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MOLECULAR INVESTIGATION OF ENTEROCOCCAL SURFACE PROTEIN (*ESP*) GENE OF *ENTEROCOCCUS FAECALIS* ISOLATED FROM ENDODONTIC PATIENTS

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МОЛЕКУЛАРНО ИСПИТИВАЊЕ ГЕНА ЕНТЕРОКОКНОГ ПОВРШИНСКОГ ПРОТЕИНА (*ESP*) *ENTEROCOCCUS FAECALIS* ИЗОЛОВАНОГ КОД ЕНДОДОНТСКИХ ПАЦИЈЕНАТА

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ABSTRACT

Objective. Enterococci are generally considered transient components of oral bacteria that may be a reason for several oral and systemic infections, particularly those related to dental root canal infections. The current study aims to examine the occurrence of Enterococcus surface protein, esp in Enterococcus faecalis, which is isolated from infected root canals.

Methods. Forty samples were collected from endodontic patients who attended the Conservative Treatment Department in the College of Dentistry/Mosul University/Dental Teaching Hospital. Materials and Methods: All samples were traditionally examined using HiCrom ™ Enterococcus faecium Agar base medium and biochemical tests. 16srRNA sequencing was performed using the polymerase chain reaction technique to confirm their identity. Then, all Enterococcus faecalis isolates were examined for the existence of esp gene coding for enterococcal surface protein using PCR assay.

Results. From 40 clinical samples obtained, 31 isolates were recognized as E. faecalis by traditional methods; unexpectedly, other non-enterococci genera were also grown on HiCrom™ Enterococcus faecium Agar base medium. The PCR products for the sequence-specific primers obtained from the full-length of 16S rRNA gene sequence, which belongs to E. faecalis, and the PCR products for specific primer of esp genes created bands at the position of 138bp and 932 bp on the agarose gel, respectively. The gene correlating with the aggregation of this bacteria on the canal walls was detected in a high proportion (91%) of the isolates.

Conclusions. PCR assay provides an accurate, rapid, and more sensitive detection of E. faecalis. A positive correlation between esp gene and enterococcal infections in root canals has been found.

Key words: *Enterococcus faecium; bacterial proteins; polymerase chain reaction.*

САЖЕТАК

Циљ. Ентерококе се генерално сматрају пролазним компонентама оралних бактерија које могу бити разлог за неколико оралних и системских инфекција, посебно оних које се односе на инфекције канала корена зуба. Садашња студија има за циљ да испита појаву површинског протеина Enterococcus, посебно Enterococcus faecalis, који је изолован из инфицираних канала корена зуба. Сакупљено је 40 узорака од ендодонтских пацијената који су похађали одељење за конзервативно лечење на Стоматолошком колеџу / Универзитету у Мосулу / Стоматолошкој болници.

Методе. Сви узорци су традиционално испитивани коришћењем HiCrom ™ Enterococcus faecium агар базне подлоге и биохемијских тестова. Секвенцирање 16srRNA је изведено коришћењем технике ланчане реакције полимеразе да би се потврдио њихов идентитет. Затим су сви изолати Enterococcus faecalis испитани на постојање esp гена, који кодира површински протеин ентерокока коришћењем PCR теста.

Резултати. Од 40 добијених клиничких узорака, 31 изолат је препознат као E. faecalis традиционалним методама; неочекивано, на бази HiCrom™ Enterococcus faecium агар базног медијума узгајани су и други родови који нису ентерококе. PCR производи за прајмере специфичне за секвенцу добијени су из пуне дужине секвенце гена 16S rRNA, која припада E. faecalis, и PCR производи за специфични прајмер esp гена створили су траке на позицији од 138bp и 932bp на агарозни гел, респективно. Ген који корелира са агрегацијом ове бактерије на зидовима канала детектован је у великом броју (91%) изолата.

Закључак. PCR тест обезбеђује тачну, брзу и осетљивију детекцију E. faecalis. Утврђена је позитивна корелација између esp гена и ентерококних инфекција у каналима корена зуба.

Кључне речи: *Enterococcus faecium, бактеријски протеини, реакција ланчане полимеризације*

INTRODUCTION

Enterococci are common bacteria that can be found and prevail in oral diseases, particularly root canal infections causing necrotic pulp and periodontitis. These bacteria can remain and resist removal by root canal preparation even if perfect mechanical is prepared. As a result, they cause clinical symptoms (persistent chronic apical periodontitis) (1). The *E. faecalis* exists in cases of dental caries, periodontitis, and tooth root infections. This bacterium is considered as one of the most resistant species found in the oral cavity and a possible source of

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root canal treatment failure (2,3). *E. faecalis* has been considered the most prevalent species in endodontic treatments. This is due to the ability of this microorganism to adhere to dentine collagen and resistance to intracanal antimicrobial procedures (4). *E. faecalis* form biofilms on the canal walls, which may be necessary for resistance to endodontic therapy (5). After adhering to the walls of root canals, *E. faecalis* can stack and compose communities organized in biofilm, which consists of a mass of bacteria stuck together (6). Biofilm protects bacteria from environmental alteration, host immune response, and antimicrobial treatments (7,8). The *esp* gene (Enterococcal surface protein) is one of several virulence factors that play a remarkable role in the aggregation of these species and initiation of dental diseases (9). This gene encoding for Enterococcus surface protein has an iterative structure leading to bacteria adhesion and biofilm formation. Esp gene is a high molecular weight protein (1873 amino acids) (10), consisting of C- the terminal domain, the Central core, and the N-terminals. It has been supposed that the N-N-terminal of this protein cooperated in interlinkage with the host. At the same time, the central core of esp plays a remarkable role in the accumulation of microorganisms and hides this protein to avoid the host immune system (6,7). Molecular genetic approaches have been used to detect Enterococcus spp. of endodontic infections accurately (1). Since traditional and cultural techniques have proved to acquire diverse determinants of bacterial recognition, it will be necessary to use more sensitive techniques to describe the microbial communities of root-filled teeth correctly. The target of our study was to check the occurrence of *E. faecalis* by using two microbiological identification techniques, 16S r RNA gene–based PCR and culture, and to investigate the presence of *esp* gene (Enterococcal Surface Protein) in *E. faecalis* isolates insulated from infected root canals.

MATERIALS AND METHODS

Traditional Diagnosis

Samples preparation: The present study included 40 endodontic patients attending the Dental Teaching Hospital; collecting the samples was done by introducing a sterile absorbent paper point in the root canal of each patient for 60 seconds, then placed in sterile brain heart infusion broth (3ml).

Bacterial isolation and identification: The samples were processed in the laboratory within two hours of collection, the transport medium containing paper point was plated onto HiCrom ^{TM} Enterococcus faecium Agar base medium supplied from Hi media (Mumbai, India), and plates were aerobically incubated at 37^0C for 24-48hrs. Isolates of *E. faecalis* were identified according to the biochemical tests: as gram stain, catalase test, capable of growth at 42^0C, and the ability to grow with 6.5 NaCl (11). Bile Esculin Agar Base (Mumbai, India) was incubated with suspected isolates at 37^0C for 24 hrs. The ability of isolates to degrade esculin and tolerate bile salt was examined (12).

Molecular diagnosis

Extraction of DNA: DNA extraction of the examined bacteria was utilized by selecting a single colony from each specimen using a sterile microbiological loop and inoculation into sterile brain heart infusion broth, then incubation at 370C for 24 hrs. Following the manufacturer's instructions, the bacterial DNA was extracted using the Add Prep Bacterial Genomic Extraction Mini kit (Add Bio, Korea). To rehydrate the DNA pellet, 100 µl of rehydration solution was added and kept at -20 °C for further attempts.

Polymerase chain reaction assay: For investigation of *E, faecalis* & *esp* gene (Table 1). Polymerase chain reaction was performed using specific primers (13). The primers were acquired from {Macrogen Co, Korea}. The PCR reaction mixtures were intended using AddBio Master Mix (2x), AddBio, Korea. The PCR mixture was implemented in 20 µl, which contained a final concentration of (1X) AddBio Master Mix, one µM of each specific primer, 2 µl of DNA template (2 ng/µl), and 10 µl of PCR grade water (Table 2). This assay was performed by utilizing a thermal cycler (T100 BioRad, USA). The cycling conditions, including time & temperature, are displayed in (Table 3).

Agarose gel electrophoresis: Gel electrophoresis was carried out to separate the amplified products by utilizing 1.5% agarose (AddBio, Korea) mixed with Gel Red dye (3 µl) (AddBio, Korea). 5µl of each PCR product was placed in the well of agarose gel. Electrophoresis was done using 300 Ma/power supply at 75 V/1 hours, and the electrophoresis tank (BioRad, USA) contains 1X (TBE buffer, GeNetBio, Korea), 100 bp DNA ladder, 6 µl (GeneDirex H3, Korea) as standard molecular weight marker.

RESULTS

Thirty-one out of 40 root canal samples (77.5 %) (31/40) showed the growth of *E. faecalis* isolates, which produced blue-color colonies on the HiCrom TM Enterococcus faecium Agar base (Figure 1A). The research result showed the possibility of the growth of diverse microorganisms on HiCrom TM Enterococcus faecium Agar, which the manufacturing company of this media did not indicate. These results found different colored colonies distinguished on this media as blue, green, white, gray, white, and brown. (Figure 1, Table 4). These different colonies were then identified by 16S rRNA gene sequencing to *Enterococcus faecalis, Enterococcus faecium, Staphylococcus epidermidis, Staphylococcus*

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Table 1. Sequencing of each primer used for amplification of specific genes of E. faecalis.

No.	Name of primer	Primer Sequence (5' – 3')	Annealing Temp.°C	PCR product size (bp)
1	Ef16S-F	CCGAGTGCTTGCACTCAATTGG	60	138
2	Ef16S-R	CTCTTATGCCATGCGGCATAAAC		
3	Esp-F	TTGCTAATGCTAGTCCACGACC	60	932
4	Esp-R	GCGTCAACACTTGCATTGCCGA		

Table 2. Reaction mixture of PCR for amplification of 16SrRNA and esp genes of E. faecalis.

Component	Volume	Final Concentration
2X Add Bio Master Mix	10µl	1X
PCR grade water	6 µl	---
F-primer (10 µM)	1 µl	1 µM
R-primer (10 µM)	1 µl	1 µM
DNA (80 ng/µl)	2 µl	2 ng/µl
Total volume	20 µl	

Table 3. PCR Cycling conditions for amplification of 16SrRNA & esp genes of E. faecalis.

No.	Step	Temp °C	Time	Cycle
1	Polymerase activation	95	10 min	1x
2	Denature	95	45 Sec	
3	Annealing	60*	45 Sec	35x
4	Extension	72	1min	
5	Final Extension	72	5 min	1x
6	Hold	4	4C°	
*Annealing Temp. was used for both 16SrRNA and *esp* genes				

Table 4. Detection of isolates by 16S rRNA gene sequencing.

Colony color on HiCrom agar	Isolate name
Blue	*Enterococcus. faecalis*
Green	*Enterococcus faecium*
White	*Staphylococcus epidermidis*
Brown	*Lactococcus lactis*
Gray-white	*Staphylococcus hominis*

hominis, and Lactococcus lactis, respectively. According to the results of PCR, Enterococcus isolates (77.5%) were detected as *E. faecalis*. Our results prove the presence of a 138 bp PCR product after comparison with the DNA ladder (Figure 2). In comparison, molecular detection of the *esp* gene, which codes for enterococcus surface protein, was performed for *E. faecalis* strains using PCR assay with 932 bp PCR product after comparison with DNA ladder (Figure 3).

DISCUSSION

HiCrom TM Enterococcus faecium agar is a chromogenic selective medium that is used for the isolation of both *E. faecium and E. faecalis*. The first species appeared as a green-colored colony, changing the color of the medium to yellow because of the ability of this species to ferment the arabinose component and cleave the chromogenic substrate, while *E. faecalis* cleaves the chromogenic substrate but does not ferment arabinose and appears as a blue colony with no change on the medium (14). In this study, other genera were grown on this medium, producing different colors. Our results agree with another study which described that *E. faecalis* was the predominant species isolated from the oral cavity. The study indicated that *E. faecalis* formed 88.7% of clinical samples, while the second species was E. durans (7,9%), followed by (1.7%) of E. faecium (15). Another study demonstrated that 126 enterococcal isolates were obtained

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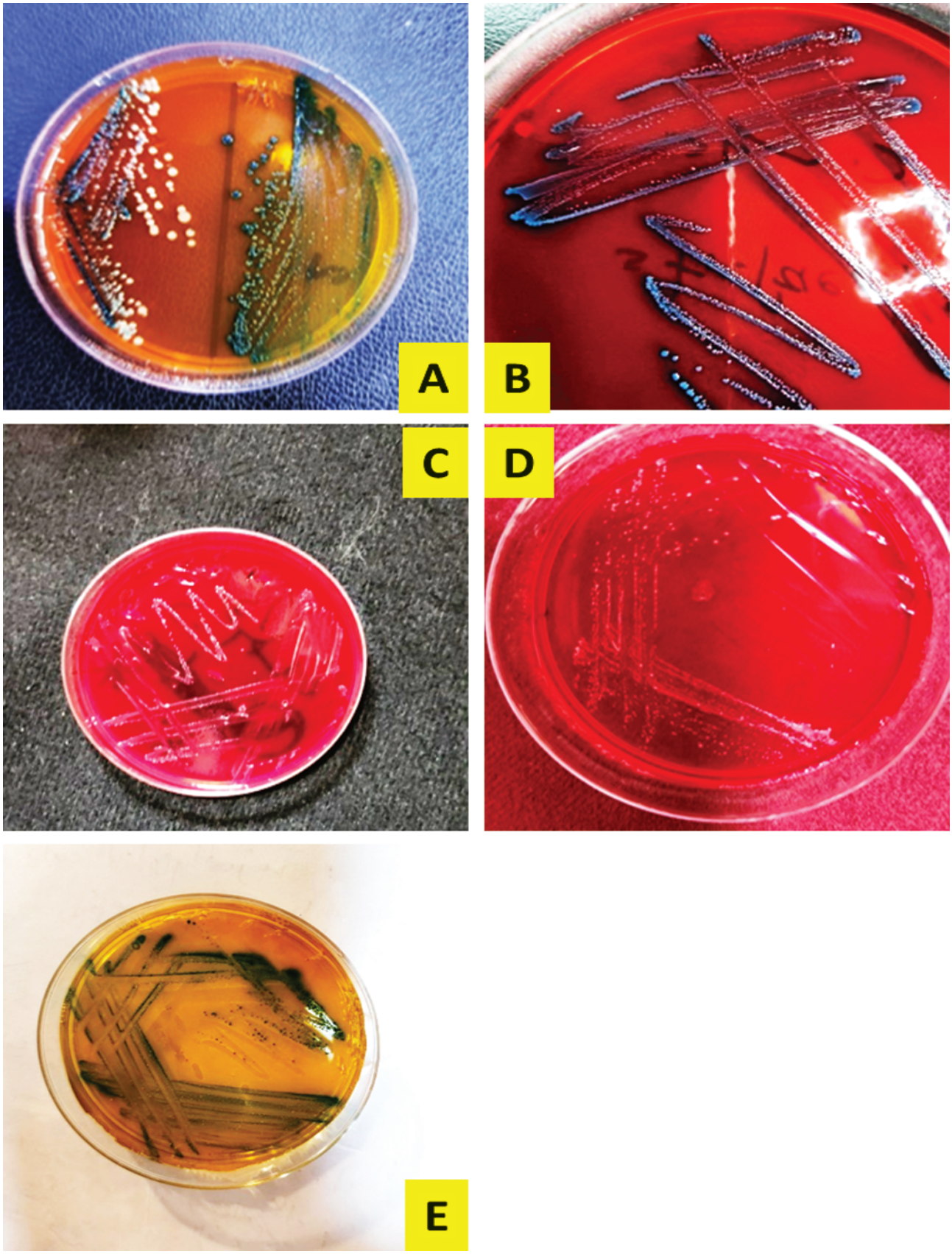

Figure 1. Several colored colonies of microorganisms grew on HiCrom agar. (A) Staph. Epidemidis (white), (B) E. faecalis (blue), (C) Staph. Hominis (gray-white), (D) Lactococcus lactis (brown), and (E) Enterococcus faecium (green).

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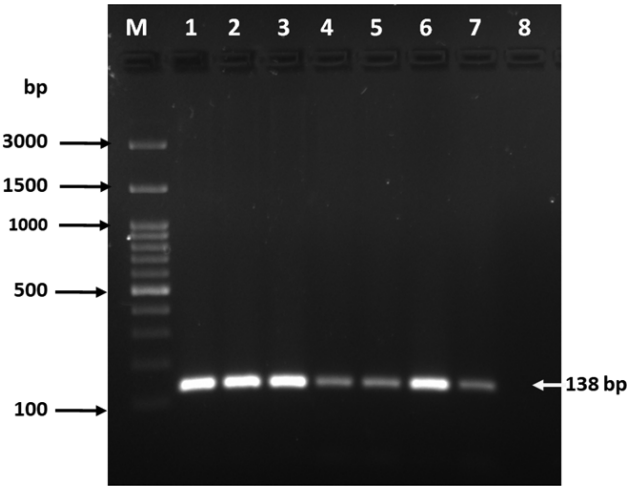

Figure 2. Polymerase chain reaction (PCR) of 16S rRNA gene of Enterococcus feacalis. Lane M: 100 bp DNA ladder. Lanes 1-7 are positive samples, and lane 8 is negative control.

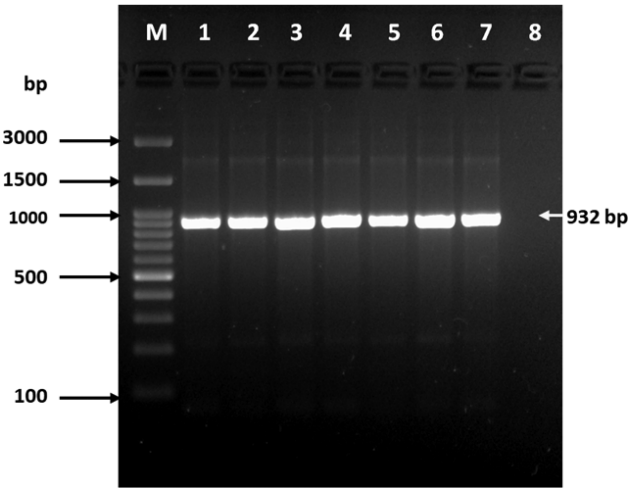

Figure 3. Polymerase chain reaction (PCR) of esp gene of Enterococcus fecalis. Lane M: 100 bp DNA ladder. Lanes 1-7 are positive samples, and lane eight is a negative control.

from the oral cavity; 72% of these samples were diagnosed as E. faecalis, and 28% were diagnosed as E. faecium (16). Most strains in this study carried the *esp* gene (91%). This result agrees with another study that found a significant relationship between the presence of this gene and the formation of biofilm among 57 VRE faecium isolates recovered from the Iranian patients (17). The *esp* gene is essential in biofilm formation, which has shown to be a very combination process. However, the participation of potential virulence factors in this critical process is very controversial (18). So, the interaction between this gene and the biofilm process may confirm the successful proportion of enterococcal infections. PCR and primers are very useful for detecting the genus or the species of Enterococci. The misidentification of uncommon strains of enterococci by traditional methods is not unforeseen, mainly when manual commercial kits have been used. The wrong recognition at the genus level and the misdiagnosis of the strains are recurrent in the clinical samples; for instance, both species (*E. faecalis* & *E. faecium*) are common problems. To detect Enterococci species, it is necessary to carry out many preparatory tests: inclusive of catalase, (6.5 % NaCL tests), (bile–esculin, (PYR), (automated device) or (commercial manual test), (pigment production), (motility), (utilization of pyruvate), (methyl-a-D-glucopyranoside ferment D-xylose fermentation), and (Litmus milk reduction). The molecular ways supply an excellent replacement for the previous physiological tests (19). Furthermore, traditional methods based on culturing take 2-3 days to yield results. Conversely, PCR can obtain delicate results in a few hours with high specificity and sensitivity and save more time than traditional methods presently applied in laboratories and hospitals (19, 20).

In conclusion, different genera can grow on HiCrom TM Enterococcus faecium Agar besides the enterococcal species. So, our results could be helpful in the appropriate identification of researchers using this medium in the future. Identifying the *esp* gene could enable the identification of infections caused by these microorganisms.

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