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Plasmid and Drug Resistance Profile of Sorbitol Nonfermenting Cefixime-Tellurite Resistant *Escherichia coli* Isolates from the Gomti River

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Enterohemorrhagic *E. coli* (EHEC), particularly those of serogroup O157 are a major worldwide threat to public health, mainly caused by consumption of contaminated food and water (Clarke et al. 2001). EHEC produce a variety of potent toxins causing severe human health problems including bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (Nataro and Kaper 1998). Cattle and other ruminants, consistently recognized as major natural reservoirs of enterohemorrhagic *E. coli*, play a significant role in the epidemiology of outbreaks and sporadic human infection (Armstrong et al. 1996; Coia 1998).

Recently, cattle waste waters contaminated the Gomti River water in Lucknow city due to failure of pumping stations upstream (D' Souza 2003). This riverine system serves as a source of water to the rural population and agriculture in the region as well as recreational purposes in Lucknow city. We have reported earlier, the presence of virulence genes (*stx1, stx2, hlyA, eae)* of *E. coli* isolates in water samples from the Gomti River at various locations (Ram et al. 2003). Global travel, increased faecal pollution of water resources and the indiscriminate use of antibiotics have spread drug-resistant microbes to all parts of the world. Internationally several rivers have become reservoirs of antibiotic resistant microbes (Pathak et al. 1993; Ash et al. 2002; Ram et al. 2003). Therefore, the present study focused on the drug resistance pattern and plasmid profile of sorbitol non-fermenting *E. coli* isolates from the Gomti River.

MATERIALS AND METHODS

Water samples were collected from four pre-identified sites of the Gomti River in Lucknow city, based on various activities including the water intake point for the water works, recreational or picnic spots and a cattle area (Figure 1). For isolation of total coliform and fecal coliform population of the Gomti River, water samples (1L) were collected in sterile, glass bottles from midstream of each selected site, stored in ice and transported to the laboratory for analysis within six hours. Water samples (500 mL) were filtered in duplicate through a membrane filter (cellulose nitrate filter of 0.45μm pore size). Each membrane filter was aseptically removed by sterile forceps, cut into 4 pieces, placed in 25 mL Erlenmeyer flasks containing 10 mL Luria broth. The flasks were incubated for 21 hr at 220 rpm on a rotary incubator (INNOVA 4230, New Brunswick, USA) at 37 $\pm$ 1°C for total coliform and 44.5$\pm$ 1°C for faecal coliform, respectively (APHA 1998).

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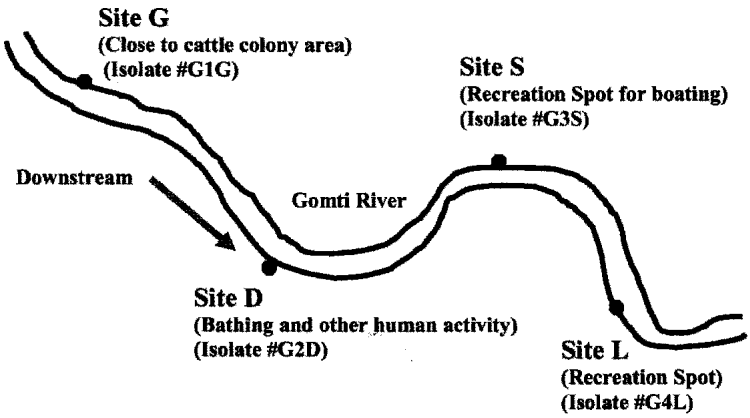

Figure 1. Locations of selected sites on the Gomti River for isolation of sorbitol non-fermenting *E. coli* strains.

E. coli strains were selected over MacConkey Agar, Hi-Chrome ECC Agar and Cefixime-Tellurite Sorbitol MacConkey Agar (Hi-Media Ltd, India). Biochemical tests such as indole production, the methyl red Voges-Proskauer reaction, citrate utilization and ECC-MUG broth were carried out for identification of *E. coli* strains. The *E. coli* strains were segregated based on antimicrobial drug resistance patterns using 15 antimicrobials (Table 1). The sensitivity of isolated organism to antimicrobials was determined by an agar diffusion test using antimicrobial impregnated paper discs (Hi-Media Ltd., India) as described by NCCLS (2000). In brief, pure culture colonies (3-4) were transferred into tubes containing 5 mL Luria broth and incubated at 35 $\pm$ 1°C for 4-6 h on a rotary shaker at 220 rpm to yield a uniform suspension of 10^6 cells per mL. The inoculum was streaked on sterile Muller Hinton Agar plates (90 mm diameter) using a sterile cotton swab. The discs for four antimicrobials were applied aseptically, 30 mm apart, on Muller Hinton Agar plates. The plates were incubated immediately at 37 $\pm$ 1°C for 16 h or later if necessary. The diameters of zones showing inhibition were measured to the nearest mm and recorded. A zone size interpretative chart was used to determine sensitivity/resistance of antimicrobials as described by NCCLS (2000). This test was performed in triplicate for each *E. coli* strain and antimicrobial. *E. coli* ATCC 25922 was used as a positive control.

The genomic DNA of *E. coli* DH5α and *E. coli* BL21 were extracted using a GenElute™ Bacterial DNA kit (Sigma, USA). In brief, for isolation of genomic DNA, the cell pellet was resuspended in 180µL lysis solution containing 20µL of proteinase K and incubated at 55 °C for 30 min, then retreated with 200µL of a second lysis solution and incubated at 55 °C for 10 min. To the lysed cells 200µL ethanol was added and then transferred to a DNA binding column. The column was spun at 6500 x g for 1 min and transferred to a new collection tube and washed with 50µL of wash solution. The genomic DNA was recovered by adding 200µL of 10mM Tris-HCL,

mM EDTA, pH 9.0 to the column spun at 6500 x g for 1min.

For relaxed and stringent plasmid amplification, 1mL of the late log culture of *E. coli* isolate (OD $_{600nm}$ of ~ 0.6) was inoculated in 25 mL of Luria broth medium containing (25µg/mL or 170µg/mL chloramphenicol) and incubated on a rotary shaker at 250 rpm at 37°C for 3hr. The cells were pelleted by centrifugation at 8000 x g for 5 min. Plasmids were isolated by the Concert ™ High Purity Plasmid Purification Midi Prep System (Invitrogen Life Technologies, Inc., USA) based on the method of Birnboim and Doly (1979). The plasmid DNA (25-35µL) was loaded on 0.8% Agarose gel in 40mM Tris Acetate and 1mM EDTA Buffer pH 8.0 and electrophoresed at 60V for 6-8 h to resolve plasmid bands.

RESULTS AND DISCUSSION

The Gomti River, a major tributary of the Ganga River traverses about 730 km through central and eastern part of Uttar Pradesh, finally merging with Ganga near the city of Varanasi. The Gomti River is major source of domestic water supply to a population of about 3.5 million in Lucknow city. The river receives from Lucknow city 450 mld of untreated domestic waste water (Singh et al. 2004). Microbiological studies conducted earlier reported high bacterial counts in the range of 4.6 x 10^3 to 2.4 x 10^9 in Gomti waters (Shrivastava et al. 2004; Singh et al. 2004).

Table 1. The antibiotics used in the study.

Classes	Antibiotics	Quantity (mcg/Disc)
Fluoroquinolones	Ciprofloxacin (Cf)	5
Phenicol	Chloramphenicol (C)	10
Folate inhibitor	Co-Trimoxazole (Co)	25
Ansamycins	Rifampicin (R)	5
Quinolone	Amikacin (Ak)	10
Cephalosporins	Cephotaxime (Ce) Norfloxacin (Nf)	10 10
Aminoglycosides	Gentamicin (G) Kanamycin (K)	10 30
Tetracycline	Tetracycline (T) Oxytetracycline (O)	30 30
ß-lactams	Penicillin G (PG) Piperacillin (Pc)	10 units 100
Metronidazole	Metronidazole (Mt)	5
Macrolides	Erythromycin (E)	15

In the present study, four sorbitol non-fermenting cefixime-tellurite resistant *E. coli* isolates were obtained, each from a different location of the Gomti River. It is now well recognized that strain of *E. coli* O157: H7 unlike the majority of *E. coli* strains do not ferment sorbitol and are resistant to cefixime and tellurite (Taylor et al. 2002). The isolates exhibited resistance to multiple drugs including erythromycin, amikacin, and penicillin among others. However, all four isolates were sensitive to norfloxacin and gentamycin (Table 2). The *E. coli* isolates G1G, G2D, G3S and G4L were

resistant to 87, 73, 67 and 60% approx. of the 15 commonly used drugs in humans. Multiple drug resistance in *E. coli* strains has been observed in other parts of the country. In a study, 49.2% of shiga toxin producing *E. coli* non-O157 strains isolated from human and cow stool samples in Calcutta, India exhibited resistance to one or more antibiotics including ampicillin, tetracycline and co-trimoxazole (Khan et al. 2002). Earlier, Chakraborty et al. (2001) reported concomitant infection of enterotoxicogenic *E. coli* during an outbreak of cholera in Ahemdabad, India in the year 2000, wherein all the *E. coli* isolates exhibited resistance to ciprofloxacin, norfloxacin and nalidixic acid. In the present study, antibiotic resistance profile of the four isolates indicates the need for careful selection of antibiotics during treatment of water borne infections observed in adjoining areas of the Gomti River.

Table 2. Antimicrobial drug resistant pattern of *E. coli* isolates.

Isolates#	Resistance pattern	Sensitive pattern
G1G	Cf, T, PG, Pc, Mt, Co, Ak, O, Ce, R, C, K, E	Nf, G
G2D	T, PG, Pc, Mt, Ak, O, Ce, R, C, K, E	Cf, Nf, G, Co
G3S	T, PG, Pc, Mt, Ak, O, R, C, K, E	Cf, Nf, G, Co, Ce
G4L	T, PG, Pc, Mt, O, R, C, K, E	Cf, Nf, G, Co, Ce, Ak

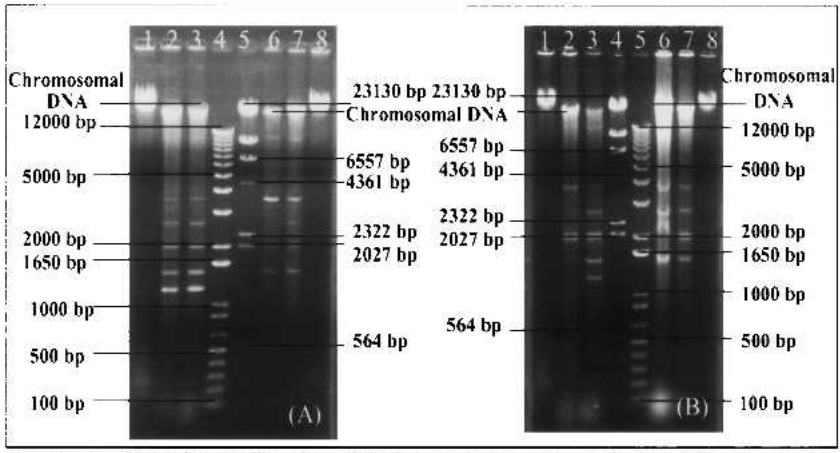

Figure 2. Plasmid profile of sorbitol non-fermenting cefixime-tellurite resistant *E. coli* isolates (A) L-R, Lane1: *E. coli* DH5α genomic DNA; Lane2: Isolate#G1Ga; Lane3: Isolate#G1Gb; Lane4: M1; Lane5: M2; Lane6: Isolate#G4La; Lane7: Isolate#G4Lb; Lane8: *E. coli* BL21genomic DNA (B) Lane1: *E. coli* DH5α genomic DNA; Lane2: Isolate#G2Da; Lane3: Isolate#G2Db; Lane4: M2; Lane5: M1; Lane6: Isolate#G3Sa; Lane7: Isolate#G3Sb; Lane8: *E. coli* BL21genomic DNA. M1: 1Kb Plus DNA Ladder (MBI Fermentas); M2: Lambda/Hind III Ladder(MBIFermentas); a: chloramphenicol (170μg/mL); b: chloramphenicol (25μg/mL)

The plasmid profile of the four isolates indicated presence of plasmids of different size between 1.2-12 kb approximately (Table 3, Figure 2). The methodology used for extraction of plasmid DNA is capable of extracting plasmids of all sizes (Birnboim and Doly 1979). We have screened the four isolates for optimal replication of relaxed and stringent plasmids using chloramphenicol (Sambrook et al. 1989; Grinsted and

Bennett 1988). Considering the downstream location of sampling sites on the water body, the observation made in the present study indicates a consistent pattern in plasmid profiles of the four drug resistant *E. coli* isolates. Further, all the isolates exhibited resistance to tellurite, a factor that has been linked to plasmids in enteric bacteria (Taylor et al. 2002). It is difficult to correlate the antibiotic resistance pattern to plasmid profiles as the same resistance pattern can be encoded by unrelated plasmid and chromosomal genes (Farrar 1983; Smith et al. 2003). However, the multiple drug resistance pattern itself is indicative of

Table 3. Plasmid profile of cefixime-tellurite resistant *E. coli* isolates from the Gomti River.

Isolate #	Chloramphenicol µg/mL	Plasmid size (kb approx.)							
		1.2	1.5	2.0	2.7	3.7	6.5	10	12
G1G	25	+	+	+	+	+	-	+	-
	170	+	+	+	+	+	-	+	-
G2D	25	-	+	+	+	+	+	+	-
	170	-	+	+	-	+	-	+	-
G3S	25	+	+	+	+	+	+	-	+
	170	-	-	+	-	+	-	+	-
G4L	25	-	+	+	+	+	+	-	+
	170	-	+	-	+	+	-	-	-

extensive transfer of resistance genes among enteric organisms thus limiting the choice of antimicrobial agents for treatment of human and veterinary infections (Mayer 1988). The observations made in the present study indicate the need to determine the profile of water-borne pathogenic bacteria for monitoring and protection of urban surface water resources.

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