REPORT

Screening for toxigenic *Escherichia coli* in stool samples of diarrhoeal patients by polymerase chain reaction

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Abstract: *Escherichia coli* (*E. coli)* are normal flora of the intestines of most animals, including humans. Most strains are harmless and beneficial to host by preventing the establishment of pathogenic bacteria within the intestine. However, some *E. coli* strains can cause a wide variety of intestinal and extra-intestinal diseases, such as diarrhoea, urinary tract infections, septicaemia, neonatal meningitis and renal complications. Several virulence factors including toxins, adhesins, serine proteases, etc. have been reported in these highly adapted clones. The present study was designed to enumerate toxin genotype through PCR assay in local clinical isolates of *E. coli*. A total of 37 *E. coli* strains were collected from different clinical laboratories of Karachi and examined for the presence of shiga toxin 1 (*stx1*) and shiga toxin 2 (*stx2*) genes of ~~E~~enterohemorrhagic *E. Coli* (EHEC) and heat stable (*st*) and healt labile (*lt*) toxin genes of enterotoxigenic *E. Coli* (ETEC). It was observed that 16 strains out of 37 carried one or more type of toxin genes. The presence of *stx1* gene was significantly higher as it was positive in 10 isolates compared to others toxins. Two in above *stx1* positive strains were also carrying for *stx2* gene. Six out of 37 isolates were positive for *lt* gene, and none of the strains are carrying *st* gene. Although, the study was carried out with fewer isolates, yet it demonstrated the trend of dispersion of toxin genes and findings can be used to correlate the gastro-intestinal infections and their complications in Pakistan.

Keywords: EHEC, ETEC, Diarrhoea, Shiga toxins, Heat-labile toxin, Heat-stable toxin.

INTRODUCTION

E. coli belong to coliform group of bacteria, which is used as an indicator for the hygienic quality of food and water samples. It is the predominant microbial flora of human and other animal's intestine, colonizes within hours of life in infant intestinal tract (Nataro and Kaper, 1998). Most strains of *E. coli* are considered as non-pathogenic, which benefit their host by preventing the colonization of pathogenic organisms (Hudault *et al.,* 2001). However, some strains are transformed into highly pathogenic forms due to the acquisition of many virulence factors. These strains are designated as diarrhoeagenic *E. coli* and among these, serotypes producing different toxins are particularly important.

Toxigenic *E. coli* consist of various serotype which release many toxins such as shiga toxin1 and 2 (Stx1, Stx2) in strains of ~~E~~enterohemorrhagic *E coli* (EHEC) and heat labile (HT) and heat stable (LT) toxin of ~~E~~enterotoxigenic *E. coli* (ETEC) strains.

EHEC, a subset of Shiga toxin-producing *E coli* (STEC) are associated with variety of disorders including a life threatening haemolytic uraemic syndrome (HUS) complication. The organism carries many putative virulence factors and Stx is believed to be the key operator for disease progression (Orth *et al.,* 2007). More than 100 serotypes of EHEC have been identified related to varying degree of disease (Khan *et al.,* 2011). The dominant serotype is EHEC O157:H7 involved in numerous outbreaks all over the world (CDC, 2013).

ETEC strains are the leading cause of traveller's diarrhoea in those travelling to developing countries and organisms are characterized by the presence of one or both groups of enterotoxin: ST and LT (Levine, 1987). LT is a member of AB_5 family of toxins and very much similar to cholera toxin in structure and functions (Beddoe *et al.,* 2010). On the other hand, ST is a small monomeric toxin produces as 72-amino acid protoxin activated after degrading into STa and STb (Jafri *et al.,* 2012).

The aim of the present study was to see the prevalence of toxin genotype through PCR assay in local clinical isolates of *E. coli*. These isolates were examined for the presence of shiga toxin 1 (*stx1*) and shiga toxin 2 (*stx2*) genes of Enterohemorrhagic *E. Coli* (EHEC) and heat stable (*st*) and heat labile (*lt*) toxin genes of enterotoxigenic *E. Coli* (ETEC).

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MATERIALS AND METHODS

Bacterial isolates

A total of 37 *E. coli* isolates were included in this study. These strains were isolated from diarrhoeal patients in different clinical laboratories of Karachi in the period of August 2012-October 2012. The isolates were initially processed to purify and confirm for their species identification. Briefly, *E. coli* isolates were streaked on Eosin Methylene Blue (EMB) agar (Oxoid, UK) plates for isolated colonies & incubated for 24hrs at 37˚C. A characteristic single colony was picked, streaked on Tryptic Soy Agar (TSA) slants (Oxoid, UK) and stored after incubation in refrigerator. All pure cultures were tested for morphological, cultural and biochemical characteristics using standard methods (Chessbrough, 1991). Morphology was determined through gram staining. Catalase test, IMViC Tests and TSI reaction were performed to check the typical reaction of *E. coli* strains.

E. coli EDL933 was used as a positive control for *stx1* and *stx2* (Khan *et al.,* 2009). For *lt* and *st*, *E. coli* AB150 ETEC strain isolated in PCSIR was used as a positive control and master mix without bacterial suspension as a negative control.

Polymerase chain reaction (PCR) for the detection of stx1, stx2, lt and st genes

Polymerase chain reaction (PCR) was used to enumerate *stx1, stx2, lt and st* genes. PCR experiments were carried out using specific primers and conditions reported earlier and described in table 1 (Schmidt *et al.*, 1999; Vidal *et al.,* 2005). Briefly, bacterial suspensions were prepared by mixing 3-4 isolated colonies in to molecular biology grade water (Promega, USA). Cell lysis was performed by heating the suspension at 95°C for 10 minutes in a water bath. This suspension was used as a DNA templete. PCR was performed separately for each gene in 25µl reaction mixture containing 2.5µl of bacterial lysate, 12.5µl of 2X GoTaq Green® Master Mix (Promega, USA), and 30pmol of each primer. All PCR tubes were placed in Thermal Cycler (BIORAD, USA) and PCR was started by initial denaturation at 94°C for 10 minutes, followed by 35 cycles respective conditions stated in table 1. The process was terminated with a final extension of 72°C for 10 minutes. PCR amplified products were analysed on 1.0% agarose gel (BIORAD, USA) and stained with ethidium bromide (Sigma, USA). Gel images were visualized under UV transilluminator and photographs were taken for records.

Table 1: PCR primers and protocols used in this study

Primer Designation	Primer sequence (5'-3')	Targets	PCR Conditions						Length of PCR Product	Reference
			Denaturing		Annealing		Extension			
			Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)		
LP30	CAGTTAATGTGGTGGCGAAGC	*stx1*	94	30	57	60	72	60	348	Schmidt *et al.,* 1999
LP31	CACCAGACAATGTAACCGCTG									
LP43	ATCCTATTCCCGGGAGTTTACG	*stx2*	94	30	57	60	72	60	584	Schmidt *et al.,* 1999
LP44	GCGTCATCGTATACACAGGAGC									
LT1	GCACACGGAGCTCCTCAGTC	*lt*	94	90	60	90	72	90	218	Vidal *et al.,* 2005
LT2	TCCTTCATCCTTTCAATGGCTTT									
ST1	AAAGGAGAGCTTCGTCACATTTT	*st*	94	90	60	90	72	90	129	Vidal *et al.,* 2005
ST2	AATGTCCGTCTTGCGTTAGGAC									

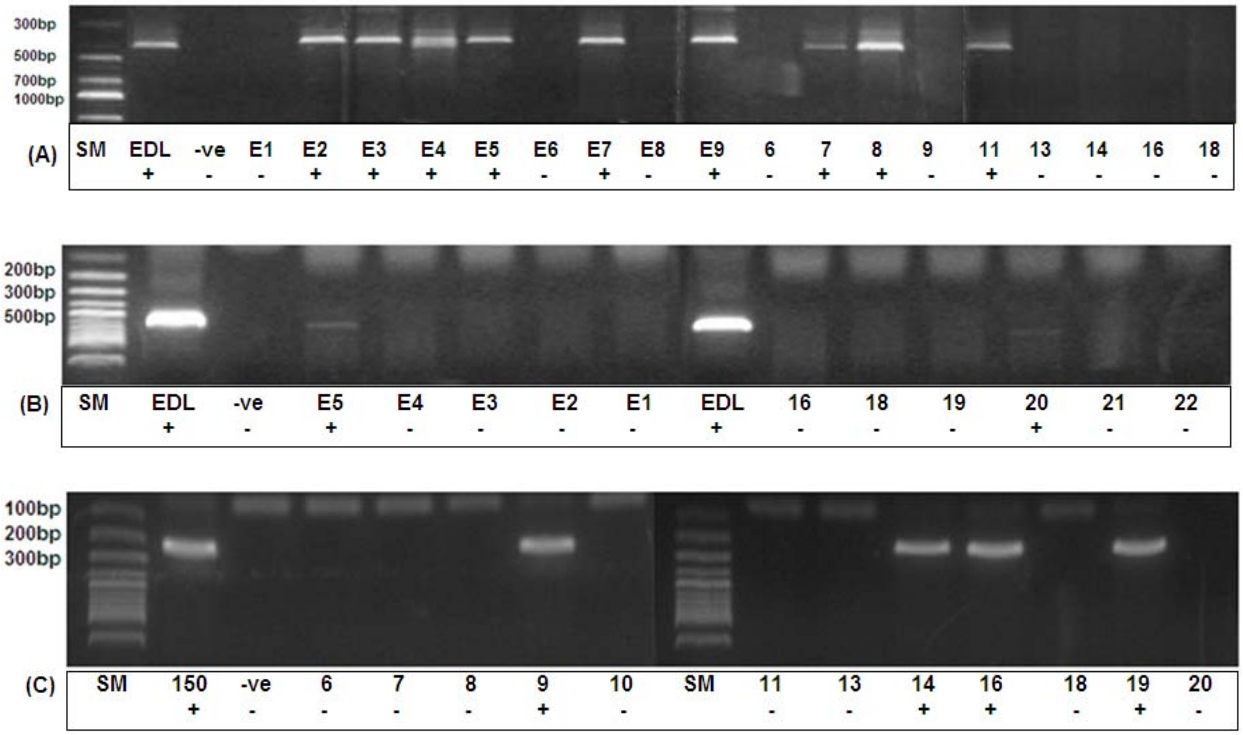

Fig. 1: Selected images of agarose gel electrophoresis of PCR products

(A) *stx1*; **(**B) *stx2*; (C) *lt*. Negative (-ve) control was master mix only and positive control was the cell suspension of *E. coli* EDL933 for *stx1* and *stx2*, and *E. coli* AB150 for *lt* and *st*. SM: 100bp and 1kb DNA size marker, *E. coli* isolates: E1-E9 and 1-28.

RESULTS

It was observed that 16 *E. coli* isolates out of 37 (43%) carried one or more type of toxin genes. The presence of *stx1* gene is more common in these *E. coli* isolates i.e., 10, compared to others toxins (fig 1A). Two strains out of these ten were also positive for *stx2* (fig 1B). None of the strains showed *stx2* gene alone. Six out of 37 isolates were positive for *lt* gene (fig 1C). However, gene for heat stable toxin (*st* gene) was not detected in our strain collection. None of the isolates have shown all genes and all toxigenic strains can be differentiated into shiga toxin-producing *E. coli* (STEC). and heat labile (*lt*) toxin carrying ETEC strains. Altogether, 27% were STEC/EHEC strains while 16% of the remaining strains were ETEC.

DISCUSSION

Gastrointestinal disease pose considerable threat to health and economy of individuals and diarrhoea alone in Pakistan cause 16% of all child deaths (Quadri *et al.*, 2013). Pathogenic strains of *E. coli* cause broad spectrum of illnesses and their pathogenecity depends on the distribution and expression of many virulence determinants like, toxins, adhesins, enzymes, ability to counter host defence, etc. In addition, they are categorized into various pathotypes based on the presence of different combinations of these virulence factors (Khan and Naim, 2011). Of these, shiga toxins (stxs) in EHEC and heat stable (st) and Heat labile (lt) toxin in ETEC are considered as the chief operators of their respective diseases (Orth *et al.*, 2007; Nataro and Kaper, 1998).

Numerous studies have been conceded through out the world to see the prevalence of these toxin genes. However, very few data is available regarding the presence of these virulence factors in local clinical isolates. In a recent study, 56% diarrhoeagenic *E. coli* strains were isolated from stool samples (Bokhari *et al.*, 2013). Of which, 29% were enterotoxigenic *E. coli* and 5% were enterohemorrhagic *E. coli* (Bokhari *et al.*, 2013). Comparing to these results, our study shows that the prevalence of STEC/EHEC strains is on the rise, which may be serious in future. These strains pose great challenge to health authorities and cause many outbreaks in recent times. It is observed that the problems are getting complicated and new clones with multiple virulent factors and broad antibiotic resistance are come in to play. It was evident in a recent outbreak in Germany by an unusual STEC serotype O104:H4 with record number of haemolytic uraemic syndrome (HUS) cases in a single outbreak (Frank *et al.*, 2012). The causative agent has been considered as enteroaggregative *E. coli*, which in

this case acquired shiga toxin gene and extended spectrum of resistance against β-lactamases (ESBL) (Werber *et al.*, 2012). It is the need of time to review the surveillance programmes for diarrhoeal incidences and consider pathogenic strains of *E. coli* as important as other causative agents.

CONCLUSION

Our study suggests that these toxin genotypes are now very well disseminated among local clinical isolates of *Escherichia coli*. The presence of these, especially shiga toxin gene is alarming as strains carrying these genes are involved in life threatening diarrhoeal-associated haemolytic uraemic syndrome. In addition, there is a high probability of horizontal gene transfer to non-pathogenic local strains. Although, number of isolates analyzed in this study was not very high, results showed a significant trend and rise of these pathogenic *E. coli* strains. The incidence rate of food borne illnesses is very high in our region, but the significance of diarrhoeagenic *E. coli* has been overlooked and the virulence characterization of local *E. coli* is yet to be investigated.

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