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Original Article

Determination of Adhesion Encoding Genes of Uropathogenic *Escherichia coli*

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Abstract

Background: The bacterial strains which cause symptomatic urinary tract infections possess diverse distinctive properties that enable them to overcome local host defences. The present study was undertaken to screen for the virulence factors of uropathogenic *Escherichia coli* (UPFC) by molecular methods and to detect the genotypic pathogenicity related sequences *pap*, *sfa*, and *afa* genes in *E. coli* strains by polymerase chain reaction (PCR).

Methods: A total of 350 *E.coli* isolates were obtained from symptomatic cases of urinary tract infections (UTI) and 60 *E. coli* faecal isolates were collected from apparently healthy individuals (during February 2014-2016) from outpatient department of a tertiary care hospital in Kerala, India. Isolates were tested for adhesive genes *pap, sfa,* and *afa*.

Results: Among the UPEC samples, 255 (72.85%) of the isolates were from females and 95 (27.14%) of them were obtained from males. The prevalence of *pap* gene was seen in 72 of the total isolates which were the highest. The *sfa* genes were shown by 56 isolates. The *afa* genes were also in 7 of the isolates. Diversity of the UPEC associated virulence genes were established in this study.

Conclusions: The *E.coli* causing UTI had greater percentage of virulence associated adhesin genes compared to *E.coli* from control stool samples.

Keywords: Uropathogenic *E. coli*, *Pap, sfa, afa* genes, Adhesins, Commensal *E.coli*

Background

Uropathogenic *Escherichia coli* (UPEC), the causative agent of urinary tract infections (UTIs) in otherwise healthy individuals, is a frequently encountered pathogen (1). The UPEC belongs to a subgroup of extraintestinal pathogenic *E. coli*'s which causes a broad spectrum of conditions including bacteraemia, UTIs, and neonatal meningitis in man and colibacillosis in poultry (2). Eighty percent of acute UTIs is associated with *E. coli*. The infection initiates when the bacteria adhere to the uroepithelial cell receptors through specific fimbrial adhesins (3). *E. coli* is part of the normal microbiota of the enteric tract of humans and animals. The mutually beneficial symbiotic association of *E. coli* with its host helps maintaining normal intestinal homeostasis and promoting the stability of the intestinal microbial flora (4).

UTI results in substantial medical costs. UPEC is associated with community acquired UTIs and nosocomial UTIs, as well as with considerable morbidity and mortality worldwide (5). The UPEC ability to cause symptomatic UTIs is dependent on the expression of broad spectrum virulence factors (6). Molecules of adhesion and toxins account for the most important mediators of pathogenicity.

UPEC receptor binding protein can be differentiated on the basis of their receptor identifying capabilities. The P-fimbriae which is encoded by the pap operon of *E. coli* will bind to digalactoside containing receptors (7). Sialic acid containing receptors are those for S-fimbriae which is encoded by *sfa* operon. Afimbrial adhesin is encoded by *afa* operon and is associated with recurrent UTIs (8). The virulence factors are helpful in colonization and persistence of bacteria in the urinary tract. Adhesins or Fimbriae, toxins, and siderophore systems are involved in initiation of UTI by colonization to host tissues (9).

Extraintestinal infections causing *E. coli* expresses mannose resistant adhesins and pap fimbriae is the most common one (10). The pap (pyelonephritis associated pilus) operon mediates galactosyl galactose specific binding to epithelial surfaces of intestine, vagina, urinary tract, and moiety of the P blood group by their tip adhesion molecule (11,12). The S fimbrial adhesins are frequently expressed in *E. coli* strains involved in extraintestinal infections (13). The S fimbriae are also mannose resistant and associated with *E. coli* strains that cause sepsis, meningitis, and ascending UTIs; they help in the bacterial spread within host tissues (14). The *E. coli*

strains expressing operons of *afa* family are found among those strains that cause human and animal infections (15). The Afa family includes fimbrial Dr and afimbrial *afa* adhesins of *E. coli*. In pyelonephritis and recurring cystitis-causing strains of UPEC, *afa* and Dr adhesins are seen (16,17). Adhesins of the *afa* family expressed in UPEC have a unique renal tropism (18). The *afa* gene shows high degree of heterogenicity (19). In a variety of *E. coli* associated diseases, toxins are the major virulence factor (20). They produce inflammatory response, resulting in the production of the symptoms of UTI. The most important toxins of *E.coli* are alpha haemolysin (*hly* D), and cytotoxic necrotizing factor (CNF) (21).

This study was carried out to detect the prevalence of adhesion protein coding *pap, sfa, and afa* genes in the UPEC in a tertiary care hospital in Palakkad, Kerala, India by amplification of genes through polymerase chain reaction (PCR).

Methods

Detection of Pathogenicity Related Sequences by Multiplex PCR

Bacterial Strains

In this study, a total of 350 *E. coli* strains isolated from UTI cases during a 2-year period (February 2014-2016, from a tertiary care hospital were included. Sixty fecal *E.coli* samples obtained from healthy volunteers were included in this study as control samples.

Growth Conditions and Characterization

Midstream urine samples were collected from suspected cases of UTI and stool samples from healthy volunteers were inoculated into MacConkey and blood agars. A colony count of 10^5 was taken as significant bacteriuria for urine samples. The *E. coli* was identified by assessing the following biochemical reactions: Nitrate was reduced to nitrites, catalase production was positive, glucose fermented, H_2S not produced, urea not hydrolysed, lysine decarboxylation positive, motile, positive for indole production, and Simmons citrate medium not utilized. Following characterization, the UPEC isolates included in the study were stored at -20°C in Luria broth medium.

For genotyping, bacteria were grown in Luria broth medium for 18 hours at 37°C. For the purpose of standardization of PCR conditions, positive strains, kindly provided by Johnson lab & Brian J from Pathos pool were used. Positive control strains include J-96 Strain positive for *pap a*nd *sfa* genes. 2-H16 strain positive for *afa* and known negative control.

Preparation of Bacterial DNA

Whole organisms' boiling was conducted to release the DNA to be amplified. Overnight broth culture of bacteria was taken and centrifuged; after centrifugation, the pellet was suspended in 200 µL of sterile water and boiled for 10 minutes at 100°C. Again, the lysate was centrifuged and this time the supernatant was obtained. This was the template DNA stock which was stored at -20°C.

Primers

Pap, sfa, afa forward and reverse primers were obtained from *Eurofins mwg*, Bangalore**.** The primers used for the amplification by the PCR conditions are described in Table 1.

Primer Mix Preparation

Stock primer is prepared by mixing primer in the powder form with nuclease free water. Then, pipette out 10 µL to 90 µL of nuclease free water to make it a working stock. dNTPs: concentration is 10mM. That is, each individual dNTP is at a concentration of 2.5 mM. Prepare 200 µmolar concentration by adding 4 µL. DNA polymerase: Sigma Aldrich 5 units/µL. PCR buffer: Concentration is 10X. Add 2.5 µL of 10X PCR buffer to single 25 µL reaction to get 1XPCR buffer. $MgCl_2$ Concentration 1.5 mM: 2 µL.Template DNA: 2 µL.

Amplification Procedure.

All amplification reactions performed in Applied Biosystem thermocycler. Following the amplification, the products were analysed by electrophoresis. The amplified DNA was run in a 2.0% submerged agar rose gel, the gel was stained with ethidium bromide and observed under UV light along with molecular weight markers.

Amplification, using thermocycler, was carried out in a total volume of 25 µL containing 2 µL of the template DNA, reaction mixture also had each of the forward and reverse primers at 0.4 µM, the 4 deoxy ribonucleotides, each at 200 µM, magnesium chloride 1.5 mM, and Taq DNA polymerase 1.2 U.

Table I. Primers Used for Genotyping UPEC

***E. coli* Virulence Factor**	**Gene**	**Primer Name**	**Oligonucleotide Primer Pairs (5´-3´)**	**Amplicon (bp)**
P fimbriae	*pap*	*pap1* *pap2*	GTGGCAGTATGAGTAATGACCGTTA ATATCCTTTCTGCAGGGATGCAATA	205 Ref (28)
S fimbriae	*sfa*	*sfa1* *sfa2*	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410 Ref (28)
afa fimbriae	*afa*	*afa1* afa2	GGCAGAGGGCCGGCAACAGGC CCCGTAACGCGCCAGCATCTC	*594* Ref (28)

MPCR amplification cycle was as follows: the initial cycle of 94°C for 60 seconds, followed by 30 cycles of denaturation at 94°C for 60 seconds, then the annealing at 63°C for 30 seconds and extension step at 72°C for 90 seconds followed by a final cycle of 72°C for 90 seconds.

For the analysis of amplified DNA, electrophoresis on 2% agarose gel and TBE buffer was done. To visualize the migration of amplified DNA during electrophoresis, 1 kb DNA ladder along with gel loading buffer was used. Staining with ethidium bromide was conducted to stain the DNA and to capture the image using UV light and camera.

A reagent blank was included in every PCR procedure. This contained all the reagents of the reaction mixture, minus the template DNA.

Statistical Methods

Chi-square test or two-tailed Fisher exact test was used to test the differences between non-continuous variables. The results were found to be statistically significant at $P < 0.05$

Results

The genotyping of 350 cases of urinary isolates of *E. coli* and 60 control samples of commensal *E. coli* from stool were carried out for its adhesins *pap, sfa a*nd *afa*. Standardisation of PCR conditions were conducted using the known strains. Ten Johnson lab strains were used as positive control strains. Of the 350 *E. coli* isolates tested, about 112 of them showed the presence of adhesive genes (Figure 1). Out of these 350 cases, 255 (72.85%) of them were females and 95 (27.14%) cases were males. The prevalence of *pap* gene was observed in 72 of the total isolates. The *sfa* genes were demonstrated by 56 isolates. The *afa* genes were also found in 7 isolates. Among the cases of control group, genes coding for adhesins were only found in 4 isolates. The *afa* genes nil, *sfa* gene in one isolate and *papC* genes among 3 isolates were observed. The presence of genes was either in combination with other genes or alone (Table 2). About 20.5% of the isolates showed *pap* gene either alone or in combination with *sfa or afa* genes. The *sfa* gene was present in 16% of the isolates. The occurrence of *afa* gene was 2%. Two isolates were positive for all the three genes. The *pap* gene was found in49 (19.2%) females and 23 (27.3%) males. The *P* value was 0.303 for *pap* gene among males and females. This result was not significant at $P < 0.05$. There was no significant association among the *sfa* genes between males and females. The age wise distribution of UPEC is shown in (Table 3). There was a significant difference among the UPEC isolates across gender in different age groups. The chi-square statistic was 22.5549. The *P* value was also 0.03. The result was significant at $P < 0.05$. There was a significant difference between different age groups, namely, below 15, 15-30, 31-40, 41-60, and >60 across the gender.

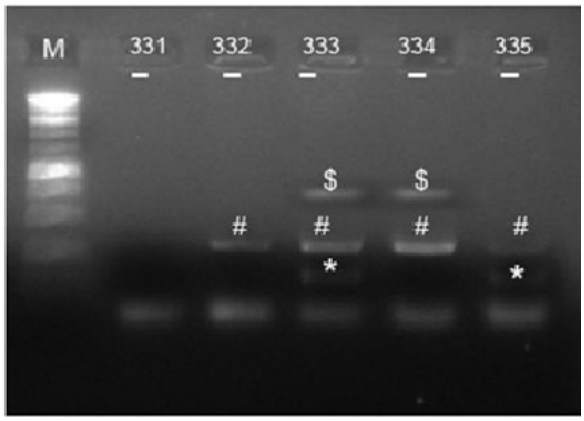

Figure I. Multiplex PCR for virulence factor genes.
Lane M: 1kb ladder; Lanes 2-6: UPEC isolates 331-335. Lane 3 (332) is positive for *sfa* gene. Lane 4 (333) positive for *pap*, *sfa* and *afa* genes. Lane 5 (334) positive for *sfa* and *afa* genes. Lane 6 (336) positive for *pap* and *sfa* genes.

Genotypic Markers Across Cases and Control

The presence of adhesion genes were compared among the *E. coli* strains obtained from cases of UTI and those from control samples (*E. coli* from stool isolated from gut flora) of healthy volunteers (Figure 2). The *pap genes* from UPEC were compared with *pap* genes from stool *E. coli* and the *P*-value was 0.003. This result was significant at $P < 0.05$. Similarly, the prevalence of *sfa* genes among UPEC and control cases was significant at $P < 0.05$, showing a *P* value of 0.003. The *chi-square* statistic was 0.85075 for this result of *afa*. The *P* value was 0.354. This result was *not* significant at $P < 0.05$. The *sfa* and *pap* genes showed significant differences across cases and control samples. Table 4 shows the *sfa* and *pap* gene distribution among UPEC and commensals. The higher prevalence of *pap, sfa* and *afa* genes among the urinary isolates of *E. coli* denotes the significance of these adhesins as a virulence factor.

Genotypic Markers in Different Age Groups

Prevalence of *afa* gene was found to be the highest among the above 60-age group. The same trend was observed for *pap* genes for above 60-age group. The *afa, sfa,* and *pap* genes were 2%, 3%, and 4% in <15 age group; while

Table 2. Virulence Genes Distribution of Uropathogenic *E.coli*

Virulence Genes of Uropathogenic *E. coli*	Cases
pap	52
sfa	37
afa	02
Two markers	
afa+pap	02
sfa+pap	16
afa+sfa	01
Three markers	
afa+pap+sfa	02

Table 3. Age Distribution of Uropathogenic *E. coli* Isolates

Age Distribution	Male	Female	Row Totals
Below 15	15[a] (14.39)[b] [0.03][c]	38 (38.61) [0.01]	53
15-30	29 (45.33) [5.88]	138 (121.67) [2.19]	167
31-40	24 (18.46) [1.66]	44 (49.54) [0.62]	68
41-6	11 (8.96) [0.47]	22 (24.04) [0.17]	33
Above 60	16 (7.87) [8.39]	13 (21.13) [3.13]	29
Column Totals	95	255	350 (Grand Total)

[a] The observed cell totals, [b] (the expected cell totals) and [c] [the chi-square statistic for each cell].

Table 4. *sfa* and *pap* Gene Distribution

	Sfa Gene		*pap* Gene		*Marginal Row Totals*
	Absent	Present	Absent	Present	
E coli from stool	59[a] (51.66)[b] [1.04][c]	1 (8.34) [6.46]	57 (49.02) [1.3]	3 (10.98) [5.8]	60
Uropathogenic *E. coli*	294 (301.34) [0.18]	56 (48.66) [1.11]	278 (285.98) [0.22]	72 (64.02) [0.99]	350
Column totals	353	5 7	335	75	410 (Grand Total)

[a] The observed cell totals, [b] (the expected cell totals) and [c] [the chi-square statistic for each cell].
For *sfa* gene the chi-square statistic was 8.7912. The *P* value is 0.003. This result was significant at $p < 0.05$.
For *pap* gene the chi-square statistic was 8.3091. The *P* value is 0.004. This result was significant at $p < 0.05$

among the age group of 15-30, the prevalence of *afa, sfa,* and *pap* genes were 1%, 7%, and 23.3%, respectively. Age wise distribution of the genes among UPEC isolates is given in Figure 3.

Validation of *pap, sfa, afa* Specific PCR Assay

Each of the primers of *pap, sfa* and *afa* yielded PCR product of the expected size, namely, *pap* 205bp, *sfa* 410bp, and *afa* 594bp. The multiplex PCR was standardized and run. The positive control strains J96 was a pyelonephritis isolate, while 2H25, 2H16, and V25 were urosepsis isolates. In addition, L31 was isolated from UTI in dogs. The human faecal isolate JJ055 was used as a negative control. In order to confirm that the products obtained by multiplex PCR are representative of the expected sequences of *pap, sfa,* and *afa,* the PCR product derived from individual PCR amplification of 3 different strains were purified using PCR purification kit and sequenced using the same primers, namely, forward and reverse *pap, sfa,* and *afa* at Applied Biosystems. The amplified product was transferred for sequencing. The products of sequencing were subjected to BLAST analysis, and the sequences showed a total of 99%-100% similarity. The sequences were submitted to Genbank and procured the following accession numbers: KU933941, KX3844965, and KU933942for *pap, sfa,* and *afa* sequences, respectively, for sequence, sequence.

BLAST Results

- The BLAST result showed that the query sequence had 100% similarity with *E. coli papC* gene (Accession No: HQ165752),
- The BLAST result also indicated that the query sequence had 99% similarity with *E. coli sfa* operon (Accession No: X16664), and finally
- The BLAST result showed that the query sequence had 99% similarity with *E. coli afa* operon (Accession No: KR338833).

Discussion

Colonization of *E. coli* strains within the urinary tract

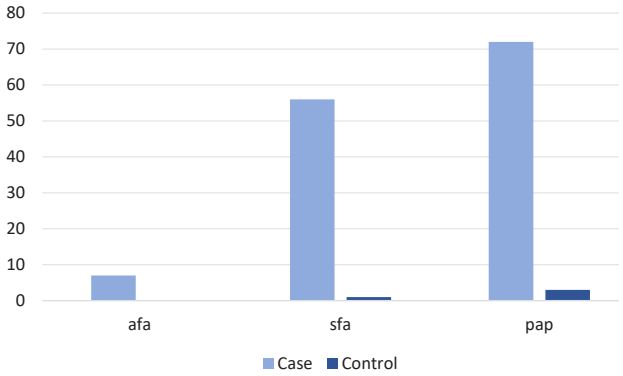

Figure 2. *E. coli* Genotypic Markers in Cases and Control.

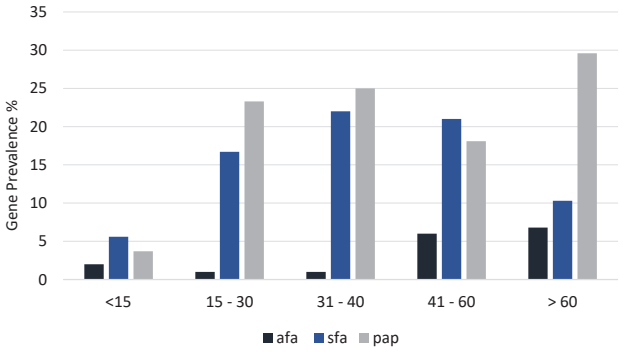

Figure 3. Genotypic Markers in Different Age Groups.

and occurrence of UTI begin with binding to epithelial surface (22). The *pap* fimbriae of *E. coli* are considered as an essential virulence factor causing pyelonephritis (23). In this study, *pap* adhesin gene was the most common VFG (20.5%). Previous studies have shown that *pap* genes are associated with different forms of UTI. The *pap* is the pyelonephritis-associated pili and there are different subsets for *pap* gene encoded by the pap A-K gene operons. The subsets of *pap* include A*, C, GII, GIII*, etc. The *pap* adhesins have been identified in 80% of pyelonephritic *E. coli* isolates (1). Literature has shown 0% to 94% variation in the prevalence of the *pap* gene (2,7). There is diversity in the frequency of *pap* gene among UPEC strains across the globe and within the same geographical region. Investigating Iranian studies it was revealed that there were variations in *pap* gene prevalence including 83.63% (8), 50.4% (9), 30% (2), 27% (10),16.6% (12), and 20.5% (11). This is because Uropathogenic strains of *E. coli* make use of a variety of adhesins to attach to the bladder urothelium. Uropathogenic strains may depend on other adhesive molecules for binding if the usual adhesins are not expressed. The diversity in frequency of *pap* gene among different studies can be due to the fact that UPEC strains utilize a variety of adhesins to bind to the urinary epithelial cells, and start the infection (12). The varied environmental niches of different hosts could be contributing to varied expression of adhesins (8). The distribution of *pap* genes were in agreement with earlier studies , which showed the higher prevalence of *pap* genes among UPEC strains (24-27).

The S fimbria is coded by the *sfa* gene operons and is associated with the PAI of *E.coli* causing UTIs and meningitis. The genetic determinants coding for *sfa* are identical in uropathogenic and meningitis causing *E. coli*. The subset of *sfa* adhesin genes showed genetic homology and there was a similarity between *sfa* and FIC as well. This was contradictory to the situation found for the P determinants (13). These are complex proteins with different biological functions. The *sfa* determinant has major and minor protein subunits. In this study, a highly conserved gene was used for *sfa S* which is a minor subunit protein coding. Several distinct patterns were found among the various isolates similar to what had been shown in the study by Yun et al (29). The *sfa* presented adhsein genes in frequencies of 26 %. Prevalence of *sfa* and *pap* genes varies from 0%-64% and from 0%-54% in different studies as in Blanco et al (3), and Yamamoto et al (30). In a study by Arachambaud et al, *sfa* prevalence was 22% (14); it was 23% in a Romanian study by Usein et al (15). Miyazaki et al showed a *sfa* prevalence of 17% (16); The isolates from a study carried out in Brazil indicated a *sfa* prevalence of 20%(17). Similarly, in a study by Abe et al*,* such a prevalence was 29.8% (18), while Tiba et al found the occurrence to be 27.8% (19). Besides, Rijavec et al in their study, observed a*sfa/foc* of 24% (20) and in a study carried out in Kermanshah, Iran, 2012, it was 20.5% (11). Florea et al studied the presence of *sfa* among UPEC strains causing UTI in children and it was found to be 54% (21), while it was only 6.8% in the study by Arisoy et al (22). There are studies where the *sfa* genes were very low in occurrence. In addition, in a study by Qin, *sfa* genes were found only in 5 out of 70 isolates (23), while in another study by Wang et al, cystitis cases showed only 8% presence of *sfa* genes and it was nil among upper UTI isolates (24).

Fimbrial and afimbrial architecture are expressed by AFA/Dr family. The common epithelial cell receptor decay accelerating factor (DAF) acts as the receptor for afa/Dr fimbriae, which is a complement cascade regulator factor (25). The *afa/Dr* genes are seen not only in *E. coli* but also in strains associated with diarrhoea (26).The *afa* genes were present in just 2% of isolates. The *afa* gene was associated with *pap* genes in 57.0% or with *sfa* genes in about 42.8%. The low prevalence of *afa* gene was in accordance with other studies. In the 2011 Russian study, involving 1 strains of *E.coli*, *afa* and *sfa* genes were absent (27). The prevalence of *afa* was 3% in the study by Rijavec et al (20), while it was 9% in Arisoy et al and Rahdar et al studies (8,22). Blanco et al and Le Bouguenec et al reported 7% and 12.4% prevalence of *afa* genes (3,28). Likewise, Yun et al (29), Tiba et al (19) and Miyazaki et al (16) reported a prevalence of 9.2%, 6.2%, and 5%, respectively (19,29,30). In the study by Oliveira et al, the prevalence of *afa*, *pap, a*nd *sfa* was 6%*,* 25%, and 26%, respectively (31). In another study in Prague, the occurrence of genes was found to be 8.3% (*afa*), 74% (*pap*) and 65% (*sfa*) in a group of 84 extraintestinal *E. coli* isolates. The occurrence of *afa* strains in UPEC strains alone was 11% (32). Prevalence depends on various conditions like clinical conditions, geographical location, and phylogenetic groups (18). Each pathotype will be possessing a unique combination of virulence factors (VFs), coded by virulence factor genes VFGs (16). These VFs usually occur within genomic pathogenicity associated islands PAIs. It has been found that UPEC may harbour multiple PAIs with a distinct cluster of VFs and can sometimes result in multiple expression of particular virulence factor. A recent study by Shetty et al showed that multiple virulence factor further increase the risk for development of UTI specifically in women and that *pap* gene was higher compared to other genes (33).

Conclusion

Adhesins are important virulence determinants of *E. coli*. They help the colonization of the bacteria within the bladder by attachment. The *Pap*, *sfa*, and *afa* are the common adhesins associated with UPEC. In this study, the distribution of these adhesins by molecular analysis of *pap, sfa, afa* genes were investigated. Briefly speaking, there was not only a significant single predictor for UTIs. There

was varied prevalence of virulence markers among UPEC isolates. Distribution of *pap* gene followed by *sfa* gene was the highest among the isolates from UTI cases while only few of the control samples showed the prevalence of *pap* and *sfa* genes. The *afa* gene distribution was only observed in UPEC strains and not in control samples. The current study shows the increased prevalence of virulence genes among *E. colis*. Strains which were more virulent might produce stronger immune response in host and might cause persistent infections. In this study, only *E. coli* isolates from acute cystitis in outpatient department were analysed. The *pap* and *sfa* genes were associated with pyelonephritis indicating the potential of these strains to persist and progress to the kidneys. Thus, the genes coding for adhesion helps providing better knowledge about the infection and its progression based on virulence. In the present study, the analysis of the gene distribution in *E. coli* did not reveal a clear determination and correlation between the adhesive genes and the complexity of the UTI. These genes could be helpful markers in future for diagnosis of UTI and for prophylactic preparations.

Conflict of Interests

Authors do not have any conflict of interest.

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