

Antimicrobial Resistance, Biofilm Formation and Virulence Factors in *Enterococcus faecalis* Strains Isolated from Urinary Tract Infection in Kermanshah, Iran

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Abstract

Aims and Objectives: This study aims to specify the antimicrobial resistance pattern and virulence genes of *Enterococcus faecalis* isolated from individuals suffering from an infected urinary tract in Kermanshah, Iran. **Patients and methods:** Isolates were gathered from 1000 urine samples of patients diagnosed with the infected urinary tract. Biofilm assays were performed by microtiter plate test through reading the OD490 following the crystal violet staining. The Kirby–Bauer disc diffusion method was performed for the antimicrobial susceptibility testing. PCR reaction was applied to study the virulence factors. **Results:** Of 1000 urine samples, *E. faecalis* was reported 5%. Strong, moderate and weak biofilm reactions reported 80%, 12%, and 8% respectively. The most resistance reported to cotrimoxazole, vancomycin and amikacin and no resistance to nitrofurantoin were reported. Prevalence of *efe A*, *ace*, *gel E*, *esp*, *cyl M*, *agg*, *cyl A* and *cyl B* in strong biofilm formation isolates was reported 100%, 87.5%, 82.5%, 80%, 60%, 57.5% 37.5% and 30% respectively. There was a significant relationship between the frequency of *efa A*, *ace*, *gel E*, *esp* genes and strong biofilm reaction ($p < 0.0001$). **Conclusion:** The presence of *E. faecalis* strains resistant to co-trimoxazole and vancomycin with some virulence factors is alarming the researchers. Since antibiotic resistance genes are probably transmitted among enterococci, and *Staphylococci*, controlling infections made by enterococci as well as the appropriate administration of antibiotics could treat the nosocomial infections effectively.

Keywords: Antibiotic Resistance; Enterococcus faecalis; Urinary Tract Infection; Virulence genes

INTRODUCTION

Enterococci as Gram-positive and catalase-negative cocci can develop along with 6.5% salts and 40% bile salts ^[1]. The most common *enterococci* found in human infection are *Enterococcus faecalis* (85-90%) and *Enterococcus faecium* (5-10%). These bacteria belong to the normal flora of the human digestive system having been used commensal bacteria in the past. *Enterococci* is capable of triggering infections in wounds such as burn wounds, urethra, and even endocarditis ^[1]. Investigations show that biofilm is produced by *E. faecalis* and biofilm growth is under the control of its quorum-sensing system ^[1]. Microbial biofilm is a bacterial mass primarily related to biotic and abiotic surfaces that trigger the cells to bind irreversibly to the surfaces by making extracellular polymers and building a matrix of alginate ^[2]. It is believed that there is an association between the development of biofilm with bacterial resistance to antibiotic treatments that probably would lead to acute problems in this area. Bacteria taking part in biofilms behave differently compared to the planktonic cells ^[3, 4]. The extracellular surface protein (*esp*) increases the ability to colonize and create bacterial biofilm *in vitro*, and it seems to be related to

the presence of biofilms *in vivo* ^[5]. Ebp protein (endocarditis and biofilm-associated pili) is one of the most important proteins encoded in the pathogenesis of *E. faecalis*. In the Ebp operon, the *Ebp A* protein plays a major role in the pathogenesis process. Also, it has been verified that *Ebp* protein is important for the formation of biofilms so that it can cause urinary tract infection and endocarditis in the experimental models ^[6]. Some enterococcal virulence factors

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have been recognized such as adhesions and secreted virulence factors. The main adhesion factors are Asa (aggregation substance), Esp (extracellular surface protein), EfaA (*E. faecalis* antigen A), Ace (adhesin of collagen from *E. faecalis*) and Ebp^[6]. Proteins like hemolysin, gelatinase, and aggregation substances are also involved in the plasmid exchange systems. The severity of endocarditis and endophthalmitis in the animal model is aggravated significantly following the production of cytolysin leading to severe enterococcal diseases in humans^[7]. The initial bonding and the formation of the *E. faecalis* biofilm are accompanied by the presence of the enterococcal surface protein. The chromosomal *gelE* gene encodes the gelatinase. This enzyme is an extracellular metalloproteinase that hydrolyzes collagen, gelatin, and small peptides; it also is involved significantly in the formation of animal endocarditis. The role of cytolysin in the onset of the disease has been verified by Epidemiological investigations^[8]. Resistance to a wide range of antibiotics is observed in enterococci, such as inherent resistance to macrolides, chloramphenicol, penicillin, tetracycline, and aminoglycosides. Multiple drug resistances are among the characteristics of enterococci^[3]. Resistance genes may be disseminated in bacteria through the interference of the horizontal gene transfer. Also, selective pressure by drugs has significant involvement in resistance dissemination; besides, enterococci are also important for wide-range antibiotic resistance. In this respect, genetic mobile elements like conjugative plasmids and transposons can spread resistance genes^[4]. This study aims to specify the antimicrobial resistance pattern and virulence genes of *Enterococcus faecalis* isolated in UTI patients in Kermanshah, Iran.

METHODS

Ethics and consent

In the current study, we tried to protect the life, health, dignity, integrity, rights to self-determination, privacy, and confidentiality of personal information of research subjects. We conform to generally accepted scientific principles based on a thorough knowledge of the scientific literature, other relevant sources of information, and adequate laboratory and, as appropriate, animal experimentation. All samples were taken from volunteer patients for this research. All ethical issues were considered and this research was performed with hospitals' permission. The name and identity, personal information and even patients' illnesses and their medical information remained secret. All of the patients voice their satisfaction to use their sample in this investigation, especially to determine antibiotic resistance in UPEC strains.

This thesis has been approved in Islamic Azad University Shahrekord branch with code 13330507962017.

Sampling, Isolation, and Identification

In this cross-sectional study, approximately 1000 urine samples were gathered from successive outpatients with a

UTI in Kermanshah, from May to August 2017. To separate the bacteria, an early morning midstream urine samples were cultured on blood agar containing 5% sheep blood under sterile conditions and incubated for 24 hr at 37°C. To verify the suspected colonies of enterococci, *E. faecalis* ATCC 29212 (Pasteur Institute of Iran) was applied as a standard strain. The pure cultures of suspected colonies were sub-cultured on Bile Esculin agar and incubated for 48 hr. at 37°C. Moreover, Gram stain, catalase test, growth at 6.5% NaCl and PYR test were performed for the early identification of enterococci. In this study, the arabinose fermentation test was employed to differentiate *E. faecalis* and *E. faecium*^[9, 10].

Microtiter Plate Assay for detection of biofilm

The modified crystal violet staining method was applied to quantify the formation of biofilm on the polystyrene microplates. For each strain, few colonies were suspended in physiological saline to 0.5 McFarland and vortexed for 1 min. 180 µL Trypticase soy broth (TSB) + 0.5% glucose and 20 µL of bacteria suspension was used to fill 96 well polystyrene Microtiter plates. All plates were performed in duplicate. Negative controls were TSB +0.5% glucose alone. Following the stationary aerobic incubation for 24 hr. at 37 °C and 5% CO₂, the broth was cautiously taken away and 300 µL of sterile phosphate-buffered saline (PBS, room temperature) was used to wash the well threefold. 150 µL methanol for 20 min was used to fix the biofilm; then, they were put to dry in the air in an inverted position in the warm room (around 30 min). To stain the biofilms, 150 µL of crystal violet solution in water (2%) was used for 15 min at room temperature; then, the wells were put under the running water tap for rinsing. To invert the Microtiter plates, they were put on a paper towel and air-dried. To specify the yield of biofilms, 150 µL of 33% acetic acid was added to each well to destain the biofilms and plates covered with lids were placed at room temperature for half an hour with no shaking. The optical density (OD) was measured at 490 nm in a spectrophotometer. This way, the isolates with OD equal to or higher than 0.216 were taken as strong biofilms, if they had an OD between 0.54 and 0.108, as weak biofilms, if they had an OD less than 0.54 as negative biofilms^[11]. DNA of the samples that were taken as positive and negative biofilms was extracted using a DNA extraction kit.

Antimicrobial Susceptibility Testing

The Kirby–Bauer disc diffusion method was applied using Mueller–Hinton agar (Merck, Germany) according to the Clinical Laboratory Standards Institute (CLSI) guidelines to conduct the antimicrobial susceptibility testing^[12]. Some sorts of antibiotics with proper disks containing vancomycin (VAN 10µg), co-trimoxazole (SXT 25µg), ceftazidime (CAZ 30µg), norfloxacin (NOR 10µg), amikacin (AN 30µg), gentamicin (GM 120µg), cefotaxime (CTX 30 µg) and nitrofurantoin (FM 300µg) (produced by PadTan-Teb, Iran).

DNA extraction and PCR Assay

A DNA extraction kit (Cinapure DNA, CinaClon, Iran) was applied according to the manufacturer's instructions to

extract genomic DNAs from *E. faecalis* isolates. Based on the method specified by Sambrook and Russell ^[13], the total DNA was measured at 260 nm optical density. PCR was applied using specifically targeted primers to detect enterococci ^[12].

Identifying the virulence genes by PCR method

PCR was applied using specific primers to identify the virulence factors genes including *gel E*, *esp*, *agg*, *ace*, cytolysin (*cylM*, *cylA* and *cyl B*) genes and *ebp A*, *ebp B*, *ebp*

C. Tables 1 and 2 show the applied primer sequence, the annealing temperature, and the used PCR program. A DNA thermal cycler was used to perform PCR, (Master Cycler Gradient, Eppendorf, Germany). Ethidium bromide was used to stain the amplicons that were then electrophoresed in 1.5% agarose gel at 80 V for 30 min. To observe and photograph PCR products, the UV doc gel documentation systems (Uvitec, UK) was used. A comparison was performed between the PCR products against a 100 bp DNA marker (Fermentas, Germany) ^[14-17].

Table 1: The oligonucleotide primers and the Multiplex PCR programs used for amplification of virulence genes of *E. faecalis* isolates

Target gene	Primer Oligonucleotide sequences (5'-3')	Accession Number	Size of amplicon (bp)	Annealing Temperature (°C)
<i>tfu</i>	F: TCAAGTACAGTTAGTCTTTATTAG R: ACGATTCAAAGCTAACTGAATCAGT	CP015883.1	112	59
<i>Ebp A</i>	F: CTAACAAAAATGATTCCGGCTCCAG R: ATCTCACGCATTTTATCTTCAACT	CP028285	517	60
<i>Ebp B</i>	F: CTGAAGGAAAAACGGTCCAA R: CTTTTCGTCGTCAGTGTGT	CP022059	1003	55
<i>Ebp C</i>	F: GATAAATATCAAGGACTGGCAGA R: AAGCATACTCTCCAGAAGTCACG	CP022059	600	58
<i>asa</i>	F: CCAGCCAACTATGGCGGAATC R: CCTGTGCGCAAGATCGACTGTA	CP22712	419	51
<i>Gel E</i>	F: ACCCCGTATCATTGGTTT R: ACGCATTGCTTTTCCATC	KU311665	629	51
<i>Cyl A</i>	F: TGGATGATAGTGATAGGAAGT R: TCTACAGTAAATCTTTCGTCA	CP015883	517	55
<i>Cyl M</i>	F: CTGATGGAAAGAAGATAGTAT R: TGAGTTGGTCTGATTACATT	AY032999	742	55
<i>Afa A</i>	F: GACAGACCCTCACGAATA R: AGTTCATCATGCTGTAGTA	KY070337	705	55
<i>Cyl B</i>	F: ATTCCTACCTATGTTCTGTTA R: AATAAACTCTTCTTTTCCAAC	KU311664	843	54
<i>ace</i>	F: GAGCAAAAGTTCAATCGTTGAC R: GTCTGTCTTTTCACTGTTTCT	AF159247	1003	54
<i>agg</i>	F: AAGAAAAAGAAGTAGACCAAC R: AAACGGCAAGACAAGTAAATA	CP002493	1553	54
<i>esp</i>	F: TTGCTAATGCTAGTCCACGACC R: GCGTCAACACTTGCATTGCCGAA	AF034779	933	54

Table 2: The Multiplex PCR programs used for amplification of *E. faecalis* isolates

Gene	PCR programme	M-PCR Volume (50 µL)
<i>tfu</i>	1 cycle: 95 °C ----- 5 min.	5 µL PCR buffer 10X 2 mM MgCl ₂ 200 µM dNTP (Fermentas) 0.4 µM of each primers F & R 1 U Taq DNA polymerase (Fermentas) 3 µL DNA template
	31 cycle: 95 °C ----- 45 s 59 °C ----- 60 s 72 °C ----- 60 s	
	1 cycle: 72 °C ----- 5 min	
<i>Ebp A</i>	1 cycle: 94 °C ----- 5 min.	5 µL PCR buffer 10X 2 mM MgCl ₂ 200 µM dNTP (Fermentas) 0.4 µM of each primers F & R 1 U Taq DNA polymerase (Fermentas) 3 µL DNA template
	32 cycle: 94 °C ----- 60 s 60 °C ----- 60 s 72 °C ----- 2 min	
	1 cycle: 72 °C ----- 5 min	
<i>Ebp B</i>	1 cycle: 94 °C ----- 5 min.	5 µL PCR buffer 10X 2 mM MgCl ₂ 200 µM dNTP (Fermentas) 0.4 µM of each primers F & R 1 U Taq DNA polymerase (Fermentas) 3 µL DNA template
	32 cycle: 94 °C ----- 60 s 55 °C ----- 60 s 72 °C ----- 2 min	
	1 cycle: 72 °C ----- 10 min	
<i>Ebp C</i>	1 cycle: 94 °C ----- 5 min.	5 µL PCR buffer 10X 2 mM MgCl ₂ 200 µM dNTP (Fermentas) 0.4 µM of each primers F & R 1 U Taq DNA polymerase (Fermentas) 3 µL DNA template
	32 cycle: 94 °C ----- 60 s 58 °C ----- 60 s 72 °C ----- 2 min	
	1 cycle: 72 °C ----- 10 min	
<i>Asa, gel E</i>	1 cycle: 95 °C ----- 5 min.	5 µL PCR buffer 10X 2.5 mM MgCl ₂ 200 µM dNTP (Fermentas) 0.5 µM of each primers F & R 2 U Taq DNA polymerase (Fermentas) 3 µL DNA template
	30 cycle: 95 °C ----- 30 s 51 °C ----- 30 s 72 °C ----- 60 s	
	1 cycle: 72 °C ----- 6 min	
<i>Cyl A, cyl M, afa A</i>	1 cycle: 95 °C ----- 6 min.	5 µL PCR buffer 10X 2.5 mM MgCl ₂ 300 µM dNTP (Fermentas) 0.4 µM of each primers F & R 2 U Taq DNA polymerase (Fermentas) 3 µL DNA template
	30 cycle: 94 °C ----- 60 s 55 °C ----- 60 s 72 °C ----- 45 s	
	1 cycle: 72 °C ----- 7 min	
<i>Cyl B, ace, agg, esp</i>	1 cycle: 94 °C ----- 6 min.	5 µL PCR buffer 10X 2.5 mM MgCl ₂ 300 µM dNTP (Fermentas) 0.4 µM of each primers F & R 2 U Taq DNA polymerase (Fermentas) 3 µL DNA template
	35 cycle: 95 °C ----- 60 s 54 °C ----- 90 s 73 °C ----- 45 s	
	1 cycle: 72 °C ----- 7 min	

Statistical analysis

SPSS software (Version 25.0) (IBM SPSS Statistics) was used for statistical analysis. Fischer exact was performed for data analysis. P-value < 0.05 was considered significant.

RESULTS

Out of 1000 Urine samples, *E. faecalis* was detected in 50 samples (5%). All samples tested positive microbiologically were tested positive in a molecular study conducted using a specific primer (Figure 1).

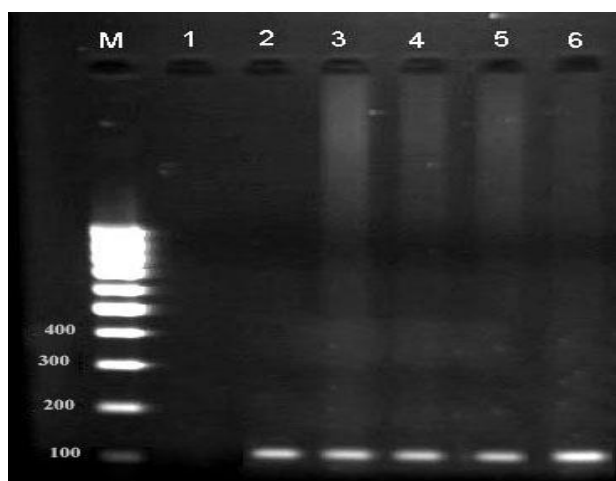


Figure 1. Result of the PCR Assay for identification of *tfu* gene in *E. faecalis* isolates. M: DNA size ladder 100 bp (Fermentas), lane 1: negative control; lane 2-6: positive samples

In the microtiter plate method, the formation of biofilm was observed in all isolates categorized as strong, moderate and weak and based on OD. In this study, the strong biofilm reaction was observed in 40 isolates (80%), moderate biofilm in 6 isolates (12%) and weak biofilm reactions were reported in 4 isolates (8%). Among the isolates producing strong biofilm, *ebp A*, *ebp B*, and *ebp C* genes were detected in 40 isolates (100%). Among the isolates producing moderate and weak biofilm *ebp A*, *ebp B* and *ebp C* genes were identified in 50% of isolates (Figure 2).

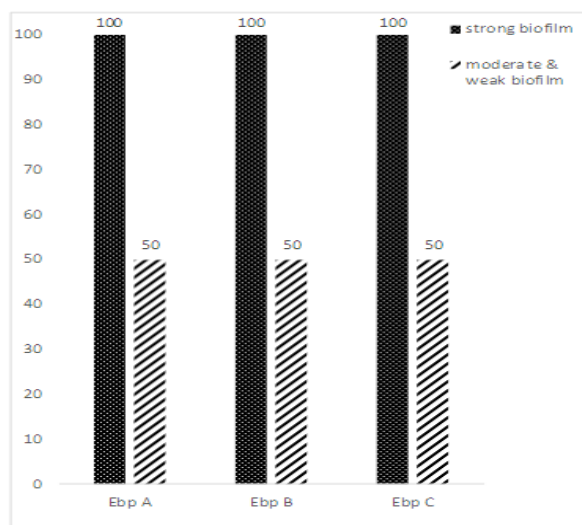


Figure 2: Prevalence of *ebp A*, *ebp B* and *ebp C* genes in biofilm-producing *E. faecalis* isolates

Figure 3 shows the resistance to the target antibiotics. The maximum resistance was against cotrimoxazole (100%), vancomycin (97.5%) and amikacin (72.5%) in strains producing a strong biofilm. Nevertheless, in moderate and weak biofilm formation strains, the maximum resistance was against cotrimoxazole (100%), vancomycin (90%), and

amikacin (60%). In this study, resistance to nitrofurantoin was not recorded. According to the statistical analysis with Fisher exact, there antibiotic resistance and type of biofilm ($p > 0.05$) were not associated significantly.

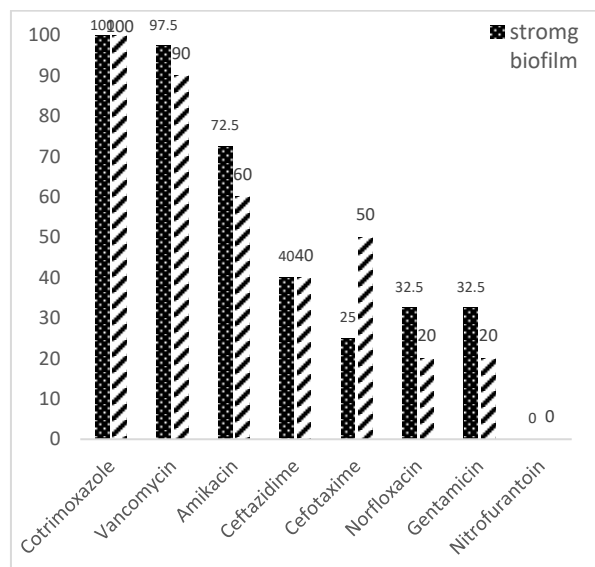


Figure 3: Antibiotic resistance pattern of the biofilm-producing *E. faecalis* isolates

Figure 4 shows how virulence genes are distributed among the clinical *E. faecalis* isolates. The most prevalence of virulence genes was in strong biofilm formation *E. faecalis* strains: *efa A* (100%), *ace* (87.5%), *gel E* (82.5%) and *esp* (80%). But in moderate and weak biofilm formation *E. faecalis* strains: *efa A* (20%), *ace* (20%), *gel E* (20%) and *esp* (10%). There was a significant relationship between the frequency of virulence genes of *efa A*, *ace*, *gel E*, *esp* and strong biofilm reaction ($P < 0.05$). However, the frequency of virulence genes and moderate and weak biofilm formation were not associated significantly.

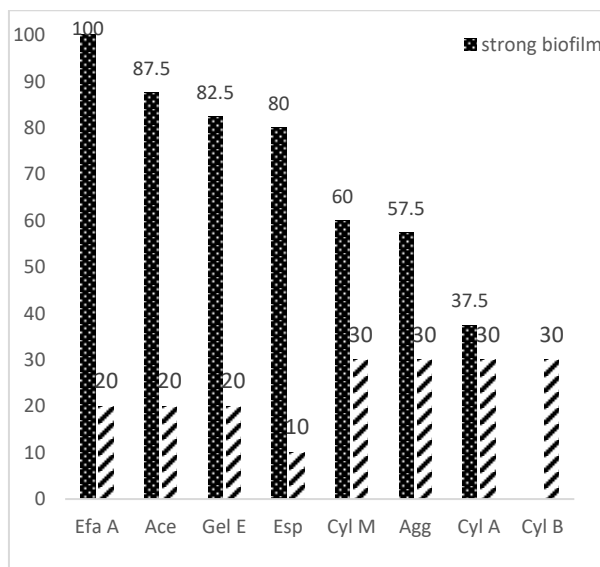


Figure 4: Prevalence of virulence genes in *E. faecalis* isolates

Result of the PCR assay for the identification of virulence genes that show in figures 5 to 8.

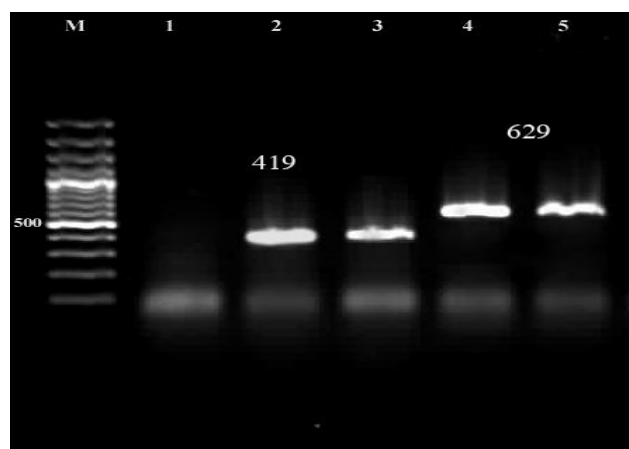


Figure 5. Result of the PCR Assay for identification of *asa* and *gel E* genes in *E. faecalis* isolates. M: DNA size ladder 100 bp (Fermentas), lane 1: negative control; lane 2,3: *asa* gene, lane 3,4: *gel E* gene

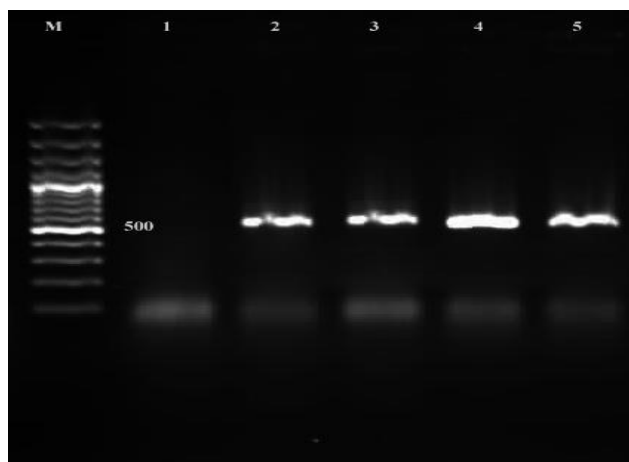


Figure 6. Result of the PCR Assay for identification of the *ebp C* gene in *E. faecalis* isolates. M: DNA size ladder 100 bp (Fermentas), lane 1: negative control; lane 2-5: *ebp C* gene

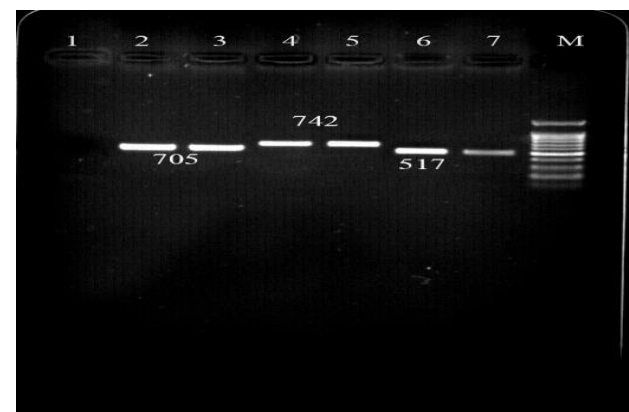


Figure 7. Result of the PCR Assay for identification of *cyl A*, *cyl M* and *afa A* genes in *E. faecalis* isolates. M: DNA size ladder 100 bp (Fermentas), lane 1: negative

control; lane 2,3: *afa A*, lane 4,5: *cyl M* and lane 6,7 *cyl A*.

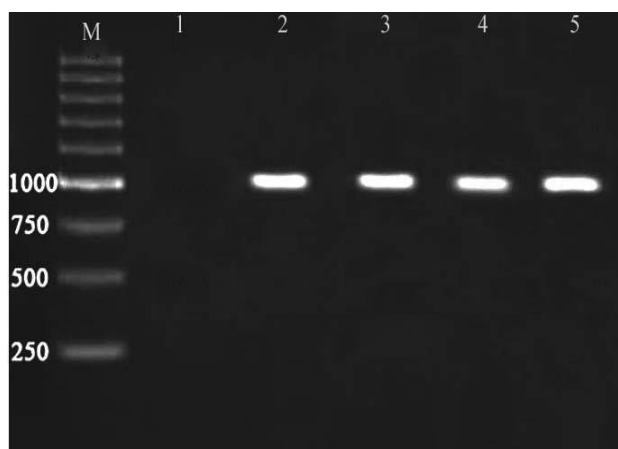


Figure 8. Result of the PCR Assay for identification of *ebp B* gene in *E. faecalis* isolates. M: DNA size ladder 100 bp (Fermentas), lane 1: negative control; lane 2-5: *ebp B*.

DISCUSSION

Enterococci are naturally resistant to several antibiotics; they are also capable of developing drug resistance either by chromosomal mutations, by transfer of plasmids, or transposon acquisition containing genetic sequences leading to resistance [18]. *E. faecalis* has come to be a common UTI pathogen. *E. faecium* outperforms *E. faecalis* in terms of drug resistance, but *E. faecalis* biofilm formation is more prevalent than that for *E. faecium*. The increased importance of enterococci as nosocomial pathogens can be to some extent justified by their intrinsic capability to acquire, accumulate, and share extrachromosomal elements encoding virulence traits or antibiotic resistance genes [19].

Our study showed a higher percentage of resistance to cotrimoxazole, vancomycin, and amikacin: 100%, 97.5%, and 72.5% in isolates that produced strong biofilm and 100%, 90%, 60% in isolates that produce moderate and weak biofilm. The high prevalence resistance to rifampicin and amikacin (86.2%) and erythromycin (73.9%) among enterococcal strains isolated from urinary tract infections have been reported by Sharifi et al. (2013) [20]. In the present study, resistance to amikacin was consistent with the results reported by Sharifi et al. In our study, resistance to nitrofurantoin was not observed which was probably due to one of the treatment options. However, Shahrakie et al. (2017) [4] reported that in the clinical multidrug resistance enterococci isolates in Southeastern Iran, resistance to nitrofurantoin was reported 7.93%. In the study by Kuhn et al. (2005) [3] resistance to vancomycin was reported 8-11%, but, our research resistance to vancomycin reported 98%. Their findings suggested significantly increased vancomycin resistance among clinical *E. faecalis* isolates from 2005 onwards. In our study, the biofilm formation of *E. faecalis* strains isolated from UTIs was explored in Kermanshah, Iran. *Enterococci*, (especially *E. faecalis*) are involved

significantly in the production of biofilm in the development of UTI. A suitable milieu is brought about by biofilms for microbial survival within the host as the organisms are protected from the host immune response, as well as antibiotics and antimicrobial agents. Several studies have focused on key virulence genes of enterococci associated with biofilm formation in these bacteria². In the present study, the strong biofilm formation was reported as 80%, moderate biofilms as 12%, and weak biofilm as 8%. Prevalence of *ebp A*, *ebp B* and *ebp C* genes in isolates producing strong biofilm was reported 100% and in isolates producing moderate and weak biofilm were 50%. Statistical analysis with Fisher exact test showed a significant correlation between biofilm formation and *ebp* genes ($p < 0.05$). In a study by Talebi et al. (2015)^[21] on 58 isolates of *E. faecalis* obtained from environmental sources and 32 isolates of *Enterococcus faecalis* obtained from two hospitals in Tehran, the ability to biofilm formation was investigated by microtiter plate. The capability of the isolates to join strongly was 62% and 71% for patient and environmental samples, in the respective order. In the hospital samples, *ebp A* was detected in 50 isolates (86%) and *ebp B* in 56 isolates (95%), and *ebp C* in 56 isolates (97%). However, in the environmental samples, *ebp A* was reported in 29 isolates (91%), *ebp B* in 30 isolates (94%) and *ebp C* in 29 isolates (91%). which is lower than our reports and previous reports. This discrepancy might be due to the presence of variable genetic make-up amongst the isolates within the *ebp* gene^[22, 23]. Studies have shown that in most cases, the bacterial isolation and stability directly depend on biofilm yield in the urinary tract^[24, 25]. More studies have revealed the pathogenesis factors of *E. faecalis*, each of which has a particular role in the development of the disease. These factors act concurrently leading to increased virulence and causing tissue degradation and invasion. The aggregation substance in the enterococci produced in response to sex pheromones that cause them to bind these bacteria together and to create a cell mass. It is also involved significantly in stimulating adhesion, cellular invasion, and degradation of myocardial and lung tissues^[26, 27]. The enterococcal superficial protein has roughly the same functions as an aggregation substance. The agent of binding to collagen is involved significantly in the binding of bacteria to collagen and cellular laminin, and its mutation leads to decreased endocarditis and UTI formation. There is a correlation between the enterococcal surface protein encoded by the chromosomal *esp* gene increased pathogenesis, colonization, persistence in the urethra and biofilm formation. It has been shown that surface proteins are involved in colonization and survival of *E. faecalis* in UTI in animal models.

Esp has been observed in large amounts in the isolates of endocarditis and bacteremia, but it is rarely seen in fecal isolates of healthy people^[5]. Some previous studies focused on the association between the virulence genes and biofilm formation and concluded that the presence of *esp* and *gel E*. *esp* help the colonization and persistence of infection within the urinary tract^[28]. In the present paper, the highest frequency of virulence genes in strong biofilm formation

isolates was observed in *efe A* (100%), *ace* (87.5%), *gel E* (82.5%) and *esp* (80%). However, in moderate and weak biofilm-producing isolates *efe A*, *ace*, *gel E* and *esp* were much less abundant. It was shown that strong biofilm formation and *efe A*, *ace*, *gel E*, and *esp* genes are correlated significantly indicating the importance of these genes in biofilm formation in *E. faecalis* isolates. Samadi Kafil et al.^[27] investigated 196 isolates of *Enterococcus* and concluded that the most prevalent genes were *efe A* (93.36%), *cyl A* and *ace* (81.63%). *Esp* and *gel E* were much less frequent compared to our research. In the present study, the presence of *esp* was not correlated with biofilm-forming ability among *Enterococcus* isolates. A significant statistical relationship was found in the study of Samadi Kafil et al. between biofilm production and *asa* and *efa A* gene, which is similar to the results of our research. Meanwhile, it was shown that the production of biofilms and the *esp* gene are not correlated. Some studies recommended that the *esp* gene is not essential nor adequate for the production of biofilm in enterococci^[29, 30]. Results on the role of the *esp* gene in biofilm formation are conflicting. Some authors believed biofilm formation is promoted by *esp* promotes biofilm formation; but, additional factors may contribute to biofilm formation in enterococci^[31, 32]. Medeiros et al. reported that there are significant correlations between strong biofilm formation and *ace* and *gelE* genes in clinical strains^[17]. Heikens et al. reported that Esp protein is not involved in biofilm production and bacterial colonization. Since cytolysin operon is closely associated with the *esp* gene, cytolysin does not have any role in biofilm production. However, some researchers believe that the *esp* gene plays a role in biofilm production^[33]. Kristich et al. (2004)^[34] reported that strains without enterococci surface protein contributed to the biofilm formation. Biofilm formation is independent of the Esp. Gelatinase increases the formation of biofilm in *E. faecalis*. It has also been shown that Esp was not necessary for biofilm formation, but its existence has been necessary for the formation of large amounts of biofilm^[35, 36]. Seno et al. (2005)^[37] reported that the presence of Esp, gelatinase, and the ability of strains to biofilm formation are correlated in the *in vitro* conditions. In a study by Gozlan et al. on 55 isolates of *E. faesium*, 41 (75%) were positive and 14 (25%) were negative for the virulence genes tested. The *esp* gene was observed in 100% of all isolates taken from urine samples^[28]. In our study, *esp* was present in 66% of all isolates. Virulence factors such as hemolysin and cytolysin exist in 32% of enterococcal species. It has been shown that the severity of endocarditis and endophthalmitis in the animal model is aggravated considerably by Cytolysin production leading to increased incidence of enterococci in humans^[3]. The chromosomal *gel E* gene encodes the gelatinase enzyme that is an extracellular metalloprotease hydrolyzing collagen, gelatin, and small peptides and is involved significantly in the endocarditis formation in animal models. Gelatinase damages the host tissue and decreases the immune response, and contributes to the activation of the autolysins and degrading the peptidoglycans, and subsequently releasing DNA and forming biofilms. *E. faecalis* with gelatinase genes have been identified in about 33% of patients with endocarditis. Sharifi

et al, (2013) [20], Heidari et al. (2016) [30] and Sabia et al. (2008) [31] reported *gel E* as the most recurrent virulence factor in *E. faecalis* strains, whereas some studies have shown the absence or low rate of *gel E* gene in enterococcal isolates [38-40]. In a study by Zhengv et al. (2018) on 113 isolates of *E. faecalis*, biofilm production was reported in 50.40%, which is lower than other studies. In this study, it was shown that *gel E* is effective in biofilm production. However, biofilm production and *esp* gene [41] were not correlated. Nonetheless, others also reported that *gel E* is not involved in the production of biofilms. In the study by Zhengv et al. the production of biofilm in *gel E* negative isolates more than in positive *gel E* isolates. In our study, there was an association between the production of biofilm and frequency of *gel E*. The *cyl A* and *ace* genes were not detected by Gozlan et al. and this is not consistent with our findings [28]. In our study, the *ace* gene was reported in 74%, *cyl M* 54%, *cyl A* 50%, and *cyl B* 22% and there was a relationship between the production of biofilm and frequency of *ace* gene. The most frequent virulence genes reported by Shokohizadeh et al. (2018) among 56 enterococci isolates in hospitalized burn patients were *gel E* and *asa* genes in *E. faecalis* (48.5%) and *E. faecium* (43%). The *cyl* gene was not identified in any *E. faecium* isolates but in *E. faecalis* reported 5.8% [29]. In our study *efa*, *A* gene was the most frequent virulence (100%), *ace* (74%), *gel E* (70 %) and *agg* (52 %). Consistent with other reports, the results of the present study indicated that biofilm formation was higher in isolates with antibiotic resistance to Cotrimoxazole, Vancomycin, Cefazidime and gentamicin suggesting a genetic association between the biofilm and these antibiotic genes. This conclusion was, however, not inclusive for all antibiotics [42, 43]. This finding shows that biofilm production in *E. faecalis* isolated from UTI in Kermanshah is higher than in other studies.

CONCLUSION

The high antibiotic resistance observed in this study likely suggests the inherent resistance of enterococci. The higher antibiotic resistance among the patient isolates could be attributed to the circulation of transposable elements carrying resistant genes in clinical isolates, indiscriminate and uncontrolled usage of antibiotics, and the presence of biofilm. In the present study, all isolates could produce biofilm. Therefore, a comparison was made between isolates producing strong, moderate, and weak biofilm, and no significant relationship was found between biofilm type and antibiotic resistance. Given the likely transmission of antibiotic resistance genes among enterococci and *Staphylococci*, managing the infections triggered by enterococci as well as the proper administration of antibiotics in patients can be good options to treat nosocomial infections effectively.

Abbreviations

Ace: Adhesin of collagen from *E. faecalis*

CLSI: Clinical and Laboratory Standards Institute

Esp: Extracellular surface protein

E. faecalis: *Enterococcus faecalis*

E. faecium: *Enterococcus faecium*

EBP: Endocarditis and Biofilm associated Pili

Efa A: *E. faecalis* antigen A

UTI: Urinary Tract Infection

Declarations

Ethics approval and consent to participate

In the current study, we tried to protect the life, health, dignity, integrity, rights to self-determination, privacy, and confidentiality of personal information of research subjects. We conform to generally accepted scientific principles based on a thorough knowledge of the scientific literature, other relevant sources of information, and adequate laboratory and, as appropriate, animal experimentation. All samples were taken from volunteer patients for this research. All ethical issues were considered and this research was performed with hospitals' permission. The name and identity, personal information and even patients' illnesses and their medical information remained secret. All of the patients voice their satisfaction to use their sample in this investigation, especially to determine antibiotic resistance in UPEC strains

Consent to Publish

Not applicable.

Availability of data and materials

All data analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

MS, ET, HM, and RR carried out the molecular genetic studies, participated in the primers sequence alignment and drafted the manuscript. MS and HM carried out the sampling and culture method. MS and ET participated in the design of the study, performed the statistical analysis and writing the manuscript. All authors read and approved the final manuscript.

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