Prevalence of Pathogenic Genes of *Escherichia coli* O157:H7 Strains from Patients with Urinary Tract Infections in Iran

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The aim of this study was to evaluate the presence and frequency of some virulent genes of *E. coli* O157:H7 in patients with urinary tract infections (UTIs). In this research, the presence of *hlyA*, eaeA, stx1, and stx2 genes in *E. coli* O157:H7 isolates was evaluated using a multiplex PCR method. Two hundred and ninety-eight positive bacterial isolates were obtained from suspected UTI samples, with 206 of the isolates (69.1%) belonging to the *E. coli* category. Of the 206 isolates that were positive for *E. coli*, 16 of them (7.8%) harbored O157 gene, and 13 of them (6.3%) had both O157 and H7 genes. For this group, multiplex PCR was conducted on stx1, stx2, eaeA, and hly genes, and the results indicated that the distribution of stx1, stx2, eaeA and hlyA genes among these isolates was as follows: eaeA (15.4%), stx1 and eaeA combined (15.4%), stx2 (7.7%), and stx2 and eaeA combined (7.7%). No hlyA genes were identified in the samples.

**Key words:** *E. coli* O157:H7; hlyA gene; eaeA gene; stx1 gene; stx2 gene; multiplex PCR.

Shiga toxin-producing *E. coli* (STEC) are the fourth most common bacterial foodborne pathogen and responsible for around 1200 illnesses per year in the UK (Tran *et al*., 2014). *E. coli* O157:H7 is a common health concern in most countries around the world (Kiranmayi *et al*., 2010). This was first recognized as a cause of illness in 1982 during an outbreak of severe bloody diarrhea traced to consumption of hamburgers at common chains of fastfood restaurants. *E. coli* O157:H7 was known to be a human pathogen for nearly 32 years (Riley *et al*., 1983). Since then, it has been reported in more than 30 countries on six continents (Brown *et al*., 1997). *E. coli* O157:H7 is highly pathogenic in humans, but it does not induce any clinical disease in cattle and other animals other than diarrhea (Ateba and Mbewe *et al*., 2014). It bears several genes, including the stx gene, the Enterohaemolysin-encoding hly gene, and the intimin-encoding eae gene (Manna *et al*., 2006).

*E. coli* is an important member of the coliform group. Based on the pathogenicity and variation in biochemical characteristics, *E. coli* has been classified into 6 categories, viz. enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EaggEC) and diffusely adherent (DEAC), of which the EHEC is considered as most important one (Kiranmayi *et al*., 2010). These STEC strains are associated with a variety of disorders in humans including non-bloody diarrhoea, haemorrhagic colitis (HC) and a very
serious complication Haemolytic Uraemic Syndrome (HUS). HUS is the major cause of acute renal failure in children and fatal in up to 2% of cases six. The diarrhoeal-associated or typical HUS is the most common form among all HUS cases and most of them are caused by enterohaemorrhagic E. coli (EHEC). EHEC are classified as a subset of STEC carrying the eae gene encoding intimin and being pathogenic to human beings (Khan et al., 2011). There are two subunits of shiga toxin that are known as ‘A’ and ‘B.’ The B subunit binds to its specific receptor on the host cell, specifically globotriaosylceramide (Gb3). Gb3 is present in greater amounts in renal epithelial tissues, so the shiga toxin may cause renal toxicity (Tran et al., 2014). The E. coli O157:H7 (EHEC) strain produces two types of toxins namely Shiga toxin 1 (Stx-1) and/or Shiga toxin 2 (Stx-2), which have been also referred as Verotoxin 1 (VT-1) and Verotoxin 2 (VT-2) respectively responsible for food borne illness (Kiramayi et al., 2010).

To the best of the author’s knowledge, this study is in Iran that urine samples of patients suspected of having UTIs were collected, bacterial isolation was conducted, and the presence of hly, eae, stx2, and stx1 genes in E. coli O157:H7 isolates was tested using the PCR method.

MATERIALS AND METHODS

Sample collection

Urine samples of individuals with suspected urinary tract infections were collected from six hospitals and clinical laboratories during a nine-month period. Two hundred and ninety-eight positive samples were isolated by culturing the samples on blood agar and EMB media and confirming the presence of urinary tract infections in the referred individuals. Subsequently, the bacteria were identified using Gram staining, catalase, oxidase, and biochemical tests. E. coli isolates were considered for E. coli O157:H7 identification.

Isolation of E. coli O157:H7 and Serological tests

Initially, E. coli bacteria were cultivated and incubated on CT-SMAC (Merck - Germany) for 24 h at 37 °C, and sorbitol-negative bacteria were identified (Brandon et al., 2009). Then, the sorbitol-negative bacteria were cultured on O157 chrome agar (High Media, India). MUG-negative bacteria were incubated on the Nutrient agar (Merck- Germany) 24 h at 37 °C. Then, using a saline test, bacteria were checked to verify that they were not members of bacteria with spontaneous agglutination. Finally, they were tested with E. coli O157 anti-serum. Agglutinated samples were reported as positive samples (Hepburn et al., 2002).

DNA extraction

The bacteria that were positive with antisemum E. coli O157:H7 were cultured in medium LB Broth (Sharlua, Spain) at 37 °C for 24 h. Then, using the DNPTM DNA Extraction Kit (Synaclone, Iran) the DNAs of the bacteria were extracted and the DNA concentrations were measured using the OD method.

PCR on gene H7, 0157

Gene O157 was screened by specific primers in the PCR assay. The conditions of the PCR and the thermal cycles were similar to those of virulent genes multiplex PCR (Paton and Paton., 1998). Another PCR amplification analysis was conducted for confirmation of the presence of the flagellar H7 gene. The PCR primers for H7 were previously described by Gannon et al. (1997). The oligonucleotide sequence of the primer and expected sizes are listed in Table 1. Both the somatic O157 and the flagellar H7 PCR assays were performed in a final volume of 50µl that contained 2.5 unit ofTaq DNA polymerase (Ferments-Germany), 0.2 mM of dNTPs, 2.5 mM MgCl2, 50 mM KCl, and 20 pmol of one of the flagellar H7 or somatic O157 primers. The PCR reactions were conducted with a GeneAmp PCR thermocycler (Techne, Germany) according to the following protocol: one cycle of initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min each, annealing for 2 min at 65 °C for H7 and at 59 °C for O157, extension at 72 °C for 2 min, and the final extension at 72 °C for 5 min. The amplified PCR products were separated on 1.5% agarose gel in TAE buffer, stained with ethidium bromide, and photographed under UV illumination (Fig. 2).

Multiplex PCR for stx1, stx2, eaeA, and hlyA

Multiplex PCR for the detection of stx1, stx2, eaeA, and EHEC hlyA genes was performed by a GeneAmp PCR thermocycler (Techne, Germany). Oligonucleotide primers for stx1, stx2,
eaeA, and hlyA were synthesized as described previously (Paton and Paton, 1998). The oligonucleotide sequence of the primers and the predicted sizes of the PCR-amplified products are listed in Table 1. Each primer pair had been determined to be specific for E. coli and had been shown not to amplify products detectable by agarose gel (Ferments-Germany) electrophoresis using DNA templates derived from a range of Gram-positive and Gram-negative bacterial species from various food and animal sources. Samples (2 ml) of each extract were amplified in 50-ml reaction mixtures containing 200 mM concentrations of deoxynucleoside triphosphates, an approximately 250 nM concentration of each primer, and 1 U of Taq polymerase in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, (Ferments-Germany) were added to the reaction mixtures. The 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 done at 72 °C for 5 min. Amplified DNA fragments were resolved by gel electrophoresis using 1.5% agarose gels in trisacetate-EDTA (TAE) buffer. Gels were stained with 0.5 µl of ethidium bromide per ml and viewed and photographed under UV illumination.

**Statistical analysis**

The data were statistically analyzed by SPSS version 15 (SPSSInc., Chicago, IL, USA). The chi-square test or the Fisher exact test was provided to compare categorical variables. P-value <0.05 was considered statistically significant.

### RESULTS AND DISCUSSION

Shiga-toxigenic E. coli (STEC), especially E. coli O157:H7 are a potential threat to public health due to the morbidity and mortality associated with its infection (Pant et al., 2014).

In this study, 10,372 urine samples from six hospitals and diagnostic laboratories were analyzed during a nine-month period. Among 298 positive isolates in patients with UTIs, 206 samples containing E. coli were detected. After cultivation on the SMAC media, the sorbitol-negative bacteria were cultured on media containing MUG. Then, 63 samples that were MUG-positive for bacteria were selected for serological tests. They were evaluated with specific anti-serum against E. coli O157, and 29 samples tested positive for E. coli bacteria with the anti-serum. By using PCR on genes O157 and H7, 16 samples were O157 positive which 13 of them were O157: H7 positive and 3 samples were H7 negative. Also, there were 13 anti-serum positive samples out of 29 which did not show the above genes, which indicated which anti-serum test for the detection of E. coli O157:H7 is not a valuable method in comparison to PCR. In the present study, E. coli O157:H7 was evaluated in suspected samples of patients with urinary tract infections. Statistical analyses of the 298 bacterial isolates from suspected UTI samples showed that 206 (69.1%) of the UTI bacterial isolates belonged to the E. coli category. Klebsiella sp. was in second place, having been identified in 36 (12.1%) of the isolates. E. coli. The data shows that the highest

### Table 1. Primers used in multiplex PCR of stx1, stx2, eae, hly, H7, and O157 genes

<table>
<thead>
<tr>
<th>Name of gene</th>
<th>Primer Oligonucleotide sequences (5’-3’)</th>
<th>Expected size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>R: CTGAATCCCCCTCCATTATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R: CCTGTCAAATCGAGCAGCAGCTTGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R: CCCCCATTCTTTTCACCGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R: CTTCACTGACCATACTAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H7 F:</td>
<td>GGCCTGTGCTGTCGAGTCTGAG</td>
<td>625 bp</td>
<td>Gannon et al. (1997)</td>
</tr>
<tr>
<td>R: CAACGGTGACTTTATCGGACCACTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R: TTGCCTATGTACAGCTAATCC</td>
<td></td>
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<td></td>
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</tbody>
</table>
Table 2. Frequency of different types of bacteria in patients with UTIs (Total number of positive samples: 298)

<table>
<thead>
<tr>
<th>Type</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria separated from women</td>
<td>67.4%</td>
</tr>
<tr>
<td>Total bacteria separated from men</td>
<td>32.6%</td>
</tr>
<tr>
<td>Samples containing blood in urine</td>
<td>52.7%</td>
</tr>
<tr>
<td><em>E. coli</em> percent</td>
<td>69.1%</td>
</tr>
<tr>
<td><em>Klebsiella</em> sp</td>
<td>12.1%</td>
</tr>
<tr>
<td>Tested positive women for <em>E. coli</em></td>
<td>86.6%</td>
</tr>
<tr>
<td>Samples containing anti-serum positive for <em>E. coli</em></td>
<td>14.1%</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>6.3%</td>
</tr>
</tbody>
</table>

percentage of urinary tract infections was observed in women. Slightly more than seventy-six percent of the women had UTIs, whereas only 32.6% of the men did. After culturing on CT-SMAC medium, transferring of sorbitol-negative *E. coli* to chrome Agar medium, and performing serological tests, a total of 14.1% of the *E. coli* isolates that gave agglutination to *E. coli* O157:H7 antisera were considered as positive isolates. Only 7.8% of the *E. coli* isolates harbored the O157 gene, and 6.3% of them had both O157 and H7 genes for which multiplex PCR was conducted on *stx1*, *stx2*, *eae*, and *hly* genes (Table 2). The results of the multiplex PCR indicated that the distributions of *stx1*, *stx2*, *eae*, and *hly* genes (Table 2). The results of the multiplex PCR indicated that the distributions of *stx1*, *stx2*, *eae*, and *hly* genes.
*eae* and *hly* genes among these isolates were as follows: *eae* alone (15.4%), *stx1* and *eae* together (15.4%), *stx2* alone (7.7%) and *stx2* and *eae* together (7.7%) (Table 2). It is important to note that the *hly* gene was not identified in any of the isolates.

Fig. 3 shows agarose gel electrophoresis of *E. coli* O157:H7 multiplex PCR products.

1) Number 1: *E. coli* O157:H7 isolate with *Stx2* gene of 790 bp.
M: Marker of 100 bp

2) Number 2: *E. coli* O157:H7 isolate with *eae* gene of 889 bp and *stx1* gene of 614 bp.

3) Number 3: *E. coli* O157:H7 isolate with *eae* gene of 889 bp and *stx2* gene of 790 bp.

4) Number 4: *E. coli* O157:H7 isolate with *eae* gene of 889 bp and *stx1* gene of 614 bp.

5) Number 5 and 6: *E. coli* O157:H7 isolate with *eae* gene of 889 bp.

Studies have shown that VTEC strain is commonly isolated in hemolytic colitis and hemolytic uremic syndrome belonging to serogroup O157:H7 (Navidinia et al., 2012).

Since most of the urinary tract infections resulted from different strains of *E. coli*, the possibility of illness caused by different strains of O157: H7 was considered. The results of this research are consistent with the findings of some other research Starr et al. (2012) and Tarr et al. (1996) also showed that O157:H7 can cause urinary tract infections. Christina et al. (2011) conducted experiments on non-O157:H7 and O157:H7 that cause disease in children and showed that these bacteria can be the cause of many diseases.

By multiplex PCR performed on the genes *eaeA*, *stx2*, *stx1* and *hlyA*, six types of *E. coli* O157:H7 bacteria were isolated from samples of UTI genes that included *stx2*, *stx1*, and *eaeA* genes, but there were no *hly* genes among them (Schmidt et al., 1999). These data were confirmed previously by culturing bacteria on blood agar, which showed no evidence of hemolysis because of the *hlyA* gene.

In his evaluation of the prevalence of *stx*-producing *E. coli* in North America, Johnson examined 597 urine samples and showed that STEC bacteria were not isolated from urine samples (Johnson et al., 2002). During a study on urine and stool samples from 6-week-old children, Starr et al. (2012) showed that *stx1* and *stx2* genes from *E. coli* were present in the urine of such patients, but the *stx* gene was not detected in their stools.

The results of this research showed that *E. coli* O157:H7 bacteria also can produce disease in the urinary tract, so it is possible that these bacteria are transferred to the urinary tract through fecal contamination. However, studies conducted by Starr et al. (2012) and Tarr et al. (1996) found no evidence to support this hypothesis. In other research projects, the relationship between urinary tract infections and gastrointestinal infections were reported (Flemming et al., 2000). In some studies, the *E. coli* O157:H7 that were isolated contained *stx1* and *stx2* genes; in other studies, the isolates contained *vtx* genes. This may explain the *E. coli* pathogenicity of *E. coli* O157:H7 in the urinary tract (Byrne et al., 2003).

Navidinia et al. (2012) isolated 378 samples there were positive for *E. coli* bacteria from 12,572 samples in children. Among them, nine samples included EHEC, and five of the EHEC positive samples contained *vtx* genes. Tarr et al. (1996) showed that the strains of shigatoxin-producing *E. coli* O103:H2 were isolated from the urine sample of six-year-old girls suffering from urinary tract infections (UTIs), while there were no signs of diarrhea in these children. In 1993 in Denmark, two UTIs caused by *E. coli* were diagnosed. The researchers proposed that *E. coli* strains isolated from UTIs can be verotoxin producers, especially while the HUS prevalence is in the clinical form (Flemming et al., 2000). Other studies that have been conducted by scientists on *E. coli* O157:H7 in foods have shown the importance of this microorganism. However, in the previous studies, the frequency and the percentage of *E. coli* O157:H7 in urinary tract infections were low, and this low frequency is very important because the infection can become exacerbated and involve the kidneys.

Bonyadian et al. (2010), in a study on patients with diarrhea, isolated 58 samples and found that 16 of the samples contained *stx1* genes, four samples had *stx2* genes, and eight samples contained both genes. *Stx* Also, 12 samples were found to contain *hly* genes. None of the *E. coli* bacteria that were isolated contained *eae* genes. They showed that STEC in this study can be the main reason for the occurrence of infections (Bonyadian et al., 2010). These bacteria are oral-fecal, so it can be said that there is a relationship.
between food poisoning and urinary tract infections caused by *E. coli* O157:H7 (Kiranmayi et al., 2010). In research conducted by Tahamtan et al. (2010) and Mazhahrei et al. (2005), the relationship between *E. coli* O157:H7 in water drinking and the occurrence of gastrointestinal infections in cattle was evaluated.

Since 67.4% of infected patients are women, the possibility of urinary tract contamination by gastrointestinal tract bacteria should be considered. Our findings supported and reinforced this possibility since we observed that 67.4% of those who had urinary tract infections were women, whereas only 32.6% of patients with this problem are men. The results as well as other studies showed that *E. coli* O157: H7 is a pathogenic factor. This bacterium can be transferred to other people by food, contaminated water, juice, vegetables and fecal (Cooley et al., 2007; Kodaka et al., 2004), therefore it is necessary to control pollution sources.

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