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Detection & characterization of necrotoxin producing *Escherichia coli* (NTEC) from patients with urinary tract infection (UTI)

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***Background & objectives*: Urinary tract infections (UTI) are a serious health problem affecting millions of people each year. Although appreciable work on various aspects of UTI including aetiology *per se* has been done, information on the emerging pathogens like necrotoxigenic *Escherichia coli* (NTEC) is largely lacking in India. In the present study *E. coli* isolates from patients with urinary tract infection from northeastern India were investigated for detection and characterization of NTEC.**

***Methods*: *E. coli* isolated and identified from urine samples of patients with UTI were serotyped. Antibiogram was determined by disc diffusion test. Plasmid profile was also determined. Virulence genes of NTEC (*cnf1, cnf2, pap, aer, sfa, hly, afa*) were detected by PCR assay. *E.coli* isolates carrying *cnf* gene (s) were identified as NTEC.**

***Results*: A total of 550 *E. coli* were isolated and tested for the presence of *cnf* genes. Of these, 84 (15.27%) belonged to NTEC. The *cnf*1 gene was present in 52 (61.9%) isolates, *cnf*2 in 23 (27.4%) and 9 (10.7%) carried both *cnf*1 and *cnf*2 genes. All the NTEC strains were found to harbour the *pap* and *aer* genes. Serogroup O4 was found to be the most common among the 12 serogroups identified amongst the NTEC isolates. Majority of the isolates (96.4%) were sensitive to furazolidone and were highly resistant to ampicillin. NTEC were found to harbour different numbers of plasmids (1 to 7). No association was observed between the number of plasmids and the antibiotic resistance of the isolates.**

***Interpretation & conclusions*: The results of the present study showed that about 15 per cent of *E. coli* isolates associated with UTI belonged to NTEC. More studies need to be done from other parts of the country.**

Key words Antibiogram - *Escherichia coli* - NTEC - plasmid profile - serotyping - UTI - virulence genes

Escherichia coli is a component of the normal gut flora in warm blooded animals, human beings and birds. However, some strains are pathogenic and cause gastrointestinal illness and extra-intestinal infections like urinary tract infection. The pathogenecity depends on the expression of an array of virulence factors produced by *E. coli.* Toxigenic strains of *E. coli* are primarily of three types - Enterotoxigenic *E. coli* (ETEC), Shiga toxigenic *E. coli* (STEC) and necrotoxigenic *E. coli* (NTEC). Two different types of NTEC have been reported: NTEC1 and NTEC2 depending on the toxin they produce[1,2]. Cytotoxic

necrotizing factor 1 (CNF1) is produced by NTEC1 and cytotoxic necrotizing factor 2 (CNF2) is produced by NTEC2. CNF1 is chromosomally encoded, whereas CNF2 is coded by genes located on the Vir plasmid[1,3].

NTEC strains were reported for the first time in neonatal enteritis[4]. Along with producing CNF1 and CNF2 these strains may produce other toxins like cytolethal distending toxin (CDT), haemolysin (*hly*), P fimbriae (*pap*), afimbrial adhesins (*afa*), S fimbriae (*sfa*) and others[5]. These CNF toxins cause enlargement and multinucleation of cultured Vero and HeLa cells and necrosis in rabbit skin. CNF2 also induces necrosis in mouse footpad and moderate fluid accumulation in rabbit illeal loops[6]. Their role in severe dysenteric syndromes, both in man and animals, is substantiated by several clinical reports[7,8]. The combined production of several powerful toxins (haemolysin, CNF, CDT) by NTEC strains makes them potentially aggressive pathogens. Moreover, NTEC1 markers from man and animals appear to be highly related according to available molecular markers, which indicate that domestic animals could constitute important reservoirs of NTEC strains which are pathogenic for humans[2].

UTI is the most common infection in patients with a chronic indwelling bladder catheter; bacteriuria is essentially unavoidable in this patient group[9]. *E. coli* are the most common cause of community-acquired urinary tract infection (UTI) and are responsible for 70-90 per cent of the estimated 150 million UTIs diagnosed annually[10]. The prevalence of CNF producing gene (*cnf*) in *E. coli* associated with UTI has been reported widely[11].

Although several studies have been conducted on *E. coli* strains isolated from UTI patients in India[12-14], but no comprehensive study has been done on the association of CNF-producing *E. coli* strains with human UTI diseases in India. Therefore, such an association was investigated in the study by searching for *cnf* genes and other associated genes among 550 *E. coli* isolates from patients with urinary tract infections by PCR, with further characterization by serogrouping, plasmid profiling and multiple drug resistance patterns.

Material & Methods

Collection of samples and isolation of E. coli: This study was conducted in the department of Biotechnology, Gauhati University, Guwahati, Assam, India. Urine samples were randomly collected aseptically from patients identified with urinary tract infection from various hospitals in Guwahati, Assam (Down Town Hospital, Gauhati Medical College Hospital and Dispur Hospital) from female patients coming from different parts of northeast India. The patients were in the age group of 18-50 yr of age. The samples were collected from January, 2009 to December, 2010. The study was approved by the Institutional Ethics Committee. A total of 573 urine samples were collected from patients with UTI and 100 from apparently healthy individuals showing no symptoms of UTI were collected as controls. The control samples were collected from healthy female attendants in the age group of 18-50 yr who were contacted when they accompanied the patients to the hospitals. Their number was restricted to 100 because most healthy individuals were reluctant to provide samples. *E. coli* was isolated from the samples as per the standard technique[15]. Colony count of *E. coli* was done as per the standard technique[15]. The isolates were identified on the basis of standard morphological and biochemical tests[16].

Preparation of E. coli DNA for PCR assay: For rapid detection of virulence genes, isolated bacterial cultures were inoculated into 2 ml Luria Bartani (L-B) broth and incubated at 37°C under constant shaking for 24 h. After incubation, 1 ml broth culture was taken in a 1.5 ml microcentrifuge tube and centrifuged at 11,200 *g* for 10 min. The pellet was washed twice in sterile normal saline solution (NSS) (0.85% NaCl) and resuspended in 400 µl of nuclease-free sterile distilled water and boiled for 10 min followed by immediate chilling. Cell debris was removed by centrifugation at 2800 *g* for 5 min. The supernatant was used as template DNA for PCR.

Detection of NTEC: A multiplex PCR was carried out using two sets of oligonucleotide primers for *cnf*1 and *cnf*2[6,17]. The PCR mixture of 25.0 µl contained 1X PCR buffer, 1.5 mM of $MgCl_2$, each primer within the 2 primer sets at a concentration of 40 nM, 200 µM each of dNTPs, 1.0 U of *Taq* DNA polymerase and 2.0 µl of template DNA. Primers and amplification conditions for the pathogenic gene coding regions used are shown in Table I. Amplified products were separated by agarose gel (2% agarose in 1X Tris-borate-EDTA buffer) electrophoresis at 5v/cm for 2 h and stained with ethidium bromide (0.5 µg/ml). Standard molecular size marker (100 bp DNA ladder) was included in each gel. DNA fragments were observed by ultraviolet transilluminator and photographed in a gel documentation system (Alpha Imager, Germany). The PCR was performed three times to ensure the

Table I. Primers and cycling conditions used in the amplification of specific gene responsible for toxin production

Target gene	Sequence	Product size (bp)	Cycling conditions	Reference
*cnf*1	F:5'-AAGATGGAGTTTCCTATGCAGGAG-3' R:5'-CATTCAGAGTCCTGCCCTCATTATT-3'	498	1 cycle of 95°C for 30 sec; 30 cycles of 95° C for 30 sec, 60 °C for 30 sec, 72°C for 30 sec; 72° C for 7 min final extension.	Falbo *et al*[6]
*cnf*2	F:5'GTGAGGCTCAACGAGATTATGCACTG-3' R:5'- CCACGCTTCTTCTTCAGTTGTTCCTC-3'	839	1 cycle of 95° C for 30 sec; 30 cycles of 95° C for 30 sec, 65° C for 30 sec, 72° C for 30 sec; 72° C for 10 min final extension.	Pass *et al*[17]
pap	F: 5'-GCAACAGCAACGCTGGTTGCATCAT-3' R:5'-AGAGAGAGCCACTCTTATACGGACA-3'	336	1 cycle of 95° C for 30 sec; 35 cycles of 95° C for 30 sec, 55° C for 30sec, 72° C for 30 sec; 72° C for 10 min final extension.	Yamamoto *et al*[18]
aer	F:5'-TACCGGATTGTCATATGCAGACCGT-3' R:5'-AATATCTTCCTCCAGTCCGGAGAAG-3'	602	1 cycle of 95° C for 30 sec; 35 cycles of 95° C for 30 sec, 55° C for 30 sec, 72° C for 30 sec; 72° C for 10 min final extension.	Herrero *et al*[19]
*afa*I	F:5'-GCTGGGCAGCAAACTGATAACTCTC-3' R:5'-CATCAAGCTGTTTGTTCGTCCGCCG-3'	750	1 cycle of 95° C for 30 sec; 35 cycles of 95° C for 30 sec, 58° C for 30 sec, 72° C for 30 sec; 72° C for 10 min final extension.	Le Bouguenec *et al*[20]
sfa	F:5'-CTCCGGAGAACTGGGTGCATCTTAC-3' R:5'-CGGAGGAGTAATTACAAACCTGGCA-3'	410	1 cycle of 95° C for 30 sec; 35 cycles of 95° C for 30 sec, 55° C for 15 sec, 72° C for 30 sec; 72° C for 10 min final extension.	Le Bouguenec *et al*[20]
hly	F:5'-AACAAGGATAAGCACTGTTCTGGCT-3' R:5'-ACCATATAAGCGGTCATTCCCGTCA-3'	1177	1 cycle of 95°C for 30 sec; 35 cycles of 95° C for 30 sec, 63° C for 30 sec, 72° C for 30 sec; 72° C for 10 min final extension.	Felmlee *et al*[21]

repeatability of the technique and to make sure that isolates were correctly assigned to respective patterns. The *E. coli* isolates that harboured either *cnf*1 and / or *cnf*2 gene were identified as NTEC and further studied by serotyping, antibiogram, presence of other virulence factors related to NTEC and plasmid profiling.

Serotyping of NTEC: *E. coli* isolates were serotyped based on their somatic (O) antigens at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India.

Antibiotic resistance patterns: NTEC isolates were tested for their susceptibility to antimicrobial drugs, by disc diffusion test[16] by using commercially available biodiscs (HiMedia, Mumbai, India) which included ampillicin (10 µg), gentamicin (10 µg), streptomycin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg), furazolidone (50 µg), neomycin (30 µg), kanamycin (30 µg) and nitrofurantoin (300 µg).

Detection of virulence genes by PCR and RAPD analysis: PCR was carried out using five sets of oligonucleotide primers for *pap*, *aer, afa, sfa* and *hly* genes for the NTEC isolates[18-21]. The PCR mixture of 25.0 µl contained 1X PCR buffer, 1.5 mM of $MgCl_2$, 40 nM of the specific primer pairs, 200 µM each of dNTPs, 1.0 U of *Taq* DNA polymerase and 2.0 µl of template DNA. Primers and amplification conditions for the virulence genes coding regions used are shown in Table I. Amplified products were separated by agarose gel (2% agarose in 1X Tris-borate-EDTA buffer) and photographed.

Plasmid profiling of NTEC isolates: Plasmid DNA was extracted and profiled by alkaline lysis method as described by Sambrook *et al*[22].

Statistical analysis: Statistical analysis was performed using SPSS software for Windows, ver.15 (SPSS, IBM, USA). Chi-square was used to evaluate the variables correlation.

Results & Discussion

Of the 573 urine samples collected from patients suffering from UTI, 550 (95.98%) were positive for *E. coli* and 23 (4.02%) were found negative for *E. coli.* Colony count of *E.coli* equaling to $>10^5$ per ml of urine sample was taken to be positive. No significant variation was found on the basis of age of the patients or the different hospitals from which the samples were obtained for the recovery of *E. coli.* No *E. coli* could be isolated from the urine samples of the apparently

healthy individuals. Of the 550 *E. coli* isolates, 84 (16.8%) carried at least one or other *cnf* genes. This percentage was less than what was reported by Landraud *et al*[11], who found 34 per cent of the isolates from UTI producing CNF. Of the 84 NTEC isolates, 52 (61.9%) harboured *cnf*1 gene (Fig. 1), 23 (27.4%) harboured *cnf*2 (Fig. 2) and nine (10.7%) carried both *cnf*1 and *cnf*2 genes. Our results showed the occurrence

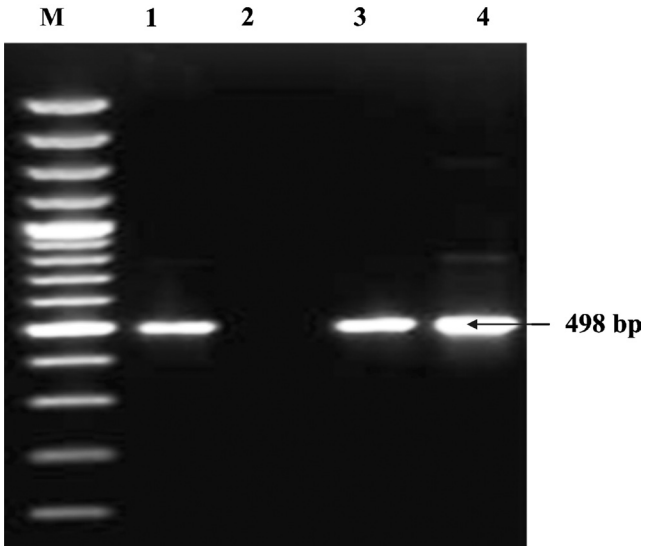

Fig. 1. Detection of *cnf*1 gene by PCR. Lane M: DNA ladder (100 bp); Lane 1: positive control (498 bp); Lane 2: Negative control; Lanes 3 & 4: Positive isolates.

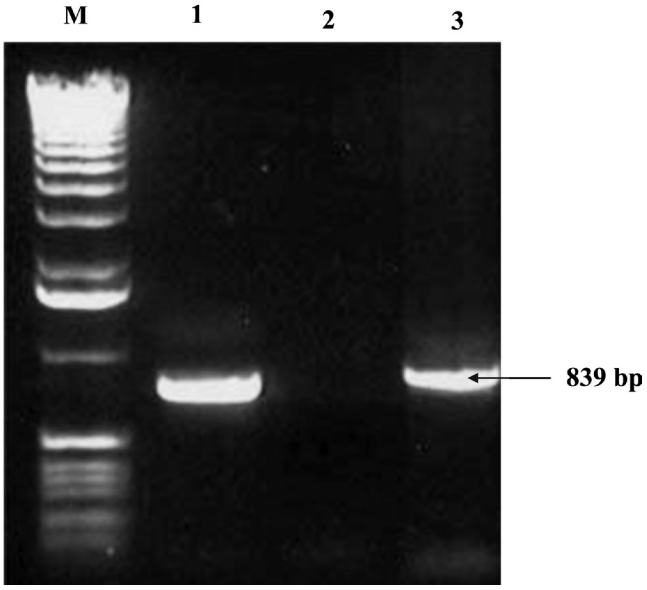

Fig. 2. Detection of *cnf*2 gene by PCR. Lane M: DNA ladder (1 kb); Lane 1: positive control (839 bp); Lane 2: Negative control; Lane 3: Positive isolates.

of *E. coli* isolates in human (95.98%) UTI cases which differed from an earlier study where *E. coli* was found in 50 to 90 per cent of UTI cases[23]. Such differences in occurrence rate may be attributed to various factors *viz*. hygienic conditions, geographical and environmental conditions.

All the 84 NTEC isolates were found to harbour the *pap* and *aer* genes. The *sfa* gene was found in 38 (45.2%) of the NTEC isolates while 11 (13.1%) carried *afa* and 32 (39.1%) carried the *hly* gene, respectively. Thirty one (59.61%) of the 52 NTEC1 isolates were found to harbour the *hly* gene whereas this gene was present in only one (3.8%) of the NTEC2 isolates. However, the presence of different combination of genes varied among different isolates of *E. coli* (Table II). The presence of these associated genes in NTEC agrees with other reports[3,5]. The combination of CNF1, α-*hly* and P-fimbriae genes has been demonstrated in the human UTI strain, J96, possibly as a pathogenicity island[24].

The NTEC isolates in our study belonged to 12 serogroups, namely O2 (8), O4 (16), O6 (7), O8 (1), O12 (14), O18 (13), O29 (11), O35 (1), O78 (8), O83 (1), O88 (3) and O123 (1). The serogroup O4 was found to be the most prominent serogroup. All the isolated serogroups, in the present study have been reported elsewhere as being among *E. coli* strains that produce CNF[1,3]. There was no association between the serotype of the isolates and the presence of virulence genes in the isolates.

The NTEC isolates were found to have different degrees of resistance towards various antimicrobial agents (Table III). All the isolates were resistant to one or more antimicrobial drugs. None of the 84

Table II. Distribution of virulence genes among necrotoxigenic *E. coli* (NTEC) isolates (n=84) from patients with UTI

Distribution of genes	No. of isolates (%)
sfa alone	38 (45.24)
afa alone	11 (13.1)
hly alone	32 (39.1)
sfa+afa	6 (7.14)
sfa+hly	13 (17.47)
afa+hly	4 (4.76)
sfa+afa+hly	2 (2.38)

Table III. Antibiotic resistance patterns among necrotoxigenic *E. coli* (NTEC) isolated from patients with UTI

Resistance to	% of isolates (n=84)
Amp	96.4
Amp+Gen	84.52
Amp+Gen+Str	73.81
Amp+Gen+Str+Cip	45.24
Amp+Gen+Str+Cip+Kan	23.81
Amp+Gen+Str+Cip+Kan+Tet	16.67
Amp+Gen+Str+Cip+Kan+Tet +Neo	3.57
Amp+Gen+Str+Cip+Kan+Tet+Neo+Nit	1.19
Amp+Gen+Str+Cip+Kan+Tet+Neo+Nit+Fur	Nil

Amp, ampillicin; Gen, gentamicin; Str, streptomycin; Tet, tetracycline; Cip, ciprofloxacin; Fur, furazolidone; Neo, neomycin; Kan, kanamycin; Nit, nitrofurontoin

isolates were found to be resistant to all the 9 agents tested. The isolates were highly resistant to ampicillin (96.4%) followed by gentamicin (86.9%), streptomycin (78.6%), ciprofloxacin (67.9%). The highest degree of sensitivity (97.6%) was shown by the isolates towards furazolidone. The isolates were less resistant to nitrofurantion (17.8 %), neomycin (19.0%), kanamycin (26.1%) and tetracycline (38.1%). Antibiotic resistance pattern of *E. coli* isolated from different sources has been reported by several workers[25,26]. There was no association between the serotype of the isolates and the multidrug resistance of the isolates.

All the NTEC isolates were found to harbour different numbers of plasmids. The number of plasmids varied from 1 to 7. The highest number of isolates, 43 (51.2%) harboured two plasmids each. However, no correlation was observed between the number of plasmids and the antibiotic resistance of the isolates.

In conclusion, the results of the present study revealed that a good proportion of *E. coli* associated with UTI belonged to NTEC. It warrants a systematic study on this important public health problem.

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