Isolation and characterization of Shiga toxin producing *Escherichia coli* isolates from raw milk and cheese by biochemical and PCR of the specific genes in Fars province, Iran

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*Escherichia coli* O157:H7 is one of the most important pathogenic bacteria known in the world. Hemolytic-Uremic Syndrome (HUS) is the most important disease caused by this bacterium. In this study, *Escherichia coli* O157:H7 was isolated from raw and pasteurized milk and cheese samples and the virulence genes were analyzed. This descriptive cross-sectional study was conducted on raw milk and cheese samples in the Fars Province, in a three-month period. The samples were cultured on Sorbitol-MacConkey agar medium. Sorbitol-negative *Escherichia coli* were confirmed with specific antiserum. By polymerase chain reaction (PCR), the presence of O157 and H7 genes was determined. By Multiplex PCR, stx1, stx2, eaeA and hlyA genes were evaluated. In this study, 187 samples were collected. After identification, seventeen *Escherichia coli* O157:H7 were isolated. Evaluation of virulence genes showed that eaeA (2.72%), stx2 (7.27%) and stx1 (5.45%) genes were the most frequent in *Escherichia coli* O157:H7 respectively. Regarding the pH of milk, it seems that this food is a carrier for intestinal pathogens. Moreover, with regard to the fact that the contamination source is livestock, controlling the sources of contamination seems necessary.

**Key words:** *E. coli* O157:H7, stx1, stx2, dairy product.

**INTRODUCTION**

Food poisoning or food-borne diseases were occurred by consumption of food which is carrier of microorganisms or their toxins. The most important bacteria transferring via food are *Escherichia coli* and *Listeria*. *Escherichia coli* are a member of human intestine microflora. *E. coli* O157:H7 is the most important intestinal pathogens belong to the Entero Hemorrhagic *Escherichia coli* (EHEC) group which causes dysentery (Mora et al., 2007; Kargar and Homayoon, 2009). Food products such as raw milk, cheese, vegetable, fruit, meat etc. are the main reservoir of this pathogen (Turutoglu et al., 2007; Kargar and Homayoon, 2009). Although, optimum pH for growth of *E. coli* O157:H7 is 5.5-7.5, it can grow in minimum pH of 4. Adaptation to acidic conditions makes this bacterium capable to survive in acidic food, also enables it passing through Stomach acidic condition and localization in intestine (Paton and Paton, 1998; Small, 2006).

Diseases are caused by *E. coli* O157:H7, include mild diarrhea, dysentery, Hemorrhagic Colitis (HC), Hemolytic Uremic Syndrome (HUS), Thrombotic Thrombocytopenia Purpura (TTP) and finally death (Lake et al., 2003). The main virulence factors of *E. coli* O157:H7 are shiga like toxin (STX1, STX2) (having cytopathic effect on intestinal epithelial), Intimin protein (responsible for attaching to the intestine), entrohemolysine (encoded by hlyA gene), catalase peroxidase and enterotoxins (Paton and Paton, 1998; Matise et al., 2003). This strain is the fourth food-borne pathogen factor in Unitate State. The outbreaks of this bacterium have been observed in United Kingdom,
Germany, Switzerland, France, Japan, Australia and Argentina (Obrig, 1997; Bidet et al., 2005). Commonly the pathogenicity of this strain was found in 6 month up to 10 years old children and Elderly people (Turutoglu et al., 2007; Wu et al., 2008).

Due to the fact that low infection dose is needed for pathogenicity of this bacterium, it could be not only transmitted by food but also spread from person to person (Turutoglu et al., 2007; Wu et al., 2008). In addition, however, effective techniques are not performed routinely in laboratory, developing of sensitive and rapid diagnostic methods, leading to timely reservoir identification and treatment is crucial. For serology detection, Sorbitol MacConkey Agar medium and Mug tests were carried out due to inability of O157:H7 to ferment sorbitol and β-D-galactosidase (Paton and Paton, 1998; Novicki et al., 2000). Detection and amplification of virulence factors genes by polymerase chain reaction (PCR) is also specific, rapid and sensitive technique to isolates pathogens in food products (Fratamico and DebRoy, 2010).

In Iran several studies were reported isolation of E. coli O157:H7 in different region. For example, the isolation rate of this isolate from hamburger samples was found 2% in mashhad, 2005. Also, at the same year, isolation rate were reported 1% in Jahrom from 500 Samples. In another study in Marvdasht, isolation rate of O157:H7 were reported as 14.1% in Stool samples of children under 5 years old (Kargar et al., 2006; Kargar and Homayoon, 2009).

According to the above, the aim of this study is evaluation of prevalence of E. coli O157:H7 in raw milk and cheese of Fars province and also comparison between serology and molecular methods to introduce best detection methods for O157:H7 serotypes among isolated bacteria.

MATERIALS AND METHODS

This Cross-sectional study were carried out during 3 months (September, October and November) in 2014 on various samples include input factory raw milk from different region in Fars province, raw milk tank factory, Pasteurized milk and cheese Of factory products and also traditionally home-made cheeses. Totally 187 samples were collected. Raw milk was collected aseptically by sterile needles after fully stirring. Randomly, pasteurized milks and cheeses were gathered monthly from market.

Isolation and Early identification of E. coli on different media

0.1 ml of diluted raw, concentrated and pasteurized milk in Ringer solution and also 0.1 gr of diluted cheese samples in Potassium hydrogen sulphate solution were inoculated on Lauryl Sulfate Broth and incubated for 24-48 h at 37°C. Gas producing samples on LSB medium were considered as Escherichia coli based on Iran National Standard, number 5234. Plate Count Agar medium were used as isolation and purification medium for positive gas producing samples from previous step. For this purpose, a loop full positive LSB samples were cultured on Plate Count Agar and incubated at 37°C for 24-48 h. Then, E. coli isolates were identified based on color pattern suggested by the medium manufacturer (Iran National Standard, number 5484). For Confirmatory test, postive samples from LSB medium were cultured on Eosin Methylene Blue Agar (EMB). Cultured media after incubation time, 24-48 h at 37°C, were evaluated and plates whit growth of metallic green colonies considered as Escherichia coli. Colonies which were growth on both media (LSB and EMB) were maintained for biochemical tests such as gram staining, catalase, oxidase, TSI, etc.

Presumptive diagnosis of E. coli O157:H7

Positively growth colonies on EMB and LSB media were cultured on Sorbitol-MacConkey Agar (SMCA) and incubated at 37°C for 24 h (Novicki et al., 2000). Sorbitol negative colonies were reported as O156:H7 serotype of E. coli. Afterwards, these colonies were examined by O157:H7 serotype antiserum (B.I.R.D Co. Iran). 1 drop of antiserum and 1 drop of normal saline (positive control) placed on two opposite side of a Glass slides and mixed them individually with sorbitol negative colonies. Clearly agglutination with antiserum was indicated as positive reaction (Pao et al., 2005). These colonies were maintained for further studies. No agglutination should be seen in normal saline mixture (positive control).

PCR amplification of H7 and O157 genes

Bacterial DNA, which was cultured on luria bertani broth (LB) for 24 h at 37°C, was extracted by DNP™ kit (sinaclon Co. Iran) according to the manufacturer instruction. Reaction mixture was consist of 2.5 units of Taq DNA polymerase (Fermentec Co. Germany), 0.2 mM of dNTPs, 2.5 mM of MgCl₂ and 20 pmol specific primer for H7 and O157 genes (Techne Co. Germany) (Table 1). The final volume of PCR mixture was 50 μl. PCR reaction conditions were as follows: primary denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 65°C for H7 gene (2min) and at 59°C for O157 gene (2 min) and extension at 72°C for 2 min (35 cycles). Also, final extension was performed at 72°C for 5 min. PCR products were electrophoresis on 1.5% Agarose gel with Tris-acetate-EDTA (TAE) buffer.
Table 1. Sequences of used primers in polymerase chain reaction (PCR).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>References</th>
</tr>
</thead>
</table>
| stx1 | F: ACACCTGGATGATCTCAGTGG
R: CTGAATCCCCCTCCATTATG | 614 bp | (Badouei et al., 2010) |
| stx2 | F: CCATGACAACGGACAGCAGTT
R: CCTGTCAACTGAGCAGCACTTTG | 779 bp | (Badouei et al., 2010) |
| eaeA | F: GTGGCGGAATCTGGCGAGACT
R: CCCCATTCTTTTTTACCGTTCG | 890 bp | (Badouei et al., 2010) |
| hlyA | F: ACGATGTGGTTTATTCTGGA
R: CTTCACGTGACCATACAT | 165 bp | (Badouei et al., 2010) |
| H7   | F: GCGCTGTCGAGTTCTATCGAG
R: CAACGGTGACTTTATCGCCATTCC | 625 bp | (Philpott and Ebel, 2003) |
| O157 | F: CGGACATCCATGTGATATGG
R: TTGCCTATGTACAGCTAATCC | 259 bp | (Philpott and Ebel, 2003) |

Table 2. The number of positive E. coli and O157:H7 serotype isolates on different media.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total number</th>
<th>LSB medium</th>
<th>PC medium</th>
<th>EMB medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>110</td>
<td>105</td>
<td>104</td>
<td>103</td>
</tr>
<tr>
<td>Raw milk tank</td>
<td>12</td>
<td>10</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Concentrated milk</td>
<td>10</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>10</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pasteurized cheese</td>
<td>35</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Home-made cheese</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3. The number of positive biochemical tests for isolated E. coli.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total number</th>
<th>Indole positive test</th>
<th>Catalase positive test</th>
<th>Oxidase negative test</th>
<th>Gram staining</th>
<th>Triple Sugar Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>110</td>
<td>104</td>
<td>105</td>
<td>104</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Raw milk tank</td>
<td>12</td>
<td>10</td>
<td>11</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Concentrated milk</td>
<td>10</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pasteurized cheese</td>
<td>35</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Home-made cheese</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 4. The number of existence of stx1, stx2, eae and hly genes in different kinds of samples.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Raw milk</th>
<th>Raw milk tank</th>
<th>Pasteurized milk</th>
<th>Concentrated milk</th>
<th>Pasteurized cheese</th>
<th>Home-made cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx1</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>stx2</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>eae</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>hly</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The gel was dyed by Ethidium Bromide and observed under UV.

Multiplex PCR of hlyA, eaeA, stx1 nad stx2 genes

Bacterial DNA was extracted as above mentioned. Multiplex PCR mixture was consist of 200 mM of dNTPs, 1 unit of Taq DNA polymerase, 10 mM of Tris-HCl, 50 mM of KCl, 2 mM of MgCl2 and 250 nM of each specific primers for hlyA, eaeA and stx1,2 genes (Table 1). PCR reaction condition was as follows: primary denaturation at 95°C for 3 min, denaturation at 95°C for 20 sec, annealing at 58°C for 40 sec and extension at 72°C for 90 sec (35 cycles). Final extension was performed at 72°C for 5 min. Multiplex PCR products were electrophoresis on 1.5% Agarose gel with TAE buffer. The gel was dyed by Ethidium Bromide and observed under UV.

RESULTS

From 178 samples, 110 ones were raw milk, 12 ones
Figure 1. Multiplex PCR amplification products of stx1, stx2, eae and hly genes on Agarose gel. M: 100bp marker. Lane 1: E.coli O157:H7 with 890bp eae and 614bp stx1 genes. Lane 2: E. coli O157:H7 with 779bp stx2 gene. Lane 3: E.coli O157:H7 with 614bp stx1 gene. Lane 4: E.coli O157:H7 with 779bp stx2 gene.

Figure 2. The contamination rate of stx1, stx2, eae and hly genes in different kind of samples.

were raw milk tank (TR), 10 ones were pasteurized milk, 10 ones were concentrated milk, 10 ones were home-made cheese and 35 ones were pasteurized cheese (different kinds of tin). According to the primary culture results on Lauryl Sulfate Broth medium, the most E. coli was isolated from raw milk (110 samples). The existence rates of E. coli in pasteurized and concentrated milk were the lowest, respectively.
The E. coli growth rates on further media (PC, EMB and SMAC) were the same as LSB, raw milk the most and pasteurized milk the least (Table 1). Also, the results of biochemical tests which were performed on positively isolated E. coli were gathered in Table 2.

Culture results on SMAC medium showed 30 number of positive E. coli O157:H7 isolates in raw milk (the most) and followed by 4 isolates in raw milk tank, 4 isolates in home-made cheese, 2 isolates in concentrated milk, 2 isolates in pasteurized cheese and non in pasteurized milk.

Multiplex PCR of selected genes (stx1, stx2, eae and hly) showed that abundance of stx1 and stx2 in O157:H7 subjects from different samples were greater than 2 other genes, 9 and 10 ones respectively. Also, no existence of hly gene was proved in positive isolates in each of different samples (Table 3) (Figure 1). In addition, the abundance rate of all 4 genes were maximum in raw milk (15.45%) (Table 4) (Figure 2).

DISCUSSION

E. coli O157:H7 is an important food pathogen which enables to causes severe diseases in human. In recent years, the number of individual who get diseases related to dairy and cheese products is increase (Tims and Lim, 2003). Moreover, since 1995, 32 outbreaks related to fresh dairy products have occurred (Cooley et al., 2007). Based on CDC report, 20 outbreaks and 634 diseases of O157:H7 serotype from fresh cheese occurred during 1998 to 2005 (Brandl and Amundson, 2008). The sources of dairy and cheese pollution are polluted water and animal fertilizer used in farmland. If cattle and other ruminants could enter to the farmland, the chance of raw milk pollution with O157:H7 serotypes will increase (Turutoglu et al., 2007; Wu et al., 2008).

Several reports were showed that the prevalence rate of this bacterium in animals such as cattle and sheep is higher in summer and early autumn. For example Kudva and his co-workers just report O157:H7 isolation in summer and Chapman et al., found the highest rate of isolation in spring and late summer. According to above, we carried out this study during September to November.

Rivas et al. (2008) isolated E. coli O157:H7 from 8% of raw milk, 8.4% of Cattle manure and 3.6% of beef carcases in Argentina (Rivas, 2008) in compare to our results, we isolated this species from 16.8% of raw milk, 2.2% of raw milk tank and home-made cheese and 1.1% of concentrated milk and pasteurized cheese. In 2005, Pao and his co-workers isolated this bacterium from sheep feces with use of CT-SMAC medium and O157 and H7 antiserum in Virginia (Pao et al., 2005). Researcher in spain (2003) also used CT-SMAC medium for bacterial isolation same as our study and detected diseases genes (stx1 and stx2) with Multiplex-PCR (Blanco et al., 2003). Jinneman et al. (2003) used Multiplex Real Time PCR for detection of stx1 and stx2 genes (Jinneman et al., 2003).

Researchers found that the stx phage status among O157:H7 serotypes are variable. This bacterium not only changes geographically, but also changes genetically. stx genes which encoded by lambda like phage, could move between different strains of E. coli and settle in O157 gene site (Manning et al., 2008).

In 2004, researchers in Austria analyzed 110 samples of E. coli O157 for diseases genes by PCR. All of them except one bacterium had eae gene. hly gene was observed in all of the subjects. From 110 bacteria, 45 ones had stx1 and atx2 genes (41%). Two O157:H7 serotypes had just stx1 (1.8%) and 57 ones had just stx2 (51.8%) (Karpman et al., 1997). In our samples 6 ones in raw milk had just stx1, 8 ones had just stx2 and 3 ones had just eae. Also 1 sample in raw milk tank had stx2. In United States, from 57 E. coli samples, 38 strains had stx1, stx2, eaeA and hly genes, 11 strains had stx1, eaeA and hly genes, 5 strains had stx2, eaeA and hly genes and 3 strains had stx1, stx2 and eaeA genes (Byrne et al., 2003). In contrary to these studies, we did not find any hly gene in our samples but the rate of stx1 and atx2 genes were more.

In Iran, Sadeghifar and his co-workers isolated this bacterium from 1557 stool samples with SMAC medium in Elam. Most people harboring EHEC were healthy and without any symptom of diarrhea. In this study from 26 samples, 18 ones had verotoxine1 (69.2%), 7 ones had verotoxine2 (26.9%) and 1 sample had both verotoxines (3.9%). Also in one sample agglutination with O157:H7 anti-serum was observed. The results of this study indicated that E. coli O157:H7 serotype is not epidemiologically prevalence in this region (Sadeghifard et al., 2002). In 2000, researchers in Mazandaran analyzed 98 samples of raw milk for O157:H7. From 98 samples were cultured on CT-SMAC 3 ones were found VTEC (3.06%) which all 3 samples after agglutination with O157-H7 anti-serum were belongs to O157 serotype (Kargar et al., 2006), but we founded 30 positive O157:H7 serotype in raw milk sample which is much more than Mazandaran study. Asgari et al. (2010) used Multiplex PCR for analysis of stx1, stx2, eaeA and hly genes from all samples, 8 ones had stx1 (5%), 3 ones had stx2 (1.9%) and eaeA and hly genes were observed with equal rate (6.3%). From 13 positive strains with PCR, dominant genotype was stx1/ eae/ hly with abundance of 53.8% (Ibekwe et al., 2002) .In total, this study on disease genes indicated 27.7% prevalence of stx2, 72.2% eaeA and 45.5% stx1 genes in isolated E. coli O157:H7.

E. coli O157:H7 could survive in a wide range of environmental conditions such as temperature, low pH and dryness. Moreover, it could stay alive in sublethal stress, but change to VBNC form. Therefore, dairy
products should be considered as a potential source of this bacterium which all groups in society use them especially children and elderly people with defective immune system. It is recommended that this kind of products should analyze routinely for E. coli O157:H7 serotype to prevent further infections.

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REFERENCES


