



ISSN: 0067-2904

Molecular detection and identification of *Enterococcus faecium* isolated from dental root canals

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Received: 5/8/2020

Accepted: 2/11/2020

Abstract:

Enterococci are usually encountered and predominate in oral infections, especially those associated with dental root canal infections of necrotic pulp and periodontitis. This study aimed to detect and identify *Enterococcus faecium* isolated from infected root canals, using polymerase chain reaction (PCR). Thirty samples were collected from patients with necrotic pulp, infected root canals, and endodontic treatment failure, attending the Conservative Treatment Department, College of Dentistry, Mosul University, Dental Teaching Hospital. The samples were obtained by inserting sterile paper points into the root canals and transferred in brain heart infusion broth vials to be inoculated in a selective M-Enterococcus Agar Base. Twenty five isolates that belong to the genus *Enterococcus* were recognized by traditional culture methods and biochemical tests. Then, DNA extractions of these isolates were carried out for identification with PCR by the amplification of *ddI* (D-Ala-D-Ala Ligase) chromosomal genes of *Enterococcus faecium*. Among the 25 isolates, twenty (80%) were identified to the level of *Enterococcus faecium* by traditional culture methods and biochemical tests, in comparison to 17 (68%) identified by molecular identification. The PCR products for the specific primer produced bands on agarose gel at the position of 658bp. The study showed that the use of PCR with primers for the *E. faecium* *ddI* gene may be the most accurate method for rapid identification of Enterococci. Molecular identification of *Enterococcus* spp. revealed a significant role of *E. faecium* in root canal infections. Also, the detection of *ddI* gene using PCR provides a definitive target that could be used for the detection of *E. faecium* from clinical samples.

Key words: *Enterococcus faecium*, root canal, polymerase chain reaction (PCR)

التحري الجزيئي للمكورات المعوية المعزولة من قنوات جذور الاسنان

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الخلاصة

تتواجد المكورات المعوية عادة وتسود في الاصابات الفموية وخاصة تلك المتعلقة بلب السن المتعفن وقناه الجذر المصابة والتهابات الأنسجة المحيطة بالسن. هدفت هذه الدراسة الى تشخيص *Enterococcus faecium* المعزولة من قنوات جذور الاسنان المصابة باستخدام تقنيته تفاعل البلمرة المتسلسل, حيث تم جمع ثلاثون نمودجا من مرضى لب السن المتعفن وقنوات الجذور المصابة وحالات المعالجة اللبية الفاشلة والذين يراجعون قسم العلاج التحفظي في المستشفى التعليمي لكلية طب الاسنان في جامعه الموصل. وتم الحصول

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على النماذج عن طريق غرس اقماح ورقية في قنوات جذور الاسنان ثم نقلها الى قناني حاوية على وسط نقيع القلب والمخ ليتم زراعتها على الوسط الانتقائي M-Enterococcus Agar Base , خمس وعشرون عزله تعود الى جنس المكورات المعوية تم تشخيصها بطرق الزرع التقليدية والاختبارات الكيموحيوية وتم استخلاص الحمض النووي DNA من هذه العزلات ليتم تحديدها بعد ذلك باستخدام تقنيه تفاعل البلمرة المتسلسل من خلال تضخيم جين (D-Ala-D-Ala Ligase) الخاص بالنوع *E. faecium* . من ضمن خمس وعشرون عزله تعود الى جنس المكورات المعوية تم تشخيص عشرين عزله (80%) لمستوى النوع *Enterococcus faecium* باستخدام طرق الزرع التقليدية والاختبارات الكيموحيوية بالمقارنة مع سبعة عشر عزله (68%) *Enterococcus faecium* شخصت بالطرق الجزيئية. واعطت نتائج تفاعل البلمرة المتسلسل حتما على الاكاروز عند الموقع 658 (زوج قواعد) واوضحت الدراسة ان تقنيه تفاعل البلمرة المتسلسل باستخدام البرايمر الخاص بجين (D-Ala-D-Ala Ligase) ربما كانت الطريقة الاكثر دقة للتحري السريع عن هذه الاجناس المعوية وان التحري الجزيئي للمكورات المعوية اظهر دورا مهما لنوع *E. faecium* في اصابات قنوات جذور الاسنان كما ان جين (D-Ala-D-Ala Ligase) *ddl* اعتبر هدفا حاسما في هذه التقنيه وبالإمكان استخدامه للكشف عن هذا النوع في العينات السريرية.

Introduction

Microorganisms have been presented in all cases associated with endodontic retreatment. *Enterococcus spp.* was reported to represent the most frequently genus detected in dental root canals [1]. Bacteria could survive in root canals even if good mechanical preparations were performed, causing clinical symptoms such as persistent chronic apical periodontitis [2]. The oral cavity may act as a reservoir for superinfection microorganisms, including *E. faecalis*, *E. faecium* and *E. coli*, as assessed by anti-biotyping studies [3]. Microbial flora that is found in the root canals after endodontic treatment failure is bounded to a small number of commonly gram positive bacterial species, especially *Enterococcus spp.* [4]. Enterococci are gram positive, facultative anaerobic, organisms that are considered as transitory constituents of the oral microbiome and may cause a diversity of oral and systemic infections [5]. In many studies, *E. faecalis* and *E. faecium* have been demonstrated as the most popular species isolated from human oral infections, with corresponding virulence factors such as formation of gelatinase, haemolysis, and biofilm formation. Also these factors were correlated with colonization of the host tissue, competition with other bacteria, alteration of the host defense mechanisms, invasion, and the formation of abscess from toxin or inflammatory processes [6]. Molecular genetic approaches have been used to identify *Enterococcus spp.* of endodontic infections; the most common molecular methods is the polymerase chain reaction [PCR] that have been widely used to identify bacteria in primary endodontic infections. Since conventional and cultivation identification methods have been proved to have several limitations with respect to microbiological diagnosis, more sensitive techniques may be necessary for accurate characterization of the microbial assembly of root-filled teeth. Species-specific PCR primers such as *ddlE. faecalis* and *ddlE. faecium* were documented as competent for enterococcal species identification by different studies. The use of PCR with these primers may be the simplest molecular approach for highly effective identification of distinct *Enterococcus spp.*, while avoiding the draw backs of commercial kits [7, 8].

Materials and methods

1- Sample collection and bacterial identification

Thirty dental root canal samples were obtained from patients attending the Dental Teaching Hospital by inserting paper points into the canals, which were then immersed in brain heart infusion broth and cultured on selective M-Enterococcus Agar Base. The Enterococcus isolates were diagnosed to the genus level by using the biochemical tests (Table1) and molecular diagnosis [9].

2- Molecular diagnosis of *Enterococcus faecium*

A- DNA extraction

DNA extraction of *Enterococcus spp.* was applied from each sample by picking a single colony with a sterile loop, which was then inoculated into sterile tubes of 5ml brain heart broth and incubated at 37°C⁰ for 24 hr. DNA of the enterococcal isolates was extracted by the use of DNA purification kit, according to the instruction of the manufacture (Jena Bioscience, Germany).

B- Polymerase chain reaction

The primers employed for the amplification of *ddl* (D-Ala-D-Ala Ligase) chromosomal genes were as follows: F 5'- TTGAGGCAGACCAGATTGACG -3' and R 5'- TATGACAGCGACTCCGATTCC-3' [8,10], provided in lyophilized forms (IDT, USA). Working stock was prepared by adding PCR grade water to the required concentration according to the supplier's recommendations. The PCR mixture was performed in 25 µl volumes that contained 2 µl of template DNA, 2 µl of specific primers of *E. faecium* (*ddlE*) (R and F), 10µl of 2 x Taq master mix, and 11µl of PCR grade water. PCR was conducted using thermal cycler (Optimus 96G, QLS, UK). DNA was amplified by a general PCR technique. An initial denaturation of 5 min at 94°C was followed by 35 cycles of denaturation at 94°C for 1min, annealing at 54°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. DNA samples were observed by using agarose gel electrophoresis (Jena Bioscience, Germany) prepared as reported by Mohanty *et al.* [11], with 1x TBE buffer (Genet Bio, Korea) and 100 bp DNA ladder (Jena Bio-science, Germany) as a standard molecular weight marker. Electrophoresis was performed using power supply of MP 300V (Major Science, UK), then the agarose gel was placed in a documentation system (Bio Doc Analyze, Germany) and examined under UV light for documentation and determination of expected bands.

Table 1- The results of the biochemical tests used for the identification of *Enterococcus faecium* isolated from

| Basic Characteristics | Results |
|-------------------------------|----------------|
| Growth in Bile esculin medium | Positive (+ve) |
| Catalase | Negative(-ve) |
| Gram staining | Gram positive |
| Growth in 6.5% NaCl | Positive(+ve) |
| Shape | Cocci |
| Growth at pH 9.6 | Positive (+ve) |
| Fermentation of sugar | |
| Lactose | (+ve) |
| Fructose | (+ve) |
| Glucose | (+ve) |
| Lactose | (+ve) |
| Arabinose | (+ve) |

Table 2-Numbers of *Enterococcus faecium* isolated by traditional methods and molecular methods

| | No of <i>Enterococcus faecium</i> isolates | Percentage % |
|---------------------|--|--------------|
| Biochemical methods | 20 | 80% |
| Molecular methods | 17 | 68% |

Results and Discussion

Out of thirty paper point samples obtained from root canals, twenty five isolates of *Enterococcus sp.* were detected according to the conventional methods and biochemical tests. Out of these 25 *Enterococcus spp.* isolates, twenty (80%) were identified to the level of *Enterococcus faecium* by the traditional culture methods and biochemical tests, in comparison to 17 (68%) isolates identified by molecular identification (Table 2). All the isolates of *Enterococcus spp.* in the present study were exposed to PCR technique with species-specific primers for *E. faecium*, based on the detection of specific gene that encodes D-alanine, namely D-alanine Ligase for *E. faecium* (*ddlE*). Based on PCR results, seventeen *Enterococcus* isolates (68%) were detected as *E. faecium*. The results confirm the presence of a 658bp PCR product when compared with the DNA ladder (Figure1).

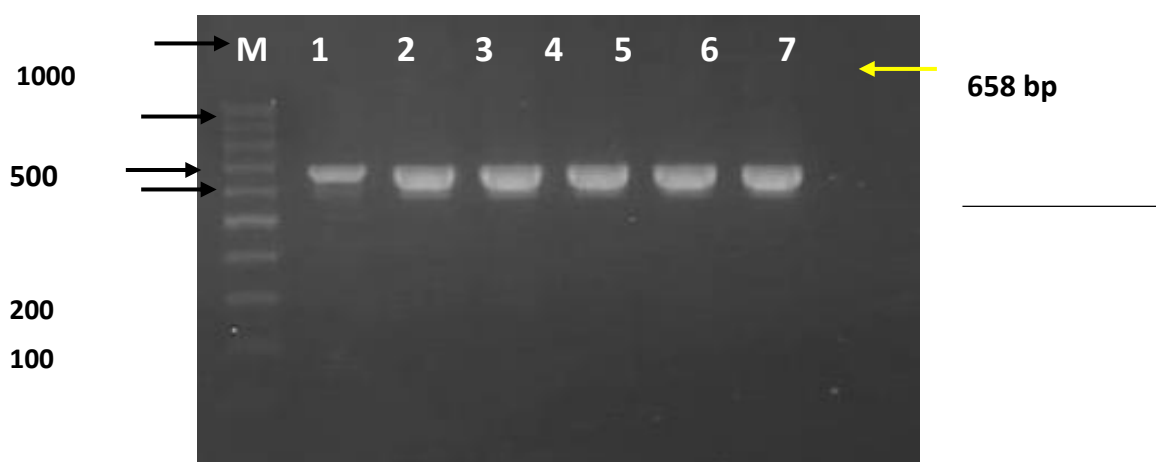


Figure 1-Polymerase chain reaction of *Enterococcus faecium*. Lane M represents 100 bp DNA marker. Wells 1-6 are positive samples, well 7 is negative control.

The significance of all *Enterococcus* species in oral infections has not been often contemplated and reported. Even less attention has been given to both phenotypic and genotypic virulent characteristics of these microbes isolated from dental infections [6]. Our study highlights the role of *E. faecium* in distinction from other *Enterococcus* species, since most previous studies focused mainly on the effects of *E. faecalis* in the pathogenesis of different oral infections, particularly in root canal infections.

The results of our study showed that *E. faecium* accounted for 68 % of root canal samples, which agreed with an earlier study that reported that, among *Enterococcal* species, *E. faecalis* and *E. faecium* were the most frequently isolated species from the oral cavity, comprising 80% of clinical samples [5]. Another study demonstrated that *E. faecium* strains from oral infections had high prevalence of many virulent determinants which play an important role in the pathogenesis of oral infections, including 33/35 (94%) of *esp* gene (gene for surface adhesion), 30/35 (86%) of *efaA* gene (gene for endocarditis), and 34/35 (97%) of *asa1* (gene for the aggregation of substances) [6].

The use of PCR and primers that target specific sequences in the *ddl* genes of the two species (*E. faecalis* and *E. faecium*) is very important for the identification of the genus and species of *Enterococcus*. The difficulty in identification of unusual enterococcal strains by phenotypic techniques is not unexpected, especially by using manual commercial kits [12,13]. Wrong identification at the genus level and the misidentification of the strains that are repeated in the clinical specimens, such as *E. faecalis* and *E. faecium*, are commonly encountered issues. In order to properly detect *Enterococcus* species, it is essential to perform many introductory tests, including catalase, bile-esculin, PYR, and 6.5 % NaCl tests. Then, automated device or commercial manual tests must be performed. However, it often appears necessary to conduct further tests, such as pigment production, motility, D-xylose fermentation, Litmus milk reduction, pyruvate utilization, and methyl-a-D-glucopyranoside fermentation. Therefore, molecular approaches could provide a good alternative to these physiological tests [14]. On the other hand, conventional culture-based methods for the identification of *Enterococcal spp.* require 2-3 days to yield results, while PCR has provided a method for culture-independent detection of *Enterococcal* bacteria in a variety of clinical specimens. This assay is capable of yielding accurate results in few hours [15]. Hence, PCR technology provides high specificity and sensitivity and is faster than the conventional methods currently used in hospitals and laboratories [16,17].

Conclusions

Since traditional phenotypic methods are not highly sufficient, it is recommended to use polymerase chain reaction technique with primers for *ddl* *E. faecium* that provides a rapid, accurate, more sensitive, and less time-consuming detection of these bacteria.

Acknowledgment

The authors would like to thank the Department of Basic Science in the College of Dentistry , University of Mosul, for supporting this study .

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