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GENOTYPING OF VIRULENCE FACTORS OF UROPATHOGENIC *Escherichia coli* FROM CHITTAGONG, BANGLADESH

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ABSTRACT

Uropathogenic *Escherichia coli* are the common cause of Urinary Tract Infection affecting 150 million people each year worldwide. In this study, we investigated the prevalence of UPEC genetic regions coding for various virulence factors. The targeted genetic determinants were those coding for pili associated with pyelonephritis (*pap*), hemolysin (*hly*), cytotoxic necrotizing factor (*cnf*) and aerobactin (*aer*). We collected 180 urine samples of UTI patients from the Chittagong region of Bangladesh and isolated UPEC. Among the studied strains the prevalence of *pap, hly, cnf* and *aer* genes were 21, 25.2, 24.3 and 7.0%, respectively. This rapid assessment of the bacterial pathogenicity may contribute to a better medical approach for the patients with urinary tract infections.

Key words: Genotyping, Uropathogenic *Escherichia coli* (UPEC), Urinary Tract Infection, Virulence factor.

INTRODUCTION

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 leading causes of morbidity and also the second most common cause of hospital visit (Ronald, 1991). It occurs in all populations and ages from the neonate to the geriatric age group (Kunin CM, 1994). However, infection is most common in women (Karki *et al.*, 2004). Uropathogenic *E. coli* (UPEC) are responsible for UTI which can be represented clinically as cystis (in bladder) or pyelonephritis (in kidney) or bacteriuria (Lloyd *et. al.*, 2007). Neonates, preschool girls, sexually active women, elderly women and men are at high risk for symptomatic urinary tract infections.

The development of UTIs depends on the integrity of host defense mechanisms, anatomical factors and the virulence pattern of the infecting organisms. Successful bacterial infection requires adhesion to host cells, colonization of tissues, and in certain cases cellular invasion followed by dissemination to other tissues, intracellular multiplication, or persistence (Nicolle, 2003). Pathogenic associated islands (PAIs) of UPEC and the gene products

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contribute to bacterial pathogenesis termed as pathogenesis factors. The pathogenesis factors are hemolysins, secreted proteins, specific lipopolysaccharides and capsule types, fimbrial adhesions and iron acquisitions (Mobley *et al.*, 2009). *P. fimbriae* encoded by the papA-K gene operon is one of the virulence factors of UPEC, which plays an important role in the pathogenesis of ascending UTIs and pyelonephritis in humans (Plos *et al.*, 1995). Bergsten *et al.*, (2005) described that these fimbriae recognize kidney glycosphingolipids carrying the Gal α (1-4) Gal determinant on renal epithelia via its papG adhesion and acts as an agonist of Toll-like receptor 4 (TLR4), a receptor involved in activation of the immune cell response. This, in turn, leads to the development of the local inflammation and pain associated with UTIs. α-Haemolysin (HlyA) is pyelonephritis causing secreted virulence factor of UTI, encoded by UPEC. At high concentrations, HlyA is able to lyse erythrocytes and nucleated host cells, damage effector immune cells and enables the UPEC to gain access to host nutrients and ironstores (Russo and Johnson, 2000). HlyA also induces the apoptosis of target host cells, neutrophils, T lymphocytes, renal cells and promote the exfoliation of bladder epithelial cells (Chen *et al.*, 2006). Approximately 50% of all cases of pyelonephritis, which leads to renal complications, are caused by Hly. The cytotoxic necrotising factor 1 (CNF1) is produced by one-third of all pyelonephritis strains and is also involved in kidney invasion. It interferes with polymorphonuclear phagocytosis and evokes apoptotic death of bladder epithelial cells (Mills *et al.*, 2000). Aerobactin is a bacterial siderophore encoded by *aer* genes and has recently been documented as a virulence factor in UPEC strains (Firoozeh *et al.*, 2014).

There is a co-relation between virulence factors and antibiotic resistance of *E. coli*, with special reference to Uropathogenic *E. coli* (Kukanur *et al.*, 2015). It was also, concluded that biofilm producing organisms are difficult to treat as they are resistant to commonly used antibiotics. Evaluation of Beta-hamolysin, biofilm and ESBL production should be employed in routine evaluation of *E. coli* isolates for early detection and prompt treatment (Kukanur *et al.*, 2015). The characterization of these virulence genes can be useful to improve our understanding of the pathogenesis of UTI, multidrug resistance and to minimize the complications, including kidney failure. But little information is available about the prevalence of virulent genotyping among UTI patients in Bangladesh. Previously Mahbub *et al* (2011) isolated some multidrug resistant *E. coli* from Urinary Tract Infection (UTI) patients. They also showed the relationship between drug resistance of *E. coli* and Curli fimbrination. Later, Khaleque *et al.*, (2017) performed the analysis of diarrheagenic potential of uropathogenic *E. coli* isolates in Dhaka, Bangladesh. By using Multiplex PCR, they assessed the presence of virulence genes related to uropathogenesis and found 42% positive for *papC* gene, 27% for *fim1*, 11% for *afa* and none was found positive for *sfa*. So far, no investigation was carried out in Chittagong region of Bangladesh for genotyping of virulence factors. The determination of the frequency of *cnf, hly, aer* and *pap* genotypes in Chittagong would be of great value to understand molecular epidemiological status of HPEC strain in Chittagong, the southeastern part of Bangladesh. Hence, this study aimed to analyse *cnf, hly, aer* and *pap* genotypes with their clinical manifestations in patients with UTI from Chittagong by using PCR.

MATERIALS AND METHODS

Sample collection, Isolation and detection of UPEC

Urine samples were collected from UTI patients of varied age groups (4-78 yrs), from the Pathology Department of Centre for Specialized Care and Research (CSCR), Chittagong, the second most populous city of Bangladesh. A total of 180 urine samples of UTI patients were tested for the detection of *E. coli* strains and 151 samples were found UPEC positive. The *E. Coli* was isolated by using Brilliant Green Bile Broth (BGBB, 2%), MacConkey agar (MAC) medium and Eosin Methylene Blue (EMB) agar media. Finally, we again confirmed the *E. coli* by PCR based detection method using 16S rRNA gene (Table 1). Among the 83.43% *E. coli* positive strains tested for phylogrouping, 42% strains are from phylogroup B2, followed by A, B1, D phylogroups, similar to report published previously (Lara *et al.*, 2017). These observations altogether indicated the B2 phylogroup as a virulent genotype.

TABLE 1: PRIMER SEQUENCES OF VIRULENT GENES

Target	Primer ID	Primer Sequences	Amplicon size (bp)
cnf1	Cnf.F	5'-AAGATGGAGTTTCCTATGCAGGAG-3'	498
	Cnf.R	5'-CATTCAGAGTCCTGCCCTCATTATT-3'	
hly	Hly.F	5'-AACAAGGATAAGCACTGTTCTGGCT-3'	1177
	Hly.R	5'-ACCATATAAGCGGTCATTCCCGTCA-3'	
aer	Aer.F	5'-TACCGGATTGTCATATGCAGACCG-3'	602
	Aer.R	5'-AATATCTTCCTCCAGTCCGGAGAAG-3'	
pap	Pap.F	5'-GACGGCTGTACTGCAGGGTGTGGCG-3'	328
	Pap.R	5'-ATATCCTTTCTGCAGGGATGCAATA-3'	

Detection of Virulent Genes

Detection of virulence factors was carried out by performing individual virulent gene specific PCR to amplify and determine the presence of virulence factors, namely, cytotoxic necrotising factor 1 (*cnf1*), a-hemolysin (*hly*), p-fimbriae (*pap*), and aerobactin (*aer*)) among the UPEC strains. The PCR was run in Q-Cycler (HAN Life Science, UK) by using template DNA, 5x GoTaq Buffer (Promega Corp.), 5 pM of each forward and reverse primer, 0.1mM of each dNTPs, 0.5mM $MgCl_2$ and 1 unit of Maximo Taq DNA Polymerase (GeneOn). The PCR protocol comprised of an initial denaturation for 5 minutes at 95°C followed by 35 cycles of denaturation at 95°C for 40 seconds, annealing at 59°C for 30 seconds for all primer except *pap* (62°C), elongation at 72°C for 1 minute for all except *hly*

(2 minutes) and a final extension at 72°C for 5 minutes. The primer sequences of virulence genes are provided in Table 1.

Agarose gel electrophoresis

The amplified PCR products of the virulence genes were subjected to electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and bands were observed under ultraviolet light in a gel documentation system (WGD-30, WiseDoc, Seoul, Korea).

RESULTS AND DISCUSSION

UTI caused by UPEC is one of the most prevalent infectious diseases leading to renal failure (Hagan *et al.*, 2007). The degree of pathogenicity of UPEC strains is dependent on the presence of virulence factors (Tiba *et al.*, 2008). In the pathogenesis of UTI, Fimbriae associated adherence is a crucial factor. *P. fimbriae* (*pap* gene) play a vital role in the development of pyelonephritis (Lloyd *et al.*, 2007). In our study we got the involvement of *pap* gene in UPEC. We observed the presence *pap* gene in 21% of the samples tested, whereas the *hlyA, cnf* and *aer* genes were present in 25.2%, 24.3% and 7% samples respectively (Figs. 1 and 2).

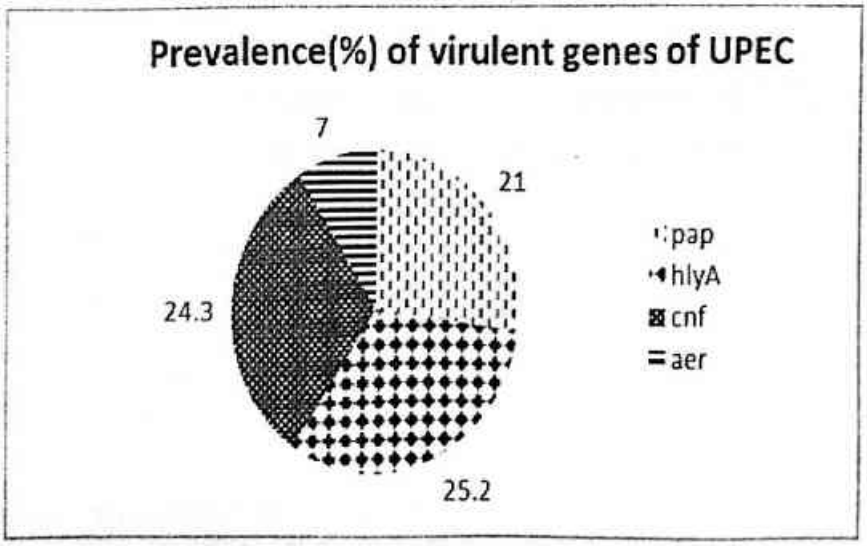

FIGURE 1: PIE CHART SHOWING THE PERCENTAGE OF PREVALENCE OF VIRULENT GENOTYPES (*pap, hlyA, cnf* and *aer*) IN UPEC FROM UTI.

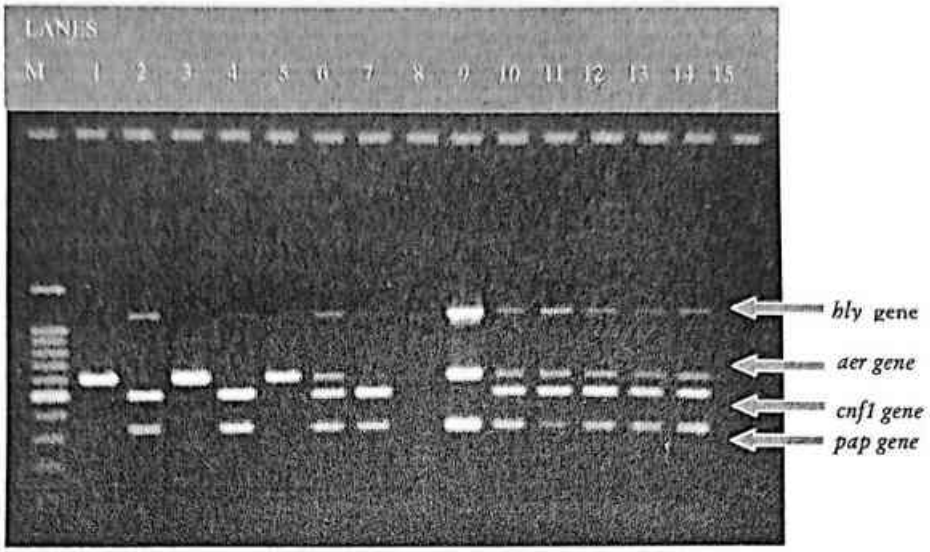

FIGURE 2: DETECTION OF *PAP, CNF1, AER* AND *HLY* GENE. (Lane M = 100 bp Ladder, Lane 1 = UPEC8, Lane 2 = UPEC9, Lane 3 = UPEC 11, Lane 4 = UPEC17, Lane 5 = UPEC22, Lane 6 = UPEC23, Lane 7 = UPEC27, Lane 8 = UPEC29, Lane 9 = UPEC 30, Lane 10 = UPEC 35, Lane 11= UPEC 36, Lane 12= UPEC 37, Lane 13= UPEC 38,Lane 14= UPEC 39).

The presence of these four virulence genes were furthermore corroborated with previous observations Mabbett *et al.* (2009), indicating that the *pap* gene is a significantly more prevalent among patients with pyelonephritis than cystitis. This is because of the role of *pap* adhesion genes in the pathophysiology of pyelonephritis caused by *E. coli* (Westerbund *et al.*, 1989; Shohreh *et al.*, 2009). Furthermore, the *pap* gene frequency may vary from 0%-77% among UTI patients worldwide (Tarchouna *et al.*, 2013; Uesin *et al.*, 2001; Neamati *et al.*, 2015; Shohreh *et al.*, 2009). Furthermore, they also found the role of two genes in the *pap* class to play more important role in the development of *E. coli* bacteremia in patients with UTI than in those with acute cholangitis. The variation in frequency of *pap* gene among different studies can be due to the fact that UPEC strains use a diverse group of adhesins to bind to the urinary epithelial cells and initiate the infection (Neamati *et al.*, 2015).

The prevalence of *hly* and *cnf* operons encoding two toxins implicated in tissue damage and dysfunction of local immune responses (Johnson, 1991). Associations of *hlyA* and *cnf* gene with pyelonephritis have been found in different studies. A common complication of *hlyA E. coli* infection is permanent renal scarring. This prototypical type 1 secreted toxin is encoded by ~50% of UPEC isolates and its expression is associated with increased clinical severity in UTI patients (Johnson, 1991; Marrs *et al.*, 2005). In our study we found higher frequency of *hlyA* and *cnf* (25.2% and 24.3%, respectively) virulent genes prevalence in UPEC isolated from Chittagong region of Bangladesh (Figs. 1-3). Although our findings partially vary from other researchers but did not show significant difference with them. Hashemizadeh *et al.*, (2017) reported the co-existence of hemolysin (*hlyA* gene) and

cytotoxic necrotizing factor type 1 (*cnf1* gene) and the prevalence of the *hlyA* gene and *cnf1* gene were 28.8% and 29.2%, respectively in UPEC isolated from Iranian patient with UTI. They also observed a statistically significant association between antimicrobial susceptibilities and presence of *hlyA* and *cnf1* genes, very similar to our observations. This is may be one of the reasons of increasing antibiotic resistances in our continent. Bacteria can transfer their pathogenic strains and transposable elements through various mechanisms including horizontal gene transfer.

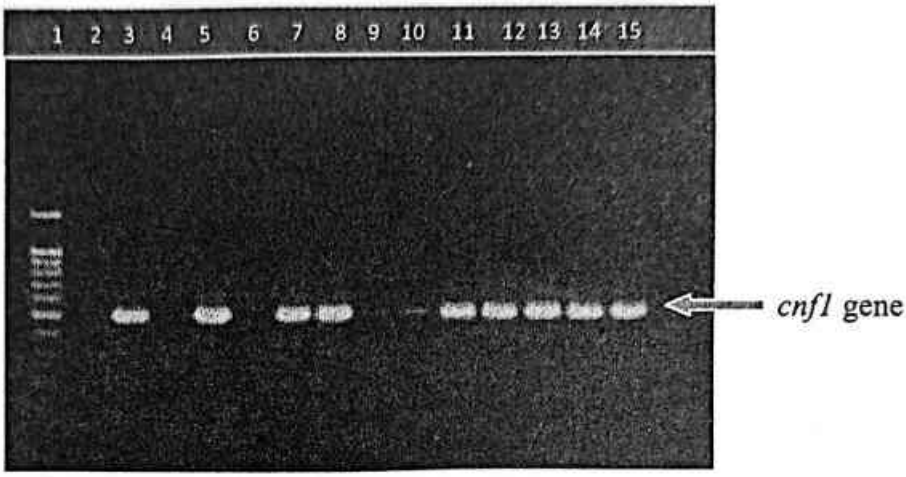

FIGURE 3: DETECTION OF *CNF1* GENE. (Lane 1 = 100 bp Ladder, Lane 2 = UPEC 8, Lane 3 = UPEC 9, Lane 4 = UPEC 11, Lane 5 = UPEC 17, Lane 6 = UPEC 22, Lane 7 = UPEC 23, Lane 8 = UPEC 27, Lane 9 = UPEC 29, Lane 10 = UPEC 30, Lane 11 = UPEC 35, Lane 12= UPEC 36, Lane 13= UPEC 37, Lane 14= UPEC 38, Lane 15= UPEC 3).

Cellular morphology and molecular biology studies have revealed that UPEC expresses siderophore production peculiar to the strains of *E. coli* causing extra intestinal infections (Vagrali, 2009). The majority of infectious *E. coli* strains possess multiple systems for ferric ion uptake, a relatively low affinity aerobactin system (*aer* operon) and two high affinity systems, yersiniabactin and enterobactin (Hancock, 2008). The *aer* operon coding for the aerobactin siderophore is broadly distributed. Munkhdelger *et al.*, (2017) reported that aer (56.1%) genes were found in UPEC of Mongolian UTI patients, indicating a putative role in iron acquisition system which is one of the main causes of UTIs. The findings of Tarchouna *et al.*, (2013), supports our findings about the prevalence of aerobactin operon aer (52%), which confers the ability to acquire iron. Usein *et al.*, (2001) also found a high percentage (42%) of *aer* in UPEC in Romania. In our study, we found less prevalence (7%) of *aer* gene in UPEC of Chittagong region of Bangladesh (Figs. 1, 2 and 4), which is smaller proportion than other studies. This is most probably because the majority of our strains did not cause parenchymatous infections (Tarchouna *et al.*, 2013).

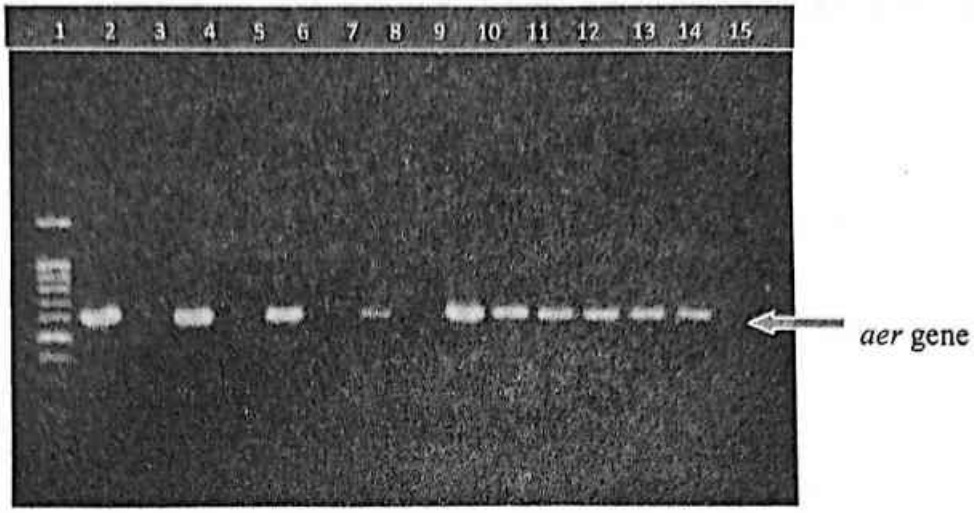

FIGURE 4: DETECTION OF *AER* GENE. (Lane 1 = 100 bp Ladder, Lane 2 = UPEC8, Lane 3 = UPEC9, Lane 4 = UPEC 11, Lane 5 = UPEC17, Lane 6 = UPEC22, Lane 7 = UPEC23,Lane 8 = UPEC27, Lane 9 = UPEC29, Lane 10 = UPEC 30, Lane 11 = UPEC 35, Lane 12= UPEC 36, Lane 13= UPEC 37, Lane 14= UPEC 38,Lane 15= UPEC 39).

In conclusion, our study found higher prevalence of pyelonephritis in the presence of virulence genes (*hly, cnf-1 and pap*) of uropathogenic *E. coli* strains and comparatively lower prevalence of *aer* genes. Detection of the genes in urine samples may help to predict the incidence of pyelonephritis. So far, this is the first molecular study of *E. coli* strains isolated from UTI in Chittagong region of Bangladesh. This is a step towards improving the knowledge regarding their virulence genetic determinants. The molecular features of *E. coli* extra intestinal strains revealed by its results may contribute to a better medical approach treating the UTI patients.

Like many pathogens, UPEC employs multiple virulence mechanism to evade and manipulate host barrier defense and innate immune responses. Our increased understanding of these host-pathogen interactions has unfolded novel therapeutic strategies that could be used to combat UPEC mediated UTI. In future more detailed study of virulent genes and their association with antibiotics sensitivity studies will be steppingstone towards epidemiological research on UTI in Bangladesh.

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