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SHORT COMMUNICATION

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

Yahya KUYUCUOĞLU * Esra ŞEKER * Barış SAREYYÜPOĞLU ** Zeki GÜRLER ***

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* Department of Microbiology, Faculty of Veterinary Medicine, Afyon Kocatepe University, ANS Campus, TR-03200 Afyonkarahisar - TURKEY
** Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, TR-06100 Ankara - TURKEY
*** Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Afyon Kocatepe University, ANS Campus, TR-03200 Afyonkarahisar - TURKEY

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Summary

The aim of this study was to detect enterohemolysin *(EhlyA)* and intimin *(eaeA)* virulence genes of 14 *Escherichia coli* O157:H7 strains isolated from 457 fecal samples (237 calves and 220 cattle) by PCR. While *EhlyA* gene was determined in 13 (92.8%) strains, the *eaeA* gene was positive in 8 (57.1%) strains. 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. This study confirmed that calves, especially diarrheic, and cattle are a reservoir of *E. coli* O157:H7 strains that may be pathogenic for human.

Keywords: *Calves, Cattle, eaeA, EhlyA, Escherichia coli O157:H7, PCR*

Afyonkarahisar'da Sığır ve Buzağılardan İzole Edilen *Escherichia coli* O157:H7 Suşlarında İntimin ve Enterohemolizin Genlerinin Belirlenmesi

Özet

Bu çalışmada, 457 dışkı örneğinden (237 buzağı ve 220 sığır) izole edilen 14 *Escherichia coli* O157:H7 suşunun enterohemolizin *(EhlyA)* ve intimin *(eaeA)* virulens genlerinin PZR ile belirlenmesi amaçlandı. On üç suşta (%92.8) *EhlyA* geni saptanırken, 8 suşta (%57.1) *eaeA* geni pozitifti. Sekiz *eaeA* geninin 4'ü (%50.0) ishalli, 2'si (%25.0) ishalsiz buzağılar ve 2'si de (%25.0) klinik olarak sağlıklı görünen sığırlarda belirlendi. Toplam 7 (%50.0) suşun hem *EhlyA* hem de *eaeA* geni taşıdığı belirlendi. Bu çalışma ile özellikle ishalli buzağıların ve sığırların, insanlar için patojenik olabilecek *E. coli* O157:H7 rezervuarı oldukları doğrulandı.

Anahtar sözcükler: *Buzağı, eaeA, EhlyA, Escherichia coli O157:H7, PCR, Sığır*

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 serotype that causes human infections [3,4]. Transmission

İletişim (Correspondence)
+90 272 2281312/143
esraseker@hotmail.com

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.

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

Tablo 1. Çalışmada kullanılan *E. coli* O157:H7 izolatlarının orijini ve *eaeA* ve *EhlyA* virulens genlerinin dağılımı

Isolates	Animal	eaeA	EhlyA
1	Cattle (H)	-	+
2	Calf (ND)	-	+
3	Cattle (H)	-	+
4	Calf (ND)	-	+
5	Cattle (H)	-	+
6	Calf (D)	+	-
7	Calf (ND)	+	+
8	Cattle (H)	+	+
9	Calf (ND)	-	+
10	Calf (D)	+	+
11	Cattle (H)	+	+
12	Calf (ND)	+	+
13	Calf (D)	+	+
14	Calf (D)	+	+
Total		**8 (57.1%)**	**13 (92.8%)**

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

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 37°C 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. Afterwards, the supernatant was discarded and pellet was resuspended 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 $MgCl_2$, 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 2.** PCR primers used in the present study*

***Tablo 2.** Çalışmada kullanılan PZR primerleri*

Gene	Primer	Oligonucleotide Sequence (5′→ 3′)	Size of Amplified Product (Base Pairs)	References
eaeA	Int-F Int-R	GGGATCGATTACCGTCAT TTTATCAGCCTTAATCTC	837	Batchelor et al.[16]
EhlyA	HlyA-F HlyA-R	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	534	Schmidt et al.[15]

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 [14] 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 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*.

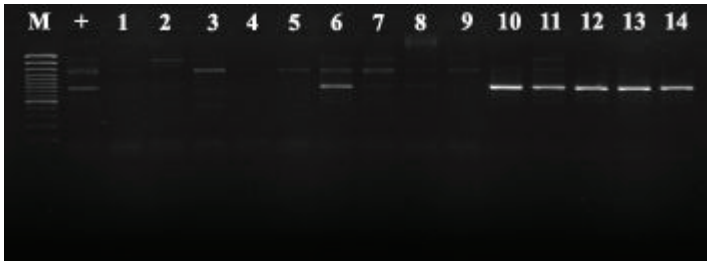

Fig 1. Detection of *eaeA* gene by PCR. M: 100 bp DNA ladder (Fermentas, Vilnius, Lithuania); +, positive control (EHEC O157:H7 strain EDL 933), lanes 6-8 and 10-14, specific bands of *eaeA* gene (837 bp)

Şekil 1. *eaeA* geninin PZR ile saptanması. M: 100 bp DNA ladder (Fermentas, Vilnius, Litvanya); +, pozitif kontrol (EHEC O157:H7 suş EDL 933); hatlar 6-8 ve 10-14, *eaeA* genine spesifik bantlar (837 bp)

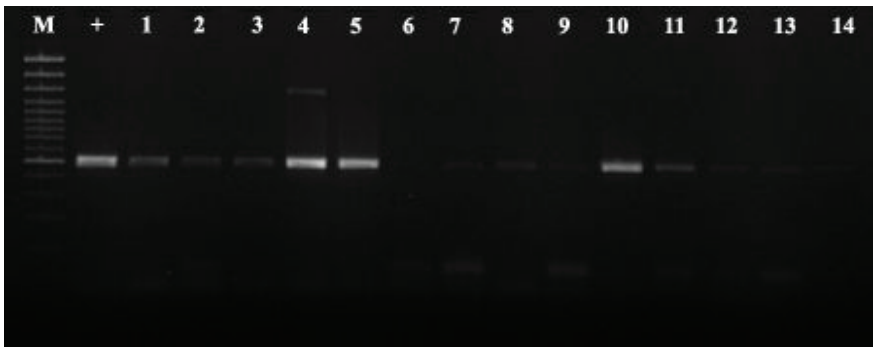

Fig 2. Detection of *EhlyA* gene by PCR. M: 100 bp DNA ladder (Fermentas, Vilnius, Lithuania); +, positive control (EHEC O157:H7 strain EDL 933); lanes 1-5 and 7-14, specific bands of *ehlyA* gene (534 bp)

Şekil 2. *EhlyA* geninin PZR ile saptanması. M: 100 bp DNA ladder (Fermentas, Vilnius, Litvanya); +, pozitif kontrol (EHEC O157:H7 suş EDL 933); hatlar 1-5 ve 7-14, *EhlyA* genine spesifik bantlar (534 bp)

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 [4,9,17]. However, this agent may be also recovered from healthy cattle [3,11]. Although several studies on the various virulence genes of *E. coli* O157:H7 strains isolated from Turkish cattle have been reported in Turkey [18,19], 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 [10]. 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 [2,20]. 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 [9,21] and healthy cattle [22]. Sandhu et al.[22] 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 [10]. However, Wieler et al.[23] 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.[22], 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 [22,23].

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

1. Gyles CL: Shiga toxin-producing *Escherichia coli:* An overview. *J Anim Sci*, 85, 45-62, 2007.

2. Karmali MA, Gannon V, Sargeant JM: Verocytotoxin-producing *Escherichia coli* (VTEC). *Vet Microbiol*, 140, 360-370, 2010.

3. Chapman PA, Siddons CA, Wright DJ, Norman P, Fox J, Crick E: Cattle as a possible source of verocytotoxin-producing *Escherichia coli* O157 infections in man. *Epidemiol Infect*, 111, 439-447, 1993.

4. Osek J, Gallien P, Protz D: Characterization of Shiga toxin-producing *Escherichia coli* strains isolated from calves in Poland. *Comp Immunol Microbiol Infect Dis*, 23, 267-276, 2000.

5. Rangel JM, Sparling PH, Crowe C, Griffin PM, Swerdlow DL: Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerg Infect Dis*, 11, 603-609, 2005.

6. Belongia E, MacDonald K, Parham G, White K, Korlath J, Lobato D, Strand S, Casale S, Osterholm M: An outbreak of *Escherichia coli* O157:H7 colitis associated with consumption of precooked meat patties. *J Infect Dis*, 164, 338-343, 1991.

7. Bielaszewska M, Janda J, Blahova K, Minarikova H, Jikova E, Karmali MA, Laubova J, Sikulova J, Preston MA, Khakhria R, Karch H, Klazorova H, Nye O: Human *Escherichia coli* O157:H7 infection associated with the consumption of unpasteurized goat's milk. *Epidemiol Infect*, 119, 299-305, 1997.

8. Nielsen EM, Scheutz F: Characterization of *Escherichia coli* 0157 isolates from Danish cattle and human patients by genotyping and presence and variants of virulence genes. *Vet Microbiol*, 88, 259-273, 2002.

9. Kang SJ, Ryu SJ, Chae, JS, Eo SK, Woo GJ, Lee JH: Occurrence and characteristics of enterohemorrhagic *Escherichia coli* O157 in calves associated with diarrhoea. *Vet Microbiol*, 98, 323-328, 2004.

10. Dean-Nystrom EA, Bosworth BT, Cray WC, Moon HW: Pathogenicity of *Escherichia coli* O157:H7 in the intestines of neonatal calves. *Infect Immun*, 65, 1842-1848, 1997.

11. Beutin L, Geiger D, Steinruck H, Zimmermann S, Karch H: Virulence markers of Shiga-like toxin-producing *Escherichia coli* strains originating from healthy domestic animals of different species. *J Clin Microbiol*, 33, 631-635, 1995.

12. Bauer ME, Welch RA: Characterization of an RTX Toxin from Enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun*, 64, 167-175, 1996.

13. Şeker E, Kuyucuoğlu Y, Sareyyüpoğlu B, Yardımcı H: PCR detection of Shiga toxins, enterohaemolysin and intimin virulence genes of *Escherichia coli* O157:H7 strains isolated from faeces of Anatolian water buffaloes in Turkey. *Zoonoses and Public Hlth,* 57, e33-e37, 2010.

14. Wells JG, Davis BR, Wachsmuth IK, Riley LW, Remis RS, Sokolow R, Morris GK: Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *J Clin Microbiol*, 18, 512-520, 1983.

15. Schmidt H, Beutin L, Karch H: Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect Immun*, 63, 1055-1061, 1995.

16. Batchelor M, Knutton S, Caprioli, Huter V, Zanial M, Dougan G, Frankel G: Development of a universal intimin antiserum and PCR primers. *J Clin Microbiol*, 37, 3822-3827, 1999.

17. Dorn CR, Francis DH, Angrich EJ, Willgohs JA, Wilson RA, Collins JE, Jenke BH, Shawd SJ: Characteristics of vero cytotoxin producing *Escherichia coli* associated with intestinal colonization and diarrhea in calves. *Vet Microbiol*, 13, 149-159, 1993.

18. Aslantaş Ö, Erdoğan S, Cantekin Z, Gülaçtı İ, Evrendilek GA: Isolation and characterization of verocytotoxin -producing *Escherichia coli* O157 from Turkish cattle. *Int J Food Microbiol,* 106, 338-342, 2006.

19. Yilmaz A, Gun H, Ugur M, Turan N, Yimaz H: Detection and frequency of VT1, VT2 and *eaeA* genes in *Escherichia coli* O157 and O157:H7 strains isolated from cattle, cattle carcasses and abattoir environment in Istanbul. *Int J Food Microbiol,* 106, 213-217, 2006.

20. Beutin L, Krause G, Zimmermann S, Kaulfuss S, Gleier K: Characterization of Shiga toxin-producing *Escherichia coli* strains isolated from human patients in Germany over a 3-year period. *J Clin Microbiol*, 42, 1099-1108, 2004.

21. Mainil JG, Jacquemin ER, Kaeckenbeeck AE: Association between the effacing (*eae*) gene and Shiga-like toxin-encoding genes in *Escherichia coli* isolates from cattle. *Am J Vet Res*, 54, 1064-1068, 1993.

22. Sandhu KS, Clark RC, McFadden K, Brouwer A, Louie M, Wilson J, Lior H, Gyles CL: Prevalence of the *eaeA* gene in verotoxigenic *Escherichia coli* strains from dairy cattle in Southwest Ontario. *Epidemiol Infect*, 116, 1-7, 1996.

23. Wieler LH, Vieler E, Erpenstein C, Schlapp T, Steinrück H, Bauerfeind R, Byomi A, Baljer G: Shiga toxin-producing *Escherichia coli* strains from bovines: Association of adhesion with carriage of *eae* and other genes. *J Clin Microbiol*, 34, 2980-2984, 1996.

24. Beutin L, Montenegro MA, Ørskov I, Ørskov F, Prada J, Zimmermann S, Stephan R: Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli. J Clin Microbiol*, 27, 2559-2564, 1989.

25. Gyles C, Johnson R, Gao A, Ziebell K, Pierard D, Aleksic S, Boerlin P: Association of enterohemorrhagic *Escherichia coli* hemolysin with serotypes of Shiga-like-toxin-producing *Escherichia coli* of human and bovine origins. *Appl Environ Microbiol*, 64, 4134-4141, 1998.