Isolation and Molecular Characterization of Shiga Toxin-Producing *Escherichia coli* in Beef Retail Markets

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**Key words**

Shiga toxin, STEC, raw ground beef, retail markets, instruments samples, molecular.

**ABSTRACT:**

Shiga toxin-producing *Escherichia coli* (STEC) are food borne pathogens cause mild to serious diseases, leading to people death. This study records the prevalence and characterization of STEC O157 and non-O157 in commercial ground beef and instruments samples (meat tables, knives, meat mincing machines) obtained from 25 retail markets in Kafir El-sheikh governorate. The STEC isolates were serotyped and virulence genes as stx1 (Shiga toxin 1), stx2 (Shiga toxin 2) and eae ( intimin) were determined. STEC O157 were identified in 11 (14.6%) of raw beef samples and 8 (10.6%) in instrument samples, while STEC non-O157 (O128, O78, O111, O26 and O119) were present in 6(8%) in raw beef samples and 9 (12%) in instruments samples. Among isolated strains, the prevalent genotype for O157 were stx1, stx2 and eae in O111, stx1 and stx2 in O26, stx1 in O128 and O119, stx2 in O78. In conclusion, cross-contamination between meat and the environment could be suspected.

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1. **INTRODUCTION.**

Shiga toxin-producing *Escherichia coli* (STEC) are a group of food and water-borne pathogens that cause mild to serious diseases. More than 70 different serotypes of Shiga toxin-producing Escherichia coli (STEC) that cause disease in humans have been described. Illnesses range from mild diarrhea to bloody diarrhea to hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS). *E. coli* O157:H7 is the STEC strain most often associated with the most severe forms of disease. However, numerous non-O157 STEC isolates have also been linked to illnesses and several outbreaks in Egypt (Ali et al., 2014) and worldwide (Karmali et al., 2010).

Shiga toxins and intimin are key virulence factors for the pathogenesis of *Escherichia coli* O157 and other Enteropathogenic *E.coli.* (EPEC) strains (Gyles, 2007), there are different cultural methods for enrichment and detection of STEC; however none of them guarantee the speed, accuracy, sensitivity, specificity and safety. On the other hand, many studies were advertising the safety, accuracy, sensitivity, and specificity of molecular methods like polymerase chain reaction (PCR) for detection of STEC (Madic et al., 2011).

Several methods used for reliable identification of STEC contaminations as PCR and the loop-mediated isothermal amplification (LAMP) assays, which may facilitate diagnosis in high-risk food commodities and also facilitate prompt diagnosis of STEC infections in clinical laboratories (Fei et al., 2012), combination of selective and differential plating media with Multiplex polymerase chain reaction (mPCR) methods have been widely used for accurate detection and identification of the major serovars of STEC and its characteristic genes (Debroy et al., 2011, Valadez et al., 2011).

Ruminants have been identified as the major reservoir of STEC (Karmali et al., 2010) and a variety of foods have been identified as vehicles of illnesses. However, approximately 52% of outbreaks have been associated with bovine products. Contamination of carcasses with STEC can occur when gut contents or fecal matter contact the meat surfaces, in addition to cross-contamination between carcasses may occur during processing (Edwards and Fung, 2006).

Contamination of fresh meat during beef processing starts with the pathogen load on the hides of cattle entering the processing plant. Several studies have identified the hide as the major source of *E. coli*
O157:H7 contamination of carcasses during processing (Barkocy-Gallagher et al., 2001). Either *E. coli* O157:H7 or other strain types associated with human illness found on the hides of cattle entering processing plants, strict hygienic precaution must be applied to prevent finished product contamination during beef processing (Terrance et al., 2014).

Retail meats, mainly ground beef, were contaminated with diverse STEC strains. The presence of atypical Enteropathogenic Escherichia coli (EPEC) strains in retail meat is also of concern due to their potential to cause human infections (Xiaodong et al., 2010).

The Centers for Disease Control and Prevention estimates that STEC are responsible for 112,000 illnesses every year in the United States (Scallan et al., 2011). In Germany, more than 60% of the STEC O-groups isolated from food were also isolated from human patients, indicating the importance of food as a potential source of human infection, relative to other forms of transmission (Erickson and Doyle, 2007).

Systematic studies should applied on local ground beef retail markets to assess the microbiological quality of meat, and verification of good hygiene practices, handlers’ habits and traceability of the raw material (Arthur et al., 2014).

The aims of this study is to detect, isolate, and characterize STEC strains (O157 and non-O157) in ground beef and instrument samples (meat tables, knives, and mincing machines) in beef retail markets.

2-Materials and methods.

2.1-Sample collections.

One hundred and fifty samples of raw ground beef meat and instruments samples (75 of each) were weekly collected from 25 retail stores at Kafer El-Sheikh governorate. Sample collections were randomly taken and covered nearly all the geographic areas of the city. Raw ground beef samples (65gm) were collected in sterile bags avoiding contamination. Instrument samples were obtained from meat contact surfaces such as meat tables (25), knives (25) and meat mincing machines (25). Concerning meat tables nearly 150 cm2 areas were sampled with a sterile sponge soaked in buffered peptone water, the entire surface of the knife blades was sponged, the meat mincing machine was disassembled and the samples were taken from the meat container. All samples were collected in sterile bags avoid contamination and were immediately transported to the laboratory in cooled ice boxes.

2.2-Culture, enrichment and isolation (De Boer and Heuelink, 2000).

The raw ground beef samples (65gm) were placed aseptically in a plastic bag with 585 mL of modified tryptic Soya broth (mTSB) with 20 mg/L of novobiocin, the sponge samples were placed aseptically in a plastic bag with 100 ml of mTSB with 20 mg/L novobiocin. After homogenizing in a stomacher, each sample was incubated at 37°C for 18 hours. 5ul streaked onto MacConkey agar (MAC), suspected colony (pink one) streaked onto Cefixime (0.05mg/litter), Tellurite (2.5mg/litter) Sorbitol MacConkey agar (C-T S MAC). Plates were incubated at 37°C for 24 hours.

2.3-Biochemical tests (Ewing and Edwards, 1986).

Positive colonies on C-T S MAC were confirmed through biochemical tests.

2.4-Serodiagnosis of *E. coli* (Kok et al., 1996).

Serological identification of *E. coli* serotypes was done in Food Analysis Center, Faculty of Veterinary Medicine, Benha University.

The isolates were serologically identified by using rapid diagnostic *E. coli* O antisera sets (DENKA SEIKEN Co., Japan) for diagnosis of the Enteropathogenic types.

2.5-Multiplex Polymerase Chain Reaction (mPCR)

Molecular characterization of *E. coli* serotypes was done in Food Analysis Center, Faculty of Veterinary Medicine, Benha University.

2.5.1. DNA preparation from bacterial culture (Shah et al., 2009).

After overnight culture on nutrient agar plates, one or two colonies were suspended in 20 ml of sterile distilled water, and the suspension was then heated at 100°C for 20 minutes. From this suspension, a 5 μl aliquot was directly used as a template for PCR amplification.

2.5.2. Application of PCR for identification of shiga toxins (stx1 & stx2) and intimin (eae) genes of *E. coli* (Sipos et al., 2007).

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) using 25 μl of PCR mixture containing 3 μl of boiled cell lysate, 250 μM of each desoxynucleotide triphosphate,
1.5 U of Taq DNA polymerase (Biotools, Madrid, Spain), buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl and 3 mM MgCl2, Biotools), 1 μM of the primers mecA-R, mecA-F; 0.8 μM of icaA-R, icaA-F and 0.8 μM of icaD-R, icaD-F. Thermocycling was performed in a Hybaid Omnigene thermocycler with simulated tube control and the following three-step PCR cycling conditions were adopted for rfb gene: an initial denaturation of 1 cycle at 95°C for 5 min, followed by 35 cycles, each consisting of 30 s at 94°C, 30 s at 66°C and 30 s at 72°C. While, Amplification conditions for the other three primers were: denaturation for 3 min at 94°C, followed by 30 cycles of 94°C for 1min, 58°C for 1 min and 72°C for 1min, with final extension at 72°C for 5 min. Amplified products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized and captured on UV transilluminator. It was performed essentially by using Primers (Pharmacia Biotech) as shown in table (1).

Table (1): Primer sequences of Stx1, Stx2 and eae (Pharmacia Biotech) used for characterization of shiga toxin-producing Escherichia coli (STEC) O157 and STEC non-O157.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide sequence (5’ → 3’)</th>
<th>Amplicon length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stx1 (F)</td>
<td>5’ ACACTGGATGATCTCAGTGG ‘3</td>
<td>614</td>
<td>Dhanashree and Mallya (2008)</td>
</tr>
<tr>
<td>Stx1 (R)</td>
<td>5’ CTGAATCCCCCTCCATTAG ‘3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx2 (F)</td>
<td>5’ CCATGACAACGGACAGCATTT ‘3</td>
<td>779</td>
<td>Dhanashree and Mallya (2008)</td>
</tr>
<tr>
<td>Stx2 (R)</td>
<td>5’ CCTGTCAACTGAGCAGCTTT ‘3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eae (F)</td>
<td>5’ GTGGCGAATACTGGCGAGACT ‘3</td>
<td>890</td>
<td>Jeshveen et al., (2013)</td>
</tr>
<tr>
<td>eae (R)</td>
<td>5’ CCCCATTTTGTTCACCGTCG ‘3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3-RESULTS and DISCUSSION

Table (2): The Prevalence of STEC O157 and Non-O157 strains isolated from Raw ground beef meat and instrument samples (serological identification).

<table>
<thead>
<tr>
<th>Sample category</th>
<th>No. of samples</th>
<th>No. (%) of STEC O157 positive samples</th>
<th>No. (%) of STEC non-O157 positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw ground beef samples</td>
<td>75</td>
<td>11 (14.6%)</td>
<td>2 (2.6%) 0 (0%) 1 (1.3%) 1 (1.3%) 2 (2.6%) 6 (8%)</td>
</tr>
<tr>
<td>Meat tables</td>
<td>25</td>
<td>3 (12%)</td>
<td>2 (2.6%) 1 (4%) 0 (0%) 0 (0%) 1 (4%) 1 (4%)</td>
</tr>
<tr>
<td>Instruments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Knives</td>
<td>25</td>
<td>0 (0%)</td>
<td>0 (0%) 0 (0%) 0 (0%)</td>
</tr>
<tr>
<td>Mincing machine</td>
<td>25</td>
<td>5 (20%)</td>
<td>1 (4%) 2 (8%) 0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>8 (10.6%)</td>
<td>3 (4%) 1 (1.3%) 3 (4%) 0 (0%) 2 (2.6%) 9 (12%)</td>
</tr>
</tbody>
</table>

Table (3), The prevalence of STEC genes (Stx1, Stx2 and eae) in STEC O157 and Non-O157 strains isolated from raw ground beef meat and instrument samples.

<table>
<thead>
<tr>
<th>Type of shiga toxin</th>
<th>E coli O157 samples</th>
<th>E coli Non-O157 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O128</td>
<td>O78</td>
</tr>
<tr>
<td>Stx1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stx2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>eae</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Photograph (1): Agarose gel electrophoresis of multiplex PCR of stx$_1$ (614 bp), stx$_2$ (779 bp) and eae (890 bp) genes for characterization of Enteropathogenic E.coli.

Lane M: 100 bp ladder as molecular size DNA marker. Lane 1: Control positive for Shiga toxin 1 (stx$_1$), Shiga toxin 2 (stx$_2$) and intimin (eae) genes. Lane 2: Control Negative for stx1, stx2 and eae genes. Lane 3 (E.coli O128): Positive strain for stx1 gene. Lane 4 (E.coli O78): Positive strain for stx2 gene. Lane 5 (E.coli O111): Positive strain for stx1, stx2 and eae genes. Lane 6 (E.coli O26): Positive strain for stx1 and stx2 genes. Lane 7 (E.coli O119): Positive strain for stx1 gene. Lane 8 (E.coli O157): Positive strain for stx1, stx2 and eae genes.

Shiga toxin–producing Escherichia coli (STEC) have emerged as important enteric food born zoonotic pathogens of considerable public health significance in Egypt (Mostafa et al., 2014, Ahmed and Shimamoto, 2015) and worldwide (Brooks et al., 2005). STEC comprise a diverse group that elaborate one or both Shiga toxins (stx1 and stx$_2$) and can cause diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in human beings (Gyles, 2007, Grant et al., 2011).

Both STEC O157 and non-O157 are bacteria that cause serious human disease outbreaks through the consumption of contaminated food products (Gyles, 2007), but most of these STEC infections are caused by E. coli O157:H7 (20–70%) throughout the world are attributed to E. coli of non-O157 (Brooks et al., 2005).

Among the 75 raw ground beef samples and 75 instrumental samples, 14.6% of raw ground beef and 10.6% of instrumental samples, were positive for STEC O157, while 8% of raw beef and 12 % of instrumental samples were positive for non-O157 STEC strains, (Table 2). From this result we can noticed that the prevalence of STEC O157 in raw ground beef samples is more than in instrumental one, this may be due to ruminants fecal contamination of food product, especially cattle, which constitute a vast reservoir for this microorganisms (Karmali et al., 2010), while the prevalence of STEC non-O157 in instrumental samples is more than in raw ground beef samples, because, STEC non-O157 have many other sources (Gyles, 2007).

In this study, among the ground beef samples, the prevalence of STEC O157 is more than the prevalence of STEC non-O157, while other researcher Hussein and Bollinger (2005) recorded that; Non-O157 STEC were found more common than STEC O157 in beef products with a range of 1.7–62.5%, and this may be due to difficult to identify the STEC O-groups as compared to E. coli O157 due to lack of differentiating culture media and detection methods. Therefore, the burden of illness in human beings due to STEC non-O157 may be higher than reported (Grant et al., 2011).

Moreover, examination of 25 meat tables, 25 knives, and 25 mincing machines samples, revealed the following percent, 12%, 0%, and 20%, respectively, were positive for STEC O157, while, 16%, 4%, and 16%, respectively, were positive for STEC non-O157 (Table 2), as mention before, the prevalence of STEC non-O157 in instrumental samples is more than raw ground beef samples, due to that STEC non-O157 have many sources of contamination than E. coli O157 (Karmali et al., 2003).

The STEC contamination level of ground beef could vary due to the sociocultural characteristics (Paton and Paton 2002). Hygienic condition in most Egyptian beef markets are very bad (Ahmed and Shimamoto, 2015), so it's easy to expect higher prevalence of Shiga toxin–producing Escherichia coli especially in raw ground beef. On the other hand, other researcher Sallam et al., (2013), recorded higher prevalence than
our results, that may be due to differences in examined samples or in the population sociocultural characteristics. As shown in table (2), Thirty-four STEC isolates (n = 19 O157, n = 15 non-O157) were characterized by biochemical test, and then serotyped. STEC O157 characterization proved that all isolates were Sorbitol negative, while all STEC non-O157 strains were Sorbitol positive. Among 19 STEC O157 isolates, 11 were isolated from raw ground beef samples and 8 were from instrumental samples, while the 15 STEC non-O157 isolates were 6 in the raw ground beef samples and 9 in the instrumental samples, these isolates were of 5 different serotypes (O128, O78, O111, O119, and O26), and it’s percent were O128 (2.6%,4%), O78 (0%,1.3%), O111 (1.3%,4%) , O119 (1.3%, 0%), and O26 (2.6%-2.6%) in raw ground beef and instrumental samples, respectively. Six O groups (O26, O45, O111, O103, O121, and O145) have been described by the Centers for Disease Control and Prevention (CDC) to be the cause of 71% of non-O157 STEC worldwide, these isolates, recovered from human beings, have been considered an adulterant in beef products (Brooks et al., 2005; Kudakwashe et al., 2013; Patricia et al., 2014).

Shiga toxin-producing Escherichia coli (STEC), encompassing E. coli O157 and non-O157 STEC, are a significant cause of food-borne illnesses and deaths in the United States and worldwide. Shiga toxins (encoded by stx) and intimin (encoded by eae) are important virulence factors for STEC strains linked to severe human illnesses (Xiaodong et al., 2010; Fei et al., 2012). These groups are important for determining potential pathogens, the presence of virulence attributes, such as stx1 and stx2 and the locus of enterocyte effacement (eae), are important parameters for pathogenicity of the strains (Johnson et al., 1996). In commercial ground beef samples of U.S. some combination of stx1, stx2, ehx, eae, subA, chuA, nleB, and nleF may be a good approach to identify samples that might harbor STEC (Joseph and Mohammad, 2011), as shown in Table(2) and Photograph (1), both Shiga toxin (stx1, stx 2) and intimin (eae) genotypes occurred among STEC O157 and non-O157 strains isolated from raw ground meat and instrument samples. The stx1 gene was found in E. coli O157, O128, O111, O119, and O26, stx2 gene was found in E. coli O157, O78, O111 and O26, while eae gene was found in E. coli O157 and O111 only. From these results, we can noticed that E.coli O157 isolates have either stx1, stx 2 and eae, while E.coli non-O157 isolates have only one or two of them except E. coli O111 has the three types. These results nearly similar to other recorded either in Egypt (Sallam et al., 2013) and other countries (Brooks et al., 2005).

Consumption of raw/undercooked ground beef is the most common route of transmission of Shiga toxin-producing E. coli (Patricia et al., 2014), so ground beef should be cooked to an internal temperature of 160°F (71°C), as measured with a food thermometer before consumption (Brooks et al. 2005).

4. CONCLUSION.
We recommended use one of many methods for inactivation of Shiga Toxin-Producing Escherichia coli (STEC) isolates which contained various combinations of the shiga toxin 1 (stx1), shiga toxin 2 (stx2), intimin (eae), and hemolysin (ehx) genes in ground beef as use gamma irradiation (Sommers et al., 2015) or High pressure processing (HPP), which consider safe and effective technology for improving food safety (Hsu et al. 2015).

5. ACKNOWLEDGMENT.
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6. REFERENCES.


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