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Detection of Endocarditis Associated Pili Genes in *Enterococcus Faecalis* Clinical Isolates

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Abstract

Out of 207 specimens,118 *Enterococcus faecalis* isolates were obtained from different sources (urine, root canal, wound, vagina, and blood). The commonest sites of infections were the root canal (50.8%), followed by the urinary tract (38.1%), wound (8.4%), and the vagina (2.5%). Urine, root canal, and wound samples from individuals aged 20–40 years old showed the highest prevalence of *Enterococcus faecalis* isolates, except for the vagina, where those over 40 years old recorded the highest rates of isolates. According to gender, females had the highest prevalence of Enterococcus faecalis isolates in urine, root canal, and vaginal samples, with the exception of the wound source, where males had a higher rate of isolates than females. The antibiotic susceptibility test results indicated that all Enterococcus isolates were resistant to these antibiotics. All Enterococcus isolates (100%) demonstrated resistance to Azithromycin, Clarithromycin, Erythromycin, Doxycycline, Minocycline, Tetracycline, and 64.7% to Gentamicin. On the other hand, 100% of the isolates showed susceptibility to Linezolid, Daptomycin, Teicoplanin, Tigecycline, and Vancomycin, while 58.8% showed susceptibility to Streptomycin, 76.4% to Ciprofloxacin, and 94.1% to Ampicillin. The PCR technique identified the presence of *epbA* and *epbC* genes in 28 *Enterococcus faecalis* isolates. The results of PCR showed that all of the isolates had genes *epbA* (111bp) and *epbC* (85bp).

Keywords: Endocarditis, Pili related to biofilm, *Enterococcus faecalis*, *EbpA, EbpB, EbpC*.

1. Introduction

Enterococci are gram-positive pathogens that can cause simple infections of the urinary tract and wounds as well as serious and life-threatening illnesses like endocarditis [1, 2]. Enterococci, which reside in the intestinal system and the human oral cavity, often lead to opportunistic infections in healthcare facilities [3]. Under a microscope, one can observe Enterococcus either individually, in pairs, or in short chains [4]. These organisms are catalase and oxidase-negative. Enterococci grow over a high-temperature range (5 °C–65 °C) and pH range (4.5–10.0) [5]. They

will also grow when 6.5% NaCl is present. These features can be distinguished from streptococci. Additionally, enterococci hydrolyze esculin in the presence of 40% bile [6].

Enterococcus faecalis is a lactic acid bacterium widely used in food fermentation, particularly fermented dairy products. In recent years, considerable quantities of E. faecalis strains have been identified in traditional dairy products. The generation of acid by enterococci because of fermentation was successful. but not gas production [7]. Clinical microbiology is increasingly focusing on enterococci due to their considerable inherent resistance to available antibiotics [8]. *E. faecalis* is responsible for up to 90% of all human enterococcal infections [9]. However, Enterococcus faecium is responsible for the remaining infections caused by *Enterococci spp*. Researchers have established that bacterial adhesion to host cells, such as Girardi heart cells and human urinary tract epithelial cells, is the first step in the pathogenesis of many illnesses [8]. In a study using a rat endocarditis model [10], Previous studies found that an *E. faecalis* OG1RF pilus-deficient disruption mutant, called endocarditis and biofilm-associated pili (Ebp), is less likely to cause infection and form biofilm. Researchers discovered attenuated Pilus-deficient mutants in both catheter-associated and non-catheter-associated UTI models. Ebp pili are also thought to play more than one part in the infection process because they help *E. faecalis* OG1RF attach to fibrinogen, collagen, and human platelets [11]. Three subunits, EbpA, EbpB, and EbpC, make up Ebp. The major pilus component is EbpC, followed by EbpB at the pilus' base and EbpA at the pilus' tip [12, 13]. The ebpABC locus upstream of ebpABC encodes EbpR, which positively regulates the three subunits [14]. The current study looks into how common biofilm-associated pili and endocarditis are in clinical enterococci isolates and uses PCR to find the genes that code for them.

2. Materials and Methods

2.1. Specimen collection

The current study collected 207 samples including 24 from healthy individuals' oral cavities, 62 from patients' oral cavities, 58 from urine, 3 from the vagina, 10 from contaminated wounds and burns, and 50 from blood from endocarditis patients. All specimens collected from private laboratories, dentist clinics, Medical City Hospital, and Yarmouk Teaching Hospital between August 2022 and October 2022. Using sterile swabs, we took samples in a sterile setting. The swabs were delivered right away to the lab, where they were implanted in the proper medium.

2.2. Isolation of *Enterococcus faecalis*

The samples were collected and cultivated for 24 hours at 45ºC on Pfizer's proprietary Enterococcus media. It acts as a selective medium for Enterococci spp. growth and isolation, and then a single colony from brown-black colonies was selected to perform additional biochemical assays that validated the identification of bacterial isolates [15, 47]. Bergy's Manual of Systematic Bacteriology, 2nd edition [16], which relies on morphology, biochemical testing [17], and the Vitek2 method, guided the identification of all *E. faecalis* isolates.

2.3. Statistical investigation

To determine the effect of various elements on research parameters, the Statistical Analysis System-SAS (2012) program was utilized. Mean, standard error, ANOVA, and LSD were used to compare differences between study groups and assays. P-values $\leq$ 0.05 were considered

significant, while p-values > 0.05 were considered non-significant. P-values ≤ 0.001 were considered highly significant.

2.4. Antibiotic susceptibility test

The susceptibility of bacterial isolates was tested by the VITEK-2 system (AST-P592). The card (AST-P592) included Ampicillin, Gentamicin High Level (synergy), Gentamicin High Level (synergy), Ciprofloxacin, Azithromycin, Clarithromycin, Erythromycin, Linezolid, Daptomycin, Teicoplanin, Vancomycin, Doxycycline, Minocycline, Tetracycline, and Tigecycline antibiotics. The isolates were grown on Pfizer Select Enterococcus Agar and then incubated for 24 hours at 37ºC. On blood agar, the ABC streaking method was used to obtain pure colonies. To achieve an inoculum density of 0.50-0.60 McFarland, the tested organism (5–10 colonies) was suspended in 5 ml of normal saline using a cotton swab. A suspension of the tested organism was manually loaded into the vitek2 system (AST-P592 card) and incubated for 6 to 8 hours. During this time, the cards were read by kinetic fluorescence measurement to check the growth of each well every 15 minutes [18].

2.5. Detection of *epbA* & *epbC* genes in by PCR Technique

2.5.1. DNA Extraction from *E. faecalis* isolates

All *E. faecalis* isolates were cultivated on Pfizer Selective Enterococcus Agar after being incubated at 37 oC for 24 hours. Then, tubes holding 5 ml of sterile brain heart infusion broth were filled with bacteria. Using the ABIO pure extraction methodology, the following steps were taken to extract genomic DNA from bacterial growth: A- For pellet cells, 1ml culture overnight at 13000 rpm for 2 minutes. The supernatant was then discarded. B- To the pellet, 100µl of Nuclease-free water and 100µl of Lysozyme solution were added and vortexed for gram-positive bacteria. C- Incubated in a 37°C water bath for 30 minutes. D- Samples were centrifuged at 13000 rpm for 2 minutes after incubation. The supernatant was then discarded. E- 20 µl of Proteinase K solution (20 mg/ml) and 200 µl of Buffer BL were added to the sample for protein digestion and cell lysis. The tube was then vigorously vortexed and incubated at 56 C for 30 minutes. F- The sample was combined with 200µl of absolute ethanol, and the combination was properly blended using a pulse vortex. G- After carefully transferring each mixture to the small column, it was centrifuged for one minute at 6,000 x g or higher (>8,000 rpm), after which the collecting tube was changed. H- The tiny column was filled with buffer BW 600µl, centrifuged for one minute at 6,000 x g above (>8,000 rpm), and the collection tube was changed. I- It was a TW 700µl from Buffer; centrifuged for 1 minute at 6,000 x g above (more than 8,000 rpm). The collecting tube's mini-column was installed in its place when the pass-through was eliminated. The mini-column was placed in a new 1.5-ml tube after being centrifuged at maximum speed (>13,000 x g) for 1 minute to remove any remaining wash buffer. K-100 µl was added to buffer AE, and it was allowed to sit at room temperature for one minute before centrifuging at 5,000 rpm for 5 minutes.

The concentration of extracted DNA was measured using a Quantus Fluorometer to determine the sample quality for subsequent uses. 200 l of diluted Quantifluor Dye were combined with 1 l of DNA. DNA concentration readings were found following a 5-minute incubation period at room temperature. At 260nm and 280nm, the concentration and purity of the isolated DNA sample were evaluated.

2.5.2. Primer preparation

Table 1. The primer preparation for *epbA* and *epbC* genes

Primer Name	Sequence 5`-3`
ebpA-F	AAAAATGATTCGGCTCCAGAA
ebpA-R	TGCCAGATTCGCTCTCAAAG
ebpC-F	CGGTCATACCGACGACCAAA
ebpC-R	TGTCACATCGCCATCGACTT

2.5.3. Protocol for Thermal Cycling and Reaction Setup

The amplification of genes epbA and epbC in monoplex PCR technique: It was conjugate to combine the isolated DNA with the primers and PCR premix. The PCR mixture was set up in a total volume of 20µl (Table 2).

Table 2. Contents of PCR reaction**.**

Materials	Stock	Final	Volume
Master Mix	2	1	10
Forward primer	10µM	0.5	1
Reverse primer	10µM	0.4	1
DNA	ng/µl		2
Nuclease Free Water			6
Aliquot per single rxn	2 µl of temp and 18 µl of master mix per tube		

2.5.4 PCR Program

PCR reaction tubes were placed into thermos cycler DNA and amplified by using PCR, as indicated in (**Tables 3 and 4**)

Table 3. A program that amplifies the *epbA* according to:

Steps	Temperature °C	Time (m:sec)	Cycle
Initial Denaturation	95	5:0	1
Denaturation		0:30	30
Annealing	58	0:30	
Extension	72	1:0	1
Final extension		7:0	
Hold	10	10:0	

Table 4. Program used to amplify the *epbC* according to:

Steps	Temperature °C	Time (m: sec)	Cycle
Initial Denaturation	95	5:0	1
Denaturation		0:30	30
Annealing	61	0:30	
Extension	72	1:0	1
Final extension		7:0	
Hold	10	10:0	

Following PCR amplification, 1.5% agarose gel stained with ethidium bromide was used to establish the occurrence of amplification using agar-gel electrophoresis. Electrical power was put on at 100 v/m Amp for 60 minutes.

3. Results and Discussion

One hundred and eighteen isolates of *E. faecalis* were collected from various sources (urine, root canal, vagina, blood). The commonest sites of infections were root canal (50.8%), followed by urinary tract (38.1%), wound (8.4%), and then vagina (2.5%), as shown in Table 5. The macroscopic and microscopic studies revealed that the bacterial isolates matched *Enterococcus* spp. The colonies on Pfizer Selective *Enterococci* media were round and grayish, and on blood agar, the isolates gave (β-hemolysis). Under a light microscope (100X), the isolates showed up as single, pair, or short chain-shaped gram-positive cocci with spherical or ovoid cells that did not form spores [19]. These isolates were catalase-negative and oxidase-negative. *Enterococci* isolates were grown at 10°C, 45°C, and pH 9.6. They can also proliferate when 6.5% NaCl is present. These features set them apart from streptococci. [6]. The biochemical test revealed that 118 (57%), out of 207 clinical samples passed the test.

Table 5. Distribution of *E. faecalis* among clinical samples.

Samples	Number of *E. facials* from each sample (%)	Number of *E. facials* from total isolated bacteria (%)
Urine	45/58 (77.5%)	45/118 (38.1%)
Root canal	60/86 (69.7%)	60/118 (50.8%)
Blood	0/50 (0%)	0/118 (0%)
Wound	10/10 (100%)	10/118 (8.4%)
Vagina	3/3 (100%)	3/118 (2.5%)
Total	118/207 (57%)	118/118 (100%)

The urine, root canal, and wound samples showed a significantly high prevalence of *E. faecalis* isolates at ages 20-40, except for the vagina, which recorded the highest rates of isolates at ages >40 (Figure 1). The [20] study found a correlation between age and *E. faecalis* isolates from endodontic infections, with patients aged 10 to 30 years accounting for about 32% of all samples overall and 47% of all samples that yielded positive results. Patients aged 31–50 years had a rate of (45%), but patients aged 51-70 years had a low incidence (14%). Researchers recognized *E. faecalis* as a significant contributor to vaginal infections and a prevalent health issue. Antibiotic-resistant vaginitis caused the highest levels of *Enterococcus* infection in women between the ages of 26 and 35, resulting in vaginal discharge [21].

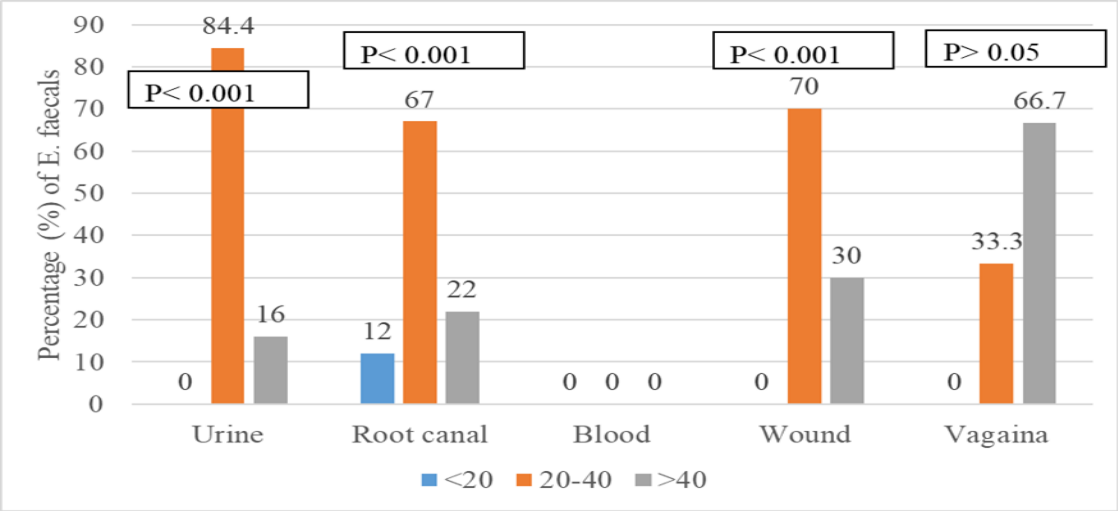

Figure 1. The prevalence of *E. faecalis* according to the age.

Differentiating between men and women, Figure 2 shows that the highest rate of *E. faecalis* isolates was found in females in urine, the root canal, and the vagina, but not in wounds. The highest rate of isolates was also found in males. [22] revealed that the percentage of vaginal *E. faecalis* isolates in swabs was 35.71%, accounting for 73.5% of the total isolates.

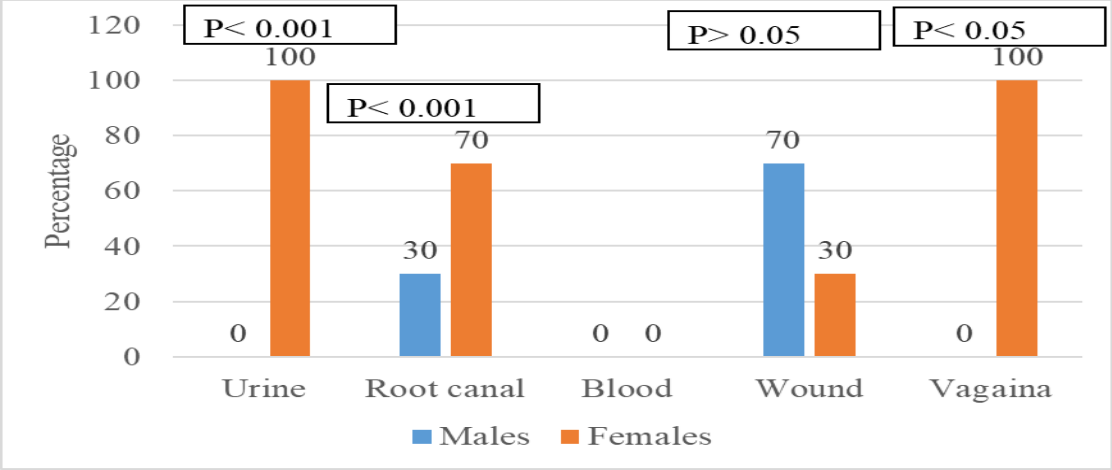

Figure 2. The prevalence of *E. faecalis* according to the gender.

3.1. Antibiotic susceptibility test (AST) of *E. faecalis*

The antibiotic susceptibility was determined for pathogenic *E. faecalis* isolates to 15 different antimicrobial agents by the VITEK-2 system (AST-P592). The antibiotic susceptibility test was determined by MIC value breakpoints [23]. All the results of AST are shown in Figure 3. The isolates showed various levels of susceptibility to different antibiotics. All of the *E. faecalis* isolates (100%) were found to be resistant to Azithromycin, Clarithromycin, Erythromycin, Doxycycline, Minocycline, and Tetracycline each, and 64.7% to Gentamicin. However, all isolates were susceptible to linezolid, daptomycin, teicoplanin, tigecycline, and Vancomycin each, 58.8% to streptomycin, 76.4% to ciprofloxacin, and 94.1% to ampicillin.

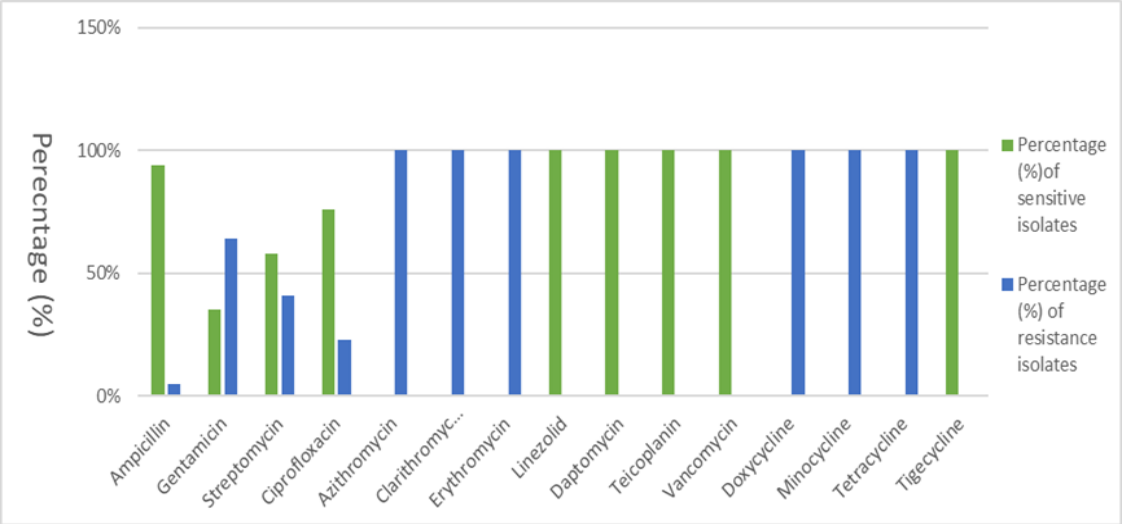

Figure 3. Antibiotic susceptibility for pathogenic *E. faecalis* isolates.

This study's erythromycin resistance was higher than that of [24] and [25], who found that 86.37% and 40.7%, respectively, were resistant to the antibiotic. A high resistance level was observed for Azithromycin and clarithromycin at 100%. This study's findings were higher as compared with the outcome of [26], which showed a 56.2% resistance rate for Azithromycin. The high level of resistance in strains of *E. faecalis* surpasses that of Erythromycin, Clarithromycin, and Azithromycin combined. The use of these medicines to treat human disorders, which may be the source of *E. faecalis*, is a significant risk factor for the development of antibiotic resistance among isolates. Therefore, monitoring the community's antibiotic usage is crucial [24]. The results showed that 100% of *E. faecalis* isolates were resistant to Tetracycline, Minocycline, and Doxycycline. One or more of the following factors typically cause tetracycline resistance: chromosomal mutations that enhance the expression of intrinsic resistance mechanisms, the acquisition of mobile genetic elements carrying tetracycline-specific resistance genes, and mutations in the ribosomal binding site. *E. faecalis* isolates were 64.7% resistant to Gentamicin. This result was lower than the findings by [27] and [28], which found that 80% and 88% of *E. faecalis* were resistant to Gentamicin. Gentamicin belongs to aminoglycosides. Cell wall formation inhibitors usually combine with gentamicin to increase its uptake, due to its low active transport through the cytoplasmic membrane. Gentamicin alone is generally regarded as insufficient for treating *Enterococci* infections. When used in conjunction with a drug that activates cell walls, such as ampicillin or vancomycin, this enzyme eliminates the synergistic activity of Gentamicin [29]. This study's sensitivity test resulted in 100% sensitivity to Vancomycin, a member of the glycopeptide group. According to the study cited in [28], 15% of *E. faecalis* were vancomycin-sensitive. As shown in [30] and [31], the percentages of vancomycin resistance were 71.43% and 90.6%, respectively. However, [32] found that the cell wall thickness of the isolate that had induced vancomycin resistance was higher than that of the sensitive isolates. Isolates of *E. faecalis* showed 100% sensitivity to Linezolid. This study's outcome was comparable to that of [33] and [34], showing 100% and 98% sensitivity to Linezolid, respectively. Linezolid is a new

oxazolidinone antimicrobial that has been approved for the treatment of infections caused by various Gram-positive bacteria. The mechanism of action of Linezolid involves a mutation that occurs in the 23S ribosomal subunit [35]. The results showed that Daptomycin, Linezolid, Tigecycline, Vancomycin, and Teicoplanin, the most effective antimicrobial agents, significantly outperformed *Enterococcus* spp. However, it is important to use this drug cautiously to prevent the emergence of bacterial resistance. The term "multidrug resistance" MDR refers to bacteria that exhibit resistance to three or more different antibiotic kinds [36]. According to [38], the emergence of MDR pathogens poses a serious threat to these classes of life-saving medications. This study revealed that 70.5% of *E. faecalis* isolates were multidrug resistant. The percentage of MDR in this result, which was similar to those reported by [36], was 66.6%. In Ethiopia, a study found that 75% of E. faecalis had multiple drug resistances [38].

3.2 Molecular identifications of *epbA* and *epbC* genes by PCR method

The PCR technique revealed the presence of the *epbA* and *epbC* genes in 28 *E. faecalis* isolates. The PCR findings revealed that all isolates had genes *epbA* (111bp) (**Figure 4**) and (**Figure 5**). Also, all the isolates possessed *epbC* (85bp) (**Figure 6**) and (**Figure 7**).

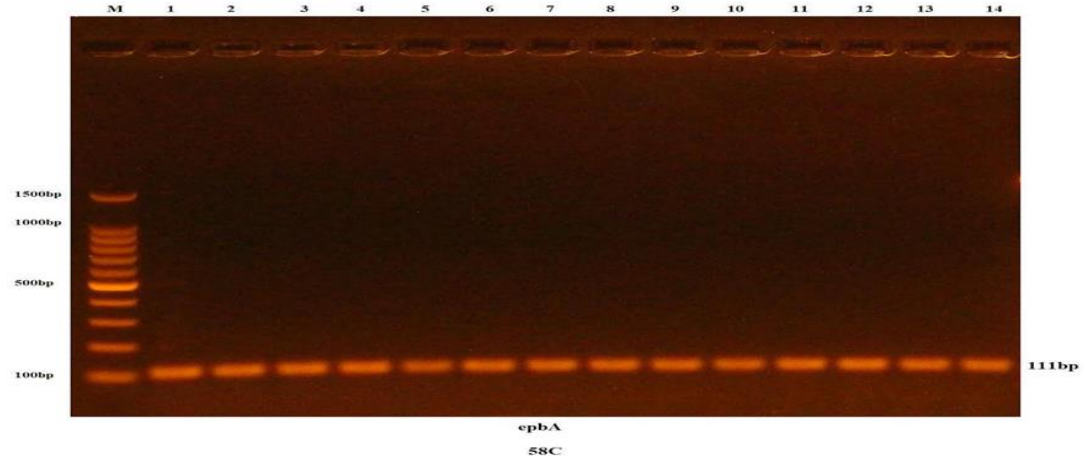

Figure 4. *E. faecalis* isolates with *ebpA* gene amplification were separated by electrophoresis on 1.5 percent agarose gel stained with 100 bp ladder marker Eth. Br. Lane's 1u-14u resembles 111bp PCR products**.**

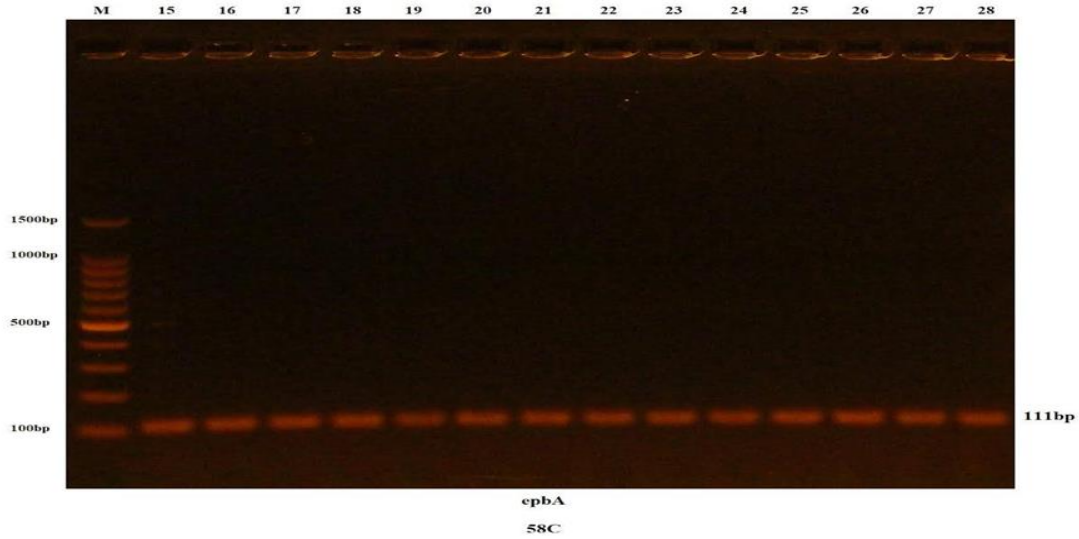

Figure 5. *E. faecalis* isolates with *ebpA* gene amplification on electrophoresis Lane's 15u-28u resemble 111bp PCR products.

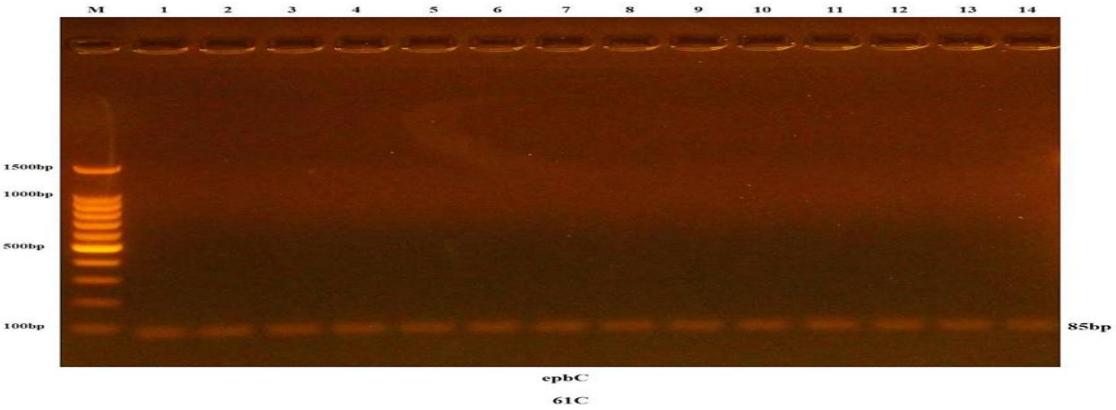

Figure 6. *E. faecalis* isolates with *ebpC* gene amplification were separated by electrophoresis on 1.5% agarose gels stained with Eth.Br. M: 100bp ladder marker.. Lane's 1u-14u resemble 85bp PCR products.

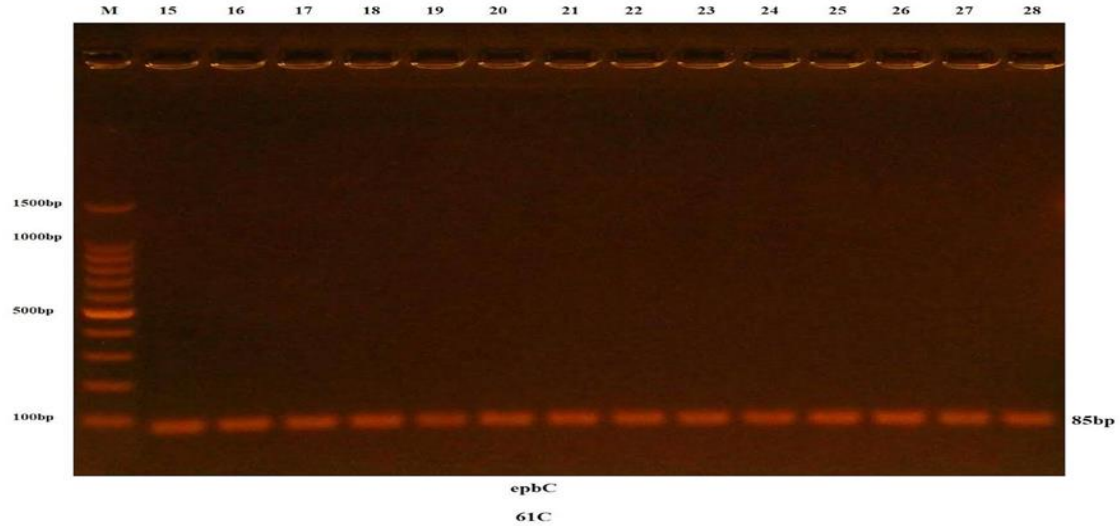

Figure 7. Results of the amplification of *ebpC* gene of *E. faecalis* isolates Lane's 15u-28u resemble 85bp PCR products**.**

Some studies targeting *epb* genes in *E. faecalis* [39] observed that the presence of *epb* was in almost all clinical *E. faecalis*, while [40] showed that among 55 (22 clinical and 33 faecal) isolates, the clinical isolates were negative for *epbA*. However, they detected the *epbA* gene coexisting with one or more virulence genes in two faecal isolates. *E. faecalis* Ebp-associated pili may promote colonization and attachment to the uroepithelium, according to a report [41]. [42] and [11] showed that *EbpA* mutations in its N-terminal domain reduced the development of Ebp-associated biofilms both in vitro and in vivo, as well as Catheter-associated Urinary Tract infection (CAUTI) in mice. The tip of EbpA, which also regulates host fibrinogen and collagen attachment, causes endocarditis, CAUTI, and UTI. In a CAUTI mouse model, the findings demonstrated the significance of the *E. faecalis* Ebp pilus and its subunits for enterococcal pathogenicity. They also demonstrated that the metal ion-dependent adhesion site (MIDAS) motif in *EbpA* is critical for Ebp to function in living organisms. The biofilm-associated pilus (Ebp) operon and endocarditis, both of which are parts of E. *faecalis'* core genome, have been shown to be crucial in the pathogenesis of the organism.; *Ebp* pili are major contributors to *E. faecalis* capacity to cling to extracellular matrix components. Research has demonstrated the crucial role of the pilus tip, *EbpA*, in the development of pilus and biofilms, as well as in experimental infections. Studies [43, 44] demonstrated that the genes *srt* (pilus-associated sortase) and *epbA, ebpB,* and *ebpC* (endocarditis and biofilm-associated pili genetic) form the ebpABC operon, which significantly influences biofilm formation in *E. faecalis* strains.

4. Conclusions

This study showed that *E. faecalis* was a significant causative agent to root canal and UTI infections, especially at 20-40 years old. All these isolates (100%) had both *ebpA* and *ebpC* genes, which indicates their essential role in the pathogenesis of *E. faecalis* infections.

Conflict of Interest

Conflict of Interest The authors declare that they have no conflicts of interest.

Funding

There is no funding for the article.

Ethical Clearance

The study was conducted following the receipt of participant consent and ethical approval from the Ethics Committee of the Biology Department at the University of Baghdad's College of Science (CSEC/0922/0117) on September 30, 2022. This procedure is in line with the guidelines set forth by the Iraqi Ministry of Health and Environment.

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