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Detection of Some Virulence Factors, Antibiotics Resistant and *esp* Gene Expression in *Enterococcus faecalis* Bacteria

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Abstract

This study aimed to identify virulence factors in *Enterococcus faecalis* and investigate the expression of the *esp* gene responsible for biofilm formation. Seventy isolates of *E. faecalis* were collected from various clinical specimens, including urine, stool, wound swabs, and uterine secretions, from several hospitals and laboratories in Baghdad, such as the Central Child Teaching Hospital, Baghdad Teaching Hospital, and the National Center for Educational Laboratories, during the period from 1 July 2022 to 1 September 2022. All isolates were confirmed as *E. faecalis* through microscopic examination, biochemical tests, and the VITEK 2 system. The virulence factors investigated were haemolysin, gelatinase, and biofilm formation. The results showed that 53.12% of isolates exhibited β-haemolysis, 90.62% were capable of forming biofilms, and 65.62% produced the enzyme gelatinase. Antibiotic susceptibility testing using the VITEK 2 system revealed that all isolates were sensitive to linezolid, teicoplanin, vancomycin, and tigecycline, while they showed 100% resistance to erythromycin. Sensitivity to other antibiotics varied among isolates. Expression of the *esp* gene, associated with biofilm formation, was analysed using real-time PCR based on the SYBR Green method, with 16S rRNA as a housekeeping control gene. When the isolates were treated with erythromycin at concentrations of 0.8 mg/L and 1.6 mg/L, no significant increase in *esp* gene expression was observed. In conclusion, *E. faecalis* isolates from various clinical sources in Baghdad exhibited several virulence factors, including haemolysin, gelatinase, and strong biofilm-forming ability. The isolates showed complete resistance to erythromycin while remaining sensitive to several other antibiotics, and treatment with erythromycin did not enhance *esp* gene expression.

Keywords: *Enterococcus faecalis*, Virulence factor, antibiotics, Gene expression.

1. Introduction

The genus *Enterococcus* is known as a gram positive, non-motile spores formation bacteria that appear in the form of single and chains cocci, most enterococci are facultative anaerobic but some species are obligated aerobic (1, 2).

E.faecalis is characterized by being cocci or oval in pairs or chains and analogous lengths, negative examine for catalase test, although a false reaction can occasionally take place after grown on a medium with blood (3). The enterococci bacteria possess many features that

permit it to survive and grow in a variation of circumstances and the ability to metabolically adapt and coexist and it is an opportunistic pathogen, including pneumonia, septicemia, meningitis and urinary tract infection, which cause vaginitis, which is one of the furthermost communal venereal diseases and influences women in puberty (4-6).

In many studies, it has been proven that the *E. faecalis* and *E.faecium* bacteria are the most communal species in human oral infection with virulent factors such as the formation of gelatenase, hemolysin and the formation of biofilms, and these factors were associated with the host tissue colonization, antagonism with other bacteria, changing the host's resistance mechanisms, invasion, and the creation of an abscess of toxin or inflammatory processes (7).

Because of some unacceptable utilization of antibiotics, which prompted numerous changes in the useful symbiotic microorganisms in the digestive tract, it is making them impervious to antibiotics, these microorganisms are portrayed as opportunistic pathogens that cause intestinal contaminations like infective endocarditis and can discharge an exotoxin substance that decays microorganisms and eukaryotic cells called cytolysin, these microorganisms are endemic to the human digestive and genitourinary tracts (8-11).

The widespread spread of bacterial strains that are resistant to many antibiotics is convert one of the main problems in the treatment of urinary system infection (12). Antimicrobial resistance converts an international problem in recent medicine that impend communal health (13). It has been demonstrated that biofilms are related with numerous infections that influence people and can be seen on clinical devices (14). Biofilm are complex aggregates of bacteria with exclusive possessions that ease host evasion immune system and invasion by adversaries (15). One of the most important benefits of biofilm status is antimicrobial resistance (16). Fecal enterococci bacteria are associated with their ability to produce many virulent factors, including cytolysin (17).

The aim of the study is to identify some of the virulence features (hemolysine, gelatenase, and biofilm formation) to study the gene expression of *esp* gene responsible for biofilm formation in *E. faecalis* bacteria.

2. Materials and Methods

2.1. Isolates collection

Seventy isolates of *E. faecalis* were collected from diverse clinical causes, containing (urine, stool, wounds, uterine secretions) from numerous hospitals clinics in Baghdad city; Central Child Teaching Hospital, Baghdad Teaching Hospital, and the National Center for Educational Laboratories, during the period 1-7-2022 until 1-9-2022, after it was cultured on blood agar and bile esculin, the bacteria then diagnosed based on microscopic examination and biochemical tests, including each of oxidase and catalase tests, for the final identification, the VITEK system was used to identify *E.faecalis* bacteria.

2.2. Detection of some virulence factors

2.2.1. Hemolysin production test

The bacterial isolates were cultured by planning method on the prepared blood agar medium and incubated at 37°C for 24 hours, the appearance of a transparent area around the growth of the colony indicates complete hemolysis-β while in the case of the appearance of a green area around the growth of the colony indicates partial hemolysis α- hemolysis while there is no hemolysis area indicating γ-hemolysis (18).

2.2.2. Gelatinase hydrolysis test

The bacterial isolates were cultured by planning method on the medium of the heart infusion (BHI) added to the mechanism of gelatin powder by 3% and incubated with 37◦C and

afterward the incubation period, a Fraser solution was added to the dish indicating the formation of a transparent area round the colony when the solution was added is evidence of the positivity of gelatinase enzyme (19).

2.2.3. Detection of biofilm formation using standard plate

The method was followed to detect biofilm composition as indicated in (19) method.

2.3. Determination of antibiotics sensitivity

Antibiotics sensitivity antibiotics was determined using VITEK2 system.

2.4. RNA extraction

The bacterial isolates (EF10 and EF11 *E. feacalis*) were grown on blood agar to obtain single colonies after incubating for 24 hours at 37◦C. After that, three single colonies were transferred to a medium and placed in the Brain heart infusion broth (as a control group), the minimum inhibitory concentration of the antibiotic under study was studied, as it was present (100-0.01) µl /ml in the Brain heart infusion broth. Then the isolates (EF10 and EF11) were grown on the mentioned medium in the presence of the antibiotic at a concentration below the minimum inhibitory concentration (Sub MIC), incubated for 24 hours at 37◦C.

A special kit was used to extract RNA from bacterial isolates according to the manufacturer's instructions (Promega, USA)

Real Time-PCR reaction was performed for the conservative gene and the *esp* gene, with a final volume of 10 µl, by mixing 5 µl of qPCR Master Mix, 0.25 µl of RT mix, 0.25 µl MgCI2, 0.5 µl of the forward and reverse primers for the conservative gene and the *esp* gene, and 2.5 µl of Nuclease free water and 1 µl of RNA. And as presented in **Table 1** the optimal conditions for the Real time PCR reaction of *esp* gene.

Table 1. Optimal conditions for Real time PCR reaction for *esp* gene.

Stage	Temperature	Time	No. of cycles
Reverse transcriptase enzyme activation	37	15 minute	One cycle
Initial denaturation	95	5 minute	
Denaturation	95	20 second	
Annealing	60	20 second	40 cycles
Extension	72	20 second	

2.5. Measurement of gene expression of the *esp* gene

esp gene expression responsible for formation of biofilm was measured by Real time PCR using *esp* and 16SrRNA primers as a control based on the SYBR green method. The gene expression of the *esp* gene responsible for biofilm formation was measured for the bacterial isolates 10, 11 of *E. faecalis* bacteria with high resistance to Erythromycin which it was treated once with erythromycin at a concentration of 0.8 ml/L for isolate (10) and a concentration of 1.6 ml/L for isolate (11) based on the minimum inhibitory concentration (MIC) and again without the antibiotic.

Calculate the amount of change in the level of gene expression as shown by the following equations:-

Folding $=2^{-\Delta\Delta CT}$

$\Delta\Delta CT = \Delta CT$ Treated - ΔCT Control

$\Delta CT =$ CT gene - CT House Keeping gene (*16SrRNA*).

3. Results

All isolates were cultured on blood agar medium and noted the presence of colonies with white to gray color and viscous texture as well as cultured on the bile esculin agar and the colonies appeared shiny and changed the color of the medium into black. The microscopic

examination of swabs stained with gram stain displayed that bacterial cells are positive gram stain. The biochemical tests also displayed that a negative consequence for oxidase and catalase tests, while the consequences of the VITEK2 system presented a positive consequence for 32 (100%) isolates. This isolates belonging to *E.faecalis* as follow: stool 6/32(18.75%), while from wounds 5/32(15.62%), urine 15/32(46.87%) and the secretions of uterine 6/32(18.75%) from as shown in **Figure 1**.

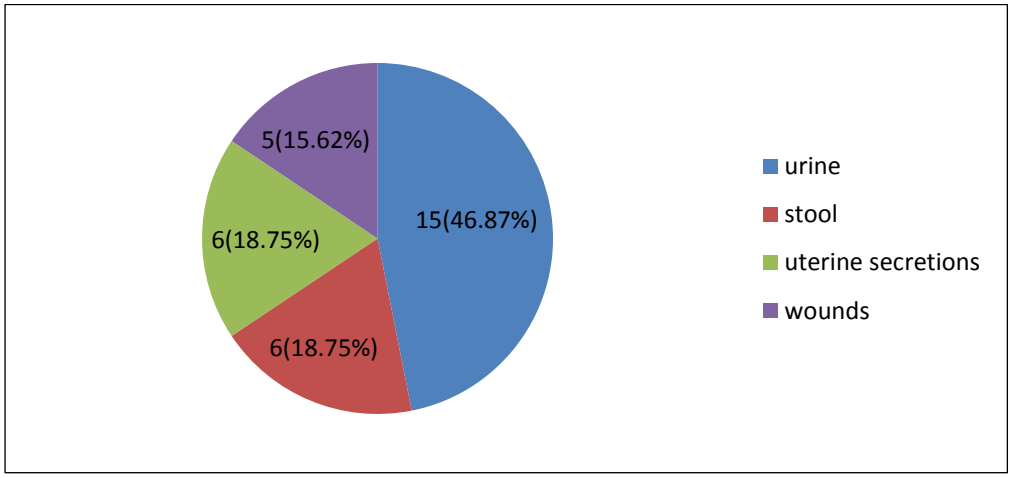

Figure 1. Percentage of *E.faecalis* isolates isolated from different clinical sources.

3.1. Detection of virulence factors

3.1.1. Hemolysin production test

The results of the detection of the production of the hemolysin enzyme for the *E. faecalis* bacteria that were cultured on the medium of blood agar, showed that the number of isolates that have the ability to analyze red blood cells in the petri dishes of blood agar was 17 isolates out of 32 isolates of *E. faecalis* bacteria by 53.12% was β-hemolysis type while the number of isolates that do not have the ability to hemolysis in the perti dishes of blood agar was 15 isolates out of 32 isolates of *E.faecalis* by percentage 46.87% as in **Figure 2**.

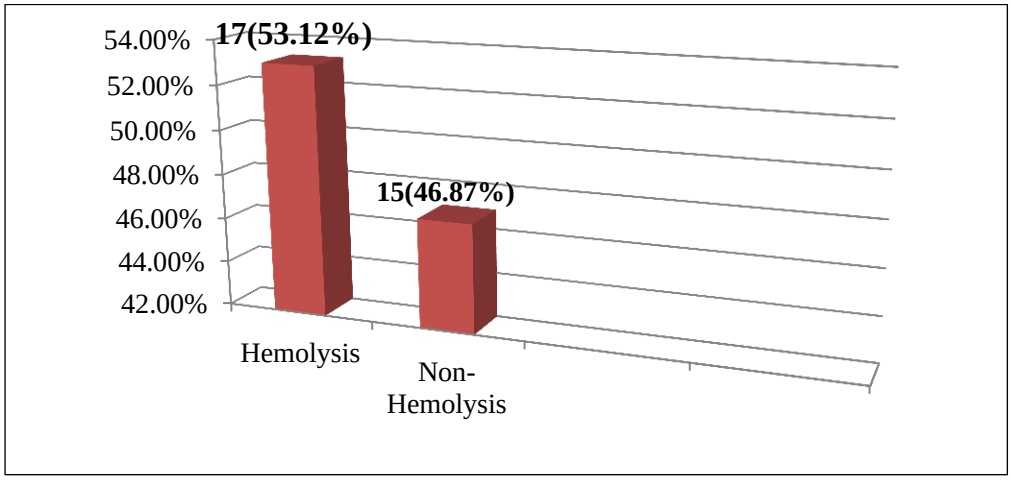

Figure 2. Percentage of *E.faecalis* susceptibility to hemolysin production.

3.1.2. Gelatin hydrolysis

The results of the detection of the production of the enzyme Gelatinase for E. faecalis bacteria showed that 21 isolates (65.62%) produced the enzyme gelatinase out of 32 isolates with the appearance of a transparent circle around the colonies evidence of the presence of gelatinase hydrolysis. The isolates that did not show transparent circles are negative for the productions of gelatinase were 11 isolates at a rate of 34.37% as in **Figure 3**.

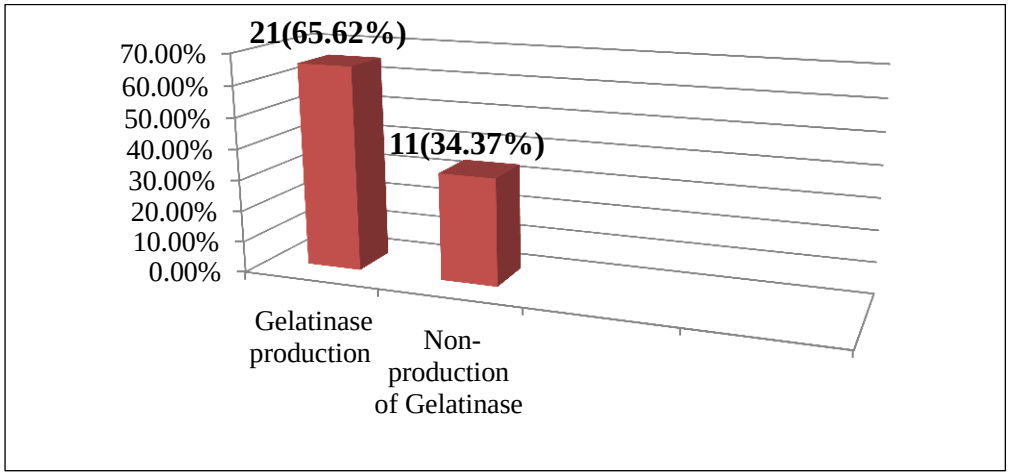

Figure 3. Percentage of *E.faecalis* susceptibility to Gelatinase production.

3.1.3. Biofilm formation

The results of the morphological examination of the susceptibility of *E.faecalis* to the formation of the biofilm by microtiter plate method showed that 29 isolates out of 32 isolates forming the biofilm, that mean, 90.62% of the bacterial isolates belonging to *E.faecalis* were form of the biofilm to different degrees.

When comparing holes results with negative control, 3 isolates were 9.37% strong adherent biofilm, 16 bacterial isolates 50% moderately adherent biofilm formation, 10 bacterial isolates 31.25% weakly adherent biofilm production and 3 isolates 9.37% non-biofilm formation as shown in **Figure 4.**

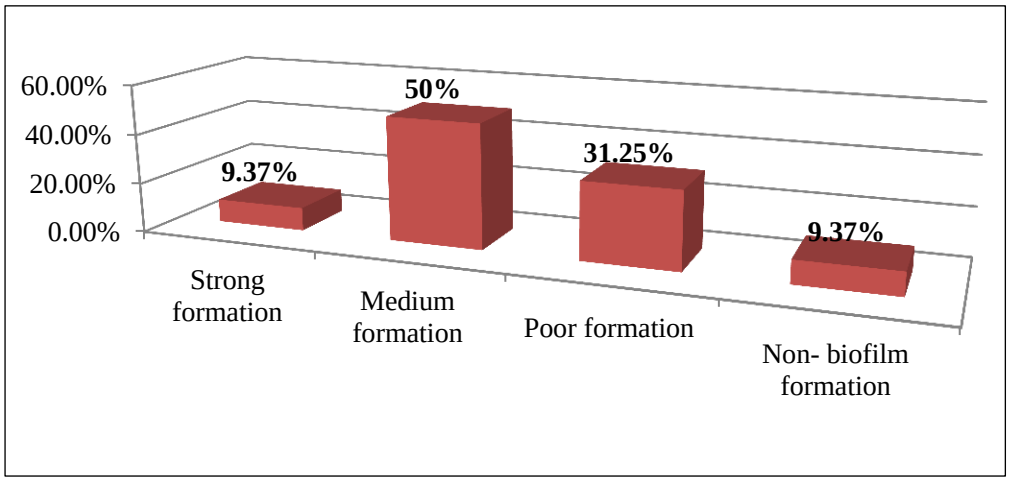

Figure 4. Percentage of *E.faecalis* susceptibility to biofilm formation.

3.2. Determination of the bacterial sensitivity to antibiotics

To find out the sensitivity and resistance of antibiotic bacterial isolates, an antibiotic sensitivity test was done by using the VITEK system, and the sensitivity of bacterial isolates was measured for 8 antibiotics and minimum inhibitory concentration (MIC) of the antibiotic and its effect on bacteria was adopted.

The results showed that all isolates were 100% sensitive to each of the antibiotics; tigecycline, vancomycin, teicoplanin and linezolid. The results showed 100% resistance to Erythromycin while some bacterial isolates differed in their resistance to other antibiotics.

Where the results of resistance to Levofloxacin 9 isolates were resistant to this Levofloxacin by 28.12%, two isolates of medium resistance by 6.25%, 21 isolates were sensitive by 65.62%, while the results of the Tetracycline showed that 29 isolates were resistant by 90.62% and three of the isolates were sensitive by 9.37% and did not show any isolates of

medium resistance, while the results of resistance to the Nitrofurantoin indicated that two isolates, with a percentage of 6.25%, were medium resistance, and 30 isolates and a percentage of 93.75% were sensitive, as displayed in **Table 2**.

Table 2. Antibiotic sensitivity of *E.faecalis* isolates using minimum inhibitory concentration (MIC).

Antibiotics	Resistance		Medium		Sensitivity	
	No	Percentage %	No	Percentage %	No	Percentage %
Levofloxacin	9	28.12	2	6.25	21	65.62
Erythromycin	32	100	–	–	–	–
Linezolid	–	–	–	–	32	100
Teicoplanin	–	–	–	–	32	100
Vancomycin	–	–	–	–	32	100
Tigecycline	-	-	-	-	32	100
Tetracycline	29	90.62	–	–	3	9.37
Nitrofurantoin	–	–	2	6.25	30	93.75

3.3. Measuring gene expression

3.3.1. Measurement of *esp* gene expression

The *esp* gene is responsible for formation of biofilm and it was measured by Real time PCR using *esp* and *16SrRNA* primers as a control based on the SYBR green method. This gene measured in the bacterial isolates 10, 11 of *E. faecalis* bacteria with high resistance to the erythromycin antibiotic.

It was treated once with an erythromycin at a concentration of 0.8 ml/L for isolate (10) and a concentration of 1.6 ml/L for isolate (11) depending on the minimum inhibitory concentration (MIC) and another without the antibiotic, the results showed that folding based on $2^{-\Delta\Delta ct}$ showed no effect of the Erythromycin and folding was (1.00) where it was considered positive control and folding was (0.23) depending on $2^{-\Delta\Delta ct}$ in the case of treatment with the erythromycin antibiotic as displayed in **Table 3** and **Table 4**.

The consequences displayed that there was no increase in gene expression when treating bacteria with the erythromycin antibiotic as in **Figure 5**.

Table 3. Gene expression values for 16SrRNA gene and *esp* gene for isolate No. 10.

Isolate 10	16SrRNA House-keeping gene CT	*esp* gene CT	ΔCT	ΔΔCT	folding
Untreated with antibiotic Positive control	11.25	13.15	1.91	0.00	1.00
Treated with antibiotic	10.34	14.37	4.03	2.12	0.23

CT (cycling threshold), ΔCT(delta cycling threshold), ΔΔCT(delta delta cycling threshold).

Table 4. Gene expression values for the 16SrRNA gene and the *esp* gene for isolate No. 11.

Isolate 11	16SrRNA House-keeping gene CT	*esp* gene CT	ΔCT	ΔΔCT	Folding
Untreated with antibiotic Positive control	10.54	14.06	3.53	0.00	1.00
Treated with antibiotic	8.91	14.41	5.51	1.98	0.25

CT (cycling threshold), ΔCT (delta cycling threshold), ΔΔCT (delta delta cycling threshold).

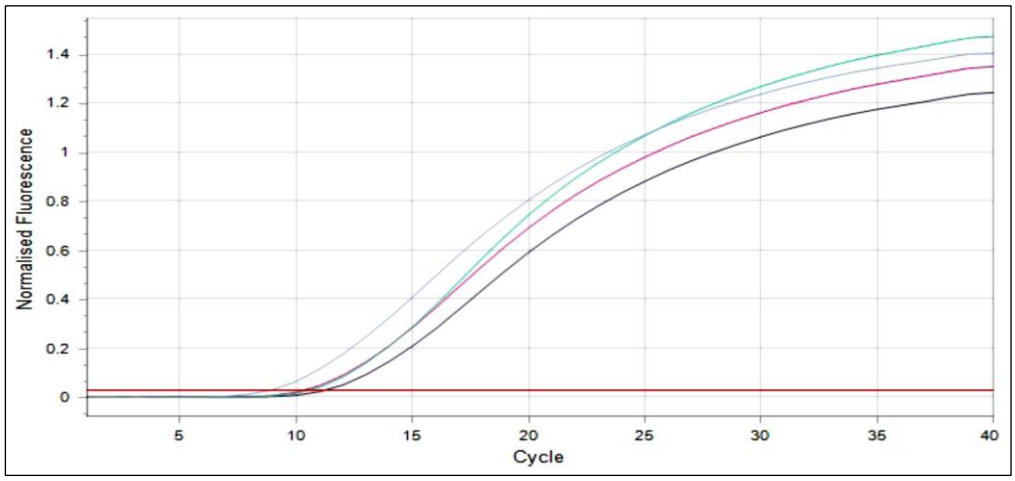

Figure 5. Quantum polymerase chain reaction curve of *esp* gene; black represents isolate No. 10 untreated with antibiotic and red isolate No. 10 treated with antibiotic, green isolate No. 11 untreated with antibiotic and blue color isolate No. 11 treated with antibiotic.

4. Discussion

The consequences of the recent study agreed with the results of the study (20) that showed 51% of the *E. faecalis* bacteria is a case of blood type β-hemolysis, while the consequences of study (21) presented that 44% of the *E. faecalis* bacteria haemolysis.

The gene responsible for the construction of β-hemolysine is the *cylA* gene, so the production or non-production of hemolysin is attributed to the acquisition of plasmid bacteria or genes encoding this enzyme may be of the type of silent genes that encode the enzyme hemolysine, which makes bacteria opportunistic pathogens (22, 23). *E. faecalis* strains producing hemolysine are inflammatory and are associated with increased severity of infection (24).

The consequences of the recent study agreed with the results of each of the researchers (25, 26), where the percentage of isolates resulting from this enzyme is 64% and 68%, respectively, while the consequences of study (19) that 22% of the *E.faecalis* bacteria are producing this enzyme, while the results of study (27) were 21% of the isolates producing this enzyme, the results of the recent study do not agree with what the study (24) reached, where the study showed any isolates of *E.faecalis* bacteria did not produce the gelatinase enzyme out of 32 isolates.

The gelatinase enzyme is a metalloprotease secreted outside the *E. faecalis* cell and works to lysis each of gelatin, collagen and casein and it is considered in animal cells as a virulence factor, the capability of gelatinase enzyme to lysis of collagen and some bioactive peptides indicates its contribution in the beginning and spread of inflammatory stages caused by *E. faecalis* bacteria.

The consequences of the recent study converged with the results of study (19), where all bacterial isolates were 100% biofilm formation, of which 62% had strong formation capacity, 25% had medium formation capacity and 13% had low formation susceptibility.

The consequences of study (27) appeared 96% of the bacterial isolates forming the biofilm included 5.5% strong biofilm formation, 42% medium biofilm formation and 48% poor biofilm formation, while the percentage of non-biofilm isolates was 4.5%, and this results were closest to the consequences of the recent study.

While the finding of the present study do not agree with the results of study (28), where the percentage of positive isolates of the biofilm is 47.2%, of which strong formation by 13.2%, medium formation by 9.1% and poor formation by 24.9%, the formation of biofilms to ease the existence and perseverance of pathogens in the host. The control disease centers now

estimate that 65% of people infections may be associated with biofilms. Diseases like infectious kidney stones, infective endocarditis and lung infections in cystic fibrosis are caused by bacteria's possession of biofilms (29).

In the current study, the percentage of resistance to the Levofloxacin due to the group of antibiotics Fluoroquinolones was 28.12%, while the percentage of sensitivity to *E. faecalis* bacteria was 65.62%, the percentage of the current study converged with (30), as the percentage of resistance to the antibiotic itself was 21.62%, while the percentage of sensitivity was 59.45%.

As for the percentage of resistance of *E.faecalis* bacteria to the Erythromycin was 100% similar to the consequences of the recent study with the results of study (31) as all isolates appeared resistant to Erythromycin and by 100%, either the resistance of bacteria to Linezolid in the current study was no resistance appeared, while the percentage of sensitivity to this antibiotic was 100%. The consequences of the present study were in line to study (32), as the sensitivity to the antibiotic was 100%.

The consequences of the recent study were agree with the results of each of (31) and (33) studies that the percentage of sensitivity to the Teicoplanin is 100%. The results showed the percentage sensitivity of *E. faecalis* bacteria to the Vancomycin in the current study was 100%, this percentage was similar to (32), where the percentage of sensitivity to the antibiotic was 100%. As for the sensitivity percentage of *E.faecalis* to the Tigecycline was 100%, this result is similar to the results of study (31), which the sensitivity rate to the antibiotic was 100%, while the percentage of bacteria resistance to the Tetracycline was 90.62%. While the sensitivity of bacteria to the antibiotic was 9.37%, the consequences of the present study converged with the results of study (34) which the resistance to the tetracycline was 93.5%, while the sensitivity of bacteria to the antibiotic was 6.5%. Whereas the sensitivity of *E. faecalis* to the Nitrofurantoin in the current study was 93.75%, while the percentage resistance to the antibiotic was 6.25%, the consequences of the present study converged with study (32), where the sensitivity to the Nitrofurantoin was 100%, while the results of study (34) for the Nitrofurantoin were 85.3% and the resistance to the antibiotic was 14.7%. 100% resistance to the Erythromycin, which belongs to the Macrolides group, which acts on 50S unit, showed resistance to ribosomes and interaction in the protein synthesis process. Another antibiotic that affects the protein synthesis process is the tetracycline groups work on 30S sub-unit in the ribosomes and thus prevents the synthesis of protein that the resistance of bacteria to this antibiotic by causing a alteration in the ribosomes location or through the enzymes production inhibiting the efficiency of tetracyclines or limiting the access of the antibiotic to the ribosomes.

Some recent studies have indicated that resistance to the antibiotics (Erythromycin and Tetracycline) is associated with bacteria's possession of virulence genes, including *gelE*, *asal*, *esp* genes that affect the virulence of bacteria and their association with antibiotic resistance, especially Tetracycline and Erythromycin (31, 35).

As for the study by (36), oxadiazole compounds were used against the gene expression of the *esp* gene, which led to a two- and three-fold decrease in the gene expression of the *esp* gene compared to control isolates. Whereas the study by (37), the antibiotics Ampicillin, vancomycin, and ceftizoxime had no influence on formation of biofilm, but the antibiotic Gentamicin stimulated formation of biofilm. Gentamicin increased the expression of each of *esp* gene by 50.9% and *efaA* gene by 33.9% and reduced the gene expression of the genes others are under study.

The study by (38) showed the isolate of *P. aeruginosa* carried biofilm gene. Was noticed a

weak correlation between the gene expression of biofilm gene and antibiotic. Another study by Maita and Boonbumrung who revealed that the biofilm formation is accompanied by drastic changes in gene regulation

5. Conclusions

The *E.faecalis* possesses some of the virulence factors, including hemolycin and gelatinase, biofilm formation, as well as all isolates appeared with their resistance to the Erythromycin antibiotic. As for the Tetracycline, most isolates have shown resistance to this antibiotic and there was no increase in *esp* gene expression after the bacteria were treated with the antibiotic erythromycin.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Ethical Clearance

The samples were obtaind according to Local Research Ethics Committee approval in Iraqi Ministry of Health No. 3223in13/6/2022.

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