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Original article

Correlation of biofilm formation of uropathogenic *Escherichia coli* (UPEC) and fimbriae genotypes

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Abstract

Objective: In the present study we try to correlate between pathogenic intrinsic factor of *Escherichia coli* (*E. coli*) presented with different fimbria genotyes and biofilm formation with host immune factor entitled interleukin-6 (IL-6) secretion as defense mechanism. **Methods**: A total of 91 pediatrics complaining of pyuria were included in the present study. In addition, 20 healthy control children were included. Full microbiological study was performed for isolated *E. coli*. PapG alleles were studied by multiple alleles PCR and biofilm formation was studied. IL-6 was measured in urine. **Results**: IL-6 had statistically significant elevation in patients' urine compared to control. From biofilm study, it was found that 19 isolated *E. coli* had formed biofilm *in vitro*. Moreover, urine samples with positive biofilm formation of *E. coli* had statistically significant lower IL-6 secretion than those with negative *E. coli* for biofilms. The distribution of fimbria genotypes showed that the frequent genotype was for allele II (34.3%) followed by mixed alleles I and II (24.1%). There was significant correlation between mixed alleles (I &II) and biofilm formation. **Conclusion**: The present study highlights the presence of significant strains of *E. coli* causing urinary tract infections capable of biofilm formation. Biofilm formation is associated with less innate immunity manifested by lower urinary IL-6. The majority of isolates had fimbria genes. It appears that mixed alleles I and II have prominent virulence effect with tendency for biofilm formation.

Keywords: Uropathogenic *Escherichia coli*; Interleukin-6 (IL-6); Urinary tract infection; Biofilm

INTRODUCTION

The urinary tract is among the most common sites of bacterial infection and *E. coli* is by far the most common infecting agent in children and adults of both sexes[1].

There is growing appreciation that formation of bacterial surface communities is a process that contributes to pathogenicity of microorganisms [2]. Formation of biofilms by this bacterium can be viewed as a developmental process [3] that is roughly divided into four steps: (i) adhesion, (ii) monolayer, (iii) microcolony, and (iv) mature biofilms [4].

It has become a common working hypothesis that the persistence of bacterial biofilms in the human body is a major cause of recurrent or chronic infections [5]. Parsek and Singh[6] proposed four basic criteria to define biofilm-associated infections: (i) Bacterial cell adherence to or association with a surface, (ii) *in vivo* observation of bacterial cell clusters, (iii) a localized infection pattern, and (iv) increased resistance to antibiotic treatment in the host compared to resistance of genetically equivalent planktonic bacteria. A role for bacterial biofilms in pathogenesis is well established for a number of infections and opportunistic pathogens; for many other infections a link between biofilms and disease has been proposed, but the evidence remains less clear [6].

P fimbriae, the virulence factor of *E. coli* most

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strains if from a single subject but exhibiting different genomic convincingly implicated in the pathogenesis of urinary tract. infection (UTI), mediate Gal(a1-4)Gal-specific binding to host surfaces via the adhesion molecule PapG[7] PapG occurs in three molecular variants (classes I-III) that are encoded by distinct alleles of the adhesion gene PapG [8]. The subtly different receptor binding specificities of the three PapG variants may confer differences in host range [9,10] or capacity for causing different clinical syndromes [11,12].

The elevation of urinary levels of cytokines, including IL-6 [13], IL-2 [14], and IL-2 inhibitor[15], has been observed in patients with UTI. IL-6 is an apparently pleiotropic cytokine that stimulates the production of acute-phase proteins [14], acts as a pyrogen [16], stimulates immunoglobulin secretion and increases immunoglobulin A (IgA) secretion in IgA-committed B cells [17]. It can be secreted by a wide variety of cell types such as renal tubular epithelial cells (1Sa). IL-6 is secreted into urine within minutes of experimental UTI, and then IL-6 levels in serum increase [15]. The rapid kinetics of the urinary IL-6 response in mice and the capability of local cells to secrete IL-6 suggested that IL-6 can be of mucosal origin.

In the present study we try to correlate between pathogenic intrinsic factor in *E. coli* presented with different fimbria genotyes and biofilm formation with host immune factor entitled IL-6 secretion as defense mechanism.

MATERIALS AND METHODS

Patients and control

A total of 91 (47 males and 44 females with age range of 4-14 years old). All patients had pyuria and none of them had urinary reflux. In addition, 20 healthy control children with age range of 5-12 years old were included and were presented to our outpatient clinic for either well-child care or with an unrelated complaint during the same period. No control child was febrile at the time of sample collection. Informed consents were given by all parents/caregivers of the children participating in the study. The study was approved by ethical committee of Mansoura University-Egypt.

Inclusion criteria

Children presented to Mansoura Paediatric Hospital from December 2006 to June 2007 with clinical signs and symptoms suggestive of UTI were screened. Children included in this study suffered from fever ≥ 38.5℃ without a focus or localization, in addition to urinary symptoms (i.e., increased frequency of micturation, dysuria, and loin pain). The mean duration of illness prior to presentation was 72 hours. Urine samples were obtained at the time of presentation prior to the administration of antibiotics. Renal dimercaptosuccinate acid (DMSA) scan was performed on all patients to discard or confirm acute pyelonephritis.

Blood and urine samples were taken from each subject. C-reactive protein was measured by a turbid meter for all subjects. Urine samples either catheterized or spontaneously voided midstream unused urine specimens were obtained. Urinalysis, including determinations of specific gravity, pH, glucose, protein, blood, leukocyte esterase, and nitrite, was done by dipstick analysis (Ames-N; Miles-Sankyo, Tokyo, Japan) as described in the manufacturer's instructions. For microscopic examination, 10 mL of the specimen was spun at 200 × g for 5 min, the supernatant was aspirated, and the urine sediment was resuspended with the residual 0.2 mL of urine. The cellular elements of 15 uL of urine were expressed as the average number of cells per high-powered field (HPF) of five separate areas (magnification, ×400).

For microbiological studies, an aliquot was inoculated into the appropriate medium and colonies were counted 24 and 48 h later. Significant bacteriuria was identified as colony forming unit (CFU) count 105/mL. Bacterial identification for isolated colonies was performed by Microscan System. The rest of the specimen was kept at −20 ℃ until needed for IL-6 assay.

For biofilm measurement, isolate was grown overnight in LB and diluted to approximately 10^8 cells/ mL in phosphate-buffered saline (PBS), and 100-μL aliquots were placed in 10 wells of a polystyrene microtiter plate. The plate was incubated at 37 ℃ for 1 h with shaking and washed with 10 times with PBS; any remaining bound cells were then resuspended in 100 μL of LB. Following growth overnight at 37 ℃ with shaking the cells were diluted to approximately 10^8 cells/ mL, and the procedure was carried out three more times. After the last incubation, the cultures were streaked for single colonies.

Assays for biofilm formation under hydrodynamic growth conditions were performed as previously described [18], with some modifications. Briefly, early-exponential-phase cultures (optical density at 600 nm [OD_{600}] = 0.2) were incubated in 100-μL volumes in a microtiter plate well for 16 h with vigorous shaking. Unbound cells were removed by inversion of the microtiter plate and tapping on absorbent paper. Adhered cells were subsequently stained by the addition of

200μL of 0.1% crystal violet. The stain was removed by thorough washing with PBS, and the wells were allowed to dry. The crystal violet was solubilized by the addition of 200 μL of ethanol-acetone (80: 20, wt/wt), and A600 was determined.

PapG genotypes

PapG genotypes were determined initially by an allele-specific polymerase chain reaction (PCR) assay [9,10]. Primers for the class I allele were j96-193f(5′-TCGTGCTCA-GGTCCGGAATTT-3′) and j96-653r (5′-TGGCATCCCCCA-ACATTATCG-3′), yielding a 461-bp product; for the class II allele, ia2-383f(5′-GGGATGAGCGGGCCTTTGAT-3′) and ia2-572r (5′-CGGGCCCCCAAGTAACTCG-3′), yielding a 190-bp product; and for the class III allele, prs-198f(5′-GGCCTGCAATGGATTTACCTGG-3′)and prs-455r(5′-CCACCAAATGACCATGCCAGAC-3′), yielding a 258-bp product[19]. Allele-specific PCR products were resolved by gel electrophoresis. All PapG genotypes were confirmed by at least one replicate determination, with discrepancies investigated further (by repeat PapG PCR testing, with or without genomic fingerprinting, of multiple isolates of the strain in question)as needed to obtain a reliable result.

IL-6 measurement

IL-6 level were measured for each sample in duplicate with the use of a standard sandwich ELISA enzyme linked immunosorbant assay according to the manufacturer's directions Pelikine compact human IL-6 ELISA kit assay. Color intensity was determined by a computerized multichanel photometer. The amount of IL-6 was calculated from the standard curve.

Principle of the Test: The Pelikine compact human IL-6 ELISA kit is a "Sandwich-type" of enzyme immunoassay in which a monoclonal anti-human IL-6 antibody bound onto polystyrene microplate wells, human IL-6 present in a measured volume of sample or standard is captured by the antibody on the microplate plate, and non-bound material is removed by washing subsequently, a biotinylated second monoclonal antibody to IL-6 is added. This antibody binds to the IL-6 antibody complex present in the microplate well.

Excess biotinylated antibody is removed by washing, followed by addition of horseradish peroxidase (HRP) conjugated streptavidin, which binds onto the biothinylated side of the IL-6 Sandwish.

After removal of non-bound HRP conjugate by washing a substrate solution is added to the wells. A colored product is formed in proportion to the amount of IL-6 present in the sample or standard. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a microplate plate reader. From the absorbance of samples and those of a standard curve, the concentration of hull-8 can be determined by interpolation with the standard curve.

Statistical Analysis

Statistical analysis was done by using SPSS (Statistical Package for Social Science) version 10 (1999). The data were parametric by using the Kolmogrov-Smirnov test. The qualitative data were presented in the form of mean, standard deviation and range. One-way ANOVA (F-test) was used for more than two groups and Student's *t*-test for two groups. The qualitative data were presented in the form of number and percentage. Correlation (Pearson) was done to study correlation between two items. Significance was considered when the *P*-value is less than 0.05, and insignificance was considered when the *P*-value was more than 0.05.

RESULTS

The study included 91 children with UTI. They were 47 males and 44 females with age range of 4-14 years old. From biofilm study, it was found that 19 (20.9%) isolated *E. coli* had formed biofilm *in vitro* study and the biofilm formation negative rate was 79.1% (72/91, 79.1%). IL-6 had statistically significant elevation in patients urine compared to control (Data not shown). Moreover, Urine samples with positive biofilm formation of *E. coli* had statistically significant lower IL-6 secretion (Mean ± SD, 124.7 ± 43.8) than those with negative *E. coli* (Mean ± SD, 145.9 ± 107.8) for biofilm ($P = 0.0001$). The distribution of fimbria genotypes showed that the frequent genotype was for allele II 34.3% followed by mixed alleles I and II 24.1%, (Table 1). There was significant correlation between mixed alleles (I &II) and biofilm formation ($r = 0.33$, $P = 0.001$).

Table 1 Alleles distributions among isolated *E. coli*.

Genotype	Distribution (*N*)	Frequency(%)
No allelels	8	5.8
Allele I	1	0.7
Allele1I	47	34.3
Allele III	2	1.5
Mixed I, II	33	24.1

DISCUSSION

Virulence factors of *E. coli* are of two main types; those produced on the surface of the cell and those produced within the cell and then exported to the site of action. Those on the surface include different sorts of fimbriae that have a role in adhesion to the surface of host cells but may also have additional roles such as tissue invasion, biofilm formation or cytokine induction. The activities of cell wall components are discussed and several exported virulence factors are described that have anti host cell activities. Others virulence factors enable the bacteria to grow in an environment of iron restriction[20].

In natural *E. coli* isolates a plethora of biofilm-forming capabilities is mediated by many genetic pathways. These may well include factors already implicated from studies on *E. coli* K-12, such as flagella, type I fimbriae, Ag43, and exopolymeric substances [colanic acid and poly(β-1,6-GlcNAc)], but certainly also adhesions not present in the MG1655 genome. The possible interplay between these factors and their varying expression levels under different environmental conditions suggests that analysis of genetic factors contributing to biofilm formation of non domesticated *E. coli* strains *in vitro* will need to be based on a case-by-case approach[21]. In the present study a biofilm forming isolates among *E. coli* was considerably representing high proportion. Previous studies have reported enhanced biofilm formation by virulent pathogens as by endocarditis isolates of Enterococcus faecalis as opposed to nonendocarditis isolates [22], whereas cystic fibrosis isolates of Pseudomonas aeruginosa did not display enhanced biofilm formation compared to other isolates [23,24]. The presence of biofilm forming strains represents a clue for persistant of infections and in vivo resistance to antibiotics therapy.

The present study revealed correlation between biofilm formation of *E. coli* responsible for urinary tract infections in children and presence of alleles I and II. Similar association between PapG alleles and different virulence factor was described by Naves et al., 2008[25].

E. coli has been an important gram-negative model organism for *in vitro* analysis of biofilm formation on abiotic surfaces [26,27]. Many cell surface components [such as flagella, type I fimbriae, outer membrane proteins, colanic acid, and poly (β-1, 6-GlcNAc)] were found to contribute to biofilm formation of K-12 strains during static growth conditions. The majority of isolates in the present study contained the PapG gene, a finding consistent with the association between P fimbriae and virulence [28,29]. Moreover PapG I and II was associated with biofilm formation. This finding ascertains that even the genotypic pattern of the fimbria influence the formation of biofilms.

Another finding of the present study was significant elevation of IL-6 in patients compared to control. Similarly, Hedges et al. 1991[30], reported that IL-6 was secreted in response to deliberate gram-negative infection of the human urinary tract. However, they reported that adhesions were not required for the stimulation of acute urinary IL-6 responses in humans. Nevertheless, it is possible that the high bacterial inoculate maximally stimulated IL-6 secretion and concealed any effects of adherence on IL-6 secretion.

Interesting finding in the present study was that bacterial isolates with tendency for formation of biofilm *in vitro* was significantly associated with lower IL-6 *in vivo*. The innate immune response is essential for defense against UTI and the associated tissue inflammation is a major cause of symptoms, leading to tissue damage and contributing to the severity of disease . The innate response is affected by the virulence of the infecting strain and many well-characterized virulence factors act by triggering inflammation. UPEC trigger these creations of epithelial cytokines and chemokines that contribute to inflammation and pathology.

Thus, this might give a clue that biofilm formation of *E. coli* leads to less innate immunological response.

The present study highlights the presence of significant strains of *E. coli* causing urinary tract infections capable of biofilm formation. Biofilm formation is associated with less innate immunity manifested by lower urinary IL-6. The majority of isolates had fimbria genes. It appears that mixed alleles I and II have prominent virulence effect with tendency for biofilm formation.

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