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ISOLATION, DETECTION AND CHARACTERIZATION OF NECROTOXIC *E. COLI* (CNF1 GENE) FROM PATIENTS WITH URINARY TRACT INFECTION

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ABSTRACT

Urinary tract infections (UTIs) are a serious health problem affecting millions of people every year. Although appreciable work on various aspects of UTI including etiology *per se* has been done, information on the emerging pathogens like necrotoxigenic *Escherichia coli* (NTEC) is largely lacking in India. In the present study total 83 urine samples were collected adult and children patients suffering from urinary tract infections from RTC Hospital, Tarnaka, Hyderabad. Among 83 samples of UTI characterized, males were 54.21%, females 33.73% and children 12.04%. Bacterial growth was observed and the percent infected were 14.45 % of males, 7.22% of female and no positive results of UTI for children. All urine samples were inoculated on selective medium CLED Medium (Cystine-Lactose-Electrolyte- Deficient) with bromo-thymol blue/Andrade indicator for selection and identification of *E. coli*. Nine *E. coli* strains based on colony morphology, biochemical test and Gram stain, nine were randomly picked up for further studies. Antibiotic susceptibility testing (AST) was carried out using 11 different antibiotics on Miller Hinton agar medium in plate culture conditions. All the nine varied in resistance and susceptibility patterns to different antibiotics tested. All the nine (967, 972, 975, 984, 994, 997, 1000, 1003, 1004) were also characterized for molecular identification of necrotoxigenic *E. coli*, cnf1 gene using PCR and two strains (972 and 984) were positive for the same.

KEYWORDS: Urinary tract infection, necrotoxigenic *E. coli*, CLED medium and Miller Hington agar medium.

1. INTRODUCTION

UTI is a common bacterial infection known to affect the different parts of the urinary tract and the occurrence is found in both males and females. Women are more vulnerable to UTI than men, because of their anatomy and reproductive system.[1] UTI is the growth and multiplication of microorganisms within the urinary tract that includes organs which collect, store, and void volume of urine from the body i.e. the kidney, bladder, urethra and ureter. The infection is usually caused as a consequence of bacterial invasion of the urinary tract including the lower and the upper urinary tract.[2] A variety of parameters are related to UTI which include age, parity, gravidity, pregnancy and association of diseases augment the condition of the infection. The diagnosis of UTI is differing based on symptoms.[3] Individually, symptoms are rarely diagnostic, but in combination their accuracy is greater. Signs and examination findings are generally unhelpful in uncomplicated cystitis, but have more value where upper UTI is possible.[4] Of all the bacterial species *E. coli* account to 80% to 85% of the infection followed by other bacteria (10-15%).[5] These organisms affect mainly external genitalia, vagina, the genital tract, rectum, and gastro-intestinal tract and associated the bladder and kidney infections.[6] Laboratory culture has historically been the benchmark for diagnosis of UTI, but studies have shown that women who respond to treatment may have bacterial counts below the traditional threshold for diagnosis.[7]

E. coli, the most prevalent facultative Gram-negative bacillus in the human fecal flora, usually inhabits the colon as an innocuous commensal. Strains of *E. coli* that cause disease outside of the gastrointestinal tract are referred to as extraintestinal pathogenic *E.coli* (ExPEC) and are divided into uropathogenic *E. coli* (UPEC) strains causing neonatal meningitis and septicemic *E. coli*. UPEC is the most common pathotype of ExPEC and is found in patients with UTIs.[8] Moreover the bacterial UTI is the common danger disease in most of the developed and developing countries.

Treatment of UTI varies with the type but is usually empirical because of the common spectrum of uropathogens. In complicated cases or if treatment has failed, a urine culture may be useful.[9] CLED medium is recommended for use in urinary bacteriology, promoting the growth of all urinary pathogens. Peptic digest of animal tissue, beef extract, casein enzymic

hydrolysate provide essential growth nutrients. The present study is aimed to isolate, detect and characterize of Necrotoxic *E. coli* (cnf1 gene) from patients with UTIs.[10]

2. MATERIALS AND METHODS

2.1. Collection of samples and isolation of bacteria

A total of 83 urine samples were collected (during March-May, 2015) from patients with UTI as recommended by the general practitioner (tab 1 & 2). The samples were streaked onto CLED agar medium with BTB indicator to see the bacterial population. Wherever, the growth was scanty, the sample was processed as such using streak plate technique to isolate different bacteria associated with patient samples of UTI. Later on, the colonies presumed to be *E. coli* based on morphological characteristics were further streaked onto CLED medium with Andrade's indicator.

2.2. Biochemical tests used to identify *E. coli*: Based on the growth of *E. coli* on the above two CLED medium, further certain specific biochemical tests were carried out to confirm the isolates as *E. coli*. The assays include TSI agar utilization, catalase, urease, oxidase and citrate utilization test.[11]

2.3. Antibiotic sensitivity assay: The Muller-Hinton agar media (contains (w/v) 30%- beef infusion, 1.75% casein hydrolysate, 0.15% starch, 1.7%- agar & pH-7 at 25^{o} C) incorporated along with 11 different commercially available antibiotics discs (CFP- cefoperazone, AK- amikacin, NT- netilmicin, NR- norfloxacin, CIP- ciprofloxacin, CFC- cefactor, CTX- ceftriaxone, G- gentamicin, NA- nalidixic acid, CD- cefadroxil, OX- olfoxacin) from Himedia were used. After 48 hours of incubation, where ever there was zone of inhibition by antibiotic, it was considered as the *E. coli* sensitive to the antibiotic used in the study. Where there was bacterial growth, in the vicinity of disc it was considered as resistant to the antibiotic used in this study (zone of inhibition were measured in mm).[12]

2.4. Genomic DNA isolation: Isolated bacterial cultures were inoculated into 2 ml LB broth and incubated at 37°C under continuous constant shaking for 24 h. After incubation, 1 ml of the broth culture was taken and centrifuged at 11,000 g for 10 min. The pellet was washed twice in sterile normal saline solution (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 genomic DNA prepared was used for detection of cnf1 gene by PCR.[13]

2.5. Detection of NTEC (cnf_1 gene)

CNF gene specific primers used in the present study were designed by primer 3 software.

cnf $_1$ (A) Forward primer TGGTTTGGCGACAAATGCAG

Reverse primer TACTTCCCCCAGCCGTATGA

cnf $_1$ (B) Forward primer TGTCGGAACACTGGAGATGC

Reverse primer GGTATTCCGACGGGAGCATT

A multiplex PCR was carried out using two sets of oligonucleotide primers for *cnf*1. The PCR mixture of 25.0 µl contained 1X PCR buffer, 1.5 mM of MgCl2, 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. 1 cycle of 95°C for 30 sec; 30 cycles of 95° C for 30 sec, 60 o C for 30 sec, 72°C for 30 sec; 72° C for 7 min final extension. 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 (0.5µg/ml). Standard molecular size marker (5kb DNA ladder) was included in each gel. DNA fragments were observed by UV-transilluminator and photographed in a gel documentation system.[14, 15]

3. RESULTS

3.1. Isolation of *E. coli* from UTI (adult and children) samples:

The urine samples were inoculated on selective medium for identification of *E. coli*. CLED Medium with BTB and the colonies that showed yellow color and yellow zone on plate were picked up for further study. Later on, where ever there is growth of bacteria, it was recorded as bacterial growth (BG) and no growth, it was recorded as no bacterial growth (NBG). Among 83 samples 54.21% of males, 33.73% of females and 12.04% of children patients were screened (Fig. 1). The bacterial growth of all samples 14.45 % of males, 7.22% of female were positive of urinary tract infection (Tab. 1). In this study, there were no positive results for children (Tab. 2).

Table 1: Growth of bacteria from UTI samples (adults) on CLED agar medium.

S. No.	Sample No.	AGE/SEX	Growth
1.	940	29/F	BG
2.	941	44/M	NBG
3.	942	26/M	NBG
4.	943	42/M	NBG
5.	944	21/M	BG
6.	945	46/M	NBG
7.	946	56/M	NBG

8.	947	24/F	NBG
9.	948	24/M	NBG
10.	949	55/F	NBG
11.	950	46/M	BG
12.	951	26/F	NBG
13.	952	48/F	NBG
14.	953	62/M	NBG
15.	954	61/F	NBG
16.	955	50/M	NBG
17.	956	58/M	NBG
18.	957	52/M	BG
19.	958	61/F	NBG
20.	959	24/F	NBG
21.	960	43/F	NBG
22.	961	34/M	NBG
23.	962	62/F	NBG
24.	963	29/F	NBG
25.	964	43/F	NBG
26.	965	34/F	NBG
27.	966	60/M	BG
28.	967	47/M	BG
29.	968	18/F	NBG
30.	969	50/M	NBG
31.	970	42/F	NBG
32.	971	24/F	BG
33.	972	23/F	BG
34.	973	32/M	NBG
35.	974	56/M	NBG
36.	975	48/M	BG
37.	976	50/F	NBG
38.	977	45/M	BG
39.	978	35/F	NBG
40.	979	45/M	NBG
41.	980	44/M	NBG
42.	981	38/M	NBG
43.	982	30/F	NBG
44.	983	28/M	NBG
45.	984	29/F	BG
46.	985	59/F	BG
47.	986	52/M	NBG
48.	987	42/M	NBG
49.	988	56/F	NBG
50.	989	48/M	NBG
51.	990	35/M	NBG
52.	991	39/M	NBG
53.	992	40/M	NBG
54.	993	31/M	NBG
55.	994	66/M	BG

56.	995	45/M	NBG
57.	996	51/F	NBG
58.	997	56/F	BG
59.	998	64/F	NBG
60.	999	56/M	NBG
61.	1000	62/M	BG
62.	1001	50/M	NBG
63.	1002	44/M	NBG
64.	1003	21/M	BG
65.	1004	24/M	BG
66.	1005	73/M	BG
67.	1006	50/M	NBG
68.	1007	65/M	NBG
69.	1008	34/M	NBG
70.	1009	52/M	NBG
71.	1010	50/F	NBG
72.	1011	45/F	NBG
73.	1012	42/M	NBG

Table 2: Growth of bacteria from UTI samples (children) on CLED agar medium.

S. No.	Sample number	Age/Sex	Bacterial growth
74	648	4/F	NBG
75	620	12/M	NBG
76	43	7/M	NBG
77	73	11/M	NBG
78	784	6/M	NBG
79	786	5/M	NBG
80	892	13/M	NBG
81	893	10/M	NBG
82	1034	3/F	NBG
83	1053	7/M	NBG

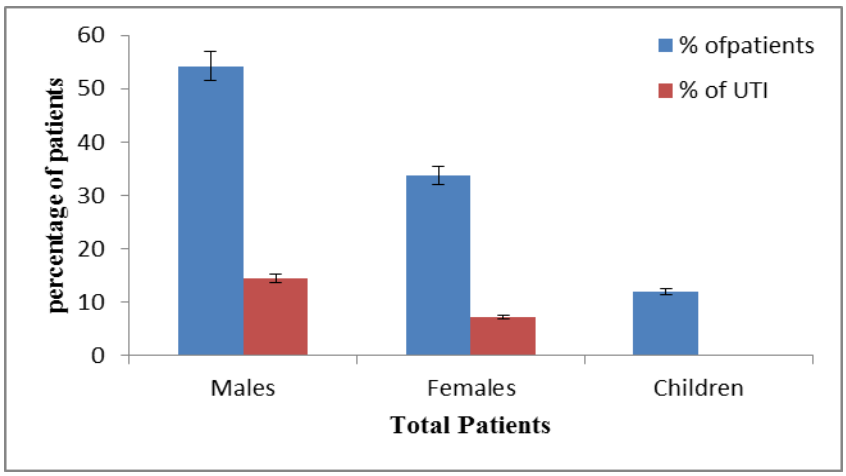

Figure 1: Percent of total patient samples and urinary tract infected samples.

Further all those yellow colonies with yellow zones were picked up considering it to be *E. coli* and plated onto CLED agar medium with Andrade's indicator.[16] The plates after incubation for 48 hours were observed for formation of pink color colonies as shown in figure 2.

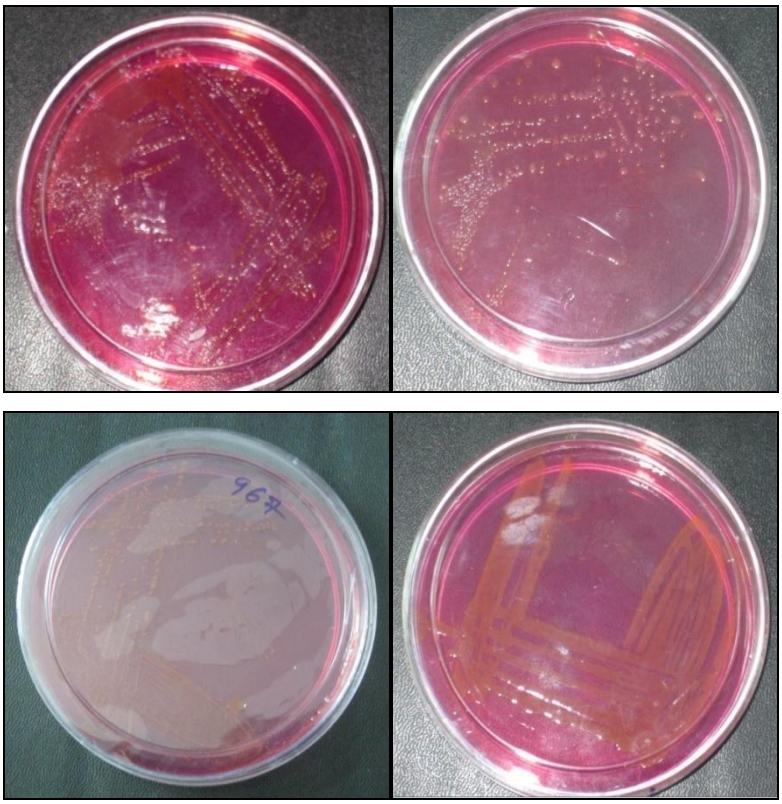

Figure 2: *E. coli* showing pink colored colonies on CLED with Andrade's indicator.

Pure culture of *E. coli* isolated from UTI: After screened of *E. coli* by using selective media from UTI patients 9 *E. coli* strains were randomly selected for further studies.

Gram staining of *E. coli*: By Gram stain procedure, all the nine colonies showed pink color indicating they are Gram negative (Fig. 3).[17]

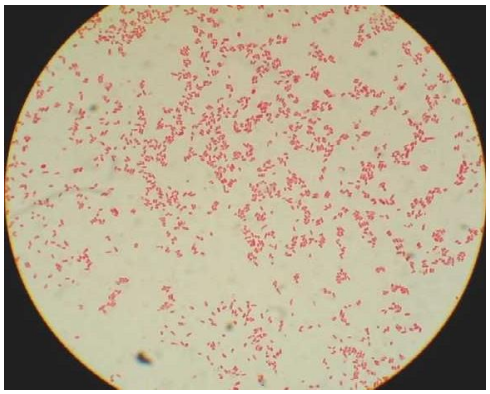

Figure 3: Gram staining of *E. coli.*

3.2. Biochemical identification of *E. coli*.

Biochemical tests revealed that the isolates were positive for Triple Sugar Iron (TSI), Indole test, catalase test (Fig. 4) and negative for Citrate utilization and Urease tests. Culture isolates were characterized by the morphological, biochemical characteristics as *E. col*i (Tab. 3).[18]

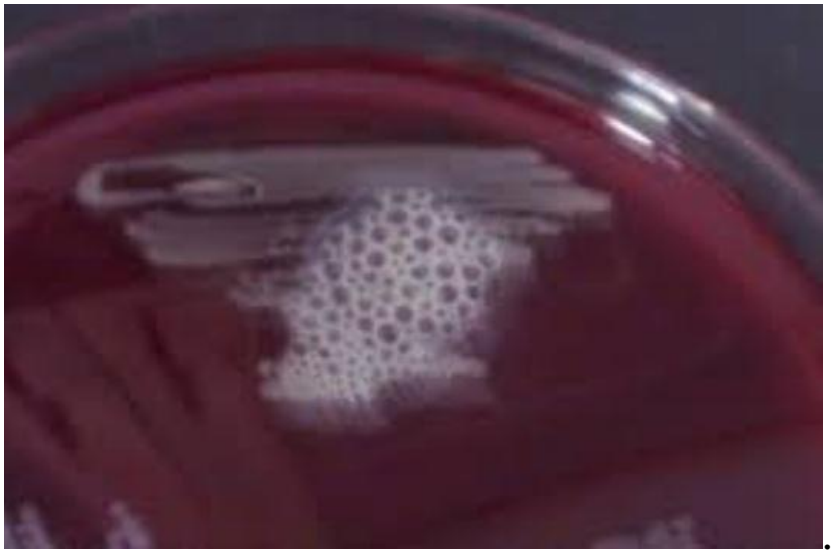

Figure 4: Gas and bubbles showing catalase positive

Table 3: Biochemical tests for the selective nine *E. coli* strains.

Isolate *E.coli*	TSI agar	Indole	Catalase	Citrate	Urease
967	+ ve	+ ve	+ ve	-ve	-ve
972	+ ve	+ ve	+ ve	-ve	-ve
975	+ ve	+ ve	+ ve	-ve	-ve
984	+ ve	+ ve	+ ve	-ve	-ve
994	+ ve	+ ve	+ ve	-ve	-ve
997	+ ve	+ ve	+ ve	-ve	-ve
1000	+ ve	+ ve	+ ve	-ve	-ve
1003	+ ve	+ ve	+ ve	-ve	-ve
1004	+ ve	+ ve	+ ve	-ve	-ve

3.3. Antibiogram of *E. coli* isolates.

It was observed that all the nine *E. coli* strains varied for antibiotic sensitivity and resistant. Most of them were sensitive to gentamicin and norfloxacin and resistant to ofloxacin (Table and Figure). As most of the strains showed resistant, it could be attributed that there could be plasmid responsible for antibiotic resistant in these *E. coli* associated with UTI.[19]

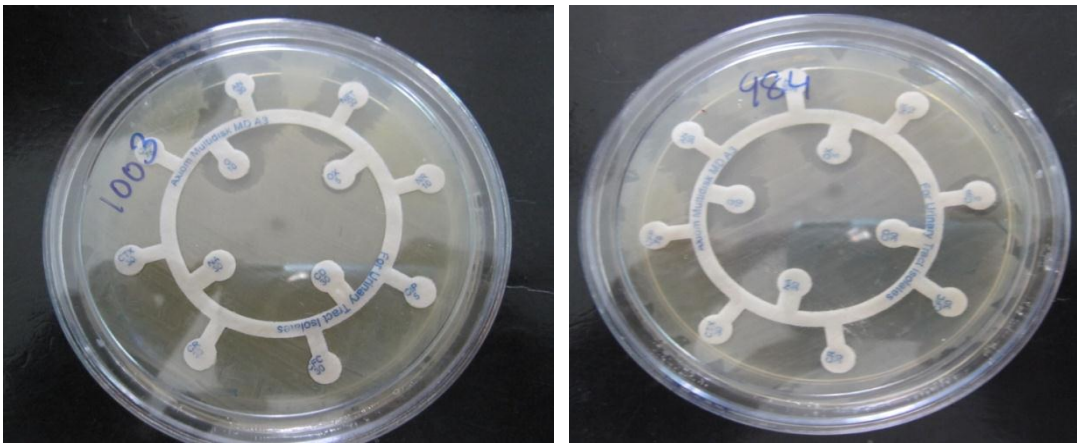

Figure 5: Antibiotic resistant and sensitive patterns of *E. coli* strains.

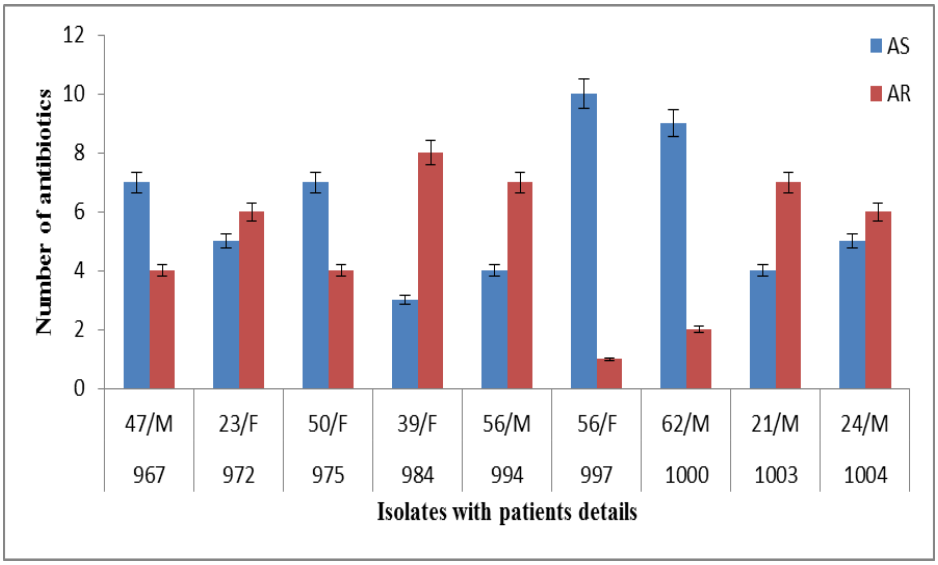

Figure 6: Graph of Antibiotic resistant and sensitive patterns of *E. coli* strains.

Table 4: Antibiotic resistance and sensitive pattern of *E. coli* strains.

Isolate *E.coli*	Age/sex	Antibiotic Sensitivity (AS)	Antibiotic Resistant (AR)
E.coli 967	47/M	G,NT,OX,CD, CFC,AK	NR, CIP, CFP, CTX.
E.coli 972	23/F	NR, CIP, CFP, CFC,AK	CTX, G,AN,NT,OX,CD
E.coli 975	50/F	NR, CIP, CFP, CTX. G,AN,NT	OX,CD, CFC,AK
E.coli 984	39/F	CIP, CFP, CTX	G, AN, NT, OX, CD, CFC, AK. NR
E.coli 994	56/M	NR, CIP, CFP, CTX	G, AN, NT, OX, CD, CFC, AK.
E.coli 997	56/F	G,AN,NT,OX,CD, CFC,AK, NR, CIP, CFP	CTX
E.coli 1000	62/M	AN,NT,OX,CD, CFC,AK, NR, CIP, CFP	G, CTX
E.coli 1003	21/M	OX,CD, CFC,AK	NR, CIP, CFP, CTX. G,AN,NT.
E.coli 1004	24/M	G, AN, NT, OX, CD, CFC.	AK, NR, CIP, CFP, CTX

Whereas,

CFP-cefoperazone, AK-amikacin, NT-netilmicin, NR-Norfloxacin, CIP-ciprofloxacin, CFC-cefactor, CTX-ceftriaxone, G-gentamicin, NA-nalidixic acid, CD-cefadroxil, OX-ofloxacin.

3.4. Genomic DNA isolation: Clear bands of genomic DNA were observed (972 and 984) which were used for further PCR studies to detect the cnf1 gene.

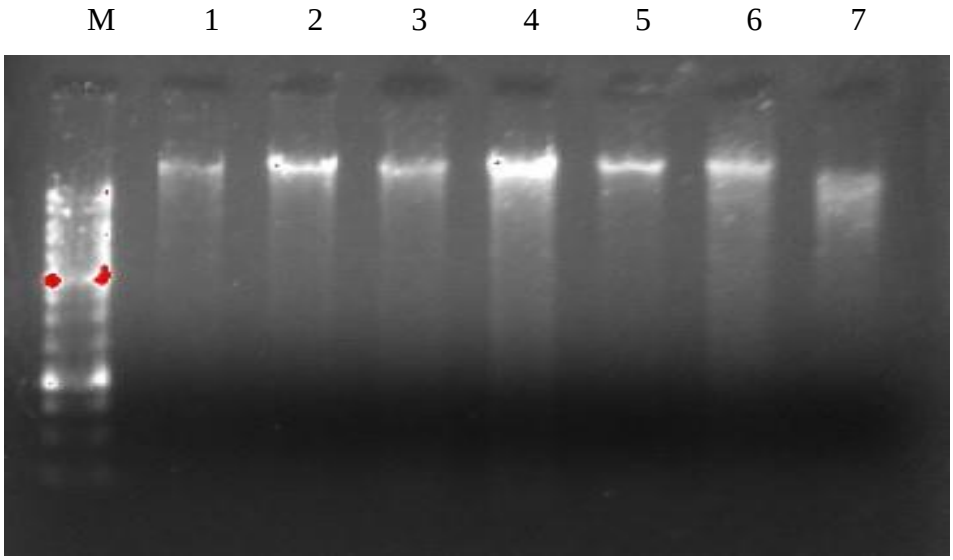

Figure 7: Genomic DNA of *E. coli* strains (967, 972, 984, 994, 997, 1000 and 1003)

M: Marker (1kb),

Wells: 1 to 7 = Genomic DNA from *E. coli.*

3.5. Identification of necrotoxic (cnf1) gene by PCR.

Primers were used for the amplification of Cnf1 from *E. coli* in this study PCR product was amplified by using primers and showed 498 bp of band corresponding to the CNF 1 gene.

Two different types of NTEC have been reported: NTEC1 and NTEC2 depending on the toxin they produce 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. Detection of NTEC indicated that cnf1 gene is present in two *E. coli* strains (972 and 984). Previous study by Rahman and Deka (2014) showed that out of the 84 isolates 52 (61.9%) harboured cnf1 gene.[20]

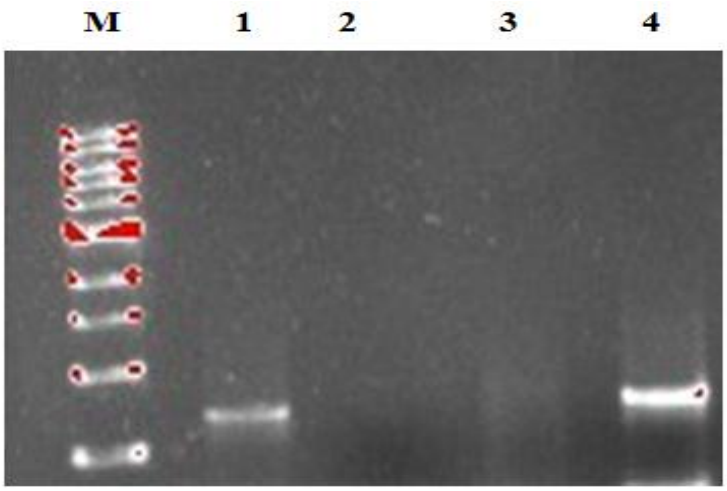

Figure 8: Detection of NTEC (cnf1 gene) in two *E. coli* strains

M= marker (5kb),

Well 1 = *E. coli* 972 (498bp) and well 4= *E. coli* 984 (498bp)

Remaining wells 2, 3, 5, 6, 7 were negative

CONCLUSION

E. coli were isolated onto selective medium (CLED agar) from different samples of UTIs. Further they were confirmed as *E. coli* using Grams stain and different biochemical tests. Nine strains of *E. coli* were selected and characterized for antibiogram test using different antibiotics (CFP, AK, NT, NR, CIP, CFC, CTX, G, NA, CD and OX). The results of the present study showed that 2 strains that were associated with UTI belonged to NTEC. However, further more studies are required with collecting of samples at different locations to confirm the presence of necrotoxic *E. coli* and also for presence of different virulent genes. This will help in diagnosis and recommendation of proper antibiotics to patients with urinary tract infections.

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