Molecular Detection and characterization of stx toxins of Escherichia coli O157:H7, Escherichia coli O26:H8, and Escherichia coli O111:H8 from diarrhoeic patients in Khartoum State, Sudan

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Research Article

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Abstract

Introduction/Background: Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is the most important serotype of *E. coli* responsible for serious intestinal and extra-intestinal disorders worldwide, but no molecular based study for EHEC as the causative agent diarrhea was performed in the Sudan. EHEC is a subset of Shiga toxin (stx) producing *E. coli* (STEC) which includes well recognized human pathogens. STEC produce high level toxins of a protein family referred to as Shiga toxin and Shiga-like toxins (SLTs; alternatively known as verocytotoxins VTEC or VTs). EHEC O26:H11 and O111:H8 have emerged as the most important non-O157:H7 EHEC.

These toxins are mediating intestinal disease in children and adults, and if the cases left untreated these toxins may contribution to the development of extra-intestinal sequelae e.g., the haemolytic uraemic syndrome and neurological disorders.

Objective: This study was conducted for the detection and molecular characterization of Shiga like toxin (*stx*) of *E. coli* O157:H7, *E. coli* O26:H11, and *E. coli* O111:H8 in Sudanese patients with diarrhea using PCR and multiplex PCR.

Materials and methods: A cross sectional laboratory-based study was carried out in Khartoum, Sudan during the period from June to October 2020. All patients with confirmed *E. coli* diarrheal disease was included in this study. The isolates have been identified by using the biochemical, serological, molecular identification and characterization tests. SPSS IBM (software version 20.0) was used for analyses.

Results: Out of 100 patients with diarrheal disease a total of 96 patients were confirmed of having *E. coli* organisms. All 96 *E. coli* bacteria were seropositive identified as members of the EHEC. Ten isolates of *E. coli* O157:H7, *E. coli* O26:H11, *E. coli* O111:H8 have been selected as follows; five, three, and two respectively.

The selected isolates followed by multiplex PCR to detect the genes of *stx* toxins of *E. coli* O157:H7, *E. coli*, O26:H11, and *E. coli* O111:H8 and comparing between them.

The multiplex PCR revealed that *E. coli* O157:H7 were carrying *stx*1 and *stx*2 genes represented by two bands, while *E. coli* O26:H11 and *E. coli* O111:H8 were carrying one *stx*2 gene represented by one band.

Conclusions: *E. coli* O157:H7 produce *stx*1 and *stx*2 while the other serotypes *E. coli* O26:H11 and *E. coli* O111:H8 produce *stx*2 only.

Effective control of pathogenic strains of *E. coli* requires a multifaceted approach. To reduce the frequency and severity of human exposure to STEC, controlling dietary sources and environmental sources such as cattle, water troughs, feed and manure is critical in breaking the cycle of infection and re-infecting with STEC.

Background
*Escherichia coli* (*E. coli*) is a Gram-negative rod-shaped bacterium commonly inhabiting the lower intestine of warm-blooded animals. UpToDate, more than 700 serotypes of *E. coli* have been identified [1]. Most *E. coli* strains are harmless, but some serotypes can cause serious gastroenteritis in humans [2]. Fecal-oral route of transmission is the major route through which those pathogenic strains cause disease [1, 3].

Based on the classification of Naruto and Kaper, 1998, diarrheagenic *E. coli* (DEC) are classified according to their virulence properties into six groups: enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) [4], enteroaggregative *E. coli* (EAEC) and diffuse adhering *E. coli* (DAEC). Enterohaemorrhagic *E. coli* (EHEC) was first recognized pathogenic strain of *E. coli* that known as EHEC O157:H7. First described as a result of an outbreak of unusual gastrointestinal illness in 1982. The outbreak was traced to contaminated hamburgers, and the illness was similar to other incidents in the United States and Japan [5, 6].

Since 1978 Shiga toxin was reported as a cytotoxic agent named as Verotoxin (VT) because it can strongly effect and kill the Vero cells (the African green monkey kidney cells) [7]. Subsequently, functional, and immunological similarities between VT and the Shiga toxin produced by Shigella dysenteriae have been revealed [8, 9]. In consequence, the VT became known as Shiga-like toxin (SLT). Two major types of SLT/VT were recognized (SLT-I and SLT-II, or VT1 and VT2). In 1996, Calderwood et al. report a proposed nomenclature of Stx1 and Stx2, to reflect the high degree of homology of the two toxins that placed them in the Shiga toxin family [10].

EHEC O157:H7 is one of the most important Viro-type Found in humans [11], cattle, and goats [12, 13]. The most famous member of this Viro-types is the O157:H7 strain that produce high level toxins of a protein family referred to as Shiga toxin and Shiga-like toxins which can provoke an intense inflammatory response [1, 14]. The Shiga toxin of the EHEC O157:H7 is known to cause hemolytic-uremic syndrome, sudden kidney failure and other sever complications.

The EHEC is a subset of Shiga toxin (stx) producing E. coli (STEC) which includes well recognized human pathogen (the O157:H7). Similarly, EHEC compose of other pathogenic strain known as EHEC Non-O157:H7 such as O26:H11, O104:H4 and O104:H21, O111:H8. The Non-O157:H7 which are also capable of produce Shiga toxin which causes cause devastating illnesses, particularly in children, of varying severity, from diarrhea (often bloody), hemorrhagic colitis but without fever, and abdominal cramps to kidney disorders [11].

The U.S. Center for Disease Control and Prevention (“CDC”) and the Food safety specialists have been describing the Six “O” groups of Non-O157 STEC to be the cause of the majority of diarrheal disease. These serotypes as well have been linked to diarrheal illnesses and outbreaks of disease [15]. The subgroups of *E. coli* O26:H11, and O111:H8cause an estimated 36,000 or more illnesses, 1,000 hospitalizations and 30 deaths in the United States alone.
Non-O157 STEC has been described to cause diarrheal disease resulting in haemolytic uraemic syndrome (HUS). It is also estimated to cause diarrheal disease at frequencies similar to other enteric bacterial pathogens, such as Salmonella and Shigella [11, 16]. EHEC O26:H11 have emerged as the most important non-O157:H7 EHEC strain, with respect to their capability to cause diarrheal disease and HUS [4].

There are over 200 STEC serotypes that produce Shiga toxin (stx), but many have not been involved in pathogenesis. Some stx variants (e.g., Stx1, Stx2, Stx2c) that contribute to the development of intestinal sequelae like hemorrhagic diarrhea, occasionally leading to severe extra-intestinal illness like kidney failure, haemolytic uraemic syndrome and neurological disorders especially in young children, elderly, and patients whose immune systems are otherwise compromised [17, 18].

There are two major antigenically distinct forms of toxin, stx1 and stx2 [9]. Whilst stx1 is homogeneous, there are numerous variants of stx2; however, stx1 and stx2 share approximately 60% DNA and amino acid homology but are immunologically distinct. These toxins are categorized as stx1 and stx2, with many subtypes and varieties depending on receptor preferences and potency of the toxin [19]. Although, most of the E. coli O157 strains produce stx2 only, amongst non-O157 STEC, the toxin phenotype is much more variable with isolates producing Stx1 alone occurring commonly. Isolates of serogroup O26 usually produce stx2 [20, 21], isolates of serogroup O111 produce stx1 and some produce stx2 in addition [22, 23]. There is considerable epidemiological evidence to indicate that STEC isolates producing stx2 are more commonly associated with serious disease than isolates producing stx1 or Stx1 and Stx2 [24]. Although, Stx1 and Stx2 have similar structures and modes of action, their toxicities appear to be distinct. Stx2 was 1000 times more cytotoxic than Stx1 towards human renal microvascular endothelial cells, the putative target of Shiga toxins in the development of HUS [25]. Similarly, in other experiments using transformed human intestinal microvascular endothelial cells showed that Stx2 was more toxic than Stx1 for these cells [26].

So, infection with pathogenic strains of E. coli has serious consequences for human health and patient safety. Especially with the increased virulence of infectious strains. Increasing virulence may lead to prolonged treatment, resulting in increased patient suffering and increased risks such as diarrheal disease or HUS, or severe complications such as irreversible renal damage or may lead to other neurological disorders.

To our knowledge, this is the first study showing that the toxin genes of the EHEC O157:H7 strains are different in length. The length of the gene of the (stx-1) toxin is different than the length of the gene (stx-2) toxin. However, the length of the gene (stx-2) toxin of O157:H7 is similar to E. coli non-O157:H7, e.g., E. coli O26:H11 and the E. coli O111:H8. Moreover, the (stx-2) toxin of E. coli non-O157:H7 is more severe than the toxin of E. coli O157:H7.

The present study was conducted to investigate the epidemiology of pathogenic strains of E. coli in diarrheagenic patients during an outbreak of diarrheal diseases in Khartoum, Sudan, and to add research
data to our previous studies in Sudan.

Patients And Methods

During the period of June to October 2020 a cross sectional laboratory-based study was conducted in different educational hospitals in Khartoum State. The participating hospitals were Khartoum Teaching hospital, Omdurman Teaching hospital, Omdurman pediatric hospital -Mohmmed Alamin Hamed-, and Bahri hospital. Institutional review board approval was obtained from the Faculty of Medical Laboratory Sciences Research Ethics Review Board, University of Khartoum, Sudan. A signed written informed consent was obtained from all subjects or from the parents or guardians in case of the participants under the 16 years of age. Sociodemographic and clinical data were obtained from each participant using structured pre-tested questionnaires.

Study Setting and Population:

Consecutive patients suffering from diarrhea and or bloody diarrhea who attended the referral clinics were approached to participate in the study. Samples were collected from patients with different age groups of both sex (males/females), and who met the inclusion criteria; signs and symptoms of diarrhea and or bloody diarrhea willingness (the participant agreed to participate). Definition of diarrhea (defecation of watery-stool three times or more/day, accompanied by at least one of the following symptoms: nausea, vomiting, abdominal cramps, or fever >38°C, and acute diarrhea that lasted ≤14 days at the time of presentation).

For all patients, an initial survey covered medical history, demographics and symptoms (as mentioned above), in addition to their culture positive results with E. coli. All patients with other etiology or negative E. coli culture were excluded from the study. See the schematic diagram of the study design Figure: 1.

Collection and Processing Specimens:

Stool samples were collected from each participant. Samples were collected using a sterile screw capped wide mouth container and processed immediately. In the medical laboratory a two or three loop full from each stool specimen was used to inoculate agar media, MacConkey's and Blood Agar (Oxoid, Basingstoke, UK). Plates were incubated aerobically at 35–37 °C for 24 h and the outcome was judged as positive/negative growth of E. coli.

Stool specimens were collected in a sterile, wide mouth, leakproof containers. Cultures were done by culturing the specimens directly onto MacConkey agar plates (Oxoid Basingstoke England), and Sorbitol-MacConkey agar (SMAC) with additional selective agents (Oxoid Basingstoke England). Culture plates were incubated aerobically at 37°C for 24 hours. All positive plates with predominant growth of E. coli, and typical colonial morphology were examined and included in this study.

Isolates of E. coli were purified by sub-culturing on MacConkey agar and incubated aerobically for 24 hours at 37°C. E. coli isolates were identified based on cultural characteristics, gram stains, biochemicals,
serological, molecular identification and characterization tests.

**Identification By using Serological Tests:**

This a not routine test. Anti-sera for the detection of the pathogenic strains of EHEC has been requested from the British Cteq Company. Serological identification tests were used for the detection of “O” and “H” antigens according to the manufacturer (the British Cteq Company) guidelines. colonies were serotyped using agglutination in polyvalent and monovalent *E. coli* “O” and “H” antisera

**Determination of DNA Concentration and Purity:**

The extracted DNA yields were determined spectrophotometrically (gene Quanta-Amersham) The DNA was diluted 1:10 with PCR-Grade distilled water (1µl DNA+ 9 µl DW). The absorbance of DNA was measured at 260 wave length while the absorbance of protein was measured at 280 wave length.

**Conventional PCR:**

Polymerase chain reaction (PCR) was done according to the method by iNtRON Biotechnology, Incorporation, Korea.

PCR is used to detect and amplify small amount of DNA to unlimited quantities. In our study PCR was done to confirm that the isolate was *E. coli* and amplified it, so the genomic DNA of *E. coli* was used as template in 25µl PCR reaction by using specific primer of *E. coli* forward 5-ATCACCCTGGTGACGCATGC-3'- reverse, 3'-CACCAGATGCTGATGTTTG-5 with length 223 bp for the 89 isolates evaluated by conventional PCR. Briefly; a 25µl reaction mixture was set by adding (4µL of PreMix, 3µL of genomic DNA, 1µL primer F, 1µL primer R mix tube, and then 16µL distilled water).

#PreMix (2.5U Taq polymerase and 2.5mm deoxyribonucleoside (dNTP) and buffer)

The amplification was done in 37 cycles in total consisted of a 2 min denaturation step at 94°C, followed by a 1 min annealing step at 55°C, and a 1 min elongation step at 72°C and a final extension step at 72°C for 15 min. *E. coli* ATCC 25922 was the control strains used in the study.

**Identification by using Multiplex PCR:**

The samples (*E. coli* organisms) were sent to the Bioneer Corporation (Seoul, Korea) for the detection of two genes of (stx-1 stx-2) toxins by Multiplex PCR Gene amplification were applied using toxin primers-the first one is stx-1 Forward primer 5-GAGCGAAATAATTTATATGTG and reverse primers TTGATGATGGCAATTCAGTAT the second one is “stx-2” forward primer 5-CCATGACAACGGACAGCAG and reverse primer 3-CCTGTCAACTGAGCACTTTGC-5 #Mango mix, (Taq DNA polymerase - dNTP mixture, -PCR buffer, - MgCl2).

**Calculation of the sample size:**

The sample size was calculated according to the following equation
\[ N = \frac{(Z)^2 P Q}{D^2} \]

N = sample size

Z = 1.96 (Rate of mortality by EHEC).

P = prevalence

Q = 100 - p

D = degree of accuracy (2-5%) So:

\[ N = \frac{(1.96)^2 \times 7 \times 93}{5^2} = 100.03526 = 100 \]

Statistics:

IBM SPSS for window (version 20.0) using Fisher's exact test. Frequency and descriptive statistical analysis were used for the demographic analysis for patients. P values < 0.05 were considered significant.

Results

During the study period, 100 subjects were initially screened, among these 11 (11%) were excluded from the study because they had missing or incomplete data. See table: 1.

Direct Culture on MacConkey's (MAC) Agar:

Of the 100 stool samples, 96 plates were positive culture growth. After matching the characteristic feature of the growth with the colonial morphology, the gram-staining and the biochemical reactions only 89 organisms were identified as *E. coli*. See the schematic diagram of the study design Figure: 1.

Sub-Culture on Sorbitol-MacConkey's (S-MAC) Agar

The 89 isolates were sub-cultured on S- MAC agar. Only 29 of them were non sorbitol fermenter *E. coli*, whereas the other 60 were sorbitol fermenter *E. coli*.

Serological Test Results:

By using the antisera for the three targeted EHEC serotypes, 25 out of the 29 non-sorbitol fermenters were positive for *E. coli* O157 H7 and four were non-typeable (O rough). Out of the 60-sorbitol fermenter *E. coli*
only 53 were found to be reactive with *E. coli* O26 H11 and seven with *E. coli* O111 H8.

**Conventional PCR Results:**

The conventional PCR was performed by using specific of *E. coli* primers, the forward gene was 5'-ATCACGGTGCTGAGCATGTCG-3'), and the reverse was 3'-CACACCGATGCCATGTCG-5 with length 223 bp for the 85 isolates evaluated by conventional PCR. See Conventional PCR showing positive results for detected of *E. coli* by using specific primer. Figure 2.

**Multiplex PCR Results:**

Ten samples were randomly selected to be tested by multiplex PCR for the presence of toxins using specific precursors of *E. coli* toxins. The first primer (*stx-1") forward primer 5'-GAGCGAAAATAATTATATGTG-3 and the reverse primer 3'-TTGATGATGCAATTCAGTAT-5 with the length of 780 bp and the second primer (*stx2) forward 5'-CCATGACAACGGACAGCAGTT-3 and reverse 3'- CCTGTCAACTGAGCAGTT-5 with the length of 510 bp. See table2.

The samples were distributed as follow: five were *E. coli* O157:H7, three were *E. coli* O26:H11 and two were *E. coli* O111:H8 but two of *E. coli* O157:H7 and 1 of *E. coli* O26:H11 were damaged or missed their genes during subcultures (as been informed by Bioneer Corporation, Korea).

So, the results showed that three of them were *E. coli* O157:H7, two of them were *E. coli* O26:H11 and two of them were *E. coli* O111:H8. See the positive results of toxins of different serotypes of *E. coli* (multiplex PCR to detect *E. coli* toxins stx). Figure 3.

The results revealed that *E. coli* O157:H7 have two genes of stx toxins (*stx-1 and stx-2) with the length of 780 bp and 510 bp respectively. While *E. coli* O26:H11 and *E. coli* O111:H8 have only one type of stx toxins gene that is (*stx-2) with the length of 510 bp.

The details of multiplex PCR results showed that *E. coli* O157:H7 gave two bands representing stx1 and stx2 while the other serotypes of *E. coli* representing O26:H11 and *E. coli* O111:H8 gave one band representing stx2.

The length of the gene of toxin belongs (*stx-2) to *E. coli* O157:H7 is different than the length of the gene of toxin (*stx-2) belongs to other serotypes of the *E. coli* O26:H11 and the *E. coli* O111:H8.

**Multiplex PCR results as following:**

ladder: lane M

*E. coli* O26:H11: lane 1: sample number 1; lane3: sample number3 and lane4: sample number4

*E. coli* O157:H7: lanes 2: sample number 2; lane 5: sample number 5; lane 6: sample number6, Lane 7: sample number 7, and lane 9: sample number 9.
Discussion

Diarrhea is one of the leading causes for morbidity and mortality especially in the developing countries. Pathogenic strains of EHEC are one of the most important pathogens that lead to diarrhea. The intestinal disease caused by an emerged O157 and Non-O157 EHEC is considered as an important issue because of their serious health problems and the extraintestinal consequences that follow the infection may lead to irreversible renal damage, or neurological disorders especially in young children, elderly, and patients with immune compromised systems [27].

This study was done during the second wave of diarrheal outbreak in Khartoum during the period from June to October 2020. This is in agreement with the previous report by Sack RB, Santosham et. al, who describes the association of diarrheal illness with seasons [28]. Most cases of diarrheal disease in children were found from May to December. The highest number of cases among adults is found during the fall season.

The main findings of this study were the isolation and identification of three different types of EHEC (i.e., O157:H7, E. coli O26:H11 and E. coli O111:H8) in children (n=40) and adults (n=60) in Khartoum by using multiplex PCR. Moreover, the identification of two types of toxins stx-1 and stx-2 that produced by E. coli O157 represented by two bands in the gel-electrophoreses. However, E. coli O26 and E. coli O111 have only one type of stx toxins gene (stx-2) that is represented by one band in the gel-electrophoreses.

Worth noting, the results describe the two stx genes of E. coli O157 (stx-1 and stx-2) with the length of 780 bp and 510 bp respectively. While E. coli O26 and E. coli O111 have only one type of stx toxins gene that is (stx-2) with the length of 510 bp. This finding indicates that not all toxigenic serotypes carry the same genes. Whereas, E. coli O157 carry two genes for one toxin but found in two forms (stx-1 and stx-2). While, both E. coli O26 and E. coli O111 carry only one toxin gene stx-2.

This study showed that EHEC were isolated from diarrheagenic adults, both E. coli O157 and E. coli non-O157 were isolated from diarrheagenic adults in Khartoum. In our study, 60% of diarrheagenic infection were adult compared to 40% of diarrheagenic cases were children.

These results differ from those obtained from Minia, Egypt [29], Tunisia [27], and the study done in Mangalore, India [30]. Mismatching of results may be due to the fact that the most common etiology of acute diarrhea in adults is viral infections which are self-limited conditions, while foodborne illness, and comorbidities that are related to bacterial causes of acute diarrhea in adults. In addition, adults do not usually visit health centers when they have diarrhoea, unless the diarrhea becomes serious [31].
In the current study, special attention was paid to investigating the presence of EHEC strains and the selected virulence factors of stx toxins in stool samples of diarrhoeagenic patients. However, this finding was typically observed in adult patients. The results observed with ETEC, in which shiga toxin–producing strains (stx-2) showed more severe disease and a stronger association with acute diarrhea than strains producing shiga toxin (stx-1), are consistent with those of other previously reported by (Vo Van Giau et al., 2015) [32] and agree with the study done by (Hessain, et al., 2015) [33]. *E. coli* O26:H11 and *E. coli* O111:H8 showed only one type of toxins stx-2 that represented by one band. These results for similar environmental isolation of a non-human source of both *E. coli* O157 and *E. coli* non-O157 suggesting an association of environmental mode of transmission.

Due to limited funding sources, this study is unable to isolate or identify other etiologies of diarrhea or presence of co-infections among study cases. In addition, the antibiotic properties of the isolated EHEC were not determined.

**Conclusion**

The result of multiplex PCR explains that there are two forms of shiga toxin produced by *E. coli* O157 while *E. coli* O26:H11 and *E. coli* O111:H8 produced one form of Shiga toxin

Similarly, the current study, detected that the length of the gene of toxin (*stx2*) belong to *E. coli* O157:H7 is different from the length of gene of toxin (*stx2*) belong to other serotypes *E. coli* O26: H11 and *E. coli* O111:H8.

**Abbreviations**

EHEC, enterohemorrhagic *E. coli*; HUS, hemolytic uremic syndrome; SLTEC, Shiga-like toxin producing *E. coli*; STEC, Shiga toxin-producing *E. coli*; Stx, Shiga toxin; VT, Verotoxin; VTEC, Verotoxin-producing *E. coli*, (P=): P-value, ng/ml: Nanograms per milliliter, SPSS: Statistical package for the social sciences.

**Declarations**

**Acknowledgment**

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**Availability of data and materials**
All the data supporting our findings are contained within this work; any other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Contributions

RA, HM, and IB carried out the study and participated in drafting the manuscript, participated in designing the study and participated in drafting the manuscript. IB coordinated in the statistical analysis. All the authors read and approved the final version.

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Ethical approval

Institutional review board approval was obtained from the Faculty of Medical Laboratory Sciences Research Ethics Review Board, University of Khartoum, Sudan.

consent to participate
A signed written informed consent was obtained from all subjects or from the parents or guardians in case of the participants under the 16 years of age. Sociodemographic and clinical data were obtained from each participant using structured pre-tested questionnaires.

**References**


### Tables

#### Table 1. Details of the participants’ gender and ages.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Frequency</th>
<th>Percentage</th>
<th>Mean age</th>
<th>Total</th>
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<tbody>
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<td>Females</td>
<td>26</td>
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<td>60</td>
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<tr>
<td></td>
<td>Males</td>
<td>34</td>
<td>34%</td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>Females</td>
<td>13</td>
<td>13%</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>27</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>100</td>
<td>100%</td>
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#### Table 2. The primers of *stx-1* and *Stx-2* genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
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<tbody>
<tr>
<td><em>Stx-1</em> forward</td>
<td>5'-GAGCGAAATAATTTATATGTG-3</td>
</tr>
<tr>
<td><em>Stx-1</em> reverse</td>
<td>3'-TTGATGATGGCAATTCAGTAT-5</td>
</tr>
<tr>
<td><em>Stx-2</em> forward</td>
<td>5'-CCATGACAACGGACAGCAGTT-3</td>
</tr>
<tr>
<td><em>Stx-2</em> reverse</td>
<td>3'-CCTGTCAACTGAGCACTTTGC-5</td>
</tr>
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### Figures
Figure 1

Schematic diagram of the study design.
Conventional PCR; Gel electrophoresis showed positive results for the detection of *E. coli* using a specific *E. coli* primer. Forward primer 5'-ATCACCGTGTTGCTGACATGTCGC-3', and reverse primer 3'-CACCAGATGCTGTTTCATCTG-5 with length of 223 bp.
Figure 3

Gel Electrophoresis showing positive results of toxins of different serotypes of *E. coli* (multiplex PCR to detect *E. coli* toxins *stx*) by using specific primers; Stx-1 forward 5'-GAGCGAAATAATTTATATGTG-3' and Stx-1 reverse primer 3'-TTGATGATGGCAATTCACTAT-5', Stx-2 forward primer 5'-CCATGACAACGGACAGCAGTT-3', Stx-2 reverse primer 3'-CCTGTCAACTGAGCACTTTGC-5'