Contents lists available at ScienceDirect

IF: 0.926

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi: 10.1016/S1995-7645(14)60182-6

Prevalence of shiga toxins (stx_1, stx_2), *eaeA* and *hly* genes of *Escherichia coli* O157:H7 strains among children with acute gastroenteritis in southern of Iran

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ARTICLE INFO

Article history:
Received 26 October 2014
Received in revised form 10 November 2014
Accepted 22 December 2014
Available online 20 January 2015

Keywords:
Escherichia coli O157:H7
Acute gastroenteritis
Virulence genes
Multiplex PCR

ABSTRACT

Objective: To survey the prevalence severe diarrhea arising from these bacteria in children under 5 years old in Marvdasht. **Methods:** In this study faecal sample from 615 children aged <5 years old who were hospitalized for gastroenteritis in Fars hospitals in Iran were collected and then enriched in *Escherichia coli (E. coli)* broth and modified tryptone soy broth with novobiocin media. Fermentation of sorbitol, lactose and β -glucoronidase activity of isolated strains was examined by CT-SMAC, VRBA and chromogenic media respectively. Then isolation of *E. coli* O157:H7 have been confirmed with the use of specific antisera and with multiplex PCR method presence of virulence genes including: stx_1, stx_2, *eaeA*, *hly* has been analyzed. **Results:** *E. coli* O157:H7 was detected in 7 (1.14%) stool specimens. A significant difference was seen between detection rate of isolated bacteria from age groups 18-23 months and other age groups (P=0.004). Out of considered virulence genes, only 1 of the isolated strains (0.16%) the stx_1 and *eaeA* genes were seen and also all isolated bacteria had resistance to penicillin, ampicillin and erythromycin antibiotics. **Conclusions:** We found that children < 2 years of age were at highest risk of infection with *E. coli* O157:H7. Regarding severity of *E. coli* O157:H7 pathogenesis, low infectious dose and lack of routine assay for detection of these bacteria in clinical laboratory, further and completed studies on diagnosis and genotyping of this *E. coli* O157:H7 strain has been recommended.

1. Introduction

Shiga toxin-producing *Escherichia coli* (*E. coli*) (STEC), also called verocytotoxin-producing *E. coli* (VTEC), have emerged as pathogens that can cause food-borne infections and severe and potentially fatal illnesses in humans, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) which is the main cause of acute renal failure in children. Most outbreaks of HC and HUS have been attributed to strains of the enterohemorrhagic serotype O157:H7[1,2]. The ability of *E. coli* O157:H7 to cause severe diseases in humans is related to their capacity to secrete shiga toxins (Stx_1 and Stx_2) or verocytotoxins (VT1 and VT2) and variants of these toxins[1,3]. Another virulence-associated factor of most STEC isolates associated with severe disease is intimin, a 94-kDa outer membrane protein, which is encoded by the *eae* gene on a ca. 34 kb chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE). This locus is associated

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with the intimate adherence of *E. coli* to epithelial cells, initiation of host signal transduction pathways, and the formation of attaching-and-effacing intestinal lesions[3]. A factor that may also affect virulence of *E. coli* O157:H7 is the enterohemolysin (Ehly), also called enterohemorrhagic *E. coli* haemolysin (EHEC- HlyA), encoded by the *hly* gene[1].

E. coli O157:H7 infection has been often associated with the consumption of contaminated ground beef, raw milk and other bovine products, thus cattle's are suspected to be a primary reservoir. But bacteria also have been isolated from domestic and wild animals. Moreover, recent outbreaks of food borne illness associated with eating fresh products have heightened concerns that these foods contaminated with STEC may be an increasing source of illness. In the past decades, outbreaks of diseases caused by STEC have been associated with the consumption of leaf lettuce, potatoes, radish sprouts and raw vegetables. Fruit-related outbreaks have also been caused by the consumption of fresh-pressed apple juice[1,4].

Detection of *E. coli* O157:H7 in the clinical laboratory depends on distinguishing the pathogenic serotypes from normal faecal flora containing commensal strains of *E. coli*. Fortunately, *E. coli* O157:H7 has two unusual biochemical markers; delayed fermentation of D-sorbitol and lack of β-D-glucuronidase activity, which help to phenotypically separate O157:H7 isolates from nonpathogenic *E. coli* strains. One of these markers (delayed sorbitol fermentation) is able to develop selective media (Sorbitol-MacConkey; SMAC) which aids in the initial recognition of suspicious colonies isolated from bloody stools. The selectivity of SMAC agar has been improved with the addition of cefixime-rhamnose (CR-SMAC), cefixime-tellurite (CT-SMAC) and 4-methylumbelliferyl- β-D-glucuronide (MSA-MUG)[4].

Fratamico *et al* described a multiplex PCR capable of detecting *stx*$_1$, *stx*$_2$, *eaeA* and EHEC *hlyA* sequences[5]. However, this PCR was not tested with faecal samples; primers for each target gene sequence showed differential sensitivities. Paton and Paton developed a multiplex PCR utilizing four PCR primer pairs for the detection of *stx*$_1$, *stx*$_2$, *eaeA* and EHEC *hlyA* in human feces and foodstuffs[6,7]. The multiplex PCR system for detecting virulence genes of STEC, reported by Paton *et al*, Pradel *et al*, Osek, Blanco *et al*, and Mohsin *et al*[8-11]. The aim of this study was to evaluate the prevalence of virulence factors and antibiotic resistance of *E. coli* O157:H7 in Fars providence, Iran.

2. Materials and methods

2.1. Sample collections

Between September 2008 and September 2009, stool samples were collected from children aged <5 years old were hospitalized with gastroenteric symptoms in Hospitals in Fars Province, Iran. A detailed history of the patients is obtained, including information on the age, sex, source of drinking water, clinical presentation and antibiotic usages.

2.2. Enrichment procedures

For *E. coli* O157:H7 detection, each faecal sample (1 g) was enriched in 5 mL *E. coli* (EC) broth (Oxoid) and modified tryptone soy broth (Difco) with 20 mg/L of novobiocin (Sigma) and incubated overnight at 37 ℃.

2.3. Culture methods

The enrichment broth was inoculated into sorbitol-MacConkey agar (Lab.M) supplemented with cefixime (0.05 mg/L) and tellurite (2.5 mg/L) (Oxoid) (CT-SMAC) for isolation (for 24 h at 37 ℃)[12,13]. Sorbitol-negative colonies were confirmed as *E. coli* by biochemical tests and then colonies transferred on chromogenic *E. coli* O157 agar and incubated at 37 ℃ for 24 h. Up to 10 colonies were tested for agglutination with *E. coli* O157:H7 latex tests[13,14].

2.4. Multiplex PCR

Presence of virulence genes were detected by multiplex PCR using specific primers for amplification of *stx*$_1$, *stx*$_2$, *eaeA* and *hly* genes (Table 1)[15] and isolates were confirmed as *E. coli* O157:H7 by using specific primers for *rfb* O157 and *flic* H7 genes[16].

Table 1

Primers used in multiplex PCR.

Primer	Oligonudeotide sequences (5′-3′)	Expected size (bp)
stx$_1$-F	ACACTGGATGATCTCAGTGG	614
stx$_1$-R	CTGAATCCCCCTCCATTATG	
stx$_2$-F	CCATGACAACGGACAGCAGTT	779
stx$_2$-R	CCTGTCAACTGAGCAGCACTTTG	
eaeA-F	GTGGCGAATACTGGCGAGACT	890
eaeA-R	CCCCATTCTTTTTCACCGTCG	
hlyA-F	ACGATGTGGTTTATTCTGGA	165
hlyA-R	CTTCACGTGACCATACATAT	

E. coli O157:H7 strain used as control was 933j. Bacterial DNA was extracted from antisera-positive samples with a DNP™ kit (CinnaGene). PCR assays were carried out in a 50 μL volume containing 4 μL of nucleic acid templates prepared from cultures. And 10 mM Tris-HCl (pH 8.4), 10 mM KCl, 3 mM $MgCl_2$, 20 pmol concentrations of each primer, 0.2 mM dNTPs, and 1 U of *Taq* DNA polymerase were added to the reaction mixtures. PCR conditions consisted of an initial 95 ℃ denaturation step for 3 min followed by 35 cycles of 95 ℃ for 20 s, 58 ℃ for 40 s and 72 ℃ for 90 s. The final extension cycle was followed by at 72 ℃ for 5 min. Amplified DNA fragments were resolved by gel electrophoresis using 1.5% agarose gels. Gels were stained with 0.5 μL of ethidium bromide (EtBr) per mL, visualized and photographed under UV illumination[4].

2.5. Antimicrobial susceptibility test

Antimicrobiol susceptibility testing was based on the disk diffusion method as recommended by the Clinical and Laboratory Standard Institute (CLSI) guidelines, with ampicillin (10 μg), penicillin (10 μg), cephalexin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), tetracycline (30 μg) and trimethoprim /sulfamethoxazole (25 μg)[4,17].

2.6. Statistic analysis

Statistical analysis were conducted with the use of SPSS version 14 and the χ^2 test, Fisher`s exact test. $P<0.05$ was considered statistically significant.

3. Results

In this study, a total of 615 samples were collected. The results showed that 77 of 89 sorbitol nonfermenting colonies from CT-SMAC were lactose ferment (12.52%) and 7(1.14%) were *E. coli* O157:H7. Out of 7 *E. coli* O157:H7 strains 42.86% were isolated from girls and 57.14% from boys. No significant correlation was found between *E. coli* O157:H7 isolation and sex. The mean age of patients was 11.7 months (range 1-24 months). A significant association was found between *E. coli* O157:H7 isolation among different age groups (P = 0.004). The highest rates of *E. coli* O157:H7 isolation (42.86%) was detected in children 18-23 months of age. Of the 7 patients infect with *E. coli* O157:H7; 4 (57.14%) reported diarrhea, 2 (28.57%) had vomiting, 1 (14.29%) had fever and 1 (14.29%) were hospitalized for 5 day and 3 (42.86%) of patients reported that they received antibiotics.

71.43% of isolates were susceptible to chloramphenicol and 57.14% to tetracycline and trimethoprim/ sulfamethoxazole. All *E. coli* O157:H7 isolated were resistant to penicillin, ampicillin and erythromycin.

As shown by PCR, 1(14.29%) of the isolates harbored stx_1 and *eaeA* genes (Figure 1) and none of the isolates had stx_2 and *hly* genes. All of these isolates confirmed to be *E. coli* O157:H7 by using specific primers for *rfb* O157 and *flic* H7 genes (Figure 2).

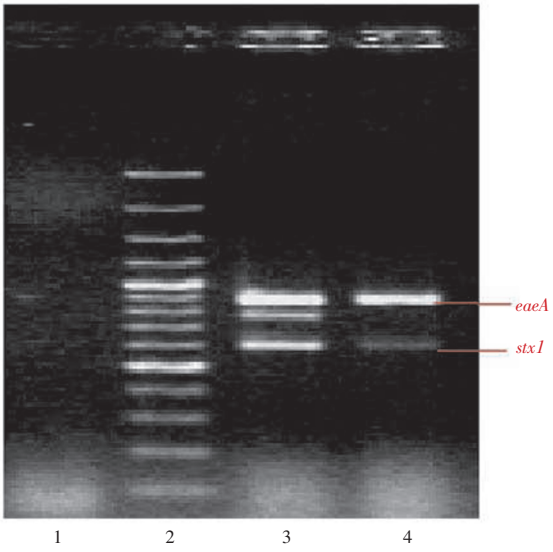

Figure 1. Multiplex PCR for detection of virulence genes *E. coli* O157:H7.
Lane 1, negative control; lane 2, ladder 100 bp; lane 3, positive control stx_1 (614 bp), stx_2 (779bp), *eaeA* (890 bp); lane 4, positive sample (stx_1, *eaeA*); Agaros gel concentration: 1.5%.

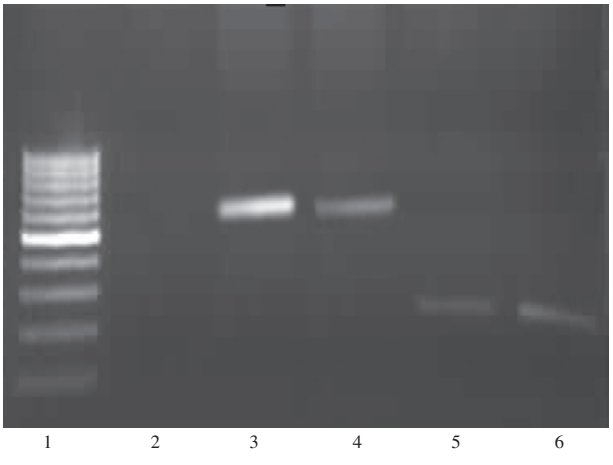

Figure 2. Multiplex PCR for detection of specific genes serotype *E. coli* O157:H7.
Lane 1, ladder 100 bp; lane 2, negative control; lane 3, 4: positive sample *fliC* H7 (259 bp; lane 5, 6: positive sample *rfb* O157 (625); Agaros gel concentration: 1.5%.

4. Discussion

Gastroenteritis is one of the most frequent diseases in the world and continues to be one of the main causes of death in developing countries. The high morbidity of *E. coli* O157:H7 around the world and its presence in five continents has focused a major public health concern. A lot of attention has been given to these pathogens in developed countries and there is a relatively clear picture regarding their prevalence[11]. STEC strains that cause human infections belong to a large number of O:H serotypes. Most outbreaks of HC and HUS have been attributed to strains of the enterohemorrhagic serotype O157:H7. However, as Non-O157 STEC is more prevalent in animals and as contaminant in foods, humans are probably more exposed to these strains[1].

In Bangladesh Shiga toxin genes (*stx*) were detected by multiplex PCR in nine samples (2.2%) from hospitalized patients and 11 samples (6.9 %) from the community patients. Two isolates were positive for the *E. coli* attaching-and-effacing (*eae*) gene and four were positive for the enterohaemolysin (*hly*EHEC) gene and enterohaemolysin production[12]. In Pakistan results showed that 11 (78.5%), 6 (42.8%), 3 (21.4%) and 11 (78.5%) STEC isolates were positive for stx_1, *eae*, *hly* and stx_2 genes respectively[11]. Seropathotypes O157:H7 stx_1-*eae*, are only observed in *E. coli* O157:H7 that cause human infections in South of Iran. Within the human disease-associated strains, those producing Shiga toxin type 2 (Stx_2) appear to be more commonly responsible for serious complications such as HUS than those producing only Shiga toxin type 1 (Stx_1)[7]. Kawano *et al*[18] conclude that *stx* genotype is one of the important factors of clinical outcome of STEC O157 infection and that pathogenicity for humans was higher in the stx_2 genotype strains. The *eae* gene, which has been shown to be necessary for attaching and effacing activity, encodes a protein which is termed intimin. Numerous investigators have underlined the strong association between the carriage of *eae* gene and the capacity of STEC strains to cause severe human illnesses, especially HUS. This important virulence gene was detected in 14.29% of *E. coli* O157:H7 in the present study. Nevertheless, production of intimin is not essential for pathogenesis, because a number of sporadic cases of HUS have been caused by *eae*-negative O157 STEC strains[1].

Antimicrobial resistance patterns were observed most commonly to ampicillin (25.4%), tetracycline (23.8%) and less frequently to cephalothin (11,1%), nalidixic acid (6.4%) in India. The USA study about antibiotic resistance showed that all isolates were resistant to tilmicosin, and most isolates were susceptible to trimethoprim/ sulfamethoxazole and ciprofoloxacin[4]. In Malaysia, resistance was observed mostly towards bacitracin (100%), ampicillin (57%), cephalothin (53%) and carbenicillin (30%). The antibiotic resistant patterns to ampicillin, fosfomycin, kanamycin and vancomycin were observed in Japan[4]. From these data, *E. coli* O157:H7 was mainly resistant to ampicillin and tetracycline. Resistance patterns of Iran isolates were approximately similar to those of the USA and Malaysia. However, antibiotics are a risk factor for HUS and their use is therefore contraindicated in patients with STEC infection[17].

In this study it was found that *E. coli* O157:H7 strains could be isolated from diarrheal as well as asymptomatic children. Epidemiologic data were not collected regarding contaminated water as a possible source of *E. coli* O157:H7 infection in the patients. Stool cultures of all patients with acute bloody diarrhea should be tested for *E. coli* O157:H7 to identify those at risk of HUS[19]. However, serotyping, cytotoxicity assays or genotyping for *E. coli* O157:H7 are not routinely performed in Iran. Further studies are needed to identify the pathogenic mechanisms of this *E. coli* O157:H7 strains and to determine the faecal carriage rate in healthy children.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors are grateful to the Islamic Azad University, Jahrom branch, for their executive support of this project.

References

[1] Mora A, Blanco M, Blanco JE, Dahbi G, lópez C, Justel P, et al. Serotypes, virulence genes and intimin types of shiga toxin (verocytotoxin)- producing *Escherichia coli* isolates from minced beef in Lugo (Spain) from 1995 through 2003. *BMC Microbiol* 2007; **7**: 1-9.

[2] Prendergast DM, Lendrum L, Pearce R, Ball C, McLernon J, O'Grady D, et al. Verocytotoxigenic *Escherichia coli* O157 in beef

and sheep abattoirs in Ireland and characterisation of isolates by Pulsed-Field Gel Electrophoresis and Multi-Locus Variable Number of Tandem Repeat Analysis. *Int J Food Microbiol* 2011; **144**: 519-527.

[3] Leotta GA, Miliwebsky ES, Chinen I, Espinosa EM, Azzopardi K, Tennant SM, et al. Characterisation of Shiga toxin-producing *Escherichia coli* O157 strains isolated from humans in Argentina, Australia and New Zealand. *BMC Microbiol* 2008; **8**: 1-8.

[4] Kim JY, Kim S, Kwon N, Bae WK, Lim JY, Koo HC, et al. Isolation and identification of *Escherichia coli* O157:H7 using different detection methods and molecular determination by multiplex PCR and RAPD. *J Vet Sci* 2005; **6**: 7-19.

[5] Fratamico PM, Sackitey SK, Wiedmann M, Deng MY. Detection of *Escherichia coli* O157:H7 by multiplex PCR. *J Clin Microbiol* 1995; **33**: 2188-2191.

[6] Fagan PK, Hornitzky MA, Bettelheim KA, Djordjevic SP. Detection of shiga-like toxin (stx_1 and stx_2), intimin (*eaeA*), and enterohemorrhagic *Escherichia coli* (EHEC) hemolysin (EHEC *hlyA*) genes in animal feces by multiplex PCR. *Appl Environ Microbiol* 1999; **65**: 868-872.

[7] Paton AW, Paton JC. Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx_1, stx_2, *eaeA*, enterohemorrhagic *E. coli hlyA*, *rfb*O111, and *rfb*O157. *J Clin Microbiol* 1998; **36**: 598-602.

[8] Paton AW, Voss E, Manning PA, Paton JC. Shiga toxin producing *Escherichia coli* isolates from cases of human disease show enhanced adherence to intestinal epithelial (Henle 407) cells. *Infect Immun* 1997; **65**: 3799-3805.

[9] Osek J. Development of a multiplex PCR approach for the identification of shiga-toxin producing *Escherichia coli* strains and their major virulence factor genes. *J Appl Microbiol* 2003; **95**: 1217-1225.

[10] Blanco M, Blanco JE, Mora A, Rey J, Alonso JM, Hermoso M, et al. Serotypes, virulance genes , and intimin types of shiga toxin (verotoxin)-producing *Escherichia coli* isolates from healthy sheep in Spain. *J Clin Microbiol* 2003; **41**: 1351-1356.

[11] Mohsin M, Hussain A, Butt MA, Bashir S, Tariq A, Babar S, et al. Prevalence of shiga toxin-producing *Escherichia coli* in diarrhoeal patients in Faisalabad region of Pakistan as determined by multiplex PCR. *J Infect Developing* 2007; **1**: 164-169.

[12] Islam MA, Heuvelink AE, de Boer E, Sturm PD, Beumer RR, Zwietering MH, et al. Shiga toxin-producing *Escherichia coli* isolated from patients with diarrhoea in Bangladesh. *J Med Microbiol* 2007; 5**6**: 380-385.

[13] Pao S, Patel D, Kalantari A, Tritschler JP, Wildeus S, Sayre BL. Detection of Salmonella strains and *E. coli* O157:H7 in faeces of small ruminants and their isolation with various media. *Appl Environ Microbiol* 2005; **71**: 2158-2161.

[14] Phan Q, Mshar P, Rabatsky-Ehr T, Welles C, Howard R, Hadler J. Laboratory-confirmed non-O157 shiga toxin -producing *Escherichia coli*-connecticut, 2000-2005. *MMWR* 2007; **56**: 29-31.

[15] Santaniello A, Gargiulo A, Borrelli L, Dipineto L, Cuomo A, Sensale M, et al. Survey of shiga-toxin producing *Escherichia coli* in urban pigeons (Columba Livia) in the City of Napoli, Italy. *Ital J Anim Sci* 2007; **6**: 313-316.

[16] Badouei MA, Zahraei Salehi T, Rabbani Khorasgani M, Tadjbakhsh H, Nikbakht Brujeni G. Occurrence and characterisation of enterohaemorrhagic *Escherichia coli* isolates from diarrhoeic calves. *Comp Clin Pathol* 2010; **19**: 295-300.

[17] Bidet P, Mariani-Kurkdjian P, Grimont F, Brahimi N, Courroux C, Grimont P, et al. Characterization of *Escherichia coli* O157:H7 isolates causing haemolytic uraemic syndrome in France. *J Med Microbiol* 2005; **54**: 71-75.

[18] Kawano K, Okada M, Haga T, Maeda K, Goto Y. Relationship between pathogenicity for humans and *stx* genotype in shiga toxin-producing *Escherichia coli* serotype O157. *Eur J Clin Microbiol Infect Dis* 2008; **27**: 227-232.

[19] Mattar S, Vasquez E. *Escherichia coli* O157:H7 infection in Colombia. *Emerg Infect Dis* 1998; **4**: 126-127.