Distribution of virulence genes and phylogenetics of uropathogenic Escherichia coli among urinary tract infection patients in Addis Ababa, Ethiopia

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Belayneh Regasa Dadi
Arba Minch University

Tamrat Abebe
Addis Ababa University School of Medicine

Lixin Zhang
Michigan State University

Adane Mihret
Addis Ababa University School of Medicine

Workeabeba Abebe
Addis Ababa University School of Medicine

Wondwossen Amogne
Addis Ababa University School of Medicine

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Abstract

Background

Urinary tract infection (UTI) is a common cause of morbidity and mortality worldwide. Uropathogenic Escherichia coli (UPEC) bacteria are the major cause of urinary tract infections. UPEC strains derive from different phylogenetic groups and possess an arsenal of virulence factors that contribute to their ability to overcome different defense mechanisms and cause disease. The objective of this study was to identify phylogroup and virulence genes of UPEC among urinary tract infection patients.

Methods

A total of 200 E. coli bacteria were isolated from 780 UTI patients using culture and conventional biochemical tests. Identification of phylogroup and genes that encodes for virulence factors was done using multiplex polymerase chain reaction (PCR). Data was processed and analyzed with SPSS version16.0 and Epi-info version 3.4.1 software.

Result

The most common urologic clinical manifestation combinations in this study were dysuria, urine urgency and urgency incontinence. The frequent UPEC virulence gene identified was fim H 164 (82%), followed by aer 109 (54.5%), hly 103 (51.5%), pap 59 (29.5%), cnf 58 (29%), sfa 50 (25%) and afa 24 (12%). There was significant association between pap gene and urine urgency (p=0.016); sfa and dysuria and urine urgency (p=0.019 and p=0.043 respectively); hly and suprapubic pain (p=0.002); aer and suprapubic pain, flank pain and fever (p=0.017, p=0.040, p=0.029 respectively). Majority of E. coli isolates were phylogroup B2 60(30%) followed by D 55(27.5%), B1 48(24%) and A 37(18.5%). There was significant association between E. coli phylogroup B2 and three virulence genes namely afa, pap, and sfa (p=0.014, p=0.002, p=0.004 respectively).

Conclusion

In this study the most frequent E. coli virulence gene was fim H, followed by aer, hly, pap, cnf, sfa and afa respectively. There was significant association between E. coli virulence genes and clinical symptoms of UTI. The phylogenetic analysis indicates majority of uropathogenic E. coli isolates were phylogroup B2 followed by phylogroup D. Phylogroup B2 carries more virulence genes. Hence,
targeting major UPEC phylogroup and virulence genes for potential vaccine candidates is essential for better management of UTI and further research has to be conducted in this area.

Background
Urinary tract infection (UTI) remains the most common bacterial infection in human population and is also one of the most frequently occurring nosocomial infections (1). Its annual global incidence is of almost 250 million (2, 3). About 20% of all UTIs cases occur in men while 50-60% of women will have at least one episode of UTI during their lifetime (4). *Escherichia coli* is the major etiologic agent in causing UTI, which accounts for up to 90% of cases (3). *Escherichia coli* strains isolated from the urinary tract are known as uropathogenic *Escherichia coli* (5).

Uropathogenic *E. coli* strains possess an arsenal of virulence factors that contribute to their ability to overcome different defense mechanisms cause disease. These virulence factors that are located in virulence genes include fimbriae (which help bacterial adherence and invasion), iron-acquisition systems (which allow bacterial survival in the iron-limited environment of the urinary tract), flagella and toxins (which promote bacterial dissemination). Virulence genes are located on transmissible genetic elements (plasmid) and/or on the chromosome (6) so that non-pathogenic strains acquire new virulence factors from accessory DNA (7).

*Escherichia coli* strains derive from different phylogenetic groups (8); phylogenetic typing in four groups: A; B1; B2, and D. The majority of strains responsible for extraintestinal infections, including urinary tract infections, belong to group B2 or, to a lesser degree, to group D, whereas commensal isolates belong to groups A and B1 (9). So identification of virulence genes and phylogenetics of uropathogenic *Escherichia coli* is important for appropriate management of urinary tract infections.

Methods
A facility (hospital) based cross sectional study was conducted in selected health facilities of Addis Ababa, Ethiopia; Namely Tikur Anbessa Specialized Hospital, Yekatit 12 Hospital and Zewditu memorial Hospital. Clinical data were collected using a well-designed questionnaire. Mid-stream urine sample was collected using sterile container from patients diagnosed with urinary tract infections. *Escherichia coli* isolates were presumptively identified by colonial morphology on MacConkey agar
(Oxoid, UK), and further identified and confirmed by conventional biochemical tests. A sample was considered as positive for UTI if a single organism was cultured at a concentration of >$10^5$ CFU (colony forming unit) per milliliter of urine (10). Patients having at least two of the following complaints: dysuria, urine urgency, frequency, incontinence, suprapubic pain, flank pain or cost vertebral angle tenderness, fever (>38°C) and chills was considered as urinary tract infection.

**Bacterial DNA extraction**

DNA extraction was performed using an alkaline heat lysis method. *Escherichia coli* strains were grown on LB medium at 37°C overnight. Bacteria colonies were inoculated and suspended in 1.5 ml centrifuge tubes containing 200 µl of 1xPBS solution, and then 800µl of 0.05M NaOH added and mixed by vortexing. The sample/mixture was incubated at 60°C for 45 minutes. After 45 minutes 240 µl 1 M Tris-Cl was added to neutralize NaOH and centrifuged at 13,000rpm for 3 minutes. One thousand microliters of the supernatant were stored at -20°C as a template DNA stock (11, 12).

**Detection of virulence genes of uropathogenic Escherichia coli**

The genetic determinants that are studied includes those coding for type 1 fimbriae [*fimH*], pili associated with pyelonephritis [*pap*], S and F1C fimbriae [*sfa* and *foc*], afimbrial adhesins [*afa*], hemolysin [*hly*], cytotoxic necrotizing factor [*cnf*], and aerobactin [*aer*](11, 13, 14, 15). Specific primers were used to amplify sequences of the [*fim*, *pap*, *sfa/foc*, *afa*, *hly*, *cnf*, and *aer* operons. Details of primer sequences and predicted sizes of the amplified products are given in Table 1.

Detection of [*fim*, *pap* and *afa*, and *sfa/foc* and *aer* sequences were done by multiplex PCR while [*hly*] and [*cnf*] detection were done by single-plex PCR (11, 15, 16). PCR amplification of bacterial DNA extracts was done in a total volume of 25µl containing 20µl of Platinum® PCR SuperMix (The mixture contains Mg++, dNTPs and recombinant Taq DNA polymerase at concentrations sufficient to allow amplification during PCR), 1.5µl template DNA and 1.5-2 µl (30 pmol of each) of the primers (11, 15). The amplification was carried out in a multiplex PCR [T100™ Thermal cycler (BIO RAD) & PTC-200
Peltier Thermal cycler (MJ Research). Conditions consisted of an initial denaturation at 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for 2 minutes, annealing at a specific temperature for 30 seconds (Multiplex PCR for fimH, afa and pap annealing temperature used was 60°C; Multiplex PCR for sfa and aer annealing temperature used was 55°C; annealing temperature of Single-plex PCR for cnf and hly was 45°C and 50°C respectively) and 72°C for 1 minute, and final extension at 72°C for 10 minutes. A 4.5µl aliquot of the PCR product was mixed with 6x blue loading dye on parafilm and loaded on 1.2% agarose gel electrophoresis stained with 10µL 10,000x GelRed. Electrophoresis was carried out for 120 minutes at 110 volt on TAE buffer system and the gel was imaged under UV light (E-gel Imager; life technologies, USA). Amplified DNA fragments of specific sizes were detected by UV-induced fluorescence and the size of the amplicons were estimated by comparing them with the 1 kb plus DNA ladder (Invitrogen™) included on the same gel (11, 15). Strain J96 was used as positive control for pap, sfa/foc, hly, cnf, and fimH sequences and the strain K10 was used as positive control for afa. The positive control for aer was J96 and Cl1212 strains (17, 18, 19).

**Phylogenetics grouping of uropathogenic Escherichia coli**

Strains responsible for extra-intestinal infection are far more likely to be members of phylogroups B2 or D than A or B1 (7, 8, 20). The clinical significance of these observations suggested that a simple method of assigning isolates to a phylogroup would be of value. This led to the development and validation of a PCR assay to detect the genes chuA and yjaA, and an anonymous DNA fragment TspE4.C2 found in *E. coli* isolates around the world (21). Based on the presence/absence of these three fragments, and *E. coli* strain could be assigned to one of the main phylogroups; A, B1, B2 or D. This triplex PCR phylogroup assignment has been used extensively as a simple and inexpensive method for assigning an *E. coli* isolate to a phylogroup and has provided further evidence that strains of the various phylogroups differ in their phenotypic and genotypic characteristics, their ecological niche, life history traits and ability to cause disease (21, 22). All PCR reactions were carried out in a
25µl volume containing 20µl of 10X buffer (supplied with Taq polymerase), 2mM each dNTP, 2U of Taq polymerase (Invitrogen™ Super mix); the amounts of primer used are 20pmol (2µl of each primers). PCR reactions (T100™ Thermal cycler, BIO RAD) were performed under the following conditions: denaturation 4 minutes at 94°C, 30 cycles of 5 seconds at 94°C and 20 seconds at 59°C, and a final extension step of 5 minutes at 72°C (22, 23).

Data analysis

SPSS version 16.0 and Epi-info version 3.4.1 softwares were used for data analysis. Regression and Chi-square test was performed to assess relationship between variables. P value <0.05 was considered as significant.

Result

Urine sample of 780 study participants who had complaints of urologic symptoms of urinary tract infections were cultured and 200 (25.6%) Escherichia coli isolates were identified by biochemical tests. Among study participants, 265 (34%) were males and 515 (66%) were females. (see Table 3).

Highest incidence of urinary tract infections was observed in the age groups 26-45. There was no significant association between gender, age and marital status, and E. coli isolation rate (P=0.392, P=0.665 and P=0.466 respectively). (see Table 4)

The most common urologic clinical manifestation combinations in this study were dysuria; urine urgency and urgency incontinence followed by dysuria and urgency incontinence (see Table 5).

Virulence genes were amplified and detected successfully in 198 (99%) E. coli isolates. The most frequent E. coli virulence gene was fimH 164 (82%), followed by aer 109 (54.5%), hly 103 (51.5%), pap 59 (29.5%), cnf 58 (29%), sfa 50 (25%) and afa 24 (12%) [see Fig 1].

The virulence genes fimH (456 bp), afa (672 bp), pap (328 bp), sfa (410 bp), aer (269 bp), cnf (693 bp) and hly (556 bp) were successfully amplified. One kilobase plus (1kb plus) DNA ladder was used to determine the base pair size (see Fig 2).

There was significant association between pap gene and urine urgency (p=0.016); sfa and dysuria and urine urgency (p=0.019 and p=0.043 respectively); hly and suprapubic pain (p=0.002); aer and suprapubic pain, flank pain and fever (p=0.017, p=0.040, p=0.029 respectively) [see Table 6].
Phylogenetics of uropathogenic Escherichia coli

The distribution of phylogenetic groups amongst Escherichia coli isolates was determined by the following genes; arp A (400bp), chu A (288bp), yja A (211bp) and an anonymous DNA fragment that is found in E. coli worldwide; TspE4C2 (152bp). One kilobase plus (1kb plus) DNA ladder was used to determine the base pair size (see Fig 3)

Phylogenetic analysis indicates majority of uropathogenic Escherichia coli isolates were group B2 60(30%) followed by group D 55(27.5%), group B1 48(24%) and group A 37(18.5%) [see Fig 4]. In this study there was significant association between Escherichia coli phylogroup B2 and three virulence genes namely afa, pap, and sfa (p-0.014, p-0.002, p-0.004 respectively). Similarly, there was significant association between Escherichia coli phylogroup D and two virulence genes namely fimH and pap (p-0.043, p-0.019 respectively). There was significant association between Escherichia coli phylogroup A and virulence genes fimH and afa (p-0.011, p-0.002 respectively). Phylogroup B1 has significant association with pap gene (p-0.001). The virulence factor that encodes pap gene has significant association with Escherichia coli phylogroup B2, D and B1 (p-0.002, p-0.019, p-0.001 respectively) [see Table 7].

Discussion

In this study higher proportion of urinary tract infections in females (66%) than in males (34%) were observed. UTI is more common in females than in males because structurally the female urethra is less effective in preventing the bacterial entry for colonization i.e. the urethra is shorter and wider. Escherichia coli is common because it is a normal flora in large intestine and can easily be acquired via faecal contamination with urinary tract especially in female it causes ascending UTI (45). Highest incidence of urinary tract infections was observed in the age groups 26-45. This could be due to the fact that this age group is sexually active. Sexual intercourse may access entry of bacteria in to bladder. Identification of virulence factors that are encoded by uropathogenic E. coli are important for pathogenesis, severity of urinary tract infection, targets for vaccine and drug development (25). The most important step to development of UTI is adherence (using fimH, pap, sfa, afa pilli) to urinary epithelia, allowing uropathogens colonization and invasion of urinary tract. Genes coding for fimbrial
adhesive systems represent the most common factors for the virulence of *E. coli* in UTI (24, 25).

In our study *fimH* adhesion gene was the most common and present in 164 (82%) uropathogenic *E. coli* isolates which is in agreement with studies conducted in Romania, 86% (15); Mongolia, 89.9% (26), Iran, 86.17% (27), 79.67% (28) and China, 87.4% (29). Targeting *fimH* as vaccine candidate is important for prevention of UTI and currently vaccine targeting *fimH* as potential vaccine candidate is under investigation. Antibodies against *fimH* prevent colonization of urinary tract by UPEC isolates (25). In this study, we found no significant association between *fimH* gene and clinical symptoms of UTI (p>0.05), but this does not mean *fimH* is not involved in pathogenesis of UTI.

Pyelonephritis associated pilli (*pap*) gene was found in 59 (29.5%) uropathogenic *E. coli* isolates which is comparable to study conducted in Iran, 30.2% (6); Mexico, 24.7% (30); Romania, 36% (15) and Brazil, 32% (31); but lower than studies conducted in Iran, 50.4% (27), 57% (32) and Egypt 54%(33).

In this study, there was significant association between presence of *pap* gene and urine urgency (*p*-0.016), which was commonly observed clinical symptom in most UTI patients. This indicates that UPEC uses *pap* genes as virulence factor to cause UTIs.

S and F1C fimbriae (*sfa* gene) was found in 50 (25%) uropathogenic *E. coli* isolates which is similar to studies conducted in Pakistan, 27% (34); Romania, 23% (15); Tunisia, 34% (11); Iran, 32% (35) and Iraq, 22.7% (36); but lower than studies conducted in Denmark, 46% (37); Iran, 81% (32) and South Korea, 100% (38) and higher than studies conducted in Mongolia, 8.8% (26) and China, 8% (29). In our study, there was significant association between presence of *sfa* gene, and dysuria and urine urgency (*p*-0.019 and *p*-0.043 respectively). This indicates that *sfa* genes are important for pathogenesis of UPEC to cause UTI and responsible for clinical symptoms of UTIs.

Afa adhesin (*afa* gene) was found in 24 (12%) uropathogenic *E. coli* isolates which is similar to studies conducted in Iran, 12% (32); Mexico, 12.8% (30); Brazil, 11% (31) and Romania, 14% (15). Afimbrial adhesins (*afa*) may favor establishment of chronic and/or recurrent urinary tract infections (51). In this study, we found no significant association between *afa* gene and clinical symptoms of UTI (p>0.05).

Uropathogenic *E. coli* secretes toxins like α-haemolysin (*hlyA*) and cytotoxic necrotizing factor 1 (*cnf1*).
*Hly* Alpha promotes bladder cell exfoliation and cell lysis, which facilitates iron and nutrient acquisition by the bacteria. *Cnf1* involved in bladder cell exfoliation and increased levels of bacterial internalization (25, 39).

In this study 103 (51.5%) uropathogenic *E. coli* isolates carries hemolysin (*hly*) gene which is comparable to studies conducted in Iran, 50.4% (27) and South Korea, 62% (38); but higher than studies conducted in Zimbabwe, 12.5% (13); Tunisia, 19% (11); Poland, 18.5% (40); Mexico, 15.4% (30) and China, 11.6% (29). In our study, hemolysin gene was significantly associated with suprapubic pain (*p*-0.002). This indicates that hemolysin may be responsible for clinical manifestation in UTI patients. Alpha-hemolysin encoded by *hlyA* is an extracellular cytolytic protein toxin that is produced by up to 50% of UPEC isolates. Alpha-hemolysin has been associated with clinical severity in UTI patients (49). Currently vaccine against *hlyA* that protect renal damage is under investigation (50).

In our study we found 58 (29%) uropathogenic *E. coli* isolates carries cytotoxic necrotizing factor 1 (*cnf1*) which is similar to studies conducted in Iran, 36.5% (41) and Pakistan, 20% (34); but higher than studies conducted in Tunisia, 3% (11); Romania, 13% (15) and Poland, 12.1% (40). In this study, we found no significant association between *cnf1* gene and clinical symptoms of UTI (*p* > 0.05).

Iron is generally required for bacterial growth during infection. Thus UPEC stains uses iron acquisition genes like aerobactin, *aer* (42). In this study we found 109 (54.5%) uropathogenic *E. coli* isolates carries aerobactin (*aer*) genes which is similar to studies conducted in Romania, 54% (15); Tunisia, 52% (11); Egypt, 51% (33) and Poland, 52.6% (40); but lower than studies conducted in South Korea, 81% (38) and Iran, 73.1% (41). Currently, Siderophore proteins are under investigation for potential vaccine candidate against UTI (25). There was significant association between *aer* gene and suprapubic pain, flank pain and fever (*p*-0.017, *p*-0.040, *p*-0.029 respectively). Thus, the high prevalence of *aer* gene in our study may be due to UPEC utilizes aerobactin virulence gene as a means of acquisition of iron and associated with clinical features of suprapubic pain, flank pain and fever which were observed in most UTI patients.

From clinical point of view UTI is classified as upper (proximal) urinary tract infection and lower (distal) urinary tract infection. Upper urinary tract infection includes pyelonephritis while lower urinary
tract infection includes cystitis and urethritis. The common clinical symptom of upper urinary includes flank pain, fever, chills and costo-vertebral angle tenderness. The common clinical symptom of lower urinary includes dysuria and frequency. The above mentioned clinical symptoms of UTI that is correlated with virulence genes should be interpreted in this aspect.

The difference in virulence genes prevalence between our study and different studies abroad may be due to sample size difference and methodology difference. Several virulence determinants are the product of different genes, which can be detected by PCR method (15, 27, 40, 41). However, when there is mutation at the level of the corresponding gene, this will lead to negative PCR. Thus negative PCR doesn’t mean absence of specific virulence gene (11).

Phylogenetic analyses have shown that virulent uropathogenic E. coli strains belonged typically to group B2 and less often to group D (37, 38). Our finding is in agreement with studies conducted in Denmark (37), Pakistan (34), South Korea (38), Poland (40) and Mexico (30) where it was found that the majority of isolates of E. coli predominantly belong to phylogenetic group B2. These findings are indicative of virulent strains of UPEC are common in study area among UTI patients and measures needs to be taken to combat these virulence strains through designing and implementing appropriate prevention and control strategies.

In our study the phylogenetic analysis indicated majority of uropathogenic E. coli isolates were group B2 60(30%) followed by group D 55(27.5%), group B1 48(24%) and group A 37(18.5%) which is in agreement with study conducted by Munkhdelger et al (26), where B2 (33.8%) was dominant strains followed by D (28.4%) strains, A (19.6%) strains and B1 (18.2%) strains. Similar study conducted by Kot et al (40), showed that 38.1% E. coli strains belonged to phylogenetic group B2, 35.3% to group D, 18.5% to group A, and 8.1% to group B1. Phylogenetic group A, represented 18% of isolates, which was higher than in studies conducted in South Korea 3.44% (38) and Iran 0.7% (43), but some studies found phylogroup A was the dominant phylogroup (9, 14, 44) suggesting that the colon may be the main reservoir for strains that cause urinary tract infections(9). In some studies, phylogroup D was the dominant strain (45, 46). These different prevalence of the phylogenetic groups may be due to health status of the host, and geographic conditions, or variations in methodology and sample size.
In our study there was significant association between \textit{E. coli} phylogroup B2 and three virulence genes namely \textit{afa}, \textit{pap}, and \textit{sfa} ($p$-0.014, $p$-0.002, $p$-0.004 respectively). This finding is explained by the fact that \textit{E. coli} strains belonging to phylogroup B2 contained a greater number of virulence genes than \textit{E. coli} than other phylogroup as reported by other studies on UPEC isolates (37, 48). There was also significant association between \textit{E. coli} phylogroup D and two virulence genes namely \textit{fimH} and \textit{pap} ($p$-0.043, $p$-0.019 respectively) which is in agreement with a study conducted in Thailand (45).

\textbf{Conclusions}

In our study the most frequent uropathogenic \textit{E. coli} virulence gene was \textit{fimH}, followed by \textit{aer}, \textit{hly}, \textit{pap}, \textit{cnf}, \textit{sfa} and \textit{afa} respectively. The most common urologic clinical manifestation combinations in this study were dysuria, urine urgency and urgency incontinence. There was significant association between \textit{pap} gene and urine urgency; \textit{sfa} and dysuria and urine urgency; \textit{hly} and suprapubic pain; \textit{aer} and suprapubic pain, flank pain and fever. The phylogenetic analysis indicates majority of uropathogenic \textit{E. coli} isolates were phylogroup B2 followed by phylogroup D. There was significant association between \textit{E. coli} phylogroup B2 and three virulence genes namely \textit{afa}, \textit{pap}, and \textit{sfa}. Hence, targeting major uropathogenic \textit{Escherichia coli} phylogroup and virulence genes for potential vaccine candidates is essential for better management of UTI and further research has to be conducted in this area.

\textbf{List Of Abbreviations}

\begin{itemize}
  \item UTI: Urinary tract infection
  \item bp: base pair
  \item IRB: Institutional Review Board
  \item NaOH: Sodium Hydroxide
  \item CFU: Colony forming unit
  \item UPEC: uropathogenic \textit{E. coli}
  \item DNA: Deoxyribonucleic acid
  \item \textit{fimH}: type1 fimbriae
  \item \textit{pap}: pili associated with pyelonephritis
  \item \textit{sfa}: S and F1C fimbriae
  \item \textit{afa}: afimbrial adhesins
  \item \textit{hly}: hemolysin
  \item \textit{cnf}: cytotoxic necrotizing factor
  \item \textit{aer}: aerobactin
  \item PCR: Polymerase chain reaction
  \item dNTPs: deoxynucleoside triphosphates
\end{itemize}

\textbf{Declarations}

\textbf{Acknowledgments}

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**Availability of data and materials**

The dataset used and analyzed during the current study is available from Belayneh Regasa at belayjanimen@gmail.com on reasonable request.

**Authors’ contributions**

BR carried out proposal development, data collection, data analysis and drafted the manuscript. TA and LZ participated in proposal development, data collection, provided resources for data collection and participated in manuscript writing. AM, WAm and WAb participated in proposal development and provided input in manuscript writing. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The proposal of this study was ethically approved by the Institutional Review Board (IRB) of Addis Ababa University, College of Health Sciences. Permission was obtained from Medical directors of Tikur anbessa specialized Hospital, Yekatit 12 Hospital and Zewditu Hospital. Written informed consent was obtained from each patient participated in the study. The study participants' age was > 1 year old. Before starting data collection, the purpose of the study was explained to all study participants and written informed consent was obtained. For age group 1-16 years old written consent was obtained from the parents or guardians.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests

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