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Molecular Detection of *Fluoroquinolone resistance Genes* in *Enterococcus spp.* Isolated From Dental Root Canals Infections

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Abstract: *Enterococcus* is among the most prevalent bacterial species identified in teeth exhibiting pulp necrosis that have not undergone prior endodontic treatment. *Enterococcus* bacteria exhibits considerable resistance to numerous root canal disinfectants, often posing a treatment challenge. This study aims to isolate and identify *Enterococcus* species using both phenotypic and molecular methods. The phenotypic patterns were determined through biochemical techniques, followed by diagnosis based on genotypes utilizing specialized primers for the *16S rRNA* and D-Ala:D-Ala ligase genes via polymerase chain reaction. The evolution of resistance strains presents substantial challenges to infection treatment, necessitating the creation of novel antibiotics. To attain this objective, it is essential to investigate the primary genes implicated in antibiotic resistance. Fifty samples were obtained from individuals of different ages and sexes suffering from root canal infections between January 2025 and March 2025, and were subsequently grown in selective and differential media. Susceptibility tests indicated that 50% of the isolates exhibited resistance to *ciprofloxacin*, 62.5% to *levofloxacin*, and 62.5% to *nofloxacin*. The results revealed 8 (16%) isolates of *E. faecalis* and no isolates of *E. faecium*. The *16s rRNA*, *ddl E. faecalis*, *GyrA*, *ParC*, and *ParE* genes were detected with a frequency of 100%, while the *GyrB* gene was detected with a prevalence of 62.5%, and the *ddl faecium* gene was absent in all samples. The presence of resistance genes enables bacteria to evade antibiotic actions and persist in the root canal, resulting in the failure of endodontic treatment.

Keywords: *E.faecalis*, *E. faecium*, *GyrA*, *GyrB*, *ParC*, *ParE*, root canal, Endodontics.

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Introduction

Enterococcus faecalis is one of the main microorganisms that infects root canals, ranking among the most prevalent microorganisms associated with endodontic treatment failure. Given its pervasive presence in persistent endodontic infections, the successful elimination of *E. faecalis* is crucial for effective endodontic treatment and retreatment (1) (35). and this bacteria are accountable for a range of illnesses in vulnerable hosts, including as bacteremia, endocarditis, mouth infections, and urinary tract infections (*UTI*) (2). this bacterium possesses various virulence genes that enhance its pathogenicity, including those associated with biofilm production and resistance to antibiotics (3). *E. faecalis* can survive in dentinal tubules post-treatment, with studies showing comparable survival rates

against different sealing materials (4). Oral infections are typically caused by *enterococci*, which are most common when they are related to periodontitis and necrotic pulp infections in dental root canals (5). *Enterococcus* species represented the genus found in dental root canals most frequently, according to reports (6). The presence of *E. faecalis* necessitates the development of more effective disinfection strategies, such as photodynamic therapy combined with laser treatment, which has shown promise in reducing bacterial counts (7). Achieving periapical healing during endodontic therapy involves limiting intracanal infection and preventing reinfection. A number of factors, including the bacterial persistence, improperly cleaned and obturated root canals, incorrect coronal seal (leakage), and untreated (missing) canals, can lead to endodontic treatment failure. *Enterococcus faecalis* and other bacterial species present in the root canal system are the primary cause of endodontic failure. These bacteria can cause a prolonged intra- or extra-radicular infection because they are more resistant to disinfectants (8). *E. faecalis* is linked to delayed healing and persistent inflammation in refractory apical periodontitis, highlighting the need for targeted therapeutic approaches (9). Accordingly, eliminating *E. faecalis* from the root canal system is crucial to improving the outcome of endodontic therapy (10).

Material and methods

Sample collection

Between January , 2025, and March 2025, a total of 50 root canal samples were collected from a specialized dental health center in Babel. These samples were obtained from patients of various ages and sexes who were experiencing root canal infections while receiving treatment through an open root canal procedure. The samples were collected by utilizing cotton to absorb the pus from within the root canal, subsequently transporting them to the laboratory using transport tube media, and then cultivating them in selective and differential media.

Isolation and Identification of *Enterococcus* bacteria

All samples were cultured in brain heart infusion broth, blood agar and bile esculin agar, and incubated for 24 hours at 37 °C. Biochemical assays utilizing the VITEK-2 system, together with colony morphology regarding size, shape, and color, were employed to identify positive growth. Positive growth in prior tests was validated at the molecular level by standard polymerase chain reaction.

Antibiotic susceptibility testing

A limited quantity of similar bacterial colonies was extracted from a new agar plate culture after 24 hours and suspended until the turbidity attained 0.5 McFarland, equating to 1.5×10^8 CFU/ml. Bacteria are inoculated into a Mueller-Hinton agar (MHA) plate, then antibiotic disks are introduced using sterile forceps. The resultant zones of inhibition were quantified using a ruler and juxtaposed with standard zones of inhibition established by the Clinical and Laboratory Standards Institute (CLSI). In this study utilized antibiotics from the *Fluoroquinolone* class.

Molecular methods DNA extraction

The DNA was extracted from *Enterococcus* isolates utilizing the Genomic DNA Extraction Mini Kit (Favorgen®, Korea). Utilize gel electrophoresis to assess the quality of DNA.

Molecular Method for Detection of *E. faecalis* and *E. faecium*

The polymerase chain reaction technique was employed to diagnose

and identify enterococcal bacteria and certain virulence genes utilizing specific primers prepared in accordance with the manufacturer's instructions. These primers contain specific sequences found in the bacterial genetic material. The primers were utilized for the detection of the following genes: *16srRNA, ddl E. faecalis, ddl E. faecium, GyrA, GyrB, ParC,* and *ParE* as detailed in Table (1).

Table (1): Sequences of primers and the sizes of products for the genes utilized in the study

Target gene	Nucleotide sequence (5'—3')	Product Size/ bp	Reference
16SrRNA	F: GGATTAGATACCCTGGTAGTCC	320	(11)
	R: TCGTTGCGGGACTTAACCCAAC		
ddl E.faecalis	F:ATCAAGTACAGTTAGTCTTTATTAG	941	(12)
	R:ACGATTCAAAGCTAACTGAATCAGT		
ddl E.faecium	F: TTGAGGCAGACCAGATTGACG	658	
	R: TATGACAGCGACTCCGATTCC		
GyrA	F: GCAATGAGTGTTATCGTCG	575	(13)
	R: TCTGGTCCAGGTAACACTTCC		
GyrB	F: TGAAATTCTTGCTGGAAAAC	492	
	R: CAACAATAGGACGCATGTAAC		
ParC	F: AATGAATAAAGATGGCAATA	191	
	R: CGCCATCCATACTTCCGTTG		
ParE	F: GGAAAATTAACACCGGCTCA	388	
	R: AAAGTGGTGGTAAGGCAATG		

The optimization of the PCR reaction component for the amplification of the (*16srRNA, ddl E. faecalis, ddl E. faecium, GyrA, GyrB, ParC,* and *parE*) genes was achieved after multiple trials; thus, the following mixtures were utilized to identify these genes in the *Enterococcus* isolate, as presented in Table (2).

Table (2): PCR Reaction Component to detect the genes of *E. faecalis* and *E. faecium*

Component	25µL (Final volume)
Masret mix	12.5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl
Distill water	9µl

Detection of *16srRNA, ddl E. faecalis* and *ddl E. faecium* genes by conventional PCR

The PCR program for amplifying the (*16srRNA, ddl E. faecalis* and *ddl E. faecium*) genes was optimized after several trials; thus, the following program were implemented for *Enterococcus* isolates, as detailed in Tables (3) and (4).

Table (3): The PCR Program to detect *ddl gene* for *E.faecalis* and *E.faecium*

No.	Step	Tm (C°)	Time	No. of cycle
1-	Initial Denaturation	94 C°	5min	1 cycle
2-	Final Denaturation	94 C°	1min	30 cycle
3-	Annealing	54 C°	45 Sec	
4-	Extension	72 C°	1min	
5-	Final Extension	72 C°	5 min	1 cycle

Table (4): The PCR program to identify the *16srRNA* gene amplification by conventional PCR.

No.	Step	Tm (C°)	Time	No. of cycle
1-	Initial Denaturation	94 C°	5min	1 cycle
2-	Final Denaturation	94 C°	1min	30 cycle
3-	Annealing	54 C°	1min	
4-	Extension	72 C°	1min	
5-	Final Extension	72 C°	5 min	1 cycle

Detection of *Fluoroquinolone* Resistance Genes *(GyrA, GyrB, ParC, and ParE*) by conventional PCR

The PCR program for amplifying the (*GyrA, GyrB, ParC and ParE,*) genes was optimized after several trials; thus, the following program were implemented *for Enterococcus* isolates, as detailed in Tables (5), (6), (7) and (8).

Table (5): The PCR program to identify the *GyrA* gene amplification by conventional PCR.

No.	Step	Tm (C°)	Time	No. of cycle
1-	Initial Denaturation	95 C°	1min	1 cycle
2-	Final Denaturation	95 C°	30sec	36 cycle
3-	Annealing	43 C°	30sec	
4-	Extension	72 C°	2min	
5-	Final Extension	72 C°	10min	1 cycle

Table (6): The PCR program to identify the *GyrB* gene amplification by conventional PCR.

No.	Step	Tm (C°)	Time	No. of cycle
1-	Initial Denaturation	95 C°	1min	1 cycle
2-	Final Denaturation	95 C°	30sec	36 cycle
3-	Annealing	48 C°	30sec	
4-	Extension	72 C°	2min	
5-	Final Extension	72 C°	10min	1 cycle

Table (7): The PCR program to identify the *ParC* gene amplification by conventional PCR.

No.	Step	Tm (C°)	Time	No. of cycle
1-	Initial Denaturation	95 C°	2min	1 cycle
2-	Final Denaturation	95 C°	1min	36 cycle
3-	Annealing	57 C°	1min	
4-	Extension	72 C°	2min	
5-	Final Extension	72 C°	10min	1 cycle

Table (8): The PCR program to identify the *ParC* gene amplification by conventional PCR.

No.	*Step*	*Tm (C°)*	*Time*	*No. of cycle*
1-	*Initial Denaturation*	*95 C°*	*2min*	*1 cycle*
2-	*Final Denaturation*	*95 C°*	*1min*	*36 cycle*
3-	*Annealing*	*51 C°*	*1min*	
4-	*Extension*	*72 C°*	*2min*	
5-	*Final Extension*	*72 C°*	*10min*	*1 cycle*

Results and Disscussion

Isolation and identification of *Enterococcus*

The obtained bacteria were identified as Gram-positive cocci, exhibiting spherical or oval shapes, and can be found singly, in pairs, or in short chains. *Enterococcus* species was isolated according to the cultural properties of the colonies, encompassing their shape, size, color, and texture. Colonies of

Enterococcus manifested on bile esculin agar as diminutive, transparent colonies with brown-black halos, resulting in the medium's coloration changing to black. Bile Esculin Agar is mostly utilized to differentiate *Enterococcus* from *Streptococcus*. Members of the *Enterococcus* genus can flourish in 40% bile (oxgall) and hydrolyze esculin into glucose and esculetin. Esculetin reacts with ferric ions to create a black complex. demonstrating the existence of bacterial proliferation in the media, as illustrated in figure (1).

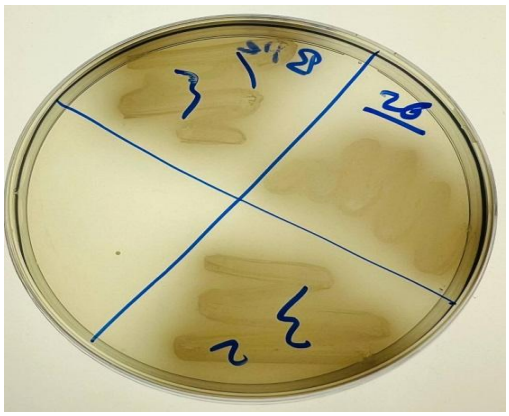

Figure: (1) *Enterococcus* colonies on bile esculin agar After 24 hours of incubation at 37°C, colonies with brown-black halos emerge on bile esculin agar.

Biochemical tests were performed on isolates cultivated on selective media using the VITEK-2 system to identify *Enterococcus* at the species level, thereby excluding other bacterial species with similar features. *Enterococci* are differentiated from other streptococci of antigenic group D by certain biochemical reactions, which are considered the diagnostic criterion for *Enterococci* in these contexts. *Enterococci* in such circumstances.

Antibiotic susceptibility testing

The findings of antibiotic resistance indicated that 50% of the isolates exhibited resistance to *Ciprofloxacin*. This outcome closely resembles the findings of Malek (21), who reported a Ciprofloxacin resistance rate of 54.65%. The findings were nearly similar with those of Al-Tarfi and Hussein (22), whose reported a Ciprofloxacin resistance rate of 60%. It contrasted with Mulkhan's findings (23), which indicated a resistance rate of 4%, and Troshianchik's results (24), which documented a resistance rate of 100%. On the other hand, the resistance rate to Levofloxacin in this study was 62.5%, which closely aligns with Khalil's findings(25) of 77.27% resistance, but diverges with Bokila's report (26) of 34.9% resistance to this antibiotic. This study documented a norfloxacin resistance rate of 62.5%. This outcome was near to Kraszewska's findings (27), which indicated a resistance rate of 51.4%, but was inconsistent with Samani's results (28), which documented a resistance rate of up to 30% for this antibiotic.

Molecular methods for identification of *Enterococcus* isolate.

The findings of this study revealed that all *Enterococcus* isolates (100%) possessed *16srRNA* and *ddl faecalis* gens, whereas *ddl faecium* gene was absent in all samples, indicating the nonexistence of the *E. faecium* genus in the tested samples. These results were completely consistent

with the results mentioned by Dhahir (14), who confirmed the absence of *E. faecium* in all dental root canal samples. Conversely, these results differed from the results of Mustafa et al. (34), which indicated the diagnosis of *E. faecium* bacteria in 68% of dental samples. The product sizes of *16srRNA* gene is 320 bp and *ddl faecalis* gene is 941 bp (Figures 2 and 3)

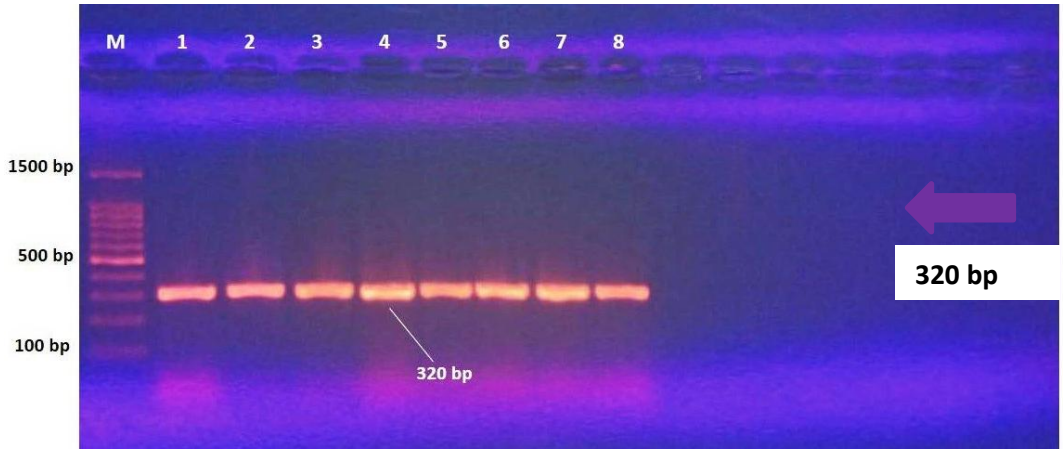

Figure (2): Gel electrophoresis of the *16S rRNA* gene of *Enterococcus* isolates was conducted using 1.5% agarose gel at 70 volts for 2 hours. M: 100 bp ladder marker; lanes 1-8 correspond to PCR products.

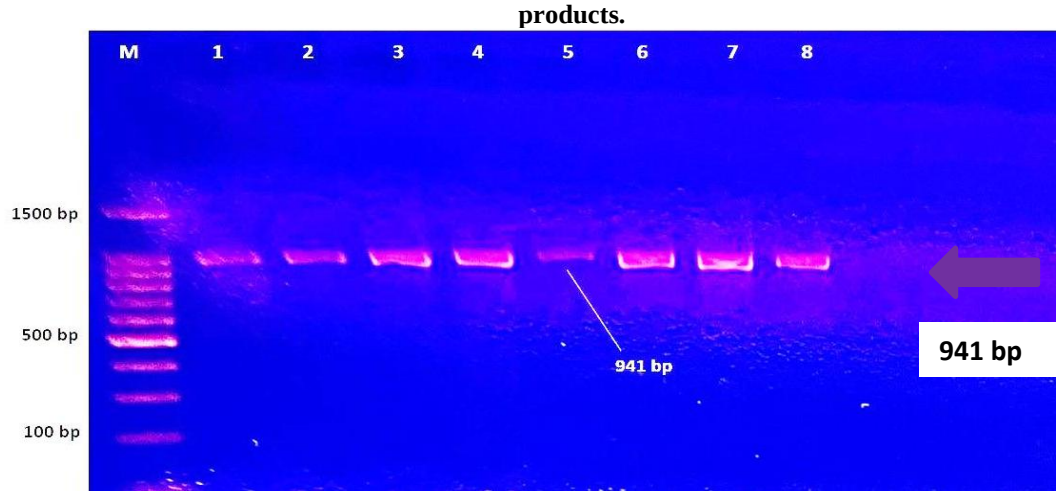

Figure (3): Gel electrophoresis of the *ddl* gene of *E*. faecalis isolates was conducted using 1.5% agarose gel at 70 volts for 2 hours. M: 100 bp ladder marker; lanes 1-8 correspond to PCR products.

The current study revealed an overall incidence of *E. faecalis* at 16%, a finding that aligns closely with Dhahir's research (14), which reported a prevalence this bacteria in reat16.6% in root canals, and Kadhem's study(15), which indicated a prevalence rate of 14%. Additionally, Mahmoudpour et al.(16) identified a 10% isolation rate of *E. faecalis* from root canals. One reason for the existence of these bacteria is their capacity to endure environmental extremes like pH and elevated salinity. While appropriate canal instrumentation and sufficient irrigation with sodium hypochlorite can reduce bacterial counts, they cannot completely eradicate *E.faecalis* from the root canal(17). All isolates were discovered to possess the *ddl* and *16SrRNA* genes, with a documented prevalence of 100% for each gene. The

current findings align with those of Alwan and Hussein (18), who employed *16SrRNA* for the detection of *E. faecalis*, and are also comparable to the results published by Al-Halaby and colleagues (19), who utilized the D-Ala:D-Ala ligase gene *(ddl faecalis*) for the molecular identification of this bacterium. D-alanine: D-alanine ligase (*ddl*) is an essential protein for the proper growth and development of bacterial cell walls. Moreover, it serves as a significant objective for the synthesis of antibacterial pharmaceutical agents (20).

Molecular Detection of *Fluoroquinolone Resistance Genes (GyrA, GyrB, ParC and ParE*)

The findings of this study revealed that all *E. faecalis* isolates (100%) possessed *GyrA*, *ParC*, and *ParE* gens whereas the *GyrB* gene was present in (62.5%) of the isolates, The product sizes for these genes were 575 bp, 191 bp, 388 bp, and 492 bp, respectively (Figures 4, 5, 6 and 7).

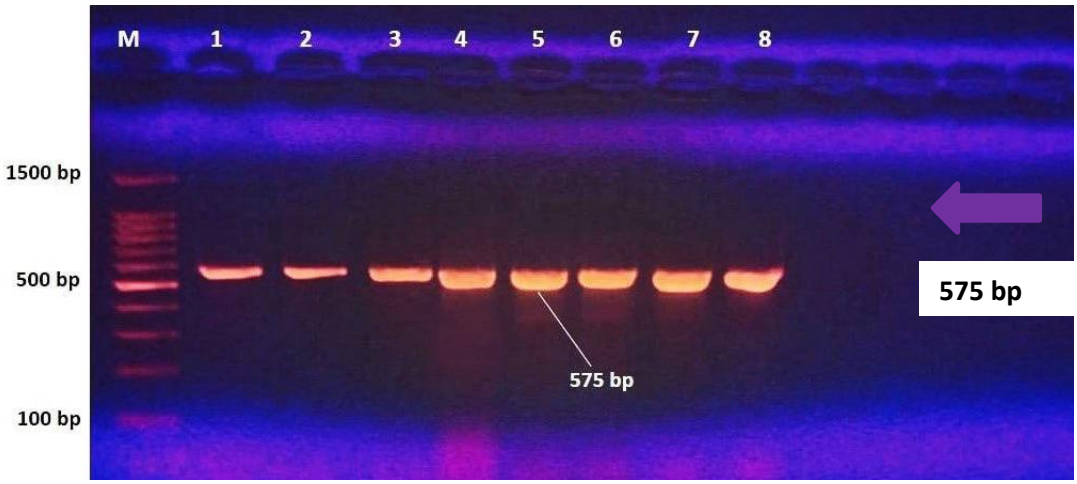

Figure (4): Gel electrophoresis of the *GyrA* gene of *Enterococcus* isolates was conducted using 1.5% agarose gel at 70 volts for 2 hours. M: 100 bp ladder marker; lanes 1-8 correspond to PCR products.

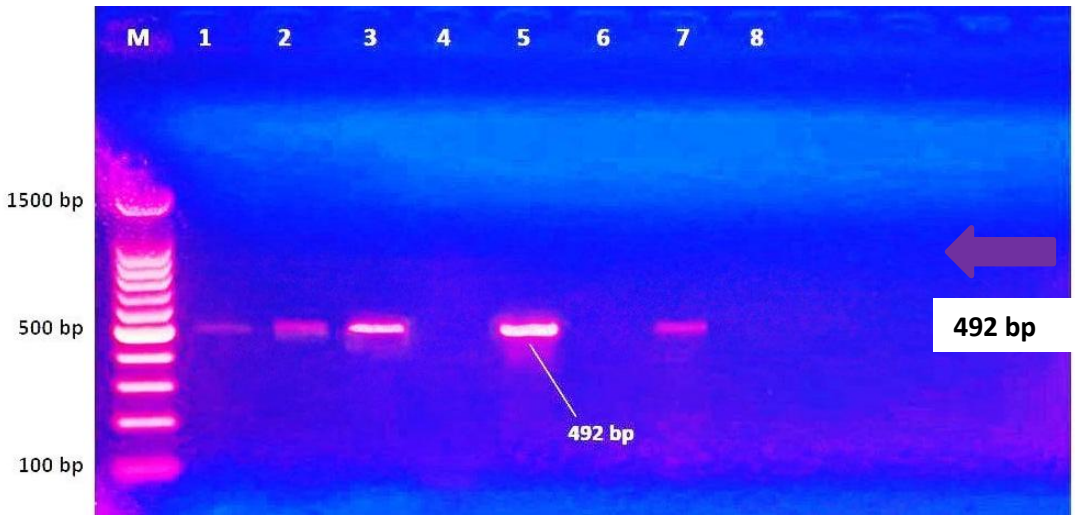

Figure (5): Gel electrophoresis of the *GyrB* gene of *Enterococcus* isolates was conducted using 1.5% agarose gel at 70 volts for 2 hours. M: 100 bp ladder marker; lanes (1,2,3,5,7): positive amplification and lane (4,6,8) negative amplification of *GyrB* gene in *E.Faecalis*.

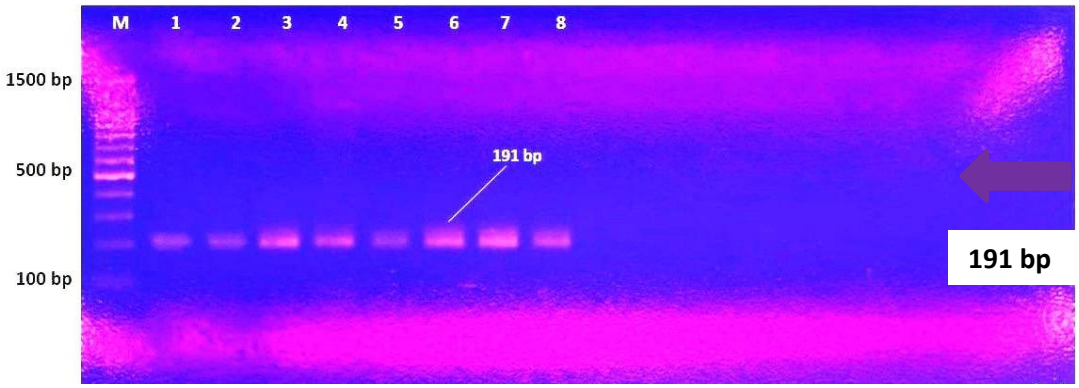

Figure (6): Gel electrophoresis of the *ParC* gene of *Enterococcus* isolates was conducted using 1.5% agarose gel at 70 volts for 2 hours. M: 100 bp ladder marker; lanes 1-8 correspond to PCR products.

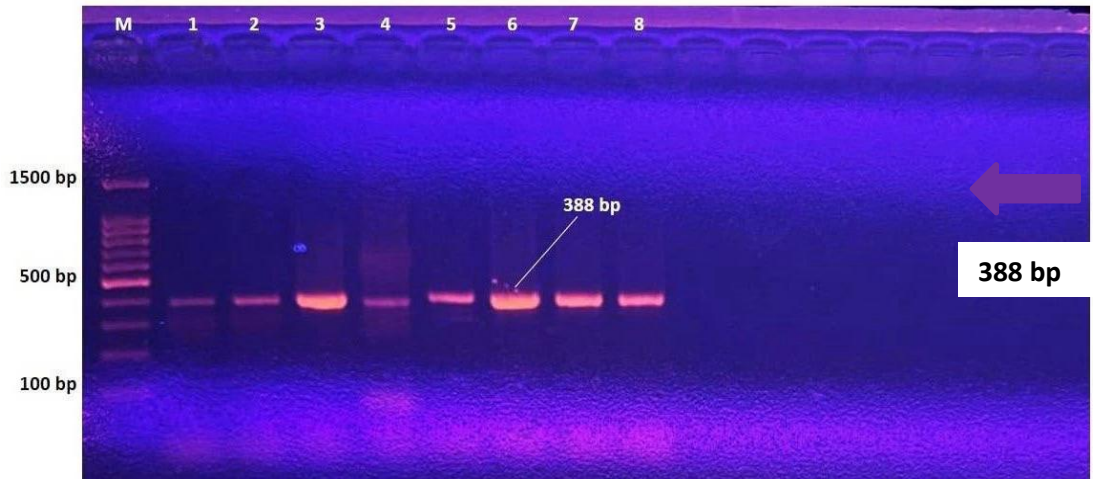

Figure (7): Gel electrophoresis of the *ParE* gene of *Enterococcus* isolates was conducted using 1.5% agarose gel at 70 volts for 2 hours. M: 100 bp ladder marker; lanes 1-8 correspond to PCR products.

The genes *GyrA*, *GyrB*, *ParC*, and *ParE* are crucial for bacterial DNA replication and play a significant role in *fluoroquinolone* resistance. These genes encode constituents of *DNA gyrase* and *topoisomerase IV*, which are crucial for preserving DNA architecture during replication and transcription. Alterations in these genes may result in resistance to *fluoroquinolones*, a category of antibiotics commonly employed to treat bacterial infections (29). The current study's results indicate that all *E. faecalis* isolates expressed the genes *(GyrA, ParC, and ParE)* at a rate of 100%. The findings align perfectly with Su's (30) results for the *GyrA* gene, which indicated a prevalence of 100% for this gene. This findings are closly to those of Al-Yassery (31), who identified this gene at a frequency of 94.1%. Nonetheless, it disagreed with the findings of Al-Tarfi and Hussein (22), who reported the existence of this gene at a frequency of 55%. The findings regarding the *ParC* gene in our study were entirely congruent with those of Su (30), who verified the presence of this gene in all *Enterococcus* isolates. This finding closely aligned with those of Alipoor and colleagues (23), who reported a gene presence rate of 88.4%, in contrast to Khosravi's (13) findings of 15.8%.

The findings regarding the *ParE* gene were entirely congruent with Khosravi's results(13), which indicated a 100% prevalence of this gene in all diagnosed isolates. Conversely, not all *Enterococcus* isolates possess the *GyrB* gene. The lack of the *GyrB* gene in certain *Enterococcus* isolates is attributable to evolutionary and functional factors associated with the traits of these bacteria. This gene encodes the B component of *DNA gyrase*. In certain Gram-positive bacteria, including *Enterococcus*, *DNA gyrase* is substituted by an enzyme known as *topoisomerase IV*, which is encoded by the *ParC* and *ParE* genes, to execute analogous activities. The absence of *GyrB* is thought to be associated with the evolutionary specialization of these bacteria, as *topoisomerase IV* became enough for their genetic requirements (33). The prevalence of the *GyrB* gene in this study was 66.2%, surpassing the findings of Khosravi (13), who reported a prevalence of 40.6%.

Conclusion

The antibiotic resistance exhibited by *E. faecalis*, stemming from modifications in essential genes such as *GyrA*, *GyrB*, *ParC*, and *ParE*, along with its resilience in various environments, plays a significant role in the colonization and infection of the root canal, ultimately leading to the unsuccessful outcomes of endodontic treatment.

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