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The correlation between some pathogenicity associated virulence factor and biofilm formation among uropathogenic *Escherichia coli* isolates in Al Najaf Al-Ashraf province

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Abstract:

Background: The linkage of virulence factors of bacteria with UTI is processed from the insight of each individual VF in separation from each other and studies of the assembly and function of UPEC virulence factors can offer a platform for the development of novel researches.
Aim of study: The study aim to investigate the incidence and prevalence of the some pathogenicity associated virulence factors (PAVF) in uropathogenic *Escherichia coli* (UPEC) such as motility (swarming and swimming), hemolysin (α and β), siderophore and kind 1 fimbria and association of them with biofilm formation capability.
Patients and Method: A total of (170) urine specimens were collected during the period from May 2014 to November 2014 from patients suffering from UTI. All Patients were hospitalized in Alzahraa and Alsadr Teaching Hospitals.
Results: **a)** Out of 170 urine samples, only 70 (41.2%) of it were UPEC positive isolates and 100 (58.8%) were negative isolates. **b)** 44 (63%) from cases showed mannose-sensitive haemagglutination (MSHA), 18 (26%) showed mannose-resistant haemagglutination (MRHA) and 8 (11%) shows negative results. **c)** 56(80%) of UPEC isolates were positive for motility and motility of both mode of motions (swarming and swimming) the results were 66(94%) and 70(100%) respectively. **d)**16 (23%) of UPEC were β-hemolysis producer, 5 (7%) of it were α -hemolysis producer and 49(70%) did not have hemolysis action. **e)** 69(99%) were positive for siderophore production **f)** 67 (96%) of UPEC isolates has positive biofilm formation. **g)** There were no significant values between biofilm production and the other virulence factors among the groups of UPEC isolates under investigation. As far as pearson correlation method, it was found that there was positive correlationship between biofilm production and virulence factors like ((hemolysin (α,β), siderophore and kind 1 fimbria)). On the other, there was negative correlationship between biofilm production and virulence factors like swarming and swimming (phenomenon).

Conclusion: In respect to the predominance occurrence of virulence factors either solely or collectively in UPEC strains this support the concept of association of UPEC with urinary pathogenicity.

Recommendation: future studies may be needed for identifying other pathogenic virulence factors among EPEC isolates necessary for emphasizing their pivotal roles in pathogenicity and to guide intervention to minimize its occurrence.
Key words: biofilm, virulence factor, UPEC.

Introduction:

A biofilm is an organized community of microorganisms (e.g. bacteria, archaea, protozoa, fungi and algae) coated by a protective and adhesive matrix. Biofilms space generally found on solid substrates or being sort floating liquid surfaces [1].

Microorganism living in a biofilm some times have completely different properties from free-floating (planktonic) microorganism of identical species, as has been shown by completely different approaches, together with microarray analysis studies [2]. In *E. coli*, biofilm formation proceeds in a biological circle consisting of five steps as i) Initial adhesion, ii) surface attachment, iii) Microcolony formation, iv) Maturation and v) Dispersion.

The initiation of a biofilm is regulated by environmental signals as nutrients, temperature, osmolarity, pH, iron and oxygen. Nutrient accessibility influences the thickness of the biofilm and dispersion. Therefore, it may be projected that the starvation response pathway plays an overall role in the management of biofilm formation. [3].

In UPEC infections kind one fimbriae through its fimH adhesin play a big role within the attachment of *E. coli* throughout infections . Also fimH mediates biofilm formation . Another feature related to kind one fimbriae is their ability to confer microorganism invasion into host cells, were expression of type one fimbriae by UPEC promotes invasion of human bladder and animal tissue cells, whereas this morphological composition wasn't determined in *fimH*-negative mutant strains [4].

In concern to motility is an important factor in concern to bacterial colonization, So many bacterial species are motile by flagella. In *E. coli* flagella drive bacterial cell swimming in liquid medium or in semisolid agar media. As a response to chemotactic external signals, *E. coli* is capable of directing its swimming movement towards a microenvironment that is ideal for it's growth and survival [5]. When the conditions for swimming change and become adverse, *E. coli* develops a different mode as swarming motility [6]. Flagella-driven coordinated motility are intermediated by cell-cell communication mechanisms like quorum sensing so, the microorganism migration activity has an intrinsically surface-linked phenomenon, leading to a shift from a single to a collective "social" behavior that permits the active exploration and colonization of surfaces [7].

In respect to α-haemolysin is a toxin that's related to uropathogenic *E. coli*. It belongs to the category of RTX toxins (repeated toxins), that all contain a synonym duplication of 9 amino

acids. This type of toxin acts by making pores within eukaryotic semi permeable membrane. Pore formation relies on the binding of Ca^{2+}. *E. coli* expressed α-hemolysin might induce epithelial tissue destruction and urinary organ constriction by the intra-renal unleash of endothelin [8].

As far as Iron is a necessary chemical compound for microorganism metabolism, survival and multiplication in specific ecological niches and depends on the potency of those organisms to scavenge these essential nutrients. Iron plays a significant role in cellular processes like energy generation, deoxyribonucleic acid replication, electron and O_2 transport, metabolism of peroxidases and protection against oxidative stress .Pathogenic bacterium, as well as ExPEC and a lot of specifically UPEC, get iron from their host by expressing associate degree iron acquisition system that utilize siderophores to scavenge iron from the surroundings and creating it offered to the microorganism cell. [9].

Aim of study: The study aimed to determine the incidence and prevalence of the some pathogenicity associated virulence factors (PAVF) in uropathogenic *Escherichia coli* (UPEC) like motility (swarming and swimming), hemolysin (α and β), siderophore and kind 1 fimbria and correlation of them with biofilm formation ability.

PATIENTS AND METHOD:

Atotal of (170) urine specimens were collected during the period from May 2014 to November 2014 from patients suffering from UTI. All urine samples were routinely cultured on MacConkey and blood agar plates. Only one bacterial isolates per patient that are characterized by purity and predominant growth was included in this study. Totally, 70 consecutive non duplicated bacterial isolates were recovered from urine samples of patients with positive bacteriuria . The isolates were identified by their cultural characteristics, Gram staining, reactions to standard biochemical tests indole, methyl red, Voges-Proskauer, triple sugar iron and cimmon citrate tests a and by API 20E system.Phenotypic analysis included special routine and advance techniques for studying of adhesion, motility, haemolysis, siderophore and biofilm productions.All experiments follow the approach of Kostakioti, (2009)[10].

A-Kind One Fimbriae Production Assays: tested by mannose heamagglutionation assay (MHAA) were the hemagglutinins were detected by agglutination of 3% 'O' blood group human RBCs, in presence or in the absence of 2% [wt/vol] D-mannose . When agglutination was observed macroscopically after manual shaking for ten minutes at room temperature, the result was considered as positive. Agglutination is inhibited in the presence of that carbohydrate; therefore showing mannose sensitive .The following controls were used: *E. Coli* for MSHA. And *Proteus mirabilis* for MRHA.

B-Motility Production Assay: capacity of bacteria to express flagella was assayed by their ability to create turbidity in semi solid manitol culture after 24 h incubation at 37°C.Motility of different types was tested: swimming motility was tested on BHI broth medium with 0.3% agar;

swarming motility was analyzed on BHI broth medium with 0.5% agar were 10µl of an overnight isolates has been spotted gently in the center of Brain Heart infusion medium . Plates were Incubated at 37% for 24hr. assess of the migration of bacteria from the point of inoculation (observed as a turbid zone in millimeters) were measured at 24 hrs. The results are the means of at least three independent experiments.

C- Hemolysin Production Assay : tested by streaking a purified bacterial isolates on blood agar base supplemented with 5 % of human blood and incubated for 24hrs at 37 ^{0}C. The result was detected by occurrence of clear zone of haemolysis around the bacterial growth which refer to β-haemolysis, While appearance of a greenish–partial-blood haemolysis refer to α- haaemolysis . γ – haemolysis indicated by absence of haemolysis.

D-Siderophore Production Assay: Organisms were inoculated into the M9 media and incubated for 24 hr. at 37 o C.The results were seen if the growth of microorganism on medium was present or not .

E-Biofilm Formation Assay: tested by the ability of isolates for adhesion and formation of biofilm slime layer . A positive result was indicated by the presence of an adherent layer of stained material on the inner surface of the tube and comprise with the negative management (tube contains BHI only) .The quantity of biofilm formation was measured by dissolving visible film with ethanol (absolute) and measured the optical density of soluble biofilm formed at 595 nm by spectrophotometer device.

Statistical Analysis:

The data were analyzed by SPSS computer programme (Version twenty-two. SPSS Inc, United States) with pearson correlation statistical system . Applied medical significance was regarded at a P value < 0.05.

RESULTS:

Out of 170 samples of UPEC from patients with urinary tract infections, the results showed 70(41.2%) are positive isolates and 100 (58.8%) are negative isolates.

In respect to the positive UPEC isolates, the results maintain that 44 (63%) from cases showed mannose-sensitive haemagglutination (MSHA), 18 (26%) showed mannose-resistant haemagglutination (MRHA) and 8 (11%) shows negative results (absence of fimbria expression).

The results also indicate that 56(80%) of UPEC are positive for motility . Analysis study of motility of both mode of motions (swarming and swimming) state that almost (70) of UPEC strains have swimming phenotype (100%) and (66) of strains possess swarming motility (94%).

The analysis also shows that, 16 (23%) of UPEC are β-hemolysis producer, 5 (7%) of it are α -hemolysis producer and 49(70%) do not have hemolysis action on blood agar medium.

The results also show, 69(99%) are positive for siderophore production and that 67 (96%) of UPEC strains has positive biofilm formation.

Table (1). Show that there were no significant values between biofilm production and the other virulence factors among the groups of UPEC isolates under investigation. As far as pearson correlation method, it was found that there was positive correlationship between biofilm production and virulence factors like ((hemolysin (α,β), siderophore and kind 1 fimbria)). On the other, there was negative correlationship between biofilm production and virulence factors like swarming and swimming (phenomenon).

Table (1). The correlations between biofilm and other virulence factors of uropathogenic *E.coli* isolates (N=70).

Virulence factors	Pearson Correlation					
	Swimming	Swarmming	Kind 1-fimberia	Siderophre	α-hemolysin	β-hemolysin
Biofilm of UPEC	-0.42	-0.75	0.80	0.71	0.16	0.86
Significant (P-value)	.733	.536	.508	.557	.899	.477

Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION:

E. coli that are linked with UTI are usually called uropathogenic isolates (UPEC), despite the fact that there is proof that various pathotypes are related to UTI . UPEC remaining the predominantly isolated species . Recent study conducted by Al-Yassery (2011) showed that *E. coli* was important nosocomial pathogens representing the first leading causes of UTI in Najaf [11].

possession of MRHA by UPEC can be regarded as one of the most important virulence factor in the pathogenesis of UTIs. This concept has been supported by many scholars such as Kauser et al. (2009) [12].who have documented the incidence of MRHA *E. coli* isolates as 30%.

Motility have been researched a lot in many research groups because of their roles in virulence and biofilm development [13]. It contributes to virulence by arranging the expression of other virulence factors [14]. Other study conducted by Mihaylova, M., (2012) who argues that most strains had swimming phenotype in 15(75%) UPEC strains. On the other hand, swarming test results where it is found eight strains possessed swarming motility (40%) [15].

The ability of UPEC to cause UTI is related to general virulence factors such as α-hemolysin together with pili-mediated adherence to uroepithelial cells (Smith Y., etal. 2006)[16]. Several studies investigate the ability of UPEC strain to produce different type of hemolysin such as Marrs, et al., **(**2005) who state that about 50 % of urinary tract infection isolates have hemolysin A and the percentage was elevated with disease severity [17].

In this respect, Reigstad, et al. (2007) showed that the classical UPEC VF and hole-producing toxin especially α-hemolysin which are highly represented in biofilm formation synthesis [18].Similarly, Garofalo, et al. (2007) argue that the absence of familiar UPEC VFs such as hemolysin in experimental infections of hamster by 15 of total 18 UPEC isolates of human identify in a UTI from clinical analytical samples, will make these strains unable to form IBCs and incapable of invading the urothelium tissue of mouse. This process highlights on role and participation of hemolysin in IBCS formation [19].

Other studies such as these conducted by Porcheron, et al.,(2013) suggested that most *E. coli* strains, including commensal strains, produce siderophore associated with pathogenic *E. coli* strains [20], also Santo, etal., (2006) foun that out of 96 pathogenic *E. coli* strains, only 76% of isolates were aerobactin producer [21].

The results conducted in this study with respect to correlation of biofilm formation with mode of motility (swimming and swarming) were several studies which verify that many regulatory protein likely c-di-GMP and CsrA are negatively impact biofilm formation. These in their role, activate different gene targets for increased swarming motility [22].

These reversely correlation did not delete the effective role of motility in biofilm development especially when several studies clearly emphasize the role of flagella in biofilm formation [23].In this respect, a temporal regulation of biofilm factors might be aided by varying conditions brought on by biofilm growth. During initiation of biofilm on abiotic surfaces, flagellum-mediated mobility allows planktonic bacteria to swim to and adhere to abiotic surfaces [24].

As soon as, early bacteria reach the surface, the flagellar encoding genes are continuously repressed [25]. The overproduction of flagellar genes whether directly or indirectly by defecting flagellar gene repressors, will reduce biofilm formation by *E. coli* [26].

Another finding in this study is that there was positive correlation between biofilm formation and kind 1 fimberia which has been verified by several investigators such as Barken,

et al. (2008) who emphasized that kind 1 fimbria has a critical role in the stability and development of the biofilm substructures in *E. coli*. kind 1 pilus-mediated adhesion was needed for firm cohesion of the biomass [24]. Marhova, et al. (2009) share the same opinion which refered that kind 1 fimbriae promotes adhesion to host epithelial cells, Thus, it is very important in the initial steps of biofilm formation[27]. Pruss et al. (2006) it was suggested that biofilm-producing UPEC isolates had an interaction with kind 1 fimbriae expression stronger than that by non biofilm-producing strains [28].

This study has also verified that there were positive correlation between Biofilm formation and siderophore production. Reigstad, et al., (2007). Show a correlation between intracellular bacterial communities (IBC) formation and induction of genes contained in acquiring iron from heme and siderophores . Genes are contained in the synthesis of the three siderophores by the UPEC strain (enterobactin, salmochelin, and yersiniabactin) which are highly represented in the IBC.The researchers keep on suggesting that the systems of UPEC of multiple redundant iron acquisition systems, whether they are in vitro or during colonization of laboratory mice are largely regulated in the IBC. A deletion mutant which lacked the heme transporter protein was widely represented in the IBC, but not elsewhere. While, it forms significantly smaller IBCs in vivo . In this respect, the same authors show the classical UPEC virulence factor and pore-forming toxin, α-hemolysin are highly expressed in IBCs [29].

Similarly, Garofalo, et al., (2007) argue that the absence of familiar UPEC virulence factors such as hemolysin in experimental infections of mice with 15 of 18 human UPEC isolates from a clinical study of UTI will make these strains unable to form IBCs and unable to invade the mouse urothelium. This process highlights the role and participation of hemolysin in IBCS formation [30]. It should be pointed that Hannan, et al., (2008) found that mutant strain of UTI89 lack the α-hemolysin gene was able to invade the urothelium and form IBCs similar to wild type [31].

Conclusion: In respect to the predominance occurrence of virulence factors either solely or collectively in UPEC strains this support the concept of association of UPEC with urinary pathogenicity.

Recommendation: In Iraq, future studies may be needed for identifying other pathogenic virulence factors among EPEC isolates necessary for emphasizing their pivotal roles in pathogenicity and to guide intervention to minimize its occurrence.

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