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Original Research Article

RAPD-PCR genotyping of *Enterococcus faecium* isolated from urinary tract infection and correlation between biofilm formation with antibiotic resistance and virulence genes

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Abstract

Purpose: *To examine the occurrence of virulence factors in Enterococcus faecium strains isolated from urinary tract infections (UTIs) and perform genotyping of the isolated strains using RAPD-PCR.*
Method: *A total of seventy-five (75) E. faecium strains isolated from UTIs underwent molecular and biochemical testing for verification. Sensitivity testing and identification of virulence factors were conducted using antimicrobial tests.*
Results: *Enterococcus faecium was detected in 53.57 % (75 out of 140) of urine samples in individuals suspected of having urinary tract infections. Additionally, all isolated strains demonstrated the ability to produce biofilm, with biofilm reactions observed in every E. faecium isolate. The strains exhibited either a strong (80 %), moderate (13.33 %) or weak (6.67 %) biofilm reaction. The highest antibiotic resistance in E. fascium isolates was related to penicillin and cotrimoxazole (83.33 and 80 %, respectively) while the lowest resistance was related to nitrofurantoin (26.67 %). The ebp C and ebp B were reported at 93.33 and 92 %, respectively, for E. faecium. The prevalence of ccf, cpd, cob and ebpA in E. fascium isolates was reported at 6.66, 13.33, 6.66, and 86.66 %, respectively. The RAPD-PCR analysis revealed the presence of 20 distinct molecular clusters, determined based on a similarity threshold of over 80 %.*
Conclusion: *This study demonstrates the spread of multidrug-resistant E. faecium strains isolated from urinary tract infections (UTI). Being a quick and cost-effective method, RAPD typing has been used to show clonal relatedness and to trace possible sources of organisms for epidemiological purposes.*

Keywords: *Antibiotic resistance, Biofilm formation, E. faecium, RAPD PCR, Enterococcus, UTI*

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INTRODUCTION

Enterococci are believed to be frequently present in the gastrointestinal tracts of both humans and animals [1]. While serving as commensals, they are also recognized as a significant contributor to community-acquired and nosocomial bacterial infections, including but not limited to surgical wound infections, urinary tract infections (UTI), pelvic infections, meningitis and intra-abdominal

infections [2]. According to the survey report from the Center for Disease Control and Prevention (CDC), Enterococci rank as the second most prevalent cause of nosocomial infections globally [3]. Lately, the escalating instances of improper antimicrobial agent usage, expanded invasive therapies and widespread application of immunosuppressants have contributed to a notable increase in clinical infections attributed to *Enterococcus spp*., notably *E. faecium*. The emergence of multiple drug resistance (MDR) in *E. faecium* has led to varying infection rates depending on the specific type of enterococci [4].

A previous report established the increasing levels of antibiotic resistance *E. faecium* over the past few years, primarily attributed to horizontal gene transfer [5]. The isolates of *E. faecium* were resistant to high-level aminoglycoside (HLAR), fluoroquinolone, vancomycin, and ampicillin [6]. Globally, the resistance to these drugs is linked to the widespread genetic lineage of CC17 (clonal complex-17), specifically correlated with resistance to quinolones and ampicillin [7].

In *E. fascium*, many genes for virulence factors have been identified and studies on cultured cells and animal models have shown how they affect the organism [8]. The genes *cpd*, *cob*, *ccf* and *cad*, responsible for sex pheromones, exhibit chemotactic properties towards human leukocytes and aid in the process of conjugation. Moreover, bacterial virulence encompasses a range of functions attributed to biofilms [9]. For instance, due to adhesion, the first stage in biofilm formation, the bacteria attach to catheters, biliary stents and silicone gastrostomy devices. Furthermore, biofilms contribute to bacterial antibiotic resistance and phagocytosis, making their eradication difficult. Many enterococcal virulence proteins have been investigated for their essential roles in biofilm. The expression of pili on the cell surface, which facilitates cell adhesion, is considered the trigger of biofilm formation [10].

The genetic locus responsible for encoding pili components, specifically associated with endocarditis and biofilm (Ebp ABC), plays a role in distinguishing the causative bacteria and clonal groups. Various techniques have been developed for this purpose. Random Amplification of Polymorphic DNA (RAPD), Pulsed-Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP), ribotyping and Multi Locus Sequence Typing (MLST) are the techniques used to study the differentiation of such organisms. Some published studies have assessed that RAPD and AFLP are among the most effective techniques suitable for epidemiological investigations of *Enterococcal spp*. These methods prove particularly advantageous when resources are limited [11]. The current investigation aims to assess the prevalence of certain virulence factors in *E. faecium* isolated from UTIs and to perform genotyping of these strains using RAPD-PCR.

EXPERIMENTAL

A total of 75 *E. faecium* strains were isolated from the 140 samples examined. All participants received comprehensive information about the study and their concerns were addressed. The study received ethical approval from the Ethics Committee of the Islamic Azad University (approval no. IR.IAU.SHK.REC.1399.055).

Sampling, isolation and identification

This cross-sectional study was conducted at Shahrekord Medical Diagnostic Laboratories, with samples collected from all affiliated branches. The isolates were cultured on blood agar containing 5 % sheep blood in sterile conditions and incubated for 24 h at 37 °C.

Enterococcus faecalis ATCC 29212 (Pasteur Institute, Iran) served as a reference strain to validate suspected enterococci colonies. The pure culture underwent subsequent sub-culturing on Bile Esculin agar and was then incubated for 48 hours at 37 °C. For early enterococci identification, Gram staining, catalase test, growth at 6.5 % NaCl and Pyrrolidonyl Arylamidase (PYR) test were conducted. Additionally, the arabinose fermentation test was employed to differentiate between *E. faecalis* and *E. faecium*.

Microtiter plate assay for biofilm formation

Biofilm formation by *Enterococcus faecium* was assessed using polystyrene microtiter plates. In brief, *Enterococcus* strains, freshly sub-cultured on blood agar plates, were inoculated into 1 mL of Brain Heart Infusion (BHI) broth containing 1 % glucose and incubated at 37 °C for 24 hours. A volume (20 μL) from a 24-hour-old bacterial culture was combined with 180 μL of fresh BHI medium, resulting in a turbidity equivalent of a 0.5 McFarland standard. The control strain (*E. faecalis* ATCC 29212) and 200 μL of the clinical isolates suspension were inoculated into flat bottom microtiter plates in duplicates and incubated at 37 °C in 5 % CO_2 for 24 h. Following incubation, the plate's contents were taken out, tapped and washed three times with phosphate-buffered saline. Methanol (150 μL)

was introduced and allowed to act for 20 minutes to dissolve the biofilm. It was inverted and allowed to air dry for about 30 minutes before being stained for 15 minutes with 0.1 % crystal violet. Excess stain was removed from the plates, followed by a rinse in distilled water. Each well received 150 μL of 33 % acetic acid and then left alone for 30 minutes. The absorbance was read at 570 nm. The isolates were classified as strong (OD_{570} > 0.24), medium (OD_{570} > 0.12 but < 0.24), weak (OD_{570} < 0.12) and non-biofilm formers (OD_{570} ≤ 0.5) based on the absorbance values.

Test for antimicrobial susceptibility

Antibacterial resistance patterns of Enterococci were assessed for 10 antimicrobial agents (PadTan-Teb, Iran), namely nitrofurantoin (FM 300 µg), gentamicin (GM 120 µg), erythromycin (E 30 µg), amikacin (AN 30 µg), tetracycline (TE 30 µg), chloramphenicol (C 30 µg), co-trimoxazole (SXT 25 µg), norfloxacin (NOR 10 µg), vancomycin (V 10 µg), ampicillin (AM 10 µg), ciprofloxacin (CRO 5 µg), rifampin (R 5 µg) and fosfomycin (F 200 µg), following the CLSI 2019 guidelines (12). The antimicrobial resistance pattern of various *E. faecium* strains was determined using a simple disk diffusion technique on Mueller-Hinton agar (Merck, Germany) [12].

DNA extraction and PCR assay

Genomic DNA from *E. faecium* isolates was extracted using a DNA extraction kit (Cinapure DNA, CinaClon, Iran), following the manufacturer's guidelines. The total DNA concentration was quantified at 260 nm optical density using the method outlined by Sambrook and Russell [13]. Polymerase chain reaction (PCR) was employed to amplify the *ddlE* gene for molecular confirmation of *E. faecium*, utilizing species-specific primers (*ddlE E. faecium* F: 5'-TTGAGGCAGACCAGATTGACG-3' and R: 5'-TATGACAGCGACTCCGATTCC-3').

PCR method for virulence gene identification

Table 1 displays the primers utilized for amplifying *E. faecium* virulence factors (*ccf*, *cpd*, *cob*, *ebpA*, *ebpB*, and *ebpC*). The Multiplex PCR programs employed for amplifying *E. faecium* isolates are outlined in Table 2.

For PCR processing, a DNA thermal cycler (Master Cycler Gradient, Eppendorf, Germany) was employed. The amplicons were stained with ethidium bromide after electrophoresis in a 1.5 % agarose gel at 80 V for 30 minutes. Ultraviolet (UV) doc gel documentation systems (Uvitec, UK) was used to view and take pictures of the PCR products. PCR products were evaluated against a 100 bp DNA marker (Fermentas, Germany).

Molecular typing

Using the primers AP4 (5'-TCA CGC TGC A-3'), RAPD typing was carried out. In accordance with the DNA isolation protocol for lactic acid bacteria and *enterococci*, DNA extraction was performed, and PCR amplification was conducted following the Banerjee (2013) protocol [14]. The UPGMA cluster analysis was used to group the RAPD-PCR patterns. For RAPD typing, the strains grouping similarity coefficients of 80 % were used.

Table 1: The oligonucleotide primers and the Multiplex PCR programs used for amplification of *ddlE* gene and virulence genes

Gene and primer	Primer Oligonucleotide sequences (5'-3')	Size of amplicon (bp)	Reference
E. faecium (ddlE)	F: TTGAGGCAGACCAGATTGACG R: TATGACAGCGACTCCGATTCC	658	19
ccf	TE53: GGG AAT TGA GTA GTG AAG AAG TE54: AGC CGC TAA AAT CGG TAA AAT	543	8
cob	TE49 AACATTCAGCAAACAAAGC TE49 TTGTCATAAAGAGTGGTCAT	1405	8
cpd	TE51 TGGTGGGTTATTTTTCAATTC TE52: TACGGCTCTGGCTTACTA	782	20
ebpA	F: CTAACAAAAATGATTCGGCTCCAG R: ATCTCACGCATTTTATCTTCAACT	517	19
ebpB	F: CTGAAGGAAAAACGGTCCAA R: CTTTTGCGTCGTCAGTGTGT	1003	19
ebpC	F: GATAAATATCAAGGACTGGCAGA R: AAGCATACTCTCCAGAAGTCACG	600	19

Table 2: Multiplex PCR programs used for amplification of *ddlE* gene and virulence genes in *E. faecium* isolates

Gene	PCR program	M-PCR Volume (50 µL)
ddlE	1 cycle: 94°C ---------- 6 min. 32 cycles: 94°C ---------- 60 s 58°C ---------- 60 s 72°C ---------- 2 min 1 cycle: 72°C ---------- 5 min	5 µL PCR buffer 10X 2 mM $MgCO_2$ 200 µM dNTP (Fermentas) 0.4 µM of each primers F & R 1 U Taq DNA polymerase (Fermentas) 3 µL DNA template
Ebp A	1 cycle: 94 °C ---------- 5 min. 32 cycles: 94°C ---------- 60 s 60°C ---------- 60 s 72°C ---------- 2 min 1 cycle: 72°C ---------- 5 min	5 µL PCR buffer 10X 2 mM Mgcl2 200 µM dNTP (Fermentas) 0.4 µM of each primers F & R 1 U Taq DNA polymerase (Fermentas) 3 µL DNA template
Ebp B	1 cycle: 94°C ---------- 5 min. 32 cycles: 94°C ---------- 60 s 55°C ---------- 60 s 72°C ---------- 2 min 1 cycle: 72°C ---------- 10 min	5 µL PCR buffer 10X 2 mM Mgcl2 200 µM dNTP (Fermentas) 0.4 µM of each primers F & R 1 U Taq DNA polymerase (Fermentas) 3 µL DNA template
Ebp C	1 cycle: 94°C ---------- 5 min. 32 cycles: 94°C ---------- 60 s 58°C ---------- 60 s 72°C ---------- 2 min 1 cycle: 72°C ---------- 10 min	5 µL PCR buffer 10X 2 mM Mgcl2 200 µM dNTP (Fermentas) 0.4 µM of each primers F & R 1 U Taq DNA polymerase (Fermentas) 3 µL DNA template
ccf, cpd, cob	1 cycle: 95°C ---------- 5 min. 30 cycles: 95°C ---------- 30 s 59°C ---------- 30 s 72°C ---------- 60 s 1 cycle: 72°C ---------- 6 min	5 µL PCR buffer 10X 2.5 mM Mgcl2 300 µM dNTP (Fermentas) 0.4 µM of each primers F & R 2 U Taq DNA polymerase (Fermentas) 3 µL DNA template

Statistical analysis

IBM SPSS Statistics' SPSS software (Version 25.0) was used for statistical analysis. *P*-values below 0.05 were statistically significant. RAPD profiles were examined by assessing the quantity and placement of significant bands using the Dice formula.

Similarity coefficients for each lane pair were calculated and a dendrogram was constructed employing the unweighted pair group method with averages (UPGMA) through Gel J software, as outlined in previous descriptions [15].

Isolates with a similarity coefficient equal to or above 80 % were clustered as identical genotypes.

RESULTS

Biofilm formation and antibiotic resistance

All samples were observed to exhibit positive results when employing a specific primer. Biofilm reactions were observed in all *E. faecium* isolates. The reported distribution of biofilm reactions indicates 80 % with a strong response, 13.33 % with a moderate response, and 6.67 % with a weak response.

The highest antibiotic resistance in *E. fascium* isolates was related to penicillin and cotrimoxazole (83.33 and 80 %), respectively, while the lowest resistance was related to nitrofurantoin (26.67 %). Tetracycline resistance was detected in 50 samples (73.33 %), ampicillin resistance in 55 samples (73.33 %), vancomycin

Table 3: Prevalence of virulence genes in *E. fascium* isolates

Virulence gene	*ccf*	*cob*	*cpd*	*ebp A*	*ebp B*	*ebp C*
E. faecium	5 (6.66%)	5 (6.66%)	10 (13.33%)	65 (86.66%)	69 (92.00%)	70 (93.33%)

resistance in 54 samples (72 %), amikacin resistance in 45 samples (60 %), gentamicin resistance in 40 samples (53.33 %), erythromycin resistance in 34 samples (42.33 %), chloramphenicol resistance in 32 samples (42.67 %), rifampin resistance in 30 samples (40 %), fosfomycin resistance in 29 samples (38.67 %), ciprofloxacin resistance in 25 samples (33.33 %) and norfloxacin resistance in 24 samples (32 %). There was a significant relationship between strong biofilm formation and resistance to ampicillin, vancomycin, amikacin and gentamicin ($p < 0.05$).

Virulence gene distribution

Table 3 illustrates the frequency and distribution of virulence genes among *E. faecium* isolates. The highest prevalence was associated with *epb* genes, with *ebpA*, *ebpB* and *ebpC* reported at 86.88 %, 92 %, and 93.33 %, respectively. Furthermore, the prevalence of *cpd*, *ccf* and *cob* genes in *E. faecium* isolates was reported at 13.33, 6.66 and 6.66 %, respectively. The frequency of *ccf, cpd, cob, ebpA, ebpB*, and *epbC* in *E. fascium* strains with strong biofilm production was reported at 60, 40, 60, 79.93, 78.26 and 85.72 %, respectively.

However, the frequency of *ccf, cpd, cob, ebpA, ebpB*, and *epbC* in *E. fascium* strains producing moderate biofilm was reported at 20, 30, 20, 15.38, 14.49, and 7.14 %, respectively. In addition, the frequency of *ccf, cpd, cob, ebpA, ebpB* and *epbC* in the strains that had weak biofilm formation was reported to be 20, 30, 30, 7.69, 7.25 and 7.14 %, respectively (Table 4). Based on Fisher's exact test, there was a statistically significant association between strong biofilm formation and the presence of virulence genes in *E. faecium* isolates ($p < 0.05$).

Table 4: Prevalence of *virulence* genes in *E. fascium* isolates based on biofilm formation

	No. (%) of biofilm forming isolates		
	Strong	Moderate	Weak
Ccf	3 (60%)	1 (20%)	1 (20%)
Cpd	4 (40%)	3 (30%)	3 (30%)
Cob	3 (60%)	1 (20%)	1 (20%)
ebp A	50 (76.93%)	10 (15.38%)	5 (7.69%)
ebp B	54 (78.26%)	10 (14.49%)	5 (7.25%)
ebp C	60 (85.72%)	5 (7.14%)	5 (7.14%)

RAPD PCR-based typing of *E. fascium*

The RAPD PCR-based typing of *E. fascium* isolates is shown in Figure 1. In addition, the profiles resulting from RAPD-PCR, along with virulence factors and antimicrobial resistance patterns in *E. faecium* strains isolated from urinary tract infections (UTIs), are presented in Table 5. The analysis of 61 isolates using the RAPD marker revealed 20 clusters of isolates, comprising 13 profiles and 7 distinct points, based on a similarity threshold of more than 80 %. Profile F was considered the dominant clone with 21 isolates. In this marker, 5 isolates were placed in a separate profile. Complete (100 %) similarity was not observed between any of the isolates.

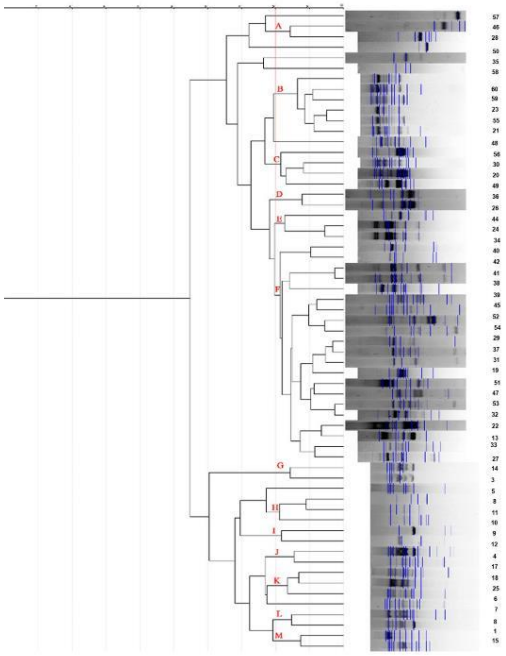

Figure 1: RAPD patterns of *E. fascium* isolated from UTI. Letters A to M indicate the number of known profiles in the analysis of isolates by RAPD method

DISCUSSION

This study focused on reporting antimicrobial resistance, characterizing virulence factors and molecular typing of *E. faecium* strains isolated from UTI samples. The prevalence of *E. faecium* is well documented in different studies, with some reporting a higher prevalence of *E. faecalis* isolated from UTIs. For instance, in a study conducted by Kraszewska and co-workers, *E.*

Table 5: Profiles obtained by RAPD-PCR, virulence factors and antimicrobial resistance pattern in *E. faecium* strains isolated from UTI

RAPD Type	Number of strains	Antibiotic resistance phenotypes	Biofilm reaction	Virulence genes
A	3, 11, 10	P, SXT, TE, AM, V, N, E, C, GM	Moderate	*ebpA, ebpB*
B	4, 2	P, SXT, TE, AM, V, N, E, C,	Moderate	*ebpA, ebpB*
C	8, 35, 32	P, SXT, TE, AM, V, N, E, C, R, F, GM, CRO, NOR, FM	Strong	*ccf, cpd, cob, ebpA, ebpB, epbC*
D	1, 5, 6, 7, 8, 12, 13, 16	P, SXT, TE, AM, V, N, E, C, R, F, GM, CRO, NOR, FM	Strong	*ccf, cpd, cob, ebpA, ebpB, epbC*
E	20, 24, 29, 30, 33, 34	P, SXT, TE, AM, V, N, E, C, R, F, GM, CRO, NOR, FM	Strong	*cpd, ebpA, ebpB, epbC*
F	14, 15, 17, 19, 23, 26, 28	P, SXT, TE, AM, V, N, E, C, F, R, GM, CRO, NOR, FM	Strong	*ebpA, ebpB, epbC*
G	24, 27	P, SXT, TE, AM, R, GM, CRO	Weak	*ebpA*

faecalis and *E. faecium* from UTI samples in Poland showed 71.8 % and 27.8 % prevalence, respectively [16]. In the present study, of the 140 urine samples analyzed, 75 isolates (53.57 %) were identified as *E. faecium*. A similar finding was also reported in Brazil and Eastern Nepal [17,18]. Some other *Enterococcus* species are documented to be linked with various diseases. For instance, *E. gallinarum* has been identified in association with autoimmune diseases, demonstrating a connection to autoantibodies [19]. This study highlighted the newly emerging role of *Enterococcus* in human health. Its translocation from the gut to the liver has been reported and its infection was frequently observed in patients with lupus erythematosus.

Enterococcus species exhibit multidrug resistance, solidifying their role as significant agents in healthcare-associated infections (HAIs). This prominence is likely attributed to their intrinsic antimicrobial resistance and their ability to acquire resistance to various new conditions. The most prevalent species in HIAs, *E. faecalis*, is even more virulent than the other known species, *E. faecium*. *Enterococcus faecium* is primarily linked to lactam vancomycin resistance [20]. In this study, the incidence of virulence determinants varied between *E. faecium* strains. All *E. faecium* strains in this study harbored multiple virulence determinants, similar to previous studies [8,21]. The reported prevalence of biofilm formation in previous studies on pathogenic isolates has shown variability. However, in this study, all strains demonstrated the capability to produce biofilm. *E. faecium* exhibited a strong positive biofilm reaction in 80 % of cases, while moderate and weak biofilm reactions were reported in 13.33 and 6.67 % of *E. faecium* isolates, respectively. In the present study, there was a significant relationship between strong biofilm production and resistance to ampicillin, vancomycin, amikacin and gentamicin in *E. fascium* isolates. Whether total or partial, the presence of this gene set has not yet been systematically examined as a predictor of the biofilm formation phenotype in any study.

In *E. faecium,* virulence factors are found to be more evident, which plays a vital role in the prognosis of infection by *Enterococcus*. Several proteins have been reported to participate in the repertoire of the pathogenicity of enterococci. Microbial surface components recognizing adhesive matrix molecules (MSCRAMM) are identified as essential for initiating infection on the host tissue surface. Numerous human studies have reported the presence of MSCRAMM genes in a majority of both *E. faecalis* and *E. faecium* strains [22]. Gene clusters of Pilin with the MSCRAMM gene are found in *E. faecium* and *E. faecalis* encoded by LPxTG-like surface protein associated with the structure of long filaments such as pili. The *ebp* (endocarditis and biofilm-associated pili) operon comprises *ebpA*, *ebpB*, *ebpC*, and an associated *srtC* gene (encoding sortase C) with an independent promoter. A disruption mutant of *ebpA* is defective in biofilm formation [23].

The occurrence of *ebpC* and *ebpB* genes in *E. faecium* was reported at 93.33 % and 92 %, respectively. The prevalence of *ccf, cpd, cob*, and *ebpA* in *E. fascium* isolates was reported at 6.66, 13.33, 6.66 and 86.66 %. There was a significant relationship between the biofilm reaction and virulence genes in *E. faecium* strains.

In a study by Hashem *et al*, *cpd* genes were found in 90 *enterococcal* isolates, or 94 % of the isolates, *ebpC* in 84 %, *ebpA* in 77 %, *ebpB* in 55 %, *cob* in 50 of the isolates [24], while in a study conducted by Sharifi *et al*, the frequency of *cpd* gene in E. faecalis and *E. faecium* isolated from clinical samples was reported as 100 and 2.63 %, respectively [21]. A high prevalence of *cpd*

gene (89.7 %) in *E. faecalis* isolates has been reported [25]. However, in this study, *cpd* gene was less commonly observed in *E. faecium* isolates. The global threat posed by *enterococci* (MDR) has increased. Additionally, *enterococcus* in food of animal origin, municipal and hospital sewage treatment plants (STPs), which could persist in the ecosystem for a long time, is the subject of intense study, mainly because these resistant pathogens could spread through these sources to the general public and hospital wards. The dissemination of vancomycin-resistant Enterococci (VRE) in hospitals poses a significant threat to patient care systems. These bacteria are not only resistant to vancomycin but also possess inherent and acquired resistance to a broad spectrum of antibiotics. They also spread to vulnerable enterococci species and other bacterial strains, including MRSA and act as a potential reservoir for resistance genes. For colonization, progression, and persistence of infections in hospitals, enterococci isolates must contain virulence determinants. It is imperative to identify virulence markers to assess the pathogenicity and severity of enterococcal diseases. The notable resistance of enterococci to vancomycin observed in the studied samples raises significant concerns, posing challenges in treating these infections. Additionally, *Enterococcus* displays resistance to trimethoprim-sulfamethoxazole [26].

In this study, we investigated the antimicrobial activity of *E. faecium* against drugs including vancomycin, cotrimoxazole, norfloxacin, amikacin, gentamicin, tetracycline, and nitrofurantoin. The highest antibiotic resistance in *E. fascium* isolates was reported to penicillin and cotrimoxazole (83.33 and 80 %), respectively, and the lowest resistance was reported to nitrofurantoin (26.67 %). This study identified a statistically significant relationship between strong biofilm formation and antibiotic resistance. Molecular typing of *E. faecium* isolates revealed the existence of 20 distinct clusters in the molecular typing analysis. This finding may show different origins of *E. fascium* isolates of the present survey. All isolates were MDR. Statistical analysis determined there was significant relationship between strong biofilm formation and resistance to ampicillin, vancomycin, amikacin and gentamicin. Conversely, a statistically significant association was observed between strong biofilm formation and the presence of virulence genes in *E. faecium* isolates. The results of this study show that biofilm plays an essential role in increasing antibiotic resistance and virulence genes.

Limitations of this study

Difficulty in identifying people with urinary tract infections and preparing samples from these people resulted in the small sample size used in this study. The limited sample size of this study may result in some biases in the conclusion of the study. In the future, a more complete and comprehensive analysis will be conducted to obtain a more definite conclusion.

CONCLUSION

E. faecium displays the highest resistance to certain antibiotics, notably showing the highest frequency of *ebpB*, *ebpC*, and *cpd* genes, and the lowest frequency of *ccf* and *cob* genes. This antimicrobial resistance pattern has been corroborated by biofilm formation assays, yielding consistent results. Further validation through RAPD-PCR analysis suggests the presence of point mutations contributing to the multidrug resistance observed in *E. faecium*. Molecular typing reveals 13 distinct profiles among the *E. faecium* isolates, implying diverse origins within the surveyed samples.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Roya

Jafavzadeh Samani - investigation, formal analysis and writing (original draft); Elahe Tajbakhsh, Hassan Momtaz, Mohsen Kabiri Samani - conceptualization, writing (review and editing), funding acquisition and supervision. All authors discussed the results and agreed on the publication of the manuscript.

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