Prevalence of shiga toxins (stx₁, stx₂), eaeA and hly genes of *Escherichia coli* O157:H7 strains among children with acute gastroenteritis in southern of Iran

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**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: stx₁, stx₂, 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 stx₁ 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 (Stx₁ and Stx₂) 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
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 °C.

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 °C)[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 °C 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 *EHEC hlyA* genes (Table 1)[15] and isolates were confirmed as *E. coli* by biochemical tests and then colonies transferred on chromogenic *E. coli* O157 agar and incubated at 37 °C for 24 h. Up to 10 colonies were tested for agglutination with *E. coli* O157:H7 latex tests[13,14].

Table 1

| Primer | Oligonucleotide sequences (5’–3’) | Expected size (bp) |
|--------|---------------------------------|-------------------|
| *stx*₁–F | ACAAATGATGATGCTGATG | 614 |
| *stx*₁–R | CTGAACTCCCTCATATG | 779 |
| *stx*₂–F | CCATGCAAGGACAAGCAGT | 890 |
| *stx*₂–R | CCTGCAACTGGCACAGT | 165 |
| eaeA–F | GTGCAATCACTGGGAGT | 614 |
| eaeA–R | CCCATTCTTTTCTTCCGTG | 779 |
| *hlyA*–F | ACATCGTGTATTTATCTG | 890 |
| *hlyA*–R | CTGCCGTGACCCTATAT | 165 |
E. coli O157:H7 strain used as control was 933j. Bacterial DNA was extracted from antisera–positive samples with a DNAPoly 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 MgCl₂, 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 °C denaturation step for 3 min followed by 35 cycles of 95 °C for 20 s, 58 °C for 40 s and 72 °C for 90 s. The final extension cycle was followed by at 72 °C 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

Antimicrobial 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 χ² 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 stx₁ and eaeA genes (Figure 1) and none of the isolates had stx₂ 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).
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 \textit{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\cite{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\cite{1}.

In Bangladesh Shiga toxin genes (\textit{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 \textit{E. coli} attaching–and–effacing (\textit{eae}) gene and four were positive for the enterohaemolysin (\textit{hly}) gene and enterohaemolysin production\cite{12}. In Pakistan results showed that 11 (78.5\%), 6 (42.8\%), 3 (21.4\%) and 11 (78.5\%) STEC isolates were positive for \textit{stx}1, \textit{eae}, \textit{hly} and \textit{stx}2 genes respectively\cite{11}. Seropathotypes O157:H7 \textit{stx}1–\textit{eae}, are only observed in \textit{E. coli} O157:H7 that cause human infections in South of Iran. Within the human disease–associated strains, those producing Shiga toxin type 2 (\textit{Stx}2) appear to be more commonly responsible for serious complications such as HUS than those producing only Shiga toxin type 1 (\textit{Stx}1)\cite{17}. Kawano \textit{et al}\cite{18} conclude that \textit{stx} genotype is one of the important factors of clinical outcome of STEC O157 infection and that pathogenicity for humans was higher in the \textit{stx}2 genotype strains. The \textit{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 \textit{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 \textit{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 \textit{eae}–negative O157 STEC strains\cite{1}.

Antimicrobial resistance patterns were observed most frequently to ampicillin (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 ciprofloxacin\cite{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\cite{4}. From these data, \textit{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\cite{17}.

In this study it was found that \textit{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 \textit{E. coli} O157:H7 infection in the patients. Stool cultures of all patients with acute bloody diarrhea should be tested for \textit{E. coli} O157:H7 to identify those at risk of HUS\cite{19}. However, serotyping, cytotoxicity assays or genotyping for \textit{E. coli} O157:H7 are not routinely performed in Iran. Further studies are needed to identify the pathogenic mechanisms of this \textit{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

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