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# Detection of virulence factors of *Enterococcus faecalis* isolated from the urinary system and evaluation of antibiotic resistance

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## ABSTRACT

**Aims:** Enterococci, which are among the leading causes of nosocomial infections, are opportunistic pathogens and cause urinary tract infections most frequently. The frequency of isolation increases especially in patients with urinary system anomalies or urological interventions. Although various virulence factors play a role in the pathogenesis of infections caused by enterococci, cytolysin, hemolysin and enterococcal surface protein (ESP) are among the frequently investigated virulence factors. In this study; It was aimed to investigate the relationship between the presence of virulence factors and antibiotic resistance in *Enteroccus faecalis (E. faecalis)* strains isolated from urine samples, as well as the effect of urinary catheter use on these factors.

**Methods:** 100 strains isolated from urine samples sent to İstanbul University İstanbul Faculty of Medicine Medical Microbiology Laboratory and identified as *E. faecalis* with the VITEK 2 (biomerioux-France) GP identification kit were included in the study. Hemolysin and gelatinase, virulence factors, were determined phenotypically. The presence of the ESP gene was investigated by PCR using ESP11 and ESP12 primers. Antibiotic sensitivities were studied by disk diffusion and gradient strip methods, and the results were evaluated in accordance with CLSI and EUCAST recommendations.

**Results:** Antibiotic resistance rates were found to be 2%, 31%, 1%, 22%, 37% for ampicillin, norfloxacin, nitrofurantoin, high-level gentamicin (HLG) and high-level streptomycin (HLS), respectively, while no strains resistant to vancomycin, linezolid and tigecycline were detected. When evaluated in terms of virulence factors; It was determined that 82% of the strains produced gelatinase, 67% produced ESP, and 35% produced hemolysin. No virulence factor was detected in eight strains.

**Conclusion:** In our study, no significant relationship was found between the presence of virulence factors and antibiotic resistance and catheter application. However, since the most detected gelatinase and ESP are virulence factors that have the ability to colonize and form biofilms on abiotic surfaces, it is thought that minimizing catheterization practices may contribute to the prevention of UTIs that may develop with enterococci.

**Keywords:** *Enterococcus faecalis*, urinary tract infection, antibiotic resistance, virulence factors

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## INTRODUCTION

Enterococci, which are a member of the gastrointestinal system flora of humans and animals, are microorganisms with low virulence. However, due to their intrinsic and acquired antimicrobial resistance properties, they have become opportunistic or nosocomial pathogens frequently isolated in community and healthcare-associated infections.[1,2]

Enterococci cause various clinical conditions such as urinary tract infection (UTI), wound, intra-abdominal, endocarditis, bloodstream infections, as well as infections associated with the use of medical devices.[3] UTI is one of the leading infections caused by enterococci, and is especially seen in patients with underlying urinary system anomalies and those who have undergone catheter and/or urinary intervention.[1,4] Risk factors for nosocomial enterococcal colonization or infection include comorbid conditions such as prolonged hospitalization or intensive care unit stay, intra-abdominal and cardiothoracic surgery, immunosuppression, and prior use of antibiotics (especially cephalosporins, vancomycin, or aminoglycosides).[1,5]

*Enterococcus faecalis (E. Faecalis)* (80-90%) and *Enterococcus faecium* (5-10%) are the most common species among healthcare-associated infectious agents worldwide.[3] However, the distribution of dominant enterococcal species varies in terms of host, environmental and hospital environment-related factors, as among the virulence factors that play a role in the pathogenesis of enterococcal infections, hemolysin/cytolysin, gelatinase and enterococcal surface protein (ESP) are frequently investigated virulence factors.[5] Hemolysis; It is a cytolytic enzyme that causes lysis in human, horse and rabbit erythrocytes and may increase the severity of the infection.[5] Gelatinase is an extracellular protease that hydrolyzes peptides such as gelatin, collagen, casein and hemoglobin that

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enable adhesion to the host cell. It has been stated in animal experiments that it may be associated with the development of endocarditis and biofilm formation.[5,7] It has been stated that ESP, another virulence factor, facilitates colonization of the host cell epithelium and subsequent infection development, and causes biofilm formation on abiotic surfaces.[5,8] It has been reported that gelatinase increases the effect of ESP and contributes to biofilm formation, especially in the development of UTI caused by *E. faecalis*.[8]

Antimicrobial resistance in enterococci may be intrinsic or acquired. Constitutive resistance is a feature encoded in the chromosomes of enterococcal species, and they are intrinsically resistant to antimicrobial drugs such as cephalosporins, aminoglycosides, lincosamides and trimethoprim-sulfamethoxazole. Acquired resistance occurs through mutations in structural DNA due to the flexibility of genome structures or by the transfer of genetic material on a plasmid or transposon. In recent years, increasing resistance rates have been reported, especially against high-level aminoglycosides (HLR), beta lactams and glycopeptides.[3,9]

The aim of this study was to determine the virulence factors and antibiotic resistance of *E. faecalis* isolated from urine samples, to investigate the relationship between the presence of virulence factors and antibiotic resistance, and to investigate the relationship between virulence factors and urinary catheter use, which constitutes a risk for the development of UTI.

## METHODS

100 *E. faecalis* strains isolated from urine samples sent to the medical microbiology laboratory from various clinics of İstanbul University İstanbul Faculty of Medicine Hospital between 22.02.2009-10.06.2010 were included in the study (Date: 01.09.2009, Decision No: 2489). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki. Urine samples were cultured on chromogenic agar (BBL Chromagar Orientation), and the cultures were evaluated after 24 and 48 hours of incubation at 37˚C. *Enterococcus* spp. are Gram-positive, catalase-negative, Gram-positive cocci that grow on media, form black colonies on bile esculin agar, and grow on media containing 6.5% NaCl. Species identification was made with the VITEK 2 (bioMerieux, France) Gram positive (GP) identification kit. For the phenotypic detection of haemolysis, which is one of the virulence factors, brain heart infusion agar containing 5% horse blood was inoculated, kept at 37˚C and 5% CO2, and evaluated after 24 and 48 hours. The formation of a beta haemolysis zone around the colonies was considered positive.[10] For phenotypic detection of gelatinase, tryptic soy agar (TSA) medium containing 1.5% skim milk was inoculated and the transparent zone formed around the colonies after 18 hours at 37˚C was considered positive.[11] The presence of the ESP gene encoding the enterococcal surface protein was investigated by polymerase chain reaction (PCR) (Amersham Ready-To-Go RT-PCR Beads, UK) using site-specific ESP11 and ESP12 primers after DNA extraction (High Pure PCR Template Preparation Kit, Roche, Germany) and the sequence of the primers are listed in Table 1.[10,12,13]

**Table 1. Primer used in PCR detection of esp**

| Gene | Primer | Sequence (5' to 3') | Amplicon size (bp) |
|---|---|---|---|
| esp | esp 11 (forward) | 5' -TTGCTAATGCTAGTCCACGACC-3' | 954 |
| | esp 12 (reverse) | 5' -GCGTCAACACTTGCATTGCCGAA-3' | |

PCR: Polymerase chain reaction

The PCR conditions were: Initial denaturation of 94˚C for 10 min, 30 cycles of: denaturation (94˚C for 45 s), annealing (63˚C for 45 s) and extension (72˚C for 60 s), added to a final extent of 72˚C for 10 min, followed by cooling the samples to 4˚C. The amplicons were analyzed by 2% agarose gel electrophoresis and 1X TBE buffer and visualized by ethidium bromür dye on the photodocumentator in UV light. In order to determine the molecular weight of the amplified DNA, the bands formed were determined by comparing the molecular weights with known bands according to the marker used. The *E. faecalis* strain MMH 594 was used as positive control in PCR detection of ESP.[10,13]

Antibiotic susceptibilities of the isolated strains were determined by Kirby-Bauer disc diffusion method according to CLSI guidelines.[14] Testing for susceptibility to vancomysin (30 µg), ampicillin (10 µg), norfloxacin (10 µg), nitrofurantoin (300 µg), linezolid (30 µg), gentamicin (120 µg) and streptomycin (300 µg) was done with use of disc (Oxoid, Basingstone, UK).[14] Testing for susceptibility to vancomysin, linezolid and tigecycline was done with use of Etest strips (AB Biodisk Sweden). Results were evaluated in accordance with CLSI and EUCAST recommendations for tigecycline.

### Statistical Analysis

Data are described using frequency and percentage. The results were evaluated with chi-square tests. Statistical significance was accepted if $p<0.05$.

## RESULTS

In this study, a total of 100 *E. faecalis* strains isolated from urine samples sent to our laboratory from various clinics and outpatient clinics of our hospital were examined. While 31 (31%) of the 100 patients examined were male and 69 (69%) were female, the age distribution of the patient group varied between four months and 87 years (mean. 4 years). The number of inpatients was determined as 15 (15%), and the number of patients followed in the outpatient clinic was determined as 85 (85%). Among the patients from whom the enterococcal strains were isolated included in the study, the number of patients who were in the risk group for UTI and had a history of using a urinary catheter at least once was 56 (56%), while the number of patients who did not have a catheter was 44 (44%).

When the antibiotic resistance rates of the tested strains were examined, no strains resistant to vancomycin, linezolid and tigecycline were detected. Resistance to nitrofurantoin was found in 1%, ampicillin in 2%, norfloxacin in 31%, and HLG and HLS in 22% and 37%, respectively. When the MIC distribution of the investigated strains is examined; MIC90 values were determined as 2, 2 and 0.19 for vancomycin,

linezolid and tigecycline, respectively. Antibiotic resistance rates are shown in Table 2.

**Table 2. Antibiotic resistance rates in *Enterococcus Faecalis* strains (%)**

| Antibiotic | Resistance (%) |
|---|---|
| Ampicillin | 2 |
| Norfloxacin | 31 |
| Nitrofurantoin | 1 |
| Vancomycin | 0 |
| Tigecycline | 0 |
| Linezolid | 0 |
| High level gentamicin | 22 |
| High level streptomycin | 37 |

When evaluated in terms of virulence factors; It was determined that a total of 82% of the strains consisted of gelatinase, 67% of them were ESP, and 35% were hemolysin. At the same time, it was determined that 37% of the strains formed *Esp* and gelatinase together, and 24% of the strains formed ESP, gelatinase and hemolysin together. No virulence factor was detected in 8 strains. No statistically significant relationship was detected between the presence of antibiotic resistance and catheter use and virulence factors (p>0.05). The presence of hemolysis and gelatinase detected in the study is shown in Figures 1 and 2. Virulence factors (%) detected in *E. faecalis* strains are shown in Table 3.

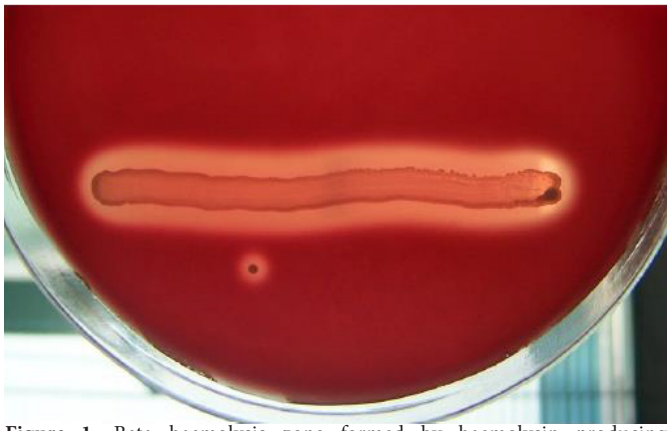

**Figure 1.** Beta haemolysis zone formed by haemolysin producing *Enterococcus faecalis*

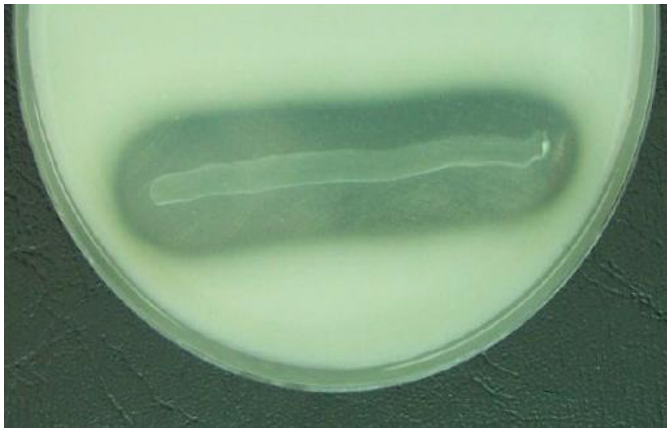

Figure 2. Transparent zone formed by gelatinase producing *Enterococcus faecalis*

**Table 3. Virulence factors detected in *Enterococcus Faecalis* strains (%)**

| Virulence factors | (%) |
|---|---|
| Gel* | 82 |
| Esp** | 67 |
| Hem*** | 35 |
| Esp/gel | 37 |
| Esp/gel/hem | 24 |
| No virulence factor | 8 |

*Gelatinase, **Enterococcal surface protein, ***Hemolysin

## DISCUSSION

In the treatment of enterococcal infections, the combination of aminoglycosides such as streptomycin and gentamicin with cell wall active inhibitors such as glycopeptides or beta-lactams is preferred. However, the emergence of highly aminoglycoside-resistant (HLAR) enterococci due to the production of enzymes that inactivate and modify aminoglycosides has created significant challenges in terms of infection management.[6] In our study, we investigated the presence of antimicrobial resistance and virulence factors in *E. faecalis* strains isolated from urine samples; Antibiotic resistance rates for ampicillin, norfloxacin, nitrofurantoin, high-level gentamicin (HLG) and high-level streptomycin (HLS) were determined as 2%, 31%, 1%, 22%, 37%, respectively, while no strains resistant to vancomycin, linezolid and tigecycline were detected.

According to the data of the European Antimicrobial Resistance Surveillance System (EARS-Net), which was established to detect the development of antimicrobial resistance in Europe; In 2014, the prevalence of *E. faecalis* in sterile samples was reported to be 28.8% on average, although there are differences between countries (8.3-76.5%). In 2019, a decrease in HLGR was noted over the years, and the resistance rate, which was 31.9% in 2015, was reported as 26.6%. In the evaluation made in terms of vancomycin resistance, while very low resistance rates were reported in *E. faecalis* strains in most countries, the resistance in *E. faecium*, which was 10.5% in 2015, increased over the years and was reported as 18.3% in 2019.[15,16] According to the 2021 Annual Epidemiological Report data of the same institution, an increase in the number of cases reported for all pathogens was reported between 2020 and 2021. Among the bacteria with the highest increase was *E. faecalis*, with an increase rate of 14%. The highest resistance increase in *E. faecalis* in 2021 was detected in HLR. Although resistance rates vary between 6.7-55.2 depending on the country, the average has been reported as 29%. However, no significant difference was observed in resistance rates between 2017 and 2021.[17]

In a study conducted in England where resistance surveillance data of *Enterococcus* species isolated from blood cultures between 2001 and 2019 were evaluated; While vancomycin resistance in *E. faecalis* remained below 4% in all years, it was determined that the resistance rate in HLG, which was 45% in 2001, decreased to 30% in 2019. It has been stated that this decrease in HLGR may be a reflection of the gentamicin and ciprofloxacin resistant clonal decline that was prevalent at the

beginning.[18] In various studies conducted abroad in which the antibiotic resistance of *E. faecalis* isolated from urine samples was investigated, the antibiotic resistance rates were; results ranging from 0-30% for ampicillin/penicillin, 20.6-94% for norfloxacin/ciprofloxacin, 8.8-54.81% for YDG, 7-16.31% for nitrofurantoin, 0-15% for vancomycin, and 0-4% for linezolid have been reported.[4,6,19-21]

In studies conducted in our country where the antibiotic resistance of *E. faecalis* was investigated in various clinical samples, the resistance rates were; ampicillin 5.6-50%, HLG 42-44.7%, HLS 37-50.4%, norfloxacin/ciprofloxacin 36-47.5%, vancomycin 1.5-2%, linezolid 0-6.5%, tigecycline 0% and 4.8-8.6% for nitrofurantoin. Wide ranges of resistance rates have been reported for some antibiotics.[22,23] In two studies investigating the resistance of *E. faecalis* in urine samples, nitrofurantoin 1.7%, ampicillin 10.6-13.6%, norfloxacin/ciprofloxacin 33.9-37.5%, HLG 14.8-22%, HLS 6.2-27.1%, vancomycin 0-1.9%, linezolid 0-3.1% and tigecycline 0-0.3% were reported.[24,25] In our study, antibiotic resistance rates were found to be 2% for ampicillin, 31% for norfloxacin, 22% for HLG, 37% for HLS and 1% for nitrofurantoin, while no strains resistant to vancomycin, linezolid and tigecycline were found. It was thought that the reason for the difference in the resistance rates obtained in the studies between the centres may be due to the diversity of clinical samples and patients as well as the change in the distribution according to years.

Although there are many virulence factors thought to contribute to the pathogenesis of enterococci, cytolysin/hemolysin, aggregation factor, ESP and gelatinase are the most researched factors. It has been stated that the presence of these factors together may cause tissue damage and deep tissue invasion. *Enterococci* have the ability to form biofilms on central venous catheters, urinary catheters and prosthetic heart valves, and especially ESP and gelatinase are held responsible for biofilm formation.[5] The most frequently detected virulence factor in our study was gelatinase with a rate of 82%. This was followed by ESP with 67% and hemolysis with 35%. No virulence factor was detected in eight strains.

In a study conducted in China,[26] where the biofilm-related virulence factors of *E. faecalis* isolated from UTI were genotypically investigated, gelatinase was found to be 41.5%, ESP 59.5% and hemolysis 57.3%. Researchers have reported that the presence of cytolysin is associated with weak biofilm formation, while the presence of aggregation factor is associated with strong biofilm formation. In another study[23] in which the virulence factors associated with biofilm formation of *E. faecalis* isolated from various clinical samples were genotypically investigated, researchers detected biofilm formation in 47.2% of the strains. They reported the presence of gelatinase, ESP and hemolysin/cytolysin, among the virulence factors, as 41.5%, 59.5%, 57.3%, respectively, and stated that there was a relationship between medium-strong biofilm formation and ESP, and weak biofilm formation and cytolysin. In a study in which the virulence factors of *E.faecalis* isolated from the urinary system were investigated phenotypically and genotypically, they found phenotypic gelatinase, hemolysin and proteinase activities to be 22%, 33% and 57%, respectively. However, researchers also stated that they detected higher rates of positivity genotypically, but this rate was not reflected in the phenotype.[28] In their study investigating the source (exogenous-endogenous) of *E. faecalis* isolated from community-acquired UTI, Ghalavand et al.[4] found ESP to be 77.8% and cytolysin/hemolysis to be 54% and they stated that these virulence factors may play a role in pathogenesis. In their studies investigating virulence genes and antibiotic resistance in *E.faecalis* isolated from the urinary system, researchers found vancomycin resistance to be 66%, gentamicin, norfloxacin and nitrofurantoin resistance to be 33.3%, 30% and 8.33%, respectively, and ESP positivity to be 66%. It has been stated that the presence of antibiotic resistance and virulence factors is higher in isolates that form strong biofilms.[12]

Baylan et al.[25] in their study investigating the relationship between antibiotic resistance and virulence factors of enterococci isolated from urine samples; Among the virulence factors of *E. faecalis*, they determined gelatinase as 22%, ESP as 35.6%, and hemolysin as 16.9%. In another study[29] in which antibiotic resistance and virulence factors of *E. faecium* and *E. faecalis* were investigated from various clinical samples, the presence of gelatinase, ESP and haemolysin in *E. faecalis* was reported as 26.5%, 79.6% and 51%, respectively. The researchers reported that *E. faecalis* had more virulence factors than *E. faecium*, but *E. faecium* had more antibiotic resistance. In this study, since the clinical characteristics of the patients from whom E. faecalis was isolated were not known, the inability to differentiate the isolates as agent/colonization/contamination and the inability to perform genotypic examination of all virulence genes investigated constitute the limitations of the study.

In their study investigating virulence factors in vancomycin-resistant *E. faecium* (VRE) and vancomycin-susceptible (VS) *E. faecalis* strains, Mete et al.[30] reported gelatinase as 52.7%, ESP as 38.9% and haemolysin as 41.1% in *E. faecalis* in urine samples and stated that VSE isolates had more virulence genes than VRE isolates. In our study, no relationship was found between the presence of virulence factors and antibiotic resistance. In addition, no statistically significant relationship was found between the presence of virulence factors and antibiotic resistance in patients with catheters ($p>0.05$).

## CONCLUSION

As a result, due to the differences observed in antimicrobial resistance profiles between centers and regions, it is thought that analyzing the antimicrobial susceptibility profiles of each center to determine their own resistance epidemiology will make a significant contribution to starting an effective treatment at an early stage. Additionally, in our study, no significant relationship was found between the presence of virulence factors and antibiotic resistance and catheter application. However, considering that the most detected virulence factors, gelatinase and *Esp*, have the ability to colonize and form biofilms on abiotic surfaces, minimizing catheterization practices may help prevent UTIs that may develop with enterococci.

## ETHICAL DECLARATIONS

### Ethics Committee Approval

The study was carried out with the permission of İstanbul University, İstanbul Faculty of Medicine Clinical Researches Ethics Committee (Date: 26.06.2009, Decision No: 2489).

### Informed Consent

Informed consent is not required for resistance studies on bacterial strains.

### Referee Evaluation Process

Externally peer-reviewed.

### Conflict of Interest Statement

The authors have no conflicts of interest to declare.

### Financial Disclosure

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### Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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