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MOLECULAR IDENTIFICATION OF (*Efa*A) IN *ENTEROCOCCUS FECALIS* AND *ENTEROCOCCUS FACIUM* AND THEIR ROLE IN BIOFILM FORMATION

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ABSTRACT

Total of (104) urine samples were collected from patients suffering from urinary tract infection with different age groups from five hospitals in Baghdad (Ibn-Albalady, Al Yarmouk, Medical city, Baghdad hospital and Al-Kandy) from the period of the beginning of September 2015 to the end of December 2015.All samples were examined by traditional methods based on cultural characteristics, biochemical test and API 20 strep. The results revealed 50 isolates to *Enterococcus* and this was confirmed by polymerase chain reaction technique (PCR) based on amplification of species specific genes. PCR were performed for *E.faecalis* and *E.faecium* in order to confirm the presence of *EfaA* genes which code for *Enterococcus faecalis* endocarditis antigen using specific primer for gene.The results showed *that Enterococcus* contain a proportion of 100% of *EfaA*.Biofilm production was detected in *E.faecalis* and *E.faecium* by using two methods: Congo red agar method and microtiter plate method.Our results show that22(44%) of *Enterococcus* isolates were strong biofilm production,25(50%)as moderate and 3(6%) as week biofilm production by use Congo red method.In microtiter plate method, our results show that 20(40%) of bacterial isolates were detected as strong, 26(52%) as moderate and 4(8%) as week biofilm production. This study aims todiagnosis of *E.faecalis* and *E.faecium* from urinary tract infection of patients by traditional and molecular methods, detection of *EfaA* gene and its role in biofilm production.

INTRODUCTION

Biofilm formation is a dynamic process involving the attachment of bacteria to a biotic or abiotic surface and encased in a hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids (1, 2). Biofilms are notoriously difficult to

eradicate and are a source of many chronic infections, approximate 80% of microbial infections occurring in the human body are biofilm-mediated (3).

More than 30 species in the genus *Enterococcus* have been described to date; the two species *Enterococcus faecalis* and *Enterococcus faecium*, have gained significance as leading opportunistic pathogens causing nosocomial infections (4, 5).Some researchers reported that *Enterococcus* have become increasingly important as nosocomial pathogens and have been found to form biofilms on several medical devices implanted in patients, such as central venous catheters, urinary catheters, intrauterine devices, and prosthetic heart valves (6,7).

Several *Enterococcu*s pathogenic factors have been identified including adhesions and secreted virulence factors (8). One of important virulence gene was *E.faecalis* antigen A *(EfaA)*which was presumed to be involved in the adhesion of *Enterococcus* to biotic and abiotic surfaces or evasion of the immune response (9). Some research report that *Enterococcus* isolates with *Asa1* and *EfaA* genes produced more biofilms than negative ones and it seems that these genes have the highest contribution in biofilm formation in the urinary tract isolates (10).In addition*EfaA* has been shown to have an important role in pathogenesis of *Enterococcus* in infective endocarditis (11,12).

MATERIALS AND METHODS

***Clinical Isolates:-**

Total of (104) urine samples were collected from patients suffering from urinary tract infection with different age groups from five hospitals in Baghdad (Ibn- Albalady, Al Yarmouk, Medical city, Baghdad hospital and Al-Kandy) from the period of the beginning of September 2015 to the end of December 2015.

*** Isolation and identification of *Enterococcus* by traditional methods:-**

-Culturing on selective media:-

The isolates were identified by characteristic colony morphology of *Enterococcus* on selective media (bile esculin agar) which gave round shape colony with slightly convex smooth edges, creamy color and convert media into black.

*** Molecular identification of *Enterococcus*:-**

-Bacterial Genomic DNA Extraction:-

Genomic DNA was extracted from the bacterial isolates using Presto Mini g DNA bacteria Kits (Geneaid, Thailand), fallowing the company instructions.

***Detection of *Enterococcus* by molecular method:-**

-Detection of *Enterococcus* speciesby using species specific primer:-

Multiplex PCR used for conformation identification of the *E.faecalis* and *E.faecium,* reactionwas conducted in 20 µl of reaction mixture containing 13µl of distilled water, PCR master mix (Bioneer Corporation), 1µl forward from each genes and 1µl reverse primer from each genes (table-1)and 3 µl of DNA (table-2).

Table (1): The Sequence of forward and reverse primers used in this study

Genes	Sequence (5' to 3')	Size	Reference
ddl*E. Faecium*	F:TTGAGGCAGACCAGATTGACG R:TATGACAGCGACTCCGATTCC	658	13
ddl *E.faecalis*	F:ATCAAGTACAGTTAGTCTTTATTAG R:ACGATTCAAAGCTAACTGAATCAGT	941	13

Table (2):The Mixture of multiplex PCR working solution for the detection of *Enterococcus* species

Component	Volume (µl)
Primer F.	2
Primer R.	2
DNA	3
water	13
Total Volume	20 µl

Amplification was conducted using a DNA thermal cycler programmed with 30 cycles included initial denaturation at 94° C for 10 min, denaturation at 94° C for 1min, annealing at 58°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min as shown in table (3).The PCR products were analyzed in Agarose gels and visualized under UV after staining with ethidium bromide.

Table (3): PCR Program for the detection of ddl*E.faecium* and ddl *E.faecalis* genes by multiplex PCR

No.	Steps	Temperature (°C)	Time
1.	Initial Denaturation	94	10min
2.	Denaturation	94	1min
3.	Annealing	58	1min
4.	Extension	72	1min
5.	Final extension	72	10min
6.	Cycles number	30	

- Detection of *EnterococcusEFaA*gene:-

PCR were used to detect of *EFaA* gene in *E.faecalis* and *E.faecium* using specific primers.

Reaction was conducted in 20 µl of reaction mixture containing 15µl of distilled water, PCR master mix (Bioneer Corporation), 1µl forward and 1µl reverse primer (the sequence of primer is mentioned in table (4)), and 3 µl of DNA were added (table-5)

Table (4): The Sequence of Forward and Reverse Primers for *EfaA* gene used in this study

Genes	Sequence (5' to 3')	Size	Reference
EFaA	F:GACAGACCCTCACGAATA R:AGTTCATCATGCTGTAGTA	705	14

Table (5): The Mixture of PCR working solution for detection of *EnterococcusEFaA* gene

Component	Volume (µl)
Primer F.	1
Primer R.	1
DNA	3
water	15
Total Volume	20 µl

Amplification was conducted using a DNA thermal cycler programmed with 30 cycles included initial denaturation at 94° C for 5 min, denaturation at 94° C for 45 sec, annealing at 52°C at 1 min, extension at 72°C for 1 min and a final extension at 72°C for 3 min as shown in table (6).Products were analyzed in agarose gels and visualized under UV after staining with ethidium bromide.

Table (6): PCR Program for the detection of *EnterococcusEfaA* gene by conventional PCR

No.	Steps	Temperature (°C)	Time
1	Initial Denaturation	94	5 min
2	Denaturation	94	45sec
3	Annealing	52	1 min
4	Extension	72	1 min
5	Final extension	72	3 min
6	Cycles number	30	

-Biofilm production test:-

1- Congo Red Agar method

A specially prepared medium known as Congo Red Agar (CRA) was used for this test. The *Enterococcus* strains were inoculated onto CRA and incubated at 37°C for 24 hours. Readings were taken after 24 hours and again after 48 hours. A positive result was indicated by black colonies with black crystalline morphology. Non-biofilm producers mostly produced pink- or red-colored colonies (15).

2- Microtiter plate methods of biofilm assay

A modified microtiter plate method was used as previously described by (16). Briefly, the wells of microtiter plate were filled with 200 µl of brain heart broth (BHB) supplemented with .5% glucose. Then, a 20 µl quantity of previously prepared bacterial suspensions with turbidity equal to 0.5 McFarland standards was added to each well (3 well for each strain). The negative control wells contained 200 µl of BHB and supplemented with 5% glucose. The plate was incubated at 37°C for 24 h before removal of the cultures, then, the cells were decanted, and each well was washed 3-times with sterile phosphate buffered saline dried in an inverted position and stained with 1% crystal violet for 20 minutes. The wells were rinsed again with

distilled water and crystal violet was solubilized in 200 µl of ethanol. The OD at a wavelength of 490 nm was determined using a micro ELISA auto reader (Bio-Rad). These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Formation of biofilm by isolates was analyzed and categorized relying on the absorbance of the crystal violet-stained attached cells table (7).

Table (7) Interpretation of biofilm production

OD value	Biofilm production
ODc < ~ ≤ 2x ODc	weak
2x ODc < ~ ≤ 4x ODc	Moderate
> 4x ODc	Strong

ODc = Optical density of negative control

RESULTS AND DISCUSSION

-Clinical Samples:-

Identification of *Enterococcus*by traditional methods:-

Fifty isolates were identified as *Enterococcus* on bile esculin agar (fig.1) depending on creamy color of colony which convert the media to black, it consist of 40% bile salt help in inhibition growth of *Streptococci* belong to group D antigen made this media useful in diagnosis of *Enterococcus* from other non-*Enterococcus* bacteria that belong to group D antigen (17)

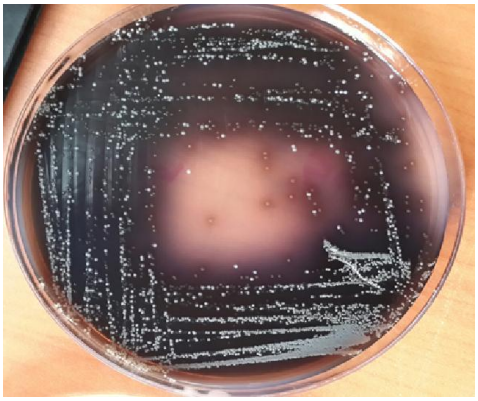

Fig. (1): Appearance of *Enterococcus* isolates on bile esculin agar.

Finally, the API 20 strep system was used for accurate identification of the isolates at generic and species level. The test gave positive results for all isolates as show in fig. (2).

Fig. (2): Biochemical identification of *Enterococcus* using API 20 strep

-Identification of *Enterococcus* species by molecular methods:

Multiplex PCR technique were used for the diagnosis of all (50) isolates which has grown on the selective media and has already been diagnosed based on their morphology characteristic on culture media and biochemical test, using species-specific primers for the D-alanine-D-alanine ligase gene (ddl *E.faecalis* and ddl *E.faecium*) which was specific for diagnosis of *E.faecalis* and *E.faecium*.It give same result of biochemical test (API 20 strep) 28bacteria isolates for *E.faecalis* and 22 bacteria isolates for *E.faecium*. Similar finding was reported by (18), piece that amplify by PCR detect by using gel electrophoresis as show in fig. (3).

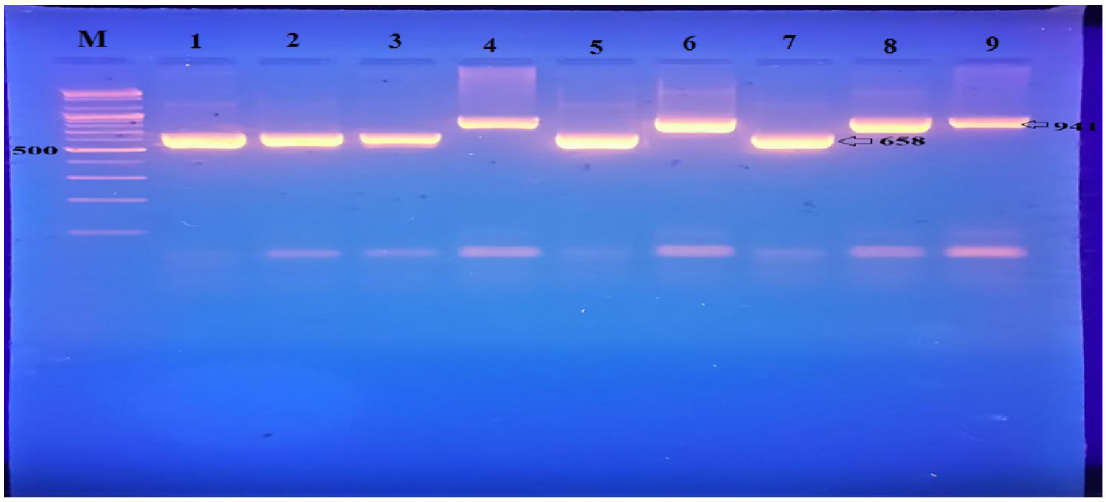

Fig. (3): Agarose gel electrophoresis of multiplex PCR for identification of*Enterococcus* species, M: marker (100pb ladder), lanes (1, 2, 3, 5, 7) positive amplification of ddl *E.faecium* gene (658) Pb, lanes (4, 6, 8, 9) positive amplification of ddl *E.faecalis* gene (941) Pb.

-Detection of *EfaA* gene in *E.faecalis* and *E.faecium* by conventional PCR:-

Several virulence and pathogenic factors have been described from *Enterococci* that enhance their ability to colonize patient's tissues, increase resistance to antibiotics, and aggravate the infection outcomes (19).Conventional PCR was performed for *E.faecalis* and *E.faecium* in order to confirm the presence of *E.faecalis* endocarditis antigen (*EfaA*) by use specific primers. Piece of DNA that amplified by PCR were detect by using gel electrophoresis as show in fig. (4**).**

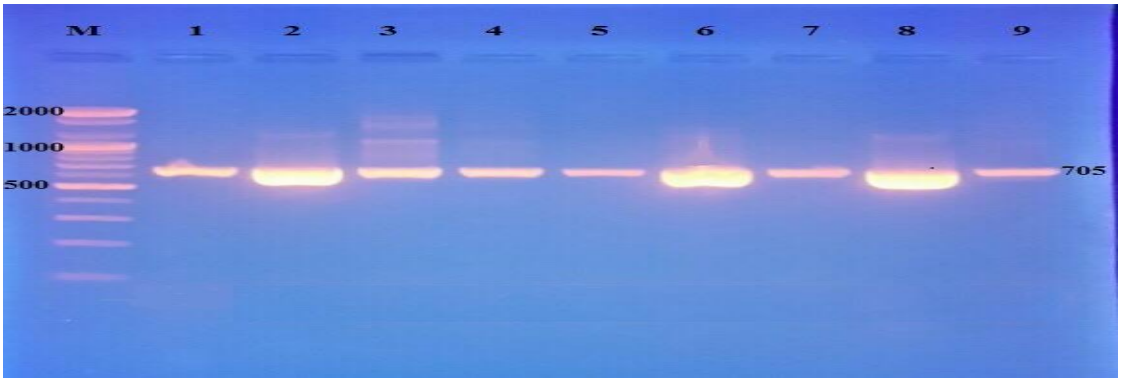

Fig. (4) Agarose gel electrophoresis of conventional PCR amplification products of *EnterococcusEfaA* gene (705pb).M: marker (100 bp ladder) lanes (1-5): positive amplification of *EfaA* gene in *E.faecalis*, lanes (6-9): positive amplification of *EfaA* gene in *E.faecium*.

Our results showed the presence*EfaA* gene 100% in *E.faecalis*. The results presented here go along nicely with the results recorded by (20) who found that *E.faecalisEFaA* gene presence 100% in urine samples.

The percentage of *EfaA* gene in *E.faecium* was 100% theseresults were matched with study of (21) who found that *EfaA* presence in 100% in *E.faecium*.The high incidence of virulence factors (*EfaA*) in urinary strains could potentially contribute to facilitate bacterial colonization and pathogenesis of *Enterococcus* in the urinary tract.

-Detection of biofilm production:-

Biofilm producing bacteria are responsible for many recalcitrant infections and are difficult to eradicate (22). *Enterococci* are one of the causative organisms of UTI.Biofilm formation allows the strain to persist in genitourinary tract for long time, and survival advantages conferred by biofilm include resistance to phagocytosis and antimicrobial agents .There are various methods to detect biofilm production;in this

study we evaluated 50 isolates by two screening methods for their ability to form biofilms, Congo red agar method [CRA] and microtitre plate method [MTP] (23).
In CRA methods all bacterial isolates were grown in Cong red agar to detect biofilm production as shown in fig. (5).

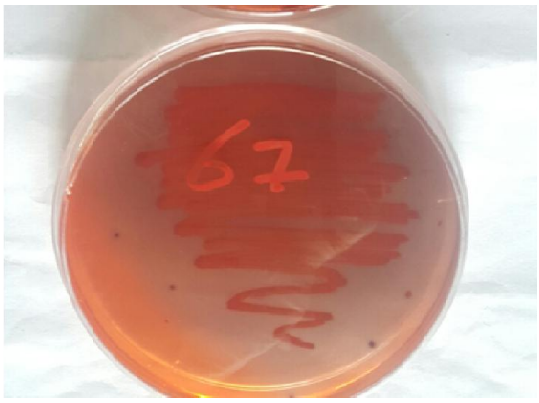

Fig. (5) Detection of *Enterococcus* biofilm production on Congo red agar Method

Our results showedthat 26(92.8%) isolates of *E.faecalis* were biofilmproducer(strong and moderate) and 2(7.1%)were weakbiofilmproduction, while 21(95%) isolates of *E.faecium*were biofilm (strong and moderate) production and1 (4.5%) weak biofilm production as show in table (8).

Table (8): percentage of *E.faecalis* and *E.faecium* biofilm production on CRA medium

***Enterococcus* species**	**Number of isolates**	**Strong**	**Moderate**	**weak**
E.faecalis	28	12(42.8%)	14(50%)	2(7.1%)
E.facium	22	10(45.4%)	11(50%)	1(4.5%)
Total	50	22(44%)	25(50%)	3(6%)

Such a high percentage of biofilm production in our results agree partially with a study obtainedby (24) who find that (85.7%) of *E.faecalis* isolates were slimes producer on CRA plates.In addition these results agree with study done by (25) who found that the ability to produce biofilm was detected in 90% of*E.faecium* isolates.In

MTP methods we used polystyrene plate of 96 wells for detection of biofilm production as shown in fig. (6).

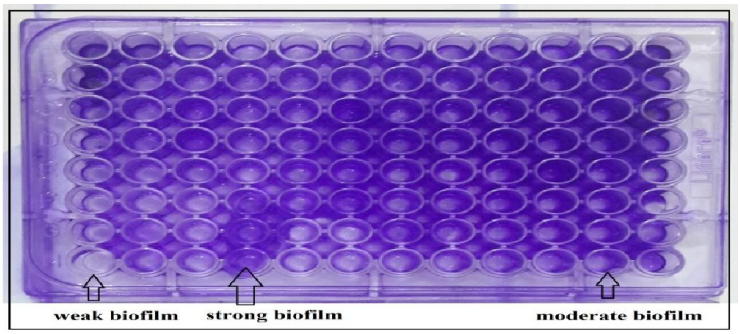

Fig. (6): polystyrene plate for detection biofilm in *Enterococcus*

Our results showed that 11(39%) isolates of *E.faecalis* detected as strong biofilm production, 14(50%) isolates as intermediate and 3(10%) as weak biofilm production, close to these results were reported by(11) who found 39% of isolates strong, , 52% moderate and 9% of isolates weak biofilm production.

The percentage of biofilm formation in *E.faecium* was 9(40%) as strong, 12(54.5%) moderate and 1(4.5%) as weak biofilm production as shown in table (9).

Table (9): percentage of *E.faecalis* and *E.faecium* biofilm production by (MTP) method

***Enterococcus* species**	**Number of isolates**	**strong**	**Moderate**	**weak**
E.faecalis	28	11(39%)	14(50%)	3(10%)
E.facium	22	9(40.9%)	12(54.5%)	1(4.5%)
Total	50	20(40%)	26(52%)	4(8%)

These results partially agree with (26) who found that 9(32.14%) fecal isolates of *E.faecium* were strong biofilm production, 3(10.7%) weak biofilm production.

On the other hand,these results weredisagreeing with study of (27) who found that *E.faecalis* 39 (25.16%) and *E.faecium* 42 (27.09%) produce biofilm.

Microtitre plate method were found to be most sensitive, accurate and reliable screening method for detection of biofilm formation when compared to CRA methods.Microtiter plate method was quantitative test and it was considered the gold

standard method for biofilm detection (28). Many studies have statistically evaluated the sensitivity and specificity between the two methods. Most of the studies recommend MTP method for general screening on biofilm formation (29) also found MTP method to be more suitable for biofilm detection as compared to CRA method. Similarly, (30) and (31) found MTP method to be superior to MTP and CRA methods.

-Comparison of the prevalence of *EfaA* genes among biofilm positive and biofilm negative in *E.faecium* and *E.faecalis* isolates:-

EfaA have a significant role in *Enterococcus* adhesion to biotic and abiotic surface which is the first step in biofilm production (9).

Our results show that *E.faecalis* have 25(89.2%)of *EfaA* gene in biofilm positive isolates and 3(10.7%) in biofilm negative isolates, while,the results of the presence of *EfaA* gene in *E.faecium*were 21(95.4%) in biofilm positive isolates and 1(4.5%) in biofilm negative isolates as shown in fig. (7). Our results demonstrate that the prevalence of *EfaA* gene in biofilm positive *Enterococcus* isolates were higher than in biofilm negative isolates.

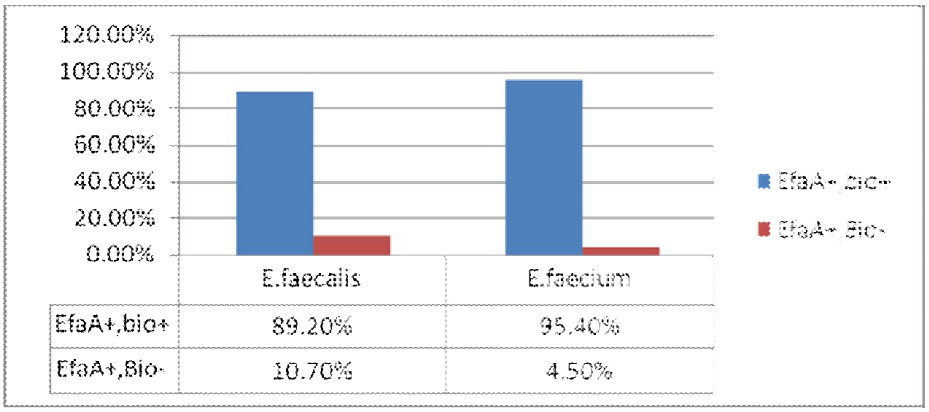

Fig. (7): percentage of *EfaA* gene among biofilm positive and negative *E.faecalis* and *E.faecium* isolates

These results were agreedwith (32) who showed that isolates with the *EfaA* gene produced more biofilms than negative ones. These results indicate the role of *EfaA* gene in biofilm production.

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الخلاصة

تم جمع (104) عينة ادرار من المرضى المصابين بالتهاب المجاري البولية لمختلف الفئات العمرية من مستشفيات مختلفة في بغداد (مستشفى ابن البلدي ،اليرموك،مدينة الطب،الكندي ومستشفى بغداد) وللفترة من بداية ايلول 2015 ولغاية نهاية كانون الاول 2015 .فحصت جميع العينات باستخدام الطرائق التقليدية بلاعتماد على الصفات الزرعية والاختبارات الكيموحيوية ونظام API 20 strep ، اظهرت نتائج التشخيص بالاعتماد على الطرائق التقليدية عائدية 50 عزلة لبكتريا المكورات المعوية وهذا ماكدته ايضا نتائج تفاعل السلسلة المتبلمرة اعتمادا على مورثات متخصصة.اجري تفاعل السلسة المتبلمرة ايضا للتحري عن وجود موروث *EFaA* المشفرالى مستضد التهاب شغاف القلب لبكتريا المكورات المعوية البرازية باستخدام بوادىء متخصصة ،اظهرت النتائج احتواء بكتريا المكورات المعوية على نسبة 100% من موروث *EFaA*. تم الكشف عن إنتاج بيوفيلم في *E.faecalis* و*E.faecium*باستخدام طريقتين: Congo red agar وطريقة microtitre plate،اظهرت النتائج ان 22(44%)من عزلات بكتريا المكورات المعوية منتجة قوية للبايوفلم، 25(50%) متوسط و3(6%)ضعيف بانتاجة للبايوفلم باستخدام طريقة CRA، اظهرت نتائج باستخدام طريقة MTP ان 20(40%)من العزلات البكتيرية منتجة قوي ،26(52%) متوسط و 4(8%) ضعيف بانتاجة للبايوفلم.تهدف هذه الدراسة الى تشخيص كل من بكتريا *E.faecalis*و *E.faecium*من مرضى التهاب المجاري البولية باستخدام طرائق تقليدية وجزيئية وكذلك الكشف عن موروث *EfaA* وعلاقتة بانتاج البايوفلم.

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