# Prevalence of Virulence Genes and Antibiotic Resistance Pattern in *Enterococcus Faecalis* Isolated from Urinary Tract Infection in Shahrekord, Iran

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## Abstract

***Background:*** This study aims to specify the antimicrobial resistance pattern and virulence genes of *Enterococcus faecalis* isolated from urinary tract infections in Shahrekord, Iran.

***Methods:*** Urine samples of 1000 people suspected of having urinary tract infections referred to Shahrekord medical diagnostic laboratories were examined. Biofilm assays were performed by microtiter plate test through reading the OD490. Polymerase Chain Reaction (PCR) was applied to study the virulence factors.

***Results:*** *Enterococcus faecalis* was detected in 60 samples. After performing microbiological tests, all samples were positive in the molecular analysis. Strong, moderate and weak biofilm reactions reported 66.67%, 25%, and 8.33% respectively. The most resistance reported to cotrimoxazole, vancomycin and amikacin and the lowest resistance to nitrofurantoin (8.33%) was reported. Statistical analysis with Fisher's exact test showed a statistically significant relationship between biofilm production and resistance to cotrimoxazole, vancomycin and cefotaxime. 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%, 62.5%, 55%, 37.5% 25% and 22.5% respectively. There was a significant relationship between the frequency of *efa A* and strong biofilm reaction.

***Conclusions:*** The presence of *E. faecalis* strains resistant to co-trimoxazole and vancomycin and present of 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

The human gut contains normal microbial flora, which includes several different bacterial species. Although many of these bacteria are harmless, non-pathogenic, and beneficial to humans, some of them, like enterococci, can cause disease. *Enterococci* (mostly *Enterococcus faecalis* and *Enterococcus faecium*) are gram-positive, fermentative bacteria that inhabit the natural gastro-intestinal tract of humans, birds, and a group of animals. They can also be found in the mouth, intestines and vagina of humans, soil, water and food. *Enterococci* can grow in environments with high salt concentrations and temperatures of 10 to 45 °C. Several reports have documented *that Staphylococcus epidermidis* and *Staphylococcus aureus* and the two most important species enterococcus are among the leading causes of opportunistic human infections,

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including urinary tract infections, infections of the surgical site and burn wound infections, bacteremia and sepsis, endocarditis, and others (1-8). The microorganism first appeared in Europe in 1986 and then spread to the United States in 1998, with a 20-fold increase in nosocomial infections between 1989 and 1998 (9-11). The prevalence of enterococcal infections worldwide is increasing in recent years, so that enterococci are reported as one of the most common nosocomial infections today. Biofilm protects Enterococci from host immune response and antibiotics. Biofilm-producing Enterococci cause recurrent, chronic, and antibiotic-resistant infections (12,13). A high mortality rate of enterococcal infections is due to increasing resistance of the organism to β-lactam antibiotics, aminoglycosides, and glycopeptides and inadequate response to the treatment (14,15). Biofilm protects Enterococci from host immune response and antibiotics. Biofilm-producing Enterococci cause recurrent, chronic, and antibiotic-resistant infections. According to the National Institute of Health, 80% of infections are related to biofilm-forming microbes apart from biofilm-forming ability, *Enterococcus* spp. are known to produce various virulence factors (16). Virulence factors of *Enterococcus* spp. may contribute to competition with other bacteria, colonization of the host, resistance against defense mechanisms of the host and production of pathological changes directly through production of toxins or indirectly through induction of inflammation (17,18). The presence of genes encoding virulence factors including collagen-binding protein (ace), aggregation substance (*asa1*), cytolysin (*cylA*), endocarditis antigen (*efaA*), enterococcal surface protein (*esp*), gelatinase (*gelE*) and hyaluronidase (*hyl*), is analyzed in recent years.

Since no research has been done on antibiotic resistance and frequency of virulence genes in *Enterococcus faecalis* isolates isolated from urinary tract infections in Shahrekord city, in this study we decided to investigate these factors.

## Materials and Methods

### Ethics approval and consent to participate
This study was done on UTI samples collected from Shahrekord medical diagnostic laboratories with patient satisfaction, so there has no ethical issue in this work. This thesis has been approved in Islamic Azad University Shahrekord branch with code IR.IAU.SHK.REC.1399.055.

### Sampling, Isolation and Identification
In this cross-sectional study, approximately 1000 urine samples from April to September 2018 were gathered from Shahrekord medical diagnostic laboratories. 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, arabinose fermentation test was employed to differentiate *E. faecalis* and *E. faecium* (19,20).

### Microtiter plate assay for biofilm formation
Polystyrene microtiter plates were used to evaluate biofilm formation of *E. faecalis*, as previously described for other gram-positive cocci (21,22). The isolates of *E. faecalis* were inoculated in 10 mL of tryptic soy broth with 0.25% glucose and incubated overnight with shaking at 37 °C. Next, the cultures were diluted 1:100, and 200 of the diluted cultures, per well, were inoculated into 96-well polystyrene microtiter plates. After 48 h incubation at 37 °C under aerobic conditions, the plates were washed three times with 300 distilled water. Subsequently, the plates were stained with 200 of 1% crystal violet, per well, for 10 min. Excess crystal violet was removed by gently washing the plate twice with distilled water. Finally, a volume of 250 of 95% ethanol solution, per well, was added to the plate and the optical density was measured at 490 nm. The absorbance of de-staining solution was measured at 490 nm in an ELISA reader (Stat fax-2100). A well with sterile TSB or LB serve as controls, whereby their ODs were subtracted from that of the experimental strains. The mean OD 570 nm value was determined using four replicates and was

considered to be adherence positive at OD 490 nm greater than or equal to 0.216 high biofilm formation, between 0.54 and 0.108 as weak biofilms. If they had an OD less than 0.54 as negative biofilms (23,24).

DNA of the samples that were taken as positive and negative biofilms was extracted using DNA extraction kit.

### Antimicrobial Susceptibility Testing

Patterns of antimicrobial resistance of the *E. faecalis* strains were studied using the simple disk diffusion technique. The Mueller–Hinton agar (Merck, Germany) medium was used for this purpose. Susceptibility of *E. faecalis* isolates were tested against several types of antibiotics with appropriate disks containing vancomycin (VAN 10µg), co-trimoxazole (SXT 25 µg), ceftazidime (CAZ 30 µg), norfloxacin (NOR 10 µg), amikacin (AN 30 µg), gentamicin (GM 120 µg), cefotaxime (CTX 30 µg) and nitrofurantoin (FM 300 µg) (produced by PadTan-Teb, Iran), according to the instruction of Clinical and Laboratory Standards Institute (25). The quality control strain used was *E. faecalis* ATCC 29212.

### 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, the total DNA was measured at 260 nm optical density. PCR was applied using specific targeted primers to detect enterococci (26).

### Identifying the virulence genes by PCR method

PCR was applied using specific primes to identify the virulence factors genes including *gel E*, *esp*, *agg*, *ace*, cytolysin (c*ylM, cylA and cyl B*) genes. Tables 1 show the applied primer sequence. A DNA thermal cycler was used to perform PCR, (Gradient Master cycler, 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 docgel documentation systems (Uvitec, UK) was used. A comparison was performed between the PCR products against a 100 bp DNA marker (Fermentas, Germany) (27, 28).

**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 (ºC) |
|---|---|---|---|---|
| *16srDNA* | RW015´-AACTGGAGGAAGGTGGGGAT-3´<br>DJ745´-AGGAGGTGATCCAACCGCA-3´ | CP015883.1 | 370 | 59 |
| *asa* | F: CCAGCCAACTATGGCGGAATC<br>R: CCTGTCGCAAGATCGACTGTA | CP22712 | 419 | 51 |
| *Gel E* | F: ACCCCGTATCATTGGTTT<br>R: ACGCATTGCTTTTCCATC | KU311665 | 629 | 51 |
| *Cyl A* | F: TGGATGATAGTGATAGGAAGT<br>R: TCTACAGTAAATCTTTCGTCA | CP015883 | 517 | 55 |
| *Cyl M* | F: CTGATGGAAAGAAGATAGTAT<br>R: TGAGTTGGTCTGATTACATTT | AY032999 | 742 | 55 |
| *efa A* | F: GACAGACCCTCACGAATA<br>R: AGTTCATCATGCTGTAGTA | KY070337 | 705 | 55 |
| *Cyl B* | F: ATTCCTACCTATGTTCTGTTA<br>R: AATAAACTCTTCTTTTCCAAC | KU311664 | 843 | 54 |
| *ace* | F: GAGCAAAAGTTCAATCGTTGAC<br>R: GTCTGTCTTTTCACTTGTTTCT | AF159247 | 1003 | 56 |
| *agg* | F: AAGAAAAAGAAGTAGACCAAC<br>R: AAACGGCAAGACAAGTAAATA | CP002493 | 1553 | 54 |
| *esp* | F: TTGCTAATGCTAGTCCACGACC<br>R: GCGTCAACACTTGCATTGCCGAA | AF034779 | 933 | 54 |

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

| Gene | PCR program | | M-PCR Volume (50 µl) |
|---|---|---|---|
| *16srDNA* | **1 cycle** | | 5 µl PCR buffer 10X |
| | 95 °C | 6 min | 2 mM MgCl2 |
| | **30 cycles** | | 200 µM dNTP (Fermentas) |
| | 95 °C | 30 s | 0.4 µM of each primer (F & R) |
| | 59°C | 30 s | 1 U Taq DNA polymerase (Fermentas) |
| | 72 °C | 60 s | 3 µl DNA template |
| | **1 cycle** | | |
| | 72 °C | 6 min | |
| *Asa, gel E* | **1 cycle** | | 5 µl PCR buffer 10X |
| | 95 °C | 5 min | 2.5 mm MgCl2 |
| | **30 cycles** | | 200 µM dNTP (Fermentas) |
| | 95 °C | 30 s | 0.5 µm of each primer (F & R) |
| | 51 °C | 30 s | 2 U Taq DNA polymerase (Fermentas) |
| | 72 °C | 60 s | 3 µl DNA template |
| | **1 cycle** | | |
| | 72 °C | 6 min | |
| *Cyl A, cyl M, afa A* | **1 cycle** | | 5 µl PCR buffer 10X |
| | 95 °C | 6 min | 2.5 mM MgCl2 |
| | **30 cycles** | | 300 µM dNTP (Fermentas) |
| | 94 °C | 60 s | 0.4 µM of each primer (F & R) |
| | 55 °C | 60 s | 2 U Taq DNA polymerase (Fermentas) |
| | 72 °C | 45 s | 3 µl DNA template |
| | **1 cycle** | | |
| | 72 °C | 7 min | |
| *Cyl B, agg, esp* | **1 cycle** | | 5 µl PCR buffer 10X |
| | 94 °C | 6 min | 2.5 mM MgCl2 |
| | **35 cycles** | | 300 µM dNTP (Fermentas) |
| | 95 °C | 60 s | 0.4 µM of each primer (F & R) |
| | 54 °C | 90 s | 2 U Taq DNA polymerase (Fermentas) |
| | 73 °C | 45 s | 3 µl DNA template |
| | **1 cycle** | | |
| | 72 °C | 7 min | |
| *ace* | **1 cycle** | | 5 µl PCR buffer 10X |
| | 94 °C | 5 min | 2.5 mm MgCl2 |
| | **35 cycles** | | 200 µM dNTP (Fermentas) |
| | 95 °C | 60 s | 0.5 µm of each primer (F & R) |
| | 56 °C | 90 s | 2 U Taq DNA polymerase (Fermentas) |
| | 73 °C | 45 s | 3 µl DNA template |
| | **1 cycle** | | |
| | 72 °C | 6 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 60 samples (16.66%). All samples tested positive microbiologically were tested positive in a molecular study conducted using a specific primer (Fig. 1).
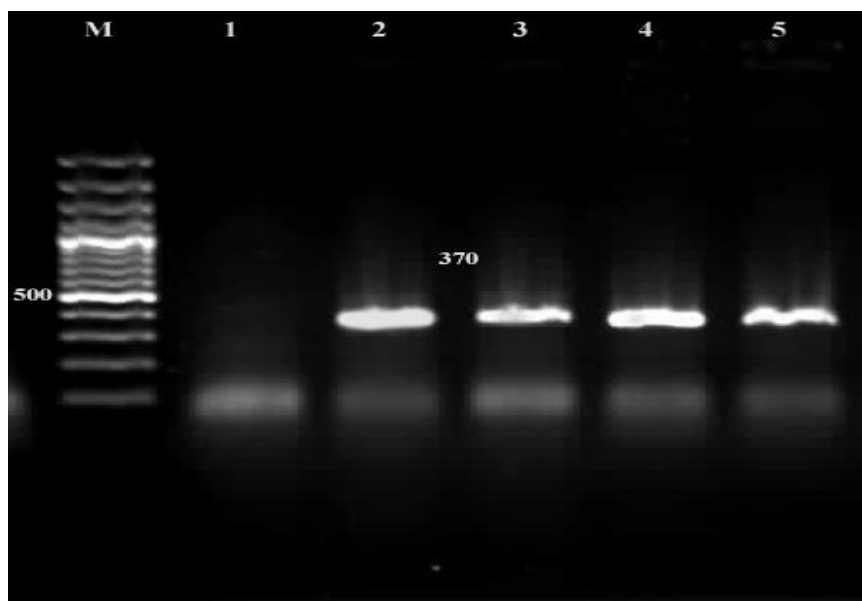
**Fig. 1.** Result of the PCR Assay for identification of *16srDNA* gene in *E. faecalis* isolates. M: DNA size ladder 100 bp (Fermentas), lane 1: negative control; lane 2-6: positive samples. Resistance to cotrimoxazole in 60 samples (100%) (The highest percentage), resistance to tetracycline in 50 samples (83.33%), resistance to vancomycin in 40 samples (66.67%), to amikacin in 30 samples (50%) Resistance to erythromycin in 28 samples (46.67%), resistance to ceftazidime in 26 samples (43.33%), resistance to gentamicin in 20 samples (33.33%), resistance ratio of cefotaxime in 20 samples (33.33%), resistance to norfloxacin in 18 samples (30%), resistance to nitrofurantoin in 5 samples (8.33%) (The lowest percentage) reported.

The biofilm production was strong in 40 (66.67%) isolates, moderate in 15 (25%) and weak in 5 (8.33%) isolates. Represents the antibiotic resistance pattern of the *E. faecalis* strains isolated from UTI samples *E. faecalis* strains harbored the highest prevalence of resistance against co-trimoxazole (100%), Tetracycline (83.33%) and vancomycin (66.67%). amikacin, erythromycin, ceftazidime, gentamicin, cefotaxime, norfloxacin, and nitrofurantoin reported: 50%, 46.67%, 43.33%, 33.33%, 33.33%, 30% and 8.33% respectively (Fig. 2).

**Table 3.** Antibiotic resistance in *Enterococcus faecalis* isolates based on biofilm formation.

| Antibiotic | Biofilm Formation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strong (N= 40) | | | | Moderate (N= 15) | | | | Weak (N= 5) | | | |
| | Resistance | | Sensitive | | Resistance | | Sensitive | | Resistance | | Sensitive | |
| Co-Trimoxazole | 38 | 95 % | 2 | 5% | 12 | 80% | 3 | 20% | 2 | 40% | 3 | 60% |
| Tetracycline | 30 | 75% | 10 | 25% | 10 | 66.67% | 5 | 33.33% | 3 | 60% | 2 | 40% |
| Vancomycin | 39 | 97.5% | 1 | 2.5% | 10 | 66.67% | 5 | 33.33% | 4 | 80% | 1 | 20% |
| Amikacin | 35 | 87.5% | 5 | 12.5% | 13 | 86.67% | 2 | 13.33% | 3 | 60% | 2 | 40% |
| Erythromycin | 29 | 72.5% | 11 | 27.5% | 10 | 66.67% | 5 | 33.33% | 1 | 20% | 4 | 80% |
| Ceftazidime | 24 | 60% | 16 | 40% | 12 | 80% | 3 | 20% | 4 | 80% | 1 | 20% |
| Cefotaxime | 36 | 90% | 4 | 10% | 14 | 93.33% | 1 | 6.67% | 2 | 40% | 3 | 60% |
| Gentamicin (GM) | 30 | 75% | 10 | 5% | 12 | 80% | 3 | 20% | 2 | 40% | 3 | 60% |

There is a statistically significant relationship between antibiotic resistance and biofilm in co-trimoxazole, vancomycin and cefotaxime using Fisher's exact test (p value< 0.05).

Prevalence of virulence factors in *E. faecalis* isolates that show in figure 3. As can be seen, the highest frequency belongs to *efa A* (86.67%) and the lowest frequency belongs to *cyl B* (16.67%).
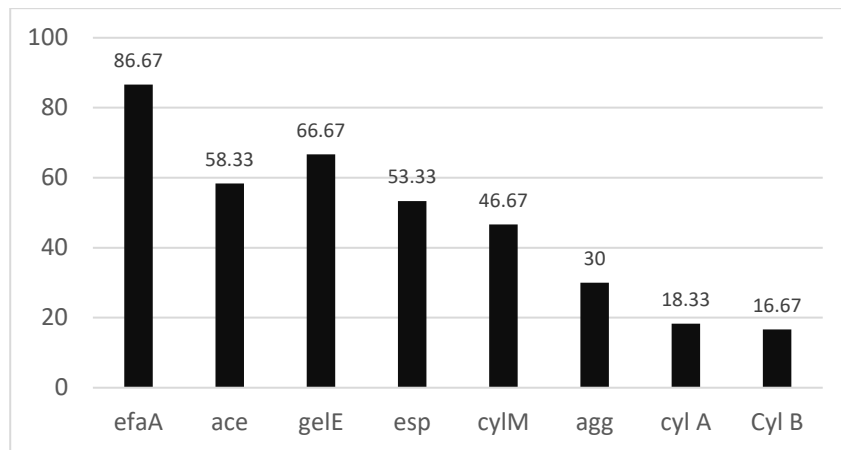


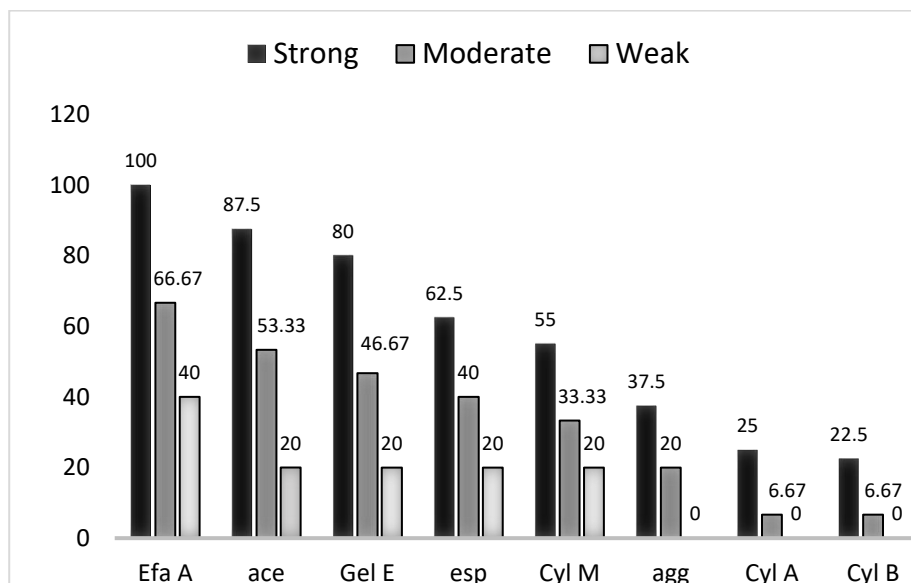**Fig. 2.** Frequency of virulence genes in *Enterococcus faecalis* isolates.



**Fig. 3.** Frequency of virulence genes in *Enterococcus faecalis* isolates based on biofilm formation.

Based on Fisher's exact test, a significant relationship was reported between the biofilm reaction and the *efa A* gene (p< 0.05). There was no statistically significant relationship between other genes and biofilm production (p value= 0.942> 0.05) (Fig. 3).

## Discussion

*Enterococcus* is one of the significant pathogens affecting all age groups. *Enterococcus* species have become problem of the world as emerging nosocomial infection and multi drug resistance bacteria (29). Among several species which belong to genus Enterococcus, *E. faecalis* is the most common isolate that has an association with 80–90% of human Enterococcal infection

(30-32). In the present study, out of 1000 urine samples, *Enterococcus faecalis* infection was detected in 50 samples. Studies show that in most cases, the establishment and stability of bacteria directly depends on the production of biofilm in the urinary tract.

Virulence factors act convergently, leading to increased virulence and tissue damage and invasion. Traits that increase pathogenicity typically include cytolysin, cumulative factor (pheromone), adhesin, extracellular superoxidase, extracellular surface protein, hemolysin, and gelatinase. Proteins such as hemolysin, gelatinase and cumulative factor are involved in plasmid exchange systems. These proteins, along with other factors, are involved in the pathogenicity of *enterococci*, although their role in infectivity remains unclear. These factors are thought to increase convergence by activating chromium sensing by bacterial virulence. The protein of the aggregate that carries the *asa1* gene in enterococci is produced in response to sex pheromones that cause these bacteria to be absorbed into each other and form a cell mass. It also plays an important role in stimulating adhesion, invading cells and destroying myocardial and lung tissues (33-35). In the present study, strong biofilm reaction was reported in 40 isolates (66.67%), moderate biofilm in 15 isolates (25%) and weak biofilm reaction in 5 isolates (8.33%). The relationship between biofilm reaction and *efa A* gene Statistically significant was reported. The increasing prevalence of enterococcal infections is associated with the use of third generation cephalosporins. Uncontrolled use of antibiotics is known to be the most important factor in the spread of resistant microorganisms that reduce the natural microflora of the human and animal gastrointestinal tract. The spread of vancomycin-resistant enterococci may be associated with the use of glycopeptides as growth promoters in animal and poultry feed. In our study, high resistance to the antibiotic vancomycin was reported (66.67%). Resistance to different antibiotics varies based on treatment patterns in different regions. In this study, the highest resistance to cotrimoxazole was 100% and the lowest resistance to nitrofurantoin was 8.33%.

Considering the comparison of different percentages of antibiogram results with similar experiments, it should be noted that regional differences in different parts of the world or even a country produce different therapeutic responses to antimicrobial drugs. The origin of these differences in different places can be considered as genetic differences of strains and differences in other fields. In the study of Joghatei et al., resistance to erythromycin, tetracycline and ciprofloxacin was 40%, 20% and 8% respectively. While in our study the resistance tetracycline, erythromycin, and norfloxacin was reported to 83.33%, 46.67% and 30% (35). Resistance to the antibiotics mentioned in our study is higher than that of Joghatei et al., Aminoglycosides are still valuable in the treatment of bacterial infections, despite their side effects and problems associated with increased resistance of microorganisms to these drugs. The three mechanisms of resistance, including changes in the ribosomal site of drug binding, decreased drug permeability, and enzymatic inactivation of the drug, are responsible for resistance to aminoglycosides. Among these, enzymatic inactivation of aminoglycosides by modifying enzymes, they are the main mechanism of resistance to these drugs in gram-negative bacteria. In this study, the aminoglycoside antibiotics amikacin, gentamicin and tetracycline were used. Resistance to amikacin (50%), resistance to gentamicin (33.33%) and resistance to tetracycline (83.33%) were reported. Biofilm production in enterococci is one of the mechanisms of resistance to antimicrobial drugs. Bacteria participating in the biofilm behave differently from planktonic cells. Today, researchers are studying genes involved in biofilm formation in enterococcal strains of various origins. Enterococcal surface protein genes increase the ability to colonize and produce bacterial biofilms *in vitro* and appear to be associated with the presence of biofilms in living organisms. Enterococcal surface protein, encoded by the chromosomal *esp* gene, is associated with increased pathogenicity, colonization, urinary tract stability, and biofilm formation (37). In this study, it was observed that

antibiotic resistance was higher in isolates capable of producing strong biofilms than in isolates producing moderate or weak biofilms. There is a statistically significant relationship between antibiotic resistance and biofilm formation in co-trimoxazole, vancomycin and cefotaxime.

In our study, virulence genes were more common in isolates producing strong biofilms than in isolates producing moderate or weak biofilms. In study conducted by Talebi et al., out of 32 *Enterococcus faecalis* isolates, obtained from two hospitals in Tehran, the ability to produce biofilm by microtiter plate was evaluated. In this study 23 strong biofilm isolates, 4 weak biofilm isolates and 5 no biofilm isolates were reported (36). In our study, *efeA* gene had the highest frequency and *cylB* gene had the lowest frequency. In statistical analysis with Fisher's exact test a significant relationship was reported between the biofilm reaction and the *efa A* gene (p< 0.05). In Gozalan et al., study of 55 *Enterococcus faecalis* isolates, 41 isolates (75%) had virulence genes and 14 isolates (25%) did not have virulence genes. *Cyl A*, *hly A*, and *ace* genes were not observed in any of the isolates. In this study, prevalence of *efaA, ace, gelE, esp, cylM and agg* reported: 100%, 74%, 70%, 66%, 54%, and 52% respectively (37). The study by Seno et al., showed that there is a strong correlation between the presence of enterococci

surface protein, gelatinase and the ability of strains to form biofilms *in vitro* (38). Heikens et al., Reported that Esp protein does not play a role in biofilm production and bacterial colonization, and since cytolysin operon is closely related to the *esp* gene, cytolysin also has no role in biofilm production (39). In our study, no statistically significant relationship was observed between the presence of *esp* and *cyl* genes and biofilm formation. In a study conducted by Zheng V et al., out of 113 *Enterococcus faecalis* isolates, biofilm production was reported in 50 isolates (40%). In this study, no correlation was observed between biofilm production and *esp* gene. In our study, no statistically significant relationship was observed between the presence of *gel E* and biofilm formation (40). Considering the comparison of different percentages of antibiogram results with similar experiments, it should be noted that regional differences in different parts of the world or even a country cause different therapeutic response to antimicrobial drugs.

## Acknowledgements

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