Detection Of Enterotoxin Producing Genes In Multidrug Resistant Strains Of *Escherichia Coli* From Cases Of Urinary Tract Infection

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Abstract

Forty-six E. coli isolates were isolated from patients suffering from UTI and serotyped. Antimicrobial susceptibility study was done for 24 different antimicrobial agents by disc diffusion method and out of 46 isolates, 42 isolates were found to be multidrug resistant, therefore 5 virulence genes viz. est, elt, stx1, stx2 and hlyA were detected by PCR method using their specific primers on those 42 multidrug resistant strains. The isolates belonged to 18 different serotypes, 7 were rough and 5 isolates were refractory to serotyping. Isolates were most susceptible to Imipenem (94.33%) followed by Nitrofurantoin (80.33%) and least effective was Pipercillin (33%).Maximum number of 5 isolates of E. coli showed resistant to 13 antimicrobial agents (48%) and PCR results showed the presence of est gene in three isolates belonging to serotypes O25, O171 and one was rough. One isolates each were found positive for stx2 (O5) and hlyA (O41).

1. Introduction

Escherichia coli is the most common colonic flora of warm blooded animals including humans (commensal organism). Some strains of *E. coli* are capable of causing disease under certain conditions when the immune system is compromised or disease may result from an environmental exposure. Subsets of *E. coli* have evolved possessing virulence properties and the association of these organisms with worldwide outbreaks of many enteric/diarrhoeal cases is well established. Pathogenic strains of *E. coli* have been divided into different pathotypes and each pathotype cause diseases using different combinations of the virulence factors, with different molecular pathways[1]. Common *E. coli* pathotypes include Shiga toxin producing *E. coli* (STEC), enterohaemorrhagic *E.coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC).

Urinary tract infections are probably the second most common bacterial infection and *E. coli* are found to be the most frequent urinary pathogens isolated from 50-90% of all uncomplicated cases of urinary tract infections[2]. Women are especially prone to UTIs for reasons that are not yet well understood. The *E. coli* normally present in the gastrointestinal tract, due to the proximity to the urinary tract ascends through the urinary passage to the urinary bladder and the kidneys to produce infections[3]. Under normal circumstances the human urinary tract is able to combat with the microbial invasion. To cause UTI the organism has to evade the host defense mechanism, which is determined by the virulence determinants. But distinct pathotypes of *E. coli* causing urinary tract infection have not been clearly defined and commonly has been termed as uropathogenic *E. coli* (UPEC). Interestingly the enteropathogenic *E. coli* has also been recovered time to time from extra intestinal sources like the urinary tract and incriminated as causative organism of UTI[4-6] and nondiarhheal (urinary tract) heamolytic uremic syndrome[7-9].

The present study was undertaken with a view to isolate and identify enteropathogenic *E. coli* causing urinary tract infection and to study their serotypings . Also susceptibility pattern of antimicrobial agents against the isolates were done since widespread and most often the misuse of antibiotics has been a cause of alarming raise in drug resistant strains, then multidrug resistant *Escherichia coli* were selected to screen the isolates for virulence determinants like *stx1*, *stx2*, *elt*, *est* and *hly* genes by polymerase chain reaction so as to determine the various enteropathogenic virulence factors in *E. coli* isolates from UTI cases using PCR amplification to have a better understanding of the uropathogenic strains and see if they really form a distinct group or have evolved from any other pathotypes.

2.Materials & Methods

2.1 Bacterial strains: Fifty nine urine samples were collected from patients with clinical cases of UTI from different hospitals in and around Guwahati, Assam, who had no previous exposure to antibiotics. The samples were processed as per the technique of Edwards and Ewing[10] for isolation and identification of *E. coli*. The isolates were serotyped at National Echerichia and Salmonella Centre, Kasauli (H. P).

2.2 Antimicrobial susceptibility testing: Antimicrobial susceptibility testing of the isolates were performed by the disk diffusion method[11] against 24 common antimicrobial agents (Hi-Media, Mumbai) *viz*. amoxycyclin/clavulanic acid, ticarcillin, ticarcillin/clavulanic acid, pipercillin, imipenem, cefazoline, cefuroxime, nalidixic acid, ciprofloxacin, gentamicin, ampicillin, amikacin, ceftazidime, cefepime, ofloxacin, tobramycin, levofloxacin, netilmycin, chloramphenicol, co-trimoxazole, norfloxacin, pipercillin tazobactum, tetracycline and nitrofurantoin on Mueller-Hinton agar (Hi-Media, Mumbai). Results were interpreted as percent sensitive (%S) and percent resistant (%R) using WHO break points. Isolates that were resistant to multiple antimicrobial agents were selected for the study.

2.3 Detection of virulence genes using PCR: PCR analysis for the detection of the five virulence genes, viz. *estI*, *eltI*, *stx1*, *stx2* and *hlyA* was carried out as per method described by Osek *et al*[12] and Khan *et al*[13].

The template DNA was prepared by the boiling method and the procedure followed for the extraction of all the five genes were similar (Rahman, 2002). A loopful of pure culture of each isolate was incubated overnight in 5ml of TSB broth at 37^0C. About 0.5ml of the broth culture was taken in a microcentrifuge tube and the bacterial cells were separated by centrifugation at 10,000 rpm for 5 min in a refrigerated centrifuge (Eppendorf) and washed twice with phosphate buffer saline (PBS, pH 7.4). The cells were finally suspended in 300µl of HPLC grade water in a microcentrifuge tube, gently vortexed and boiled for 10 min. After boiling, the cell suspensions were cooled in an ice bath and immediately tested for the presence of the *stx*2, *elt* and *est* genes by PCR amplification using their specific primers.

The detection of *stx*2 gene by PCR was carried out as per the method described by Rahman (2002), while the *elt* and *est* genes detection were carried out as per the method described by Osek (1999). The amplification reaction was carried out in a final volume of 25µl containing 12.5µl Master mix (Bangalore GeNei, India) which contains 2.5µl Taq DNA polymerase, 200µM each of dATP, dCTP, dTTP and dGTP and PCR buffer, 5µl (1µM) each of forward and reverse primers and 2.5µl of template DNA. The PCR incubation was carried out in a thermocycler (Eppendorf) in 30 cycles.

The PCR product was visualized in a horizontal submarine agarose gel electrophoresis as described by Sambrock *et al*. (1989). A 1% agarose gel containing 0.5µl/ml ethidium bromide in TAE buffer (1X) was used. About 5µl of PCR product was mixed with 2µl of gel loading dye (0.5% bromophenol blue) was loaded into the wells of the gel. A standard DNA marker 100bp DNA ladder (Bangalore GeNei, India) was also loaded into the wells with the gel loading dye. Electrophoresis was carried out at 70V for 60 min till the bromophenol blue of the gel loading buffer migrated more than $4/5^{th}$ the length of the gel. At the end of electrophoresis, the gel was stained with ethidium bromide (0.5µl/ml) for 15 min and was visualized

in the gel documentation system (Vilber Lourmat) and photographed.

3. Results & Discussion

A total of 46 isolates of *E. coli* were isolated from 59 samples (77.97%). The isolation percentage was within the generally reported isolation percentage limits of 50-90%[2]. Of the 46 isolates, 36 were typable and belonged to 18 different serotypes. The frequency of serotypes isolated in descending order was O25 (6), O1 (5), O6, O8, O171 (3 each), O11, O20, O41 (2 each), O5, O62, O86, O111, O117, O130, O131, O140, O141, O153 (1 each). Six isolates were found refractory to typing and 4 were rough. All the serotypes isolated in this study and many more have been isolated by different workers from cases of UTI across India[5,14].

The isolates showed considerable degree of variation in their sensitivity towards these antimicrobial agents. Out of the 46 isolates of *E. coli* tested 4 isolates (8.64%) were found to be susceptible to all the 24 antimicrobial agents. Rest of the 42 isolates (91.36%) showed varine degree resistance to the antimicrobial agents. These 42 multidrug resistant isolates were used in this study.

Antibiogram of the isoaltes against 24 different antimicrobial agents showed that Imipenem (94.33%) was the most effective antimicrobial agent followed by nitrofurantoin(80.33%), pipercillin tazobactum (75.31%), amikacin (74.33%), levofloxacin, ofloxacin and ciprofloxacin (68.65% each), netilmycin (64.45%), norfloxacin (62.25%), amoxicillin/clavulanic acid (61.24%), nalidixic acid (59%), cefepime (54.57%), gentamicin (53.83%). Less than 50% of strains showed sensitive to tobramycin, cefazoline, chloramphenicol, ticarcillin/clavulanic acid, ceftazidime, ampicillin, ticarcillin, tetracycline, cefuroxime, co-trimoxazole and pipercillin (49.63%, 45.67%, 43.46%, 39.26%, 37%, 36%, 31.36%, 30.62%, 28.65%, 24% and 23%) . Antibiotic resistant pattern revealed that isolates were highly resistant to Pipercillin (77%) followed by Co-trimoxazole (76%), Cefuroxime (71.33%), Tetracycline (69.33%), Ticarcillin (68.67%), Ampicillin (64%), Ceftazidime (63%), Ticarcillin/Clavulanic Acid (60.67%), Chloramphenicol (56.67%), Cefepime, norfloxacin, amoxyclav and ciprofloxacin were also found less effective against the isolates . Similar results were reported by Zakaria[15] for sensitivity and resistance pattern. Steady increase in ciprofloxacin resistant strains has been observed in various parts of the world[16-18]. 5 isolates of *E. coli* showed resistant to 13 antimicrobial agents followed by 6, 3, 2 and 26 to 10, 9, 6 and 4 antimicrobial agents. The wide spread and most often use of antimicrobial drugs, inappropriate prescribing of antibiotics and poor infection control strategies have led to a general rise in the emergence of resistant bacteria particularly to ciprofloxacin[19]. Use of quinolones as a routine should be discouraged and their use restricted only to grave situations associated with multiple drug resistant strains after their proper antibacterial sensitivity data are available. It must be emphasized that changes evolving in increasing drug resistance in bacterial pathogens has become a true hinderance to successful therapy of UTI and require important reassessment of local empirical choices for managing UTI. Among the other genes tested, *hly*A and *stx*2 genes were detected in one isolate each belonging to serotype O41 and O5, respectively. Among *E. coli* strains causing UTI, the production of hemolysin is often associated with other factors assumed to contribute to virulence. Although *E. coli* isolates from UTI cases positive for *hly*A gene has been reported by many other studies[20-22] but very few reports suggest the incrimination of Shiga toxin (Stx) producing *E. coli* in cases of UTI. Rathore *et al*[6] reported that 44.4% of *E. coli* isolated from UTI cases belonging to 4 different serotypes (O9, O5, 100, O172) could produce Shiga toxins (Stx). Tarr *et al*[7] described a case with nondiarrheal HUS and acute pyelonephritis caused by STEC O103. Starr *et al*[8] reported a case of UTI with an O5 STEC positive for *stx*1, *stx*2 and *hly* genes detected by DNA hybridization was detected as the causative organism. *E. coli* serotype O91 positive for *stx*2 gene was found responsible for UTI infection followed by HUS[9]. Hacker *et al*[23] attributed the emergence of uropathogenic STEC in one of two ways: from an inherently uropathogenic strain of *E. coli*, with the ability to colonize and replicate in the urinary tract, that became lysogenized with one or more Stx-encoding or from an EHEC strain that acquired elements, such as P-pili, that would enhance its ability to colonize the urinary tract was probably of fecal origin and not primarily a uropathogenic.

The association of serotypes and enterotoxin is well established but their ability to cause UTI is not well defined. Given that enterotoxigenic genes have been detected in *E. coli* from UTI cases as this study suggests, it is highly likely that the *E. coli* strains recovered from patient's urine originated in their gastrointestinal tract. Assuming that *E. coli* strains causing UTIs are not a distinctly different pathotype, widely called the uropathogenic *E. coli*, but any other enteropathogenic pathotypes (ETEC, STEC, EHEC) can cause UTI by evolving mechanism to invade and colonize the urinary tract and cause infections after reaching the urinary tract from the gastrointestinal system. Future research effort should emphasize in studies to trace the natural history and the evolutionary pattern of the organism, its mechanism of transmission, the impact of enteropathogenic *E. coli* strains to the urinary tract and evolving changes in drug resistance, should they become frequent pathogens of the urinary tract in near future as these strains are not the natural inhabitants of the urinary tract.

4.References

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