Detection of Enterohemolysin and Intimin Genes in *Escherichia coli* O157:H7 Strains Isolated from Calves and Cattle in Afyonkarahisar - Turkey [1]

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[1] This study was supported by the Scientific Research Projects Coordination Unit of Afyon Kocatepe University, Turkey, Project Number 06.VF.18, 2009

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**INTRODUCTION**

*Escherichia coli* O157:H7 serotype, which belongs to Enterohemorrhagic *Escherichia coli* (EHEC) group, has been considered to be one of the most important pathogens of food-borne infections in the world in recent years. This serotype is a major cause of bloody diarrhea, hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in humans 1,2.

Domestic cattle, especially young animals, have been implicated as a principal reservoir of *E. coli* O157:H7 strains that may be pathogenic for human.

**Keywords:** Calves, Cattle, eaeA, EhlyA, Escherichia coli O157:H7, PCR
occurs through consumption of raw or undercooked ground beef, unpasteurized dairy products and vegetables or water contaminated feces of carriers. Person-to-person transmission has also been documented 5-7.

Shiga toxins (Stx1, Stx2) are well-known potential virulence marker of E. coli O157:H7 serotype 8,9. In addition to toxin production, another virulence-associated factor is a protein called intimin (eae), which is responsible for intimate attachment of the agent to the intestinal epithelial cells, causing attaching and effacing (A/E) lesions in the intestinal mucosa 10. Intimin is encoded by the chromosomal gene eaeA and severe diarrhea (especially in HC) and HUS are closely associated with E. coli O157:H7 serotype carrying eaeA gene 11. Apart from Shiga toxins and intimins, E. coli O157:H7 may has a putative virulence factor such as enterohemolysin (Ehly), also called enterohemorrhagic E. coli hemolysin, which is encoded ehxA gene. Although many E. coli O157 carry this gene, the role of enterohemolysin in the pathogenesis of HC or HUS is uncertain 12.

Although several studies on the various virulence genes of E. coli O157:H7 strains have been reported in the various countries 4,8,9,11,13, researches on the ecology and prevalence of major virulence genes in E. coli O157:H7 strains isolated from domestic ruminants, especially calves, are limited in Western Turkey. Therefore, we investigated that the EhlyA and eaeA virulence genes in the E. coli O157:H7 strains previously isolated from fecal samples belong to calves and cattle in the Afyonkarahisar province of Western Turkey.

**MATERIAL and METHODS**

**Bacterial Isolates**

A total of 14 E. coli O157:H7 isolates were used in this study were previously isolated from 457 fecal samples (237 calves and 220 cattle) in Afyonkarahisar. Of the 14 isolates, 9 were obtained from calves (4 isolates from diarrheic calves, 5 isolates from non-diarrheic calves), and 5 from healthy cattle (Table 1). Previously characterized EHEC O157:H7 strain EDL 933 14 was used as positive control strain in all tests.

**Extraction of DNA**

DNA purification kit (MBI, Fermentas, Lithunia) was used the extraction of DNA from both control and test strains according to the manufacturer protocols. Briefly, a single bacterial colony grown on Tryptone Soya agar (TSA) (Oxoid Basingstoke, Hampshire, UK) was inoculated into Tryptone Soy broth (TSB) (Oxoid Basingstoke, Hampshire, UK) and incubated at 37ºC for 18 h. 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 4,000 rpm for 2 min. After wards, 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.

**PCR Amplification**

The primers used in this study purchased from TIB MOLBIOL Syntheselabor (Eresburgstraβe, D-12103 Berlin, Germany), and they were shown in Table 2. Singleplex PCRs were used for the detection of eaeA and EhlyA genes 15,16. 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, 2 U Taq DNA polymerase (MBI Fermentas, Lithunai), 2 μl target DNA and the final volume

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Animal</th>
<th>eaeA</th>
<th>EhlyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cattle (H)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Calf (ND)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Cattle (H)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Calf (ND)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Cattle (H)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Calf (D)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Calf (ND)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Cattle (H)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Calf (ND)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Calf (D)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Cattle (H)</td>
<td>+</td>
<td>+</td>
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<tr>
<td>12</td>
<td>Calf (ND)</td>
<td>+</td>
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<tr>
<td>13</td>
<td>Calf (D)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>Calf (D)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>8 (57.1%)</td>
<td>13 (92.8%)</td>
</tr>
</tbody>
</table>

H: Healthy; D: Diarrheic; ND: Non-diarrheic

**Table 1.** The origin of E. coli O157:H7 isolates used in this study and distribution of eaeA and EhlyA virulence genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Oligonucleotide Sequence (5’ → 3’)</th>
<th>Size of Amplified Product (Base Pairs)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>eaeA</td>
<td>Int-F</td>
<td>GGGATCGATTACCGTCATTTTATCAGCCTTAATCTC</td>
<td>837</td>
<td>Batchelor et al.16</td>
</tr>
<tr>
<td></td>
<td>Int-R</td>
<td>TTATACGCTATTTATCGCTTAATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EhlyA</td>
<td>HlyA-F</td>
<td>GCATCATCAGGGTACGTCC</td>
<td>534</td>
<td>Schmidt et al.15</td>
</tr>
<tr>
<td></td>
<td>HlyA-R</td>
<td>AATGAGCCAAGCTGGTTAAGCT</td>
<td></td>
<td></td>
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</table>

**Table 2.** PCR primers used in the present study

**Table 2.** Çalışmada kullanılan PZR primerleri
of 50 μl was adjusted by the addition of deionized water. DNAs of *E. coli* ATCC 25922 (Oxoid, Hampshire, England) and EHEC O157:H7 strain EDL 933 were used as negative and positive control, respectively. The PCR amplification conditions for *EhlyA* and *eaeA* genes consisted an initial denaturation step at 95ºC for 1 min, followed by 30 cycles of 95ºC for 30 sec (denaturation), 57ºC for 30 sec (annealing), 72ºC for 30 sec (extension) and a final step at 72ºC for 7 min. All PCR products were analyzed by using 1.5% agarose gel electrophoresis and visualized by using ethidium bromide under UV light. Product sizes were determined by using DNA size marker (100-bp DNA ladder, Fermentas, Lithuania). The 837-bp and 534-bp bands were accepted as positive for *eaeA* and *EhlyA* genes, respectively.

**RESULTS**

In our study, the *eaeA* and *EhlyA* genes were detected in 8 (57.1%) and in 13 (92.8%) of the isolates, respectively. Of the 8 *eaeA* genes, 4 (50.0%) were obtained from diarrheic calves, 2 (25.0%) from non-diarrheic calves, and 2 (25.0%) from healthy cattle. A total of 7 (50.0%) strains were determined to harbour both of the *EhlyA* and *eaeA* genes. Enterohemolysin was found as the predominant virulence factor (Table 1). Amplification of *EhlyA* and *eaeA* genes in the *E. coli* O157:H7 strains by PCR were shown in Fig. 1 and 2.

**DISCUSSION**

In the present study, we investigated the *eaeA* and *EhlyA* virulence genes in the *E. coli* O157:H7 strains previously isolated from diarrheic and non-diarrheic calves and healthy cattle in Western Turkey. *E. coli* O157:H7 strains have been implicated as an etiological factor of calf diarrhea, and these animals have been considered as primer reservoir of *E. coli* O157:H7 for human infections. However, this agent may be also recovered from healthy cattle. Although several studies on the various virulence genes of *E. coli* O157:H7 strains isolated from Turkish cattle have been reported in Turkey, researches on the prevalence of major virulence genes in *E. coli* O157:H7 strains isolated from calves are limited.

Intimin gene, which has been shown to be necessary for attaching and effacing activity, encodes a 94- to 97 kDa outer membrane protein (OMP), which is termed intimin. Several researchers were indicated the strong association between carrying *eaeA* and the capacity of *E. coli* O157:H7 to cause severe human disease, especially HUS. The high and low prevalence of *eaeA* gene were reported in *E. coli* O157:H7 strains isolated from diarrheic as well as non-diarrheic calves and healthy cattle. Sandhu et al. emphasized that *eaeA* gene is more frequently found in *E. coli* O157:H7 strains from calves compared with *E. coli* O157:H7 from adult cattle. It has also been reported that the *eaeA* gene have a defined role in causing the A/E lesions in calves. However, Wieler et al. indicated that low prevalence of *eaeA* gene is typical for EHEC O157:H7 isolated from healthy cattle. This virulence gene was detected in 57.1% of *E. coli* O157:H7 isolates in this study.

Similar to opinion of Sandhu et al., of the 8 *eaeA* genes, 6 (75.0%) were belonged to strains isolated from calves. This rate was higher in diarrheic calves (50.0%) than in non-diarrheic calves (25.0%). This finding is consistent with other researcher’s results.

Enterohemolysin is distinct from alpha hemolysin of...
E. coli and is encoded by 60MDa virulence plasmid of EHEC [24]. The role of this virulence factor is unclear, although it lyses erythrocytes and leukocytes in cattle, which provides a mechanism for iron acquisition and subsequent bacterial growth [12]. It was reported that *EhlyA* gene was found in most *E. coli* O157:H7 strains associated with HUS and in EHEC O157:H7 field isolates from ruminants [25]. *EhlyA* gene was similarly found as the predominant virulence factor in our study. A total of 13 strains (92.8%) were determined to harbour *EhlyA* gene, remaining one strain obtained from diarrheic calf did not harbour this gene. This result shows that enterohemolysin may be an important virulence factor for human infections.

In conclusion, the major virulence genes of *E. coli* O157:H7 such as *eaeA* and *EhlyA* were detected in the strains isolated from calves and cattle using PCR. Of the 14 *E. coli* O157:H7 strains tested, 7 (50.0%) harboured two virulence genes. Although an outbreak or individual case connected to *E. coli* O157:H7 has not been reported in Turkey so far, it should be considered that calves, especially diarrheic, and cattle can be a potential reservoir for *E. coli* O157:H7 infections in human. According to our results, it may be also considered that *E. coli* O157:H7 strains carrying *eaeA* and *EhlyA* genes may be more pathogenic for human.

**REFERENCES**