

# High Incidence of Virulence Factors among Clinical *Enterococcus faecalis* Isolates in Southwestern Iran

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**Background:** Over the past two decades, enterococci have emerged as an important agent responsible for hospital acquired infection. Several virulence factors contribute to the adherence, colonization, evasion of the host immune response, and pathogenicity and severity of the infection. *Enterococcus faecalis* is the most common and virulent species causing infections in hospitalized patients. The aim of the present study was to examine the prevalence of genes encoding virulence factors and antimicrobial resistance patterns of *E. faecalis* strains isolated from hospitalized patients in Shiraz, south west of Iran.

**Materials and Methods:** A total of 51 *E. faecalis* isolates from the urine, blood, pleural fluid, peritoneal fluid, eye discharge, endotracheal tube (ETT) and transjugular intrahepatic portosystemic shunt (TIPS) specimens of patients were identified by phenotypic and genotypic methods. Antimicrobial sensitivity tests and detection of virulence factors were performed using standard methods.

**Results:** The *efa* and *asa1* were the most frequently detected gene (100%) among the isolates, followed by *esp* (94.1%), *ace* (90.2%), *gelE* (80.4%), *cyIA* (64.7%), and *hyl* (51%). More than half of the isolates (52.9%) were high level gentamicin resistant (HLGR). Vancomycin resistance was observed among 23 (45.1%) isolates. The lowest antimicrobial activity was related to erythromycin (3.9%), tetracycline (5.9%) and ciprofloxacin (9.8%). No isolate was found resistant to fosfomycin and linezolid.

**Conclusion:** Our data indicated a high incidence of virulence factors among *E. faecalis* strains isolated from clinical samples. Colonization of drug resistant virulent isolates in hospital environment may lead to life threatening infection in hospitalized patients. Therefore, infection control procedures should be performed.

**Key Words:** *Enterococcus faecalis*; Virulence Factors; Iran

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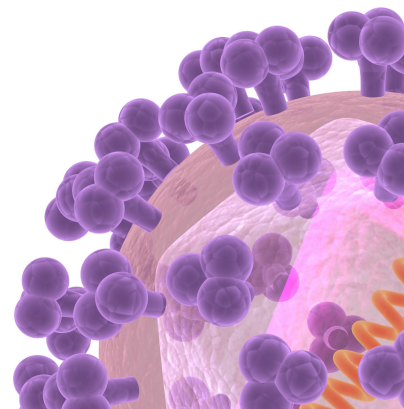
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## Introduction

*Enterococcus* is a Gram-positive and common commensal bacterium of human gastrointestinal and genitourinary tracts [1]. Over the past two decades, enterococci have emerged as an important agent responsible for hospital acquired infection, particularly bacteremia, sepsis, endocarditis, urinary tract infection (UTI), and abdominal and pelvic infections [2]. Certain conditions, such as prolonged hospitalization, immunodeficiency and antibiotic therapy, causes a high risk for acquiring of enterococcal infections in patients [3, 4].

Several virulence factors including enterococcal surface protein (Esp), hyaluronidase (Hyl), gelatinase (GelE), aggregation substance (AS) proteins (Asa1), collagen-binding protein (Ace), and cytolysin (CylA) contribute to the adherence, colonization, evasion of the host immune response, extracellular production of enzymes, pathogenicity, and severity of the infection [5, 6]. Moreover, treatment of enterococcal infections has been clinically challenging due to increasing resistance to a broad range of antimicrobial agents, including  $\beta$ -lactams, macrolides, fluoroquinolones, glycopeptides, and aminoglycosides [4, 7].

Enterococci possess efficient genetic exchange systems. The genes encoding virulence determinants can be transferred to resistant strains via these systems [4, 8]. Emergence of antimicrobial resistant virulent enterococci is a serious problem in the treatment and control of nosocomial infections [9].

Several studies have shown that *Enterococcus faecalis* is an important and virulent species causing various infections [4, 6, 10, 11]. There are few data regarding the enterococcal virulence determinants in hospitalized patients in south-west of Iran. Therefore, the aim of the present study was to examine the prevalence of genes encoding virulence factors and antimicrobial resistance patterns of *E. faecalis* strains isolated from hospitalized patients in Shiraz, Iran.

## Materials and Methods

### 1. Bacterial isolates

A total of 51 *E. faecalis* isolates were collected from specimens of patients hospitalized in Nemazee hospital (the main hospital affiliated to Shiraz University of Medical Sciences with 1,000 beds and 50,000 inpatients per year) during September 2015 to March 2016. They were obtained from the urine (n = 38), blood (n = 4), pleural fluid (n = 4), peritoneal fluid (n = 2), eye discharge (n = 1), endotracheal tube (ETT) (n = 1), and

transjugular intrahepatic portosystemic shunt (TIPS) (n = 1). Only one isolate per patient was included. Identification of enterococcal isolates was performed based on conventional microbiological tests (Gram staining, catalase test, growth on Brain Heart Infusion agar (Conda, Madrid, Spain) with 6.5% NaCl, and bile-esculin test) [12, 13]. The *ddlE* gene was targeted by polymerase chain reaction (PCR) using species specific primers (*ddlE. faecalis* F- 5'- ATCAAGTACAGTTAGTCT-3' and R-5'-ACGATTCAAAGCTAACTG-3') for molecular confirmation of *E. faecalis* [4, 14]. The PCR protocol consisted of a pre-denaturation step at 95 °C for 5 min, followed by 30 cycles of 60s at 95°C, 45s at 45°C and 50s at 72°C. A final extension step was performed at 72°C for 5 min.

### 2. Antimicrobial susceptibility testing

Antimicrobial sensitivity tests were performed by disc diffusion method on the Mueller- Hinton Agar (Merck Co., Darmstadt, Germany) based on Clinical and Laboratory Standards Institute (CLSI) guideline [15]. The tested antibiotics (Mast Group Ltd., UK.) were penicillin (10 units), ampicillin (10 µg), ciprofloxacin (5 µg), erythromycin (15 µg), tetracycline (30 µg), nitrofurantoin (300 µg), rifampin (5 µg), fosfomycin (200 µg), quinupristin- dalfopristin (15 µg), linezolid (5 µg), vancomycin, (30 µg) and teicoplanin (30 µg). High-level gentamicin resistant (HLGR) isolates were also detected by disc diffusion method (with 120 µg gentamicin disks) on the Mueller- Hinton Agar (Merck Co., Darmstadt, Germany) [15]. *E. faecalis* ATCC 29212 was used as the reference strain for antibiotic susceptibility testing.

### 3. DNA extraction and detection of virulence genes

Genomic DNA was extracted from fresh colonies as described previously [4]. PCR was done for detection of the genes encoding virulence factors (*esp*, *cylA*, *hyl*, *gelE*, *asa1*, *ace*, *efa*) [4, 8]. The products were separated by electrophoresis in 1.5% agarose gels with 1 X TAE (Tris/Acetate/EDTA) buffer, stained with KBC power load dye (CinnaGen Co. Tehran, Iran) and visualized under ultraviolet illumination.

### 4. Statistical analysis

The distribution of virulence genes among HLGR and non-HLGR isolates was calculated by Chi-square and Fisher's exact test for each gene. In addition, differences in the incidence of virulence genes with respect to the origin of specimen were also analyzed by Chi-square test. A *P*-value of  $\leq 0.05$  was considered as statistically significant.

## Results

### 1. Antimicrobial resistance pattern

During 6 months of the study, a total of 51 *E. faecalis* strains were collected randomly with the possibility of true infections. No isolate was found to be resistant to fosfomycin and linezolid. Vancomycin resistance was observed among 23 (45.1%) isolates (Table 1). More than half of the isolates (52.9%) were HLGR (Table 2).

### 2. Distribution of virulence genes

The *efa* and *asa1* were the most frequently detected gene (100%) among isolates, followed by *esp* (94.1%), *ace* (90.2%), *gelE* (80.4%), *cylA* (64.7%), and *hyl* (51%) (Table 2). The frequency of genes encoding virulence factors among HLGR and non-HLGR isolates is shown in Table 3. There was no significant difference between the distribution of virulence genes among HLGR and non-HLGR isolates ( $P > 0.05$ ).

## Discussion

Enterococci, particularly *E. faecalis*, are common pathogens that can cause severe nosocomial infections [7]. In our study, *E. faecalis* isolates were obtained from various clinical specimens. Similar to other reports from Iran, noticeable resistance

**Table 1.** Antimicrobial resistance rates among *Enterococcus faecalis* isolates

Antimicrobial agents	No. (%) of isolates		
	S	I	R
PEN	36 (70.6)	-	15 (29.4)
AMP	33 (64.7)	-	18 (35.3)
CIP	5 (9.8)	21 (41.2)	25 (49)
ERY	2 (3.9)	8 (15.7)	41 (80.4)
TET	3 (5.9)	1 (2)	47 (92.1)
NIT	40 (78.4)	6 (11.8)	5 (9.8)
RIF	20 (39.2)	10 (19.6)	21 (41.2)
FOF	51 (100)	0 (0)	0 (0)
QDA	11 (21.6)	2 (3.9)	38 (74.5)
LZD	51 (100)	0 (0)	0 (0)
TEC	42 (82.3)	1 (2)	8 (15.7)
VAN	9 (17.6)	19 (37.3)	23 (45.1)

S, susceptible; I, intermediate; R, resistant; PEN, penicillin; AMP, ampicillin; CIP, ciprofloxacin; ERY, erythromycin; TET, tetracycline; NIT, nitrofurantoin; RIF, rifampin; FOF, fosfomycin; QDA, quinupristin-dalfopristin; LZD, linezolid; TEC, teicoplanin; VAN, vancomycin.

**Table 2.** The distribution of virulence genes and high level resistance to gentamicin among *Enterococcus faecalis* isolates

Clinical samples (N)	Virulence genes	HLGR / non-HLGR
Urine (38)	<i>efa, asa1, esp, ace, cylA, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	non-HLGR
	<i>efa, asa1, esp, ace, cylA, hyl</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, cylA, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	non-HLGR
	<i>efa, asa1, esp, ace, cylA, hyl</i>	non-HLGR
	<i>efa, asa1, esp, gelE</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, hyl</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, hyl</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	non-HLGR
	<i>efa, asa1, esp, gelE</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	non-HLGR
	<i>efa, asa1, esp, ace, cylA</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, hyl</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, hyl</i>	non-HLGR
	<i>efa, asa1, esp, gelE, cylA</i>	HLGR
	<i>efa, asa1, ace, gelE, cylA</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	non-HLGR
	<i>efa, asa1, ace, gelE, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, cylA</i>	HLGR
	<i>efa, asa1, esp, gelE</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	non-HLGR
	<i>efa, asa1, esp, ace, cylA, hyl</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	HLGR
	<i>efa, asa1, esp, ace, gelE</i>	HLGR
	<i>efa, asa1, esp, ace, gelE</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	non-HLGR
	<i>efa, asa1, esp, ace, cylA, hyl</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	HLGR
<i>efa, asa1, ace, gelE</i>	HLGR	
Blood (4)	<i>efa, asa1, esp, ace, gelE</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	non-HLGR
	<i>efa, asa1, esp, ace, gelE, cylA</i>	non-HLGR
	<i>efa, asa1, ace, gelE</i>	HLGR
Pleural (4)	<i>efa, asa1, esp, ace, gelE</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, cylA, hyl</i>	non-HLGR
	<i>efa, asa1, esp</i>	non-HLGR
Peritoneal (2)	<i>efa, asa1, esp, ace, gelE, cylA</i>	HLGR
	<i>efa, asa1, esp, ace, gelE, hyl</i>	non-HLGR
Eye (1)	<i>efa, asa1, esp, ace, gelE, hyl</i>	HLGR
ETT (1)	<i>efa, asa1, esp, ace, hyl</i>	non-HLGR
TIPS (1)	<i>efa, asa1, esp, ace, gelE, cylA</i>	HLGR

ETT, endotracheal tube; TIPS, transjugular intrahepatic portosystemic shunt; HLGR, high level gentamicin resistant.

**Table 3.** Distribution of genes encoding virulence factors among HLGR and non-HLGR isolates

	Virulence gene				
	<i>hyl</i> N(%)	<i>cylA</i> N(%)	<i>gelE</i> N(%)	<i>ace</i> N(%)	<i>esp</i> N(%)
HLGR 27(52.9)	11 (40.7)	18 (66.6)	23 (85.2)	24 (88.8)	25 (92.6)
Non-HLGR 24(47.1)	15 (62.5)	15 (52.5)	18 (75)	22 (91.6)	23 (95.8)
Total (51)	26 (51)	33 (64.7)	41 (80.4)	46 (90.2)	48 (94.1)

HLGR, High level gentamicin resistant.

to several antimicrobial agents among *E. faecalis* strains was observed [4, 16]. Nearly half of the isolates were resistant to vancomycin (45.1%). Similarly, high prevalence of vancomycin-resistant enterococci has been previously reported from southwestern Iran [13, 17]. All of isolates were susceptible to fosfomycin and linezolid (Table 1). Our findings indicate that these agents are efficient choices against enterococcal infections.

More than 52% of the strains were HLGR. Resistance to high concentrations of gentamicin among *E. faecalis* isolates has been reported in previous studies [8, 9] Presence of genes encoding aminoglycoside-modifying enzymes and transmission among enterococcal strains lead to high levels resistance to gentamicin [18]. It seems that gentamicin has become an ineffective antibiotic against hospital-acquired enterococcal infections.

In addition to antimicrobial resistance, the presence of virulence factors is another concern about enterococci that may contribute to the severity of enterococcal infections [8]. In the present study, the distribution of virulence genes among *E. faecalis* strains was determined.

All of the isolates harbored *efa* and *asa1* genes (Table 2). In various studies, similar frequencies of the *efa* have been reported [4, 8, 9]. In a study conducted by Gaglio et al., the *efa* gene was detected among 25% of the strains isolated from dairy products [19]. It appears that the *efa* gene is always present in clinical *E. faecalis* strains. In the current study, the prevalence of *asa1* was more than that observed in previous studies [4, 8, 19-21]. AS encoded by *asa1* causes clumping of *E. faecalis* strains and mediates efficient DNA exchange between donor and recipient bacteria [5, 22]. The presence of *asa1* gene among all of our isolates can facilitate exchange of resistance and virulence associated genes in hospital setting.

The *esp* was the second most detected gene among the isolates (94.1%). The prevalence of *esp* was also 94.7% in strains

isolated from UTI. High frequency of *esp* among UTI causative *E. faecalis* isolates is consistent with other studies [6, 23]. The enterococcal surface protein is encoded by the *esp* gene facilitates colonization and persistence of *E. faecalis* in acute urinary tract infections [5, 24].

We found that the prevalence of *ace*, *gelE*, *cylA* and *hyl* genes was 90.2%, 80.4%, 64.7%, and 51%. Our results indicated that *E. faecalis* isolates carried more virulence genes in comparison to various reports [4, 9, 18, 19, 25-28]. These differences probably reflect variability in the types of samples or geographic regions.

There was no significant difference between the distribution of virulence factors among HLGR and non-HLGR isolates, although *cylA* and *gelE* were more prevalent in HLGR strains (Table 3). This result is in contrast with that of a previous study which has reported that the *hyl* gene is significantly the most frequent virulence gene in HLGR isolates [4].

In the current study, the antimicrobial resistant isolate obtained from TIPS had 6 virulence genes. Colonization of same isolates in hospital environment may lead to life threatening infection in hospitalized patients. Therefore, to prevent colonization of such drug resistant virulent isolates, infection control procedures should be performed.

To the best of our knowledge, this study is the first investigation regarding enterococcal virulence determinants in Shiraz, the south west of Iran. Moreover, high virulence strains were identified. Different profiles of virulence factors can be important for understanding the enterococcal pathogenicity and their effects on the host.

There were some limitations related to the present study. First, small number of isolates were investigated. Second, to identification of the source of infection and take preventive measures in hospital setting molecular analysis was required.

In conclusion, a high incidence of genes encoding virulence factors among *E. faecalis* strains isolated from clinical samples was observed. Our results indicated that all of the isolates carried *efa* and *asa1* genes. The *esp* was the second most prevalent one. Gene encoding Hyl (*hyl*) was the least frequent one that presented in more than half of the isolates. To prevent colonization of such virulent strains in hospital environment, infection control procedures are recommended to be performed.

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## Conflicts of Interest

No conflicts of interest.

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