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Molecular characterization of potentially virulent multidrug-resistant *Enterococcus faecalis* isolated from acquired urinary tract infections in Egyptian patients

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Abstract: In this study, 154 urine samples were collected from urinary tract infections (UTIs) of hospitalized patients, with 43 (28%) positive for the presence of *Enterococcus faecalis* without detection of *Enterococcus faecium*. Based on the demographic data associated with UTI patients, the prevalence of *E. faecalis* among females was higher than among males in the age group ≥41 years; cases from rural areas showed higher infection than from urban localities. Patients not treated with antibiotics were 2.8-fold more likely to be infected with *E. faecalis* than patients who received antibiotics. At the molecular level, the genes encoding the virulence determinants in *E. faecalis,* including cytolysins (*cyl*A, *cyl*B and *cyl*M), gelatinase (*gel*E), *E. faecalis* antigen A (*efa*A), extracellular surface protein (*esp*), aggregation substance (*asa*) and collagen binding adhesion (*ace*) were determined using SYBR green real-time PCR. Antibiotic susceptibility testing showed that almost all strains were multidrug-resistant, with an average multiple antibiotic resistance (MAR) index of 0.55. The colorimetric microtiter plate assay showed that 56% of the strains were biofilm producers. A significant correlation was observed between strong biofilm formation and the presence of *cyl*B and *cyl*M genes. Multidrug-resistant *E. faecalis* and its virulence potential and biofilm formation ability pose a risk to UTI patients.

Keywords: *Enterococcus faecalis;* multidrug resistance; virulence determinants

Abbreviations: Urinary tract infections (UTIs); *Enterococcus faecalis* (*E. faecalis*); multiple antibiotic resistance (MAR); enterococcal surface protein (Esp); aggregation substance (As); collagen-binding protein (Ace); gelatinase (GelE); hyaluronidase (Hyl); intensive care unit (ICU); buffered peptone water (BPW); *E. faecalis* antigen A (*efa*A); optical density (OD); cutoff value (ODc); standard deviation (SD); penicillin-binding proteins (PBPs); cytolysin (Cyl); real-time polymerase chain reaction (RT-PCR)

INTRODUCTION

Urinary tract infection (UTI), one of the most prevalent hospital-acquired infections, is caused by various pathogenic Gram-positive and Gram-negative bacteria, among which are *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Staphylococcus saprophyticus* [1]. *E. faecalis*, particularly antibiotic-resistant strains, are recognized as important major nosocomial pathogens causing bacteremia, sepsis, endocarditis, wound infection [2] and commonly isolated from surgical site infections [3]. About 80% of enterococcal infections in humans are caused by *E. faecalis*, while only about 20% are caused by *E. faecium* [4].

Treating enterococcal infections is difficult because enterococci have intrinsic and acquired resistance to many antimicrobial agents. They have been reported to exhibit intrinsic resistance to aminoglycosides and β-lactams as they carry several resistance genes [5] in addition to the acquired resistance to macrolides and glycopeptides that results from DNA mutation and acquisition of new genes *via* gene transfer [6]. Furthermore, prolonged hospitalization, immunodeficiency, and uncontrolled antibiotic therapy are the most important causes of acquiring enterococcal infections in patients [3,7]. The survival of enterococci in the hospital environment results from their ability to acquire specific genetic traits (virulence and antibiotic-resistant determinants) [8,9]. Therefore, adhesion and

invasion factors such as enterococcal surface protein (Esp), aggregation substance (As) protein (Asa1), collagen-binding protein (Ace), gelatinase (GelE), hyaluronidase (Hyl), and cytolysin (CylA) have a role in the pathogenicity and virulence of enterococcal infections [8,9].

The attachment of bacterial cells on biotic and abiotic surfaces forms a biofilm that is surrounded by a hydrated matrix of exopolymeric substances, nucleic acids, polysaccharides, and proteins [10]. *Enterococcus* spp.-producing biofilms are more resistant to antibiotics with concentrations 10-1000 times higher than non-biofilm-producing bacteria [11].

Due to the prevalence and risk factors of multidrug-resistant enterococci in several Egyptian hospitals in different geographic locations [7,12-15], characterization of adhesions and invasion factors of clinical *Enterococcus* spp., especially *E. faecalis*, could be helpful to improve our understanding and assessment of their pathogenicity. Therefore, in the present study, we investigated the molecular characterization of *E. faecalis* associated with UTIs in hospitalized patients in the Sharkia district, Egypt. This was manifested by the identification of their antibiotic-resistance patterns, virulence potential, and biofilm formation ability.

MATERIALS AND METHODS

Sample collection

A total of 154 urine samples were collected from July to December 2017 from intensive care unit (ICU) patients with gastrointestinal tract and urogenital infections after obtaining informed verbal/written consent for participation. As this study only focused on *Enterococcus faecalis* strains and their molecular identification, multidrug-resistance, virulence potential, and biofilm formation ability and did not use any human/patient material, the Review Board of the Ethics Committee of The Zagazig University Hospital exempted this study from review and waived the need for informed consent. All methods were carried out in accordance with relevant guidelines and regulations. The samples were collected from three large hospitals in Zagazig, Sharkia Governorate, Egypt: Al-Mabarrah, Al-Ahrar, and The Zagazig University hospitals.

Bacteriological examination

One mL from each urine sample was added to 9 mL of buffered peptone water (BPW, HIMedia, M614-500G) and incubated at 37°C overnight. A loopful from the pre-enriched broth was streaked onto bile esculine azide agar (HIMedia, M340) plates and incubated at 37°C overnight. A colony was picked from each plate with suspected growth and streaked onto plates containing nutrient agar (CM0003, Oxoid, UK) and incubated for 16-18 h at 37°C. The isolates were then microscopically examined and subjected to biochemical characterization [16].

Molecular identification of *Enterococcus* spp.

Bacterial DNA was extracted using the QIAamp DNA Mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's guidelines. Primers specific to 16S rRNA were used to identify *Enterococcus* isolates [17]. The primer sets used in PCR amplification were synthesized by Midland Certified Reagent Company (Oligos, USA). These primers are listed in Supplementary Table S1. The rRNA gene was amplified using the PCR technique in which each primer was incorporated in the reaction mixture containing Emerald Amp GT PCR Master Mix (Takara, Korea). A negative control (reaction mixture without DNA) and a positive control (provided by the Reference Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Giza, Egypt) were run in the reaction. After amplification of each product, 1.5% agarose gel was prepared in 1× tris-borate EDTA (TBE) and stained with 5 M ethidium bromide (Sigma, USA). The PCR products (10 µL each) were mixed with loading buffer (3 µL) and loaded in the gel with 5 µL of 100 bp DNA ladder (Qiagen, USA). The gel was then run in 1×TBE and 5 µM ethidium bromide for 45 min at 100 V and exposed to the ultraviolet light of an ultraviolet transilluminator (Gel Documentation System, Consort, Belgium). The isolates confirmed as *Enterococcus* species were further subjected to amplification of the 16S rRNA gene (specific for *E. faecalis*, [18]) and *sod*A gene (specific for *E. faecium*, [19]).

Molecular identification of virulence-associated genes

The isolates confirmed as *Enterococcus* species were further subjected to amplification of gelatinase (*gel*E), *Enterococcal* surface protein (*esp*), and cytolysin activator *(cyl*A, *cyl*B, and *cyl*M) as previously published [20]. Moreover, aggregation substance (*asa*), *E. faecalis* antigen A (*efa*A) gene, and adhesion of collagen from *E. faecalis* (*ace*) genes were also investigated [22]. PCR amplifications were performed by real-time PCR (RT-PCR; Mx3005P, Stratagene System, Qiagen, USA) using primer sets (Alpha DNA, Canada) specific for each gene. The sequences of the primers are listed in Supplementary Table S1. The amplification mixture of 25 µL contained 5 µL DNA template, 0.3 µM of each primer, 12.5 µL SYBR Green I ready-made master mix QuantiTect® SYBR® Green PCR kits (Qiagen) (containing HotStarTaq DNA Polymerase, Quantitect SYBR Green I PCR Buffer [Tris-Cl, KCl, $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, pH 8.7], 0.2 mM dNTP mix, SYBR Green I dye, ROX dye), 0.1 units AmpErase [Uracil N-glycosylase] (Applied Biosystems, Life Technologies, Thermo Fisher Scientific corporation, USA). Nuclease-free water was added to a final volume of 25 µL. The reaction conditions were 50°C for 2 min to activate UNG, 95°C for 10 min, then 40 cycles at 95°C for 15 s, at 60°C for 60 s, and at 72°C for 60 s, followed by plate read for fluorescence acquisition. A temperature gradient between 55°C and 95°C was run to obtain the dissociation curve to separate the specific product from non-specific products and primer dimers. SYBR Green I fluorogenic signal was collected, and the cycle threshold of the reactions was detected by MJ OpticonMonitor™ Analysis software version 3.1 (Bio-Rad, USA). Each isolate was tested in duplicate, and positive and negative controls were included in all analyses.

Antibiotic sensitivity test and antibiotic-resistant genes detection

All encountered *E. faecalis* strains (*n*=43) were screened for their phenotypic susceptibility against 13 antibacterial antibiotic agents. These agents included the following classes: penicillins (penicillin G), amino-benzyl penicillins (ampicillin), glycopeptides (vancomycin), macrolides (erythromycin), tetracyclines (tetracycline, doxycycline), fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin, and gatifloxacin), ansamycins (rifampin), nitrofurantoins (nitrofurantoin) and phenicols (chloramphenicol). These antimicrobial agents were selected based on their importance for treating human enterococcal infections. The standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial sensitivity profiles based on clinical and laboratory standards institute guidelines [25] on Mueller-Hinton agar (M173, HIMedia, PA, USA). The multiple antibiotic resistance (MAR) index for the strains was determined as the ratio of the number of antibiotics to which the strains displayed resistance to the number of drugs for which the strains were examined [26]. Multidrug resistance was defined as the resistance of a strain to at least one agent in three or more antibiotic classes [27]. Antibiotic-resistance genes viz. *van*A [23], *van*B [24], *tet*L, *tet*M, *tet*S, *pbp*5, *gyr*A [20] were amplified as described earlier. Their primer sequences are listed in Supplementary Table S1.

Biofilm formation ability

The ability of biofilm formation by *E. faecalis* strains was evaluated using microtiter plates with 96 wells [28]. The optical density (OD) was determined using an ELISA reader (Tecan, Sunrise R4, Austria) at OD620 nm following adjustment of the negative control to zero. The experiment was performed in triplicate, and the data were represented as the mean±standard deviation (SD). The cutoff value (ODc) was calculated using the formula:

ODc=Average OD of negative control+
(3x standard deviation of negative control)

The mean OD value obtained from the media control well was deduced from all the test OD values (biofilm OD=OD1-ODc). The strains were classified according to their potential for biofilm production based on Saxena et al. [29], as follows: OD≤ODc (non-biofilm producer); ODc<OD≤2×ODc (weak biofilm producer); 2×ODc<OD≤4×ODc (moderate biofilm producer); 4×ODc<OD (strong biofilm producer).

Statistical analyses

Kruskal-Wallis H one-way analysis of variance (ANOVA) and post hoc Bonferroni correction were used to analyze the differences in biofilm degrees

between the strains. The test results were calculated by SPSS version 22 (IBM Corp. 2013, Armonk, NY, USA). Data are presented as the mean±SD, and significance was considered at P<0.05. The Spearman correlation test was used to determine the association between biofilm production and the presence of biofilm-associated genes (*cyl*A, *cyl*B, *cyl*M, *gel*E and *esp*).

RESULTS

Identification of *Enterococcus* species

Suspected colonies of *Enterococcus* spp. isolated from the urine samples were identified by the colony morphology on bile esculin azide agar. The colonies appeared white, surrounded by darker media as enterococcal growth hydrolyzes esculin to products that react with ferric citrate in the medium and produce insoluble iron salts, resulting in the blackening of the medium. Of the examined samples, 43 (28%) were suspected to be *Enterococcus* spp. based on the growth on the selective medium. The suspected bacterial isolates were then subjected to Gram staining and further characterized by biochemical tests (Supplementary Table S2). Microscopic examination showed that all bacterial isolates were Gram-positive cocci or diplococci non-spore formers. All *Enterococcus* isolates encountered showed positive results for nitrate reduction and Voges-Proskauer, and negative results for all other tests except blood hemolysis with variable results obtained (Supplementary Table S2).

Molecular identification of *Enterococcus* isolates

The forty-three biochemically identified isolates were subjected to amplification of the 16S rRNA gene specific for *Enterococcus* spp with an amplicon size of 337 bp (Supplementary Fig. S1a). All the examined isolates (*n*=43) were confirmed as *Enterococcus* species. Further typing to the species level was carried out, and all the *Enterococcus* isolates were identified as *E. faecalis* by the amplification of the *E. faecalis* 16S rRNA gene producing a 310-bp fragment (Supplementary Fig. S1b). No *E. faecium* isolates were detected, as confirmed by specific *E. faecium sod*A PCR (Supplementary Fig. S1c).

Risk factors for *E. faecalis* infection

Of the 154 hospitalized patients involved in the study, 41.6% were male, and the age group ranged from one month to 84 years, with the majority ≥41 (58.4%, *n*=90) (Table 1). Most of the participants were from rural areas (85.7%), and the majority were without occupation (61.04%) and illiterate (87%). All the participants had a disease history, and 59.7% received antibiotic treatment. Based on the demographic data, *E. faecalis* infection was most common in ages ≥41(30%), with females (30%) being more susceptible to infection than males (25%). Patients from rural regions (28.8%) tended to be more frequently infected with *E. faecalis* than those from urban areas (22.7%). However, age, gender, and residence had no significance for the likelihood of infection. Meanwhile, patients without

Table 1. Demographic data associated with *E. faecalis* infection in patients with UTIs.

Risk factors		No. of participants	*E. faecalis*		COR (CI95%: lower-upper)	*P*- value
			Positive	Negative		
Age	≤ 15	9 (5.8%)	2 (22.2%)	7 (77.8%)	0.667 (0.13-3.42)	0.627
	16-25	14 (9.1%)	3 (21.4%)	11 (78.6%)	0.636 (0.16-2.46)	0.513
	26-40	41 (26.6%)	11(26.8%)	30 (73.2%)	0.856 (0.38-1.95)	0.711
	≥ 41	90 (58.4%)	27 (30%)	63 (70%)	1	
Gender	Male	64 (41.6%)	16 (25%)	48 (75%)	0.778 (0.377-1.603)	0.496
	Female	90 (58.4%)	27 (30%)	63 (70%)	1	
Education	Educated	20 (13%)	3 (15%)	17 (85%)	1	0.178
	Illiterate	134 (87%)	40 (29.9%)	94 (70.1%)	0.415 (0.115-1.494)	
Residence	Rural	132 (85.7%)	38 (28.8%)	94 (71.2%)	1	0.559
	Urban	22 (14.3%)	5 (22.7%)	17 (77.3%)	1.374 (0.473-3.991)	
Occupation	Workers	60 (39%)	15 (25%)	45 (75%)	1	0.519
	Non-working	94 (61%)	28 (29.8%)	66 (70.2%)	0.786 (0.378-1.635)	
Treatment	Antibiotic	92 (59.7%)	11 (12%)	81 (88%)	1	0.001*
	Others	62 (40.3%)	25 (40.3%)	37 (59.7%)	2.8 (1.348-5.724)	

antibiotic treatment were 2.8-fold more likely to be infected with *E. faecalis* than those who received antibiotics (OR=2.8, 95% CI: 1.35-5.72, P<0.05).

Of the 43 patients infected with *E. faecalis*, there were 12 (27.91%) cases of kidney failure, 8 (18.6%) cases of diabetes, 7 (16.3%) cases of coma, 3 (6.98%) cases with brain clots, 3 (6.98%) cases with acute anemia, 2 (4.65%) cases with spleen and liver disease and one case each of a drop in blood circulation, hemophilia, ulcers, water retention, encephalopathy, pneumonia, liver problems, and lupus (Supplementary Table S3). In these hospitalized patients, all cases of kidney failure were treated with dialysis, except one, which was treated with ceftriaxone (cephalosporin/cephamycin beta-lactam antibiotic). Diabetes cases were treated with insulin. In the case of coma, some cases were treated with ceftriaxone or unasyn (ampicillin/sulbactam) only; other cases were treated either with a combination of unasyn and cefotaxime (cephalosporin) or unasyn and ceftriaxone. Cases of brain clots were treated with ceftriaxone and unasyn and with ceftriaxone and alfacef (levofloxacin). All cases of acute anemia were treated with blood transfusions (Supplementary Table S3).

Virulence-associated genes and antibiotic resistance in *E. faecalis* strains

The genes encoding the virulence determinants were determined using the SYBR green RT-PCR assay (Table 2). Data indicated that the examined strains (*n*=43) harbored different virulence-associated genes; the highest carrier percentage was for the *esp* gene (83.7%), followed by *gel*E (67.4%), *asa* (44.2%), and *ace* (41.9%). The other investigated genes showed a detection rate of 23.3%-34.9% (Table 2).

The antimicrobial resistance pattern of the 43 *E. faecalis* strains against 13 antimicrobial agents is shown in Table 3. Specifically, 93% of the strains were resistant to rifampin. A percentage of strains was resistant to erythromycin and tetracycline (88.4% each), while a high percentage of strains was sensitive to ampicillin (93%), followed by vancomycin (88.4%), and penicillin (83.7%).

Table 2. Distribution of virulence-associated genes in *E. faecalis* isolates (*n*=43) as determined by the RT-PCR assay.

Total	*cyl*A	*cyl*B	*cyl*M	*gel*E	*esp*	*efa*A	*asa*	*ace*
No	10	13	15	29	36	13	19	18
%	(23.3%)	(30.2%)	(34.9%)	(67.4%)	(83.7%)	(30.2%)	(44.2%)	(41.9%)

Table 3. Resistance of *E. faecalis* strains (*n*=43) against antimicrobial agents.

Antimicrobial agents	Conc. (µg per disk)	Number of strains (%)		
		R *n*(%)	I *n*(%)	S *n*(%)
Penicillin G	10	7(16.3%)	0	36(83.7%)
Ampicillin	10	3(7%)	0	40(93%)
Vancomycin	30	0	5(11.6%)	38(88.4%)
Erythromycin	15	38(88.4%)	5(11.6%)	-
Tetracycline	30	38(88.4%)	-	5(11.6%)
Doxycycline	30	8(18.6%)	14(32.6%)	21(48.8%)
Ciprofloxacin	5	14(32.6%)	17(39.5%)	12(27.9%)
Levofloxacin	5	10(23.3%)	1(2.3%)	32(74.4%)
Norfloxacin	5	13(30.2%)	7(16.3%)	23(53.5%)
Gatifloxacin	5	7(16.3%)	3(7%)	33(76.7%)
Nitrofurantoin	300	6(14%)	8(18.6%)	29(67.4%)
Rifampin	5	40(93%)	2(4.7%)	1(2.3%)
Chloramphenicol	30	8(18.6%)	6(14%)	29(67.4%)

R, Resistant; I, Intermediate; S, Sensitive.

A multidrug-resistance pattern to at least three or more antimicrobial agents was observed in 42 strains of *E. faecalis* (97.7%), as shown in Table 4. The majority of the strains were resistant to five antibiotics (20.9%), followed by six antibiotics (16.3%), then nine antibiotics (13.9%). The MAR index of the strains ranged from 0.23-0.92, with an average of 0.55.

Table 4. Frequency distribution of multidrug-resistant *E. faecalis* strains.

Resistance pattern	No. of *E. faecalis* strains	Percentage of *E. faecalis* isolates	MAR index
3 antibiotics	3	6.9%	0.23
4 antibiotics	7	16.3%	0.31
5 antibiotics	9	20.9%	0.38
6 antibiotics	7	16.3%	0.46
7 antibiotics	4	9.3%	0.54
8 antibiotics	3	6.9%	0.62
9 antibiotics	6	13.9%	0.69
10 antibiotics	1	2.3%	0.77
12 antibiotics	2	4.7%	0.92

*Average MAR=0.55

The frequency of antibiotic-resistance genes among *E. faecalis* strains was detected (Table 5). The *tet*L, *tet*M, and *tet*S genes conferring resistance to tetracycline were present in 88%, 51.2%, and 32.6% of strains, respectively; *van*A and *van*B were detected

in vancomycin-resistant *E. faecalis* strains in an equal percentage (25.6%). A satisfactory percentage of *gyr*A conferring resistance to gyrase A was present in the examined strains (53.5%); *pbp*5 was detected in penicillin-resistant *E. faecalis* strains in the lowest percentage (23.6%).

Phenotypic biofilm formation ability

The distribution characteristics of the biofilm phenotype of *E. faecalis* strains were determined by the microtiter plate method (Table 6). Data showed that 44.2% (19/43) of the strains were non-biofilm producers. Among the biofilm-forming strains, 9.3% (4/43) had a strong biofilm phenotype, and 23.3% (10/43) had a moderate biofilm phenotype as well as a weak biofilm phenotype. A statistically significant difference (P<0.05) among the different degrees of biofilm formation produced by the strains was obtained.

Correlation between biofilm production and the presence of biofilm-associated genes

Data in Table 7 show that there is no significant correlation between the overall ability of biofilm production and the *esp* and *cyl*A genes. A significant correlation was observed between strong biofilm formation and the presence of *cyl*B (P<0.05), and *cyl*M (P<0.01). Moreover, the correlation between moderate biofilm formation and *gel*E gene was significant (P<0.05).

DISCUSSION

Enterococci have emerged as the most important cause of nosocomial UTI in hospitals [8]. Infections are mainly caused by the retrograde ascent of fecal flora to the bladder and kidney through the urethra, especially in females [30]. These bacterial species are notorious due to their antibiotic resistance. They are also among the most frequently reported opportunistic pathogens with intrinsic and acquired drug resistance [3]. Therefore, enterococci, particularly *E. faecalis*, have garnered a growing interest. In this study, 43 enterococcal isolates were recovered from 154 urine samples of Egyptian patients on esculin azide agar, which was used as a selective medium for isolating *Enterococcus* spp. as described previously [3,13,14]. The results of Gram staining, morphological and biochemical characterization of the isolated enterococcal colonies were confirmed based on the diagnostic key of Bergey's Manual of Systematic of Archaea and Bacteria [31]. All the examined isolates were confirmed as *E. faecalis* based on the amplification of the *E. faecalis* 16S rRNA gene and a specific *E. faecium sod*A gene, while no *E. faecium* was detected. The frequency of *E. faecalis* isolates was 79.6%. In the literature, the frequency of enterococci isolation was variable. In this regard, *E. faecalis* has been reported as the predominant *Enterococcus* spp., accounting for 80-90% of clinical isolates, followed by *E. faecium* (5-15%) [32]. Abdelkareem et al. [7] reported that

Table 5. Number and percentage of antibiotic resistance genes among *E. faecalis* strains.

Antibiotic resistance genes	No. of *E. faecalis* strains (*n*=43)	Percentage of *E. faecalis* strains
***van*A**	11	25.6%
***van*B**	11	25.6%
***tet*L**	38	88.4%
***tet*M**	22	51.2%
***tet*S**	14	32.6%
***gyr*A**	23	53.5%
***pbp*5**	10	23.3%

Table 6. Biofilm formation profile of *E. faecalis* strains.

Biofilm phenotype	Number (%)	Average O.D± SD
Non-biofilm	19(44.2%)	0.015±0.016[a]
Weak	10(23.3%)	0.015±0.153[b]
Moderate	10(23.3%)	0.015±0.015[b]
Strong	4(9.3%)	0.007±0.011[c]

Means±SD with different superscripts in the column of OD are considered statistically different among the biofilm phenotype (P<0.05).

Table 7. Correlation between biofilm production and the presence of biofilm-associated genes in the examined *E. faecalis* strains (*n*=43).

Phenotypic biofilm production		Biofilm associated genes				
		***cyl*A**	***cyl*B**	***cyl*M**	***gel*E**	***esp***
Overall	*r*[#]	0.046	0.178	0.062	-0.218	-0.012
	P[§]	0.768	0.254	0.694	0.159	0.940
Strong	*r*[#]	0.203	0.312	0.438	0.223	-0.076
	P[§]	0.192	0.042[*]	0.003[**]	0.152	0.630
Moderate	*r*[#]	-0.173	-0.003	-0.056	0.322	-0.055
	P[§]	0.268	0.986	0.719	0.035[*]	0.724
Weak	*r*[#]	0.088	-0.003	-0.172	-0.087	0.094
	P[§]	0.575	0.986	0.270	0.577	0.550

[#]*r*: Pearson correlation, [§]*P*: P value, *significant correlation, **high significant correlation.

E. faecalis was isolated at a frequency of 19% in 300 Egyptian patients with UTI. In Iran, *E. faecalis* was identified in urine samples at frequencies of 85.9% [5] and 56.19% [33]. The variability in *Enterococcus* isolation rates can be attributed to the differences in the control measures of infections and in the demographics of the examined patients. Moreover, variation in the methods employed for detecting enterococci is an additional explanation [34].

The demographic results based on *E. faecalis* infection showed that females (30%) were more susceptible to infection than males (25%). Abdelkareem et al. [7] found that the isolation rate of *E. faecalis* from Egyptian patients with UTI was greater in females (22.5%) than in males (15%). The higher frequency of UTI in females might be due to the anatomical variations between males and females, exemplified by a shorter urethra and shorter distance between the urinary system on one side and the genital/intestinal system on the other side that can cause immediate bacterial penetration into the urinary tract [35]. In this study, the prevalence rate of *E. faecalis* in UTI patients was elevated in the age group ≥41 years (30%) compared to other age groups. Comparable results were reported among Egyptian patients, where the highest prevalence rate was recorded in the age group >50 years (25%) [7]. The elevated incidence in older people may be attributed to their increased immunodeficiency.

It is worth mentioning that our hospitalized patients with *E. faecalis* infection had a history of disease, and 39.5% received treatment with antibiotics. In this regard, the enterococcal population was reported to be higher in hospitalized patients receiving antibiotics compared to hospitalized patients without antibiotics and healthy volunteers [34]. *E. faecalis* was reported to cause various nosocomial infections, of which UTIs are the most common. Moreover, the uropathogenic strains exhibit tropism for the kidneys in the urinary tracts of mice [36]. Since fecal flora, notably *E. faecalis*, are opportunistic microorganisms, this pathogen harbored within urothelial cells was shed from the bladder in patients with lower urinary tract symptoms [30]. The present study showed that 18.6% of diabetic patients had *E. faecalis* UTI infection. Comparable results were previously cited, where 15.6% of diabetic patients with *E. faecalis* UTI infections were recorded in India [37]. A higher incidence of UTIs was recorded in diabetic patients than in non-diabetics [38]. The nerve damage produced by high blood glucose levels consequently influences the ability of the bladder to sense the presence of urine; the probability of infection increases due to the prolonged persistence of urine in the bladder; in addition, the high glucose concentration in urine facilitates the growth of the bacteria [39].

Enterococci produces various virulence factors involved in adhesion and invasion processes, biofilm development, and histological damage (CylA, CylB, CylM, GelE, Esp, EfaA, Asa1, Ace) [5,8,9]. The greatest number of virulence factors was reported in *E. faecalis* isolated from urine [8]. The *asa* gene involved in the aggregation phenotype was identified in 44.2% of analyzed *E. faecalis* strains. Similarly, Kafil and Mobarez [33] documented the presence of the *asa* virulence gene in 84.2% of enterococcal isolates recovered from urine samples. The *ace* gene involved in adhesive properties [40] was identified in 41.9% of strains in this study. This percentage was lower than that reported in a study performed on urine samples in Bulgaria (64.8%) [41]. The *efa*A gene is also involved in adhesion, and the current study showed that 30.2% of strains harbored this gene. Similarly, 21.4% of *E. faecalis* strains in Kuwait were positive for this gene [42]. Plasmid conjugation can lead to the transfer of the sex pheromones and the extracellular surface protein (*esp*) gene between *E. faecium* strains by chromosome-to-chromosome transmission [6]. The prevalence rate of the *esp* in analyzed *E. faecalis* strains was 83.7%. A comparable percentage of 84.2% was reported in Iran [36]. It was reported that this gene has a role in the pathogenesis of *E. faecalis* [8].

Cytolysin is a bacteriocin-type exotoxin that has bactericidal effects on Gram-negative bacteria and causes β-hemolysis of blood cells [43]. The cytolysins (*cyl*A, *cyl*B, and *cyl*M) are associated with increased toxicity in human infection. The prevalence rate of the three cytolysins in the examined strains was 23.3%, 30.2%, and 34.9%, respectively. In a previous study, 15% of the *E. faecalis* isolates from UTIs were positive for *cyl*A [42]. In the current study, the gelatinase virulence gene (*gel*E) was detected in 67.4% of strains. The gene was reported in clinical and food enterococcal strains [20]. It was previously identified in *E. faecalis* of Bulgarian isolates at a percentage of 82.2% [41]. Variations in the prevalence rate of the virulence genes of enterococci might be due to the difference in the isolation methods, detection methodology, or the number and types of examined samples.

Enterococci have acquired resistance against several types of antimicrobial antibiotics such as tetracycline, rifampin, erythromycin, ciprofloxacin, and penicillin G [5]. *E. faecalis* strains in the current study exhibited the highest resistance to rifampin, followed by tetracycline and erythromycin at percentages of 93%, 88.4%, and 88.4%, respectively. In this regard, resistance to rifampin was reported to be widespread, occurring in 71.4% of *E. faecalis* isolates from Iran [5]. Rifampin resistance arises from various mutations in the *rpo*B gene that encodes for the β'-subunit of RNA polymerase [44]. The widespread use of tetracycline in human treatment and growth promoters in animals has caused an increase in the number of acquired resistance [45]. The percentage of erythromycin resistance in our study was higher than that recorded in a previous study in Egypt (70.2%, [7]). High resistance rates of enterococci to erythromycin could be due to uncontrolled treatment with the drug in hospitals. Nitrofurantoin has been recommended to treat UTIs caused by enterococci, although it has side effects, such as vomiting, nausea, and anorexia [47]. The data showed that the susceptibility pattern of *E. faecalis* against nitrofurantoin was 67.4%. A high rate of nitrofurantoin sensitivity of *E. faecalis* isolates recovered from Egyptian patients with UTI was previously reported [7]. Though vancomycin is an effective antibiotic, the worldwide emergence of vancomycin-resistant enterococci (VRE) is one of the most challenging recent issues [5]. Our study revealed that 88.4% of *E. faecalis* strains were susceptible to vancomycin. In this regard, 98.13% of *E. faecalis* isolates from UTIs displayed susceptibility to vancomycin [35].

Current data indicated that all *E. faecalis* strains had a MAR index ranging from 0.23 to 0.92, with an average of 0.55. Accordingly, *E. faecalis* isolates from UTI patients in India showed a MAR index of 0.6 [47]. There are no criteria for the MAR index for enterococci [48]. Krumperman [26] suggested that a MAR index of 0.2 differentiates between low and high-risk contamination. Based on these criteria, our strains are all from high-risk-of-contamination sources (hospitalized patients), thus posing a risk to individuals in contact with these patients.

E. faecalis isolates with phenotypic tetracycline resistance harbored one or more tetracycline resistance genes [49]. We identified the tetracycline resistance genes, *tet*L, *tet*M, and *tet*S, of *E. faecalis* strains at the following percentages: 88.4%, 51.2%, and 32.6%, respectively. In a previous study, the respective genes were identified as the most dominant tetracycline resistance gene determinants in enterococci, with frequencies of 28.7%, 68.4%, and 2.7%, respectively [49]. It was reported that all enterococci that exhibit decreased susceptibility to penicillin and ampicillin, as well as a high level of resistance to most cephalosporins and all semi-synthetic penicillins, express low-affinity penicillin-binding proteins (PBPs) [50]. *E. faecalis* produces five PBPs, including four high molecular weight PBPs and one low molecular weight PBP [51]. In this study, we identified *pbp*5 in 23.3% of the *E. faecalis* strains. Both *van*A and *van*B showed an equal frequency of 25.6% in our *E. faecalis* strains. In an American study, the two genes were identified in *E. faecalis* isolates at percentages of 83.8% and 16.2%, respectively [52]. A significant concern about the emergence and rapid spread of acquired fluoroquinolones resistance (including ciprofloxacin) in enterococci has already been reported [53]. The *E. faecalis* strains examined in this study exhibited a 53.5% frequency of *gyr*A. Mutation in the *gyr*A gene, which encodes the A subunit of DNA gyrase, contributes to fluoroquinolone resistance in *E. faecalis* [54]. Additionally, it was found that 7 of 13 ciprofloxacin-resistant enterococcal strains (53.84%) harbored various mutations in *gyr*A [53].

Based on the formation ability of the biofilm phenotype, *E. faecalis* strains were differentiated into strong (9.3%), moderate (23.3%), weak (23.3%), and non-biofilm-forming (44.2%) strains. The ability of enterococci to form biofilms was found to be closely related to UTIs [55]. A Japanese study reported that *E. faecalis* strains from UTIs exhibited strong, medium, and weak biofilm formation at frequencies of 18.2%, 44.3%, and 37.5%, respectively [56]; in a Chinese study, the biofilm formation frequencies of *E. faecalis* isolates were 13.3%, 13.3%, and 23.9%, respectively [55]. Interestingly, no association was observed between biofilm formation and the presence of biofilm-associated genes (*cyl*A, *cyl*B, *cyl*M, *gel*E, and *esp*) in our study; this was consistent with a previous study [57]. The *esp* gene is not an obligatory genetic component for producing biofilm, but when present, it may enhance biofilm formation [58]. On the chromosome of enterococci, the cytolysin operon is close to the *esp* gene, however; no significant association with biofilm formation and cytolysin was reported [33]. Moreover, no correlation was reported between gelatinase and biofilm formation in *E. faecalis* isolates [59].

CONCLUSIONS

Enterococcus faecalis was isolated from 43 urine samples of 154 hospitalized Egyptian patients with UTIs. Almost all *E. faecalis* strains were multidrug-resistant, and the genes encoding the virulence determinants were molecularly characterized. The obtained findings show that the strains pose a risk to other individuals in contact with these patients. The biofilm formation ability of *E. faecalis* strains (56%) is another virulence determinant. Monitoring enterococci infection and antibiotic resistance patterns is essential in determining control and prevention measures. Future epidemiological investigations need to be regularly conducted to assess the prevalence rates.

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Author contributions: H.A. Ahmed, E.Y.T. Elariny and R.M. Ahmed conceived and designed the study; N. Mostafa acquired the data; H.A. Ahmed and E.Y.T. Elariny analyzed and interpreted the data; H.A. Ahmed, R.M. Ahmed and N. Mostafa Drafted the manuscript; R.M. Ahmed, H.A. Ahmed and E.Y.T. Elariny revised the manuscript; N. Mostafa performed the statistical analysis. H.A. Ahmed, E.Y.T. Elariny and R.M. Ahmed offered administrative, technical, and material support; H.A.Ahmed, E.Y.T. Elariny and R.M. Ahmed supervised the study. All authors read and approved the manuscript.

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Data availability: All data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Ahmed%20et%20al_Dataset.pdf

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Primer sequences used in PCR analysis.

Genes	Primer sequence (5'-3')	Reference
Enterococcus 16S rRNA*	For: ATCAGAGGGGGATAACACTT Rev: ACTCTCATCCTTGTTCTTCTC	[17]
E. faecalis 16s rRNA*	For: GTTTATGCCGCAT GGCATAAGAG Rev: CCGTCAGGGGACGTTCAG	[18]
*E. faecium sod*A*	For: GAAAAAACAATAGAAGAATTAT Rev: TGCTTTTTTGAATTCTTCTTTA	[19]
gel*E*	For: TCAGTGGTGTCAGCAGCCTTT Rev: TGGTTTACCTGAATGTCTTCTTTAGC	[20]
esp	For: CTTTCGACGTGGATGTAGAGTTTC Rev: GGTACGTATGTTGCATCATTTTCC	[20]
*cyl*A	For: CAAGTTGCTGGAGTAATAGACACGAT Rev: TCCCATCCATCACCTTGTAAGA	[20]
*cyl*B	For: CATGGTACACAAGTTGCTGGAGTAA Rev: CCCATCCATCACCTTGTAAGAATT	[20]
*cyl*M	For: GTATTTAGAATCACTAGGATTCTTTGTAGGAA Rev: GGAATTTCAGAATCTAGGTTTCTCAATAA	[20]
asa	For: GATACAAAGCCAATGTCGTTCCT Rev: TAAAGAGTCGCCACGTTTCACA	[6]
*efa*A	For: TGGGACAGACCCTCACGAATA Rev: CGCCTGTTTCTAAGTTCAAGCC	[21]
ace	For: CGGCGACTCAACGTTTGAC Rev: TCCAGCCAAATCGCCTACTT	[22]
*van*A	For: CATGACGTATCGGTAAAATC Rev: ACCGGGCAGRGTATTGAC	[23]
*van*B	For: GTGACAAACCGGAGGCGAGGA Rev: CCGCCATCCTCCTGCAAAAAA	[24]
*tet*L	For: GGGTAAAGCATTTGGTCTTATTGG Rev: ATCGCTGGACCGACTCCTT	[20]
*tet*M	For: GCAGAATATACCATTCACATCGAAGT Rev: GCAGAATATACCATTCACATCGAAGT	[20]
*tet*S	For: CCATTGATATCGAAGTACCTCCAA Rev: AGGAAGTGGTGTTACAGATAAACCAA	[20]
*pbp*5	For: GTTCTGATCGAACATGAAGTTCAAA Rev: TGTGCCTTCGGATCGATTG	[20]
*gyr*A	For: CGGATGAACGAATTGGGTGTGA Rev: AATTTTACTCATACGTGCTT	[20]

*Primers used for conventional PCR

Supplementary Table S2. Biochemical characterization of *Enterococcus* isolates.

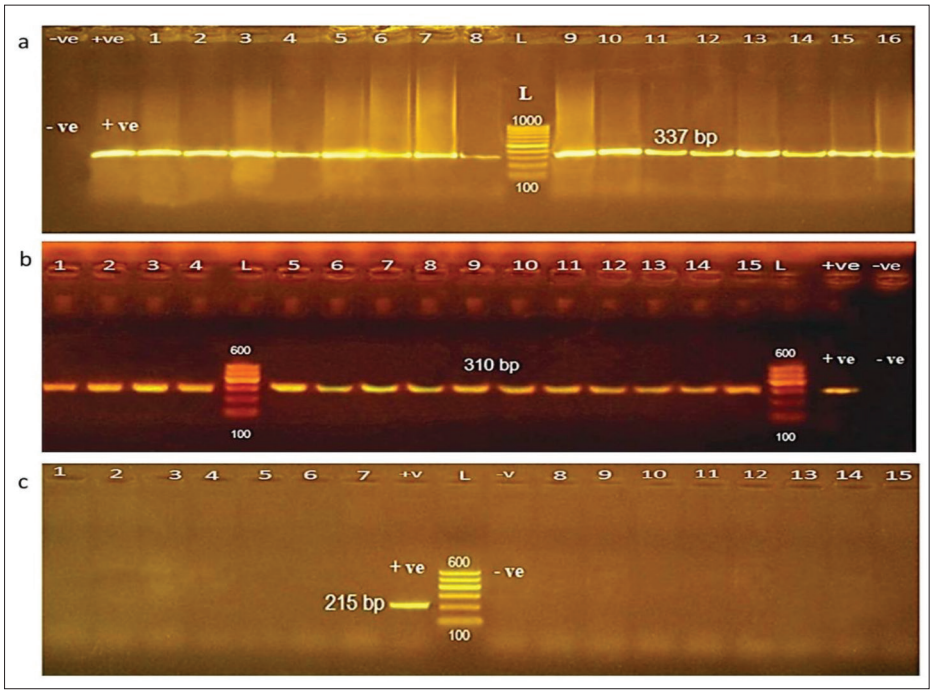
Test	Result
Catalase	–
Oxidase	–
Blood hemolysis	V[1]
Methyl red	–
Voges-Proskauer	+
Citrate utilization	–

Test	Result
Urease	–
Nitrate reduction	+
H_2S production	–
Indole	–
Motility	–

[1]V, variable

Supplementary Table S3. Diagnosis and treatment of UTI patients infected with *E. faecalis*.

Treatment	Diagnosis	Gender	Age (years or months)	Code	Isolate SN.
Cefotax	Spleen, liver disease, and internal bleeding	M	61	UGE 5	**1**
Ceftriaxone	Spleen, liver disease, and water on lungs	F	52	UGE 7	**2**
Under observation	Falling in blood circulation	F	30	UGE 8	**3**
Under observation	Hemophilia	M	40	UGE 15	**4**
Blood transfusion	Acute anemia	F	66	UGE 16	**5**
Ceftriaxone	Kidney failure	M	65	UGE 33	**6**
Dialysis	Kidney failure	M	62	UGE 34	**7**
Unasyn	Ulcers	M	65	UGE 35	**8**
Water disposal	Water retention	M	66	UGE 36	**9**
Dialysis	Kidney failure	M	61	UGE 40	**10**
Blood transfusion	Acute anemia	M	84	UGE 42	**11**
Ceftriaxone and Alfacef	Frequent brain clots	M	64	UGE 47	**12**
Insulin	Diabetes	F	55	UGE 51	**13**
Ceftriaxone	Coma	F	69	UGE 52	**14**
Ceftriaxone	Encephalopathy	M	72	UGE 55	**15**
Vancomycin	Pneumonia	M	2 months	UGE 97	**16**
Dialysis	Kidney failure	F	32	AICU 100	**17**
Dialysis	Kidney failure	F	65	UUT 101	**18**
Unasyn	Coma	M	84	UUT 102	**19**
Dialysis	Kidney failure	M	51	UUT 103	**20**
Cefotax	Liver problems	F	48	UUT 104	**21**
Insulin	Diabetes	F	44	UUT 105	**22**
Unasyn and cefotax	Coma	F	80	UUT 106	**23**
Dialysis	Kidney failure	F	62	UUT 107	**24**
Unasyn and cefotax	Lupus	F	55	UUT 108	**25**
Insulin	Diabetes	F	50	UUT 109	**26**
Blood transfusion	Acute anemia	F	49	UUT 111	**27**
Dialysis	Kidney failure	F	27	UUT 112	**28**
Insulin	Diabetes	F	42	UUT 113	**29**
Unasyn and cefotax	Coma	F	29	UUT 117	**30**
Unasyn and cefotax	Coma	F	35	UUT 119	**31**
Insulin	Diabetes	F	16	UUT 122	**32**
Dialysis	Kidney failure	F	19	UUT 124	**33**
Insulin	Diabetes	F	56	UUT 125	**34**
Unasyn and cefotax	Coma	F	15	UUT 128	**35**
Ceftriaxone	Coma	F	22	UUT 130	**36**
Dialysis	Kidney failure	M	26	UUT 132	**37**
Insulin	Diabetes	F	30	UUT 133	**38**
Ceftriaxone and unasyn 1.5gm	Brain clot	M	34	UUT 134	**39**
Ceftriaxone and unasyn 1.5gm	Brain clot	F	30	UUT 135	**40**
Dialysis	Kidney failure	F	26	UUT 137	**41**
Insulin	Diabetes	F	55	UUT 146	**42**
Dialysis	Kidney failure	M	20	UUT 147	**43**



Supplementary Fig. S1. Agarose gel electrophoresis patterns; **a –** amplification of 16S rRNA gene specific for *Enterococcus* spp. (337 bp), lanes (1-16), positive samples; **b** –amplification of 16S rRNA specific for *E. faecalis* (310 bp), lanes (1-15), positive samples; **c –** amplification of *sod*A gene specific for *E. faecium* (215 bp), lanes (1-15), negative samples. L: DNA molecular size marker (100 bp), +ve: positive control, -ve: negative control.