Prevalence of Drug Resistant Enterotoxigenic *Escherichia Coli* in Potable Water of Gwalior City

Rajesh Singh Tomar, Mohit Agarwal, and Anurag Jyoti

***Abstract*—** Enterotoxigenic *Escherichia coli* (ETEC) is one of the major causative agents of diarrhea. Humans and non-humans can acquire infections primarily through consumption of contaminated water. In India, ETEC has been concerned as a foremost cause of travelers' diarrhea. ETEC is responsible for 200 million diarrheal episodes and 300,000 to 400,000 deaths, primarily in children under the age of 5 years. The pathogen harbors *LT1* signature gene responsible for its virulence. The prevalence of drug resistance ETEC in water is of great concern. The present study aims to isolate and characterise drug resistant virulent isolates of ETEC in potable water collected from different locations (n=6) of urban settings in Gwalior city. All the samples have been characterised using conventional culture and advanced molecular methods. Primer pair specific to signature gene *LT1* were used to identify ETEC from environment samples. All identified ETEC isolates have been subjected to antibiotic susceptibility testing. Our study indicates the existence of pathogenic drug-resistant isolates in potable water of Gwalior. Site#1 exhibited maximum intermediate isolates for antibiotics which is alarming and of great concern where as site#4 exhibited maximum resistance isolates of ETEC. This study may help in risk assessment posed by the infections due to persistence of antibiotic resistant bacteria in potable waters.

***Keywords*—** Enterotoxigenic *E. coli*, Diarrhea, Drug resistance bacteria, Potable water, Virulence determinants.

I. Introduction

Rapid industrialization and urbanization has often lead to the deterioration of microbiological quality of water. Even in urban settings the drinking water has been contaminated. *Escherichia coli* and its pathotypes are one of the major habitats of water. Among them, enterotoxigenic *Escherichia coli* (ETEC) is mainly responsible for infectious diarrhea and also for other enteric diseases [1]. Contaminated water sources, surface and potable water are one of the main reservoirs of ETEC. ETEC strains cause diarrhea in infants and in adults by the production and release of enterotoxins, LT (heat labile enterotoxin) and ST (heat-stable enterotoxin). Children's under the age of five having poor immunity are most susceptible for ETEC infections [2], [3].

Development and usage of antibiotics have been successful in the management of infectious diseases to some extent killing pathogenic bacteria since decades [4]. Although, the disease is managed, the pathogen still persists in the environment. These pathogens have slowly developed the resistance against existing antibiotics through horizontal gene transfer and other means. The persistence and emergence of antimicrobial resistance associated with plasmid is of great concern as it leads to the dissemination of resistant strains [5], [6]. Fluoroquinolone is a group of antimicrobials which is used primarily for the therapeutics of intestinal infections.

The exhaustive and over usage of drugs to cure infections has leads the development of resistance mechanism in pathogenic strains. Ultimately, the environment including water has been heavily contaminated with these resistant strains [7]. Multi drug resistant strains through poor and leaky water distribution system mixed with sewage pipelines reach the households in urban settings. Household waste as well as industrial effluents get discharged into the river. [8]. However, the emergence of Fluoroquinolone resistant bacteria has kept challenges before clinics and health personals.

Therefore, the study on antibiotics resistance pattern both in terms of resistance and intermediate is of great concern for understanding their spread and emergence. Due to inadequate treatment plants the pathogenic bacteria harbouring antimicrobial resistant gene still persist in drinking water. This water supplied in households is used for both drinking and bathing purpose. The persistence of antibiotic resistant microbes in potable water further pose challenges for the management of infectious diseases [7]. In urban environment, the water pipelines are often leaky and water gets contaminated with pathogenic microbes leading to possible waterborne disease outbreaks [9].

In India and other developing countries, pathogen diagnostics based on antimicrobial agent resistance and virulence gene profiles of ETEC of potable water resources is not well established [10]. Therefore, antibiotic resistance pattern of potable water isolates of ETEC will have immense impact on management of dissemination of antibiotic resistance bacteria in environment [11], [12].

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In the present study, we report the presence of antimicrobial-resistant ETEC in the potable water samples collected from different sites of Gwalior as a major city of Northern India.

II. Materials And Methods

A. Bacterial Isolates and strains

Different reference strains were used in this study. The reference strains (Enterotoxigenic *Escherichia coli* MTCC 723) were procured from Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology (IMTECH), Chandigarh, India. Enterotoxigenic *Escherichia coli* MTCC 723 was used as positive control for PCR.

B. Sampling and identification of ETEC

Potable water samples were collected from the different sites located in the city. Samples (2 liters) were collected in sterilized bottles from six different sites and were processed immediately in laboratory.

Samples from each site were filtered through membrane filter (cellulose nitrate filter 0.22 µm pore size; Millipore, USA). The membrane filters immobilized with bacteria were aseptically removed and placed on culture media containing Eosine Methylene Blue agar (Hi-media, Mumbai) and incubated for further 12-15 h. The isolates confirmed as ETEC were maintained at –70°C supplemented with 15% (vol. /vol.) glycerol.

C. Isolation of Genomic DNA

DNA was extracted by boiling prep method. Briefly, *E. coli* isolates were incubated at 37^0C for 16 hours. An aliquot (1ml) of the overnight culture was transferred to 1.5 ml tubes and centrifuged at 6000 x g for 3 min. The supernatant was removed, and the pellet was resuspended by vortexing in 200 µl of sterile double distilled water. The suspension was boiled for 30 min, followed by precipitation using sodium acetate (0.3 M, pH 5.2) and ethanol. The precipitated DNA was resuspended in 100 µl TE (pH 8.0) [13].

D. Primers

Primers for *LT-1* gene were adopted from Ram et al. 2008 (Table I) [14]. The specificity of adopted primers were further analyzed using NCBI-BLAST against the known microbial genome sequences to check the cross homology. Further, specificity of the primer set was validated using reference strain enterotoxigenic *Escherichia coli* MTCC 723.

TABLE I
PRIMER SEQUENCES OF *LT-1* GENE FOR THE DETECTION OF ETEC

Gene	Primer Sequence (5-3)	Amplicon
LT1	GGCAGGCAAAAGAGAAATGG	150 bp
	TTGGTCTCGGTCAGATATGTG	

E. Detection of virulence signatures of ETEC:

We selected 5 random *E. coli* isolates from each site for the presence of signature virulent gene *LT-1* (heat labile enterotoxin) using primers (Table 2) and thermal cyclic conditions. We used *E. coli* MTCC 723 as template in the positive control for amplification of *LT1* gene using defined thermal conditions (Table II). All the assays were done in duplicate. Amplicons were analyzed on 1.7% agarose gel, visualized and recorded.

TABLE II
AMPLIFICATION CONDITIONS FOR PCR OF *Lt1* GENE OF ETEC

Steps	Temperature (^{0}C)	Time	Cycles
Initial Denaturation	95	3 min	1
Denaturation	94	30 sec	35
Annealing	55.8	45 sec	
Extension	72	30 sec	
Final Extension	72	7 min	1

F. Antimicrobial drug susceptibility

Four random isolates were selected for screening against antimicrobials from six identified sites. Total 24 isolates were used against eleven antibiotics from seven different classes as per CLSI guidelines. These are β-lactams: Oxaciline 1 µg/disc, Macrolides: Erythromycin 15 µg/disc, Aminoglycosides: Amikacin, 10 µg/disc, Phenicols: Chloramphenicol, 10 µg/disc, Fluoroquinolones: Ciprofloxacin 5 µg/disc, Folate inhibitors: Co-trimoxazole, 25 µg/disc. Data for antibiotic resistance of each isolate have been reported as resistant (R), isolates with reduced susceptibility (Intermediates) or sensitive (S), based on Clinical and Laboratory Standards Institute guidelines.

III. Results And Discussion

A. Bacterial Isolates & Strain:

In this study from 24 isolates of six different sites, four sites were positive for ETEC which were further characterised molecular method using PCR.

B. In-silico specificity of primers:

The specificity of oligonucleotides was determined against the known microbial genomes by NCBI-BLAST. The BLAST analysis showed no cross homology in other genera or species [14].

C. Identification of virulent signatures of ETEC isolates:

Out of six sites four were positive for the presence of ETEC, harboring *LT-1*. The 150 bp amplicon were observed in these samples on gel electrophoresis after PCR (Figure 1). In this study, four isolates were taken from each of the six different sites. The selected 24 isolates were checked for the specificity of virulent gene, out of which 4 sites were found to be positive. Remaining sites were negative for the presence of *LT1* gene. Our interpretation on virulence markers specify that the potable water of Gwalior city is contaminated by ETEC isolates exhibiting *LT1* gene. Contaminated water and poor hygiene are the reasons for diarrheal diseases throughout the world [15]. Fifteen countries contribute three quarters of

childhood deaths due to diarrhea in children under five years of age worldwide out of which India ranks first [16]. In present study, we found potable water samples contaminated with strains of *E. coli*. The presence of ETEC in potable water suggests the possibility of contamination of water supplies [17].

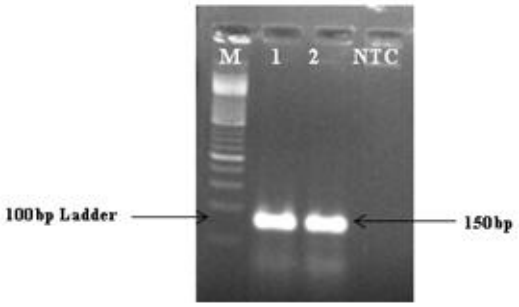

Fig. 1 Agarose gel electrophoresis of amplicon (150 bp)
M=DNA Ladder (100 bp)
1= Positive control (*E. coli* MTCC 723)
2= ETEC isolate, NTC= Non-template control

D. Antimicrobial Susceptibility Test

β-lactams: Oxaciline 1 µg/disc, Macrolides: Erythromycin 15 µg/disc, Aminoglycosides: Amikacin, 10 µg/disc, Phenicols: Chloramphenicol, 10 µg/disc, Fluoroquinolones: Ciprofloxacin 5 µg/disc, Folate inhibitors: Co-trimoxazole, 25 µg/disc were used. ETEC is one of the important pathogens in water. Aquatic ecosystem could serve as a reservoir of antibiotic resistant pathogen through horizontal gene transfer mechanism. These selected Drug resistant strains showed high resistance against Oxacilline and Erythromycin (Table III).

TABLE III
MEAN ZONE OF INHIBITION OF ETEC AGAINST ANTIBIOTICS

S. No.	Antibiotics	Results (R/I/S)
1	Ox^{1} Oxacilline	Resistance
2	E^{15} Erythromycin	Resistance
3	AMK^{10} Amikacine	Intermediate
4	C^{10} Chloramphenicol	Intermediate
5	CIP^{5} Ciprofloxin	Sensitive
6	Cot^{25} Co-trimoxazol	Intermediate

A similar resistance pattern has also been observed in case of *Salmonella* also which has also been isolated form potable water in Agarwal *et al* (2015).

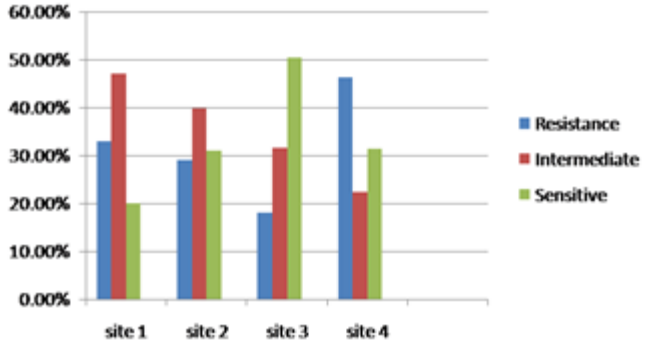

Fig. 2: Percentage of Resistance, Intermediate and Sensitive against ETEC isolates of different sites.

Some of the strains were found to be sensitive against Ciprofloxin. The lesser number of antibiotics having bactericidal effect as compared to more antibiotics showing ineffectiveness against ETEC isolates is crucial and alarming.

It was found that site 4 showed high resistance pattern (46.22%) as compared to site 1 (32.87%), site 2 (29.09%) and site 3 (18%). Site 1 has high intermediate pattern (47.12%) as compared to other sites (Figure 2).

IV. CONCLUSION

The occurrence of drug resistant isolates of ETEC in potable water signify the contribution of virulent and resistance genes into aquatic environments. This can lead to potential health hazard to human and animals which are dependent on water sources. This could play a major role in the epidemiology of antibiotic resistance in Gwalior for the emergence of emerging and re-emerging diarrheagenic diseases.

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