Egyptian Journal of Medical Microbiology Volume 26 / No.4 / October 2017 113-120

ORIGINAL ARTICLE

Virulence Determinants of Uropathogenic *Escherichia coli* versus Commensal Fecal *Escherichia coli*

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Key words:
Uropathogenic E. coli; urinary tract infections; asymptomatic bacteruria; α-hly; cnf 1; In vitro biofilm; p fimbrae

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ABSTRACT

Background: *Uropathogenic E. coli (UPEC) is by far the most common cause of urinary tract infections (UTIs) worldwide. The degree of pathogenicity is dependent on the presence of virulence factors; however the clinical aspects of such factors are not yet fully understood.* ***Objectives:*** *The aim of this study was to assess the prevalence of virulence determinants in UPEC of urinary tract infected subjects and commensal fecal E. coli isolates of healthy individuals.* ***Methodology:*** *A total of 208 patients with clinically diagnosed UTIs were included in the study, 170/208 yielded significant bacterial growth. One hundred fifty five E. coli isolates (100 UPEC from patients with suspected UTIs, 50 commensal fecal E. coli and 5 urinary E. coli isolates from apparently healthy individuals with significant asymptomatic bacteriuria) were investigated phenotypically for α-hly, cnf 1, in vitro biofilm formation and P fimbrae. Four urovirulence genes (hly A, cnf 1, aer and pap C) were investigated by PCR.* ***Results:*** *α-Hly & cnf 1 virulence factors and their corresponding genes were significantly(P= 0.001) detected in UPEC isolates compared with fecal E. coli isolates, with insignificant difference in phenotypic expression of p fimbrae and PCR assay of aer & pap C genes.* ***Conclusion:*** *the studied virulence determinants are involved in both pathogenesis of UTI and in the ability of E.coli to survive either in intestine or in the new ecological niches such as the human urinary tract.*

INTRODUCTION

Escherichia coli comprises a wide diversity of strains belonging to the commensal intestinal flora of humans and warm blooded animals. On the other hand, *E. coli* is one of the major causes of extraintestinal infections such as UTIs, neonatal meningitis and Gram-negative bacteraemia[1]. Urinary tract infections became a real threat and a significant cause of morbidity that is attributed to the synergistic action of several virulence factors including capsules, toxins, iron acquisition systems and others [2]. Such virulence factors are usually encoded on large chromosomal or plasmid blocks termed pathogenicity islands (PAIs)[3]. It is of a great value to find correlation between the expression of urovirulence genes and the disease especially in case of recurrent UTIs. This correlation can be used as a predictor of the course of disease which may be a guide for clinician to use special regimen of chemotherapeutic agents or other new strategies of therapy for management of such cases 4. Currently, increasing rates of antibiotic resistance and high recurrences of UTIs caused by UPEC, result in considerable economic and public health burdens. Yet, many promising approaches are being developed based on what we have learned about basic biology of UTI pathogenesis to specially target virulence pathways.

The aim of the present study was to assess the prevalence of virulence determinants in uropathogenic *E. coli* isolates of urinary tract infected subjects and fecal *E. coli* isolates of healthy individuals.

METHODOLOGY

The present study was conducted in Microbiology department, Faculty of Medicine for Girls, AL-Azhar University. This study was conducted between March and October 2014.

The study included 208 patients suspected of having UTIs with age ranged from 22 to 60 years, urine samples collected from patients were immediately transferred to the laboratory for microbiological examination. In parallel, urine and stool samples were collected from healthy individuals. The study was approved by the Institutional Human Ethical Committee of Faculty of Medicine – Al-Azhar University and all of the participants gave their informed consent before collection of samples.

Isolation and Identification of *E. coli*:

E. coli was isolated from both types of samples using Cystine Lactose Electrolyte Deficient (CLED) and MacConkey's media. Colonies were identified by biochemical test[4] including citrate utilization, urease, triple sugar iron and Motility-Indole-Ornithine. Antibiotic susceptibility test was carried out using disc diffusion method according to CLSI, 2010[5]**.** The following antimicrobials were tested: Ampicillin, Amikacin, Ceftriaxone, Nitrofurantion, Gentamicin, Trimethprim-Sulphamethoxazole, Ciprofloxacin and Imipenem. ATCC 25922 *E. coli* strain kindly supplied by NAMRU-3, was used as a quality control strain in biochemical & antibiotic susceptibility testing.

Phenotypic assay of virulence factors of *E. coli*:

One hundred UPEC were obtained from patients with symptomatic UTI. In parallel, 50 commensal fecal *E. coli* isolates and 5urinary *E. coli* isolates were obtained from apparently heathy individuals. *E. coli* isolates from both sources were investigated phenotypically:

α- Haemolysin (Hly A):

Toxin production by inoculating the pure *E. coli* isolate onto 5% sheep blood agar (Oxoid) and incubation at 37 ◦C overnight. Haemolysin production was detected by presence of clear zone of lysis around colonies [6]**.**

Cytotoxic necrotizing factor 1 (cnf 1)[7]

Production: Pure *E. coli* isolates were inoculated onto 5 ml Trypticase soy broth and incubated for 48. Bacterial culture ***was*** ultrasonically disintegrated for 60 seconds and cell lysate was clarified by centrifugation (6,000 xg for 15 min). The supernatant was filtered separately using 0.22-µm-pore size sterile syringe filter. Twenty four well microtiter tissue culture plates were seeded with 1ml /well of trypsinized cell suspension (4×105 cells per ml of cell culture medium). The plates were incubated at 37ºC in 5% CO2 atmosphere for 24 hrs to form confluent sheet. The growth medium was discarded and replaced by a freshly prepared cell culture DMEM 2% fetal bovine serum (0.5 ml per well) and cells were inoculated with 0.05 ml of sterile undiluted cell lysate. In each plate, two wells were used for each cell lysate clarified from each isolate, two wells were seeded by cells alone without cell lysate to be used as a cell control, 2 wells for control positive and 2 wells for control negative clarified cell lysates. Plates were incubated at 37ºC in 5% CO2 and observed after 24 hrs under an inverted microscope.

Phenotypic assay of in-vitro biofilm formation by tube adherence method:

A qualitative assessment of slime production was determined by tube method according to Christensen et al. [8] by inoculating 2 ml of brain heart infusion broth supplemented by 1% glucose in polystyrene Falcon tubes with a loopful of the tested isolate and incubating the tubes for 2 days aerobically at 37°C without shaking. Tubes were then decanted of their contents, washed three times with PBS, allowed to air dry in inverted position and stained with 0.1% crystal violet for 30 minutes, washed with tap water three times and allowed to air dry in an inverted position. Control negative and control positive were used with each test and the test is performed in triplicate to validate the results.

Phenotypic assay of P-fimbriae:

E. coli isolates were inoculated into 5 ml of Muller Hinton broth at 37 ◦C for 5 days. The formed pellicle was sub-cultured onto Muller Hinton agar and incubated overnight at 37ºC[9]**.**

Forty microliter of the 3% erythrocyte suspension was added to 40µl of PBS in a well of microtiter plate. In the second well, 40µl of D-mannose (3% in PBS) was added to 40µl of erythrocyte suspension. Two colonies from each isolate were mixed in both wells with shaking for 5 min and the haem-agglutination reactions were recorded. Mannose resistant haemagglutination was considered when it occurred in presence of D- mannose.

Detection of urovirulence genes of *E. coli* by PCR:

Bacterial DNA was extracted from *E. coli* isolates subcultured overnight at 37ºC on MacConkey agar. Five colonies were emulsified in 200µl of sterile distilled water that was heated at100◦C for 15 minutes then chilled on ice for 5 minutes and centrifuged at 10000g for 5 minutes. The supernatant was used as template for subsequent amplification. PCR amplification of urovirulence genes (papC, cnf 1,aer and hly) was performed using the protocol of Oliveira et al.[1] with a readymade green master mix (Thermo Scientific) used in a final volume 50 µl using the primers in Table 1. Amplicons were detected by gel electrophoresis in 2% agarose gel stained with ethidium bromide and visualized in an ultraviolet trans-illuminator.

Table 1: Sequence of the primers of urovirulence genes

Gene	Primer	Primer sequence	Amplicon size
papC	F1	5'- GACGGCTGTACTGCAGGGTGTGGCG -3'	328bp
R1		5'- ATATCCTTTCTGCAGGGATGCAATA -3'	
cnf 1	F2	5'- AAGATGGAGTTTCCTATGCAGGAG -3'	498bp
R2		5'-CATTCAGAGTCCTGCCCTCATTATT -3'	
aer	F3	5'- TACCGGATTGTCATATGCAGACCGT -3'	602bp
R3		5'AATATCTTCCTCCAGTCCGGAGAAG -3'	
hly	F4	5'- AACAAGGATAAGCACTGTTCTGGCT -3'	1,177bp
R4		5'-ACCATATAAGCGGTCATTCCCGTCA- 3'	

Statistical analysis

Analysis was conducted through SPSS program version 16. Analytical statistics was performed by using test of significance as Chi-square test ($\chi 2$) for comparison of qualitative data. The level of significance was taken at $P \leq 0.05$, P-value <0.01 was considered highly significant, while P-value > 0.05 was considered statistically not significant.

RESULTS

Among 208 urine samples collected from patients with clinically diagnosed urinary tract infection, one hundred and seventy (170/208, 81.7%) yielded significant bacterial growth. Of the isolated uropathogens, E. coli was the most common organism isolated from 100 (100/170, 58.8 %), klebsiella species from 34 (20%) and pseudomonas species from 11 cases (6.5%) (Fig.1). In parallel, 50 commensal fecal *E. coli* isolates and 5urinary *E. coli* isolates were identified from apparently healthy individuals based on biochemical tests.

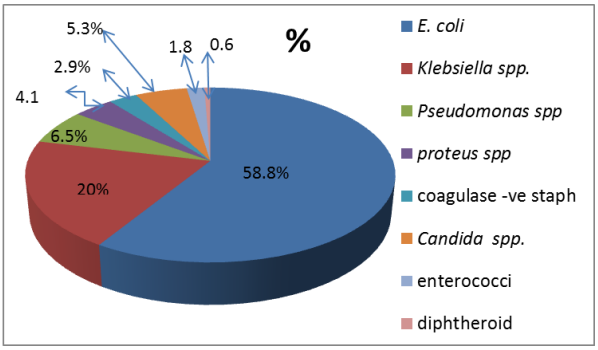

Fig. 1: Frequency of uropathogens isolated from patients with clinically diagnosed UTI and yielded significant bacterial count.

One hundred UPEC (58.8%) were identified. In parallel, 50 commensal fecal *E. coli* isolates and 5urinary *E. coli* isolates were identified from apparently heathy individuals based on biochemical tests.

Antimicrobial susceptibility

Out of 100 UPEC isolates, only 6 % were sensitive to all tested antibiotics and 96% were sensitive to nitrofuran , 70% of the isolates were multidrug resistant. Out of 50 fecal E.coli isolates, 42% resistance was observed to Ampicillin and Trimethoprim/ sulfamethoxazole followed by 24% resistance to Ampicillin alone. None of the fecal *E. coli* isolates were found to be sensitive to all tested antibiotics.

Virulence factors of *E. coli* isolates by phenotypic tests.

α-Haemolysin was significantly (P=0.001)expressed in UPEC isolates (38%) while none of fecal *E. coli* isolates (0%) were producing α-haemolysin toxin. Cytotoxic necrotizing factor 1 was also significantly (P=0.001) expressed in UPEC isolates from patients (19%) in which the clarified lysates of UPEC isolates produced cytopathic effects in vero cells (Fig. 2), while none of fecal E. coli isolates (0%) were producing cnf1 toxin. In vitro biofilm production was significantly (P=0.005) lower in fecal E. coli isolates (34%) when compared with UPEC isolates (51%) by the tube adherence method (Fig. 3). The phenotypic expression of P- fimbriae was nearly similar in fecal E. coli isolates (34%) when compared with UPEC isolates (38%) with statistically insignificant difference (Fig. 4), suggesting that α-hly & cnf 1 are more important for virulence while biofilm formation & p fimbrae are more important for the colonization process.

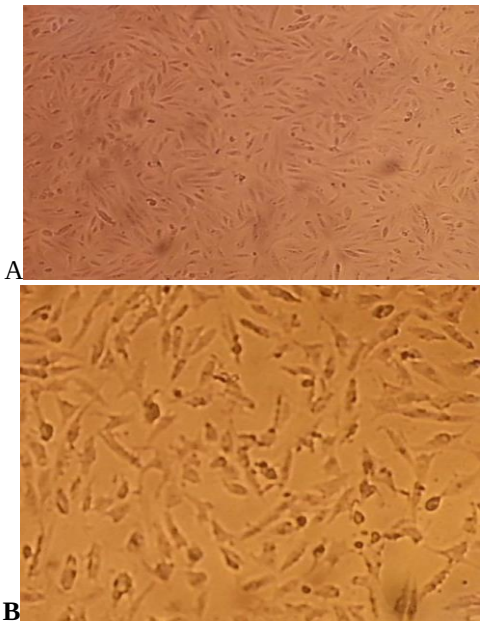

Fig 2: Phenotypic toxicity assay for detection of cnf 1 toxin production by E .coli isolates. (A) Normal cell control of vero cell line. (B) Cytopathic effect (altered Vero cell morphology and detachment by different degrees) exerted by cnf 1 extracts on vero cell cultures after 24 hrs of inoculation.

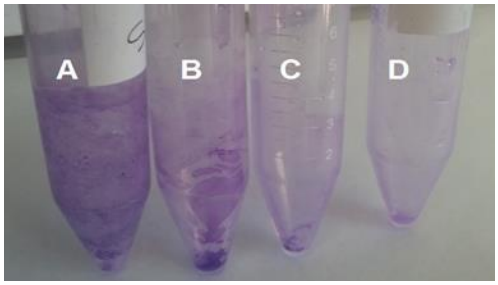

Fig 3: In vitro biofilm formation by tube method. (A) Strong positive result, (B) Moderate positive result, (C) Weak positive result while (D) Negative result.

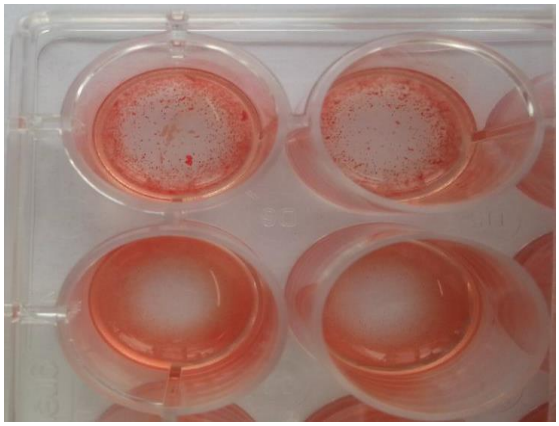

Fig 4: Phenotypic assay of mannose resistant haemagglutination for detection of P fimbrea expression. A) MRHA, B) Negative result.

Virulence genes in *E. coli* isolates by multiplex PCR

The virulence genes hly A (1,177bp); cnf 1 (498bp); aer (602bp) and pap C (328bp) were successfully amplified in *E. coli* isolates (Fig 5). hly A gene was detected in 40% of isolates, cnf 1 gene was detected in 21%, whereas aer and pap C genes were detected in 60% and 42% of the isolates respectively (Table 2).

Table 2: Prevalence of the detected virulence genes among UPEC isolates from patients with UTI

Virulence genes	*UPEC isolates NO=100 (100%)*
hly +ve	40(40.0%)
Cnf +ve	21 (21.0%)
Aer + ve	60 (60.0%)
papC +ve	42 (42.0%)

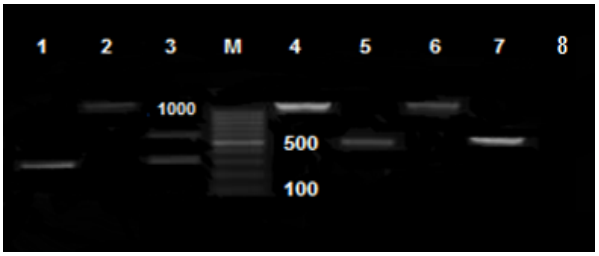

Fig. 5: Agarose gel electrophoresis of multiplex PCR for detecting virulence associated genes: lane 1. pap C (328bp): lanes 2,6. hly A(1,177bp):lane3. aer (602bp):lanes 5,7. cnf 1(498bp): lane 4, positive control; lane 8, negative control; M, Molecular Marker.

As regard the 5 urinary *E. coli* isolates from the healthy individuals, virulence determinants were more prevalent in the urinary isolates and completely absent in their corresponding fecal isolates as regard haemolysin and biofilm formation. However both fecal and urinary *E. coli* isolates showed the same distribution as regard P fimbrae expression and pap C gene detection.

DISCUSSION

E. coli was the most frequently isolated organism 100/170 (58.8%) from the examined urine samples followed by klebsiella spp. 34/170 (20%), Pseudomonas spp. 11/170 (6.5%), Proteus spp. 6/170 (4.1%), Enterococci spp. 3/170 (1.8%), coagulative negative staphylococci 5/170 (2.9%), diphtheroid 1/170 (0.6%) and Candida spp. 9/170 (5.3%) that was similar to the findings of Hassan[10].

The most frequent antimicrobial resistance found was against ampicillin (93%). This result was in agreement or even higher than those reported by other studies that found ampicillin resistance ranging from 30 to 58%[11,12]. The second most frequent resistance

observed in their work was to trimethoprim/ sulfamethoxazole (44%), again a common finding, with frequencies varying from 10.9 to 36% world-wide. Resistance to ciprofloxacin, frequently used in the treatment of UTI, was found in 13% of their isolates; other studies found frequencies to ciprofloxacin resistance of 3% *E. coli* isolates[12], 9% by Houdouin et al., [11] and 17% by Arslan et al.[13].

Although a higher percentage of our UPEC isolates showed resistance to antimicrobials than fecal *E. coli* isolates, both shared the most identified pattern of antimicrobial resistance (in UPEC 20% and in fecal E.coli 42%) which was determined by resistance to ampicillin and Trimethprim-Sulphamethoxazole.

Similar results were observed by Logue et al.[14]. Such observation showed that antimicrobial susceptibility patterns failed to provide a clear distinction between UPEC and human fecal *E. coli* isolates despite the source that UPEC isolates were harvested from urinary tract infected patients while fecal *E. coli* isolates were harvested from stool samples of healthy individuals.

A lower percentage of UPEC (4%) and fecal *E. coli* isolates (0%) was surprisingly exhibited resistance to nitrofuran. Nitrofuran takes the advantage of being urinary antiseptic, it effectively lowers bacterial count in urine and thus greatly diminish the symptoms of LUTI and only used in management of UTIs, an observation which may be of great value to be used as an alternative antimicrobial in case of resistance to other commonly used antibiotics [15].

All virulence factors were detected among E.coli isolates in the present study. The difference between UPEC and fecal *E. coli* isolates for the production of haemolysin was highly significant. Our results were completely matched with Kuhar et al[16]. The absence of haemolysin production in fecal *E. coli* isolates indicates that the examined isolates do not harbor a larger pathogenicity island. This result was also in agreement with the finding of study conducted by Raksha et al.[17] regarding UPEC isolates only. As regard fecal *E. coli* isolates, our results were in contrast with the previous study, where haemolysin was found to be produced by 41% of the urinary and 12% of the fecal isolates while none of our fecal isolates were hemolytic which can be explained by possibility that such fecal strains of *E. coli* in other studies may be UPEC colonizing the intestine.

In our study cnf 1 toxin was detected in 19% of UPEC compared significantly to fecal *E. coli* isolates in which none of them produced cnf 1(0%). Our finding were in agreement with Caprioli et al.[18], who reported that cnf 1toxin is also found more often in UTI strains than in fecal strains.

In the present study, UPEC isolates were significantly (P=0.005) in vitro biofilm producers when compared with fecal *E. coli* isolates. Similar results were detected by Niveditha et al.[19], who reported biofilm production in (44%) of UPEC isolates.

On the other hand, the results of our study were in contrast to results reported by Sabitha et al[20], who found a significant difference in P fimbriae expression between UPEC and fecal E.coli isolates (35.5%, 6% respectively) with high prevelance among UPEC isolates. Our results may be explained by the hypothesis that P fimbriae adherence is essential for colonization and survival of *E.coli* regardless the site they harboured either in the colon or urinary tract. P fimbriae play an important role in the survival and pathogenesis of UPEC, firstly in colonization of the GIT, and then invasion of the UT[21].

As regard virulence genes, aer gene was the most common detected gene among UPEC isolates (60%). Our results showed to some extent lower prevalence of aerobactin encoding sequence (60%) among the investigated UPEC isolates, compared to 79% reported by Houdouin et al.,[11] and 78% reported by Moreno et al.[22], while all other studies reported lower prevalence[23,24], 67%, 55% respectively that are completely matched with our findings.

In our results, pap C gene was detected in 42% of UPEC isolates compared to(54%) reported by Alabsi et al.,[25,26] among UPEC isolates from Egyptian patients attending the Microbiology Department of Alexandria Medical Research Institute. On the other hand, hly gene was detected in 40% of our UPEC isolates, while cnf 1 was detected in 21%. Prevalence of these genes vary according to the phylogenetic group, clinical conditions of host and geographical localization. Geographical differences regarding virulence determinants could be due to variability in host immune response and different antibiotic regimes used. Frequencies ranging from 0 to 32% were found for cnf1; and from 0 to 44% for hly[27-30].

In the present study, unexpectedly high prevalence of UPEC isolates possessed none of the studied virulence genes (24%), and 22% could not express any of the studied virulence factors. These negative results were higher than those detected by Oliveira et al.,[1], who reported that in 10% of the analyzed isolates, none of the studied UPEC associated virulence genes were detected.

Similar finding but more higher prevalence was detected in an Egyptian study conducted by Massoud et al.,[31], they reported that fifty (50%) of UPEC isolates lack the three virulence genes tested (papC, cnf1, hlyA), the majority of them (80%) were associated with lower UTI. It is conceivable that these isolates actually possessed other genes that compensate for their lack of the studied virulence genes. Lack of virulence markers was also observed in another study conducted by Blanco et al[27], who reported that 41 % of their UPEC isolates lack the presence of hlyA, cnf1 and pap genes. These findings reflect the heterogeneity in the distribution of virulence genes among UPEC strains and the complex and multifactorial characteristic of UPEC virulence. In addition, since most urovirulence genes only exist in a

portion of DNA of *E. coli* isolates, such isolates may not have harbored these potential virulence genes[21,28].

In our study, asymptomatic bacteriuria was detected in 5 out of 50(10%) of *E. coli* isolates from healthy individuals with a high variation between fecal and urinary *E. coli* isolates as regard virulence determinants. Our results were in agreement with those reported by Bergsten et al.[32], who detected that ABU occurs in 2-20% of the population, depending on age and gender, and the bacteria may persist in the host for months or years.

Our observation on comparing fecal and urinary *E. coli* isolates in healthy individuals as regard virulence determinants were completely matched with results conducted by Salvador et al.[33]. They characterized differences in the genome content of community-acquired ABU isolates relative to commensal *E. coli* isolates. Generally, the extra-intestinal pathogenic *E. coli* (ExPEC) virulence gene content was higher in ABU isolates than in commensal isolates.

In our study, virulence factors namely haemolysin and biofilm formation were more prevalent in urinary isolates from healthy individuals with ASB, and completely absent in fecal *E. coli* isolates with the same results as regard hly A and aer genes. However both fecal and urinary *E. coli* isolates showed the same distribution as regard P fimbrae expression and pap C gene detection.

Similarly, Salvador et al.[33], reported that the prevalence of virulence genes differed significantly among the two groups of isolates. Virulence genes were significantly more prevalent in ABU isolates than in commensal strains, i.e. those coding for the exotoxins α-haemolysin (hlyA) and cytotoxic necrotizing factor (cnf1) and the aerobactin siderophore receptors.

Our results regarding equal distribution of P fimbriae in both ABU and fecal *E. coli* isolates have been supported by the observations speculated by previous studies [34,35]. They reported that P fimbriae have been confirmed to be an independent virulence factor in the human urinary tract infection, and the mechanism of tissue attack has been explained, in part, by their glycolipid receptor specificity and the pathogen-specific signaling pathway that they activate in host cells.

Our observations have been supported the hypothesis that evolution toward commensalism rather than virulence is favored during asymptomatic bladder colonization and also support the hypothesis that bacterial adaptation in vivo is in part driven by positive selection of UPEC variants with a reduced ability to activate the innate immune response which is crucial to avoid overt activation of the host response and pathogen-specific inflammatory pathways that trigger many aspects of symptomatic UTI. On the basis of this attenuation, which may result from virulence gene inactivation and loss of gene expression either by accumulation of point mutation or by deletions, such strains achieve long-term carriage in urinary bladder of health individuals without eliciting any manifestations[32,36,37].

In conclusion, virulence potential of E.coli isolates from the stools of healthy individuals is lower than that of E.coli isolates producing symptomatic urinary tract infections or asymptomatic bacteriuria , however, some fecal E.coli isolates appeared to be potentially virulent:

- Indicating that intestinal flora acts as a reservoir for UPEC isolates and,
- Reinforcing the idea that the studied virulence determinants are involved in both pathogenesis of UTI and in the ability of E.coli to survive either in intestine or in the new ecological niches such as the human urinary tract.

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