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Assessment of Biofilm Production and Antibiotic Pattern in *E. faecium* and *E. faecalis* isolated from Some UTI Iraqi Patients

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A B S T R A C T

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 showed the revealed of 50 isolates to *Enterococcus* and this confirmed by polymerase chain reaction technique based on amplification of species specific genes. Antimicrobial susceptibility testing of the fifty isolates were screened by disc diffusion method on Mueller-Hinton agar using 10 types of antibiotics which differ in their action includes (Ampicillin, Cefepime, Imipenem, Vancomycin, Rifampicin, Erythromycin, Ofloxacin, Oxacillin Oxytetracycline, and Streptomycin),results show 100% of isolates resist to six types of antibiotics (Cefepime, vancomycin, rifampicin, erythromycin, oxacillin and oxytetracyclin) and showed different pattern of resistance to the others four antibiotics. On the other hand we use two methods, Congo red agar(CRA) and microtitre plate method (MTP) to detect *Enterococcus* biofilm production. Results of (CRA) methods show that 22(44%) detect as strong, 25(50%) as moderate and 3(6%) as week biofilm production, while for (MTP) method results show that 20(40%) detect as strong, 26(52%) as moderate and 4(8%) as week biofilm production. This study aimed to describe antibiotic resistance of *E. faecalis* and *E. faecium* and it role in biofilm formation from bacterial isolates.

Introduction

Urinary tract infections (UTIs) comprise one of the largest classes of infections occurring in both hospital and community (Peleg and Hooper, 2010; Broeren *et al*., 2011). UTI are classified as uncomplicated or complicated (Stamm *et al*., 2001). Uncomplicated UTIs occur in sexually active healthy female patients with structurally and functionally normalurinary tracts.

Complicated UTIs are those that are associated with comorbid conditions that prolong the need for treatment or increase the chances for therapeutic failure. These conditions include abnormalities of the urinary tract that impede urine flow, the existence of a foreign body (e.g., indwelling catheter, stone), or infection with multidrug resistant pathogens (Hooton, 2000; Stapleton, 2003).

Enterococcus are intrinsically resistant to many antibiotics and are able to acquire drug resistance either by chromosome, transfer of plasmid or transposon acquisition containing genetic sequences that confer resistance in other bacteria (Belgacem *et al*., 2010; Hammerum *et al*., 2010).

Biofilms are an important factor in the pathogenesis of *Enterococcus* infections (Mohamed *et al*., 2004). Biofilms are not only resistant to antibiotics but a variety of disinfectants (Chenand Wen, 2011).

Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic into biofilm and expression of possible resistance genes (Lewis, 2001).

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 extraction Genomic DNA, Purification depending on instruction of manufacturing company (Geneaid, Thailand).

Detection of *Enterococcus* by Molecular Method

Detection of *Enterococcus* speciesby 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).

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.

Antibiotics Susceptibility Test (11)

Few colonies of identical bacteria were picked up from an agar plate (fresh culture 24 h old) and suspended until a turbidity equivalent to 0.5 McFarland. Sterile swab was dipped into inoculums tube, streaked over the surface of the Mueller-Hinton medium then left to dry. A maximum of five

antibiotic disks were placed on the plate, incubated for 24 hour at 37^{o}C.The resulting zones of inhibition were measured by a ruler and compared with the zones of inhibition determined by CLSI (2013).

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 24h. Readings were taken after 24h and again after 48h. A positive result was indicated by black colonies with black crystalline morphology. Non-biofilm producers mostly produced pink- or red-colored colonies (Freeman *et al*., 1989).

Microtiter plate methods

A modified tissue culture plate method was used as described by Mirzaee *et al*., (2014). 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 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 was measured at 490 nm using a micro ELISA auto reader and considered as an index of bacteria adhering to surface and forming biofilms (table-4).

Results and Discussion

Clinical Samples

Identification of *Enterococcus* by Traditional methods

Fifty isolates identify as *Enterococcus* on bile esculin agar Fig(1) depend on creamy color of colony which conversion of 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 (MacFaddin, 2000).

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).

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*, similar finding was reported by Comerlato *et al*. (2013),piece that amplify by PCR detect by using gel electrophoresis as show in fig(3).

Antibiotics Susceptibility Test

Antibiotic sensitivity test was conducted for 50 *Enterococcus* isolates (twenty eight

isolates of *E.faecalis* and twenty two isolates for *E.facium*) using 10types of antibiotics with different action, the percentage of resistance show in table (5).

The results showed that (100%) of *E.faecalis* and *E.faecium* were resist to (cefepime, vancomycin, rifampicin, erythromycin, oxacillin and oxytetracycline), while 95.4% of *E.faecium* and 89.2%of *E.faecalis* were resist to ampicillin and ofloxacin respectively.

On the other hand 77.2% of *E.faecium* and 78.5% of *E.faecalis* were resist to impenem, 81.1% of *E.faecium* and 92.8% of *E.faecalis* were resist to streptomycin.

This matching of results correlated with the previous study in Iraq (Al-Shamary, 2011 and AL-Marjani, 2013) and other study in the world (Al-Ruwaili *et al*., 2012 and Sharifi *et al*., 2012).

Detection of biofilm production

All isolates of bacteria grow in Congo red agar for detection biofilm production as show in fig.(4). The results show that 26(92.8%) of *E.faecalis* produce biofilm (strong and moderate) while 2(7.1%) were weak biofilm production, 21(95%) isolates of *E.faecium* were biofilm production (strong and moderate) while one isolate (4.5%) was weak biofilm production as show in table (6).

Such a high percentage of biofilm production in our results was agree partially with study obtain by Mohamad and El Shalakan (2016) who find that (85.7%) of *E.faecalis* were slimes producer on CRA plates, also this results agree with study done by Sieńko *et al*.(2015) who find that the ability to produce biofilm was detected in 90% of *E.faecium*.

In MTP methods we use polystyrene plate of 96 wells for detection of biofilm production as show in fig. (5).

Our results show 11(39%) isolates of *E.faecalis* detect as strong biofilm production, 14(50%) isolates as intermediate and 3(10%) as weak biofilm production, close to these results was reported by Mohamed *et al*., (2004) 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 one isolate (4.5%) as weak biofilm production as show in table (7).

These results partially agree with Diani *et al*., (2014) who find 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 (2015) 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 (Mathur *et al*., 2006). 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*., (2002) also found MTP method to be more suitable for biofilm detection as compared to CRA method. Similarly, Hittinahalli *et al.*, (2012) and Ira *et al*., (2013) found MTP method to be superior to MTP and CRA methods.

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	Sharifi *et al.*, (2012)
ddl *E.faecalis*	F:ATCAAGTACAGTTAGTCTTTATTAG R:ACGATTCAAAGCTAACTGAATCAGT	941	Sharifi *et al.*, (2012)

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

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	

Table.4 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

Table.5 Percentage of antibiotic resistant in *Enterococcus* species

Antibiotic	*E.faecium*	*E.faecalis*	Total resist %
Ampicillin(AM)	95.4 %	89.2%	92%
Cefepime(FEP)	100%	100%	100%
Imipenem(IMP)	77.2 %	78.5 %	78 %
Vancomycin(VA)	100 %	100%	100 %
Rifampicin(RA)	100%	100%	100%
Erythromycin(E)	100%	100%	100%
Ofloxacin(OFX)	95.4 %	89.2 %	92 %
Oxacillin(OX)	100%	100%	100%
Oxtetracycline(OXY)	100%	100%	100%
Streptomycin(S)	81.8 %	92.8 %	88%

Table.6 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.faecium	22	10(45.4%)	11(50%)	1(4.5%)
Total	50	22(44%)	25(50%)	3(6%)

Table.7 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%)

Fig.1 Appearance of *Enterococcus* isolates on Bile Esculin Agar.

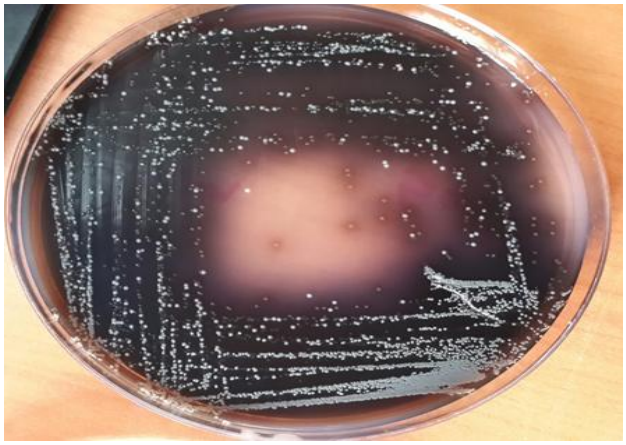


Fig.2 Biochemical identification of *Enterococcus* using API 20 strep

Fig.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.

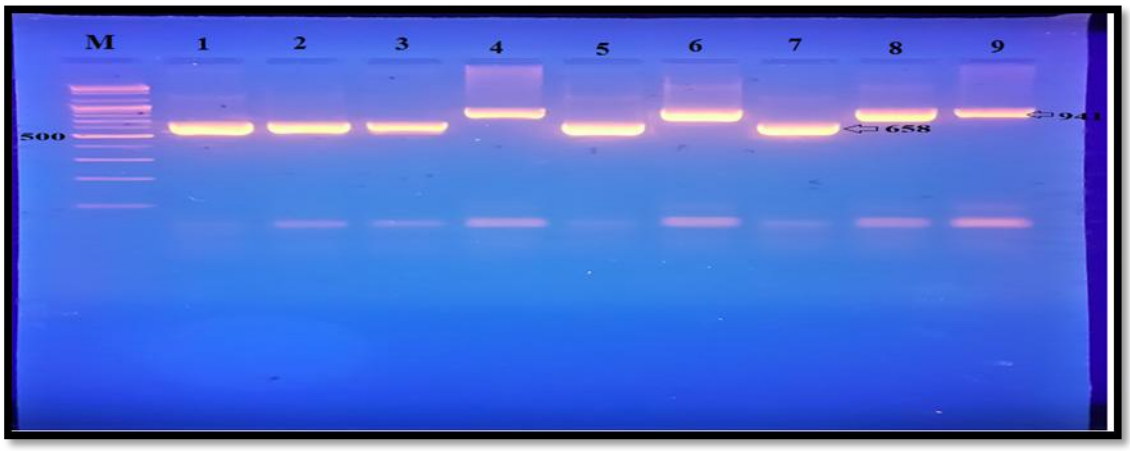


Fig.4 Detection of *Enterococcus* biofilm production on Congo red agar method

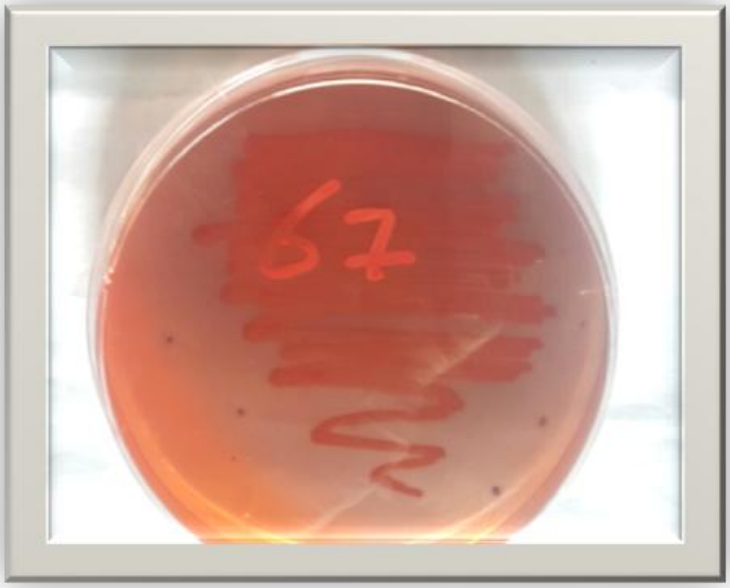


Fig.5 polystyrene plate for detection biofilm in *Enterococcus*

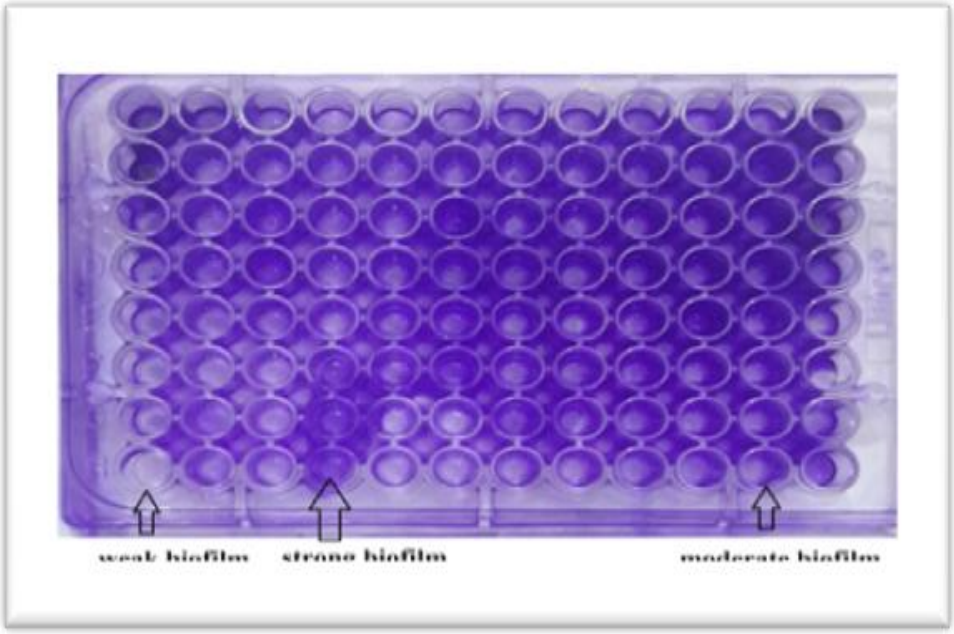
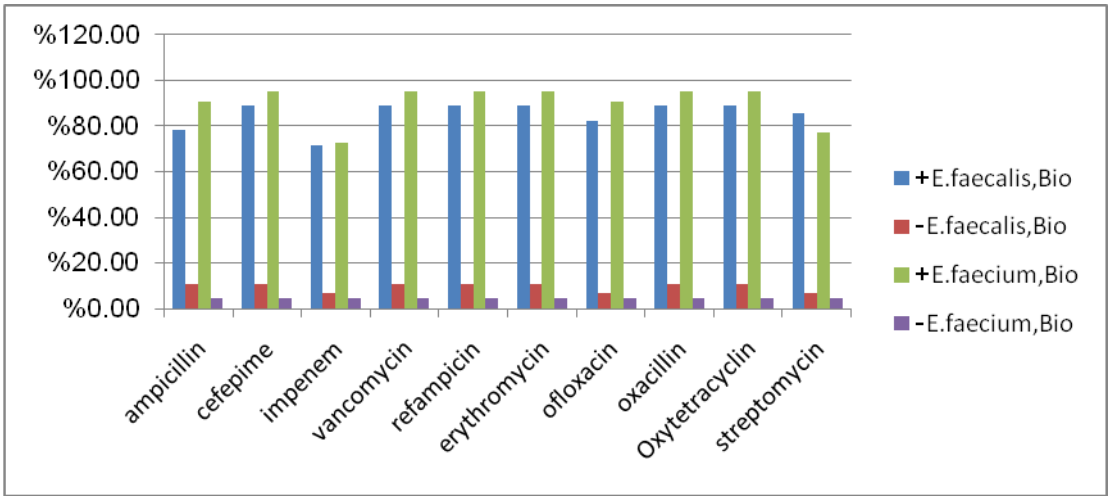


Fig.6 Relation between *Enterococcus* antibiotic resist and biofilm production



Our results show high antibiotic resist isolates among biofilm positive isolates compere to biofilm negative isolates as show in fig.(6).

Importance of biofilm formation has been described in the control of microbial infection in several areas because the biofilm can increase resistance to various physical and chemical agents, especially antibiotics (Murray and Weinstock, 1999).

Biofilm exhibits more resistance to broad spectrum antibiotics (Mathur *et al*., 2006), this supports that biofilm adds to the virulence profile of microorganism (Suman *et al.*, 2007).

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