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Original Article

Bioinformatics Analysis of Genetic Variations and Clonal Relationships of Clinical Enterococci Species in the Context of Antibiotic Resistance in Iraq

Raid Razzaq Ojaimi[1*]

[1]Medical Biotechnology Department, College of Biotechnology, Al-Qasim Green University, Babylon 51013, Iraq

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***Corresponding author:**
Raid Razzaq Ojaimi,
Email: rayid.r@biotech.uoqasim.edu.iq

Abstract

Background: Antibiotic-resistant enterococci have turned into major pathogens in humans in Iraq. Therefore, this study examined genetic variations and clonal relationships among *Enterococcus* strains concerning antibiotic resistance in Al-Diwaniya, Iraq, using RAPD-PCR.

Methods: Overall, 250 clinical samples were collected under sterilized conditions between December 2020 and May 2021. Using standard methods, enterococci isolates were identified and evaluated for antibacterial susceptibility. Moreover, molecular techniques were utilized to examine genetic variations and clonal relationships of *Enterococcus* spp. and detect *EsβL* genes. The random amplified polymorphic deoxyribonucleic acid-polymerase chain reaction (RAPD-PCR) results were analyzed by BioNumerics software.

Results: Thirty-four enterococci strains were isolated and identified by amplifying and sequencing the *16S–23S rRNA* gene. Of these strains, 82.3%, 8.8%, 5.8%, and 2.9% were identified as *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum*, respectively. The highest antibiotic resistance among *E. faecium* isolates was associated with ampicillin, cefotaxime, ceftriaxone, and gentamicin (100%), while the lowest resistance belonged to chloramphenicol (66.60%). Multidrug-resistant and extensively drug-resistant strains were present in 50% and 5.8% of the enterococci isolates, respectively. The presence of the *bla-TEM*, *bla-SHV*, and *bla-CTX-M2* genes in several isolates at frequencies of 44.1%, 38.2%, and 11.7%, respectively, indicated the spread of antibiotic resistance mechanisms. RAPD results confirmed a relationship between RAPD patterns, isolate sources, and antibiotic resistance patterns.

Conclusion: Our findings demonstrated a distinct genotypic variety of the *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* strains isolated from various clinical cases. RAPD analysis proved to be highly effective in determining the genetic variation among *Enterococcus* species.

Keywords: BioNumerics software, *Enterococcus* spp., Extended spectrum β-lactamases, Random amplification polymorphism DNA, Multi-drug resistance

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Introduction

Enterococcus spp. is a bacterial genus of gram-positive, facultative anaerobic cocci, adapted through long years of evolution to a commensal role in the digestive system and, to a lesser degree, in the vagina and oral cavities of humans and animals (1). In addition, it is a leading cause of opportunistic nosocomial infections, causing various diseases, including endocarditis, bacteremia, infections in the urinary tract and abdominal cavity, meningitis leading to the central nervous system, and the like. Among the many species of enterococci, *Enterococcus faecalis* and *Enterococcus faecium* are the main infectious agents for humans (2). The *E. faecalis-E. faecium* infection ratio has changed during the past two decades, with *E. faecium* emerging as the predominant agent. The considerable genetic flexibility and the capacity of species to exhibit widespread antimicrobial resistance are two reasons for this change (3). Notably, enterococci rank second in urinary tract infections (UTIs), third in bacterial diseases, and fourth among nosocomial infectious agents (4). Concerns about the global dissemination of antibiotic resistance have arisen due to enterococci's resistance to a wide variety of antibiotic groups, such as cephalosporins, aminoglycosides, lincosamides, and trimethoprim-sulfamethoxazole; nonetheless, their genomes exhibit remarkable adaptability, allowing for the acquisition of novel resistance determinants (5). Bacteria have developed three major patterns of resistance: multi-drug resistance (MDR) is defined as non-susceptibility to at least one antibiotic in three or more antibacterial classes; extensive-drug resistance (XDR) is characterized by resistance to at least one antibiotic in all except two or fewer antibacterial classes. Further, pan-drug resistance is defined as resistance to all classes of antibacterial agents (6).

Understanding the genetic variation and relationships among bacterial strains associated with various infections is essential for epidemiological investigations. It further establishes a foundation for estimating the pathogenic potential of bacteria. Random amplified polymorphic deoxyribonucleic acid-polymerase chain reaction (RAPD-PCR) is a simple and cost-effective genotyping technique used to differentiate bacterial strains. A short primer, typically 10 nucleotides long, amplifies multiple genomic loci, identified through agarose gel electrophoresis as strain-specific profiles (RAPD-PCR genotypes). The main advantage of the RAPD-PCR technique is that prior knowledge of the DNA sequence of the tested microorganisms is unnecessary (7). Furthermore, it can be effectively applied to evaluate the similarity among enterococci species isolated from samples (8). Knowledge of the molecular analysis and assessment of genetic variation in *E. faecium*, *E. faecalis*, *E. casseliflavus*, and *E. gallinarum* is an important step for epidemiological surveillance, characterization, and determination of isolates. Moreover, it is useful for controlling infections due to the above bacterium. Therefore, this study aims to apply RAPD-PCR, a relatively cheap and fast method for detecting genetic similarities among some antibiotic-resistant enterococci species isolated from different clinical cases in the hospitals of Iraq.

Materials and Methods

Design of the Study

This investigation was conducted in the laboratory of the Women and Children Teaching Hospital in Al-Diwaniya, central Iraq. Two hundred fifty clinical samples were gathered under sterilized conditions between December 2020 and May 2021. These samples were collected from the patient's urine, diarrhea, and cerebrospinal fluid (CSF) using leak-proof containers. In addition, sterile cotton swabs were used to collect vaginal samples.

Bacteriological Analysis

All clinical samples were cultured on conventional culture media using a sterile standard loop, including nutrient and blood agars (Oxoid, Basingstoke, UK). After incubation at 37 ˚C for 24 hours, they were cultured on selective media, such as MacConkey agar N2 (HiMedia, India) and Rapid HiEnterococci agar (HiMedia, India). Then, the plates were incubated for 24 hours at 37˚C. The isolates were biochemically identified using Gram staining and catalase, motility, bile esculin hydrolysis, pigment tests, and a salt tolerance test (6.5% NaCl). Other testing methods, including growth in highly alkaline conditions (pH = 9.6) and at high temperatures, were used to identify *Enterococcus* spp. In total, 34 Enterococci isolates were investigated in this study, including 3 (8.8%), 10 (29.4%), and 11 (32.3%) from CSF, the urine, and the vagina, respectively. All enterococci isolates were stored in Tryptic soy broth (Lab M, Lancashire, UK) containing 20% glycerol at –80 ˚C in cryogenic vials for other tests (9).

Antibacterial Susceptibility Assay

The Kirby-Bauer disc diffusion method was used to assess the antibiotic resistance of the antimicrobial agents against clinical enterococci isolates. To determine antibiotic resistance in the isolates, 25 µg of ampicillin (AM), 30 µg of cefotaxime (CTX), 30 µg of ceftriaxone (CRO), 10 µg of gentamicin (GN), 300 µg of nitrofurantoin (F), 30 µg of chloramphenicol (CAM), and 30 µg vancomycin (VA) antibiotic discs were utilized (all purchased from Becton Becton Dickinson, Heidelberg, Germany). After incubation, the resulting diameters of the inhibition zones formed around the antibiotic discs were classified as susceptible or resistant according to the diameters and breakpoints reported in Clinical and Laboratory Standards Institute documents (10). MDR refers to bacteria that have developed resistance to at least three antibiotics, and XDR refers to bacteria that have resistance to all antibiotics except two (11).

Molecular Study

The PCR technique was applied to amplify the *16S–23S rRNA* and *ESβL* gene. In addition, using the random primers (Table 1), the RAPD-PCR technique was performed to genotype enterococci strains.

Gel Electrophoresis

The *16S–23S rRNA* gene and RAPD-PCR products were separated by electrophoresis at 100 V for 1 hour in a 2% agarose gel. Further, the PCR products of the *ESβL* gene were separated in a 1.5% agarose gel electrophoresis at 100 V for 30 minutes, stained with ethidium bromide, and visualized under UV light (molecular size marker: 100 bp DNA ladder; Fermentas, Germany). The images were saved in a TIFF file for subsequent analysis.

Deoxyribonucleic Acid Sequences

Purified PCR products were sent for *16S–23S rRNA* gene sequencing at Macrogen (South Korea). The initial sequence analysis was performed using the Finch TV program (version 1.4; https://finchtv.software.informer.com/1.4), which allowed us to manually corroborate the sequences obtained from the sequencing process of each sample and generate a consensus sequence. Each sequence was analyzed with the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm its identity. Subsequently, the sequences were aligned by the Clustal Omega program (version 7; https://www.ebi.ac.uk/Tools/msa/clustalo). Furthermore, the MEGA program (version 11; http://www.megasoftware.net/mega.php) was used to construct the phylogenetic tree. Finally, the newly generated sequences for the *16S-23S rRNA* gene were deposited in GenBank.

Calculation of a Discrimination Index

A single numerical index of discrimination (D), described by Hunter and Gaston, was utilized to evaluate the discrimination power of the RAPD-PCR technique for genotyping *Enterococcus* spp. The index is based on the probability that two independent isolates, sampled randomly from the tested population, are classified into different genotypes. The genotypes were determined based on 100% similarity of obtained patterns (19).

Statistical Analysis

The RAPD-PCR patterns were analyzed using BioNumerics, version 8.0 (Applied Math, Sint-Martens-Latem, Belgium). An unweighted pair group method with arithmetic averages was used to build a dendrogram, and Dice's coefficient (80%) was employed to calculate the similarity percentage among enterococci strains. Additionally, the RAPD-PCR results were correlated with the phenotypic and genotypic determination of antibiotic resistance through PCR and the disk diffusion test (19).

Results

Identification of Enterococcus spp.

In general, 34 presumptive enterococci strains were isolated from various clinical sources (10 from urine, 10 from diarrhea, 11 from the vagina, and 3 from CSF). All isolates appeared as red or pink colonies on MacConkey agar N2 because of the abilities of the lactose sugar fermenter (Figure 1A). In addition, they all appeared as small greenish-blue colonies when streaked on the

Table 1. The Primers Sequences and Optimal Conditions for the PCR and RAPD-PCR

Gene Name		Primer sequence (5´- 3´)	Size (bp)	Conditions	References
		PCR Programming			
16s-23s rRNA	F	CAAGGCATCCACCGT	300	Step 1: 94 °C, 5 minutes	Clementino et al (12)
				Step 2: 94 °C, 1 minute	
				Step 3: 56 °C, 1 minute	
	R	GAAGTCGTAACAAGG		Step 4: 72 °C, 1 minute	
				Step 5: 72 °C, 5 minutes	
bla-CTX-$_{M-2}$	F	ACGCTACCCCTGCTATTT	200	Step 1: 97 °C, 5 minutes	Shibata et al (13)
	R	CCTTTCCGCCTTCTGCTC		Step 2: 94 °C, 30 seconds	
bla-TEM	F	TTTCGTGTCGCCCTTATTCC	500	Step 3: 54 °C, 30 seconds	Sharif et al (14).
	R	CCGGCTCCAGATTTATCAGC		Step 4: 72 °C, 2 minutes	
bla-SHV	F	ATCGTTGTCAGAAGTAAGTTGG	1200	Step 5: 72 °C, 10 minutes	Yagi et al (15)
	R	TTTATGGCGTTACCTTTGACC			
		RAPD-PCR Programming			
RAPD-640		CGTGGGGCCT	300-800	Step 1: 95°C, 5 minutes	Ashayeri et al (16)
RAPD-2		GTTTCGCTCC	400-800	Step 2: 95°C, 30 seconds	Chen et al (17)
RAPD-4		AAGACGCCGT	300-750	Step 3: 40°C, 30 seconds	Deschaght et al (18)
				Step 4: 72°C, 5 minutes	
				Step 5: 72°C, 5 minutes	

Note. RAPD-PCR: Random amplified polymorphic deoxyribonucleic acid-polymerase chain reaction; F: Forward; R: Reverse.

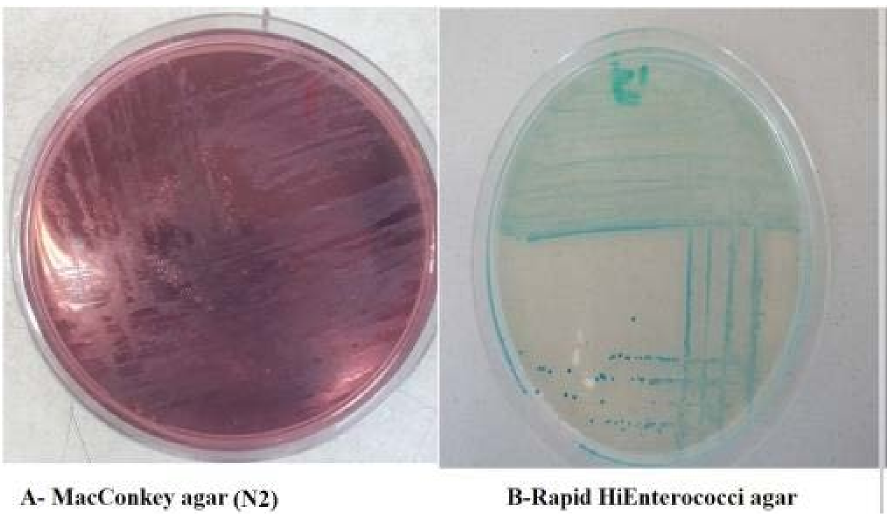

Figure 1. Cultural Characterization of *Enterococcus* spp. on **(A)** MacConkey Agar (N2) and **(B)** Rapid HiEnterococci Agar

selective Rapid HiEnterococci agar (Figure 1B). Under the microscope, they were detected as Gram-positive cocci and grouped in short chains or pairs. Overall, 24 (70.5%) isolates were female and 10 (29.4%) were male.

Two molecular techniques were employed to identify enterococcal species: PCR utilizing genus-specific and species-specific primers and sequencing of the *16S-23S rRNA* gene.

Based on the electrophoresis and sequencing of the *16S-23S rRNA* gene, four species were identified: *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum*. The greatest species diversity was related to diarrhea, as four species were identified: 40% *E. faecalis*, 30% *E. faecium*, 20% *E. casseliflavus*, and 10% *E. gallinarum*. In contrast, all isolates from the vagina, urine, and CSF were identified as *E. faecalis* (Figure 2).

GenBank Accession Number

The nucleotide sequences of the *16S–23S rRNA* gene for *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* were deposited in the GenBank database under the accession numbers OK144136.1, OK144139.1, OK144140.1, OK144137.1, OK144138.1, and OK265106.1.

Antibiotic Resistance Pattern in Enterococci Species

Enterococci species isolated from clinical samples exhibited various levels of antibiotic resistance (Figure 3). High levels of resistance to CRO (100%), CTX (100%), and GN (91.1%) were found in *Enterococcus* isolates. *E. faecium* had a higher rate of antibiotic resistance than other species. Moreover, high sensitivity to VA, AM, CAM, and nitrofurantoin was noted in this investigation.

Overall, 19 out of 34 *Enterococcus* spp. (17%) were resistant to three or more classes of antibiotics, classifying them as MDR bacteria, and 2 (5.8%) species were also resistant to all classes of antibiotics except for two classes of antibiotics, qualifying them as XDR. However, no isolate demonstrated pan-drug resistance.

Molecular Detection of Extended-Spectrum Beta-Lactamase Genes

The molecular detection results for β-lactam-resistance genes in enterococci species are summarized in Table 2. The distribution of these genes is illustrated in Figure 4. Based on the results, *bla-TEM*, *bla-CTX*$_{-M2}$, and *bla-SHV* were detected in 20.5%, 11.7%, and 38.2% of strains, respectively, while they were not found in *E. gallinarum*.

Random Amplified Polymorphic Deoxyribonucleic Acid-Polymerase Chain Reaction Analysis

In this research, the RAPD-PCR technique was used to assess genotypic polymorphism and genetic relatedness among 34 enterococci isolates obtained from urine, vagina, diarrhea, and CSF using three distinct primers (RAPD-2, RAPD-4, and RAPD-640). The primers produced several polymorphic amplicons ranging from > 300 to > 900 bp (Figure 5). The electrophoretic patterns of RAPD-2, RAPD-4, and RAPD-640 primers were compared, revealing that 52 isolates were clustered into 14 (41%), 14 (41%), and 24 (70.5%) groups, respectively, with similar genotypic patterns among the *Enterococcus* strains. All UTIs caused by enterococci strains that shared identical

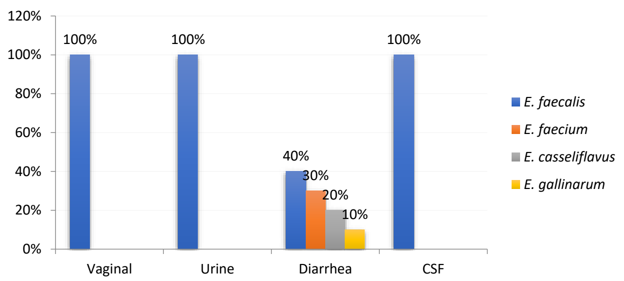

Figure 2. Percentages of Enterococci Species Isolated From Various Clinical Sources. *Note*. CSF: Cerebrospinal fluid

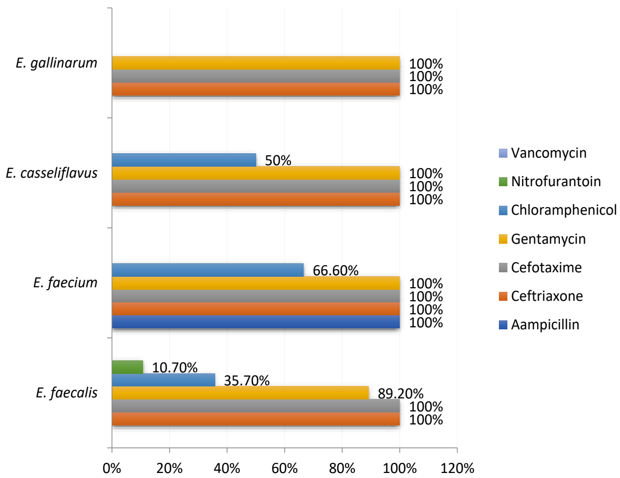

Figure 3. Prevalence of Antibiotic Resistance Among *Enterococcus* Species Isolates From Various Clinical Samples

Table 2. Prevalence of β-Lactam-Resistance Genes Among *Enterococcus* spp. Based on Isolated Sources

Organism	Sources	Number of isolates tested	Number of positives	Detection rate (%)	*ESβL* genes
E. faecalis	Urine	28	4	14.2	*bla-TEM* and *bla-SHV*
	Vagina		3	10.7	*bla-TEM* and *bla-SHV*
	Diarrhea		2	7.1	*bla-SHV* and *bla-CTX*$_{-M2}$
	CSF		2	7.1	*bla-TEM* and *bla-SHV*
E. faecium	diarrhea	3	3	100	*bla-SHV* and *bla-CTX*$_{-M2}$
E. casseliflavus	diarrhea	2	2	100	*bla-TEM* and *bla-SHV*

Note. ESβL: Extended-spectrum beta-lactamase; CSF: Cerebrospinal fluid.

RAPD profiles, antibiotic resistance, and *ESβL*-gene production—compared to strains isolated from diarrhea, vaginosis, and CSF samples from the same patients—were classified as endogenous infections. Conversely, exogenous infection was identified in 50 isolates with different genotypic patterns.

The RAPD-2 primer agarose electrophoresis results revealed high discriminatory power for assessing similarities among enterococci strains. Due to the number of amplicons obtained by Primer RAPD-2, 117 amplicons were generated, distributed among 44 amplicons within a molecular weight range of 300–700 bp, 30 amplicons within 300–800 bp, 13 amplicons within 300–750 bp, and 11 amplicons within 300–900 bp, in *E. faecalis* isolated from vagina, urine, diarrhea, and CSF, respectively. Additionally, primer RAPD-2 produced 9 amplicons in *E. faecium*, 8 amplicons in *E. casseliflavus*, and 2 amplicons in *E. gallinarum*, all isolated from diarrhea samples (Figure 5 and Table 3). For the RAPD-4 primer, the number of amplicons obtained under standard conditions was low and did not provide satisfactory discriminatory power among strains. As RAPD-4 produced 84 amplicons, distributed as 32 in *E. faecalis* from vagina samples and 26 from urine samples, both within the molecular weight range of 400–800 bp. It also showed 22 amplicons in enterococci isolated from diarrhea, with 8 amplicons each in *E. faecalis* and *E. faecium* within the same weight range, along with 4 amplicons in *E. casseliflavus* ranging from 600 bp to 750 bp, and 2 amplicons in *E. gallinarum* at 600 bp and 750 bp. In addition, four amplicons were found in *E. faecalis* isolated from CSF (Figure 5 and Table 3). Meanwhile, the gel electrophoresis yielded sufficient discriminatory power to evaluate similarity among enterococci strains with primer RAPD-640, which produced 92 amplicons in *E. faecalis* isolates from vagina, urine, diarrhea, and CSF samples, with 30, 26, 13, and 9 amplicons, respectively. Additionally, *E. faecium* isolates from diarrhea produced 6 amplicons within the 600–700 bp range. Further, *E. casseliflavus* isolates from diarrhea demonstrated 8 amplicons within 400–700 bp, and *E. gallinarum* isolates from diarrhea contained only 2 amplicons within 650–700 bp (Figure 5 and Table 3). Most enterococci strains represented the same RAPD-PCR patterns, highlighting their origin from an epidemic

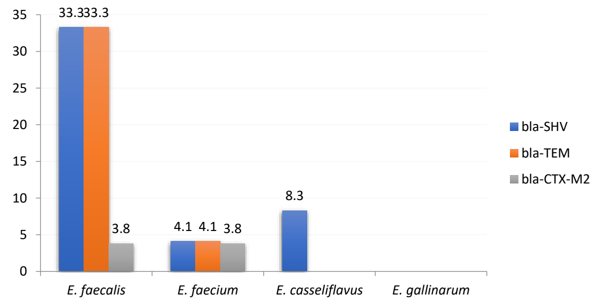

Figure 4. Distribution of *ESβL* Genes Among Clinical Enterococci Species. *Note*. ESβL: Extended-spectrum beta-lactamase

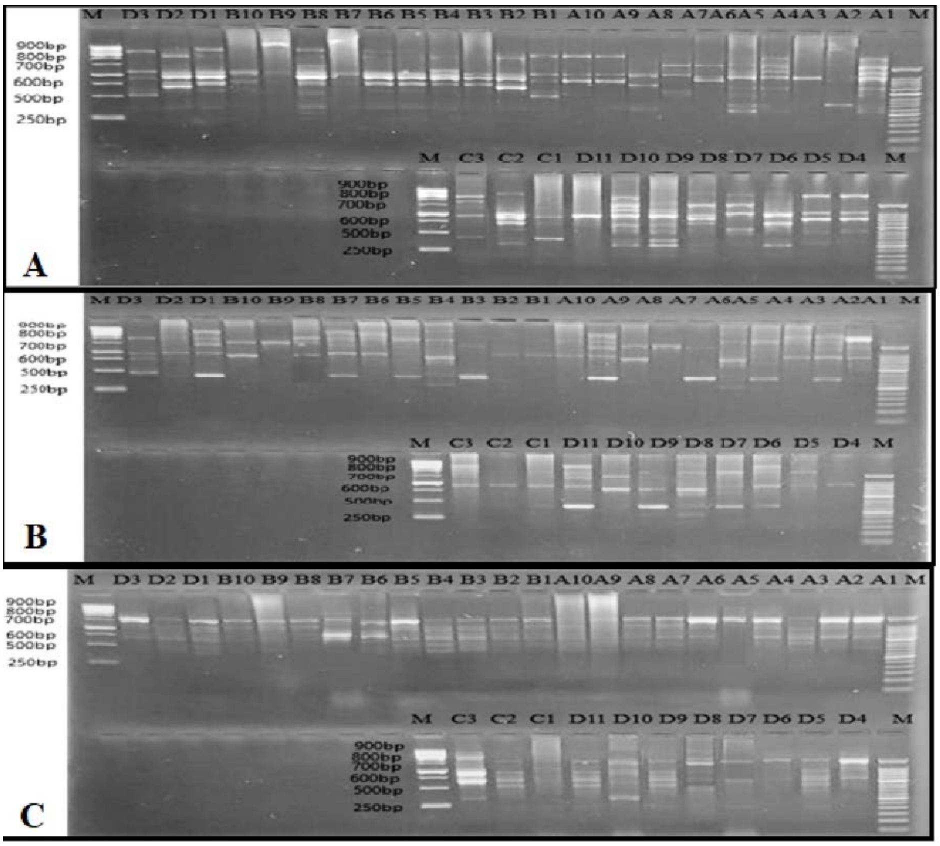

Figure 5. RAPD-PCR Amplification Products in Agarose Gel Electrophoresis Using Primers A-RAPD-2, B-RAPD-4, and C-RAPD-640. *Note*. RAPD-PCR: Random amplified polymorphic deoxyribonucleic acid-polymerase chain reaction; CSF: Cerebrospinal fluid. A1-A10: Urine isolates, B1-B10: Diarrhea isolates, D1-D11: Vagina isolates, and C1-C3: CSF isolates. M: DNA ladder

outbreak and the usefulness of the applied methodology for epidemiology studies.

According to the BioNumerics analysis of RAPD-PCR products on gel electrophoresis, one to seven clear amplicons were obtained from RAPD analysis using primer RAPD-2. Moreover, genomic typification by RAPD-PCR revealed three major enterococci genotypes (A-C) at a 100% similarity level, representing genetically indistinguishable isolates identified by RAPD-2 (Figure 6 and Table 3). Additionally, the A genotype included three *E. faecalis* isolates from females with diarrhea, which were genetically close to other isolates of *E. faecalis* and *E. casseliflavus*, and additional *E. faecalis* isolates from the vagina, diarrhea, and urine, respectively, with similarity percentages of 75%, 73%, 62%, and 53%. The B genotype contained three isolates—two of *E. faecium* from male diarrhea and one of *E. faecalis* from the vagina—which were comparable to *E. faecalis* isolated from female CSF at 50%. In addition, two isolates fell into the genotype C of *E. faecalis*, known to cause UTIs in females, and were related to the *E. casseliflavus* isolate from female diarrhea, sharing 75% similarity. The remaining twenty isolates displayed unique genetic patterns, classifying them as distinct. This analysis highlights the genomic diversity among the isolated enterococci species.

Table 3. Results of RAPD-PCR Analysis With RAPD-2, RAPD-4, and RAPD-640 Primers on Thirty-Four Isolates of *Enterococcus* spp.

	Results of RAPD-PCR			
Number of amplicons	**RAPD-2 n = 34 isolates (%)**	**RAPD-640 n = 34 isolates (%)**	**RAPD-4 n = 34 isolates (%)**	**Combination of 3 primers N = 102 isolates (%)**
One	5 (14.70)	3 (8.82)	8 (23.52)	16/102 (15.68)
Two	5 (14.70)	10 (29.41)	14 (41.17)	29/102 (28.43)
Three	7 (20.58)	14 (41.17)	7 (20.58)	28/102 (27.45)
Four	10 (29.41)	8 (23.52)	4 (11.76)	22/102 (21.56)
Five	4 (11.76)	0 (0)	2 (5.88)	6/102 (5.88)
Six	0 (0)	0 (0)	0 (0)	0/102 (0)
Seven	3 (8.82)	0 (0)	0 (0)	3/102 (2.49)
Source of isolations	**RAPD-2 n = 117 amplicons (%)**	**RAPD-640 n = 92 amplicons (%)**	**RAPD-4 n = 84 amplicons (%)**	**Combination of 3 primers N = 293 amplicons**
Urine	30 (25.64)	26 (28.26)	26 (30.90)	82/293 (27.98)
Vagina	44 (37.6)	30 (32.60)	32 (38)	106/293 (36.17)
Diarrhea	32 (27.35)	27 (29.34)	22 (26.19)	81/293 (27.64)

Note. RAPD-PCR: Random amplified polymorphic deoxyribonucleic acid-polymerase chain reaction.

Figure 6. Dendrogram of Primer RAPD-PCR (RAPD-2) Patterns Using BioNumerics for 34 Clinical Enterococci Isolates. *Note*. RAPD-PCR: Random amplified polymorphic deoxyribonucleic acid-polymerase chain reaction

As shown in Figure 6, the tree diagram includes the genetic analysis of RAPD-PCR patterns, antibiotic resistance testing, and the presence of *ESβL* genes. The three *E. faecalis* isolates with 100% similarity in genotype A demonstrated resistance to CRO, CTX, and GN. Additionally, they carried the *bla-TEM*, *bla-CTX-M2*, and *bla-SHV* genes. Conversely, they showed similarity to the other three *E. faecalis* isolates (two from the vagina and one from urine) and one *E. casseliflavus* isolate from diarrhea, all representing resistance to CRO, CTX, GN, and CAM. Of these, only two isolates (i.e., *E. faecalis* and *E. casseliflavus*) possessed the *bla-SHV* gene. In genotype B, there were three other isolates (two *E. faecium* from male diarrhea and one *E. faecalis* from the vagina), which were comparable with the *E. faecalis* isolated from female CSF at 50%. These isolates displayed a single pattern of resistance to CRO, CTX, and GN, and harbored the *bla-SHV*, *bla-CTX-M2*, and *bla-TEM* genes. Genotype C included two similar *E. faecalis* isolates resistant to CRO, CTX, GN, CAM, and nitrofurantoin, along with one *E. casseliflavus* isolate resistant to AM, CRO, CTX, and GN. The isolates of this type are characterized by the lack of genes.

Based on the results, one to five amplicons were produced using the primer RAPD-4. The 34 enterococci isolates were divided into five genotypes (A-E) of genetically identical isolates with 100% similarity based on RAPD-4 (Figure 7 and Table 3). Genotype A included three *E. faecalis* isolates (two from CSF and one from the vagina). Two *E. faecalis* isolates belonged to genotype B (one from CSF and one from the vagina), similar to two other *E. faecalis* isolates from the vagina, with 58.3% similarity. Furthermore, genotype C contained two isolates: one *E. casseliflavus* and one *E. faecalis* from diarrhea, with 50% similarity to *E. faecalis* from the vagina. Likewise, genotype D included two *E. faecalis* isolates causing UTIs, while genotype E had two isolates: one *E. casseliflavus* from male diarrhea and one *E. faecalis* from female urine. Overall, 20 isolates from various sources were obtained, which were distinguished by their specific genetic patterns compared to others.

The clinical isolates of enterococci in genotype A showed similar resistance patterns to CRO, CTX, and GN. The isolates from CSF uniquely carried only the *bla-TEM*- and *bla-SHV*-resistant genes. As shown in Figure 7, genotype B *E. faecalis* isolates exhibited resistance similarities to CRO, CTX, GN, and CAM while lacking *ESβL* genes. Moreover, genotype C isolates—*E. casseliflavus* and *E. faecalis* from diarrhea—demonstrated resistance to AM, CRO, CTX, and GN and carried *bla-SHV* and *bla-TEM* genes. Additionally, genotype D uropathogenic *E. faecalis* isolates displayed resistance to CRO, CTX, GN, and nitrofurantoin, without any *ESβL* genes. Finally, *E. casseliflavus* and *E. faecalis* isolates of genotype E were distinguished by their resistance to CRO, CTX, GN, and CAM, as well as the presence of *bla-SHV*, *bla-CTX-M2*, and *bla-TEM* genes (Figure 7).

Figure 8 illustrates the dendrogram based on the RAPD-PCR analysis of *Enterococcus* strains. Considering genetic relationships between strains with more than 100% similarity of the RAPD genotypes, seven genotypes (A–G) were distinguished using the RAPD-640 primer (Figure 8 and Table 3). Genotype A contained four isolates (two *E. faecium* isolates from male diarrhea, one *E. gallinarum* isolate from female diarrhea, and one E. faecalis isolate from male urine), which were possibly related to two *E. faecalis* isolates from the vagina in the same genotype (66.7% and 63.3%, respectively). Genotype B grouped three *E. faecalis* isolates (two from the vagina and one from male CSF), similar to an isolate of *E. faecalis* from female urine, with 86.7% similarity. In addition, genotype C included two isolates (one *E. casseliflavus* isolate from female diarrhea and one *E. faecalis* isolate from male urine), which were similar to two isolates of *E. faecalis*—one from female diarrhea and one from female urine—with 75% similarity. Additionally, it displayed 58.8% similarity to another *E. faecalis* strain isolated from female urine. Further, genotype D contained two *E. faecalis* isolates (one from male diarrhea and another from male urine), similar to *E. faecalis* from the vagina and *E. faecium* from male diarrhea, with 66.7% and 55.6%, respectively. Furthermore, genotype E consisted of two *E. faecalis* isolates (one from the vagina and another from female diarrhea), similar to an *E. casseliflavus* isolate from female diarrhea (with 60% similarity). Moreover, genotype F included two *E. faecalis* isolates (one from the vagina and one from male urine), similar to *E. faecalis* from the vagina (with 50% similarity). Genotype G contained two *E. faecalis* isolates (one from female CSF and one from female urine), which were similar to *E. faecalis* isolated from female diarrhea (with 45.8% similarity).

The results (Figure 8) confirmed a similarity among enterococci isolates in their patterns of antibiotic resistance and the presence of resistance genes. The four genetically similar isolates of genotype A showed high resistance to various antibiotics, such as CRO, CTX, and GN, with *E. faecium* isolates distinguished by carrying only the *bla-SHV* and *bla-CTX-M2* genes. In contrast, genotype B isolates exhibited similar resistance to CRO, CTX, and GN and carried the *bla-SHV* gene. The genotype C isolates, which were also similar, displayed multiple antibiotic resistances to AM, CRO, CTX, GN, and CAM, with the *E. casseliflavus* isolate possessing the *bla-TEM* gene. Similarly, the six *E. faecalis* isolates obtained from urine, diarrhea, vagina, and CSF, belonging to genotypes D, E, and G, demonstrated comparable resistance to CRO, CTX, GN, and nitrofurantoin; three of these isolates carried both the *bla-SHV* and *bla-TEM* genes. Meanwhile, genotype F isolates depicted similar resistance to CRO, CTX, and GN and contained the same *bla-SHV* and *bla-TEM* genes. Moreover, isolates with different genetic profiles displayed varied antibiotic resistance patterns and differed in their possession of resistance genes.

MDR and XDR isolates were distributed among all

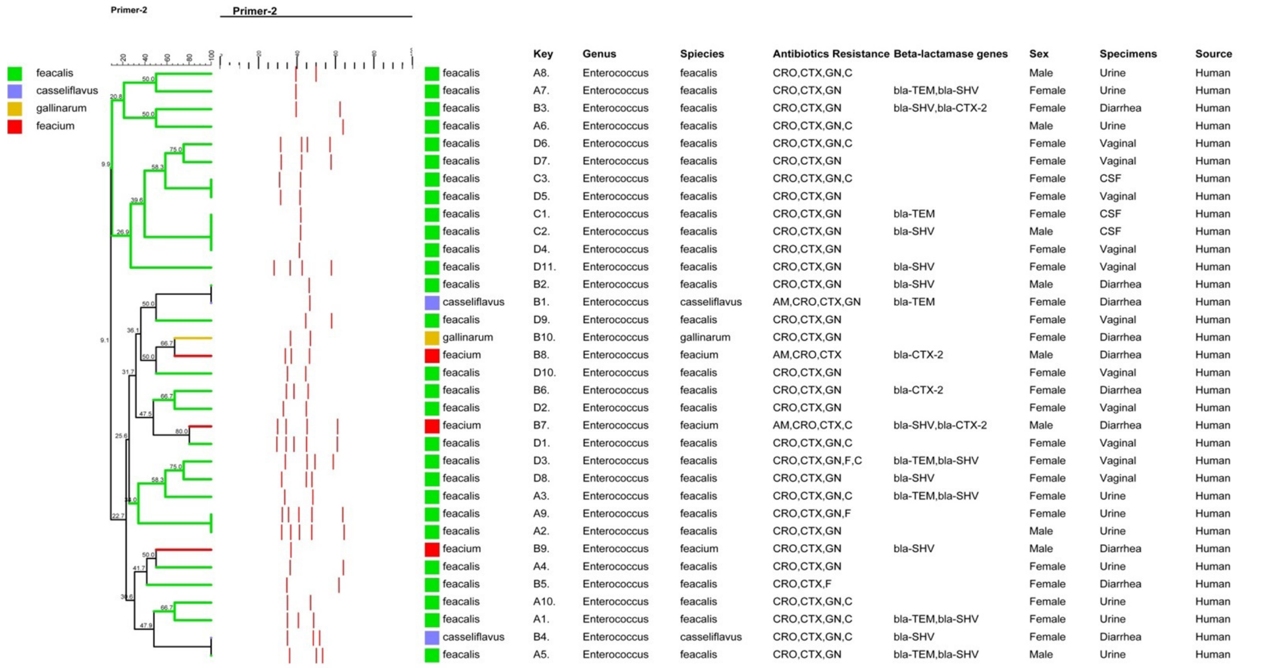

Figure 7. Dendrogram of Primer RAPD-PCR (RAPD-4) Patterns Using BioNumerics for 34 Clinical Enterococci Isolates. *Note*. RAPD-PCR: Random amplified polymorphic deoxyribonucleic acid-polymerase chain reaction

Figure 8. Dendrogram of Primer RAPD-PCR (RAPD-640) Patterns Using BioNumerics for 34 Clinical Enterococci Isolates. *Note*. RAPD-PCR: Random amplified polymorphic deoxyribonucleic acid-polymerase chain reaction

Discussion

In recent years, enterococci have gained importance as nosocomial pathogens (20). These bacteria arise in medical facilities, particularly in intensive care units, and can spread through direct contact between people or through contaminated medical equipment (21). In addition, they can live and spread in hospitals due to their inherent tolerance to harsh surroundings and antibacterial medications. Our findings revealed a high distribution of

of which were resistant to beta-lactam, aminoglycoside, and CAM antibiotics. CRO and CTX had the highest resistance rates (100%), and only *E. faecium* isolates showed high resistance to AM (100%).

Furthermore, the high prevalence of resistance to gentamycin was reported at 100% in *E. faecium*, *E. casseliflavus*, and *E. gallinarum*, while *E. faecalis* was resistant at 89.2%. The propensity of enterococci to develop new resistance mechanisms, particularly to AM, aminoglycosides, and glycopeptides, makes them intrinsically resistant to various kinds of antibiotics that impair cell wall formation (23).

Enterococci species can be categorized to help distinguish between possible variances in their properties and the methods by which these bacteria circulate among various hosts and sources using numerous phenotypic and genotypic approaches, such as RAPD and RFLP-PCR, as well as pulsed-field gel electrophoresis, multi-locus sequence typing, and biotyping for typing *Enterococcus* spp. Currently, clonal relatedness among strains, strain origin, and epidemiological investigations are all successfully conducted using PCR-based typing methods. Among these methods, RAPD-PCR typing is a straightforward, practical, and reasonably priced method that has been extensively utilized to distinguish bacterial isolates (24).

The RAPD-PCR analysis in this study revealed the genetic polymorphism of the enterococci strains (Figures 6, 7, and 8). The 34 enterococci isolated from various clinical cases were divided into three, five, and seven groups of the same clone showing similar patterns of genotypic traits, antibiotic resistance, and *ESβL*-gene production among isolates from urine, vaginosis, diarrhea, and CSF; these were considered endogenous infections. According to RAPD-PCR results and using the three primers RAPD-2, RAPD-4, and RAPD-640 means that the Enterococcus isolates that clustered at 100% similarity level are genetically identical.

The results of RAPD-PCR indicated that 52 isolates shared similar molecular patterns across urine, vagina, CSF, and diarrhea specimens from each patient. Conversely, 50 isolates were uniquely genotyped with RAPD-2, RAPD-4, and RAPD-640 primers. Isolates from UTIs showed greater diversity compared to those from other infection sites. These findings align with earlier reports (25), suggesting hospital epidemiology driven by the spread of endemic MDR *E. faecalis* and *E. faecium*. Through horizontal gene transfer, these bacteria could increase their virulence and antibiotic resistance. Some amplicons in the RAPD-PCR patterns were present in all specimens, while others were absent (Table 3). The primer RAPD-2 produced seven amplicons in three isolates: two *E. faecalis* (one from the vagina and one from urine) and one *E. faecium* from diarrhea, achieving maximum amplification. Most polymorphisms and strain variability were observed in vaginal isolates (106 profiles by three primers), while CSF infection isolates had the lowest levels of variability (24 profiles by three primers).

The dendrogram revealed clusters, with strains and enterococci species clustering together, which could be from various hosts. Specific genotypes (genotype B in RAPD-2, genotype A in RAPD-4, and genotypes A and B in RAPD-640) displayed similar molecular and antibiotic resistance patterns and produced *ESβL*-genes in urine, vagina, diarrhea, and CSF isolates within each patient, highlighting that these are endogenous strains capable of causing endogenous infections in the same patient. Only *E. faecalis* isolates from the same sources were included in genotype A of RAPD-2, indicating that these isolates may derive from a single clone. The findings demonstrated that all enterococci isolates could be typed using RAPD-2, RAPD-4, and RAPD-640 primers in RAPD-PCR analysis. Our results confirmed that RAPD-PCR is an effective method for determining and assessing the genetic relationships among *Enterococcus* species, such as *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum*, isolated from different clinical samples, which is consistent with previous reports (26,27).

Conclusion

The results highlight the critical need for constantly updating the laboratory protocols for appropriate and up-to-date antibiotic susceptibility testing and enhanced infection control practices in health centers to address the growing threat of antibiotic resistance. In addition, this research offers valuable insights into resistance mechanisms in hospital settings and supports the development of strategies to manage and trace the spread of resistant bacteria in Iraq. The RAPD technique serves as a valuable tool for identifying genetic differences among *Enterococcus* species as well as for tracing the origins of infections and in forming treatment strategies.

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Competing Interests

The author does not have any conflict of interests.

Ethical Approval

The present study followed the guidelines of the Declaration of Helsinki (1975), revised in 2013, and all procedures performed in this study were in accordance with national and international guidelines. A written informed consent form was obtained from every patient before the study, and the patients were completely anonymized by the researchers. Ethical approval was not sought for the present study because of the nature of the study (in vitro only). The samples were obtained based on standard diagnostic and therapeutic protocols for managing gastrointestinal infections. Raid Razzaq Ojaimi ensure that this study is Health Insurance Portability and Accountability Act (1996) compliant. Raid Razzaq Ojaimi followed every mandatory (health and safety) procedure.

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