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Molecular Identification of Some Antibiotic-Resistance Genes and Antibiotic Susceptibility Profile of *Enterococcus faecalis* Isolated from Clinical Samples

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Abstract

Enterococcus spp. are a commensal bacteria and opportunistic pathogens that seriously threaten public health. *Enterococcus faecalis* and *Enterococcus faecium* are responsible for about 90% of the enterococcal infections. The ability of *E. faecalis* to resist many of the utilized antibiotics, which makes the treatment hard, together with its ability to form a biofilm that allows it to cause severe infections, make these bacteria a threat to public health. The study aimed to identify the resistance patterns and species distribution of *E. faecalis* strains isolated from clinical samples. Conventional methods were used to identify 52 *E. faecalis* isolates from patients at Al-Imam al-Sadiq teaching hospital in HIilla / Iraq, and a species-specific PCR assay was used for molecular identification for confirmation. The Kirby Bauer test was used to test antibiotic susceptibility to 15 antibiotics and analyze the distribution of two different resistance genes among the isolates (*bla*-TEM and *GyrA*). Statistical analysis showed significant differences (P<0.0001) for the antibiotic type and the response's nature. Among *E. faecalis*, the most prevalent was resistance to oxacillin, penicillin, erythromycin, Rifampicin, clindamycin, and Vancomycin, with a 100% ratio. While the percentage of the resistant isolates to the other antibiotics sorted in descending order was ceftazidime (85%), ampicillin (84.6%), amoxicillin-clavulanic acid (61%), Ciprofloxacin (23%), co-trimoxazole (13.46%), Gentamicin (7.69%), and Chloramphenicol (7.69%). PCR results detected the presence of the *gyrA* gene in 57.6% (30/52) of the isolates, while the *bla* TEM appeared in 78.8% of them (41/52). According to the current study, *E. faecalis* was found to be resistant to a number of antibiotics and to have genes that resist antibiotics, such as *bla*-TEM and *gyr*A.

Keywords: *Enterococcus faecalis,* antimicrobial susceptibility, PCR.

لبكتيريا المكورات المعوية البرازية *Enterococcus faecalis* المعزولة من عينات سريرية

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الخلاصة

هدفت هذه الدراسة إلى تحديد أنماط المقاومة للمضادات الحيوية، بالإضافة إلى توزيع الأنواع لسلالات *E. faecalis* المعزولة من عينات سريرية. تم التعرف على 52 عزلة باستخدام الطرق التقليدية من مرضى في مستشفى الإمام الصادق التعليمي في الحلة/العراق، كما تم تأكيد التشخيص باستخدام تفاعل البلمرة المتسلسل النوعي .(PCR) واعتمد اختبار كيربي–باور (Kirby–Bauer) لتقييم حساسية العزلات تجاه 15 نوعًا من المضادات الحيوية، إلى جانب تحليل توزع جيني المقاومة *bla*–TEM و *gyr*A بين العزلات .أظهرت نتائج التحليل الإحصائي وجود فروق معنوية عالية ($P<0.0001$) تبعًا لنوع المضاد الحيوي وطبيعة الاستجابة البكتيرية. وقد سُجلت أعلى نسب المقاومة في سلالات *E. faecalis* تجاه الأوكساسيلين، والبنسلين، والإريثروميسين، والريفامبيسين، والكليندامايسين، والفانكومايسين، بنسبة بلغت 100% لكل منها. في حين تراوحت نسب المقاومة لبقية المضادات الحيوية بترتيب تنازلي على النحو الآتي: السيفتازيديم (85%)، الأمبيسيلين (84.6%)، الأموكسيسيلين–حمض الكلافولانيك (61%)، السيبروفلوكساسين (23%)، الكوتريموكسازول (13.46%)، الجنتامايسين (7.69%)، والكلورامفينيكول (7.69%) كما أظهرت نتائج تفاعل PCR وجود جين *gyr*A في 57.6% من العزلات، في حين تم الكشف عن جين *bla*–TEM في 78.8% منها. وتشير هذه النتائج إلى أن سلالات *E. faecalis* المدروسة تمتلك مستويات مرتفعة من المقاومة للمضادات الحيوية، بالإضافة إلى احتوائها على جينات مقاومة مهمة مثل *bla*–TEM و*gyr*A، مما يعزز من خطورته السريرية.

Introduction

Enterococci are important hospital-acquired infectious agents, which are Gram-positive facultative anaerobic bacteria that live in the gastrointestinal microbiota of both humans and animals. Although enterococcus has many species, *Enterococcus faecalis* and *Enterococcus faecium* are the most frequently found species in humans [1].

The enterococcal species that is the most frequently isolated from clinical specimens is *E. faecalis;* therefore, it holds specific importance in regard to human health and represents a threat that is emphasized by its low susceptibility to most of the used antibiotics [2]. It can cause several infections, including teeth infections [3], endocarditis [4], and urinary tract infections [5]. Antimicrobial resistance (AMR) is a serious global health issue that is becoming worse quickly [6].

Bacteria can resist antibiotic treatments by using several mechanisms; for example, one class of antibiotics, beta-lactamase, which is a bactericidal that works on disrupting and inhibiting the biosynthesis of the bacterial cell wall by binding to and inhibiting specific proteins called the penicillin-binding proteins (PBPs) which carry out the last stage of the biosynthesis of the cell wall [7].

The mechanism used by enterococci to resist this type of cell wall targeting antibiotic is low-affinity PBPs production [2], and they are considered intrinsically resistant to some of the antibiotics belonging to the β-lactam category antibiotics like the cephalosporins and carbapenems; however, penicillin resistance is either acquired by mutations in the PBPs or though rare and less frequent, by the production of an enzyme called β-lactamase [8]. Humans can contract enterococci, which carry resistance genes in a number of ways, including direct contact with farm workers, contact with animal-derived food, drinking it, or coming into contact with wastewater and surface water. Environmental and water samples are frequently contaminated with enterococci [9]. Among nosocomial pathogens, enterococci, which is resistant to antibiotics, plays a significant role. In addition to species-specific natural resistance, enterococci have acquired antibiotic resistance, which makes it difficult to design effective treatment protocols for patients. Enterococcus species have been reported to be becoming more resistant in recent years, particularly to high-level aminoglycosides, beta-

lactams, and glycopeptides [10]. Furthermore, because enterococci carry a large number of conjugative plasmids and transposons that can spread widely to other bacteria, they are well-known reservoirs of antibiotic-resistance genes [11].

The *gyrA* gene encodes the gyrA subunit of the enzyme known as DNA gyrase. This enzyme is the target of the quinolones antibiotics; therefore, any mutation in the *gyr* A gene is related to the resistance to quinolone [12].

Materials and Methods

Sample Collection

A total of 150 urine samples were collected with an incidence of UTI (100 (66.6%) from women and 50 (33.33%) from men) from some of the local hospitals in Hilla province. The bacteria were identified using a variety of biochemical and microscopical testing.

Culture and Identification

Using standard bacteriological and biochemical techniques, all samples were phenotypically identified to the species level after being cultivated on blood and selective agar media. The isolates were subsequently verified as *E. faecalis* using the Vitek 2 compact system and molecular identification by targeting the *ddI* gene, which encoded for D-alanine-D-alanine ligase with specific primers for the identification of *E. faecalis* isolates, as demonstrated in Table 1 [13].

Molecular Characterization

Using a genomic DNA extraction kit (Promega, Madison, Wisconsin), bacterial DNA was isolated from *E. faecalis* isolates according to the manufacturer's protocol. The quantity of DNA was confirmed by Nanodrop (Eppendorf AG, Germany), and DNA was stored at −20°C. Table 1 contains a list of primer sequences that were used to amplify genes related to identification and antibiotic resistance. 2.0µl (10pmol) of each primer, 5.5µl of free nuclease water, 3µl of DNA template, and 12.5µl of master mix (2X, Promega) were included in each 25µl PCR reaction. A thermal cycler (Applied biosystem-USA) was used to do PCR amplification using a conventional PCR procedure, as shown in Table 2.

The PCR amplification products were visible by performing gel electrophoresis on 1.5–2% agarose gel for 45 minutes at 100 volts. Its size was ascertained by comparing the amplicons to the 100 bp ladder molecular marker.

Table 1: Primers sequences and product size used for identifying *E. faecalis* and its genes for antibiotic resistance.

Primer	Sequence (5' - 3')	Amplicon size
ddl E. faecalis (D-Ala:D-Ala) [13]	F/ ATCAAGTACAGTTAGTCTTTATTAG R/ ACGATTCAAAGCTAACTGAATCAGT	941bp
Gyr A [14]	F: GCAATGAGTGTTATCGTCG R: TCTGGTCCAGGTAACACTTCC	575 bp
Bla-Tem[15]	F: TCAACATTTTCGTGTCGCCC R: AACTACGATACGGGAGGGCT	766 bp

Table 2 : The PCR thermo cycling conditions programs.

	Temperature (C/Time)					Cycles Number
	Initial denaturation	Cycling conditions			Final extension	
		Denaturation	Annealing	Extension		
ddl	94/5min	94/1min	54/1min	72/1min	72/10min	30
***gyr*A**	95/5min	95/30s	50.5/30s	72/45s	72/5min	35
***Bla*-Tem**	95/5min	95/30s	56/45s	72/60s	72/5min	35

Antibiotics susceptibility test

The Kirby-Bauer technique was utilized in compliance with the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) to assess *E. faecalis* resistance to different antibiotics. Following an overnight culture of the *E. faecalis* isolates, the bacterial concentration was adjusted to yield a turbidity standard equivalent to a 0.5 McFarland standard. Commercially prepared antibiotic discs such as Oxacillin, Penicillin, Erythromycin, Rifampicin, Clindamycin, Vancomycin, Chloramphenicol, Amoxicillin / clavulanic acid, Ampicillin, Ciprofloxacin, Co-trimoxazole, Gentamicin, Ceftazidime, Norfloxacin, and Tetracycline were used for testing antibiotic susceptibility on Mueller-Hinton agar (MHA) plates.

The MHA plates underwent an 18–24-hour incubation period at 37°C, after being infused with the standardized bacterial suspensions and coated with antibiotic discs. The *E. faecalis* isolates were categorized as susceptible, intermediate, or resistant to each corresponding antibiotic using CLSI breakpoints, which were obtained by measuring and examining the diameters of the inhibition zones surrounding different antibiotic discs.

Statistical analysis

A chi-square test for independence (Two-way Chi-squared test) was conducted using SPSS version 26.

Results

All isolated bacteria have been identified based on colonial morphology and biochemical characteristics. The phenotypic characteristics of *E. faecalis* revealed that the colonies are small to medium in size, gray or white in color, smooth, and non-hemolytic (γ- hemolytic). In rare cases, some strains produce a narrow zone of β- or α- hemolysis around colonies. These biochemical characteristics and colonial morphology have been used to identify these bacteria.

Fifty-two (34.6%, 52/150) isolates of *E. faecalis* were found out of 150 clinical samples that were cultured and confirmed using specific primers for the *ddl* gene*, as* shown in Figure 1.

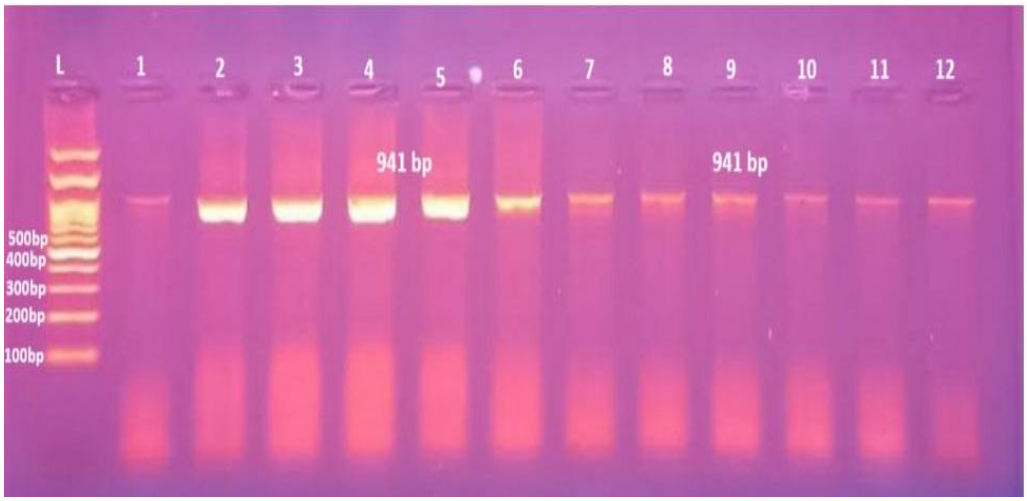

Figure 1 : Gel electrophoresis results for *ddI* gene amplification (941 bp). Lines 1 through 12 represent *E. faecalis* isolates. Line L: DNA marker; gel was stained with ethidium bromide and migrated at 75 volts for 80 minutes.

All the 52 isolates of *E. faecalis* recovered from the patient were tested for their antibiotic resistance to 15 antibiotics. Table 3 provides an overview of the antimicrobial potency of specific antibiotics against *E. faecalis*.

Table 3 : The Resistance rate of 52 *E. faecalis* isolates to 15 antibiotics

Antibiotic Name	Sensitive	Intermediate	Resistance
Ciprofloxacin	65% (34/52)	11.5% (6/52)	23% (12/52)
Oxacillin	0%	0%	100% (52/52)
Penicillin	0%	0%	100% (52/52)
Erythromycin	0%	0%	100% (52/52)
Chloramphenicol	84.6%(44/52)	7.69% (4/52)	7.69% (4/52)
Co-trimoxazole	53.85% (28/52)	32.69% (17/52)	13.46% (7/52)
Rifampicin	0%	0%	100% (52/52)
Ceftazidime	0%	15%(8/52)	85%(44/52)
Ampicillin	7.69%(4/52)	7.69(4/52)	84.6 %(44/52)
Norafloxacin	96% (50/52)	4% (2/52)	0%
Clindamycin	0%	0%	100%(52/52)
Vancomycin	0%	0%	100% (52/52)
Gentamicin	88.4% (46/52)	3.84%(2/52)	7.69% (4/52)
Amoxicillin Clavulanate	50% (26/52)	0%	50% (26/52)
Tetracycline	0%	0%	100% (52/52%)
Two-way Chi-squared test	Chi-square value: 667.434 DF:28 Significance level: P < 0.0001		

Statistical analysis showed significant differences (P<0.0001) for the type of antibiotic and the nature of the response. The results showed that, generally, the isolates are resistant to beta-lactams. The highest resistance recorded was to Penicillin (100%) and Oxacillin (100%), followed by Ceftazidime, Ampicillin, and Amoxicillin/Clavulanic acid (85%), (84.6%), (50%), respectively. Also, results showed a low resistance rate to Gentamicin, an aminoglycosides antibiotic. Only 7.69% (4/52) of *E. faecalis* isolates resisted Gentamicin. The following were the outcomes of fluoroquinolone resistance of the 52 isolates: 23% (12/23) were resistant to Ciprofloxacin. The Bacterial isolates also revealed that none of the 52 were resistant to Norfloxacin.

It was observed through this study that 100% of the isolates (n=52) showed resistance to Vancomycin, which is a big jump in the resistance rate of *E. faecalis* to Vancomycin. Only 7.69% of the 52 *E. faecalis* isolates (4/52) were resistant to Chloramphenicol, and only 13.46% (7/52) were resistant to Co-trimoxazole. Most of the isolates were susceptible to these antibiotics.

In order to identify some antibiotic-resistant genes, the genotypic characteristics of every *E. faecalis* isolate in this investigation were examined. The existence of the *gyr*A and *bla*-TEM genes was checked using the particular primers.
All isolates (100%) were resistant to Rifampicin, Erythromycin, Tetracycline, and Clindamycin. The PCR amplification results of the *bla*-TEM and *gyrA gene*s were disclosed. Forty-one isolates of *E. faecalis* were positive for *bla*-TEM *(*78.8%, 41/52), and 30 out of the 52 *E. faecalis* isolates appeared to harbor the *gyr* A gene (57.6%, 30/52), as shown in Figures 1 and 2.

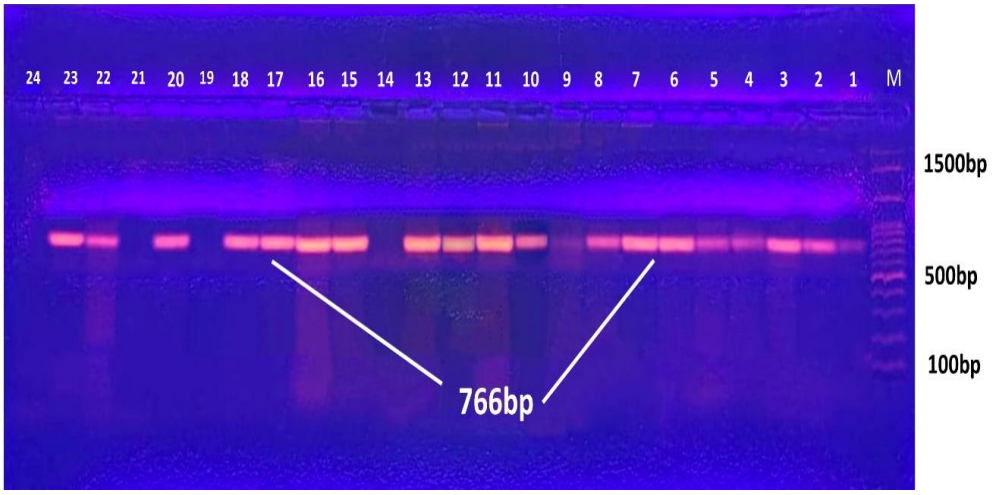

Figure 2: Gel electrophoresis results for *bla* TEM gene amplification (766 bp). Lines 1 through 24 represent *E. faecalis* isolates. Line M: DNA marker; gel was stained with ethidium bromide and migrated at 75 volts for 80 minutes.

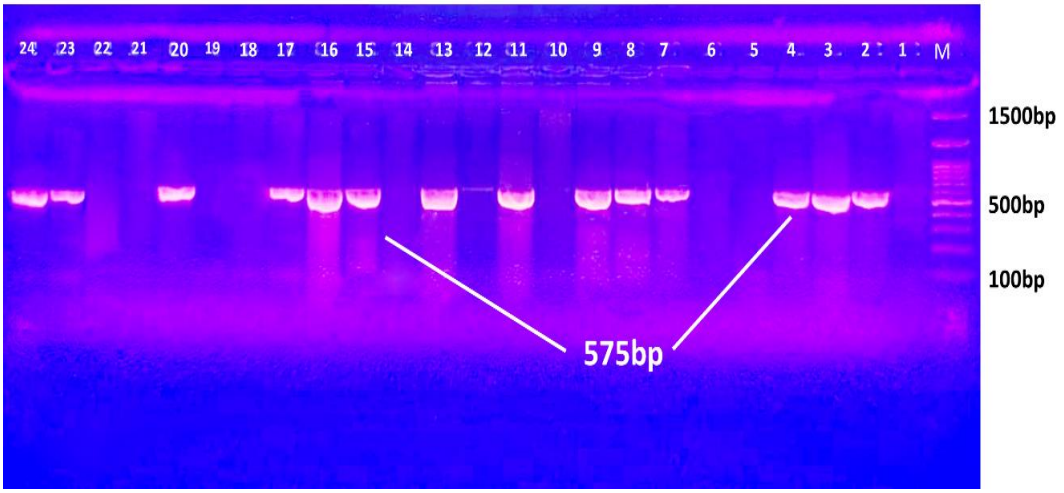

Figure 3 : Gel electrophoresis results for *gyr* A gene amplification (575 bp). Lines 1 through 24 represent *E. faecalis* isolates. Line M: DNA marker; gel was stained with ethidium bromide and migrated at 75 volts for 80 minutes.

Discussion:

The most frequently isolated species from clinical samples of humans is *E. faecalis,* which is followed by *E. faecium* [16]. Numerous prior studies conducted both domestically and internationally have shown that *E. faecalis* isolates are a major cause of infection, particularly in the human urinary tract [17,18].

Most *E. faecalis* are considered Penicillin-susceptible, but since the 1980s, Penicillin-resistant, Ampicillin-susceptible *E. faecalis* have been found (PRASEF) [8]. In a study carried out in South Korea that involved 295 patients suffering from bloodstream infections caused by *E. faecalis*, 22.7% of recovered isolates appeared to be Penicillin-resistant, Ampicillin-susceptible by the use of the CLSI breakpoints [19].

Although the widespread use of Vancomycin and broad-spectrum antimicrobials led to the emergence of vancomycin-resistant enterococci (VRE), and the rate of resistance increased rapidly from 0% to more than 83% by 2016, most of these VRE were other enterococci species such as *E. faecium* while only 9% of the *E. faecalis* species exhibited the VRE phenotype over the same time [20].

Generally, Vancomycin proved effective against *E. faecalis* with a 0% resistance rate [21, 22]. Fluoroquinolones are a class of antibiotics that has been frequently used to treat

infections caused by *E. faecalis*. The emergence of *E. faecalis* strains with resistance to fluoroquinolones has been reported in several countries recently [23]. This class of antibiotics exerts its inhibitory effect by inhibiting the DNA gyrase and topoisomerase enzymes [24].
According to previous studies, the resistance rate to each mentioned antibiotic was as follows: fifty-seven percent (57%) of *E. faecalis* isolates were resistant to Ciprofloxacin (40/70) [25], while two studies done more recently in 2018 & 2020 found that the percentage of the Ciprofloxacin resistant isolates was 27.5%, 23% respectively [26, 21].
As demonstrated in a study by Albaayit *et al*., the transformed *E. coli* cells by *P. aeruginosa* plasmids showed a significant increase in their resistance to Ciprofloxacin ($p<0.05$) compared to the non-transformed parental *E. coli*. This suggests that the resistance variation to fluoroquinolones may be caused by extrachromosomal genes (i.e., plasmid resistance) [27].
Enterococci are mostly susceptible to Tetracycline [28], although acquired resistance to this antibiotic has been reported as widespread [11,29]. Among 232 *E. faecalis* isolates, the highest antibiotic resistance was seen against Tetracycline (93.5%), followed by Erythromycin (87%) and Ciprofloxacin (80%) [30].
Another study showed that 25% (n=12) of *E. faecalis* isolated from human feces were resistant to Tetracycline [21], while 95% out of 232 *E. faecalis* isolates (41%) were resistant to Rifampicin [30]. The study's findings revealed that a significant proportion (78.8%) of *E. faecalis* isolates possessed the *bla*-TEM gene. Similarly, a study by Jia *et al.,* found that 70% of enterococcus isolates possessed the *bla*-TEM gene [31].

In a different study, Anderson *et al.,* found that the *bla*-TEM gene was correlated with the origin of *E. faecalis* in 30 percent of the endodontic isolates and 53.3% of the isolates from nosocomial infections [32]. However, according to Adeniji *et al.,* 0% of the enterococci isolates carried the *bla*-TEM gene [33]. *E. faecalis* uses a number of strategies to avoid the effects of antibiotics. Among its many virulence factors are lytic enzymes like β-lactamases, which are encoded by multiple genes, including the *bla*-TEM gene, which codes for extended-spectrum β-lactamase (ESBL). By hydrolyzing the beta-lactam ring, β-lactamases play a key role in resistance mechanisms by rendering the beta-lactam antibiotic inactive before it binds to the target [34,35].

The percentage of *E. faecalis* isolates with the *gyr* A gene present in this study was 57.6 %, which is lower than the results reported by Khosravi *et al.,* [14], which showed that 94.1% of isolates carried this gene. On the other hand, another study found that only 0% of isolates had the *gyr*A gene [17]. Different studies done in several countries reported the emergence of resistance to fluoroquinolones, which is mediated either by the chromosomes or the plasmid. This comprises mutations in the gene that encodes for the enzymes targeted by those antibiotics: gyrA, *gyr*B, *par*C, and *par*E [23].

Ethical approval

Approval for carrying out this research has been obtained from the ethical committee, College of Science for Women, University of Babylon, and according to document number 50. This study was approved by the Ministry of Health, Babylon Health Directorate, training and Human Development Center, and research committee on the 21st of May 2024.

Conflict of Interest

The authors report having no conflicts of interest

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