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Isolation and molecular detection of *Enterococcus faecium* from infected root canals

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Abstract:

Background and Objectives: *Enterococcus* is a lactic acid bacterium that, according to the Lancefield method of serology-based classification, was formerly classified as a group D *Streptococcus* until 1984. The current study aimed to isolate and molecularly detect *E. faecium* (*EF*) from the infected root canal, and to assess the antimicrobial susceptibility of the isolates.

Material and Methods: Samples were collected from 50 people who had a single root canal tooth with radiographic evidence of apical periodontitis. The transport media were preincubated at 37°C for 30 minutes and vigorously agitated for 60s before use and were then cultured on primary media. Aerotolerance, Gram-staining, synthesis of catalase, trypsin, and indole were used in the preliminary characterization stage. For species-level identification after preservation at 37°C, a variety of biochemical tests were used, including motility tests, carbohydrate fermentation, and the detection of enzymes. DNA extraction was achieved using the Presto™ Mini gDNA bacteria kit. Amplification of *ddl* chromosomal genes from *EF* was accomplished using DNA extracted from these isolates. Antimicrobial sensitivity tests were conducted to investigate the sensitivity and resistance of the target bacteria against 7 antimicrobial agents. **Results:** The analysis of data revealed that the prevalence of *EF* was 70% (in 35 of the samples collected). Seventy-four percent of the *Enterococcus* isolates tested positive for *EF* according to PCR

findings. The antibiotic sensitivity test results showed that vancomycin and erythromycin resistance was observed in all samples of *EF*. As many as 94.3% of isolates were resistant to amikacin, 88.7% were resistant to gentamycin, and 91.4 % were resistant to azithromycin. Doxycycline (94.3%) and nitrofurantoin (97.1%) were more effective against the *E. faecium* isolated from the root canals**.**

Conclusions: The study concludes that the *EF* bacteria is an important pathogen of root canal infection which is resistant to many antibiotics.

Keywords: antibiotics, *Enterococcus faecium***,** root canals

Abbreviations:

AS – aggregation substance
BHI – brain heart infusion
Ebp – biofilm-associated Pili
EF – *Enterococcus faecium*
GelE – gelatinase
PCR – polymerase chain reaction
VBNC – viable but non-cultivable

INTRODUCTION

Enterococcus is a lactic acid bacterium that was formerly classified as a group D *Streptococcus* until 1984 [1,2]. Because it was often found in fecal waste or sewage, *EF* has been referred to as the "Streptococcus of fecal origin" from its initial description in 1906 [3]. Eighty years after that, the genus *Enterococcus* could ultimately be defined and recognized thanks to improved molecular methods and genetic evidence [4].

Its remarkable capacity to resist antimicrobial drugs, acquire and spread resistance to antibiotic determinants, and most significantly, adaption to environmental changes, are the key pathogenic factors of this phenotype [5].

Treatment failures have been linked to the bacterium *Enterococci*, which is often found in infected endodontically treated teeth and allegedly capable of resisting root canal

treatment, including the alkaline pH provided by calcium hydroxide-based inter-appointment dressing [6]. In studies using culture-based detection techniques, its prevalence in endodontically treated teeth varies from 24-70 percent, and between 66 and 77 percent when molecular methods are used [7]. *EF* had a median prevalence of 9 percent (with a range between 4-24 percent) in microbiological investigations of primary endodontic infections [8].

For long-term survival in root-filled canals, this bacterium's most essential features are probably inherent resistance to antiseptics and endodontic medicaments combined with the capacity to down-regulate its metabolism, entering an inactive or even a "viable but non-cultivable (VBNC) condition" [9]. A large number of *EF* disease-associated virulence factors have been identified, including aggregation substance (AS), gelatinase (GelE), biofilm-associated Pili (Ebp), and biofilm formation [10]. Microorganisms' elements such as lipopolysaccharides lipoteichoic acid may induce periapical inflammation by increasing the level of cytokines [11-13].

The current study aimed to isolate and perform the molecular detection of *EF* from infected dental root canals and to assess the antimicrobial susceptibility of these isolates.

MATERIALS AND METHODS

Samples were collected from 50 people who had a single root canal tooth with radiographic evidence of apical periodontitis. A detailed dental and medical history were recorded for each patient. Those who had recently had antibiotic therapy or who had a general illness were ruled out.

Access cavity preparation was achieved utilizing a sterile turbine bur. The target teeth and the area nearby were cleaned with 2.5% NaOCl with a sufficient suction system. An initial patency check was obtained using a #15 stainless steel K-files (Dentsply Maillefer, Switzerland) to a length 2 mm short of the radiographical apex.

Ethical Approval

This study was reviewed and approved by the Research Ethical Approval Committee of the Dental School of the University of Babylon (Babylon, Iraq), with reference number

(29) on (29/5/2024). Prior to sample collection, each patient completed a valid consent form and was enrolled in the study. This was done according to the ethical approval outlined in the Helsinki Declaration.

Microbiological procedures

Sterile tweezers were used with sterile paper points (five/canal) introduced inside the root canal up to approximate 3 mm short of apex, and each paper point was kept inside the canal for 10 seconds to ensure saturation. A sterile physiological saline solution was utilized to prevent the canal dehydration. The second sample was collected by injecting a tiny amount of sterile physiological saline, then use a Hedstrom file to smooth down the canal walls and a sterile paper point to collect the sample. After that, the five paper points were placed in a sterile plain tube with five milliliters of brain heart infusion (BHI) broth inside of it. The samples were moved to a microbiological laboratory within four hours, while whenever feasible, an instantaneous transfer was favored. Before being used, each tube was stored for 72 hours at 37°C in an anaerobic incubator and then will be cultured on primary media.

Gram staining, aerotolerance, and the synthesis of trypsin, catalase, and indole were all used in the preliminary characterization stage. For species-level identification after preservation at 37°C, various biochemical tests were performed, including motility tests and carbohydrate fermentation as well as the detection of enzymes such as glycosidase and aminopeptidase enzymes that had already been produced. Unknown streptococcal isolates were detected using a commercial test panel on bile-esculin agar plates (Difco) [14].

Molecular detection

Separation of DNA from EF

DNA extraction was achieved using the DNA extraction kit (Presto™ Mini) according to the manufacturer's instructions. The primer (**Error! Reference source not found.**) was then dissolved and the contents were mixed. Subsequently, the mixture was then transferred to an 1.5% agarose gel. After cooling to 50^{0}C, ethidium bromide was added at a concentration of 0.5 μg per milliliter, and the mixture was then transferred to a PCR machine under at 70 volt for 60 minutes with a particular primer.

Detection of ddI E. faecium Gene

Conventional PCR was achieved in volumes of 25 µl reactions that contained DNA purity 20–200 ng (assessed by nanodrop Thermo Scientific), 0.5 µM of one µl of each particular primer for *ddI* gene, 200 µM of each dNTP, 1.5 mM MgCl2, and 1× PCR buffer and 2µ DNA.

Antibiotic susceptibility test

This was conducted using the Kirby Bauer method to identify isolates to 7 antimicrobials, including amikacin (10µg), azithromycin (15µg), doxycycline (10µg), erythromycin(10µg), gentamycin (10µg), nitrofurantoin (100µg), and vancomycin (30µg).

RESULTS

The results of present study showed the presence of *EF* according to media culture. The samples were grown on a number of culture media such as blood agar, MacConky agar, Mitis Salivarius agar, biochemical activity and confirmed by conventional PCR. The results showed that *EF* was found in 35 of the samples collected (70%), as shown in .

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According to the PCR findings, the *Enterococcus* isolates tested positive for *EF* in 37 of the samples collected (74%). The result of agarose gel electrophoresis of *ddl* products showed that isolates had positive results for implication where amplicon with molecular weight 658 appeared (

and **Error! Reference source not found.**).

Thirty-five *EF* isolates were tested for antibiotic sensitivity with seven antibiotics. The proportions exhibiting resistance were found and are shown in ..

DISCUSSION

The ultimate goal of a successful endodontic treatment is to ensure the eradication of all pathogenic microorganisms from the root canal system [15]. Various types of irrigants,

chemical products and natural products, have been used to clean and disinfect the root canals [16, 17]. Therefore, it is of crucial importance for endodontists to specify the pathogens present and their growth capacity in the root canal system.

Media culture, biochemical activity, and PCR (both conventional and real-time) were used to detect the presence of different pathogens in the endodontic environment [18].

In the current study, the existence of *EF* in infected root canal systems was shown utilizing a media culture method and biochemical testing. It was further confirmed with the aid of conventional PCR, which provides slightly more sensitive results than the culture method [15, 18-20]. Our results as shown in Table-2 agree with those of Mustafa et al. [21] who found *EF* in 68-80% of the culture-positive cases.

The ddI genes of both bacterial species (*E. faecium* and *E. faecalis)* that detected by PCR and primers are crucial for an identification of *Enterococcus* species. This highlights the importance of using genetic methods instead of phenotypic ones for uncommon enterococcal strains [22, 23].

In contrast, traditional techniques that used a culture media for the identification of *Enterococcus* spp. may take a couple of days for delivering the findings, while a genetic approach based on PCR offers the potential to provide accurate findings in a short period [24]. As a result, PCR offers more specificity and sensitivity than presently employed techniques in hospitals and laboratories, while also being quicker [25, 26].

The present findings from the antibiotic sensitivity test were similar to those published by Zavaryani et al. [27], who showed that the isolates were resistant to vancomycin and gentamycin. Furthermore, [28, 29] found that *EF* isolates had high resistance to ampicillin and erythromycin.

The above-mentioned results add to the bank of information available about bacterial species present in the root canal environment and further facilitate the management of infections related to these species.

CONCLUSION

The conventional PCR technique is sufficient to detect the presence of the *Enterococcus faecium* bacteria. This bacterium is sensitive to doxycycline, imipenem, and nitrofurantoin and resistant to amoxicillin, erythromycin, azithromycin, tetracycline, amikacin, and gentamycin.

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Conflicts of interest:

There are no conflicts of interest

Author contributions: Qasim Mohammed and Ahmed Alhelal designed the study. Qasim Mohammed, Ahmed Alhelal and Aqeel Al Jothery had achieved the practical work and analyzed the data. Ahmed Alhelal wrote the paper and further edited by all authors.

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Table *1* Specific primers sequence and amplicon size.

Primer	Primer sequences	Product size	References
Forward	**5'TTGAGGCAGACCAGATTGACG -3'**	**658 bp**	**12**
Reverse	**5'-TATGACAGCGACTCCGATTCC-3'**		

Table 2 EF detected by culture among the samples.

N	No. of *EF* isolates	Percentage
50	**35**	**70%**

Table 3 EF detected by molecular method.

N	No. of *EF* isolates	Percentage
50	**37**	**74%**

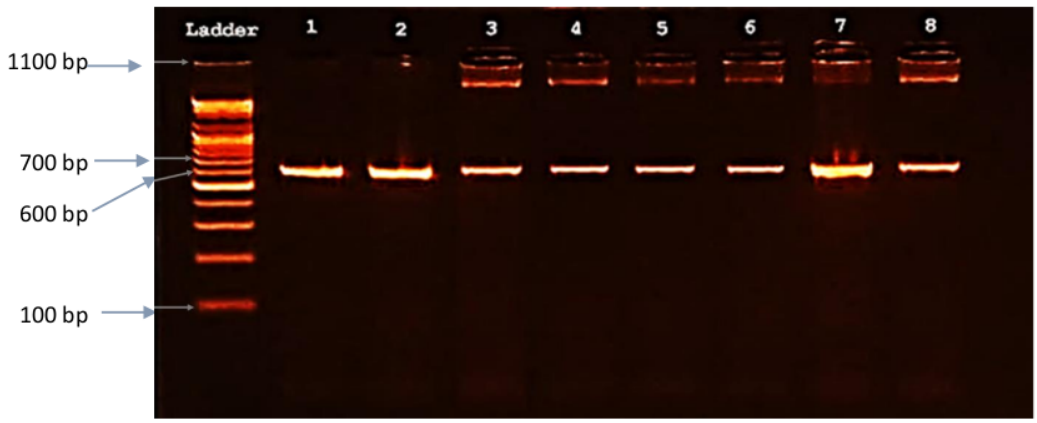

Figure 1 PCR of EF. represents a 100 bp DNA marker. Wells 1-8 are positive samples.

Table 4 Antibiotic sensitivity test for EF isolates.

Antibiotics	No. of resistant samples	Percentage	No. of sensitive samples	Percentage
Amikacin	**33**	**94.3**	**2**	**5.7**
Azithromycin	**32**	**91.4**	**3**	**8.6**
Doxycycline	**2**	**5.7**	**33**	**94.3**
Erythromycin	**35**	**100**	**0**	**0**
Gentamycin	**31**	**88.7**	**4**	**11.3**
Nitrofurantoin	**1**	**2.9**	**34**	**97.1**
Vancomycin	**35**	**100**	**0**	**0**