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RESEARCH ARTICLE

Evaluation of Uropathogenic Virulence Genes in Escherichia coli Isolated from Children with Urinary Tract Infection

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Abstract

Background: Urinary tract infection is a major health problem. *Escherichia coli* (*E. coli*) is a major pathogen causing UTI.

Aim: The aim of this study was to evaluate the prevalence of different virulence genes among *E. coli* strains isolated from the urine of children with urinary tract infection (UTI), and to look at the correlation between the genetic virulence genes and the type of UTI.

Method: The study included 91 isolates of *E. coli* isolated from children with urinary tract infection. Clinical isolates were subjected to study of virulence genes *form, pap, sofa/foc, Ufa, holy, knife,* and *air* by polymerase chain reaction and the formation of biofilm.

The results: Regarding virulence genes in *E. coli*, the most prevalent genes were adhesions genes *fimH* (65.9%), *pap* (63.7%), *sfa* (56%) and *afa* (54.9%) followed by genes coding for toxin production *aer* (53.8%) and *hly* (53.8%) and iron binding gene *cnf* (42.9%). Biofilm formation was found in 20.9% of isolated *E. coli.* There was statistically significant formation of biofilm among *E. coli* associated with acute pyelonephritis (p=0. 01) compared to that of *E. coli* associated with acute cystitis.

Conclusion In conclusion, our study demonstrated that virulence genes are common among *E. coli* strains causing urinary tract infection. The adhesion genes *fimH, sfa* and *afa* are the most common among those uropathogenic *E. coli* strains. Biofilm formation is another virulence factor associated with uropathogenic *E. coli* and increased mainly in *E. coli* strains causing pyelonephritis. Further studies are needed to identify *E. coli* virulence factors responsible for UTI and to determine the physiopathology of these infections to consider possible prevention measures and means.

INTRODUCTION

Urinary tract infection (UTI) is an important worldwide public health problem. *Escherichia coli* (*E. coli*) are being the etiologic agent in the majority of cases representing around 50–80% of the causative pathogen. In children urinary tract infection remains a significant cause of serious bacterial infection in young children and infants (Farell et al.,2003, Matute et al., 2004). The principal objective of early recognition and treatment of UTI is the prevention of renal parenchymal damage and subsequent renal scarring (Zhanel et al., 2006). The pathogenic role of *E. coli* depends mainly on the presence of various virulence factors.

Many virulence factors contribute to the pathogenic role of *E. coli* strains responsible for UTI that is termed uropathogenic *E. coli* (UPEC) (Johnson,1991). These factors include production of toxins, siderophores and adhesions. The hypothesis of the presence of UPEC is that UPEC evolved from non-pathogenic strains by acquiring new virulence factors from accessory DNA horizontal transfer located in the plasmid or chromosomes (Johnson et

al., 2006). Multiple genes encode urovirulent factors such as hemolysin (hly gene), cytotoxic nercotizing factor type 1 (cnf1 gene), pyelonephritis associated pili (pap genes) and S-family adhesions (sfa gene) (Mobley et al., 1994, Yamamoto et al., 1995, Johnson et al., 1997, Zhanel et al., 2006).

Virulence factors of UPEC are associated with colonization and survival in the normal urinary tract that affects the pathogenicity of symptomatic UTIs (Bonacorsi et al., 2006, Cheng et al., 2007, **Chiou** et al., 2010, Bien et al., 2012). These virulence factors play a crucial role in the adherence to urinary epithelia allowing the bacteria to persist in the urinary tract against flushing by urine flow and activation of host signaling pathways leading to the development of UTI, (Le Bougue´nec et al., 1992, Crepin et al.,2012). The important step in the growth of the invading pathogenic bacteria is the presence of iron, so, UPEC has several pathways for obtaining iron from the host through the expression of iron-acquisition systems (Zgur-Bertok et al.,1990).

The utilization of various toxins secreted by UPEC strains is well recognized. Among these toxins is a-hemolysin, an extracellular cytolytic protein toxin that has been associated with clinical severity in UTI patients (Garcia et al.,2013).

Another virulence factor associated with UPEC is the formation of biofilm. Biofilm structure usually is formed within the bladder and is considered as a reservoir for recurrent or/and persistent infection. Biofilm is formed from multiple adherent colonies surrounded by polysaccharide matrix that protect bacterial pathogens from an innate immune response like phagocytosis. The identification of UPEC strains capable of forming biofilm is an important issue for adequate control of infection (Anderson et al., 2004, Trautner et al., 2004, Suman et al., 2005,Soto et al., 2006).

These virulence factors increase the suitability of UPEC for the renal environment and aid them to resist elimination by the host defense. Through their interactions with host cells, the virulence factors stimulate the innate immune response, leading to clinical symptoms such as fever and flank pain. Progress in molecular technology has facilitated studies on UPEC (Farmer et al.,1999, Johnson et al., 2000). Urovirulence factors of *E. coli* can be detected by multiplex PCR proven to be accurate markers for the detection of uropathogenic *E. coli* (Oliveira et al.,2011).

The aim of this study was to evaluate the prevalence of different virulence genes among *E. coli* strains isolated from the urine of children with UTI and in vitro ability of UPEC strains to form biofilm. Moreover, to look at the correlation between the genetic virulence genes and the type of UTI.

Materials and Methods

Patients

Children below 18 years of age complaining of symptomatic UTI in general were enrolled in the present study. The study was carried out at Mansoura University Children's Hospital from January 2013 to May 2014. The patients were assigned a clinical diagnosis of acute cystitis based on the presence of symptoms like dysuria, frequency of masturbation and the presence or absence of suprapubic pain, a temperature <38.0°C and no flank pain. Patients who also had loin pain associated with fever (>38.0°C) were diagnosed clinically as having acute cystitis with upper urinary tract involvement. All enrolled children had significant bacteriuria (> 10^5 cfu/ml). Informed consent was obtained from the children's parents before the commencement of the study. There was no any anatomical nor functional deficiencies in urinary tract function. The study was approved by the ethical committee of Mansoura Faculty of Medicine, Egypt.

Urine cultures

Midstream urine samples were obtained at diagnosis when applicable for children. For infants adhesive bags were utilized for accumulation of urine samples. Quantitative urine cultures were performed. Amassed urine samples (10 µl) from cases of suspected UTIs in infants and adolescent children were inoculated with brain-heart infusion (BHI) agar plates as well as MacConkey ((biometrics, Inc., 100 Rodolphe Street, Durham, NC 27712) agar plates, and then incubated overnight at 370C. Bacterial colonies were counted and colony identification was utilized API20E kits (biometrics, Inc., 100 Rodolphe Street, Durham, NC 27712) Isolates were stored in 5% trypticase broth at –70°C.

Polymerase Chain Reaction for virulence factors in *E. coli*

Preparation of bacterial DNA.

DNA to be amplified was prepared from *E. coli* colonies by boiling. Bacteria were harvested from 1 ml of an overnight broth culture, resuspended

At 300 microns of sterile water, and incubated at 100°C for 10 min. Following centrifugation of the lysate, a 150 microns of the supernatant was stored at -20°C to be used for amplification.

Amplification and Detection of virulence genes of *E. coli*

Primers were chosen to amplify sequences of the *fim, pap, sfa/foc, afa, hly, cnf*, and *aer* genes. The sequences of the primers are summarized in table 1. Detection of *pap, sfa/foc*, and *afa* sequences were done by multiplex PCR

(Le Bougue´nec et al.,1992). Amplification of bacterial DNA was done in a total volume of 50 µl containing 10 µl of DNA, 30 pmol of each of the primers, 200 µM of dNTP, and 1.25 U Taq DNA polymerase (Promega) in 1X PCR buffer containing 1.5 mM MgCl2 (Le Bougue´nec et al.,1992)..

The amplification was carried out on a Perkin Elmer Thermal Cycler model 2400 (Perkin Elmer Cetus, Norwalk, Conn.). Conditions consisted of an initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 2 min, annealing at a categorical temperature for 30 s (Table 1), and extension at 72 °C for 1 min. A 10-µl aliquot of the PCR product underwent gel electrophoresis on 1.5% agarose, followed by staining with ethidium bromide solution. Amplified DNA fragments of categorical sizes were detected by UV-induced fluorescence and the size of the amplicons was estimated by comparing them with the 1 kb DNA ladder (Promega) included on the same gel (Tarchouna et al., 2013).

Biofim formation

Biofilm was examined by the semi-quantitative determination of biofilm formation in 96-well flat bottom plates. Bacterial suspensions from isolated *E. coli* were prepared in tryptic soya broth media from overnight cultures and adjusted for optical density OD_{600} of 0.1 (~ 10^7CFU/mL). 100 µL aliquots of bacterial suspension were driven to the wells of a 96-well flat-bottomed polystyrene plate and incubated 37°C for 24 hours. After incubation, plates were washed with phosphate buffered saline (PBS; pH 7.4) and stained with 100 µL of 0.1% Crystal Violet (Sigma-Aldrich, St. Louis, MO) delivered into each well for 30 min at room temperature. After the incubation crystal violet was removed by washing and 100 microns of 95% ethyl alcohol was delivered into each well. Biofilm was quantified by measuring the corresponding optical density (OD) at 570nm and well was reported positive with an OD ≥0. 5 (Cassat et al.,2007, Sanchez et al.,2013).

Statistical Analysis

The chi - square test or the Fisher's exact test was used. P <0.05 was considered statistically significant (two-tailed).

Results

The present study included 91 children affected with urinary tract infections due to *E. coli*. The majority were females (62.6%). The mean age± SD of the children was 6.0± 3.5 years. The main UTI was cystitis (67%). Females are more susceptible than male.

Our results show a higher frequency of adhesion genes *fimH* (65.9%), *pap* (63.7%) and *sfa* (56%) genes compared with the rest of the studied genes.

In our study, the analysis of the urovirulence genes prevalence in *E. coli* strains did not allow the clear discrimination between pyelonephritis and cystitis.

There was statistically significant formation of biofilm among *E. coli* associated with acute pyelonephritis (P=0. 01) compared to that of *E. coli* associated with acute cystitis, with high optical density of bioifilm formation for *E. coli* strains isolated from pyelonephritis compared to that isolated from cystitis, table 3, figure 1.

In our study, *E. coli*, showed the highest susceptibility rate to amikacin 79.1% and Cefazolin (68.1%), table 4.

Table (1): Primers used for the amplification

Virulence factor	Target genes	Name	Primer sequence (5′–3′)	Size of amplicon (bp)	Annealing temperature (^{0}C)	Ref.
Type 1 fimbriae	*fimH*	fimH-f fimH-r	AACAGCGATGATTTCCAGTTTGTGTG ATTGCGTACCAGCATTAGCAATGTCC	465	65^{0}C	Farmer,1999
fimbriae	*papC*	pap1 pap2	GACGGCTgTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328	65^{0}C	Soto et al.,2011
S and FIC fimbriae	*sfa/focDEh* region	Sfa1 Sfa2	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410	65^{0}C	Soto et al.,2011
Afa	*afaCc*	afa-f	CGGCTTTTCTGCTgAACTGGCAGGC	672	65^{0}C	Soto et

adhesions		**afa-r**	**CCGTCAGCCCCCACGGCAGACC**			**al.,2011**
Emolysin	***hlyCA* region**	**hly s** **hly as**	**AGATTCTTGGGCATGTATCCT** **TTGCTTTGCAGACTGTAGTGT**	**556**	**65^{0}C**	Codruta-Romanita et al.,**2001**
Aerobactin	***iucC***	**aer s** **aer as**	**AAACCTGGCTTACGCAACTGT** **ACCCGTCTGCAAATCATGGAT**	**269**	**60^{0}C**	Codruta-Romanita et al.,**2001**
Cytotoxic necrotizing factor	***cnf***	**cnf s** **cnf as**	**TTATATAGTCGTCAAGATGGA** **CACTAAGCTTTACAATATTGA**	**693**	**58^{0}C**	Licznar et al., 2003

Table (2): Demographic, clinical and laboratory data of study patients

Data	Parameter
Age (mean± SD)	6.0± 3.5
Sex Male Female	 34(37.4%) 57(62.6%)
Diagnosis Pyelonephritis Cystitis	 30(33%) 61(67%)
Biofilm formation	19(20.9%)
fimH	60(65.9%)
aer	49(53.8%)
Pap	58(63.7%)
sfa	51 (56%)
afa	50(54.9%)
hly	49(53.8%)
cnf	39 (42.9%)

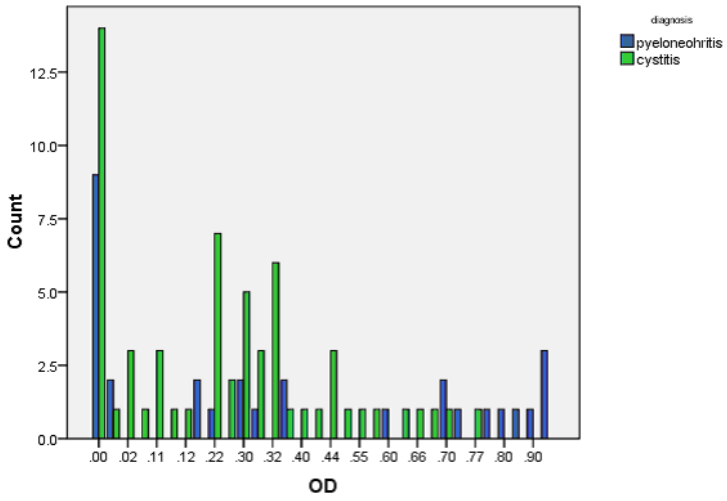

Figure (1): Optical density of biofilm in *E. coli* isolates from patients with pyelonephritis and cystitis

Table (3): Optical density of biofilm in *E. coli* isolates from patients with pyelonephritis and cystitis

	Diagnosis	
OD	Pyeloneohritis N0. %	Cystitisis N0. . %
0	9 (30%)	14(22.9%)
0.01	2 (6.7%)	1(1.6%)
0.02	0(0%)	3(4.9%)
0.1	0(0%)	1(1.6%)
0.11	0(0%)	3(4.9%)
0.112	0(0%)	1(1.6%)
0.12	0(0%)	1(1.6%)
0.2	2 (6.7%)	0(0%)
0.22	1(3.3%)	7(11.5%)
0.23	0(0%)	2(3.4%)
0.3	2(6.7%)	5(8.2%)
0.31	1(3.3 %)	3(4.9%)
0.32	0(0%)	6(9.8%)
0.33	2(6.7%)	1(1.6%)
0.4	0(0%)	1(1.6%)
0.42	0(0%)	1(1.6%)
0.44	0(0%)	3(4.9%)
0.54	0(0%)	1(1.6%)
0.55	0(0%)	1(1.6%)
0.58	0(0%)	1(1.6%)
0.6	1(3.3 %)	0(0%)
0.63	0(0%)	1(1.6%)
0.66	0(0%)	1(1.6%)
0.67	0(0%)	1(1.6%)
0.7	2(6.7%)	1(1.6%)
0.76	1(3.3 %)	0(0%)
0.77	0(0%)	1(1.6%)
0.78	1(3.3%)	0(0%)
0.8	1(3.3%)	0(0%)
0.88	1(3.3 %)	0(0%)
0.9	1(3.3 %)	0(0%)
1.0	3(10%)	0(0%)
	30(100%)	61(100%)

Table (4) Antibiotic susceptibility of isolated *E. coli*

Antibiotics	No. %

Amikacin	72	79.1%
Cefazolin	62	68.1%
Ceftazidim	49	53.8%
Ceftriaxion	49	53.8%
Cefotaxim	46	50.1%
Amox/clav	47	51.6%
Amp/Sulbactam	47	51.6%
Cefipim	43	47.3%
Ampicillin	11	12.1%

Discussion

UTI is a prevalent public health problem that varies from cystitis to pyelonephritis. The major pathogen associated with this infection is *E. coli*. This infection depends on the virulence factors of the infecting strains and on the susceptibility of the host, especially if there is an associated urological anomaly (Soto et al.,2011, Sanchez et al.,2013).

The main UTI was cystitis (67%). Females are more susceptible than male. this may be explained on the basis that these pathogens may be emerging from gastrointestinal flora through fecal contamination and may gain entry into the female urethra because of the close proximity of the anus to the urethra in females (Awaness et al.,2000). The cystitis is more common in girls than boys in children and this may be due to cystitis cystica due to the bladder wall changes due to recurrent cystitis. (Milosević D et al.,2010).

A better understanding of the virulence factors of UPEC will increase our capacity to detect the degree of the pathogenesis of the organism. To our knowledge, this study is the first to demonstrate associations between *E. coli* virulence genes and UTI in some Egyptian children. Numerous virulence factors contribute to the pathogenicity of *E. coli* in UTI. The virulence factors are the results of different genes which can be detected by PCR method (Le Bougue´nec et al.,1992, Codruta-Romanita et al.,2001, Licznar et al.,2003).

Our results show a higher frequency of adhesion genes *fimH* (65.9%), *pap* (63.7%) and *sfa* (56%) genes compared with the rest of the studied genes. These results highlight a crucial role of these virulence genes in *E. coli* causing UTI in children. Among the virulence factors, adhesion of E. coli to the uroepithelium is a basic factor that protects the bacteria from urinary discharge and promotes their ability to multiply and invade renal tissue (Soto et al.,2011). The uropathogenic E. coli adherence factors are mainly called pili or fimbriae. The two principle pili (type 1 and P) found in patients with UTIs, with their differing only in their detection method (Svanborg & Godaly,1997). Different other adhesion factors are mediated by specific bacterial proteins called adhesions which may or may not be associated with the presence of fimbriae. These proteins are nominated as hemagglutinin and are encoded by operons, P, S and Afa leads to formation of Pap (pyelonephritis associated pili), sfa (S fimbrial adhesion) and afa (afimbrial adhesion) respectively (Sokurenko et al.,1998, Zhanel et al.,2006).

Generally, genes responsible for fimbrial adhesive systems represent the most important factors for the virulence of UPEC. The presence of these genes in isolated strains in the present study is in concordance with previous reports (Sokurenko et al., 1998, Donnenberg ,&Welch, 1996, Codruta-Romanita et al.,2001).

Our finding demonstrated that *pap* gene is common in species isolated from strains of *E. coli* causing UTI in children. A recent study carried by Shetty et al., 2014 reported similar results. There is a crucial role of pap adhesion genes in the pathophysiology of pyelonephritis caused by *E. coli* that has been reported in other studies (Westerbund et al.,1989, Donnenberg & Welch 1996).

Despite the growing importance attributed to the *afa* fimbrial adhesions in the development of chronic interstitial nephritis some studies reported a minute percentage of *afa* PCR-positive strains in cases of chronic pyelonephritis (Goluszko et al.,1997). In a comparative study between children and those with cystitis adhesive genes *fimH*, *sfa* and *afa* were increased with in *E. coli* isolates associated with acute pyelonephritis (73.3%, 63.3%, 66.6%

respectively) compared to *E. coli* isolated from children with cystitis (data not shown). These findings are higher than that reported previously (Shohreh & Fatemeh , 2009). Previous studies have established that *fimH* is most frequent in isolates from a variety of forms of UTI(Mabbett et al., 2009, Cheng et al., 2010, Wang et al., 2013). These findings may denote that the presence of adhesion genes in uropathogenic *E. coli* is a clue for development of pyelonephritis.

The prevalence of *aer* (53.8%) *hly* (53.8%) and *cnf* (42.9%) among our clinical isolates are similar to those found by other investigators (29, 36), and in higher proportions than in other studies (Johnson,1991, Westerbund et al., 1989). This is most probably because the majority of our strains was isolated from children with cystitis

In our study, the analysis of the urovirulence genes prevalence in *E. coli* strains did not allow the clear discrimination between pyelonephritis and cystitis. Some studies have been able to establish a correlation between distribution of urovirulence genes and the seriousness of the UTI by different models of gene association (Codruta-Romanita et al., 2001). Additionally, there is always the possibility of mutation of the corresponding gene so it can not be detected by PCR. Therefore, a positive PCR shows the presence of the virulence gene, but a negative PCR does not omit the presence of the corresponding gene.

There was statistically significant formation of biofilm among *E. coli* associated with acute pyelonephritis (P=0. 01) compared to that of *E. coli* associated with acute cystitis, with high optical density of bioifilm formation for *E. coli* strains isolated from pyelonephritis compared to that isolated from cystitis.

It has been postulated that biofilm formation by UPEC strains is associated with both recurrence and persistence of urinary tract infections. It seems that the properties of UPEC strains required for effective biofilm growth on in vitro studies are used for establishment of pyelonephritis, but not for cystitis. (Tapiainen et al.,2014).

In our study, *E. coli*, showed the highest susceptibility rate to amikacin 79.1% and Cefazolin (68.1%), table 4. This is in agreement to Musa- Aisien and Colleagues 2003 (Musa-Aisien et al.,2003).

In conclusion, our study demonstrated that virulence genes are mundane among *E. coli* strains causing urinary tract infection. The adhesion genes *fimH, sfa and afa* are the most prevalent among those uropathogenic E. coli strains. Biofilm formation is another virulence factor associated with uropathogenic *E. coli* and incremented mainly in *E. coli* strains causing pyelonephritis. Further studies are needed to identify *E. coli* virulence factors responsible for UTI and to determine the physiopathology of these infections to consider possible aversion measures and denotes.

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