The Chittagong Univ. J. B. Sci. 9(1&2): 29-38, 2019

PCR BASED DETECTION AND MOLECULAR TYPING OF UROPATHOGENIC *Escherichia coli* ISOLATED FROM PATIENTS IN CHITTAGONG, BANGLADESH

LAILA KHALEDA, BASHUDEV RUDRA, INZAMAMUL ISMAIL SHAWON, MD. JIBRAN ALAM AND MOHAMMAD AL-FORKAN*

Department of Genetic Engineering and Biotechnology, University of Chittagong, Chittagong-4331, Bangladesh.

ABSTRACT

The uropathogenic *Escherichia coli* (UPEC) are causative agents of urinary tract infection (UTI) throughout the world. This study aimed at analyzing the prevalence of UTI among different age and gender in Chittagong, the second most populous city of Bangladesh. We performed a PCR based UPEC detection method by using 16S rRNA and phylotyping by multiplex PCR. We found *E.coli* in 83.43% of UTI samples in this study. Among these, 42% of UPEC belongs to B2 phylogroup along with A, B1, C, D, E and F. Moreover, there was a significant association of UPEC infection with age and gender. To be more specific, 45.03% of males and 54.97% of females UTI patients were infected with UPEC. Therefore, we believe that this study would play a crucial role in detecting and managing UPEC induced UTI by understanding the prevalence of UPEC and their phylotype in the Chittagong region of Bangladesh.

Key Words: Uropathogenic *E. coli*, Urinary Tract Infection, Phylogrouping, PCR, Molecular typing.

INTRODUCTION

Urinary tract infection (UTI) is delineated as the invasion of pathogens to the urinary tract tissues extending from the renal cortex to the urethra which includes prostate, urinary bladder, kidney (Kunin, 1979). In every year almost 150 million peoples are infected with UTI worldwide (Sanjee *et al.*, 2017). It may occur in both the upper part (pyelonephritis or kidney infection) and the lower part (Cystitis) of the urinary tract. The infection may vary according to age and sex. This is accompanied with the syndrome which ranged from asymptomatic bacteriuria to perinephric abscess with sepsis or even death (Sanjee *et al.*, 2017). *E. coli* is the cause of 80-85% of urinary tract infections, with *Staphylococcus saprophyticus* being the cause in 5-10% (Nicolle, 2003). Human urine can support the growth of several different strains of bacteria by its favorable chemical composition (Asscher *et al.*, 1966). Although *E. coli* is well known as a normal gut microflora, there are some pathogenic strains which can cause a wide variety of intestinal and extra-intestinal diseases (Marrs *et al.*, 2005).

* Corresponding author E-mail: alforkangeb@gmail.com

In Bangladesh, each year many women are infected with acute UTI and approximately, 60% of all women experience at least one UTI within their lifetime. About 20-30% women suffer from repeated infections (Rahman *et al.*, 2014). In the United States, the UPEC strains are responsible for 70-90% of the seven million cases of acute cystitis and 250,000 cases of pyelonephritis reported annually (Buckles *et al.*, 2004). Day-by-day multidrug resistant strains of *E. coli* are causing UTI and invasive infections. Furthermore, the prevention and control of the spread of UPEC infections are hampered greatly by a poor understanding of the population biology of this pathogen (Lau *et al.*, 2008). Also it is important to know the molecular profiling of pathogenic islands for proper understanding of infections and their effective treatment. However, it is noteworthy that bacteria can change the virulence pattern to adopt in different environment as a process of evolution (Gal-Mor and Finlay, 2006).

Molecular tools based on PCR for accurate detection and diagnosis of UTI have been used worldwide. It has huge impact on the characterization, detection, diagnosis and taxonomy of the infectious disease agents (Siripattanapipong *et al.*, 2010). PCR-based technique has also been used for the identification of phylogenetic groups of the *E. coli* species. Phylo-groups vary in their ecological niches, life-history characteristics and propensity to cause disease (Gordon *et al.*, 2008). Because of UTIs are very common in Bangladesh, so it is important to know the molecular characteristics of UPEC for taking proper preventive steps against UTI. Akter *et al.* (2018) from Chattagram Maa Shishu-O-General Hospital Chittagong investigated the prevalence of UTI in Patients with Indwelling Catheter in Chittagong Medical College Hospital. They found that 30% developed bacteriuria or UTI with catheter. They isolated *E. coli, Klebsella, Proteas* and *Pseudomonas aeroginosa* by culturing in MacConkey's agar and blood agar.

A study was also carried out by Shahina *et al.*, (2011) on antibacterial susceptibility and resistance pattern of *E. coli* causing urinary tract infection in Chittagong, Bangladesh. They asserted that *E. coli* was the major causative agent of UTI. They also concluded that females are more susceptible than males. Mazed *et al.*, (2008) also analysed the antibiotic susceptibility profile of bacteria causing urinary tract infections at Chittagong Medical College Hospital. The most common pathogens isolated by culturing into selective growth media, were *E. coli* (66, 37.71%), *Klebsiella* species (60, 34.29%), *Proteus* species (17, 9.71%) and *Pseudomonas* species (16, 9.14%). Khaleque *et al.*, (2017) performed the analysis of diarrheagenic potential of uropathogenic *E. coli* isolates in Dhaka, Bangladesh. To the best of our knowledge no molecular detections, prevalence of UPEC and profiling were carried out in Chittagong region so far. So, we aimed to carry out this research to detect the UPEC by biochemical and molecular methods from UTI patients. We also aimed to elucidate the frequency of prevalent UPEC pathotypes (Phylogrouping) within the population diagnosed with UTI.

MATERIALS AND METHODS

Sample collection and Isolation of UPEC

Urine samples were collected from UTI patients of ages 0-75 yrs), from the Pathology Department of Centre for Specialized Care and Research (CSCR), Chittagong, Bangladesh.

A total of 150 urine samples were tested for the detection of presence of *E. coli* strains. At first, urine samples were inoculated into Brilliant Green Bile Broth (BGBB, 2%) and incubated at 37°C for 24 hours to separate facultative anaerobe, gram-negative bacteria of Eterobacteriacae family which produces CO_2 by fermentation. BGBB fermentation positive cultures were then streaked onto MacConkey agar (MAC) medium and incubated at 37°C for 24 hours to get single colony coliform bacteria. Relatively large, red single colonies surrounded by turbid zone were picked up from MAC agar medium and streaked onto Eosin Methylene Blue (EMB) agar media in order to isolate *E. coli*. The plates were incubated for 24 hours at 37°C. *E. coli* exhibiting characteristic green metallic sheen in reflected light on EMB agar media after incubation were selected. The isolated cultures of *E. coli* were then inoculated into Luria-Bertani (LB) broth, incubated for 6 hours at 37°C and transferred to cryo-vials containing 50% glycerol prior to cryopreservation at ultra-low temperature.

Extraction of DNA

Genomic DNA was isolated from the cultured *E. coli* cells by using Boiling method of DNA extraction. DNA samples were quantified by NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA) at 260/280 nm wavelength with purity ranging between 1.8-2.0 and varied range of concentration (ng/µl).

Molecular Detection of E. coli

Molecular detection of UPEC was carried out in Q-Cycler (HAN Life Science, UK) by using *Escherichia* genus specific primer to amplify 16S rRNA gene of *E. coli*. In this reaction sequence of forward primer: 5'-GACCTCGGTTTAGTTCACAGA -3', and reverse primer: 5'-CACACGCTGACGCTGACCA -3' were used. Total reaction volume was 25 µl. Each reaction mixture contained 5x GoTaq Flexi Buffer (Promega Corp.), 5pM of each forward and reverse primer, 0.1mM of each dNTPs, 2mM $MgCl_2$ and ½ unit of GoTaq DNA Polymerase (Promega Corp.). An initial denaturation was at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, elongation at 72°C for 45 seconds and a final elongation at 72°C for 5 minutes.

Phylogrouping

Phylogrouping of *E. coli* strains was done by Quadruplex PCR according to the new Clermont phylo-typing method described by Clermont *et al.*, (2013) using gene targets of *chuA* (F-5′-ATG GTA CCG GAC GAA CCA AC-3′, R-5′-TGC CGC CAG TAC CAA AGA CA-3′) amplicon size 288, *yjaA* (F-5′-CAA ACG TGA AGT GTC AGG AG-3′, R-5′ AAT GCG TTC CTC AAC CTG TG-3′) amplicon size 211, *TspE4.C2* (F-5′-CAC TAT TCG TAA GGT CAT CC-3′, R-5′ AGT TTA TCG CTG CGG GTC GC-3′) amplicon size 152, *arpA* (F-5′-AAC GCT ATT CGC CAG CTT GC-3′, R-5′TCT CCC CAT ACC GTA CGC TA-3′) amplicon size 400. Single reaction volume was 20µl. Each reaction mixture contained 5x GoTaq Flexi Buffer (Promega Corp.), 2.5pM of each forward and reverse primer of 4 pair primers for four genes (*chuA, yjaA, TspE4.C2 and arpA*), 0.025 mM of

each dNTPs, 2mM $MgCl_2$ and 1.5 unit of GoTaq DNA Polymerase (Promega Corp.). Cycling conditions: An initial denaturation of 5 minutes at 95°C followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C for 30 seconds, elongation at 72°C for 30 seconds and a final elongation at 72°C for 5 minutes were provided for PCR amplification.The amplified PCR products were then electrophoresed on 1.5% agarose gel with 100 bp DNA ladder (GeneDirex, DM001-R500) and stained with ethidium bromide (500 ng/mL) to visualize the bands under a gel documentation system (WGD-30, WiseDoc). *E. coli* standard DNA was used as positive control. Nuclease free water (Invitrogen, 10977-015) was used as negative control.

RESULTS AND DISCUSSION

Urinary Tract Infection (UTI) is the most common bacterial infection accounting for 25% of all infections in many developing countries like Bangladesh where proper sanitation is not maintained adequately. It is one of the most important causes of morbidity and also the second most common cause of hospital visit (Ronald, 1991).

Isolation of E. coli from Urine Culture

Brilliant green bile broth (BGBB) media facilitates growth of coliform bacteria exclusively inhibiting growth of gram-positive and most of the gram-negative bacteria. Positive selection of *E. coli* by BGBB was done by observing production of gas in Durham's tube due to lactose fermentation. Eosin Methylene Blue (EMB) media is highly efficient for isolation of pathogenic Enterobacteriaceae and confirms *E. coli* selection with greenish metallic sheen in reflected light and blue-black centered in transmitted light. Among the collected specimens (180), 150 samples were positive for all the three tests (BGBB, MAC, EMB) while the rest were partially positive or negative for these tests (Fig. 1).

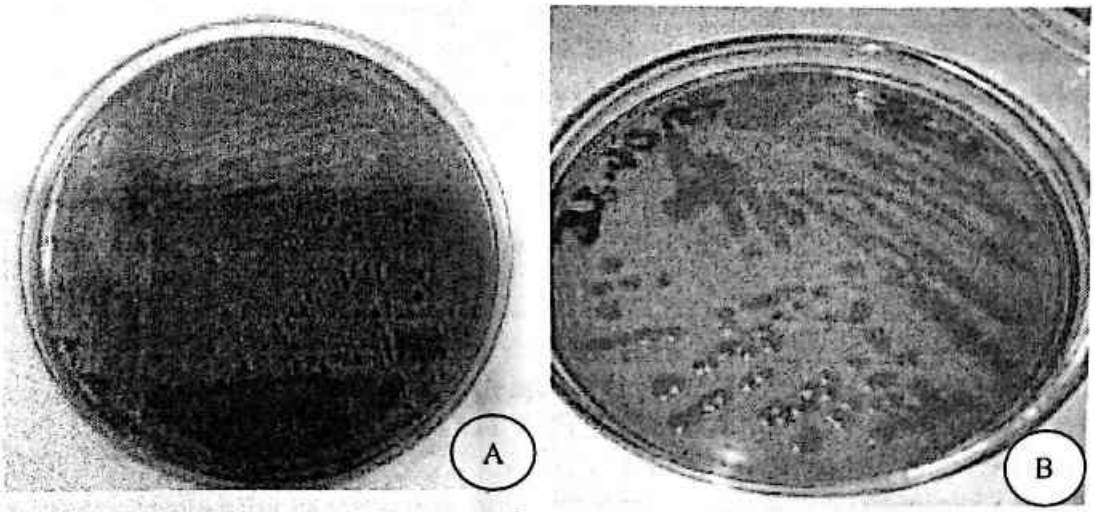

FIGURE 1: UROPATHOGENIC *E. coli* IN (A) EMB AND (B) MACCONKEY AGAR MEDIA

Molecular Detection of UPEC

16S rRNA gene of bacteria is highly genus specific and reliable for molecular detection of uropathogenic *E. coli*. DNA extracted from *E. coli* isolates was used in the PCR assay. PCR primers targeting 16S rRNA gene of *E. coli* amplified 585 bp fragments of DNA which confirmed the identity of *E. coli* (Fig. 2).

Many organisms are responsible for the UTI infections like *Pseudomonas aeruginosa, Staphylococcus aureus, Ecscherichia coli* and *Proteus* spp. Saber *et al.*, (2010) reported that the most common organism implicated in UTIs (80-85%) is *E. coli*. In our study, we got 83.43% *E. coli* infections in UTI samples. Bhowmic *et al.*, (2004) and Bova *et al.*, (1985) also asserted that *E. coli* was the prevalent organism in UTI. Until now a number of studies showed the prevalence of *E. coli* infection and their detection by biochemical and culture methods in Bangladesh which is time consuming. We are expecting that this molecular detection will help the diagnostics, research centers and hospitals to detect UTI infections in a very efficient, convenient and rapid manner.

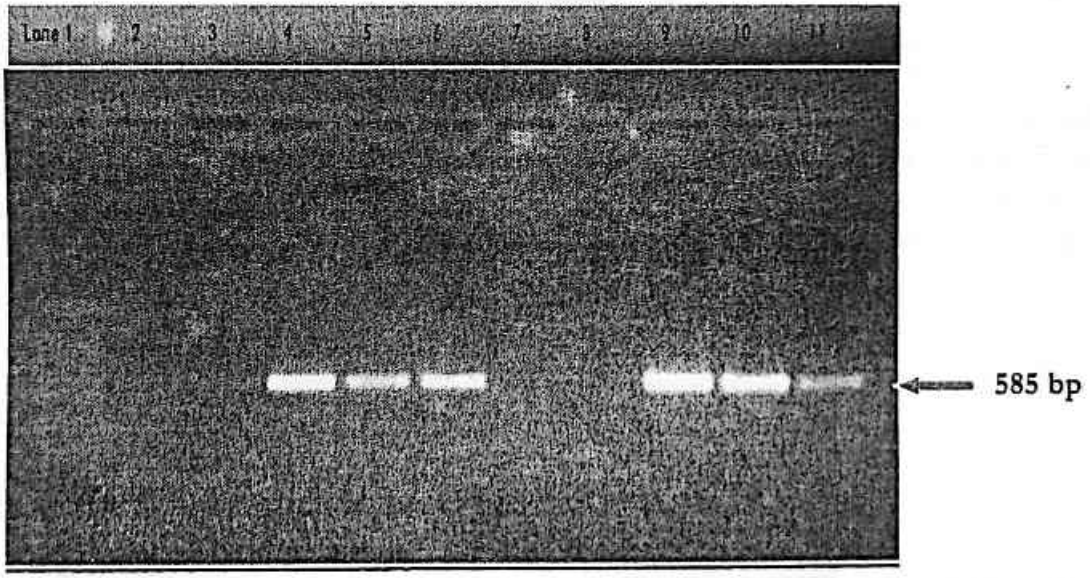

FIGURE 2: MOLECULAR DETECTION OF *E COLI* BY 16S RRNA GENE (585BP). (Lane M = 100 bp Ladder, Lane 1 = UPEC1, Lane 2 = UPEC2, Lane 3 = UPEC3, Lane 4 = UPEC4, Lane 5 = UPEC6, Lane 6 = UPEC7, Lane 7 = UPEC8, Lane 8 = UPEC9, Lane 9 = UPEC 10, Lane 10 = UPEC 11, Lane 11= UPEC 12)

Phylogrouping

Phylogrouping of strains of *E. coli* enables us to distinguish between pathogenic and commensal strains. By following the quadruplex PCR method adopted by Clermont *et al.*, (2013), it is much easier to categorize various strains according to their distinct banding pattern. For phylogrouping of *E. coli*, a quadruplex PCR assay was used in order to detect the genes *chuA, yjaA, arpA* and a DNA fragment of TspE4.C2 gene. Clermont *et al.*, (2013), classified *E. Coli* strain into seven recognized phylo-groups (A, B1, B2, C, D, E, F) based on the presence or absence of these following fragments (Table 1).

TABLE 1: PHYLOGROUPING ACCORDING TO CLERMONT *et al.* (2013).

arpA (400 bp)	*chuA* (288 bp)	*yjaA* (211 bp)	*TspE4.C2* (152 bp)	Phylogroup
+	-	-	-	A
+	-	-	+	B1
-	+	-	-	F
-	+	+	-	B2
-	+	+	+	B2
-	+	-	+	B2
+	-	+	-	A/C
+	+	-	-	D/E
+	+	-	+	D/E
+	+	+	-	E

We found 42% of UPEC belongs to B_2 phylogroup (Figure 3). Other phylogroups are A, B1 which constituted 21% of each, C phylogroup covered about 14%. The least numbers are found in the phylogroup F, which was only 2%. Doumith *et al.*, (2012) asserted that pathogenic and commensals strains of *E. coli* are classified into four phylogenetic groups as A, B1, B2 and D. Lara *et al.*, (2017) also added that extraintestinal pathogenic *E. coli* strains are sorted into phylogroups B2 or D. Epidemiological studies supported the hypothesis that commensal strains are categorized as phylogroup A or B1. Mechanisms of horizontal genetic transfer allow the exchange of VGs among phylogroups, which may promote the sporadic emergence of highly virulent strains belonging to commensal phylogroups A or B1. Therefore, it is important to characterize the virulence patterns within each phylogroups.

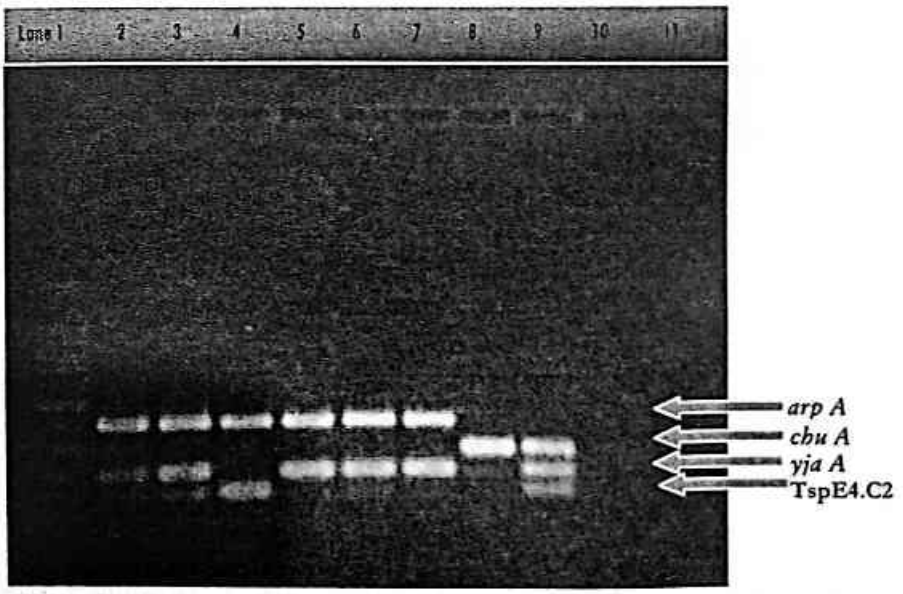

FIGURE 3: *E COLI* PHYLOGROUPING BY 16S BY QUADRUPLEX PCR. (Lane 1 = 100 bp Ladder, Lane 2 = UPEC1, Lane 3 = UPEC2, Lane 4 = UPEC3, Lane 5 = UPEC4, Lane 6= UPEC6, Lane 7 = UPEC7, Lane 8 = UPEC8, Lane 9 = UPEC9, Lane 10 = UPEC 10, Lane 11= UPEC 11. *Arp A*(400bp), *chuA* (288bp), *yja A*(211bp), TspE4.C2 (152 bp)).

Study Population and Association of UPEC with age and gender

Out of 150 subjects enrolled in this study, 68 (45.03%) were males and 83 (54.97%) were females. From the experimental data it is clearly seen that females are more susceptible than males to UPEC infections. Therefore it can be concluded that there is a significant association of UPEC with UTI patients (Table 2). We also observed the varied association of frequency of UPEC in different aged group patients. Most of the patients are belong to 50 to 75 years age group. The other two groups as 0 to 25 and 26 to 49, the infections frequency is almost equal although 26 to 49 age groups patients are more susceptible to UTI infections (Fig. 4).

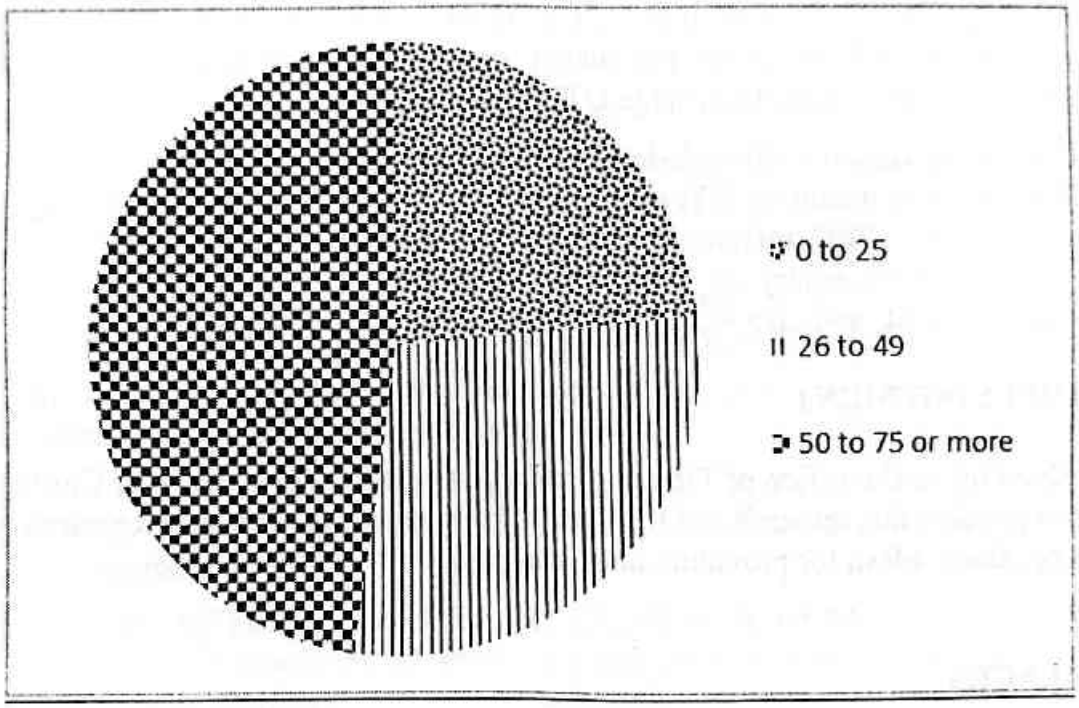

FIGURE 4: FREQUENCY OF POSITIVE UPEC IN DIFFERENT AGE GROUP UTI PATIENTS.

TABLE 2: ASSOCIATION OF UPEC INFECTION WITH SEX.

Characteristics	Number of Positive UPEC	Frequency (%)
Gender		
Male	68	45.03
Female	83	54.97
Total	150	100

Karki *et al.*, (2004) and Hooton TM (2000) also supported our findings and maintained that infection is the most common in women. They also stated that women are more susceptible than men due to several clinical factors including anatomic differences, hormonal effects and behavioral patterns. In Bangladesh, Saber *et al.*, 2010 reported 16.4% UTI in the female garments workers of Dhaka. The prevalence of UTI (growth positive

cases) was recorded higher in females than in males. Females were predominant with UTI showing 90.10% of urine culture positivity whereas the male subjects showed only 9.90% of culture positivity. Magliano *et al.*, (2012) stated that both patients' age and gender are significant factors in determining UTIs etiology; they can increase accuracy in defining the causative uropathogen as well as providing useful guidance to empiric treatment. They found *Escherichia coli*, was to be less prevalent in the youngest and oldest male subjects (51.3% and 52.2% respectively) and more frequent in female patients aged 15 years or older (approximately 71%). Findings Kiffer *et al.*, (2007) collaborate with our findings. They showed a lower percentage of *E. coli* isolation in patients younger than 13 years or older than 60 years (69.0% and 68.8%, respectively) as compared to the age group 13-60 years (79.7%). This molecular approach to detect UPEC induced UTI could play an important role to understand the prevalence of UPEC and their phylotype which will ultimately help the physician to manage UTI properly.

In the Chittagong region of Bangladesh, UTI is more prevalent among the females than males. The causative agents of UTI are mostly UPEC. The people aged above 45 years are prone to recurrent UPEC infections. Most of the UPEC belongs to B2 phylogroup. In future study, it is necessary to screen the virulence genes profiling among different phylogroups of UPEC and also to study their antibiotic sensitivity patterns.

ACKNOWLEDGEMENT

We are thankful to the office of Planning and Development, University of Chittagong for funding to conduct this research and the Centre for Specialized Care and Research (CSCR), Chittagong, Bangladesh for providing urine samples.

REFERENCES

AKTER, R., HASSAN, M. U. AND BISWAS, R.S.R. 2018. Prevalence of Urinary Tract Infection in Patient with Indwelling Catheter in Chittagong Medical College Hospital. *Chattagram Maa-O-Shishu Hospital Medical College Journal* **17(1)**: 42-45.

ASSCHER, A.W., SUSSMAN, M., WATERS, W.E., DAVIS R.H., CHICK, S. 1966. Urine as a medium for bacterial growth. *Lancet* **11:** 1037-41.

BHOWMICK, B.K. AND RASHID, H. 2004. Prevalence and antibiotic susceptibility of *E. coli* Isolated from Urinary tract infections (UTI) in Bangladesh. *Pak. J. Biol. Sci.* **7 (5):** 717-720.

BOVA, J.G., POTTER, J.L., AREVALOS, E., HOPENS, T., GOLDSTEIN, H.M. AND RADWIN, H.M. 1985. Renal and perirenal infection: to the role of computerized tomography. *J Urol.* **133:** 375-8.

BUCKLES, E.L., BAHRANI-MOUGEOT, F.K., MOLINA, A., LOCKATELL, C.V., JOHNSON, D.E., DRACHENBERG, C.B., BURLAND, V., BLATTNER, F.R. AND DONNENBERG, M.S. 2004. Identification and characterization of a novel uropathogenic *Escherichia coli*-associated fimbrial gene cluster. *Infection and immunity* **72(7):** 3890-3901.

CLERMONT, O., CHRISTENSON, J.K., DENAMUR, E. AND GORDON, D.M. 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environmental microbiology reports* **5(1):** 58-65.

DOUMITH, M., DAY M, J., HOPE, R., WAIN, J. AND WOODFORD, N. 2012. Improved multiplex PCR strategy for rapid assignment of the four major *Escherichia coli* phylogenetic groups. *J. Clin. Microbiol.* **50:** 3108-3110.

GAL-MOR, O. AND FINLAY, B.B. 2006. Pathogenicity islands: a molecular toolbox for bacterial virulence. *Cellular microbiology.* **8(11):** 1707-1719.

GORDON, D.M., CLERMONT, O., TOLLEY H. AND DENAMUR, E. 2008. Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. *Environmental Microbiology* **10(10):** 2484-2496.

HOOTON, T.M. 2000. Pathogenesis of urinary tract infections: an update. *Journal of antimicrobial chemotherapy* **46**(suppl 1): 1-7.

KARKI, A., TIWARI, B.R., PRADHAN, S.B. 2004. Study of bacteria Isolated from Urinary Tract Infections and Their Sensitivity Pattern. *Journal of Nepal Medical Association*. **43:** 200-203.

KHALEQUE M, AKTER S, AKHTER H, KHAN S, BEGUM A. 2017. Analysis of diarrheagenic potential of uropathogenic *Escherichia coli* isolates in Dhaka, Bangladesh. *J Infect Dev Ctries* **11:** 459-469.

KIFFER, C.R.V., MENDES, C. OPLUSTIL, C.P. AND SAMPAIO, J.L. 2007. Antibiotic resistance and trend of urinary pathogens in general outpatients from a major urban city. *International Brazilian Journal of Urology.* **33(1):** 42-48.

KUNIN C.M. 1979. Detection, Prevention and Management of Urinary Tract Infections, ed. 3, *Philadelphia,* Lea and Febiger p. 293.

LARA, F., NERY, D.R., DE OLIVEIRA, P.M., ARAUJO, M.L., CARVALHO, F.R., MESSIAS-SILVA, L.C., FERREIRA, L.B., FARIA-JUNIOR, C. AND PEREIRA, A.L., 2017. Virulence markers and phylogenetic analysis of Escherichia coli strains with hybrid EAEC/UPEC genotypes recovered from sporadic cases of extraintestinal infections. *Frontiers in microbiology*, **8, p.146.**

LAU, S.H. REDDY, S. CHEESBROUGH, J. BOLTON, F.J. WILLSHAW, G. CHEASTY, T. FOX, A.J., AND UPTON M. 2008. Major Uropathogenic *Escherichia coli* Strain Isolated in the Northwest of England Identified by Multilocus Sequence Typing. *J Clin Microbiol.* **46(3):** 1076-1080.

MAGLIANO, E., GRAZIOLI, V., DEFLORIO, L., LEUCI, A.I., MATTINA, R., ROMANO, P. AND COCUZZA, C.E. 2012. Gender and age-dependent etiology of community-acquired urinary tract infections. *The Scientific World Journal* **2012:34957.**

MARRS, C.F., ZHANG, L. AND FOXMAN, B. 2005. Escherichia coli mediated urinary tract infections: are there distinct uropathogenic *E. coli* (UPEC) pathotypes?. *FEMS Microbiology Letters* **252(2):** 183-190.

MAZED, M.A., HUSSAIN, A., AKTER, N., SULTAN, T., DEWANJE, A.K., BAUER, A.W. AND KIRBY, W.M.M. 2008. Pattern of Bacteria Causing Urinary Tract Infections and Their Antibiotic Susceptibility Profile at Chittagong Medical College Hospital. *Bangladesh J Med Microbiol.* **2 (2):** 17-21.

NICOLLE, L.E. 2003. Urinary tract infection: traditional pharmacologic therapies. *Dis Mon.* **49:** 111-28.

RAHMAN, S.R., AHMED, M.F., BEGUM, A. 2014. Occurrence of Urinary Tract Infection in Adolescent and Adult Women of Shanty Town in Dhaka City, Bangladesh. *Ethiopian Journal of Health Sciences* **24(2):** 145-152.

RONALD, A.R. 1991. The natural history of urinary infection in adults. *Med Clin North Am.* **75:** 299-312.

SABER MH, BARAI L, HAQ JA, JILANI MSA, BEGUM J. 2010 The pattern of organism causing urinary tract infection in diabetic and non-diabetic patients in Bangladesh. *Bangladesh J Med Microbiol.*;4(1):6–8.

SANJEE, S.A., KARIM, M.E., AKTER, T., PARVEZ, M.A.K., HOSSAIN, M., JANNAT, B. AND PERVIN, S. 2017. Prevalence and Antibiogram of Bacterial Uropathogens of Urinary Tract Infections from a Tertiary Care Hospital of Bangladesh. *Journal of Scientific Research.* **9(3):** 317-328.

SHAHINA, Z., ISLAM, M.J., ABEDIN, J., CHOWDHURY, A.H.M.I. AND ARIFUZZAMAN, M. 2011. A study of antibacterial susceptibility and resistance pattern of *E. coli* causing urinary tract infection in Chittagong, Bangladesh. *Asian Journal of Biological Sciences* **4(7):** 548-555.

SIRIPATTANAPIPONG, S., MUNGTHINN, M., TAN-ARIYA, P., AND LEELAYOOVA, S. 2010. Multilocuse sequence typing (MLST) and its aplications in epidemiological studies for parasitic infections. In. parasitology research trend. Ed. De Bruyn O. and Peeters, 99-117.

Manuscript received on 02.05.2019; Accepted on 22.07.2019

The Chittagong University Journal of Biological Sciences, Vol. 9 (1 & 2). Page No. 29-38