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Molecular Characterization of *Escherichia coli* Strains Isolated from Drinking Water Sources in Jammu, India

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Fifty seven isolates of thermotolerant E. coli were recovered from 125 drinking water sources, 47 (82.45%) were typable of which 9 (26.3%) were pathogenic serotypes. The isolated *E. coli* belonged to 13 serogroups on 'O' antigen basis with O20 (12.28%) being predominant followed by O68 (10.52%). PCR using specific primers for stx_1, stx_2, eae and *hlyA* genes detected STEC, EPEC and EHEC in 23.2, 4 and 2.4 per cent of the samples respectively.

Key words: Thermotolerant *E. coli*, EHEC, EPEC, STEC.

Water intended for human consumption should be safe, wholesome, easily accessible, adequate in quantity, free from contamination and readily available. The drinking water has become greatly affected by pollution of rivers, lakes and oceans. The widespread pollution of water has rendered readily accessible sources of water unsuitable for human and animal consumption. Major hazard associated with drinking water supplies is microbial contamination arising as a result of agricultural land wash, domestic sewage, livestock and Industrial effluents, improper storage and handling (W.H.O, 2006; Saha *et al.*, 2006). Besides a normal intestinal flora of man and animals, certain strains of *E. coli* are highly pathogenic. Serotyping is a common method used for the characterization of clinical isolates of *E. coli* and has a broad use in epidemiology and also in medical diagnosis. The existing association between serotype and pathotype makes this method a valuable tool for typing *E. coli* and other bacterial species. The newly emerged pathogen 0157:H7 is an example of a strong association of specific serotype with a pathotype (Day, Scotland, Cheasty, & Rowe, 1983). It could be diagnosed before any virulence properties of this strain were known. Bonnet *et al*. (1998) shown that *E. coli* serotypes other than 0157:H can also cause serious disease. Water-borne diseases are usually considered as a result of widespread occurrence of pathogens, parasites and disease vectors. Poor drinking water quality also causes diseases to livestock and poultry. Cattle drinking water is a source of on-farm *Escherichia coli* O157:H7 transmission. Many diseases can be transmitted to the bird flock through the drinking water contaminated by faeces and secretions of sick

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birds, pathogens originating from other animal species and the man (Jafari *et al*., 2006).

Public health is facing new challenges, due to increase in bacterial resistance to most of the existing antibacterial agents besides the growing number of immuno-suppressed patients makes it imperative to develop new concepts making their drinking-water safe. Many bacteria have evolved strategies which influence their infectivity and pathogenicity, thus threatening public health. The ecology of water borne pathogens thus should be assessed in relation to modern agricultural practices vis-a-vis anthropogenic activities. Genetic and phenotypic characterization of pathogenic bacteria is needed to elucidate the zoonotic relationships with their animal hosts and factors influencing the water borne transmission of human origin diseases (WHO, 1993; WHO, 2003).

As is seen elsewhere in India, the water resources in Jammu are also contaminated due to inadequate facilities for human excreta and waste water disposal. The increasing cases of waterborne diseases in recent times necessitated the investigation of drinking water sources of Jammu region. The magnitude of problem associated with unsafe drinking water has not been systematically assessed in this part of state; although newspapers occasionally bring in focus some reported water borne illnesses. The assessment of hygienic status of drinking water goes a long way in reducing the burden of water borne diseases. Keeping in view the widespread pollution of water, the assessment of hygienic quality in different drinking water sources of Jammu was studied.

MATERIALS AND METHODS

Sampling

The sample collection was carried out following the standard procedure as outlined by World Health Organisation (2008). A total of 125 water samples were collected from Tawi river (n=25), post filtration water (n=25), household supplies (n=25), tubewells (n=20), filling stations (n=15) and drinking water sources of livestock/poultry (n=15) from rural and urban areas of Jammu division, India. Water samples (300 ml) were collected in sterile glass containers (Hi Media, Ltd Mumbai, India) and transferred to laboratory on ice. Isolation and identification of the organisms was carried out as per the method described by Cowan and Steel (1993). The samples were processed using bromo-cresol purple MacConkey's bile broth.To confirm the presence of faecal coliforms, a loopful of the culture from the tubes showing positive for production of both acid and gas were streaked on Eosine Methylene Blue Agar plate (EMB) as well as on McConkey's agar plates. The plates were incubated at 37ºC for 24 hrs. The appearance of typical colonies with metallic sheen and small pink lactose fermenting colonies on EMB and McConkey's agar respectively constituted a positive test. After incubation at 35-37ºC for 24±2 hours, Gram's-staining was done. The demonstration of Gram negative, non-spore-forming bacilli in the stained smears on microscopic examination confirmed the presence of *E.coli.*

Detection of virulence genes by multiplex Polymerase Chain Reaction

E.coli isolates were subjected to polymerase chain reaction (m-PCR) for detection of *stx*$_1$, *stx*$_2$, *eae* and *hlyA* genes as described by Paton and Paton (1998).

Preparation of DNA template

The loopful of colonies was taken from MLA plate, characterized biochemically by IMViC Test were suspended in 200 µl of PBS (pH, 0.1 M, 7.2). Suspension was prepared by gently vortexing the tubes. The suspension in the microcentrifuge tubes was allowed to boil for 10 minutes followed by snap chilling on ice for 10 minutes and centrifugation at 14,000 rpm for 5 min. The supernatant was transferred to autoclaved nuclease free tubes and was used as template for PCR reaction.

Multiplex PCR for detection of stx_1, stx_2, eae and hlyA genes

stx$_1$, *stx*$_2$, *eae* and *hly*A genes were amplified by PCR as described by Paton & Paton (1998) with slight modifications. PCR was carried out in a final reaction volume of 25μl (Table 1) using 0.2 ml thin wall sterile and nuclease free PCR tubes (Eppendorf, Germany). The PCR mixture contained a final concentration of 3.0 mM $MgCl_2$, 0.20 mM concentrations of each 2'-deoxynucleoside 5'-triphosphate (dNTPs), 3.0 μl of 10x PCR buffer, 1.0 μl of forward and reverse primers, 3.0 μl template DNA and 1.0 U of Fastaq DNA Polymerase (Chromous Biotech Pvt. Ltd,

Bangalore, India). Oligonucleotide primers used in the present study were synthesised from Chromous Biotech Pvt. Ltd, Bangalore, India. Amplification was carried out in a thermal cycler (Eppendorf Master Cycler Gradient, Germany). Primer sequences used in the study and predicted size of the PCR amplicon is given in Table 2.

The amplification cycle consisted of initial denaturation at 94ºC for 2 min, followed by 35 cycles, each consisting of initial denaturation at 94ºC for 10 s, annealing at 56ºC for 10 s and extension at 72ºC for 5 s which was followed by final extension at 72ºC for 5 min.

Agarose Gel Electrophoresis

The confirmation of PCR product was done by electrophoresis of amplified products in 1.0 per cent agarose gel in horizontal electrophoresis unit (Biometra, Germany). Agarose gel was prepared by boiling low electroendo-osmosis (EEO) agarose (Bangalore Genei), 1.0 per cent in 0.5X Tris-borate-EDTA buffer (TBE) and heated to dissolve it completely. After cooling to about 50ºC, ethidium bromide was added to the agarose solution to a final concentration of 0.5μg per ml. The gel casting tray was placed on a leveled surface after sealing the open sides with rubber plugs. The comb was then placed across the gel casting tray. The molten agarose was poured onto the gel casting tray and it was kept undisturbed until solidified. The comb was taken out and rubber plugs were removed. The gel casting tray including solidified gel was then submerged in the electrophoresis tank. 10μl of PCR product was mixed with 2μl of 6X orange loading dye (Bangalore Genei) and loaded into the wells. The samples were run for 1 hr at 7 V/cm in horizontal gel electrophoresis apparatus till the indicator dye reached the last third of the gel. DNA ladders (Bangalore Genei) of size 100bp was used to check the size of the amplicon. This gel was visualized under BioDocAnalyse (Biometra) and photographed. Molecular sizes of PCR products were estimated by comparison of their mobility with respect to that of standard molecular size markers.

Table 1. Reaction mixture for PCR based detection of *stx$_1$*, *stx$_2$*, *eae and hlyA* genes

PCR Ingredient	Volume in ml
PCR Assay buffer (10 X) (without $MgCl_2$)	3.0 ml
dNTP mix (2.0 mM each)	2.0 ml
$MgCl_2$ (25 mM)	3.0 µl
Forward Primer (25 pM/ /ml)	1.0 ml
Reverse primer (25 pM/ /ml)	1.0ml
Taq polymerase (3 units/ml)	0.33 ml
DNA template	3.0 ml
Nuclease free water	11.67 ml
Total	25.00 ml

RESULTS

Serotyping of *E. coli* isolates

Fifty seven isolates of thermotolerant *E. coli* were recovered from 125 drinking water sources. The serotyping was based on O-antigenic character. Most of the serotypes of *E. coli* recovered from contaminated different water sources (Table 3). Out of 57 *E.coli* isolates, 47 (82.45%) were grouped into 13 different (O)-groups) with 1 rough and 9 untypeable strains. Among serotypes, *E. coli* belongs to serotype O20

Table 2. List of oligonucleotide primers used for detection of *stx$_1$*, *stx$_2$*, *eaeA*, and *hlyA* gene (Paton & Paton (1998)

Primer	Sequence (52 –32)	Target gene	Amplicon size
stx$_1$-F	ATAAATCGCCATTCGTTGACTAC	*Stx$_1$*	180 bp
stx$_1$-R	AGAACGCCCACTGAGATCATC		
stx$_2$-F	GCACTGTCTGAAACTGCTCC	*Stx$_2$*	255bp
stx$_2$-R	TCGCCAGTTATCTGACATTCTG		
eae-F	GACCCGGCACAAGCATAAGC	*eae*	384bp
eae-R	CCACCTGCAGCAACAAGAGG		
hlyA-F	GCATCATCAAGCGTACGTTCC	*hlyA*	534bp
hlyA-R	AATGAGCCAAGCTGGTTAAGCT		

F: Forward primer R: Reverse primer

Table 3. Serogroups of *E. coli* isolated from different sources of water

Source	No of samples analysed	No. of Isolates	Most Common Serotypes
Inputs to filtrationplants (Tawi River)	25	25	O89(4),O102(3),O145(3),O130(2),O60(2),O68(2), O20(2),O84(2), O153(1), Rough(1) , UT(3)
Post filtration Water	25	5	O68(2), O20(1), O89(1) ,UT(1)
Household supply	25	7	O20(2),O153(2), O69(1) UT(2)
Tube wells	20	07	O117(3), O1(1), O6(1), O69(1) , UT(1)
Filling stations	15	05	O68(2), O102(2), O20(1)
Livestock & poultry water supply	15	08	O20(2), O69(2),O145(2), UT(2)
Total	125	57	

UT=untypeable, R = Rough, figures in parenthesis indicates total isolates

Table 4. Overall number and percentage of *E. coli* serogroups isolated in water

S.No.	Serogroups	No. of strains	Percentage
1	O20	7	12.28
2	O68	6	10.52
3	O89	5	8.77
4	O102	5	8.77
5	O145	5	8.77
6	O69	4	7.01
7	O153	3	5.26
8	O117	3	5.26
9	O130	2	3.50
10	O60	2	3.50
11	O84	2	3.50
12	O6	2	3.50
13	O1	1	1.75
14	Rough*	1	1.75
15	UT*	9	15.78
	Total	57	

UT=untypeable, R = Rough

was predominant (Table 4).

Characterization of *E.coli* by m-PCR

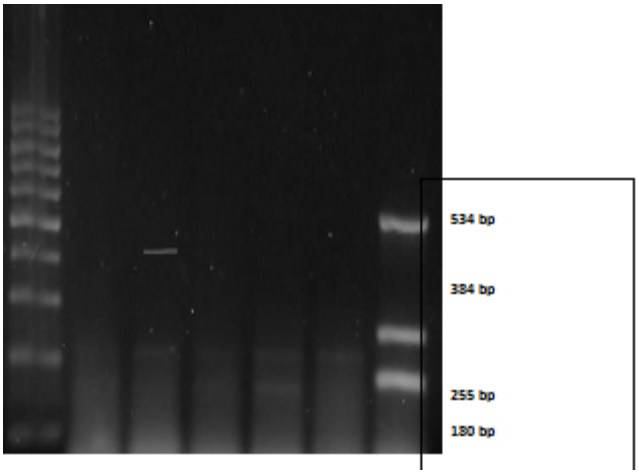
All the 57 *E.coli* isolates were subjected to polymerase chain reaction (m-PCR) for detection of stx_1, stx_2, *eaeA* and *hlyA* genes as described by Paton and Paton (1998). Stx_1, stx_2, *eaeA* and *EHEC-hlyA* genes were present in 12.8, 8.8, 4 and 2.4 per cent of the samples respectively (Table 5 Fig 1). Twenty nine of 57 (23.2%) isolates were possessing stx_1 and or stx_2 genes as detected by PCR and designated as STEC. Out of the 57 isolates 5(4%) showed the presence of only *eaeA* gene but not *stx* gene and were designated as Enteropathogenic *Escherichia coli* **(**EPEC).

DISCUSSION

In the present investigation, 57 of 125 (45.60%) samples revealed the presence of *E.coli* and were serogrouped into 13 different serogroups,

Table 5. Occurance of Shiga toxin-producing *E. coli* (STEC) and Enteropathogenic *E. coli* (EPEC) from water

Source	No of samples analysed	No. of Isolates	Prevalence (%)					
			Stx_1	Stx_2	Stx_1&Stx_2	STEC (%)	EPEC(%)	*EHEC-hlyA*
Inputs to filtration Plants (Tawi River)	25	25	8(32)	6(24)	1(4)	15(60)	3(12)	2(8)
Post filtration Water	25	5	-	-	-	-	-	-
Household supply	25	7	2(8)	1(4)	-	3(12)	-	-
Tube wells	20	07	1(5)	1(5)	-	2(10)	-	-
Filling stations	15	05	1(6.6)	-	-	1(6.6)	-	-
Livestock/ poultry water supply	15	08	4(26.66)	3(20)	-	8(53.33)	2(13.3)	1(12.5)
Total	125	57	16(12.8)	11(8.8)	1(0.8)	29(23.2)	5(4)	3 (2.4)



Lane M: 100 bp molecular weight marker
Lane 1,3, 4, 5= Negative samples
Lane2= Amplified product of *384 bp of eae* gene
Lane 6= Amplified product of 180 bp, 255 bp and 534 bp for *stx* $_1$, *stx*$_2$, *and hlyA* genes

Fig 1. Agarose gel showing the amplification product of m-PCR performed on *E. coli* isolates for the detection of *stx*$_1$, *stx*$_2$, eae and hlyA genes

the predominant serogroup was O20 followed by O68. The present findings are in agreement with the findings of (Santiago-Mercado and Hazen, 1987 and Laura *et al*., 2009) whileas, Ramteke and Tewari (2006) recovered O4, O25, O8, O86, O103, O113 and O157 serotypes of *E. coli* from different drinking water sources and Willayat *et al*. (2005) isolated O157, O141, O09 and O19 serotypes of *E. coli* from various drinking water sources of Srinagar city. Similar findings were also reported by (McCarthy *et al*., 2001; Boyce *et al.,* 1999; Hatha *et al*., 2004).

PCR using specific primers for *stx*$_1$, *stx*$_2$, eae and *hlyA* genes detected STEC, EPEC and EHEC of the different water samples. The present findings concurred with the findings of (Jakee *et al*., 2009; Rao *et al.,* 2011). Our study show that potentially pathogenic *E. coli* isolates could be found ubiquitously in the drinking water sources of Jammu which are in agreement with the results of a previous study of contamination of the drinking water in France by Loukiadis *et al.* (2006). The present findings suggest that STEC and *eae*-positive *E. coli* can persist during all stages of water treatment and are able to adapt to environmental stress in aquatic systems and can survive there. As a consequence, our study indicates that the drinking water is a reservoir for human-pathogenic *E. coli* isolates, which has consequences for public health. Shiga-like toxin producing *E.coli* (STEC) cause a broad range of symptoms in human including hemorrhagic colitis and the often deadly hemorrhagic uremic syndrome. EPEC represent major cause of diarrhoea throughout the world. A wide range of animal species are known to carry STEC and EHEC strains, but ruminants are the most important natural reservoirs and excrete these bacteria with their faeces. Moreover, EHEC and STEC can persist and remain infectious for several weeks in slurries, farmyard manure and sewage sludge as well as on pasture land.

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