Detection of cnf1 gene for the presence of cytotoxic necrotizing factor among clinical isolates of Escherichia coli

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***Abstract*: Escherichia coli (E.coli) is a gram-negative, facultative aneaerobic rod-shaped bacteria that is a commensal of the human gut. They are beneficial to the host by producing Vitamin K (k_6) and prevent pathogenic bacteria from colonizing. Uropathogenic strains of E.coli is also a commensal of the gut. Toxigenic strains of *E. coli* are of three types such as Enterotoxigenic *E. coli* (ETEC), Shiga toxigenic *E. coli* (STEC) and necrotoxigenic *E. coli* (NTEC). Two different types of NTEC have been reported they are NTEC1 and NTEC2 depending on the toxin they produce [1] 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. A sum of 20 urinary isolates of E. coli were subjected to antibiotic susceptibility testing and detected for cnf1 gene by PCR. We found increased percentage of isolates were shown to be resistant to different antibiotics and 70% of them were resistant to imipenem drug. 60% of them were harboring cnf1 gene by PCR. This indicates that most of the strains of Uropathogenic E. coli were found to harbor cnf1 gene, is associated with the implication of UTI in humans. Certain strains of E.coli are known to produce amyloid material which are similar to those seen in Alzhiemer's disease [14]. The versatility and the ease of handling this organism makes it easier to do extensive studies. Futher studies need to be conducted to gain better understanding about its ecology and natural history.**

***Keywords*: E. coli, cnf1 gene, PCR, cytotoxic necrotizing factor, Alzhiemer's disease**

Introduction:

Escherichia coli is one of the normal gut flora in human beings.) is a gram-negative, facultative aneaerobic rod-shaped bacteria that is a commensal of the human gut. They are beneficial to the host by producing Vitamin K (k6) and prevent pathogenic bacteria from colonizing. However, some strains are pathogenic and cause gastrointestinal illness and extra-intestinal infections like urinary tract infection. Some strains are also known to cause cancer [17, 18, 20]. The pathogenecity depends on the expression of an array of virulence factors produced by *E. coli*. They contains a various virulence factors such as fimbria or pilli which helps them adhere to the vaginal and uroepithelial cells making them resistant to bactericidal activity of human serum. [15,16]. Toxigenic strains of *E. coli* are of three types such as Enterotoxigenic *E. coli* (ETEC), Shiga toxigenic *E. coli* (STEC) and necrotoxigenic *E. coli* (NTEC). Depending on the toxin they produce two different types of NTEC have been reported they are NTEC1 and NTEC2 [1], 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 [2]. CNF1is a cytoplasmic protein and has the ability to cross blood-brain barrier and penetrate into the brain leading to meningitis [19].

NTEC strains were first reported in neonatal enteritis [3]. Along with CNF1 and CNF2 production these strains may also produce other toxins like cytolethal distending toxin (CDT), haemolysin (*hly*), *P* fimbriae (*pap*), afimbrial adhesins (*afa*), S fimbriae (*sfa*) and others [4]. 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 [5]. The combined production of several toxins (haemolysin, CNF, CDT) by NTEC strains making them potentially aggressive pathogens. Moreover, NTEC1 markers from man and animals found to be highly related according to available molecular markers, which indicate that domestic animals could constitute important reservoirs of NTEC strains which become pathogenic for humans [6]. Certain strains of E.coli are found to produce amyloid material (curli) similar to those found in Alzheimer's disease [14]. In an in-vitro study conducted on transgenic mice homozygous for human ApoE4 gene, it was found that CNF1 decreased th levels of beta amyloid accumulation thereby increasing the memory performances in Alzhiemer's disease [21]. A study conducted by Viviana G et al, on rats, concluded that retinal disease such as retinitis pigmentosa can be treated using CNF1 [22].

UTI is the most common infection in patients with a chronic indwelling catheter; bacteriuria is unavoidable in this patient group [7]. *E. coli* are the most common cause of community-acquired urinary tract infection (UTI) and are responsible for 70-90 % of the 150 million UTIs diagnosed cases annually [8]. The prevalence of CNF producing gene (*cnf*) in *E. coli* associated with UTI has been reported widely [9]. Based on this information, we have undertaken this study to detect the cnf1 gene among our clinical isolates of E. coli.

Materials and methods:

Bacterial isolates:

A total of 20 non repetitive urinary isolates of Escherichia coli were collected from Saveetha Medical College and Hospitals, Chennai. They were processed for a battery of standard biochemical tests and confirmed. Isolates were preserved in semisolid trypticase soy broth stock and were stored at 4 ºC until further use.

Antibiotic susceptibility testing:

Antibiotic susceptibility test was determined for these isolates to routinely used antibiotics such as ampicillin, amoxicillin, amikacin, norfloxacin, ceftazimide, cefotaxime, ciprofloxacin and gentamicin, imipenem as by Kirby Bauer disc diffusion method [10].

Detection of cnf1 gene in E.coli:

Escherichia coli isolates were detected for the presence of cnf1gene by PCR analysis. Detection of the gene was carried out using primer as depicted in table 1. Bacterial DNA was extracted by boiling lysis method. 1 µL of DNA extract was used as template for PCR reaction. The reaction mixture contained 1mM of $Mgcl_2$0.2mM dNTP mix and 0.8µM of cnf1 gene with 0.5U of Taq polymerase (New England Biolabs) in a 1x PCR buffered reaction. A positive control of E.coli with cnf1gene was also included in this study. PCR amplification was carried out using thermal cycler (Eppendorf) with the following cycling condition. Initial denaturation at 97ºC for 5 min and 35 cycles for 30s, 69ºC for 30s and 68º C for 60s, followed by a final extension of 5 min at 75ºC. PCR products were resolved in 1.5% agarose gel. A 100bp ladder was including in all the gel analysis [11].

Table 1: Gene sequencing of cnf1 gene

Primer	Primer sequence	Product size
cnf1	AAGATGGAG TTT CCT ATGCAGGAG CAT TCA GAG TCC TGC CCT CAT TAT T	501bp

Results:

Sample wise distribution of clinical isolates of E.coli:

Of the 20 clinical isolates of E.coli, 12/20 (60%) were from acute urinary tract infections and 8/20 (40%) were from chronic urinary tract infections. Figure 1 depicts the sample wise distribution of clinical isolates of E.coli.

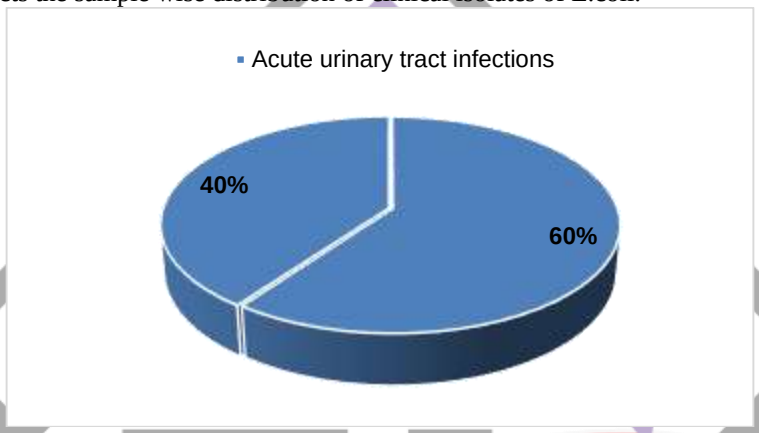


Figure 1: Sample wise distribution of urinary isolates of *E.coli*

Antibiotic susceptibility testing:

In our isolates, we have found increased percentage 14/20 (70%) of isolates showed sensitivity to amikacin followed by gentamicin, which showed sensitivity of 9/20 (45%). 80- 90% of E.coli isolates showed resistance to cephalosporin group of drugs. 6/20 (30%) were found to be resistant to imipenem. However, we have observed an elevated level of resistance to other routinely used antibiotics. The detailed resistant pattern of E.coli isolates is shown in table 2. Several studies have shown a rise in the resistance to ciprofloxacin, amoxicillin/clavulanic acid [23], ampicillin, sulfonamide, trimethoprim and gentamicin [24]. There has been an increase in the emergence of multidrug resistant bacteria which poses a major threat in treating disease. [25,26].

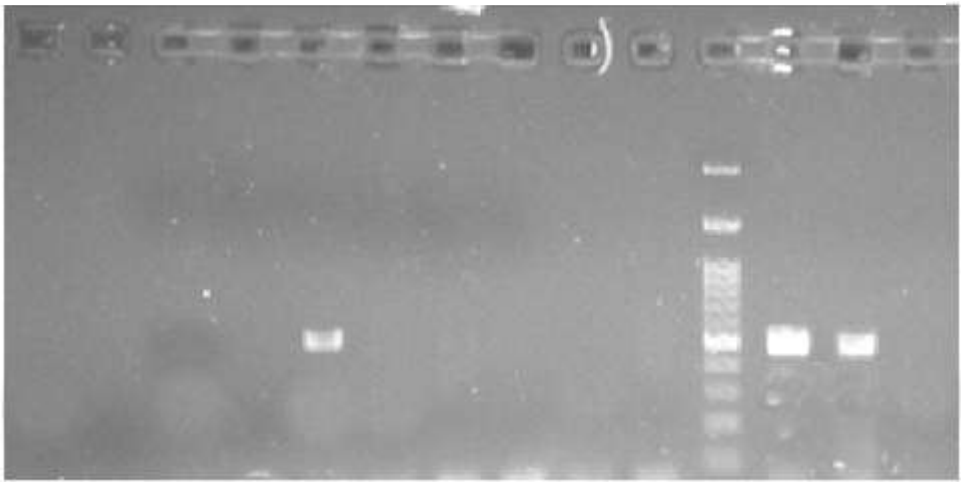
Antibiotics	Sensitivity(20) (%)	Intermediate (20) (%)	Resistant(20) (%)
Ampicillin	5	0	95
Amoxicillin	5	0	95
Ceftazidime	10	10	80
Cefotaxime	5	5	90
Amikacin	70	10	20
Gentamicin	45	20	35
Norfloxacin	15	15	70
Ciprofloxacin	20	5	75
Imipenem	70	0	30

Table 2: Showing antibiotic sensitivity pattern of E.coli

Result of cnf1gene in E.coli:
12/20 (60%) clinical isolate of urinary isolates of E.coli was found to harbour cnf1 gene.

Figure 2: Representative gel picture showing presence of cnf1 gene.

L1 L2 L3 L4 L5 L6 L7 L8 L9 L10 L11 L12 L13 L14



L11-100bp ladder; L5, L12. L13 –cnf1 gene positive

Discussion:
We have observed 12/20 (60%) of isolates were found to show positive for cnf1 gene. Similar kind of study done by Rahman in 2014 found that, of the 550 *E. coli* isolates, 84 (16.8%) carried at least one or other *cnf* genes [12]. This percentage was less than the study reported by Landraud *et al* [9], who found 34 % of the isolates from UTI producing CNF. Of the 84 NTEC isolates, 52 (61.9%) harboured *cnf1* gene, 23 (27.4%) harboured *cnf2* and nine (10.7%) carried both *cnf1* and *cnf2* genes. However, in our study did not use cnf2 gene. Rahman results showed the occurrence of *E. coli* isolates in human (95.98%) UTI cases which differed from an earlier study where *E. coli* was found in 50-90 % of UTI cases [13]. We have also found such kind of results in our study as we used only urinary isolates of E.coli from humans. Such differences in occurrence rate may be attributed to various factors *such as* hygienic conditions, geographical and environmental conditions.

Conclusion:
This indicates that most of the strains of Uropathogenic E. coli were found to harbor cnf1 gene, is associated with the implication of UTI in humans. Further study is needed to establish this fact with more number of isolates from different samples and from different populations. There has been extensive studies on CNF1 emphasisng its clinical application in treating various diseases such as Alzhiemer's disease and retinitis pigmentosa. Hence by understanding the pathogenesis of various diseases caused by e.coli, we can apply it in treating various other diseases by eliminating the virulence factor. Another aspect of this study shows the increase in the trend of multi drug resustant bacteria which poses a major threat in treating various disease. The versatility and the ease of handling this organism makes it easier to do extensive studies. Futher studies need to be conducted to gain better understanding about its ecology and natural history.

Acknowledgment:
We thank Dr. Kalyani, Professor and Head of the Department of Microbiology, Saveetha Medical College, Chennai for kindly providing the clinical isolates to carry out our research work fruitfully.

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