Virulence genes of Shiga toxin-producing *Escherichia coli* O157:H7 strains isolated from calves and cattle

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**Summary:** The aim of this study was to detect *E. coli* O157:H7 serotype in the faeces samples collected from calves and cattle farms located of Afyonkarahisar province in Turkey and to determine the stx(1) (Shiga toxin 1) and stx(2) (Shiga toxin 2) virulence genes in the strains of *E. coli* O157:H7 by multiplex PCR (mPCR). In this study, *E. coli* O157:H7 was isolated from the 3.1% (14 out 457 faeces samples) of the calves and cattle examined, in particular 2.3% of the cattle, 2.6% of non-diarrhoeic calves and 10.6% of diarrhoeic calves were positive for *E. coli* O157:H7. The stx(1) and stx(2) genes were detected in 6 out of 14 (42.8%) DNA samples extracted from STEC O157:H7 strains. This study demonstrated of *E. coli* O157:H7 serotype in cattle and calves, which represent an important reservoir for strains that a potential risk for human infections.

Keywords: Calves, cattle, *Escherichia coli* O157:H7, mPCR, stx(1) and stx(2) genes

**Introduction**

It has been widely reported that Shiga toxin-producing *Escherichia coli* (STEC) is linked to life threatening human disease such as haemorrhagic colitis (HC) and the haemolytic uremic syndrome (HUS) (20, 27, 34). *E. coli* O157:H7 was first recognized as a human pathogen in 1982 (35). As it was associated with consumption of undercooked 'hamburgers', it became known as 'the hamburger bug'. As it has subsequently been found that healthy cattle can harbour the bacterium, ruminants are now regarded as its main reservoir, though STEC O157:H7 has been isolated from other animal species such as sheep, pigs, geese, gulls and pet animals (17). Especially undercooked meat of bovine origin but also unpasteurized milk and other dairy products have been implicated in transmitting STEC O157:H7 to humans. (2, 9, 10, 19, 20, 22). Another route for acquiring the infection is direct transmission from cattle, especially calves, for instance on 'open farms' where groups of children are welcome to visit. As the bacterium survives well in the environment, drinking water, vegetables irrigated with contaminated water, and public outdoor swimming pools have been mentioned as sources of community outbreaks (17).

*E. coli* O157:H7 has been shown to have several virulence factors such as Shiga toxin 1 and 2 (encoded by stx(1) and stx(2) genes), enterohaemolysin (encoded by E-hlyA genes) and intimin (encoded by bacterial eaeA genes) (29). *E. coli* O157:H7 produced verotoxins are very similar to cytotoxins called stxs. However, stx(1) and stx(2) are encoded by different sets of genes. Therefore, stx(1) and stx(2) are different proteins but their active molecular structure and biological functions are identical to the other Shiga toxins (23). As mentioned above, the family of stxs has been classified into two prominent classes, stx(1) and stx(2). The stx(1) family is...
very homogenous while stx(2) has several variants (16). Shiga toxins inhibit protein synthesis by inactivating ribosomal RNA and they induce mortality in host cells. It has been shown that several techniques including immunoassays, verocell assay, PCR, multiplex PCR could be used to detect Shiga toxins (2, 24, 32, 33, 42).

The aim of this study was to detect \textit{E. coli} O157:H7 serotypes in the faeces samples collected from calves and cattle in dairy cattle enterprises located in small villages and towns in Afyonkarahisar province in Turkey and to determine the stx(1) and stx(2) virulence genes in the strains of \textit{E. coli} O157:H7 by mPCR.

**Materials and Methods**

\textbf{Samples:} Faeces samples were collected from 237 calves (38 of them having diarrhea) and 220 cattle with different ages in dairy cattle enterprises in villages, towns and counties of Afyonkarahisar province, Turkey. The sample collection sites and the number of samples are shown in Table 3 and the distribution of samples according to age shown in Table 2. The rectal samples were collected from the rectum and stored in disposable sterile plastic faeces sample container. Separate rectal gloves were used for each animal to avoid cross contamination. Samples were then placed in an ice–pack container and immediately transported to the laboratory.

\begin{table}
\centering
\caption{Distribution of \textit{E. coli} O157:H7 strains isolated from faecal samples according to age of animals}
\begin{tabular}{|l|l|}
\hline
Age & Positivity/n \% \\
\hline
1-4 weeks old & 5/80 \hspace{0.5cm} 6.2 \\
1-2 months old & 3/120 \hspace{0.5cm} 2.5 \\
2-3 months old & 1/37 \hspace{0.5cm} 2.7 \\
1 year old and older & 5/220 \hspace{0.5cm} 2.3 \\
Total & 14/457 \hspace{0.5cm} 3.1 \\
\hline
\end{tabular}
\end{table}

\textbf{Isolation of \textit{E. coli} O157:H7:} A 10% suspension was prepared by homogenizing faeces into 10 ml modified tryptone soya broth (mTSB) (Oxoid Basingstoke, Hampshire, UK) containing 20 mg/l novobiocin (Oxoid Basingstoke, Hampshire, UK). Suspension was incubated at 37°C for 6-12 h. Fifty microliters of the samples from mTSB were transferred into Cefixime-Tellurite Sorbitol MacConkey agar (CT- SMAC) containing 0.05 mg/l cefixime, 2.5 mg/l tellurite (Oxoid Basingstoke, Hampshire, UK), and 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (BCIG). The plates were then incubated at 37°C under aerobic conditions for 18-24 h. Both sorbitol fermentation and β-glucuronidase enzyme activity negative colonies (39) were subjected to agglutination with a latex reagent (Oxoid, Basingstoke, UK) for detecting serogroup O157. Moreover, H7 determination was performed with antisera (Denka Seiken, Tokyo, Japan). Biochemical assays of isolates were completed with triple sugar iron, phenylalanine deaminase, maltose, mannitol, indole, methyl red, Voges-Proskauer, urease, and citrate (18). STEC O157:H7 strain EDL 933 (38) was used as positive control strain and \textit{E. coli} ATCC 25922 (Oxoid) as negative control in all tests.

\textbf{Extraction of DNA:} DNA purification kit (MBI, Fermentas, Lithuania) was employed to extract DNA from both control and test strains according to the manufacturer protocols. Briefly, a single bacterial colony grown on TSA (Oxoid Basingstoke, Hampshire, UK) was inoculated into TSB (Oxoid Basingstoke, Hampshire, UK) and incubated at 37°C for 18h. After incubation, aliquots of one ml was taken from TSB and transferred into sterile DNase and RNase free 1.5 ml eppendorf tubes. Tubes were then centrifuged at 4000 rpm for 2 min. Afterwards, the supernatant was discarded and pellet was re-suspended in 200 μl sterile deionized water. The extraction was completed following the steps as indicated in the kit’s manual.

\textbf{PCR amplification:} The nucleotide sequences and the product sizes of the primers, purchased from TIB MOLBIOL Synthesize labor (Eresburgstrafße, D-12103 Berlin, Germany), were given in Table 1. PCR was used for the detection of stx(1) and stx(2) genes (33). The PCR mixture contained 5 μl of 10x PCR buffer, 2.5 mM MgCl₂, 0.2 mM from each of dNTPs, 0.25 mM from each primer, 2U Taq DNA polymerase (MBI Fermentas, Lithunia), 2 μl target DNA and the final volume of 50 μl was adjusted by the addition of deionized water. DNA of \textit{E. coli} ATCC 25922 dissolved in deionized water kept as a negative control, while strain carrying virulence genes were employed as positive controls. The PCR amplification conditions for stx(1) and stx(2) genes consisted an initial denaturation step at 95°C for min, followed by 30 cycles of 95°C for 30 sec (denaturation), 57°C for 30 sec (annealing),
Table 3. The sampling source, origin of animals and distribution of stx genes detected by mPCR

<table>
<thead>
<tr>
<th>Farm</th>
<th>No. animals sampled/ (%)</th>
<th>No.</th>
<th>Animal</th>
<th>stx1</th>
<th>stx2</th>
<th>stx1+stx2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Private farm A</td>
<td>5/150 (3.3)</td>
<td>1</td>
<td>Cattle</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Calf ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Cattle</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>Calf ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>Cattle</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Private farm B</td>
<td>2/85 (2.4)</td>
<td>6</td>
<td>Calf D</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>Calf ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Private farm C</td>
<td>2/72 (2.8)</td>
<td>8</td>
<td>Cattle</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>Calf ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Private farm D</td>
<td>3/110 (2.7)</td>
<td>10</td>
<td>Calf D</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>Cattle</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Private farm E</td>
<td>2/20 (10)</td>
<td>13</td>
<td>Calf D</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>Calf D</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Private farm F</td>
<td>0/20 (0)</td>
<td>8</td>
<td>Calf D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>Calf D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>14/457 (3.1)</td>
<td>7</td>
<td></td>
<td>(50%)</td>
<td>10</td>
<td>(71.4%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td></td>
<td>(42.8%)</td>
<td></td>
<td></td>
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</tbody>
</table>

H: Healthy; D: Diarrhoeic; ND: Non-diarrhoeic

Figure 1. PCR amplicons obtained with E.coli O157:H7 strains as tested in mPCR. Lane M, 100 bp DNA marker; Lane +, STEC O157:H7 EDL 933, 1-14 stx1 (210 bp) and stx2 (484 bp) genes were detected in E.coli O157:H7 strains isolated from calves and cattle faeces samples.
72 °C for 30 sec (extension) and a final step at 72°C for 7 min. All PCR products were analyzed by using 1.5 % agarose gel electrophoresis and visualized by using ethidiun bromide under UV light. Product sizes were determined by using DNA size marker (100 bp DNA ladder, Fermentas, Lithuania).

Statistical analysis: Chi-square test (Minitab version 13.2; Minitab Inc. 2000) was used to detect differences in the prevalence of E. coli O157:H7 for age groups at a significance level of 0.05.

**Results**

The prevalence of E. coli O157:H7 positive samples: Escherichia coli O157:H7 was detected with a ratio of 3.1 % (14 out 457 faeces samples) in calves and cattle by the study. Sources of isolation of E. coli O157:H7 strains were those: 4 calves out of 199 non-diarrhoeic calves (2.6%) and 5 out of 38 diarrhoeic calves (10.6%) and 5 out 220 cattle (2.3%). The isolation rate of E. coli O157:H7 from calves 1-4 weeks old (6.2%) was higher than other age groups. Distribution of E. coli O157:H7 strains isolated from faecal samples according to age are summarized in Table 2.

The distribution of virulence genes: Amplification of stx(1) and stx(2) genes in E. coli O157:H7 strains by mPCR are shown in Figure 1. The stx(1) and stx(2) genes were detected in 6 out of 14 (42.8%) DNA samples extracted from E. coli O157:H7 strains isolated from faeces samples (Table 3), whereas the stx(1) gene was detected in a total of 7 (50%) strains (Figure 1).

**Discussion and Conclusion**

Shiga toxin-producing Escherichia coli O157:H7 serotypes are known to be the most important emerged group of pathogens (3, 5, 10, 19, 32). It has been reported that cattle are main reservoir for STEC (2, 20, 26). Investigations carried out in different part of Europe, Asia, North America have showed that 10-80% cattle were infected with STEC (1, 2, 5, 8, 27, 31, 37). Kang et al. (19) reported that stx(1) and stx(2) appear to play a major role in the pathogenesis of haemorrhagic colitis and haemolytic-uremic syndrome and STEC strains predominantly carries either stx(1) or stx(2) or both genes. The distribution and the origin of Shiga toxigenic genes including stx(1), stx(2) and eaeA gene in toxigenic but in non-toxigenic E. coli O157 and O157:H7 isolated from faeces and carcass of cattle could be determined by multiplex PCR (42). The mPCR analysis shows that stx(2) gene is more frequently found than that of stx(1) in STEC strains (33). Similar findings are reported by different researchers by various countries (1, 7, 28, 31, 43). Keen and Elder (21) reported that the frequency of stx(2) genes could be 93.1 %, whereas the frequency of stx(1) 0 % in any isolated strains. In another study conducted by Chapman et al. (13), stx(2) and intimin gene were found in 71.3 % of isolated E. coli O157:H7 strains. In this study, mPCR amplified genes isolated from E. coli O157:H7 are shown in Figure 1 showing that stx(2) (71.4%) is found in higher frequency than that of stx(1) gene alone (42.8%) or stx(1) gene in combination (50%) (Table 3).

Blanco et al. (10) demonstrated that the strains of STEC can easily be isolated from healthy animals in comparison to animals having diarrhea since this strain present in the normal flora of intestine. According to Blanco et al. (10) stx(1), pathogenic strains could only be isolated from animals having diarrhea, while both stx(1) and stx(2), could be isolated from healthy calves. For instance, stx(1) gene was found significantly higher in diarrheic calves than healthy calves (40). Leomil et al. (24) reported that the diarrheic calves carry the higher frequency of stx was than that of non-diarrheic animals. Aslantas et al. (4) showed that E. coli 157H7 containing either stx(2) or both stx(1) and stx(2), could be isolated predominantly in 1- to 3- year old cattle group studied in Turkey. In the present study, stx(1) was more prevalent in diarrhoeic calves in comparison to other studies (Table 3).
In conclusion, E. coli O157:H7 virulence genes such as stx(1) and stx(2) were detected in faeces samples collected from calves and cattle using mPCR. Therefore, we believe that the E. coli O157:H7 serotypes isolated and analyzed in Turkey will be beneficial to the many research and researcher in this field. This study demonstrated of E. coli O157:H7 serotype in cattle and calves, which represent an important reservoir for strains that a potential risk for human infections.

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References


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