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Molecular Detection of *Esp* and *Ace* Genes in *Enterococcus faecalis* Isolated from Endodontic Infections

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Abstract: *Enterococcus faecalis* is a natural inhabitant of the human gastrointestinal tract but can become dominant and cause infections when the intestinal homeostasis is disrupted. *Enterococcal* bacteria are considered one of the main reasons for the failure of endodontic treatment. This study aim to isolation and identification of *E.faecalis* depended on phenotype and molecular method, the phenotypic patterns using traditional biochemical methods, and then diagnosed it based on the genotypes and using specialized primers for *16srRNA* and *D-Ala: D-Ala ligase* genes using polymerase chain reaction, In order to achieve successful treatment, it is necessary to study the bacterial behavior within the root canal system together with their resistance and defensive systems in an endeavor to accomplish the eradication of microorganisms and better control their engagement with the tooth, sixty samples taken from patients of different ages suffering from dental root canal infections from the beginning of December 2023 to the end of January 2024. Then it is grown in selective and differential media, The result showed 10 (16.6%) isolates belonging to *E. faecalis*, the gene of *16srRNA*, *ddl E.faecalis*, *Ace* and *Esp* detected in all isolates of *E.faecalis* with percentage recorded 100% for all genes, the presence of *Esp* and *Ace* genes and resistance to harsh environmental conditions contributed to the virulence and proliferation of *E. faecalis* bacteria in the tooth root canal in addition to the failure of endodontic treatment.

Keywords: *E. faecalis*, *Esp*, *Ace*, root canal, Endodontics.

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Introduction

A variety of systemic and oral illnesses can be brought on by *enterococci*, which are gram-positive, facultative anaerobic bacteria that are thought to be transient components of the oral microbiome (1). 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). It also has the ability to produce some virulence factors (3). 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 (4). One of the aims of root canal treatment is to eliminate the bacteria, their products and substance from the root canal system. Mechanical debridment of the root canals plays a fundamental role in a chieving endodontic success. In spite of all attempts, attaining complete sterility of the canal system is still extremely difficult (5). *Enterococcus species*

represented the genus found in dental root canals most frequently, according to reports (6). 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 (7). Bacterial contamination can be removed partially by mechanical instruments and cleaning, but not completely (8). More endodontic treatment-resistant bacteria, like *E. faecalis*, are typically present and lower the therapy's effectiveness rate. Several studies have shown that the microbiota present in the root canals of endodontically treated teeth differs from the microbiota typically found in untreated teeth. These microorganisms may have entered the root canals through crown filtration in teeth with filled roots, or they may have survived biochemical procedures (9). Accordingly, eliminating *E. faecalis* from the root canal system is crucial to improving the outcome of endodontic therapy (10).

Materials and methods

Sample collection

During the period from first of December 2023 to the end of January 2024, 60 root canal sample were collected from the specialized health dental center in Al Mahmoudiya and private dental clinics from patients of different ages and of both sexes suffering from root canal infection while undergoing a root canal open for the purpose of treatment. The samples were obtained by taking the fine paper or cotton used to dry the pus inside the root canal, transporting it to the laboratory by transport tube media, and then cultivating it in selective and differential media.

Isolation and Identification of *E. faecalis*

All specimens were cultured on brain heart infusion broth, bile esculin agar base, and chromogenic agar and incubated for 24 hours at 37 °C. Biochemical assays and colony morphology the size, shape, and color of the colonies were used to identify the positive growth.and also catalase test, analysis of growth in the presence of 6.5% sodium chloride and growth examination at 10°C, 45°C , the VITEK-2 system, and Positive growth in previous tests was confirmed at the molecular level using conventional polymerase chain reaction.

Molecular methods

DNA extraction

The DNA was obtained from *E. faecalis* isolates using the Genomic DNA Extraction Mini Kit (Favorgen®, Korea). and use gel electrophoresis to analyse DNA quality.

Diagnosis of *E.faecalis* using PCR technology

The polymerase chain reaction technique was used to diagnose and identify *enterococcal* bacteria and some virulence genes using specific primers prepared according to the manufacturer's instructions, the composition of which includes specific sequences present in the bacterial genetic material, the primer were used for detection of (*16srRNA, ddl E.faecalis, Esp, Ace*) genes were described in Table (1).

Table (1): Primer sequences and product size of the genes used in the study

Target gene	sequence of primer (5'—3')	Product Size/ bp	Reference
16SrRNA	F: GGATTAGATACCCTGGTAGTCC	**320**	(11)
	R: TCGTTGCGGGACTTAACCCAAC		
ddl E.faecalis	F:ATCAAGTACAGTTAGTCTTTATTAG	**941**	(12)
	R:ACGATTCAAAGCTAACTGAATCAGT		
ESP	F:AGATTTCATCTTTGATTCTTGG	**510**	(13)
	R:AATTGATTCTTTAGCATCTGG		
Ace	F: CAGGCCAACATCAAGCAACA	**125**	(14)
	R: GCTTGCCTCGCCTTCTACAA		

Optimization of PCR master mix for amplification of (*16srRNA, ddl E.faecalis, Esp, Ace*) gene was accomplished after several trails; thus, the following mixtures were adopted for detect this genes of *E.faecalis* isolate as in Table (2).

Table (2): PCR master mix to detect the genes of *E.faecalis* isolates.

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 *ddl E.faecalis, 16srRNA, Esp, Ace* genes by conventional PCR

Optimization of PCR program for amplification of (*16srRNA, ddl E.faecalis, Esp, Ace*) gene was accomplished after several trails; thus, the following program were adopted for *E.faecalis* isolates, as in Table (3), (4), and (5).

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

No.	Phase	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): PCR program to identify the *16srRNA* gene amplification by conventional PCR.

No.	Phase	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

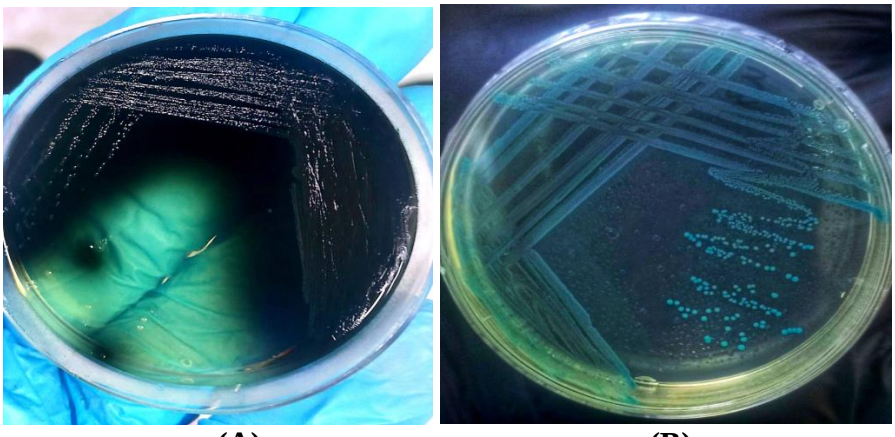
Table (5): PCR program to identify the *Ace* and *Esp* genes amplification by conventional PCR.

No.	Phase	Tm (C°)	Time	No. of cycle
1-	Initial Denaturation	95 C°	5min	1 cycle
2-	Final Denaturation	94 C°	1min	30 cycle
3-	Annealing	56 C°	1min	
4-	Extension	72 C°	1min	
5-	Final Extension	72 C°	10min	1 cycle

Results and discussion

Isolation and identification of *E.faecalis*

The isolated bacteria were determined to be Gram-positive cocci, which had spherical or oval forms and may be observed alone, in pairs, or in brief chains. *Enterococcus faecalis* was isolated based on the cultural characteristics of the colonies, including the shape, size, color, and texture of the colonies. Colonies of *E. faecalis* appeared on bile esculin agar in the form of small, transparent colonies with brown-black halos, and the color of the medium turned black, Bile Esculin Agar is mostly used to distinguish *Enterococcus* from *Streptococcus*. Members of the *Enterococcus* genus may thrive in 40% bile (oxgall) and hydrolyze esculin into glucose and esculetin. Esculetin interacts with ferric ions to form a black complex. indicating the presence of bacterial growth in the medium figure (1A). and On chromogenic agar medium figure (1B), small-sized bacterial colonies with a transparent blue color appeared depending on the chromogenic substrates present in the medium, where one of the chromogenic substrates is cleaved by the *β-glucosida*se that it possesses *Enterococci*, leading to the formation of blue colonies biochemical analyses were conducted on isolates grown on selective media to diagnose *enterococci* at the species level, in order to exclude other bacterial species that share some characteristics.



(A) **(B)**

Figure: (1A) Colonies of *E.faecalis* on bile esculin agar after 24 hours of incubation at 37°C appear colonies with brown-black halos on bile esculin agar . (1B) Colonies of *E.faecalis* on chromogenic agar after 24 hours of incubation at 37°C appear blue color on chromogenic agar.

As a result of these tests, the production of the catalase enzyme, which releases oxygen gas from hydrogen peroxide in the form of gas bubbles, was absent in ten isolates. Nonetheless, the capacity to thrive at temperatures between 10 C°and 45C° was demonstrated by these 10 isolates. It was also able to grow in a medium with a basic pH of up to 9.6 and in a liquid medium with a salt concentration of 6.5% sodium chloride. *Enterococci* are distinguished from other *streptococci* of antigenic group D by these biochemical responses, which are thought to constitute the diagnostic key for *enterococci* in such circumstances.

Molecular methods for identification of *Enterococcus faecalis* isolate.

The results of this investigation showed that all *E. faecalis* isolates (100%) had the *ddl*, *16srRNA*, *Esp*, and *Ace* genes, with respective product sizes of 941bp, 320bp, 510bp, and 125bp respectivelly (Figures 2, 3, 4, 5).

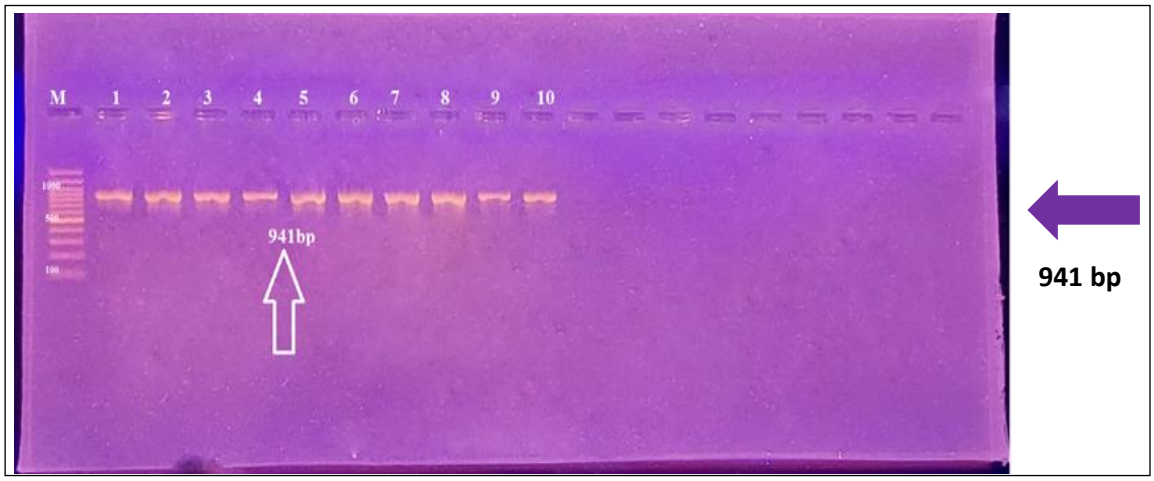


Figure (2): Gel electrophoresis of *ddl gene* of *E.faecalis* isolates using 1.5% agarose gel electrophoresis (70 volt for 2 hours). M :100 pb ladder marker, lane 1-10 resemble PCR products.

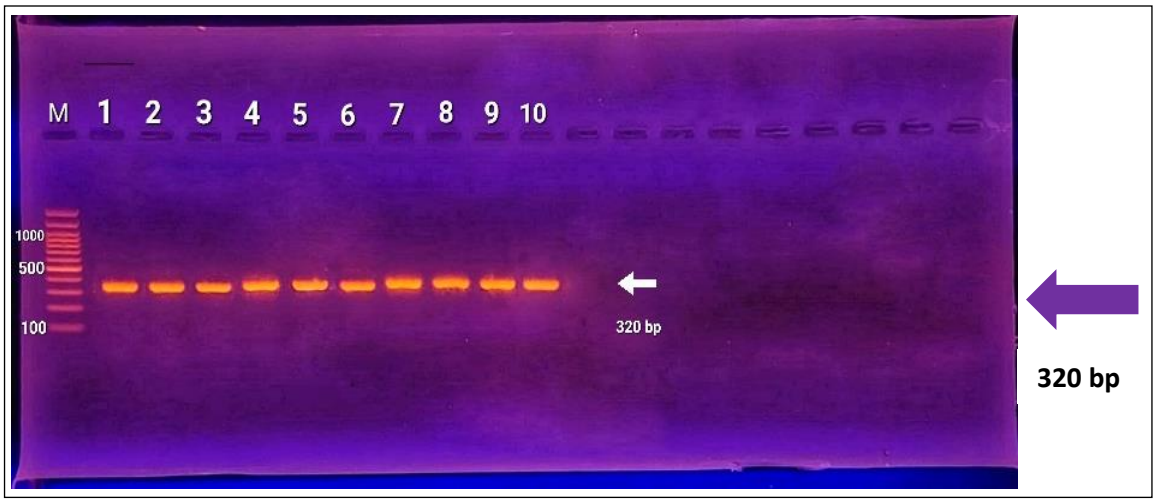


Figure (3): Gel electrophoresis of *16srRNA* gene of *E.faecalis* isolates using 1.5% agarose gel electrophoresis (70 volt for 2 hours). M :100 pb ladder marker, lane 1-10 resemble PCR products.

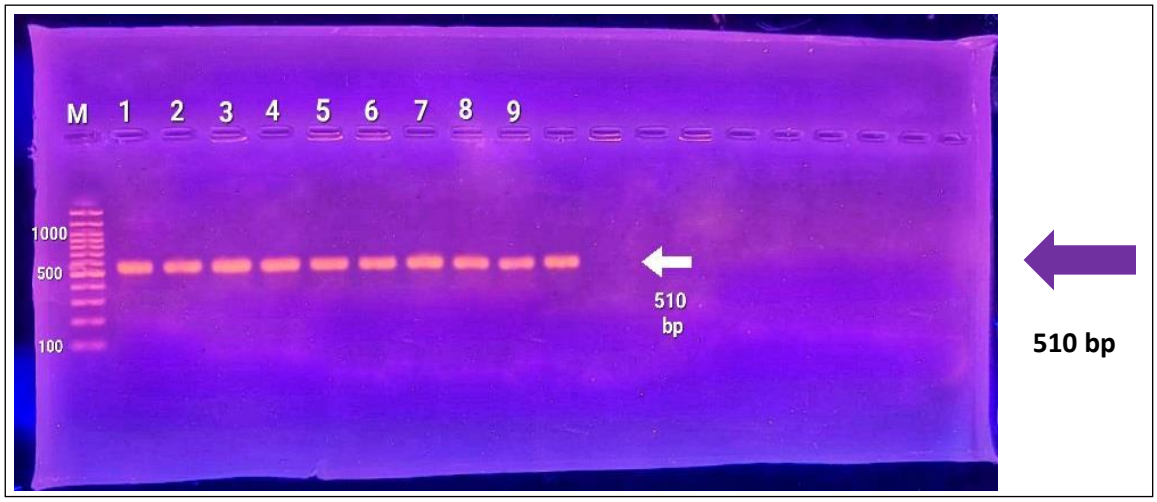


Figure (4): Gel electrophoresis of *Esp* gene of *E.faecalis* isolates using 1.5% agarose gel electrophoresis (70 volt for 2 hours). M :100 pb ladder marker, lane 1-10 resemble PCR products.

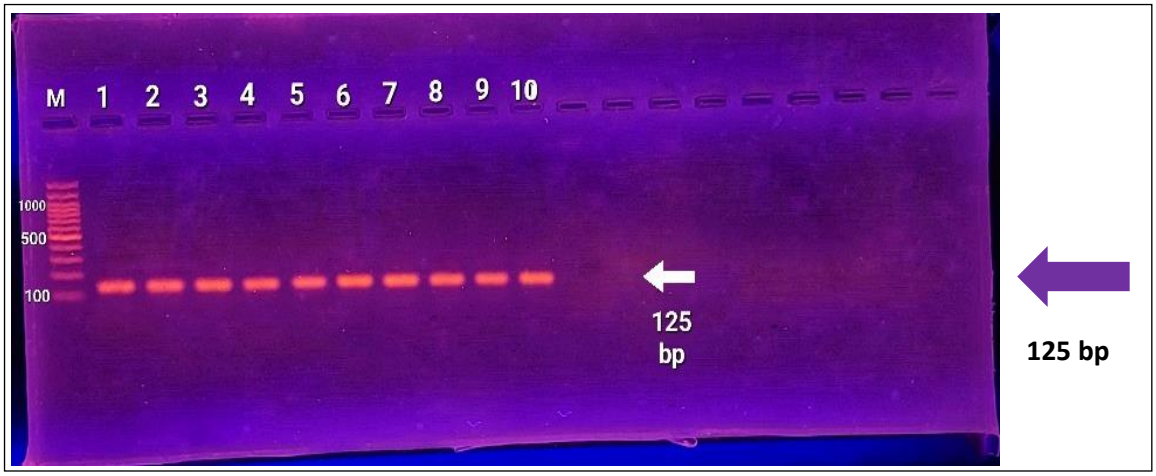


Figure (5): Gel electrophoresis of *Ace* gene of *E.faecalis* isolates using 1.5% agarose gel electrophoresis (70 volt for 2 hours). M :100 pb ladder marker, lane 1-10 resemble PCR products.

In the present study, the overall incidence percentage of *E.faecalis* were 10 (16.6 %) and this result was close to that report conduceted by Kadhem's research (15) who reported the percentage of *E.faecalis* was 14%, Mahmoudpour *et al*.(16) found 10 % of *E.faecalis* isolate from root canal, Among the reasons that may explain the presence of these bacteria is their ability to withstand environmental conditions such as pH and high salinity, and the bacteria possess virulence factors that enable them to form biofilm. Although proper canal instrumentation and adequate irrigation with sodium hypochlorite can decrease the number of bacteria, it cannot remove *Enterococcus faecalis* from the root canal entirely (17). While, Preethee *et al*. (18) reported 46.87 % of *E.faecalis* was isolated from root canal. In total, all the isolates were found to carry (*ddl*, *16srRNA*, *Esp* and *Ace*) genes with percentage recorded (100 %) for each one. The present results are similar to those of Alwan and Hussein (19), who used *16SrRNA* for detection *E.faecalis* and also similar to those reported by Al-Halaby and his colleagues (20)

who used *D-Ala:D-Ala ligase* gene (*ddl faecalis*) for Molecular detection of this bacteria. A necessary protein for the healthy growth and development of bacterial cell walls is *D-alanine: D-alanine ligase* (*ddl*). Additionally, it is an important target for the synthesis of antibacterial medicinal compounds(21).

An agent that forms biofilms in *E. faecalis* is the high-molecular-weight surface protein known as *enterococcal surface protein* (*Esp*) (22). and *Esp* binds to epithelial cells and is encoded on a pathogenicity island in *E.faecalis* and *E.faecium* (23). According to the study's findings, every *E.faecalis* isolate produced an *esp* gene (10 isolate) 100% that was perfectly compatible with study of Kadhem (15) and also similar to the results of the study of Karim and Abdullah (24), However, the results of this investigation were not the same as the outcomes obtained by Akhondnezhad *et al*. (25) who showed that isolates (18.6%) have this gene. The results of molecular detection for *Ace* gene by conventional PCR were fairly close to the results of the research Francisco *et al.* (26) as it was found that the percentage of the presence of the *Ace* gene in the isolates reached 68%, while the results of the research under study showed the percentage of the presence of this gene in the isolates amounted to (10 isolate) 100 % , and The results were also similar to the study Zoletti *et al.* (27) which showed that the percentage of presence of the gene reached 90%. The results are completely identical to the study Salah *et al.* (28) as the percentage of presence of the gene *Ace* in the study isolates is 100%. Attachment of *E.faecalis* isolates to extracellular proteins, including laminin and collagen I and IV , The *Ace* gene is involved in binding to type I collagen and its encoded protein possesses characteristics of bacterial adhesion. Since endodontic infections and recurring root canal infections have long been linked to *E. faecalis* Illnesses (29).

Conclusion

The possession of *E.faecalis* for various virulence factors, including *Esp*, *Ace* and their resistant nature to difficult environmental conditions contributes to the colonization and infection of *E.faecalis* in the tooth root canal, as well as the failure of endodontic treatment.

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