Risk of Shiga Toxin-Producing *Escherichia coli* Infection in Humans Due to Consuming Unpasteurized Dairy Products

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Abstract

Background: Cattle transiently harbor Shiga-toxigenic *Escherichia coli* (STEC) in their gastrointestinal tracts, and many human infections result from ingestion of contaminated dairy products. The occurrence of STEC infections in human ranges from mild watery diarrhea to life-threatening conditions such as thrombotic thrombocytopenic purpura, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS).

Objective: Isolation of STEC from unpasteurized dairy products as a source of human infections is the aim of this research.

Materials and Methods: In this study, after collecting 150 samples of unpasteurized dairy products from different parts of Ahvaz, primary enrichment, selective enrichment and conventional biochemical tests were done and the suspected DNA isolates were extracted by boiling. Confirmation of being toxigenic isolates was performed by multiplex polymerase chain reaction (mPCR) assay. The stx₁ and stx₂-specific primers were used in m-PCR.

Results: Out of 75 isolates with lactose-fermentation ability, 11 *E. coli* strains were confirmed by biochemical tests. Two isolates (18.18%) were detected as carriers of stx genes by PCR.

Conclusion: Because of low infective dose, the presence of a low percentage of toxigenic *E. coli* in dairy products could be a grand public health risk, while the bacteria other than *E. coli* could be producing Shiga toxin which should not be ignored.

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Background

Pathogenic *Escherichia coli* strains are classified into pathotypes; that is groups of strains with remarkable assortments and common virulence factors.¹ Shiga-toxigenic *E. coli* (STEC) pathotypes are proactive factors for certain severe clinical syndromes in humans such as hemorrhagic colitis (HC), thrombotic thrombocytopenic purpura and Hemolytic Uremic Syndrome (HUS).

The most common aggression factors in this pathotype are 2 phage-encoded SLT, and SLT₂ cytoxins which are encoded by stx₁ and stx₂ genes, respectively.² The high fatality rate, low infective dose and severity of the signs make it a harmful threat to food safety.¹³ It appears that ruminants are a reservoir for STEC in the environment.⁴⁻⁶ A large variety of foods have been found to be contaminated with STEC, and unpasteurized or traditional dairy products including cheeses and raw milk in particular, could contain these strains.⁶⁻⁹ Consumption of undercooked hamburgers was associated with the first outbreak of STEC; the next outbreaks have imputed to both plant and foods of animal origin.¹⁰ Raw milk is presumably contaminated with STEC during milking via fecal contamination.¹¹ However, these organisms have been reported to be directly excreted from infected udders.¹² Fermented dairy products that are produced from raw milk contaminated with STEC could be considered as a serious threat to public hygiene because of surveillance of this pathogen after insufficient heating in manufacturing fermented products or contamination of products after successful heating step. Survival of this pathotype has been well documented in lactic cheeses made from raw goat milk,¹³ aged Cheddar cheese made from raw milk,¹⁴ Feta cheese,¹⁵ and even yogurt.¹⁶ Based on a review of the resources, until now, no study on the prevalence of Shiga-toxin producing *E. coli* in the raw milk supply has been done in Ahvaz, whilst there are several studies regarding the presence of Shiga toxin-producing *E. coli* O157:H7 in raw milk and dairy products in Iran and other countries.¹⁷⁻²⁰

Objectives

The purpose of this study was to detect STEC in several dairy products, including raw milk, unpasteurized cheese,
butter, ice cream, “Sarshir” (same as cream but with a different manufacturing procedure) and “Shirberenj” (rice cooked in milk) in Ahvaz.

**Materials and Methods**

**Collection of Samples**

One hundred fifty samples of dairy products including raw milk (39), cheese (24), butter (25), Sarshir (16), ice cream (29) and Shirbrenj (17) were collected in a time span of 6 months from various locations in Ahvaz, and they were examined for the presence of *E. coli*. Samples were transferred to sterile plastic bags under sterile conditions. Transportation of samples to the laboratory was done under cold conditions in short time after sampling. The samples were kept at -20°C until bacteriological analysis.21

**Bacteriological Analysis**

From the center of each sample, a portion (4-5 g or mL) was removed and homogenized aseptically in 40 mL of sterile lactose broth (Merck, Germany) as enrichment broth. Primary enrichment stage was done for 24 hours at 37°C.21

**Media and Growth Conditions**

In order to isolate and identify *E. coli*, a loop full of enriched samples was cultured in MacConkey (Mac) agar (Merck, Germany) as selective medium and incubated for 24 hours at 37°C. Doubtful pinkish colonies (4 colonies per plate) as lactose-positive strains were plated on blood agar for further identification. In addition, suspected isolates were cultured in eosin methylene blue (EMB) agar (Merck, Germany) as selective medium, for producing metallic sheen.21

**Physiological and Biochemical Examination**

From each bacterial plate, 4 to 5 suspected colonies were selected, subcultured, and then confirmed as *E. coli* by different biochemical tests such as gram staining, oxidative test, various sugar fermentation tests, indole, nitrate reduction, methyl red, Voges-Proskauer test, Simmon citrate agar, and urease production (Merck, Germany).21

**Multiplex Polymerase Chain Reaction**

The isolates confirmed as *E. coli* were examined by multiplex polymerase chain reaction (m-PCR) in order to examine the presence of studied genes (*stx1* and *stx2*). Shiga toxin-producing *Escherichia coli* O157:H7 (ATCC-43894) and sterile distilled water were used as the positive and negative control, respectively. A bacterial suspension of each confirmed *E. coli* isolate was prepared in sterile TE (Tris-EDTA) buffer with 2% two-mercaptoethanol. The suspension was heated in a boiling water bath for 10 minutes to make a bacterial lysate. For precipitation of cellular debris, centrifugation was done for 3 minutes at 13000 rpm (Eppendorf, Germany). The supernatant was collected in a new sterile microtube and stored at -20°C as a template in m-PCR. The used primer (SinaGen, Iran) sequence was as follows *stx1* gene encoding the Shiga-like toxin1 (SLT1) and *stx2*, which encodes the Shiga-like toxin 2 (SLT2) (Table 1).21 The temperature and conditions of amplification in this research was as described before by Brenjchi et al.17 The total volume of each reaction mixture was 25 μL which contained 0.5 μM of each primer (1 μL), and 5 μL of the template and 12.5 μL 2X Mastermix (SinaGen, Iran). The initial denaturation was done by incubation at 94°C for 5 minutes and followed by 35 cycles consisting of denaturation at 94°C, annealing at 52°C and elongation at 72°C for 60, 30 and 60 seconds, respectively and final extension at 72°C for 10 minutes. The products of PCR were analyzed by electrophoresis in 1.5% agarose gel in TAE (tris-acetate-EDTA) buffer, and they were visualized by safe-staining (SinaGen, Iran), then illuminated by a UV transilluminator (Uvitech, Germany) and documented afterward by a gel documentation apparatus. 100 bp DNA ladder was used as a marker for m-PCR assay.17

**Results**

Out of 150 dairy product samples, 367 lactose-fermenting colonies were isolated, after enrichment and selective plating. Based on the reaction in EMB medium, 172 colonies were selected. Then, 35 oxidase and gram-negative medium size colonies were isolated. Finally, 11 strains were confirmed as *E. coli* by gram negative rod shape and catalase positive, oxidase negative, urease and indole negative, and methyl red positive reactions, lactose fermentation, and nitrate reduction. In other words, 7.3% of the samples (11/150) were confirmed as *E. coli*. These strains were isolated from ice cream (1), raw milk (4), Shirbrenj (2), and butter (4). Using *stx1*- and *stx2*-specific primers showed that the 2 isolates (18.18%) were harboring Shiga-like toxin (*stx2*). Toxigenic bacteria detected by PCR were isolated from raw milk and Shirbrenj samples (Figure 1). There were no significant differences (P > 0.05) in the level of contamination with *E. coli* among different types of the samples and the production of *stx2* was dominant.

**Discussion**

On the whole, the existence of various strains of *E. coli*, as a probable causative agent of food-borne disease, in dairy products is not significant if *E. coli* is considered as a ubiquitous organism.21 However, in case of the presence of pathogenic strains, they could be harmful to consumers.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence</th>
<th>Size (bp)</th>
</tr>
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<tbody>
<tr>
<td><em>stx1</em></td>
<td>F: 5'-ACA CTA GAT GAT CTC AGT GG-3' R: 5'-CTG AAC TCA AGC TTA GG-3'</td>
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</tr>
<tr>
<td><em>stx2</em></td>
<td>F: 5’-CCA GTA CAA CGG ACA GCA GCT-3’ R: 5’-CCT GTC AAG TGA GCA CGT-3’</td>
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Dairy products such as cheese, butter, ice cream, Shirbrenj and Sarshir as similar as milk are the sources of nutrients and protein as well as casein because of the presence of the 9 essential nutrients. These milk products are of great importance in the Iranian diet, thus, their contamination can bring about different health hazards. Dairy products are prepared as pasteurized or unpasteurized (traditional) and used in different regions of Ahvaz on a daily basis. The production, maintenance and presentation of traditional products were entirely unpasteurized. Traditional methods can create a suitable environment for bacterial contamination.

The most well-known serotype in STEC pathotype is O157:H7 (O157) and other serotypes of STEC called as non-O157. Previous studies have shown that the prevalence of O157, O26, O114, O44, O124, O111, O55, and O119 serotypes are the most common serotypes of E. coli isolates. Depending on the geographical location, the most common serotype is different. For instance, O157:H7 has been introduced as the most common serotype in the United States, Europe, and Japan. Seasonal distribution of STEC serotypes O157:H7 have been reported before, so that the highest and the lowest occurrence were reported in summer and winter, respectively. Therefore, there is the possibility of a higher percentage of contamination than 18.18% in other seasons; and that is due to the fact that the samples of the present study were collected during the fall and winter months. Up until now, several studies have been conducted in Iran and other countries on the presence of O157:H7 in food. Escherichia coli created subclinical mastitis in bovine could reduce milk quality for human consumption. The regulations of inspection and control of milk are of greater importance in cases where milk is consumed raw. The milk of animals with mastitis, unsanitary milk collection and milking machine, methods of processing and milking, and prevention of the contamination of raw milk with extrinsic factors like the staff, dust, and insects, as well as the primary hygiene of milk are important in contamination of milk with STEC strains. Several disease outbreaks due to E. coli have shown that these strains are the common sources of poisoning in milk.

In this study, STEC contamination of different dairy products was evaluated by culture and m-PCR and a relatively significant contamination rate (7.3%) was observed, and 18.18% of isolates were toxigenic (SLT2 producing). Based on previous studies, most strains of this organism produce Stx2, a number of them produce both Stx1 and Stx2, and a few produce solely Stx1. In a study by the present author (unpublished), E. coli O157:H7 contamination of raw milk (on the farm) was evaluated in Khuzestan province. In the present research, 13.3% (20/150) of the samples were carriers of O157, some of which were toxigenic. Some studies have indicated that there is a probability of the presence of antibiotic resistance gene along with other virulence genes such as eae, Ehl1, stx2, and stx1, in STEC strains and this could be alarming for public health. Based on the results of this research, uncompromising preventive measures are recommended to produce uncontaminated dairy products, which subsequently promote public health.

Authors’ Contributions
This study was designed by NMB and AF. Sampling was done by AF and THJ. Treatment, isolation, and biochemical and molecular identifications were done by NMB and THJ.

Ethical Approval
We hereby declare that all ethical standards have been respected in the preparation of the article.

Conflict of Interest Disclosures
The authors declare that they have no conflict of interests.

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References


