Original Article

Prevalence of Shiga Toxin-Producing *Escherichia coli* in diarrheal patients in Faisalabad region of Pakistan as determined by multiplex PCR

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Abstract

Background: Strains of Shiga toxin producing *Escherichia coli* (STEC) have been associated with outbreaks of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) in humans around the world. In this study we investigated the prevalence of STEC in hospitalized diarrhea patients (children) in Faisalabad region of Pakistan.

Methodology: We used a multiplex polymerase chain reaction to characterize local isolates of STEC from stool samples of 200 children during the summer season of 2005. The targeted genes were *stx1*, *stx2*, *hly*, and *eae*, representing Shiga toxin 1, Shiga toxin 2, enterohemolysin and intimin respectively. Presence of *stx1* and *stx2* was confirmed by PCR followed by restriction analysis with Tail and HaeIII respectively. Serological examination was carried out by using polyvalent antisera followed by monovalent antisera for O26 and O157.

Results: Multiplex PCR results showed that 22 (11%) of the patients were positive for *stx* genes. Among other virulence factors, plasmid borne *hly* and *eae* genes were detected in 13 (6.5 %) and 16 (8.0 %) patients respectively. However, only 14 STEC isolates were obtained. Among these 11(78.5%), 11(78.5%), 3(21.4%) and 6 (42.8%) were positive for *stx1*, *stx2*, *hly* and *eae* genes respectively. Only 3 of these isolates belonged to serotype O157.

Conclusions: Our results show that the prevalence of STEC is significant in this area and has potential to become a major health problem in the future. It was also observed that serotype O157 was not the major pathogen.

Key Words: Diarrhea, Shiga toxin-producing *Escherichia coli* (STEC), Pakistan.

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an emerging group of human enteric pathogens which are capable of producing a number of diseases ranging from self-limiting, sporadic, and epidemic diarrhea to more complicated conditions lsuch as hemorrhagic colitis and life-threatening hemolytic uremic syndrome (HUS) [1,2].

The STEC are characterized by production of Shiga toxins. Beside E coli, these toxins are also produced by Shigella dysenteriae type I [3]. STEC strains produce one or both of two major types of Shiga toxins, designated *stx1* and *stx2*. These toxins have a cytotoxic effect on intestinal epithelial cells that perhaps causes the characteristic bloody diarrhea [4].

The STEC family is very diverse and a broad range of O:H serotypes have been found to be associated with human diseases [5]. However, epidemiological evidence indicates that certain STEC subsets, for example O157:H7, account for a disproportionately large number of serious infections. *Escherichia coli* O157:H7, the most frequently identified STEC serotype, alone causes an estimated 60 deaths and 73,000 illnesses annually in the United States [6,7].

In a large PCR-based study conducted in Belgium, of more than 10,000 stool specimens, STEC were the third most prevalent enteric pathogen after Campylobacter and Salmonella [8]. This large morbidity caused by STEC illustrates the threat that these organisms present to public health globally [9].

E. coli O157:H7 does not ferment sorbitol thus the use of a selective medium, such as sorbitol MacConkey (SMAC) agar is of great value [10]. But in the past decade, many other STEC serotypes (non-O157) that ferment sorbitol have

caused large outbreaks [11] and emerged as a common cause of STEC diseases [12,13].

PCR can effectively overcome this diagnostic problem as it can efficiently detect Shiga toxins 1 and 2 in both O157 and non-O157 STEC serotypes. Direct PCR analysis is increasingly being used for the detection of STEC in primary cultures of feces or foods. A positive reaction with primers specific for *stx1* or *stx2* is sufficient to confirm the presence of STEC in a stool sample [14,15]. The use of primers capable of detecting accessory virulence genes such as *eae* [present on a pathogenicity island named the locus for enterocyte effacement (LEE) on the chromosome of some STEC] and *hly* that encodes for intimin and EHEC enterohemolysin provides additional clinically suitable information of significant epidemiological value [16, 17].

Diarrhea is the most common disease in children in Pakistan. It is very relevant to assess the frequency of different diarrhea causing pathogens and characterize them. No STEC study has been done in Pakistan. This objective of this study was to find the share of STEC in diarrheal diseases and association of related genes with these pathogens.

Materials and Methods

Selection of patients

Two hundred stool samples were collected from children (less than 10 years of age) admitted to the Children Ward, Allied Hospital Faisalabad, Pakistan, during April to September 2005. These children were clinically diagnosed with diarrhea and typically suffered from acute diarrhea, vomiting, abdominal cramps, and fever.

Sample collection

Clinical stool samples were collected in 10 ml of transport medium (1 M Tris, 70 mM CaCl2, pH 7.2) in sterile plastic bottles and kept at 4°C. These samples were transported to the laboratory under refrigerated conditions.

Cultivation of bacteria from stool samples

After centrifugation of medium containing stool samples at 4000 rpm for 5 min, 1 ml of supernatant was poured on MacConkey agar (Merck, Germany) and incubated at 37°C for 24 hours. Trypticase soy broth (TSB) (Merck, Germany) containing 20% glycerol was poured onto these plates and kept at room temperature for 4 hours with gentle shaking. The suspended growth was then aliquoted (1 ml) in small Eppendorff tubes and stored at -20°C until further use.

DNA extraction from mixed culture

One hundred µl of thawed stored culture were inoculated into 3 mL of TSB and kept at 37°C overnight. The DNA was extracted by conventional phenol/chloroform method [18]. The integrity of the DNA was checked by electrophoresis on 0.8% agarose gel.

Primers

Four primer pairs were used simultaneously to target genes *stx1* [19], *stx2* [20], *eaeA* [21] and *hlyA* [8]. The oligonucleotide sequences along with the targeted gene regions and amplified products sizes are listed in Table 1.

Table 1. PCR primers and genes targeted.

Target Gene	Primer	Sequence (5'-3')	Position (nucleotides)	Amplicon size (bp)	Reference
stx1	*stx*1F *stx*1R	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	454-633 of A subunit	180	Jackson et al (19)
stx2	*stx*2F *stx*2R	GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	nt 603-857 of A subunit	255	Jackson et al (20)
eae	*eae*AF *eae*AR	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	27-410	384	Yu and Kaper (21)
hly	*hly*AF *hly*A	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGC	70-603	534	Schmidt et al (8)

Multiplex PCR analysis

A 100 µl PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 100 pmol of each primer, 50 nmol of each dNTP, 1.0 µl of Taq polymerase (Fermentas, USA), 20 µl of template, and distilled water to make the volume.

The thermal cycler (MasterCycler; Eppendorf, Hamburg, Germany) conditions were as follows:

95°C for 5 minutes for initial denaturation of the DNA, followed by 30 cycles each of 94°C for 30 seconds (denaturation), 48°C for 1 minute (primer annealing) and 72°C for 30 seconds (extension), and a final extension at 72°C for 10 minutes.

The amplified products in each case were separated by gel electrophoresis on 2% agarose gels at 100 V for 60 minutes and photographed using Eagle Eye (Stratagene, USA).

Isolation of STEC colonies

The multiplex PCR positive STEC samples were streaked onto Sorbitol MacConkey agar (SMAC) (OXOID, England). White colonies were selected for isolation of non-sorbitol fermenting O157:H7. In addition, five pink colonies were also picked from each plate for sorbitol fermenting STEC. All selected colonies were inoculated into TSB for enrichment.

Detection of stx1 and stx2 by regular PCR

For confirmation of presence of *stx* genes, 100 µl of bacterial growth was suspended in 900 µl Tris buffer (10mM at pH 8.0), boiled at 100°C for 30 minutes and centrifuged at 12000 rpm for 10 seconds. The supernatant was directly used as a template in the PCR for *stx1* and *stx2* genes.

The PCR for amplification of both genes was conducted under similar conditions. Briefly, 100 µl of a PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 75 pmol of each primer, 50 nmol of each dNTP, 1 µl of Taq polymerase (Fermentas, USA), 20 µl of template, and distilled water to make the volume.

Samples were subjected to 35 PCR cycles (MasterCycler; Eppendorf, Hamburg, Germany), each consisting of 1 minute of denaturation at 95°C, 1 minute of annealing at 50°C and 1.5 minutes of elongation at 72°C followed by a final extension at 72°C for 10 minutes.

The amplified products were separated by gel electrophoresis on 2% agarose gels at 100 V for 60 minutes and photographed using Eagle Eye (Stratagene, USA).

Restriction analysis

Amplified products of *stx1* and *stx2* (5 µl each containing 0.5 and 1 µg DNA for complete and partial digestion respectively) were digested with 10 U of restriction endonucleases Tail (Fermentas, USA) and Hae111 (Fermentas, USA) respectively for 24 hours at 37°C in a final volume of 20 µl as recommended by the manufacturer. The digested products in each case were separated by gel electrophoresis on 2% agarose gels at 100 V for 60 minutes and photographed using Eagle Eye (Stratagene, USA).

Serotyping

Serotypes of the STEC positive samples were determined by slide agglutination with commercially available O polyvalent and monovalent antisera (Statens Serum Institut, Copenhagen, Denmark). Polyvalent Pool 1 which includes O26, O103, O111, O145, and O157 was used for screening. In addition, monovalent antisera for O26 and O157 were also used. The procedures were carried out according to manufacturer's recommendations.

Table 2. Clinical picture of STEC positive patients.

STEC isolate	Age	Sex	Fever	Vomiting	Diarrhea			Clinical Diagnosis
					Mucus	Watery	Bloody	
Ms 4	10 month	F	- ve	- ve	- ve	✓	- ve	Acute gastroenteritis
Ms 19a	2 month	M	✓	✓	✓	- ve	- ve	Sepsis
Ms 19c	2 month	M	✓	✓	✓	- ve	- ve	Sepsis
Ms 29	8 month	F	✓	✓	✓	- ve	- ve	Acute gastroenteritis
Ms 43	2 years	F	✓	✓	- ve	- ve	✓	Acute gastroenteritis
Ms 60a	6 month	F	✓	✓	- ve	✓	- ve	Acute gastroenteritis
Ms 60c	6 month	F	✓	✓	- ve	✓	- ve	Acute gastroenteritis
Ms 70	10 month	F	✓	✓	- ve	- ve	✓	Acute gastroenteritis
Ms 92	2 years	F	✓	✓	- ve	✓	- ve	Dehydration
Ms 119	8 month	M	✓	- ve	- ve	✓	- ve	Acute gastroenteritis
Ms 121	2 years	M	✓	✓	- ve	✓	- ve	Acute gastroenteritis
Ms 126	2 month	F	✓	✓	- ve	✓	- ve	Acute gastroenteritis
Ms 168	10 years	F	- ve	✓	- ve	✓	✓	Acute gastroenteritis
Ms 169	2 years	F	✓	✓	- ve	✓	✓	Acute gastroenteritis

✓: positive; - ve: negative, NOTE : Numerals indicate patient number. Alphabets were added when more than one isolate was obtained from a patient.

Results

Prevalence of STEC as determined by multiplex PCR

In our study, a total of 200 samples from patients suffering with diarrhea were examined for *stx* genes along with other virulence factors. Multiplex PCR (Figure 1) showed that in 22 (11%) patients one or both Shiga toxin genes (*stx*) were detectable. However, only 14 STEC isolates from 12 patients were obtained by cultivation on MacConkey agar and Sorbitol MacConkey agar which were subsequently confirmed by PCR for

stx1 and *stx2* followed by restriction analysis as described below. This was understandable because it was not possible to check all colonies on culture plates. Only 5 colonies were selected in each case. Among these isolates, 11 (78.5%) each carried either the *stx1* or *stx2* gene while 8 (57.1%) harbored both *stx1* and *stx2*. Multiple isolates from one patient were found to have different gene profiles (Table 3). The presence of other virulence factors in these STEC as determined by multiplex PCR is also shown in Table 3. Only two isolates showed the presence of all four genes.

Table 3. Distribution of various virulence gene of STEC.

STEC strains	*Stx1*	*Stx2*	*hly*	*eae*	Serotypes
Ms 4	-	+	-	-	O26
Ms 19a	+	+	-	+	O157
Ms 19c	+	+	+	+	O157
Ms 29	-	+	-	-	ND
Ms 43	-	+	-	+	ND
Ms 60a	+	-	-	-	ND
Ms 60c	+	+	-	-	ND
Ms 70	+	+	+	+	O157
Ms 92	+	+	+	-	ND
Ms 119	+	+	-	-	ND
Ms 121	+	+	-	-	Pool A
Ms 126	+	+	-	-	ND
Ms 168	+	-	-	+	O26
Ms 169	+	-	-	+	ND

ND: Not determined; +: positive; -: negative.

PCR for stx1 and stx2

After multiplex PCR, presence of *stx1* and *stx2* was confirmed by PCR. The results are shown in Figure 1.

Confirmation of stx genes by restriction analysis

The amplification products obtained by PCR of *stx1* and *stx2* were restricted with Tail endonuclease and Hae111 endonuclease respectively. Products of 126 bp and 54 bp for *stx1* (180bp), and 167bp and 88 bp for *stx2* (255bp) were obtained (Figure 2).

Serotyping

The 14 STEC isolates were screened by Polyvalent Pool 1 (Statens Serum Institut, Copenhagen, Denmark) that includes serotypes O26, O103, O111, O145, and O157. Six of the isolates gave positive agglutination reaction. Each of these isolates was further screened by monovalent antisera for 026 and 0157. Two of these isolates tested positive for O26 and three for O157. One of the isolates gave no reaction with these antisera.

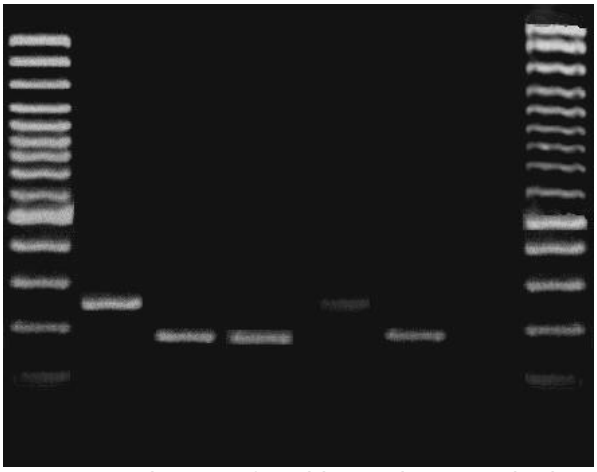

Figure 1. Regular PCR for Shiga toxin genes (*stx*).
Electrophoresis pattern on 2% Agarose gel. Lane 1 and 8: Molecular weight marker (SM0323S) showing 3000, 2000, 1031, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bps markers in descending order; Lane 3, 4, and 6: Isolates Ms 92, Ms 168 and Ms 169. Amplification of *stx1* gene (180 bps); Lane 2 and 5: Isolates Ms 4 and Ms 29. Amplification product of *stx2* gene (255 bps). Lane 7: Negative control without DNA.

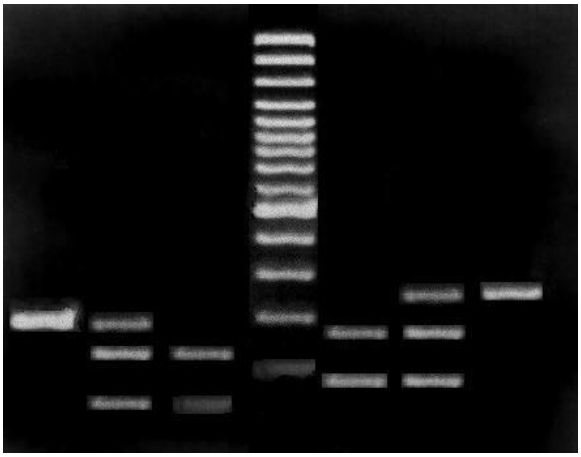

Figure 2. Restriction analysis for *stx1* and *stx2* genes
Electrophoresis pattern on 2% Agarose gel.
Lane 4: Molecular weight marker (SM0323S) showing 3000, 2000, 1031, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bps markers in descending order. Lane 1: Isolate Ms 92. Unrestricted amplification product for *stx1* (180 bps) Lane 2: Isolate Ms 92. Restriction of *stx1* amplification product by Tail showing original product (180 bps) and two restriction products of 126 bps and 54 bps (substrate concentration 1.0 µg). Lane 3: Isolate Ms 92. Restriction of *stx1* amplification product by Tail showing two restriction products of 126 bps and 54 bps (substrate concentration 0.5 µg). Lane 5: Isolate Ms 4: Restriction of *stx2* amplification product by Hae III showing two restriction products of 167 bps and 88 bps (substrate concentration 0.5 µg). Lane 6: Isolate Ms 4: Restriction of *stx2* amplification product by Hae III showing original product (255 bps) and two restriction products of 167 bps and 88 bps (substrate concentration 1.0 µg). Lane 7: Isolate Ms 4. Unrestricted amplification product for *stx2* (255 bps).

Prevalence of hly and eae in diarrhea patients

All the 200 samples were studied for virulence genes enterohemolysin (*hly*) and intimin (*eae*). The

hlyA and *eae* gene were found in 13 (6.5%) and 16 (8%) of the patients studied respectively. Among the 14 isolates, only three (21.4%) were positive for enterohemolysin (*hly*) gene. However, intimin (*eae*) gene was much more frequent, being detectable in 6 (42.8%) isolates.

Discussion

Shiga toxin producing *Escherichia coli* (STEC) are important emerging pathogens associated with different clinical manifestations such as bloody and non-bloody diarrhea, acute gastroenteritis and advance cases of hemolytic uremic syndrome (HUS). STEC were first implicated in disease in the early 1980s. The high morbidity of STEC around the world and its presence in five continents has focused a major public health concern. In fact, according to some experts, *stx*-positive *E. coli* are perhaps the most dangerous enteric pathogens that clinical microbiologists in developed countries are likely to encounter in future [22]. They are not always associated with symptoms as they have been isolated from stools of healthy individuals [23] as well.

A lot of attention has been given to these pathogens in developed countries and there is a relatively clear picture regarding their prevalence. For example, incidence of STEC has been reported to be 1.6% in Australia. The incidence was 2.5 times more in samples that contained blood [24]. In continental Europe, the incidence of STEC is low (<1/105), but in the United Kingdom the incidence has been as high as 2.7/105 (in 1997) [25]. In contrast, from most of the developing countries in which STEC is more relevant, very little scientific data is available [26]. No such report is available from Pakistan.

We found that incidence of STEC was significantly higher than that reported in developed countries. Twenty-two (11%) patients harbored one or both Shiga toxin genes as indicated by multiplex PCR. However, we could isolate only 14 STEC after cultivation. It is understandable because it was not possible to check every colony of the pool culture.

Historically O157:H7 is a dominant STEC serotype in many parts of the world and has been the type most commonly associated with large outbreaks [27]. However, now it is known that a large number of other serotypes are involved. In Finland, 56 non-O157 isolates collected from 1990 to 2000 were characterized and found to be belonging to 29 different serotypes [28]. Similarly, continental Europe is faced with emerging non-O157-infections (e.g., O111, O103, and O26 [25].

Our results show that O157:H7 serotype which is generally considered to be most important in North America and Western Europe, was almost insignificant among our isolates, being present in only 3 (21.4%) cases.

It has been reported that *stx2* is more common as compared with *stx1*. Ekland et al. [28] found that most (n = 63) of the 71 STEC strains isolated carried the *stx2* gene only; five carried the *stx1* gene only; and three carried both genes. The *eaeA* gene was detected in all other isolates except five non-O157 strains. Our results were different. We found equal incidence of *stx1* and *stx2* genes (11 out of 14) in our isolates, whereas 8 harbored both *stx1* and *stx2*. We also found that *eaeA* gene was not frequently detected (6 out of 14). This may be attributed to the fact that most of our isolates were not O157:H7. In these 14 patients, only three (21.4%) isolates were positive for enterohemolysin (*hly*) gene.

Our study shows that the occurrence of STEC in this region is significantly high. It is anticipated that similar situations exist in other parts of Pakistan as well. It is concluded that STEC infection has the potential to become a serious health problem. More extensive studies should be conducted to map the features of local isolates so that meaningful measures can be taken to control the spread of these organisms.

References

1. Besser RE, Griffin PM, Slutsker L (1999) *Escherichia coli* 0157:H7 gastroenteritis and the hemolytic uremic syndrome: an emerging infectious disease. Annu. Rev. Med 50: 355-367.
2. Martin D, MacDonald K, White K, Soler J, Osterholm M (1990) The epidemiology and clinical aspects of the hemolytic uremic syndrome in Minnesota. N. Engl. J. Med 323: 1161-1167.
3. Herold S, Karch H, Schmidt H (2004) Shiga toxin-encoding bacteriophages-genomes in motion. Int J Med Microbiol. 294(2-3):115-121.
4. Boerlin P, McEwen SA, Boerlin-Petzold F, Wilson JB, Johnson RP, Gyles CL (1999) Associations between virulence factors of Shiga toxin producing *Escherichia coli* and disease in humans. J. Clin. Microbiol 37: 497–503.
5. Karmali MA (1989) Infection by verotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev 2: 15–38.
6. Mead PS., Slutsker L,. Dietz V, McCaig LF, Breese JS, Shapiro C, Griffin PM, Tauxe RV (1999) Food-

related illness and death in the United States. Emerg. Infect. Dis 5: 607-625.
7. Siegler RL, Pavia AT, Christofferson RD, Milligan MK (1994) A 20-year population-based study of postdiarrheal hemolytic uremic syndrome in Utah. Pediatrics 94: 35-40.
8. Schmidt H, Beutin L, Karch H (1995) Molecular analysis of the plasmid- encoded hemolysin of *Escherichia coli* O157:H7 strain EDL933. Infect. Immun 63: 1055-1061.
9. Griffin PM, Bell BP, Cieslak PR, Tuttle J, Barrett TJ, Doyle MP, McNamara AM, Shefer AM, Wells JG (1994) Large outbreak of *Escherichia coli* O157:H7 infections in the western United States: the big picture, p. 7-12. In M. A. Karmali, and A. G. Goglio (ed.), Recent advances in verocytotoxin-producing *Escherichia coli* infections. Elsevier Science B.V., Amsterdam, The Netherlands.
10. March SB, Ratnam S (1986) Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. J. Clin. Microbiol 23: 869-872.
11. Liesegang A, Sachse U, Prager R, Claus H, Steinrück H, Aleksic S, Rabsch W, Voigt W,. Fruth A, Karch H, Bockemühl J, Tschäpe H (2000) Clonal diversity of Shiga toxin-producing *Escherichia coli* O157:H7/H in Germany—a ten-year study. Int. J. Med. Microbiol 290: 269-278.
12. Caprioli A, Tozzi AE, Rizzoni G, Karch H (1997) Non-O157 shiga toxin- producing *Escherichia coli* infections in Europe. Emerg. Infect. Dis 3: 578-579.
13. Fey PD, Wickert RS, Rupp ME, Safranek TJ, Hinrichs SH (2000) Prevalence of non-O157:H7 shiga toxin-producing *Escherichia coli* in diarrheal stool samples from Nebraska. Emerg. Infect. Dis 6: 530-533.
14. Paton AW, Paton JC (1998) Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli hlyA*, rfbO111, and rfbO157. J. Clin. Microbiol. 36: 598–602.
15. Aranda KR, Fagundes-Neto U, Scaletsky IC (2004) Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic Escherichia coli and Shigella spp J Clin Microbiol. (12): 5849-5853.
16. McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB (1995) A genetic locus for enterocyte effacement conserved among diverse enterobacterial pathogens. Proc. Natl. Acad. Sci USA 92: 1664-1668.
17. Schmidt H, Karch H, (1996) Enterohemolytic phenotypes and genotypes of Shiga-toxin-producing *Escherichia coli* O111 strains from patients with diarrhea and hemolytic-uremic syndrome. J. Clin. Microbiol. 34: 2364- 2367.
18. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a Laboratory Manual, 2nd edn. New York: Cold Spring Harbor Laboratory Press.
19. Jackson MP, Newland JW, Holmes RK, O'Brien AD (1987) Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli*. Microb. Pathog 2: 147-153.
20. Jackson MP, Neill RJ, O'Brien AD, Holmes RK, Newland JW (1987) Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli*. FEMS Microbiol. Lett. 44: 109-114.
21. Yu J, Kaper JB (1992) Cloning and characterization of the *eae* gene of enterohaemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol 6: 411- 417.
22. Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11: 142-201.
23. Boerlin P, McEwen SA, Boerlin-Petzold F, Wilson JB, Johnson RP,Carlton LG (1999) Associations between Virulence Factors of Shiga Toxin-Producing *Escherichia coli* and Disease in Humans. J. Clin. Microbiol. 37:497-503.
24. Bennett-Wood VR, Russell J, Bordun A, Johnson PDR, Robins-Browne RM (2004) Detection of enterohaemorrhagic *Escherichia coli* in patients attending hospital in Melbourne, Australia. Pathology. 36: 345-351.
25. Overview of EHEC Infections and Hemolytic Uremic Syndrome (HUS) in Europe. 1999. Emerg. Infect. Dis. Volume 5.
26. Khan A, Yamasaki S, Sato T, Ramamurthy T, Pal A, Datta S, Chowdhury NR, Das SC, Sikdar A, Tsukamoto T, Bhattacharya SK, Takeda Y, Nair GB (2002) Prevalence and Genetic Profiling of Virulence Determinants of Non-O157 Shiga Toxin-Producing *Escherichia coli* Isolated from Cattle, Beef, and Humans, Calcutta, India. Emerg. Infect. Dis 8(1): 54-62.
27. Paton JC, Paton AC (1998) Pathogenesis and Diagnosis of Shiga Toxin-Producing *Escherichia coli* Infections. Clin. Microbiol. Rev.11:450-479.
28. Eklund M, Scheutz F, Siitonen A (2001) Clinical Isolates of Non-O157 Shiga Toxin-Producing *Escherichia coli*: Serotypes, Virulence Characteristics, and Molecular Profiles of Strains of the Same Serotype. J. Clin. Microbiol. 39: 2829-2834.

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