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Molecular Detection of *Enterococcus* Surface Protein (*Esp*) Gene in *E.faecalis* and *E.faecium* 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 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 showed the revealed of 50 isolates to Enterococcus and this confirmed by polymerase chain reaction technique based on amplification of species specific genes. PCR were performed for *E.faecalis* and *E.faecium* in order to confirm the presence of *Esp* gene which coding for Enterococcus surface protein using specific primer for gene, the results showed Enterococcus contain a proportion of 54% of *Esp*. Biofilm production was detected in *E.faecalis* and *E.faecium* by use two methods: Congo red agar method and microtiter plate method, our results showed 22(44%) of Enterococcus isolates was strong biofilm production, 25(50%)as moderate and 3(6%) as week biofilm production. By use Congo red method, in microtiter plate method results showed that 20(40%) of bacterial isolates was detected as strong, 26(52%) as moderate and 4(8%) as week biofilm production.

Key words: *E.faecalis*, *E.faecium*, *Esp*, 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 *Enterococcus* pathogenic factors have been identified including adhesions and secreted virulence factors (8). One of the Enterococcus virulence gene was *Esp* which encoded by the *Esp* gene, seems to contribute to the colonization and persistence of *E. faecalis* strains in ascending infections of the urinary tract. In addition, *Esp*

may mediate the interaction with primary surfaces and participate in biofilm formation (9), which substantially enhances bacterial survival in biopolymers and may also be involved in antimicrobial resistance (10).

This study aims to diagnosis of *E.faecalis* and *E.faecium* from urinary tract infection patients by traditional and molecular methods, detection of *Esp* gene and it role in biofilm production.

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 to the end of December 2015.

Isolation and identification of *Enterococcus* by traditional methods

Culturing on selective media

The isolates were culture on different media such as (blood agar, MacConkey agar) and 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 extraction Genomic DNA, Purification depending on instruction of manufacturing company(Geneaid, Thailand).

Detection of *Enterococcus* by molecular method

Detection of *Enterococcus* species by use species specific primer

Multiplex PCR used for conformation identification of the *E.faecalis* and *E.faecium,* reaction was 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, the sequence of primer mention in table (1), finely 3 µl of DNA added(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	11
ddl *E.faecalis*	F:ATCAAGTACAGTTAGTCTTTATTAG R:ACGATTCAAAGCTAACTGAATCAGT	941	11

Table (2): The Mixture of multiplex PCR working solution for 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 show in table (3), for PCR products were analyzed in Agarose gels and visualized under UV after staining with ethidium bromide.

Table (3): PCR Program for detection of ddl *E.faecium* and ddl *E.faecalis* genes amplification 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 *Enterococcus Esp* gene

PCR were used for detection of *Esp* gene in *E.faecalis* and *E.faecium* using specific primer. 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 mention in (Table 4), finely 3 µl of DNA added (Table5).

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

Genes	Sequence(5' to 3')	Size	Reference
Esp	F:AGATTTCATCTTTGATTCTTGG R:AATTGATTCTTTAGCATCTGG	510	12

Table (5): The Mixture of PCR working solution for detection of Enterococcus *Esp* 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 95° C for 5 min, denaturation at 94° C for 1 mint, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min as show in table (6), products were analyzed in Agarose gels and visualized under UV after staining with ethidium bromide.

Table (6): PCR Program for detection of Enterococcus *Esp* gene amplification by conventional PCR.

No.	Steps	Temperature (°C)	Time
1	Initial Denaturation	95	5 min
2	Denaturation	94	1 min
3	Annealing	56	1 min
4	Extension	72	1 min
5	Final extension	72	10 min
6	Cycles number	30	

Biofilm production test

Congo Red Agar method

A specially prepared medium known as Congo Red Agar (CRA) is 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 (13).

Microtiter plate methods of biofilm assay

A modified microtitre plate method was used as described by Mirzaee *et al*. (14). Briefly, the wells of microtitre 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 supplemented with 5% glucose. Incubation 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 stain 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

Samples of urine were culture on different media such as (blood agar , MacConkey agar, and bile esculin agar), on the blood agar colony appear as white to gray color, sticky textures while in MacConkey agar colony appear as small size, smooth and circular shape with a pink color due to its ability to ferment lactose(15).While on selective media (bile esculin agar) gave round shape colony with slightly convex smooth edges, white or creamy color and convert media color into black as show in (Figure 1).

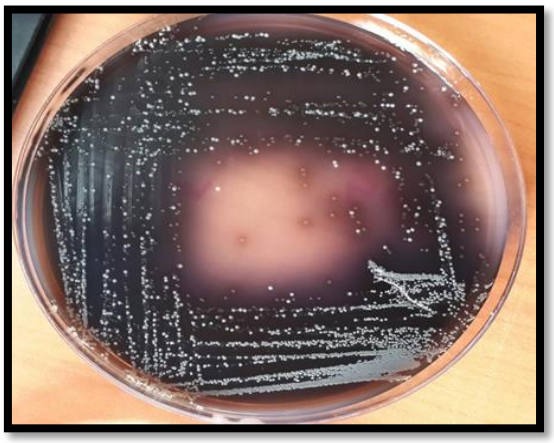


Figure (1): colony of Enterococcus on bile esculin agar.

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 (Figure 2).

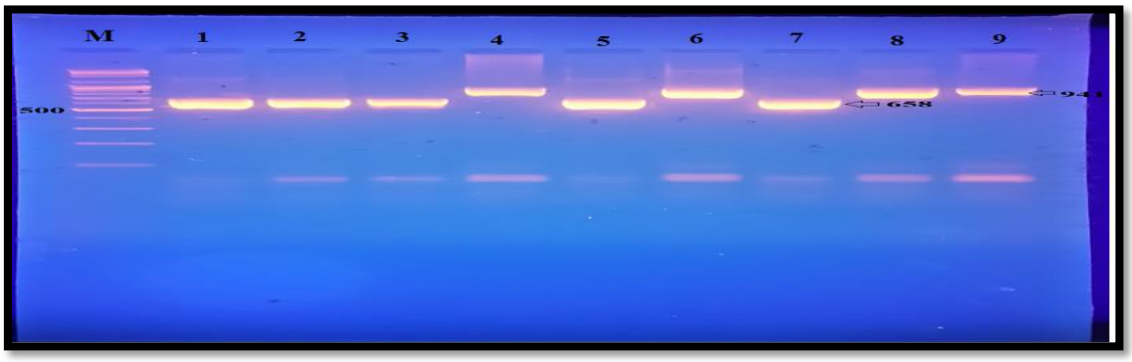
Figure (2): Biochemical identification of Enterococcus using API 20 strep.

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, use 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* (table 8), similar finding was reported by Comerlato *et al*.(16),piece that amplify by PCR detect by using gel electrophoresis as show in (Figure 3).

Table (8): Number and percentage of Enterococcus species

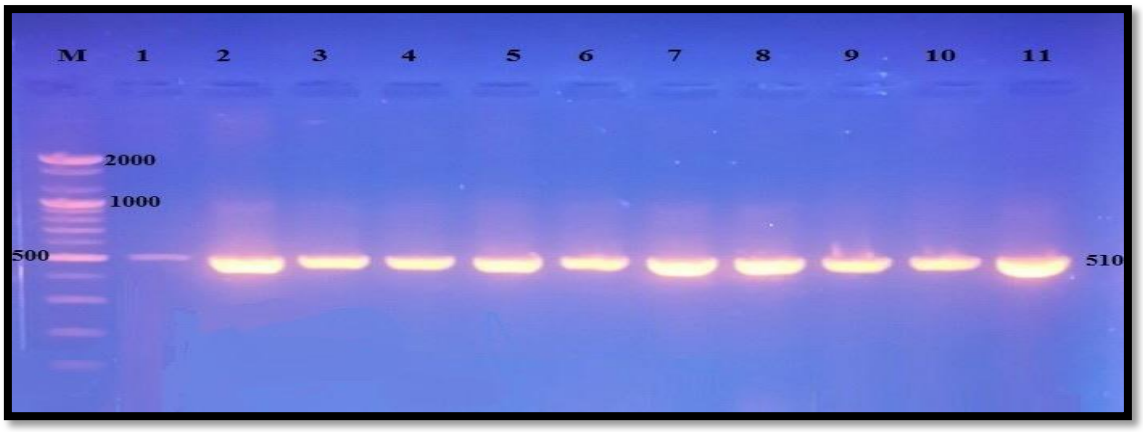
Enterococcus species	Number of isolates	Rate of isolation
E.faecalis	28	56%
E.faecium	22	44%
Total	50	100



Figure(3): Agarose gel electrophoresis of multiplex PCR for identification 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 *Esp* gene in *E.faecalis* and *E.faecium* by conventional PCR

Several virulence and pathogenicity factors have been described from Enterococci that enhance their ability to colonize patient's tissues, increase resistance to antibiotics, and aggravate the infection outcomes (17). Conventional PCR amplification was performed for *E.faecalis* and *E.faecium* in order to confirm the presence of Enterococcus surface protein (Esp) by use specific primer, piece of DNA that amplify by PCR detect by using gel electrophoresis as show in (Figure 4**).**



Figure(4): Agarose gel electrophoresis of conventional PCR amplification products of Enterococcus species *Esp* gene (510pb).M: marker (100pb ladder) lanes (1-6): positive amplification of *Esp* gene in *E.faecalis*, lanes (7-11): positive amplification of *Esp* gene in *E.faecium*.

The percentage of *Esp* gene in our study for *E.faecalis* was 15 (53.5%), such a low percentage coincide with study by Sharifi *et al*.(18) who study virulence and antimicrobial resistance in Enterococci isolated from urinary tract infections.

Occurrence of *Esp* genes in *E.faecium* was 12(54.5%), in comparing the current results with other research, it can be concluded that there was similarity with that of Sieńko *et al*. (19) who observed that biofilm non-producing *E.faecium* harboring *Esp* gene in 55%.Our results of presence *Esp* gene in Enterococcus isolates was disagreement with study of Eaton and Gasson (20) whom study molecular screening of Enterococcus virulence determinants and potential for genetic exchange between Food and medical isolates.

Detection of biofilm production

Biofilm producing bacteria are responsible for many recalcitrant infections and are difficult to eradicate (21). 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] (22).

In CRA methods all bacterial isolates was grow in Cong red agar for detect biofilm production as show in (Figure 5).

Figure(5): Detection of Enterococcus biofilm production on Congo red agar Method A: negative results, B: positive results.

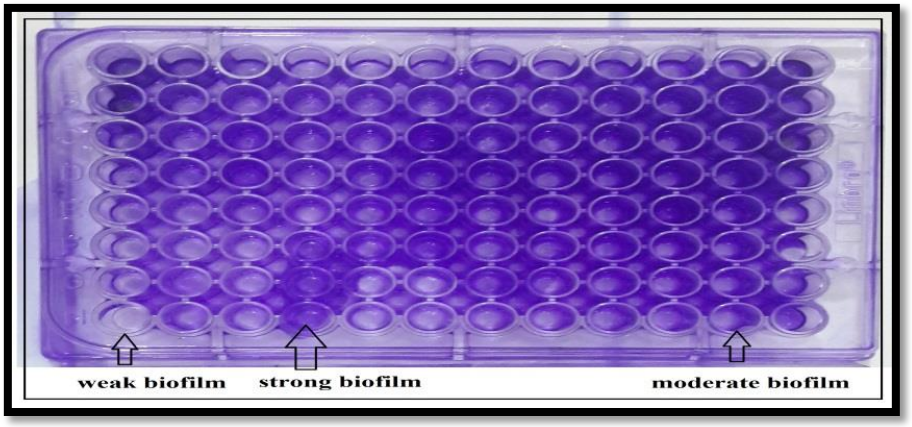
Our results showed that 26(92.8%) isolates of *E.faecalis* were biofilm producer (strong and moderate) and 2(7.1%) were weak biofilm production, while 21(95%) isolates of *E.faecium* were biofilm producers and1 (4.5%) weak biofilm producers as show in (Table 9).

Table (9): 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 was agree partially with study obtain by Mohamad and El Shalakan (23) who found that (85.7%) of *E.faecalis* isolates were slimes producer on CRA plates. Also this results agree with study done by Sieńko *et al.*(19) who found that the ability to produce biofilm was detected in 90% of *E.faecium* isolates.

In MTP methods we use polystyrene plate of 96 wells for detection of biofilm production as show in (Figure 6).



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

Our results showed that 11(39%) isolates of *E.faecalis* detect as strong biofilm producers, 14(50%) isolates as intermediate and 3(10%) as weak biofilm producers, close to these results was reported by Mohamed *et al*. (25) who find 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 show in (Table 10).

Table (10): 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 Diani *et al*. (24) 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 were disagreeing with study of Banerjee and Anupurba (26) whom 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, microtitre plate method was quantitative test method and it was considered the gold standard method for biofilm detection (27). 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. Knobloch *et al.* (28) also found MTP method to be more suitable for biofilm detection as compared to CRA method. Similarly, Hittinahalli *et al.* (29) and Ira *et al.* (30) found that MTP best method than CRA method.

Comparison of the prevalence of *Esp* genes among biofilm positive and negative production in *E.faecium* and *E.faecalis* isolates

Expression of *Esp* was correlated with initial adherence to polystyrene and biofilm formation (31).

Our results showed that *E.faecalis* have (53.5%) of *Esp* gene in biofilm positive isolates and zero in biofilm negative isolates, while, the results of the presence of *Esp* gene in *E.faecium* were (50%) in biofilm positive isolates and (5%) in biofilm negative isolates as shown in fig.(7). Our results demonstrate that the prevalence of *Esp* gene in biofilm positive Enterococcus isolates was higher than in biofilm negative isolates. These results indicate the role of *Esp* gene in biofilm production. Our results agree with study of Moniri *et al*.(32) how found *E. faecalis* isolates that have both *Esp* and *Aac (6')/aph (2")* genes form strong biofilms in the urinary tract.

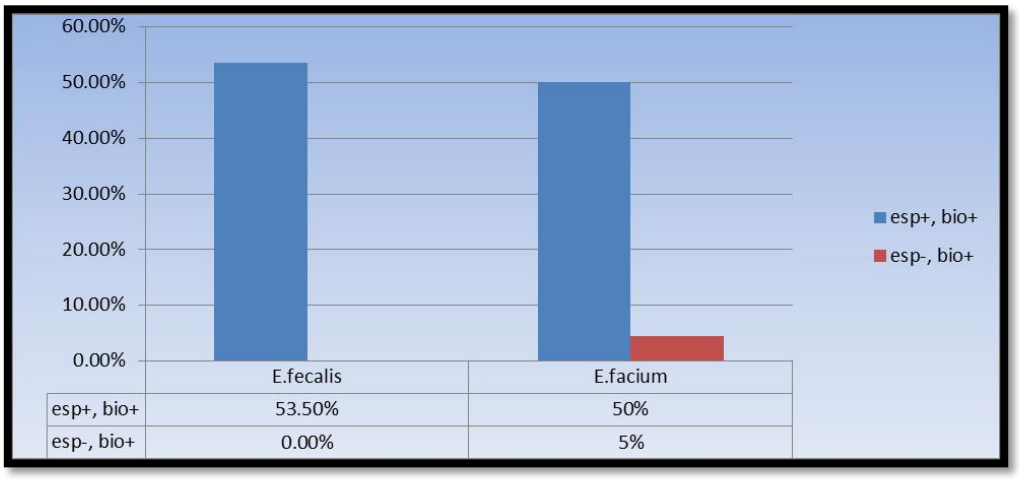


Figure (7): percentage of *Esp* gene among biofilm positive and negative *E.faecalis* and *E.faecium* isolates.

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