ISOLATION AND IDENTIFICATION OF BIOFILM PRODUCING *ENTEROCOCCUS FAECALIS* FROM ROOT CANAL

A. M. Salih   **G. M. S. Al-Quraishi**
Researcher   **Prof.**
Dept. Biology, Coll. Sci., University of Baghdad, Baghdad, Iraq
3ly95mahdi@gmail.com   ghada90m@gmail.com

ABSTRACT
This study was aimed to one of the most prevalent causes for endodontic treatment failure is the presence of *Enterococcus faecalis* bacterium within teeth root canals. To achieve successful treatment, it is so important to study *E. faecalis* behavior. The aim of study was to investigate biofilm production and antibiotic sensitivity of *E. faecalis* isolated from root canals. Results showed isolation of *E. feacalis* (65%) of samples, identified by specific gene by PCR technique. Most isolates were sensitive to Imipenem and resistant to Erythromycin, Clindamycin, Tetracycline and Trimethoprim. Strong biofilm production was detected among 29.5% of highest antibiotic resistant isolates. The results may indicate that infected root canals with *E. feacalis* may lead to serious complication for patients.
Key words: bacterial infections, Polymerase chain reaction, teeth, antibiotic sensitivity.

عزل وتشخيص بكتريا المكورات المعوية البرازية المنتجة للغشاء الحيوي من قنوات جذور الاسنان

علي مهدي صالح   غادة محمد صالح القريشي

باحث   استاذ

قسم علوم الحياة، كلية العلوم، جامعة بغداد، العراق

المستخلص

احدى اكثر الاسباب الشائعة لفشل علاج الالتهابات اللبية لجذور الاسنان هي وجود بكتريا *Enterococcus faecalis* (المكورات المعوية البرازية) في هذه القنوات. ولتحقيق علاج ناجح من المهم دراسة سلوك هذه البكتريا. الهدف من الدراسة هو للتحري عن انتاج الغشاء الحيوي وحساسية بكتريا المكورات المعوية البرازية للمضادات الحيوية والمعزولة من قنوات جذور الاسنان . أظهرت النتائج عزل بكتريا المكورات المعوية البرازية بنسبة 65% من العينات ، وتم تشخيصها جزئيا بأستخدام جين متخصص بواسطة تقنية تفاعل البلمرة المتسلسل (PCR). معظم العزلات كانت حساسة Imipenem ومقاومة لكل من Trimethoprim, Tetracycline Clindamycin, Erythromycin. العزلات لبكتريا المكورات المعوية البرازية ذات الانتاج العالي للاغشية الحيوية وجدت في 29.5% من العزلات الاكثر مقاومة للمضادات الحيوية. تشير النتائج الى ان قنوات الجذور للاسنان المصابة بالمكورات المعوية البرازية ممكن ان تؤدي الى مضاعفات خطيرة للمرضى المصابين بها.

الكلمات المفتاحية: الالتهابات البكتيرية، الاسنان، تفاعل البلمرة المتسلسل ، الحساسية للمضادات الحيوية.

INTRODUCTION

Enterococci bacterium belong to the group of Gram-positive bacteria and most of the species of this genus have Lysine-D-asparagin type of peptidoglycan except for *E. faecalis*, which has the lysine-alanin type (7). They are catalase negative and some species can show hemolytic activity. Enterococci belong to facultative anaerobic bacteria with the preference of anaerobic environment; however, they may live in aerobic environment (26). *Enterococcus faecalis* is one of the etiological agents linked to failed endodontic treatments and is regularly isolated from infected root canals with its ability to thrive in an environment lack of nutrients and in the highly alkaline tooth environment following treatment with intracanal medicaments, it is frequently discovered in unsuccessful endodontic therapies (21). Several features have been hypothesized to improve *E. faecalis* ability to survive in the nutrient- and oxygen-depleted root canal environment and induce inflammatory reactions around the root end, known as apical periodontitis. The colonization of the root canal space is likely aided by adhesion factors such as *Enterococcus faecalis* antigen A (efaA), aggregation material, and adhesin to collagen (*ace*) (25). Enterococcal surface protein (esp) and gelatinase (gelE), two possible virulence factors, have been highlighted in the pathogenesis of apical periodontitis by enhancing biofilm development and worsening tissue destruction and bone resorption around the root end (30). The ability of Enterococci to form biofilms may confer an ecological advantage in certain situations. For example, clinical strains of *E. faecalis* isolated from infective endocarditis patients were significantly associated with the greater biofilm formation than nonendocarditis clinical isolates; this may be attributable in part to specific virulence factors in Enterococci. Adhesions and secreted virulence factors have both been identified as Enterococcal virulence factors, Enterococci pathogenic factors that are secreted and have a role in pathogenesis. Which has been related to biofilm production in *E. faecalis* (20). This study design to evaluate biofilm production of *E. faecalis* isolated from root canal samples.

MATERIALS AND METHODS

A. Bacterial isolation and identification: -

During the period of October 2019 to march 2021, (60) root canal specimens were collected from patients in Dentists clinics by sterile paper point and special files, the paper points and files were collected in sterile Eppendorf tubes for each with 1 ml of sterile brain heart broth and placed in a cooled box and transported to microbiology laboratory as soon as possible for further experiments.

1. Macro and microscopical examination: All samples were inoculated on Pfizer specific *Enterococcus* media, which is a selective medium for the isolation and growth of *Enterococci spp.*, and incubated anaerobically for 24 hours at 37°C. The form, size, and color of the isolates were all investigated. Other biochemical assays were performed on a single pure isolated colony to corroborate the identification of *Enterococcus spp.* isolates. The isolates stained by Gram stain were examined under a light microscope to determine their shape, stain, and arrangement.

2. Biochemical tests: Different biochemical tests were performed to identify bacteria, for catalase test bacteria were transfered on a glass slide from Pfizer selective Enterococcus plates, and few colonies were combined with a drop of 3% H_2O_2, Positive results show immediate bubbling (18). Growth Test in 10 Cº and 45 Cº was detected through incubating bacterial isolates in Brain Heart infusion broth and incubated anaerobically for 24 hours at 10 Cº and 45 Cº. After incubation, the presence of growth was considered a positive result (10). Growth in medium with 6.5% NaCl to detect tolerance of bacterial isolates in high salt concentration in brain heart infusion broth medium containing 6.5% Sodium Chloride (NaCl) and incubated anaerobically at 37Cº for 24 hr., Positive result show appearance of growth (23). The Ability of growth in pH 9.6 the bacterial isolate was inoculated into Brain Heart infusion broth medium with adjusting the PH to 9.6 then incubated anaerobically at 37°C for 24 hours. After incubation, the presence of growth was considered positive result (10).

3. Molecular detection: Molecular technique was carried out for bacteria isolates. The DNA of pure bacterial colonies was extracted by

Genomic DNA mini extraction kit, according to the manufacture protocol, and then the polymerase chain reaction (PCR) was used to amplify bacterial DNA. The primers of *E.faecalis* selected for this study was **F**:TCAAGTACAGTTAGTCTTTATTAG, **R**:ACGATTCAAAGCTAACTGAATCAGT, with 940bp these primers were provided in lyophilized form, dissolved in sterile deionized distilled water (according to the manufacturer instruction) to give final concentration of 100pmol/μl and stored in deep freezer until used in PCR amplification (14).

B. Antibiotic sensitivity test (Disk diffusion method): The Kirby-Bauer method was adopted as follows to detect the antibacterial activity against the antibiotics. Antibiotic discs used in this study is Imipenem, Gentamicin, Tetracycline, Cephalothin, Chloramphenicol, Ciprofloxacin, Rifampin, Trimethoprim, Vancomycin, Erythromycin, Clindamycin, Ampicillin. Mueller-Hinton agar (MHA) was placed into plates and allowed to set to a depth of approximately 4 mm. The plates were kept at 4°C until they were used. To make the inoculums, colonies from overnight cultures of tested isolates were transferred to a tube containing 5 ml of normal saline and adjusted to McFarland standard tube No. 0.5 to generate a culture with $1.5*10^8$ CFU/ml, by dipping a sterile swab into the inoculums and then streaking the MHA plate. Rotating the plate through at a 60-degree angle after each application. Finally, the swab was wrapped around the agar surface's edge. After that, the inoculums were left to dry at room temperature for a few minutes with the lid closed. The antibiotic discs (table 1) were placed on the inoculation plate using sterile forceps. The discs should be placed on the agar surface and gently pressed down. The plates were incubated anaerobically for 18-24 hr. at 37ºC (6). The inhibition zones were measured in millimeters (mm) after incubation. By comparing the width of each antimicrobial disk to the standard inhibition zone (8), the diameter of the inhibition zone was translated into sensitive (S), intermediate (I), and resistant (R) categories.

C. Biofilm detection of *Enterococcus faecalis*
The ability of *E.faecalis* isolates to produce biofilm was evaluated by crystal violet staining technique in polystyrene micro-titer plate. The optical density (OD) was measured at 490nm. The overnight culture of *E.faecalis* was subculture in Tryptic soy broth (TSB) supplemented with 1% glucose and incubated at 37°C for 18hr. After incubation growth was diluted to 1.5×10^8 CFU/ml. A volume of 200μl of each bacterial isolate suspension was transferred to its corresponding well in a flat-bottomed 96 well polystyrene plate. The plate was incubated at 37°C for 24hr. Then wells washed 3 times with 200μl of sterile phosphate buffer saline (ph:7.2). biofilm fixed with heating at 60°C for 15minates then 200μl of crystal violet solution (0.1% wt./vo.) was added to all wells and left for 15min. excess crystal violet was rinsed with distilled water and air dried overnight. Bounded crystal violet was released by adding 200μl of 96% ethanol (27). Absorbance was measured by spectrophotometrically at 630nm (A_{630}) and was proportional to biofilm biomass. Negative control well contained TSB only. Calculation of biofilm formation was accomplished according to Table (1).

Table 1. Calculation of biofilm formation by bacterial isolates

OD Value	Biofilm formation
<OD	Non
ODc < ODt < 2ODc	Weak
2ODc < ODt < 4ODc	Moderate
4ODc < ODt	High

RESULTS AND DISCUSSION
A. Bacterial isolation and identification:
Isolates that were identified as *E.faecalis* which showed positive growth on Pfizer selective ager , the colonies appeared circular, grey colonies of 2mm in diameter, have black point in the center of colony and bordered by black zones (Figure1), also all isolates were examined under microscope, the isolate showed Gram-positive ovoid or spherical cell that arranged singly, pair or in short chain (Figure 2), and finally the bacterial isolates gave negative results for catalase and have ability to grow in 6.5% NaCl, pH 9.6, as well as growth at 10 and 45ºC

Figure 1. *E.faecalis* **on Pfizer Selective Enterococci medium**

Figure 2. *Enterococcus* **under microscope (100x) as Gram positive cocci**
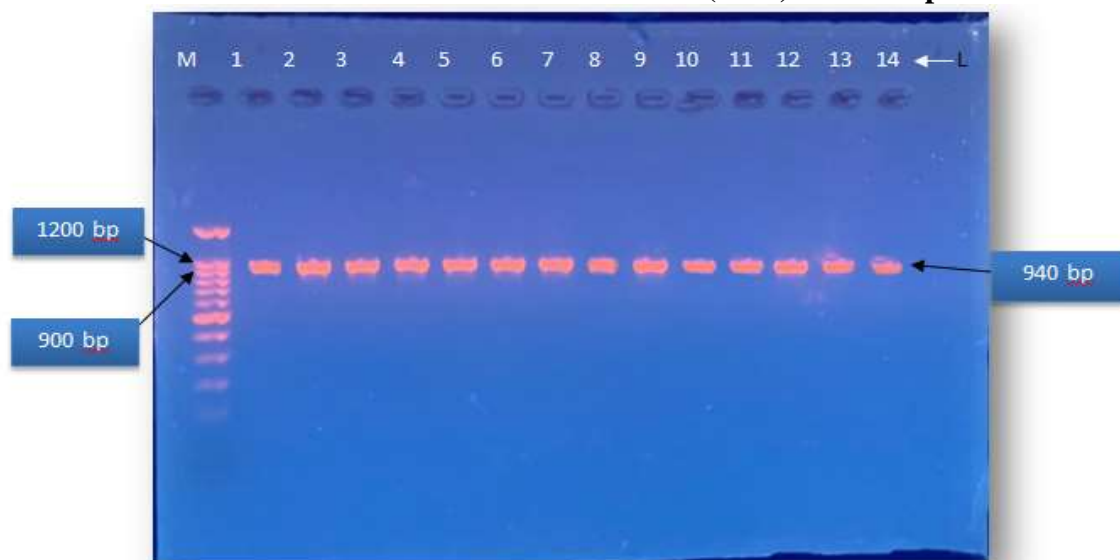

Figure3. Agarose gel 1.5% electrophoresis of PCR amplified products for *E. faecalis* gene stained with ethidium bromide (75volt/cm for 50min). Lane M: l500bp Ladder. Lane L 1-14: positive result with positive bands of 940bp of *E. faecalis* gene

The results of molecular detection by PCR showed that 39(65%) from 60 isolates had *E. faecalis* gene. The positive result of *E. faecalis* gene was confirmed by 1.5% agarose gel electrophoresis stained with ethidium bromide, electrophoresed in 75 Volt. For 50min., and photographed under (UV) trans illuminator. The result showed that *E. faecalis* gene bonds detected a 940bp region when compared to the DNA ladder. The bands looked sharp, single not dispersed and no smear which might result from DNA degradation. As shows in (Figure 3). Study targeting the same gene was also detected by Dilsah Cogulu *et al.,* (9) found 32% of samples have *E. faecalis* gene and Hossein S. K. and Ashraf M. M. (20) found 56.12% of isolates were *E. faecalis.*

B. Antibiotic sensitivity test: - (Disk diffusion method): The result showed that 100% of *E. faecalis* isolates were sensitive to Imipenem, whereas 100% of *E. faecalis* isolate were resistant to Erythromycin, Clindamycin, Tetracycline and Trimethoprim. Other antibiotics sensitivity showed significant difference ($P<0.05$) as most *E. faecalis* were resistant to Rifampin, Cephalothin, Gentamicin except for a very few that were intermediate. As well as 76% of *E. faecalis* isolates showed sensitivity to Vancomycin and the residual 24% isolates was intermediate, also 76% of *E. faecalis* isolates showed sensitivity to Chloramphenicol and the other are resistant, finally 65% of *E. faecalis* isolates showed resistant to Ciprofloxacin and 29% are sensitive and the rest are intermediate (Figure 4).
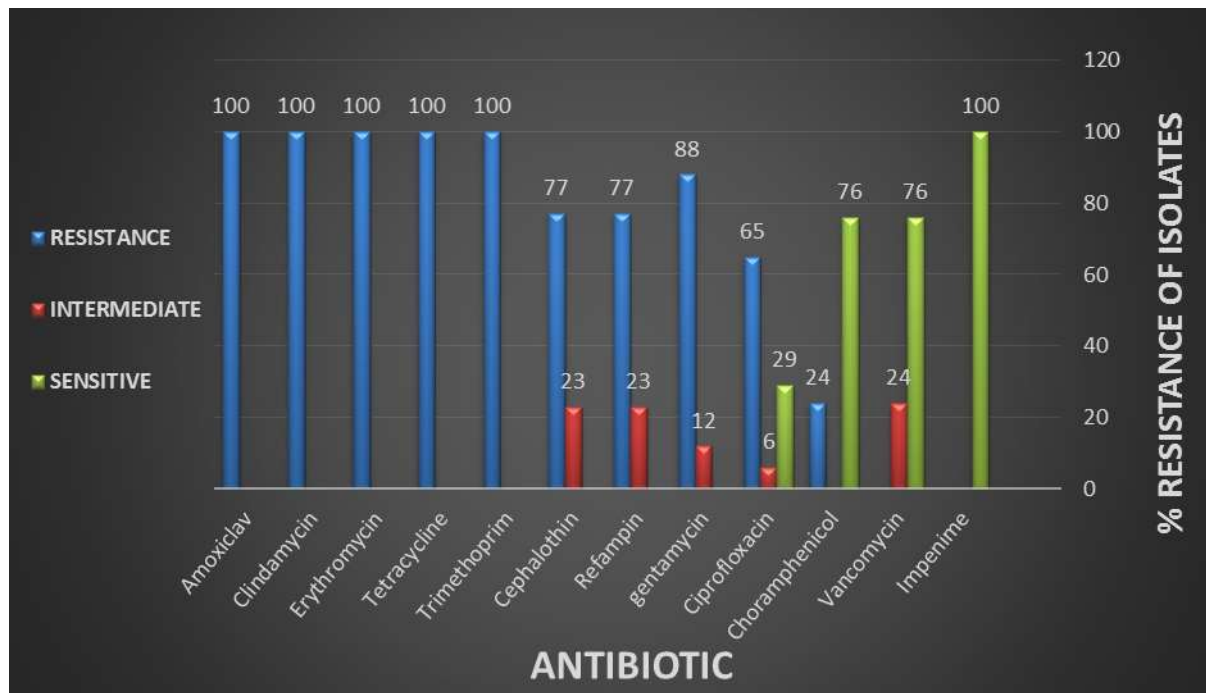
Figure 4. Antibiotic susceptibility for *E. faecalis* isolates from root canal sample against antibiotics

Results of antibiotic susceptibility confirmed that 100% resistant to Amoxiclav (Amoxicillin-clavulanic acid) complex which belongs to β-lactams antibiotics, this complex is a larger molecule than Amoxicillin, may experience greater difficulty in permeability and overall transport across the microbial cell wall/membrane barrier, Beta-Lactamase inhibitors such as clavulanate are used to extend the spectrum of penicillins against b-lactamase producing organisms (2). The result of this study is similar to that done by Haider S., (17) that showed 100% resistance to Amoxiclave. Also all *E.facealis* showed resistance to both Clindamycin and Trimethoprim by (100%), because the Enterococci acquire exogenous factors (Exo gene) that help the bacteria to resist (28). The results of this study are partially similar with the study of Al-Saadi (3), where the researcher obtained isolates that were resistant by (100%) to each of the Clindamycin and Trimethoprim, Also with the results of the study of Al-Ta'i (4), as most of the isolates in the researcher's study were resistant to the Trimethoprim with a percentage of (91%). Enterococci isolates showed resistance 100% to Tetracycline, this resistance is achieved by 2 mechanism: by pumping the antibiotics out of the cell and protecting the ribosome by changing the configuration so this preventing the binding the antibiotics (28). This result was in

agreement with results of some local and international studies, where Hayes et al., (19) obtained resistant isolates by 94%, Dupre et al., (13) indicated that the percentage of resistance of isolates of this bacteria isolated from different clinical cases to Tetracycline antagonists amounted to (73%) and these results were partially consistent with what we found in the current study. Enterococci isolates in the current study showed resistance to Rifampicin (77%), and resistance occurs as a result of a mutation that leads to a change in the structure of the RNA polymerase enzyme and thus reduces the binding affinity (5). Also 88% of *E. faecalis* in study showed intrinsically resistant to low levels of Gentamicin belong to aminoglycosides due to inefficient active transport across the cytoplasmic membrane. Thus, aminoglycosides alone are considered inactive in the treatment of Enterococcal infections and are usually combined with inhibitors of cell wall synthesis which may facilitate their uptake (12). *Enterococcus faecalis* show low resistant to Vancomycin (24 % intermediate resistance) that belong to glycopeptide, the main mechanism of glycopeptide resistance in Enterococci involves the alteration of the peptidoglycan synthesis pathway. Such alterations can lead to variable expressions of glycopeptide resistance. The ability to induce such alterations is related to several genes

harbored on mobile genetic elements and/or chromosomally encoded regions of different *Enterococcus* species (22). The results also showed 77% of *E. faecalis* isolates was resistant to the Cephalexin which is a first generation cephalosporin's, *E. faecalis* are intrinsically resistant to Cephalosporin, antibiotics that target cell wall biosynthesis (20). While Imipenem susceptibility percentage was 100% of *E. faecalis* isolates, Imipenem is related to Carbapenems class which are unique class, because they are relatively resistant to hydrolysis by most β-lactamases, in some cases act as "slow substrates" or inhibitors of β-lactamases, and still target penicillin binding proteins. Mechanisms of resistance to Carbapenems include production of β-lactamases, and mutations that alter the expression and/or function of PBPs. In Gram-positive cocci, Carbapenem resistance is typically the result of substitutions in amino acid sequences of PBPs or acquisition production of a new Carbapenem-resistant PBP (24).

C. biofilm detection of Enterococcus feacalis
The ability of *E. faecalis* isolates to form biofilm were evaluated using pre-sterilized 96-well polystyrene micro-titer plates and then absorbance was determined at 630nm for the determination of the degree of biofilm for studied isolates that adhered on the surface of the micro-titer well. The results indicated that each isolate showed a different potential to form biofilm under the same conditions of experimentation. Only seventeen samples that showed high antibiotic resistance were chosen from the latter 39 isolates to preform biofilm test for the detection of biofilm formation. The results obtained from root canal samples are shows in Figure 5. About 23.5% of the tested isolates were weakly producers of biofilm, while 47% were moderate and 29.5% were strongly biofilm producers, with significant differences at (P<0.01).
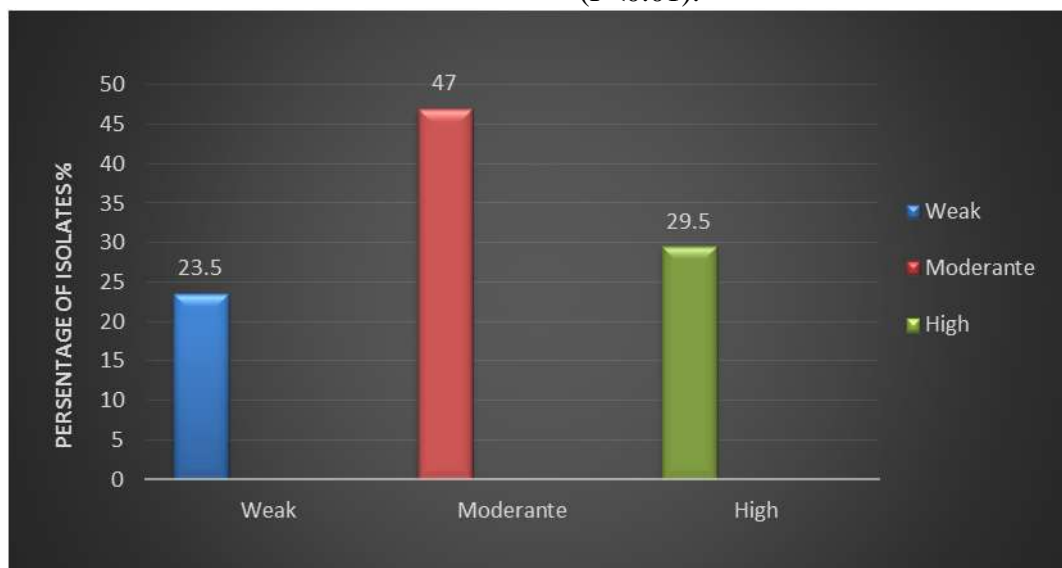

Figure 5. Distribution of *E. feacalis* among weak, moderate and high groups of biofilm production form root canal isolates

A study by M. Akbari Aghdam et al., (1) presented 49% of the isolates as strong biofilm producer, and 42% displayed moderate biofilm formation while, in 9 % weak or no biofilm was observed, which is different from the results of current study, while Gustavo O. et al., (16) Collected 20 isolate, *E. faecalis* strains were classified as follows: 6 (30%) weak, 8(40%) moderate and 6(30%) strong biofilm producers. which agreed with result of current study. The importance of biofilm development in bacterial pathogenicity is obvious from the research. Biofilms serve a key role in colonization during infection, giving bacteria the chance to build medication resistance. Biofilm-forming bacteria are encased in a well-hydrated matrix made up of secreted exopolymeric compounds, proteins, and nucleic acids from dead-lysed cells, which protects them from immune clearance and antibiotic treatment (15). Bacterial biofilms can form on body implant devices as well as tissue epithelia. It's possible that the increased use of antibiotics and implant devices in

hospitals contributes to the enrichment of features in clinical pathogens that promote biofilm formation (11).

Conclusion

From all previous founding and results of this study could be conclude that the biofilm production by *E. faecalis* isolated from root canal had a very important role in increasing the antibacterial activity and causing infection of root canal that could be hard to treat, that lead to more complications for patients with infected root canal.

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