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Investigating the Presence and Expression of *stx1* Gene in *Escherichia coli* Isolated From Women With Urinary Tract Infection Using Real-Time PCR in Tabriz, Iran

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Abstract

Background: Urinary tract infection (UTI) is one of the most common diseases in all the age categories around the world.

Objective: The current study aimed to investigate the presence and expression of the *stx1* gene in *Escherichia coli* isolated from women with UTI in Tabriz.

Materials and Methods: In this descriptive cross-sectional study, 25 *E. coli* isolates were assessed for the presence of the *stx1* gene by polymerase chain reaction (PCR) method after DNA extraction. Then, they were evaluated in terms of *stx1* gene expression by the real-time PCR.

Results: Six (24%) out of 25 tested *E. coli* isolates harbored *stx1* gene. In addition, 2 samples out of these 6 isolates containing the *stx1* gene showed more gene expression compared to the control samples.

Conclusion: Based on the prevalence of UTI with *E. coli* among the community and diffusion of its pathogenesis factors, fast and accurate identification of *E. coli* and its resistance factors is necessary for curring this infection.

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Background

Escherichia coli is the most common cause of urinary tract infections (UTIs), especially in young women.[1] *E. coli* is responsible for more than 80%-90% of UTI among the community and 30%-50% in the hospitals. In addition, this bacterium is one of the main causes of hospitalization with significant complications and high health care costs.[2] The verotoxigenic *E. coli* (VTEC) strains secrete a type of toxin which is called verotoxin due to its ability in killing Vero cells. Further, because of the similarity of verotoxin to the Shiga neurotoxin which is secreted by *Shigella dysenteriae*, type I is called Shiga-like toxin and the strains which secrete it are referred to as Shiga toxin-producing *E. coli* (STEC).[3] Furthermore, the verotoxins are categorized into *stx1* and *stx2* groups. These toxins inhibit the synthesis of proteins through affecting the ribosomal RNA, and the types of *E. coli* that produce these toxins are called STEC. The STEC strains are an important group of zoonotic and foodborne pathogens in human that can cause various diseases such as bloody diarrhea, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP).[4] A number of current studies have shown that about 20 000 instances of infection and 250 cases of death are annually reported due to the consumption of contaminated foods by STEC in the United States.[5] Today, it is well known that STEC is intermittently isolated from cattle and sheep stools, and as a result it can contaminate the meat of these animals.[6]

Objectives

This study sought to examine the presence and expression of the *stx1* gene in *E. coli* isolated from women with UTI in Tabriz.

Materials and Methods

Sample Collection

A total of 25 *E. coli* isolates were evaluated in this study, which were identified in our previous studies by the biochemical tests administered to women with UTI. The *E. coli* samples were isolated from women with UTI who referred to Asadabadi hospital in Tabriz. The collected

urine samples were cultured in EMB (eosin methylene blue) agar (Merck, Germany) medium. Then, the obtained bacteria colonies were evaluated and biochemical tests including IMViC and triple sugar iron (TSI) agar were used to identify the *E. coli* strains. Next, the *E. coli* isolates were inoculated in tryptic soy broth (TSB) medium containing 40% glycerol and were kept at -20˚C until DNA and RNA were extracted.

Extraction of DNA

Genomic DNA was extracted using the salting-out method. Brain heart infusion (BHI) medium (Merck, Germany) containing *E. coli* was transferred to 2 mL tubes and centrifuged at 10 000 g for 5 minutes. The supernatant was poured out and then 800 µL of lysis buffer solution was added to each of the tubes. The tubes were respectively placed in a bain-marie at 85°C (30 minutes), in a freezer at -70°C (10 minutes), and again in a bain-marie at 85°C (5-6 minutes). Then, 700 µL of chloroform-isoamyl alcohol solution containing chloroform (24 mL) and isoamyl alcohol (1 mL) was added to each of the tubes, and they were stirred slowly. Next, the tubes were centrifuged at 12 000 g for 5 minutes and the supernatant containing DNA was transferred to the new tubes. Subsequently, cold isopropanol (1.5 times of the samples' volume) was added to the new tubes, and the tubes were stirred slowly and placed in a freezer at -70°C for the whole day. The next day, the samples were removed from the freezer and put at the room temperature for defreezing. Then, the tubes were centrifuged at 12 000 g for 5 minutes and the supernatant was poured out. Afterward, the tubes were thoroughly dried using a sterile napkin so that the tubes did not smell alcohol. Finally, 50 µL of deionized distilled water (DNase free) was added to each of the tubes[4] and then the optical density (OD) and concentration of the extracted DNA were measured using a NanoDrop instrument.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) method was used to confirm the presence of *stx1* gene in isolated *E. coli* from the UTI. The PCR test was performed in 25 µL volume using the following materials: the PCR buffer (2.5 µL), extracted DNA (1 µG), each of reverse and forward praimers (25 pmol), Taq DNA polymerase (1.5 unit), dNTP (0.1 mmol), and Mgcl2 (1.5 mmol/L). The PCR condition was as follows: 1 cycle initial denaturation (95°C for 4 minutes), 35 cycles denaturation (94°C for 1 minute), 35 cycles annealing (53°C for 1 minute), 35 cycles extension (72°C for 1 minute), and 1 cycle final extension (72°C for 10 minutes).[7] The PCR products were electrophoresed on 2% agarose and photographed using a gel document instrument.

Primer Design

The sequence of the 16s rRNA gene and the related data were obtained from the National Center for Biotechnology Information (NCBI) database. The specific primers for amplification of the 16s rRNA gene were designed using Oligo software, version 5. Moreover, the primer sequence was synthesized in Takapo Zist Company, Iran. The primer sequences and their characteristics are provided in Table 1.

Extraction of RNA

The positive samples related to the presence of *stx1* gene were cultured in Mueller Hinton broth medium and centrifuged. First, 500 µL of Trizol solution was added to each of the bacterial sediment and kept at 45°C for 30 minutes. Then, 200 µL of chloroform was added to each of the tubes and stirred slowly and placed at room temperature for 5 minutes. The tubes were then centrifuged at 12 000 g for 10 minutes and the supernatant (without interrupting the middle phase) was carefully transferred to the new tubes. Next, cold isopropanol (2.5 times of the samples' volume) was added to the new tubes, and the tubes were stirred slowly and placed in a freezer at -70°C for the whole day. The next day, the samples were removed from the freezer and brought to the room temperature for defreezing. Subsequently, the tubes were centrifuged at 12 000 g for 10 minutes and supernatant was poured out. Then, the tubes were thoroughly dried using a sterile napkin so that the tubes did not smell alcohol. Next, 20 µL of diethyl pyrocarbonate (DEPC) was added to each of the tubes and they were placed at room temperature for 10 minutes.[9] Eventually, the OD and concentration of extracted RNA were measured applying a NanoDrop instrument.

cDNA Synthesis and Real-Time Quantitative PCR

The real-time quantitative PCR method was implemented to evaluate the expression of the *stx1* gene in *E. coli* isolated from UTI. Additionally, the fold changes were normalized to 16s rRNA. The cDNA was synthesized at 20 µL volume as follows: total RNA (500 ng), universal hexamer primer 0.2 Mm (1 µL), dNTP 10 mM (1 µL),

Table 1. The Sequences and Characteristics of the Primers

Product Size	Primer Sequence	Gene	Reference
180 bp	F: 5`-ATAAATCGCCATTCGTTGACTAC-3` R: 5`-AGAACGCCCACTGAGATCATC-3`	stx1	8
90 bp	F: 5`-ACTCTGTTATTAGGGAAGAA-3` R: 5`-AACGCTTGCCACCTACGTAT-3`	16s rRNA Designed primer*	

* The primer design for 16s rRNA gene was performed using Oligo software.

and DEPC water were mixed and incubated at 65°C for 5 minutes. The obtained solution was placed on the ice and then reverse transcription enzyme (5 units) (M-MLV), RNase inhibitor (1 unit/µL), and M-MLV RT buffer 1x were added. The PCR condition was as follows: 25°C for 10 minutes, 42°C for 60 minutes and, 72°C for 10 minutes. The real-time PCR (RT-PCR) was performed as triplet repeats at 20 µL volume as cDNA (1 µL) and forward primer 0.2 µM (1 µL), reverse primer 0.2 µM (1 µL), DEPC (7 µL), and Real-time mastermix 1x (10 µL) were added to the special RT-PCR tubes. The PCR was conducted under the following condition: 1 cycle initial denaturation (94°C for 10 minutes), 40 cycles denaturation (94°C for 15 seconds), 40 cycles annealing (53°C for 30 seconds), 40 cycles extension (72°C for 25 seconds), and 1 cycle final extension (72°C for 5 minutes). To obtain further details about the primer sequences and their characteristics refer to Table 1.

Analysis of Gene Expression Information

The threshold cycle (CT) was determined for each sample. The expression of the *stx1* gene was compared to that of the control group and the obtained data were normalized by 16s rRNA housekeeping gene. The expression ratio and production of Shiga toxin were calculated using the ratio of Michael Pfaffl's mathematical method:

Ratio = $(E_{target})\ \Delta CP_{target}$ (control - sample) / $(E_{ref})\ \Delta CP_{ref}$ (control - sample).[10]

Results

Presence of *stx1* Gene

The obtained results demonstrated that only 6 out of 25 *E. coli* isolates (24%) harbored the *stx1* gene (Figure 1).

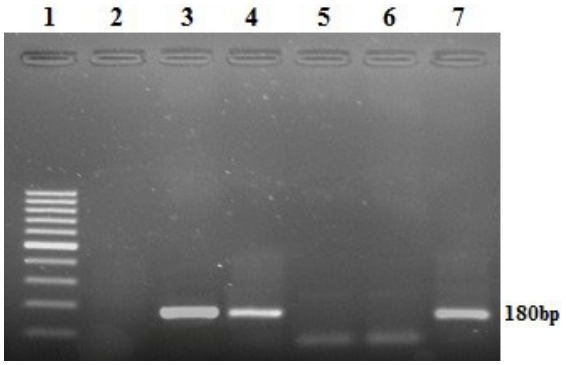

Figure 1. The Electrophoresis of PCR Products on 2% Agarose. (1) Ladder 100 bp; (2) negative control (distilled water); (3) positive control (*E. coli* O157: H7 PTCC43889); (4 & 7) presence of *stx1* gene.

Expression of *stx1* Gene

The expression of the *stx1* gene in the studied *E. coli* isolates varied from 0.125 to 4.99. In addition, *stx1* gene expression in 2 of the *E. coli* isolates was three times higher than the standard strain (Figure 2).

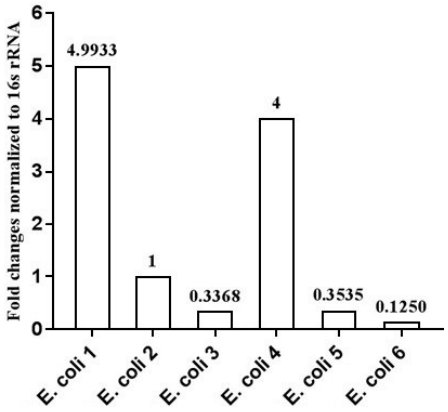

Figure 2. The *stx1* Gene Expression of the Isolated *Escherichia coli* Samples.

Discussion

The results indicated that 6 (24%) *E. coli* isolates contained the *stx1* genes. Adeli et al reported that 1% of *E. coli* isolates from patients with UTI harbored the *stx1* gene.[11] Further, Mansouri et al and Mahdavi found that the prevalence of *stx1* gene in 146 samples of *E. coli* isolated from patients with UTI and *E. coli* isolates from the local cheese was 0%.[12,14] However, According to Nazemi et al, the prevalence of *stx1* gene in *E. coli* bacteria isolated from patients with UTI was 10%. Similarly, base on a report by Beni et al., 11.1% of *E. coli* isolates from the lambs' meat contained *stx1* gene.[15] Except for *E. coli* O157, other Shiga toxin producing *E. coli* serotypes are the cause of 60% of the STEC infections in many parts of the world such as Argentina, Australia, Spain, Denmark, Chile, and Germany.[16] The results of this study compared to those of previous studies revealed the difference in dispersion of the virulence genes of *E. coli*. This may be due to geographical differences as well as differences in the ecological origin of the isolated strains (e.g., milk, human, and animals). In the present study, we may have lost the *stx1* gene in some *E. coli* samples due to repeated passages of the cultures or mutation. In a similar study, strains with three-fold increase in gene expression were considered positive while those with 2 or fewer times of increase were regarded as negative compared to the standard strain. Furthermore, strains with 2-3-fold increase in gene expression are called borderline.[17] In this study, the expression range of the *stx1* gene in *E. coli* isolates was between 0.125 and 4.99, and the average increase in gene expression was 1.79 times higher than that of the *E. coli* O157 (H7 PTCC43889 as the standard strain). Moreover, the expression of the *stx1* gene in 2 *E. coli* isolates was three times more than that of the standard strain. It was revealed that Shiga toxin can be coded by phage, chromosome, or plasmid. If Shiga toxin-encoding gene was located on a phage or plasmid, it could be distributed between different strains of a bacterium or among different bacteria strains.[18] Shiga toxin is found to have apoptotic effects on some of the cancer cells, and the clinical use of this toxin in some

cancers is at its early stages.[19,20] If the aim is to use Shiga toxin for therapeutic purposes, it is advisable to select the strain that has the highest production of Shiga toxin. Additionally, as there may be a difference between the expression of phage, chromosomal, or plasmid genes, it is necessary to determine the genetic origin of Shiga toxin in a variety of clinical isolates. In addition, if the Shiga toxin-encoding gene is located on the phage or plasmid, it is possible to transfer it between the strains. Therefore, having knowledge regarding the genetic origin of clinical isolates is essential for providing basic information to counteract the release of toxin genes among the strains.

Conclusion

Generally, the presence of the *stx1* gene in *E. coli* isolates from women with UTI was 24% which is considerably high. Further, all the isolates containing this gene could express it. Accordingly, the PCR method can be used as a routine test in reference laboratories due to its sensitivity and specificity. Furthermore, by employing this method, verotoxigenic strains can quickly and appropriately be detected in order to determine the type of secreted toxin by each strain and to increase the possibility of direct application on clinical samples.

Authors' Contributions

This article was extracted from the MSc project of ET where SM supervised this project and suggested the problem; All authors had an equal role in design, work, statistical analysis, and manuscript writing. All authors read and approved the final manuscript.

Ethical Approval

All participants were informed about the study and signed a consent form according to the Declaration of Helsinki ethical standards.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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None.

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