of *E. faecium* (30%). This finding contradicts the findings of other studies in which the prevalence of *Enterococcus* species isolated from clinical specimens has been reported in favor of *E. faecium* [16-18]. Haghi et al. (2019) in northwestern Iran reported that *E. faecalis* isolates were the predominant enterococci isolated from urine samples, which is similar to the results of the current study [14]. Another study in Iran indicated that *E. faecalis* isolates were more prevalent among enterococci derived from various clinical samples [3]. These results show that the prevalence of *Enterococcus* species varies in different clinical samples and geographical regions.

MDR enterococci as the main pathogens have become a serious problem in nosocomial infections [14]. In this study, 82% of *Enterococcus* isolates were resistant to one or more antibiotics. The prevalence of antibiotic resistance among *E. faecalis* isolates was more than in *E. faecium* isolates; also, the results showed a high prevalence of MDR *Enterococcus* isolates in urine specimens. Most *Enterococcus* isolates were sensitive to linezolid and

tigecyclin (97% for each). Previous studies in Iran have shown a high frequency of antibiotic resistance among *Enterococcus* spp., except linezolid, tigecycline, and fosfomycin [11, 14]. A study in China reported a high prevalence of resistance to rifampicin, penicillin, ampicillin, fosfomycin, ciprofloxacin, chloramphenicol, levofloxacin, erythromycin, quinupristin/dalfopristin, minocycline, and tetracycline among *Enterococcus* spp., while the prevalence of resistance to vancomycin, teicoplanin, and linezolid was low in *E. faecium* and *E. faecalis* isolates [19]. Screening of antimicrobial resistance indicated that 75% of the isolates were resistant to gentamycin, which is similar to the results of recent studies indicating that the prevalence of gentamycin resistance ranges from 50 to 65% [20, 21]. In the current study, resistance to vancomycin in *E. faecium* and *E. faecalis* isolates was 43 and 12%, respectively. Another study in Iran showed that the prevalence of vancomycin resistance in *E. faecium* isolates was more than in *E. faecalis* isolates [3]. In a study in Turkey, Çopur et al. (2016) reported a high frequency of vancomycin resistance among *E. faecium* isolates (95.6%) compared to *E. faecalis* isolates (4.3%), and most VRE strains were isolated from specimens of surgery clinics and intensive care units [22]. A higher prevalence rate of VR among *E. faecium* isolates was also reported in a study in Saudi Arabia (62.3%) [23]. In this study, 8% of VRE isolates were isolated from clinical samples of the internal ward, and 6% were isolated from samples of OPD and surgery rooms. A previous study reported that the high prevalence of antibiotic resistance detected in ICU and burn hospital wards may be attributed to immunodeficiency, long-term antibiotic use, and patients' critical illness [19].

In this study, the *gelE* gene was detected in 30% of *E. faecalis* and 14% of *E. faecium*

isolates, this finding is consistent with the finding of a previous Indian study documenting a high frequency of *gelE* among *E. faecalis* compared to *E. faecium* [7]. In another study in Iran, most *Enterococcus* spp. (79.7%) isolated from clinical samples carried the *gelE* gene; also, 82% out of 128 *E. faecalis* isolates and 60% out of 15 *E. faecium* isolates harbored *gelE* [24]. Banerjee and Anupurba (2015) reported that among enterococci strains isolated from clinical samples, the *gelE* gene was detected in 9.6 and 8.3% of *E. faecalis* and *E. faecium* isolates, respectively; also, most virulence factors were associated with biofilm formation [25]. For invasive *Enterococcus* isolates (infective endocarditis), virulence biomarkers may be more relevant to other traits than adherence, such as collagen and gelatin degradation (by *gelE* gene) which may be relevant to dissemination and invasion [26].

Cytolysin, encoded by *cylA*, is a bacteriocin-type exotoxin with hemolytic activity towards eukaryotic cells. This exotoxin exhibits toxic properties against erythrocytes, leukocytes, and macrophages and bactericidal properties towards Gram-negative bacteria. Cytolysin-encoding sequences (*cyl*) have been detected in *Enterococcus* strains isolated from both invasive and non-invasive infections [27]. Arshadi et al. (2018) in Iran reported that 7.1, 6.2, and 0% of *E. faecium* intestinal isolates, clinical isolates, and environmental isolates possessed hemolysin gene, respectively [28]. In the present study, the frequency of *cylA* gene among *Enterococcus* strains isolated from clinical specimens was 48% (60 and 19% among *E. faecalis* and *E. faecium* strains, respectively). A study in Brazil reported the presence of *cyl* genes in 54.4% of clinical enterococcal strains [29].

Conclusion

This study data indicate that *E. faecalis* isolates carry more virulence genes than *E. faecium*. Thus, we are faced with MDR *E. faecalis*

strains with virulence and antimicrobial resistance genes which enable them to adapt and survive in hospital settings and cause severe infections. Infections caused by VRE and MDR isolates could be associated with high mortality in patients. Given that most of the isolates were sensitive to linezolid and tigecycline, it is suggested that therapeutic strategies be reviewed according to new antimicrobial resistance patterns.

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Authors' Contribution: Conceptualization: KA, AH, DT; data curation: KA, DT; formal analysis: KA, DT; funding Acquisition: DT; investigation: KA, AH, DT; methodology: KA, AH, DT; project administration: KA; resources: DT; software: DT; supervision: KA, AH; writing of the original draft: KA, AH; writing-review and editing: KA, AH, DT.

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Consent to participate: Written informed consents were obtained from participants.

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