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Expression of Pili Genes (*ebp*A and *ebp*C) Associated with Multi Drug Resistant *Enterococcus faecalis* Isolates

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Abstract

Enterococcus faecalis is a commensal opportunistic pathogen that frequently causes persistent infections linked to antibiotic resistance. This study was conducted to determine the expression of *ebp*A and *ebp*C genes in multi drug resistant *E. faecalis* isolates. The antibiotic susceptibility was determined for 118 clinical *E. faecalis* isolates, the isolates showed various levels of susceptibilities to different antibiotics and 70.5% of these isolates were multidrug resistant. The relative expression of *ebp*A gene in six multidrug resistance *E. faecalis* isolates was determined in comparison with antibiotic-sensitive isolates selected as a control sample. The isolate 21 that isolated from root canal (multi drug resistance isolate) showed increasing in expression in comparison with antibiotic sensitive isolates of *E. faecalis*. The relative expression of *ebp*C gene in multidrug resistance *E. faecalis* isolates was determined in comparison with antibiotic sensitive isolates which were selected as control samples. The isolate 21 from root canal and the isolate 19 from urine (multi drug resistance) showed high expression in comparison with antibiotic sensitive isolates of *E. faecalis*. The expression of *ebp*A and *ebp*C genes in multi drug resistance isolates in relation to their sources showed that urine group gave a high rate of expression of *ebp*C followed by root canal in comparison with *ebp*A expression. In contrast, the vagina group gave a higher expression of *ebp*A than *ebp*C. The findings of the present investigation revealed a significant correlation ($p = 0.05$) between the multi-drug resistance and gene expression of *ebp*A gene, while there is no significant ($p = 0.05$) correlation with *ebp*C.

Keywords: *ebp*A, *ebp*C, *E. faecalis*, Endocarditis and biofilm-associated pili, Gene expression, Multi drug resistance.

التعبير عن جينات الشعيرات (*ebp*C و *ebp*A) المرتبط في عزلات *Enterococcus faecalis* المتعدده المقاومه للادويه

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الخلاصه

المكورات المعوية البرازية هي أحد مسببات الأمراض الانتهازية المتعايشة والتي تسبب في كثير من الأحيان التهابات مستمرة مرتبطة بمقاومة المضادات الحيوية . تم اجراء هذه الدراسة لتحديد التعبير عن جينات *ebp*A و *ebp*C في عزلات *E. faecalis* المتعددة المقاومة للادوية . تم تحديد حساسية ١١٨ عزلة سريرية من بكتريا *E. faecalis* للمضادات الحيوية ، أظهرت العزلات مستويات متباينة من الحساسية للمضادات الحيوية المختلفة و ٧٠,٥٪ من هذه العزلات كانت متعددة المقاومة للأدوية. تم تحديد التعبير النسبي لجين *ebp*A في ستة عزلات *E. faecalis* المتعددة المقاومة للأدوية بالمقارنة مع العزلات الحساسة للمضادات الحيوية التي تم اختيارها كعينة سيطرة . أظهرت العزلة ٢١ المعزولة من قناة الجذر (عزلة متعددة المقاومة للأدوية) زيادة في التعبير بالمقارنة مع عزلات *E. faecalis* الحساسة للمضادات الحيوية. كما تم تحديد التعبير النسبي لجين *ebp*C في عزلات *E. faecalis* المتعددة المقاومة للأدوية بالمقارنة مع عزلات السيطرة . أظهرت العزلة ٢١ من قناة الجذر والعزلة ١٩ من الادرار (المتعددة المقاومة للأدوية) تعبيراً عالياً بالمقارنة مع عزلات *E. faecalis* الحساسة للمضادات الحيوية.أظهر التعبير عن جينات *ebp*A و *ebp*C في العزلات المقاومة للأدوية المتعددة فيما يتعلق بمصادرها أن مجموعة البول أعطت معدلًا عاليًا من التعبير عن *ebp*C متبوعًا بقناة الجذر مقارنة بتعبير *ebp*A. في المقابل ، أعطت المجموعة المهبلية تعبيرًا أعلى لـ *ebp*A من *ebp*C. كشفت نتائج الدراسة الحالية عن وجود علاقة معنوية ($p = 0.05$) بين مقاومة الأدوية المتعددة والتعبير الجيني لجين ebpA ، بينما لا توجد علاقة معنوية ($p = 0.05$) مع *ebp*C.

الكلمات المفتاحيه: *ebpA* ، *ebpC* ، المكورات المعوية البرازية ، الشعيرات المرتبطه بالغشاء الحيوي والتهاب شغاف القلب ، التعبير الجيني ، المقاومه المتعدده للادوية.

Introduction

Enterococcus faecalis is a Gram-positive opportunistic pathogen that is ordinarily found in the mammalian gastrointestinal (GI) tract and frequently causes persistent infections linked to antibiotic resistance [(1)]. It is typically found in water and soil [(2)] and can grow in both anaerobic and aerobic conditions, even in the presence of 6.5% NaCl[(3)]. Enterococci produce lactic acid as a result of fermentation and can survive a wide range of pH (4.5–10.0) and temperature (5 °C to 65 °C) [(4)]. Despite the non-pathogenic nature of enterococci, they are among the most frequent organisms found in nosocomial infections [(5)]. *E. faecalis* causes urinary tract infections, bacteremia, prosthetic joint infection, abdominal-pelvic infections, and endocarditis [(6)]. *E. faecalis* encodes a large number of adhesins that aid in colonization and adhesion to various host habitats. Its capacity to form biofilms and its intrinsic and acquired resistance to antibiotics permits it to be both a pathogen and a commensal [(7)].

The global public health threat posed by antibiotic-resistant bacteria is becoming more and more significant. Intrinsic resistance is the term used to describe the existence of bacterial resistance to an antibiotic in all strains of a certain bacterial species. In contrast, acquired resistance is thought to exist when a strain of a generally sensitive species is resistant to a specific antibiotic [(8)]. Enterococci are resistant to many antimicrobial substances developed by both intrinsic and acquired mechanisms [(9)]. They are resistance to cephalosporins, aminoglycosides, and low concentrations of clindamycin intrinsically [(10)].

The development of acquired resistance (High Level Resistance) in enterococci, is caused by genetic mutation, the acquisition of foreign genetic material, or the horizontal transfer of antimicrobial resistance (AMR) genes by plasmids, transposons, or integrative and conjugative elements [(11)]. The presence of these genes is a major threat as they can be passed on to opportunistic and dangerous bacteria [(12)]. Furthermore, enterococci are becoming increasingly resistant to various antimicrobials, including glycopeptides, lactam antibiotics, and high-level aminoglycosides [(13)].

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 [(14)]. *E. faecalis* has many adhesins that help with adhesion and disease establishment, like endocarditis- and biofilm-associated pili (Ebp) [(15)]. Three subunits make up Ebp: EbpA, EbpB, and EbpC, The major pilus component is EbpC, followed by EbpB at the pilus' base and EbpA at the pilus' tip [(16)]. EbpR positively regulates the three subunits and is encoded at the ebpABC locus upstream of ebpABC , Ebp pili appear to play various roles during infection especially, endocarditis and urinary tract infections since they help *E. faecalis* bind to host fibrinogen, collagen, and human platelets [(17)]. The objective of this research was to study the expression of *ebp*A and *ebp*C genes and their potential association with multi drug resistance in *E. faecalis* isolates.

Materials and Method

Enterococcus faecalis isolates

One hundred and eighteen of *E. faecalis* were isolated from various sources (45 from urine, 60 from root canal, 10 from wound and 3 from vagina).These isolates were identified by biochemical tests [(18,19)] also confirms by Vitek2 system.

Antibiotic susceptibility test

The susceptibility of bacterial isolates was tested by VITEK-2 system (AST-P592). The card (AST- P592) which included Ampicillin, Gentamicin, 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 a blood agar, the ABC streaking method was used to obtain pure colonies. To achieve an inoculum density of 0.50-0.60 McFarland, the test organism (5–10 Colonies) was suspended in 5 ml of normal saline using a cotton swab. A suspension of the test 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 [(20)].

Pili ebpA and ebpC gene expression in E. faecalis isolates

The gene expression of pili *epbA*, *epbC* genes were detected in six multidrug resistance isolates of *E. faecalis*. 23s rRNA was used as housekeeping gene. As the calibrator two antibiotic sensitive *E. faecalis* isolates one from wound (3W) and the second from root canal (31R) were used. All isolates were activated on Brain heart infusion broth that taken colonies from selective Pfizer enterococcus medium and incubated at 37 °C for 24 hr. under aerobic conditions.

RNA purification

RNA was extracted from the sample in accordance with the instructions for the TRIzol™ Reagent [(21)]:

A- Sampling lysis: 1.4 ml of the cell culture was centrifuged for 2 minutes at 13000 rpm to precipitate

the cells. The TRIzol™ Reagent (0.75 mL) was added to the pellet. Then, the lysate was homogenized by repeated pipetting up and down motions.

B- For the separation of three phases: Each tube's lysate was mixed with 0.2 mL of chloroform. After incubation for 2–3 minutes, all mixtures were centrifuged for 10 minutes at 12,000 rpm to separate them into three phases. A fresh tube was used to hold the RNA-containing aqueous phase.

C- RNA precipitation

1- 0.5 mL of isopropanol was added to the aqueous phase and the mixture was centrifuged at 12,000 rpm for 10 minutes. After total RNA precipitated, the supernatant was then removed and solidified into a pellet that resembled white gel at the bottom of the tube.

2- 0.5 mL of 70% ethanol was added to each tube, vortexed for a brief period of time, and then centrifuged for 5 minutes at 10,000 rpm. The pellet was then aspirated with ethanol and dried by air.

3- After being rehydrated in 50 µl of nuclease-free water, the pellet was incubated for 10–15 minutes in a water bath or heat block set to 55–60 C.

4- The concentration of extracted RNA was detected using a Quantus Fluorometer.

Protocol for Thermal Cycling and Reaction Setup (One Step RT-PCR)

The primers used in this study were shown in Table 1.

Table 1. Primers for gene expression

Primer Name	Sequence 5`-3`	Reference
ebpA-F	AAAAATGATTCGGCTCCAGAA	Bourgogne *et al.*[17]
ebpA-R	TGCCAGATTCGCTCTCAAAG	
ebpC-F	CGGTCATACCGACGACCAAA	Afonina *et al.* [22]
ebpC-R	TGTCACATCGCCATCGACTT	
Entero.faec._23S-F	CCTATCGGCCTCGGCTTAG	This study
Entero.faec._23S-R	AGCGAAAGACAGGTGAGAATCC	

The extracted RNA, Primers, and RT-qPCR master mix were mixed well by vortex. Reaction mixture (10µl) was prepared. 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 comparative CT (ΔΔCt) approach was utilized to assess the relative expression of two genes [23].

Relative quantification:

$$\Delta Ct = Ct\ gene - Ct\ House\ Keeping\ gene$$

$$\Delta\Delta Ct = \Delta Ct\ Test - \Delta Ct\ Control$$

$$Folding = 2\text{^}(-\Delta\Delta Ct)$$

Results and Discussion

Antibiotic susceptibility test (AST) of E. faecalis

The antibiotics susceptibility was determined for pathogenic *E. faecalis* isolates to 15 different antimicrobial agents by VITEK-2 system (AST-P592). The antibiotic susceptibility test was determined by MIC value breakpoints [24]. All of the *E. faecalis* isolates (100%) were found to be resistance to Azithromycin, Clarithromycin, Erythromycin, Doxycycline, minocycline, Tetracycline, and 64.7% to Gentamicin, However, all isolates were susceptible to linezolid, daptomycin, teicoplanin, and tigecycline, Vancomycin each, 58.8% to Streptomycin, 76.4% for ciprofloxacin, and 94.1% to Ampicillin. Al-Taie *et al* [25] indicated high resistance levels in Enterococcus spp. isolated from dental caries to amoxicillin (68.7 %), vancomycin (56.2%), and tetracycline (50%). Khadem and Flayyih [26] found that 80% of *E. faecalis* were resistant to Gentamicin. According to the study cited by Salih [27], 15% of *E. faecalis* were vancomycin sensitive. While Chabuck *et al* [28], Praharaj *et al* [29] demonstrated that the corresponding percentages of vancomycin resistance were 71.43% and 90.6%. Abdrabaa and Flayyih [30] reported that the cell wall thickness of the isolate with induced vancomycin resistance was higher than that of the sensitive isolates. According to Al-shawi and Al-Quraishi [31], all root canal *E. faecalis* isolates were 100% susceptible to Imipenem and Linezolid but 100% resistant to Tetracycline and Trimethoprim.

The findings of Kouidhi[32] demonstrated the presence of enterococci (*E. faecalis* and *E. faecium*) resistant to a variety of antibiotics, including erythromycin, streptomycin, bacitracin, nalidixic acid, ciprofloxacin, ofloxacin, and nitroxolin, as well as penicillin, ticarcillin, cefsulodin, ceftazidime, amikacin and tobramycin. Resistant Enterococci to currently available antibiotics pose real therapeutic difficulties [33].

The term "multidrug resistance" Bacteria that are resistant to three or more different antibiotic

kinds are referred to as (MDR) [34], the emergence of MDR pathogens poses a serious threat to these classes of life-saving medications [35]. This study revealed that 70.5% of *E. faecalis* isolates were multidrug resistant. The percentage of MDR in this result, was similar to those reported by Al-Jarousha *et al* [34], was 66.6%. According to Yilema *et al* [36], 75% of the isolates were multiple drug-resistant enterococci. However, this result was more than the 67.92% found in the study conducted in India [37]. This disparity may be the result of the MDR strains gradually changing as a result of the selection pressure. The outcome was also inferior to a research conducted in Iraq, where 100% of the isolates were MDR [31]. This change may be the result of different study participants and specimens from the last study, which only included hospitalized patients with UTI. Farman *et al.* [38] found that 96% of *E. faecalis* isolates had traits of multiple drug resistance (MDR), which is similar to the 82.35% reported in Ethiopia[33] . Esmail *et al*.'s study revealed that all *E. faecalis* isolates (100%) showed MDR, along with high-level resistance to gentamicin, erythromycin, and vancomycin. [39]. The spread of antimicrobial resistance among Enterococcal species in Iraq has posed a significant challenge for the medical community. Sadly, inadequate knowledge of antibiotic resistance, particularly glycopeptide resistance among endemic enterococci, is leading to an increase in treatment failures for enterococcal infections. Such knowledge is necessary for the proper treatment of patients with enterococcal infections, which are the second most prevalent cause of UTI and the third most common cause of bacteremia, respectively [40,26].

Real Time-qPCR

Six isolates of *E. faecalis* were chosen for studying the gene expression of *ebp*A and *ebp*C in comparison with antibiotic sensitive isolates which selected as control. In order to assess the quality of samples for later uses, the concentration of extracted RNA was detected using a Quantus Fluorometer, the RNA concentration ranged from 109-207ng/µl. Quantitative reverse transcription polymerase chain reaction(qPCR) was performed. 23s rRNA was used as housekeeping gene. The melting point for *ebp*A and *ebp*C 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).

The relative expression of *ebp*A gene in six multidrug resistance *E. faecalis* isolates were determined as fold change in comparison with antibiotic sensitive isolates which selected as control sample. The *E. faecalis* isolate denoted with number 21, which obtained from a root canal and exhibiting multi-drug resistance, demonstrated a 4.5-fold increase in expression compared to the antibiotic-sensitive *E. faecalis* isolates as shown in Table 2.

Table 2. Gene expression of *ebp*A in multidrug resistance *E. faecalis* isolates

Group	Samples	H.K	*ebp*A(Ct)	ΔCt *ebp*A	ΔΔCt *ebp*A	Fold
Vagina	231	10.28	20.70	10.42	2.19	0.22
Root canal	52	9.98	21.38	11.40	3.17	0.111
Root canal	21	10.21	15.70	6.06	-2.17	4.5↑
Urine	42	8.91	19.51	10.60	2.37	0.193
Urine	47	11.36	23.85	12.48	4.25	0.053
Urine	19	8.63	16.98	8.35	0.12	0.920
Control	3 W & 31R*	9.37	17.60	8.23	0	1

* The average of *ebp*A (Ct) values of antibiotic-sensitive *E. faecalis* isolates (control) was used for calibration.

The relative expression of *ebp*C gene in multidrug resistance *E. faecalis* isolates were determined as fold change in compare with antibiotic sensitive isolates which selected as control sample. When compared to antibiotic-sensitive *E. faecalis* isolates, the expression levels of isolate with number 21 from root canal and isolate 19 from urine (both of which are multi-drug resistant) were found to be 4.723-fold and 7.11-fold higher, respectively.as shown in Table 3.

The expression of *ebp*A and *ebp*C genes represented as mean of fold change of multi-drug resistance isolates in a relation with their sources showed that urine group gave a high rate expression of *ebp*C followed by root canal in compare with *ebp*A expression, while vagina group gave high expression of *ebp*A than *ebp*C Figure1.

Table 3. Gene expression of *ebpC* in multidrug resistance *E. faecalis* isolates

Group	Samples	H.K	*ebp*C(Ct)	ΔCt *ebp*C	ΔΔCt *ebp*C	Fold
Vagina	231	10.28	21.94	11.66	3.36	0.097
Root canal	52	9.98	21.02	11.04	2.71	0.153
Root canal	21	10.21	16.26	6.06	-2.24	4.723↑
Urine	42	8.91	18.77	9.86	1.5	0.353
Urine	47	11.36	22.82	11.46	3.16	0.112
Urine	19	8.63	14.10	5.47	-2.83	7.11↑
Control	3 W & 31R*	9.37	17.67	8.3	0	1

* The average of *ebp*C (Ct) values of antibiotic-sensitive *E. faecalis* isolates (control) was used for calibration

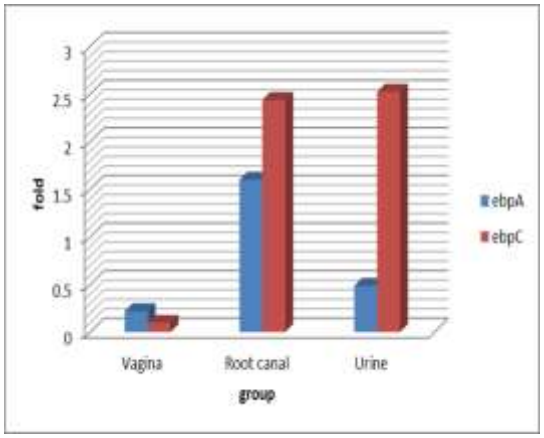

Figure 1. Gene expression of *ebpA* and *epbC* in multidrug resistance *E. faecalis* isolates according to their sources

The findings of this study revealed a significant (p=0.05) correlation between the multi-drug resistance and gene expression of *ebpA* gene (r= 0.8003), while there is no significant (p=0.05) correlation with *ebpC* (r= 0.5514), Figure. 2 and Figure. 3.

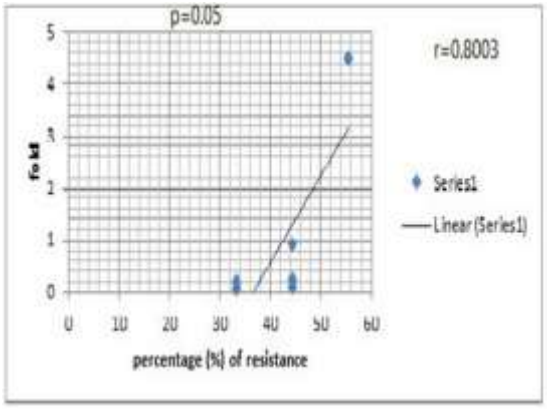

Figure 2. The correlation between gene expression of *ebpA* gene and multidrug resistance

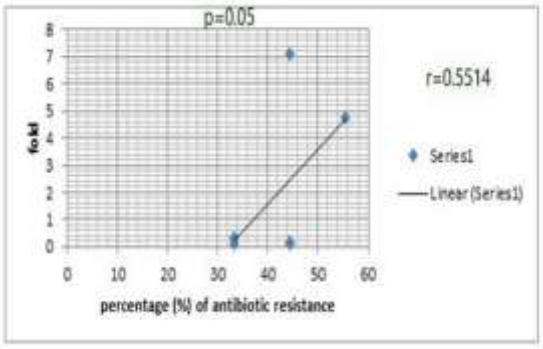

Figure 3.The correlation between gene expression of *ebpC* gene and multidrug resistance

According to Kot *et al.*, poorly adherent strains expressed the *icaA* and *icaD* genes at much lower levels than those of planktonic strains, but not by a meaningful amount. The result indicates that the isolates, cell form and growth period affect the expression of the genes [41]. Atshan *et al.* [42] also observed differences in the gene expression levels of genes related to biofilm formation among four MRSA isolates.

The high resistance to drugs and the formation of strong biofilms by Enterococci suggest that these factors could be greatly influential in enterococcal infections [32]. Hashem *et al.*'s research showed that enterococcal clinical isolates from Egyptian hospitals often formed biofilms. 31 isolates screened by PCR the *ebp*C, *ebp*A, and *ebp*B genes were present in 84%, 77%, and 55% respectively [43]. Although *E. faecalis* produces a variety of adhesins, these adhesins are not all expressed at the same time. Variations in expression may be the consequence of cross-regulation [22], which could account for the greater expression of the gene *ebpC* than *ebpA* in multidrug resistance isolates.

The results of the study revealed that there is a correlation between *ebp*A and *ebp*C expression and multidrug resistance. The study of Bhardwaj [44] demonstrated a significant association of biofilm with multiple drug resistance. It has been suggested that the complexity of the bacterial community in the biofilm may play a role in bacterial resistance,

another study showed that enterococci in biofilms are more highly resistant to antibiotics than planktonically growing strains [45]. Marghmalek *et al*. [46] demonstrated that the clinical and environmental isolates of *E. faecalis* and *E. faecium* showed high levels of antibiotic resistance. Moreover, the MDR isolates of enterococci included in the study were a major source of genes for biofilm and antibiotic resistance. These genetic elements can be transferred to additional bacteria, thereby increasing the amount of antibiotic resistance in a population of healthy individuals and in hospital environmental microorganisms.

Conclusion

The current study shows *ebp*A gene expression was high in multidrug-resistant *E.faecalis* isolated from the root canal and *ebpC* gene expression was at a high level in root canal and urine isolates. The urine group gave a high rate expression of *ebp*C followed by root canal in comparison with *ebp*A expression, while the vagina group gave a high expression of *ebp*A than *ebp*C.

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Conflicts of Interest

The authors declare no conflict of interest

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Ethics Statements

The study was conducted after receiving agreement from the participants and ethical approval from the biology department's ethics committee at the University of Baghdad's College of Science.

Author Contribution

The authors confirm contribution to the paper as follows: study conception and design: FMT; data collection: NAA; analysis and interpretation of results: FMT; draft manuscript preparation: NAA. All authors reviewed the results and approved the final version of the manuscript.

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