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EXPRESSION OF ENDOCARDITIS ASSOCIATED GENES (EBPA AND EBPC) IN BIOFILM PRODUCER ENTEROCOCCUS FAECALIS ISOLATES

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Abstract

One hundred and eighteen isolates of *E. faecalis* were isolated from different sources (urine, root canal, vagina, blood).The ability of *E. faecalis* isolates to produce a slime layer was examined by culturing the bacteria on Congo-red agar plates. The results showed that 37% of *E. faecalis* isolates were slime producers; 19% of *E. faecalis* isolates were moderate slime producers, and 44% of the isolates were weak-non slime producers. Biofilm formation by *E. faecalis* isolates was achieved by a microtiter assay. Out of one hundred and eighteen isolates of *E. faecalis*, 69 isolates were biofilm producers; about 4% of isolates were strong biofilm producers; 19% were moderate biofilm producers and 35% were weak producers of biofilm. The expression of the *ebpA* and *ebpC* genes was detected in eight *E. faecalis* isolates. The *E. faecalis* isolates showed differences in the expression of both *ebpA* and *ebpC* genes. In biofilm producer isolates, the relative expression of the *ebpA* and *ebpC* genes was evaluated in comparison with the biofilm non-producer isolate which was selected as a control sample. The isolate (21R) from the root canal (a biofilm producer isolate) showed increasing in expression of the *ebpA* gene (6.681-fold change) , while the biofilm producer isolates (21R) from root canal and (19U) from urine showed higher expression of the *ebpC* gene , 1.765 and 3.411-fold change respectively. According to the clinical sources, the root canal group gave a high expression rate of the *ebpA* gene in comparison with the urine and vagina groups. While, the urine group gave a high expression rate of the *ebpC* gene, followed by the root canal and then the vagina groups.

Keywords: Endocarditis, Pili, *Enterococcus Faecalis*, EbpA, EbpC, Biofilm

INTRODUCTION

Enterococcus faecalis is a common gram-positive organism that causes nosocomial and community-based illnesses that is linked to significant morbidity and death. It is characterized by high environmental adaptation and developing antibiotic resistance (1,2) . There are no proteins or lipids in the cell wall of *E. faecalis* , but there is teichoic acid, which is resistant to drying, and allows the bacteria to survive in unfavorable environments, like those prevalent in a hospital (3,4) . There are many virulence-related elements of *E. faecalis*, it has been proposed that a significant element in the pathophysiology of enterococci infections is their ability to produce biofilm (5). *E. faecalis* attaches well to both biotic and abiotic surfaces like many other pathogenic bacteria, and has developed an antibiotic-resistant biofilm. Clinical *E. faecalis* strains identified from infected endocarditis patients were substantially more likely to form biofilms than clinical isolates from patients without endocarditis (6) .

E. faecalis has many adhesins that help with adhesion and disease establishment, like endocarditis- and biofilm-associated pili (Ebp) and aggregation substance (AS) (7) . Ebp consists of three subunits: EbpA, EbpB, and EbpC. EbpC is the main subunit of pilus , with EbpB at the pilus' base and EbpA at the tip . Ebp pili appear to play various roles during infection especially, endocarditis and urinary tract infections, activating the expression of the endocarditis and biofilm-associated pilus operon (*ebpABC*) can enhance biofilm formation (8-10).

Since the Ebp pili has a role in the pathogenicity of Enterococci, this study aimed to evaluate the expression of endocarditis and biofilm-associated pili among *E. faecalis* biofilm producer isolates in comparsion with nonbiofim producer isolate.

MATERIALS AND METHOD

Enterococcus faecalis isolation

The samples (207 specimens) which include, 24 oral cavity specimens from healthy people, 62 oral cavity specimens from patients, 58 urine, 50 blood, 3 vagina and 10 from contaminated wounds and burns were collected and cultivated for 24 hours at 45 °C on Pfizer's proprietary enterococcus media. Then a single colony from brown-black colonies was selected to perform additional biochemical assays(11) . All *E. faecalis* isolates were identified according to Harley and Prescott (12) , based on morphology, biochemical testing[13] and the vitek2 method .

Ethical approval

The study was conducted after receiving agreement from the participants and ethical approval from the College of Scence research ethics committee at the University of Baghdad (Ref.: CSEC/0922/0117) .

Determination of bacteria's capacity to form biofilm

A. Congo-Red Media

Isolates were cultured on Congo red agar (CRA) plates to study slime layer producion at 37°C for 24h . The black colonies on CRA, were considered slime-producing isolates, while the red colonies were categorized as non slime- producer and the gray classified as moderate slime producing isolates(14).

B. Icro-Titer Plate

A semi-quantitative adhesion assay was used to measure the biofilm formed by the bacterial isolates (15) . The bacterial isolates from overnight Brain Heart infusion (BHI) broth established at 37°C were diluted to 1:100 in BHI with 2% glucose (w/v) . 200µl of the cell suspensions was placed in a 96-well micro titer plate with a U-bottom. For negative control, sterile BHI alone was used , and then the plates were incubated aerobically at 37°C for 24 hours. The experiment was done in triplicates . The micro titer wells were rinsed twice with phosphate-buffered saline (PBS) and 95% ethanol was used to fix adherent bacteria, and 1% (w/v) crystal violet solution was used to stain them for 5 minutes. The microtiter plate was washed and dried then the optical density at 570 nm (OD_{570}) was measured using an automated ElISA reader ; the isolates adherence was classified according to Atshan et al. (16) . The cutoff value(average OD_{570} of negative control + 3× standard deviation (SD) of negative control) was 0.048 , the isolates were considered as strong biofilm producer ($OD_{570} > 0.192$) , moderate biofilm producer ($0.096 < OD_{570} \leq 0.192$) , weak biofilm producer ($0.048 < OD_{570} \leq 0.096$) and non- biofilm producer ($OD_{570} \leq 0.048$) depending on their obsorbance at570 nm .

Detection *ebpA* and *ebpC* genes in *E. faecalis* isolates

DNA was extracted from *E. faecalis* isolates , The primers : epbA (F: AAAAATGATTCGGCTCCAGAA , R: TGCCAGATTCGCTCTCAAAG), epbC (F: CGGTCATACCGACGACCAAA, R: TGTCACATCGCCATCGACTT) were used .The genes *epbA & epbC* were amplified by monoplex PCR technique.

Expression of *ebpA* and *ebpC* genes by *E. faecalis* isolates

The expression of *epbA*, *epbC* genes was detected in seven biofilm producer isolates of *E. faecalis* and non-biofilm producer *E. faecalis* isolate from wound , which was used as a calibrator . All isolates were activated on Brain Heart infusion broth , incubated at 37 °C for 24 hr. under aerobic conditions. The TRIzolTM Reagent's protocol was followed in order to extract RNA from the sample.The concentration of extracted RNA was detected using a Quantus Fluorometer .

Real Time-qPCR

The expression of *epbA* and *epbC* genes was measured by using One Step RT-qPCR.The primers: *epbA* (F: AAAAATGATTCGGCTCCAGAA, R: TGCCAGATTCGCTCTCAAAG), *epbC* (F: CGGTCATACCGACGACCAAA, R: TGTCACATCGCCATCGACTT) and 23srRNA, housekeeping gene (F: CCTATCGGCCTCGGCTTAG, R: AGCGAAAGACAGGTGAGAATCC) were used.

The extracted RNA, Primers, and RT- qPCR master mix were mixed well by vortex. Reaction mixture (10µl) . The qPCR was prepared using (GoTaq® 1-Step RT-qPCR System). This was done under adequate thermocycling condition which were : 37°C for15 minutes to RT enzyme activation and 95°C for 5 minutes as first denaturation(1 cycle for each), followed by forty cycles of (95°C for 20 seconds for denaturation and 60°C for 20 seconds for Annealing, and 72°C for 20 seconds for Extension).

Analysis Gene Expression using Livak Method

The expression of two genes were assesed by using the comparative CT method 2-ΔΔCT (17).

RESULTS AND DISCUSSION

Enterococcus faecalis isolation

One hundred and eighteen of *E. faecalis* were isolated from various sources (urine, root canal, vagina,wounds, blood). The macroscopic and microscopic studies showed that the bacterial isolates were matched with Enterococcus spp, The colonies on Pfizer Selective Enterococci media were round, and grayish and on blood agar , the isolates gave (β-hemolysis). The results of the biochemical tests showed that out of 207 clinical specimens 118(57%) were *E. faecalis* . These results were confirmed by Vitek2 system. The commonest sites of infections were root canal 60/118(50.8%) followed by urinary tract 45/118(38.1%), wound 10/118(8.4%) and then vagina 3/118(2.5%) as shown in Figure (1) .

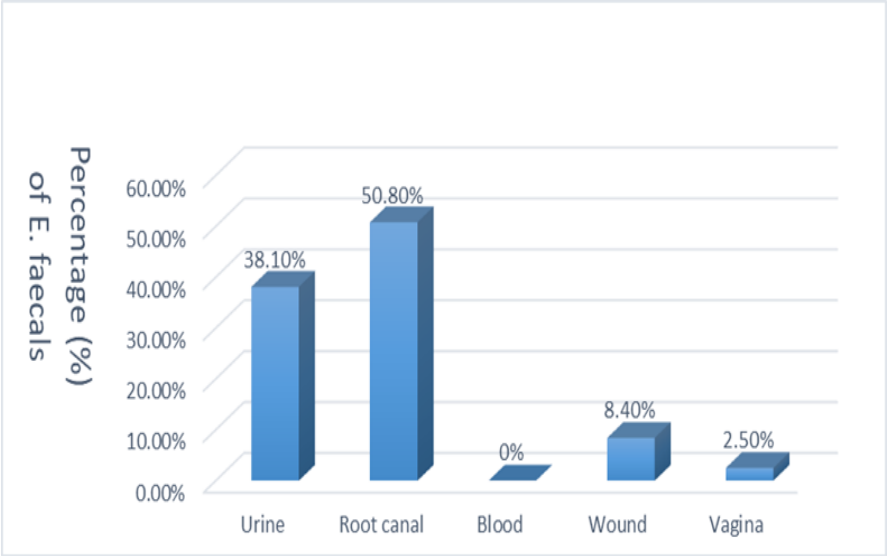

Figure 1: Distribution of *E. faecalis* among clinical samples

Enterococcus faecalis is associated with some serious diseases like urinary tract infections , bacteremia and endocarditis due to their virulence factors . The current study showed that out of 207 clinical specimens, 118(57%) were *E. faecalis* . This outcome exceeded the research findings by Hadi (18) which found the percentage from urine were 20% . Kadhem and Flayyih(19) , revealed that the percentage from urine was 35% . Yilema et al. (20) , found the *E. faecalis* prevalence rate from urine was 41.6%, while Praharaj et al.(21) and AL-Jmor (22) , reported that approximately 59% and 20.6% of *E. faecalis* were isolated from urine.

Detection of slime layer producing by *E. faecalis* isolates

According to the procedure outlined by Kouidhi *et al*.[14], slime layer producion by *E. faecalis* isolates was examined. All tested bacteria were cultured on CRA plates. The results showed that 37% of *E. feacalis* were slime producers (Table 1) indicating by forming of black colonies with dry consistency on CRA plates as showen in Figure (2) ; 19% were moderate slime producers of *E. faecalis* isolates (gray colonies) and 44% of *E. faecalis* isolates were weak-non slime producers (pink colonies).

Table 1: Slime Layer producing *E. feacalis* isolates

Charcater of isolate	Number	Percentage
Slime Producer	44/118	37%
Moderate Slime Producer	22/118	19%
Weak-non Slime Producer	52/118	44%

Figure 2: Slime Layer producting by *E. faecalis* on Congo-red agar medium (CRA)

The results showed that 37% of *E. feacalis* were slime producers . This study findings were higher than those of Hassan et al. (23) , who found that 11.7% of *E. faecalis* isolates produced slime. Kouidhi et al.(24) showed that 12 strains (71%) of the 17 identified *E. faecalis* produced slime ; while AL-Jmor (22) showed that all *E. faecalis* isolates from the vagina (100%) were slime producers. These variations in the results may be attributed to the number of specimens and isolates, the origins of the isolates, and the infection site . The biofilm development and slime formation appear to play a role in survival under challenging conditions, as evidenced by the availability of an extra glucose source in the medium or by an iron deficit (15).

Detection of Biofilm Formation by *E. faecalis* isolates

The potential of *E. faecalis* isolates to produce biofilm was demonstrated by the microtiter assay . Out of one hundred and eighteen isolates of *E. faecalis*, 69 isolates were biofilm producer. The results showed that 4% of the isolates were strong producers , 19% were moderate producers and 35% were weak producers (Table 2).

Table 2: Biofilm Production by *E. faecalis* isolates

Character of isolate	Number	Percentage
Strong Biofilm Producer	5/118	4%
Moderate biofilm Producer	23/118	19%
Weak Biofilm Producer	41/118	35%
Non-Biofilm producer	49/118	42%

In the current study, out of one hundred and eighteen isolates of *E. faecalis*, 69 isolates produced biofilms . A study by Aghdam et al. (25) showed significant biofilm formation in 49% of the isolates. Salih [(26) showed 36% of the tested urine isolates were strong biofilm producers and about 8% were weak biofilm producers , while about 29.5% were strong biofilm producers from root canal samples. Flayyih and Al-Hasani (27) concluded that both MRSA and MR-CONS isolates have the ability to produce a slime layer in different amounts of production, at the same time the two groups of isolates showed high and strong biofilm formation by microtiter assay method.

Detection and expression of *ebpA* and *ebpC* genes by *E. faecalis* isolates

Our study showed that both biofilm producer and non-biofilm producer *E. faecalis* isolates from urine , root canals , wound and vagina had *ebpA* and *ebpC* genes as shown in Table (3) .

Table 3: Characters of *E. faecalis* isolates

Sources of isolates	No.	*epbA* gene	*epbC* gene	biofilm prodction
Urine	3	+	+	Strong
	9	+	+	Moderate
	3	+	+	Weak
	3	+	+	Moderate
Root canal	1	+	+	Strong
	2	+	+	Moderate
	1	+	+	Weak
	2	+	+	Non
	1	+	+	Moderate
	1	+	+	Non
Wound	1	+	+	Non
Vagina	1	+	+	Moderate

Eight isolates of *E. faecalis* were chosen for studying the expression of *ebpA* and *ebpC* genes (Table .4). qPCR was performed and 23s rRNA was used as housekeeping gene. The melting point for *ebpA* and *ebpC* genes product exhibited one peak in melting point curve in all processes that mean it was pure in all of them, it ranged between 72°C and 95°C at 0.3°C/s. The amplification accuracy of genes product was noticed by the value of cycle threshold (Ct).

Table 4: *E. faecalis* isolates selected for real time PCR

isolates	Source	Characters
3 W	Wound	Non-producer for biofilm
19 U	Urine	Moderate producer for biofilm.
47 U	Urine	Strong producer for biofilm
42 U	Urine	Strong producer for biofilm.
31 R	Root canal	Moderate producer for biofilm
52 R	Root canal	Strong producer for biofilm
21 R	Root canal	Moderate for biofilm
231 V	vagina	Moderate producer for biofilm

The *E. faecalis* isolates showed differences in the expression of both *ebpA* and *ebpC* .The expression of *ebpA* gene in biofilm producer isolates was determined as fold change in comparison with biofilm non-producer isolate which was selected as a control sample. The isolate 21R from the root canal (biofilm producer) showed 6.681-fold change increase in comparison with the control isolate as shown in Table (5) .

Table 5: Gene expression of *ebpA* in biofilm producer *E. faecalis* isolates

Group	Isolate	H.K	*ebpA*	dct *ebpA*	ddct	Fold
Control	3	10.79	18.35	7.55	0	1
Vagina	231	10.28	20.70	10.42	2.87	0.137
Root canal	52	9.98	21.38	11.40	3.85	0.069
Root canal	31	7.95	16.85	8.90	1.35	0.392
Root canal	21	10.21	15.70	5.49	-2.74	6.681
Urine	42	8.91	19.51	10.60	3.05	0.121
Urine	47	11.36	23.85	12.48	4.93	0.032
Urine	19	8.63	16.98	8.35	0.8	0.574

H.K = housekeeping gene

The results of expression of *ebpA* gene of biofilm producer isolates in relation to the clinical sources showed that the root canal isolates group gave a high rate of expression in comparison with the urine and vagina groups (Figure 3).

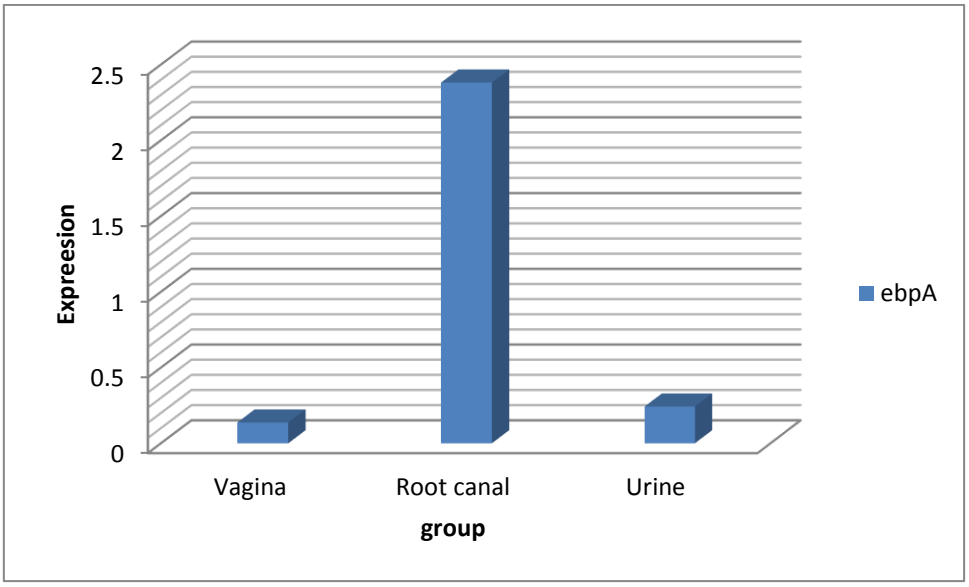

Figure 3: Eexpression of *ebpA* gene in biofilm producer *E. faecalis* isolates according to the clinical groups

Also, the *ebpC* gene expression in biofilm producer isolates was determined after normalization with the housekeeping gene and calibration with the control sample (biofilm non- producer isolate). The biofilm producer isolates 21R from the root canal and 19U from urine showed 1.765 and 3.411- fold change increase respectively , in comparison with the non-producer isolate of *E. faecalis* as shown in Table (6) .

Table 6: Gene expression of *ebpC* in biofilm producer *E. faecalis* isolates

Group	Samples	H.K	*ebpC*	dct *ebpC*	ddct	Fold
Control	3	10.79	18.04	7.24	0	1
Vagina	231	10.28	21.94	11.66	4.42	0.047
Root canal	52	9.98	21.02	11.04	3.8	0.072
Root canal	31	7.95	17.30	9.34	2.1	0.233
Root canal	21	10.21	16.26	6.06	-0.82	1.765
Urine	42	8.91	18.77	9.86	2.62	0.163
Urine	47	11.36	22.82	11.46	4.22	0.054
Urine	19	8.63	14.10	5.47	-1.77	3.411

H.K = housekeeping gene

The results of expression of *ebpC* gene of biofilm producer isolates in relation to the clinical sources showed that the urine isolates group gave a high rate of expression followed by the root canal and then the vagina groups (Figure 4).

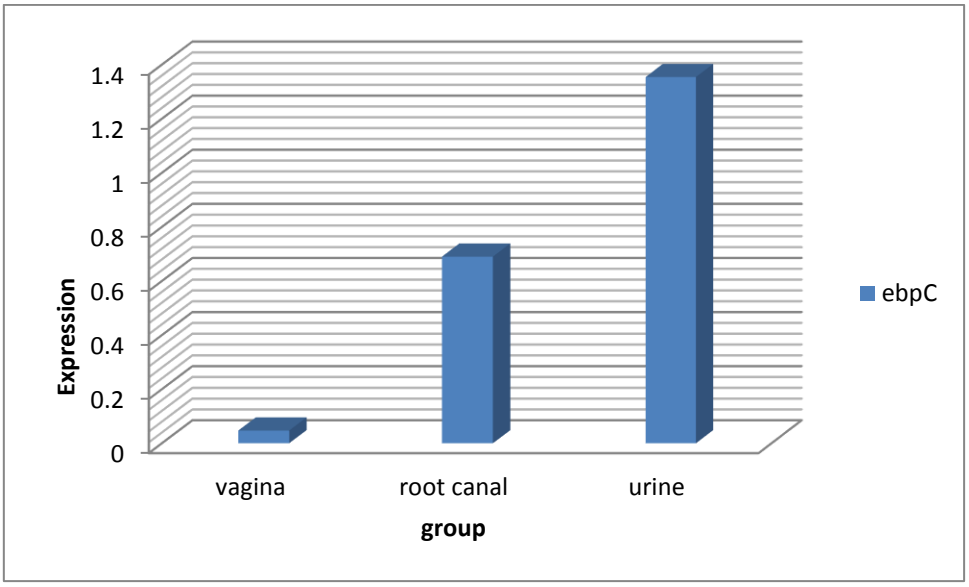

Figure 4: Expression of *ebpC* gene in biofilm producer *E. faecalis* isolates according to the clinical groups

Enterococcus faecalis has several virulence factors which have a role in biofilm formation[28] . It is clear from the research that biofilm growth is crucial for bacterial pathogenicity. When an infection occurs, biofilms play an essential role in colonization, giving bacteria the chance to develop drug resistance. Ebp pili aid in the formation and structure of biofilms, and synergizes with aggregation substance (AS) to promote maximal biofilm formation, during biofilm development(29, 30). According to Singh et al. (31) , the ebp pili promote colonization and adhesion to uroepithelium in a manner that is comparable to that of the fimbriae of *Proteus mirabilis* and *Escherichia coli*. The Ebp mutant showed a decrease in the effects in the animal endocarditis and UTI models due to the decrease in the biofilm formation than the parent strain. Also, EbpA mutations in its N-terminal region inhibited the development of Ebp-associated biofilms in mice, both in vivo and in vitro(32). Sillanpää et al.(33) showed that deletion of *ebpB*, which did not significantly change pilus polymerization, had little to no effect on biofilm formation . In contrast, e*bpA* and, to a lesser extent, *ebpC* are both necessary for full biofilm development.

E. faecalis encodes many adhesins that aid surface attachment and host colonization, but these adhesins are not expressed at the same time. Variation in expression can arise from cross-regulation; this explains the reason for the higher expression of a gene *ebpA* over a gene *ebpC* (34). Kot et al.(35) investigated the levels of expression of biofilm formation genes for two strains of MRSA , the first strain was a strong biofilm producer from wounds and the second one was a weak producer from the anus. The planktonic cells of the MRSA strain (moderate biofilm producer from the respiratory tract) were utilized as calibrator samples , the results demonstrated that gene expression levels varied between strains, suggesting that the biofilm expression regulation is affected by the nature of the bacterial strains. The expression levels of these genes in the biofilm for the strongly adhering strain were noticeably higher than for the weakly adhering strain, and the peak expression for some adhesin genes was primarily observed during the first 6 hours of development. With time, the high expression of genes producing binding factors in the first stages of biofilm formation diminished, perhaps as a result of decreased metabolism caused by food depletion

and unfavorable oxygen concentrations. In their investigation of the expression of genes involved in biofilm formation, Atshan et al.(36) also noted variations in the gene expression levels between four MRSA isolates. Ranjith et al. (37) revealed that the expression of several genes associated with biofilm formation, drug resistance, and virulence was upregulated in the *C. albicans* isolate that form biofilm in comparison to the *C. albicans* isolate that does not form biofilm.

CONCLUSION

The clinical *E. faecalis* that isolated from various sources (urine, root canal, vagina, blood) showed variations in their ability to produce slime layer and biofilm formation. The *E. faecalis* isolates showed differences in expression of both *ebpA* and *ebpC* genes .The *ebpA* gene of biofilm producer isolate from root canal up regulated in comparison with non-producer isolate of *E. faecalis* and the *ebpC* gene of biofilm producer isolates from root canal and urine up regulated in comparison with non-producer isolate of *E. faecalis*.

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