doi: 10.17221/45/2016-VETMED

Molecular characterisation of Shiga toxin-producing *Escherichia coli* O157:H7 isolates from cattle in eastern Turkey

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ABSTRACT: Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is an important foodborne pathogen worldwide. In Turkey, little is known about the molecular characteristics of STEC O157:H7 isolates. The aim of this study was to examine the genetic diversity of STEC O157:H7 isolates from cattle in Turkey using pulsed-field gel electrophoresis (PFGE). A total of 18 STEC O157:H7 strains were isolated from 540 rectal swab samples of slaughtered cattle in two abattoirs (coded A and B) located in eastern Turkey. The presence of virulence genes (*stx1*, *stx2*, *hlyA* and *eae*) was detected using the polymerase chain reaction. Of the 18 STEC O157:H7 isolates, 14 had s*tx2*, *hlyA*, and *eae* genes. Four of the isolates were positive for *stx1*, s*tx2*, *hlyA*, and *eae* genes. Thirteen different PFGE patterns were observed among the STEC O157:H7 isolates. The most common PFGE pattern was X1, which was detected in six STEC O157:H7 isolates. These six isolates contained the same virulence genes (*stx2*, *eae* and *hlyA*). However, some of the isolates containing the same virulence genes showed different PFGE profiles. Eleven different PFGE patterns were detected among the isolates from abattoir B, and two different PFGE patterns were detected among the isolates from abattoir A. The results of PFGE revealed a wide genetic diversity among the STEC O157:H7 isolates from cattle in Turkey. This is the first study on PFGE typing of STEC O157:H7 isolates from cattle in Turkey and further studies are needed.

Keywords: pulsed-field gel electrophoresis; virulence genes; bovine; rectal swabs; *E. coli* strains

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is one of the most important foodborne pathogens worldwide (Mora et al. 2004; Kawano et al. 2012). Domestic ruminants, especially cattle, are considered to be the main reservoir of STEC O157:H7. STEC O157:H7 is commonly found in the intestines of cattle and it can contaminate carcasses at slaughter (Hussein and Bollinger 2005). Humans can be infected by consumption of food and water contaminated with animal faeces (Ferreira et al. 2014). STEC O157:H7 causes diarrhoea, haemorrhagic colitis, haemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura in humans (D'Astek et al. 2012; Chui et al. 2015). It may lead to death in some people (Money et al. 2010). Paediatric and elderly patients are more sensitive to STEC O157:H7 infection (Hiroi et al. 2012). Virulence factors contribute to the pathogenicity of this bacterium (Kawano et al. 2012). The most important virulence factors include shiga toxins encoded by the *stx1* and *stx2* genes, intimin encoded by the *eae* gene and enterohaemorrhagic *E. coli* (EHEC) haemolysin encoded by the EHEC-*hlyA* gene (Sanchez et al. 2010; Amezquita-Lopez et al. 2012). Several molecular techniques have been used to determine the genotypes of human, food and animal *E. coli* isolates, and pulsed-field gel electrophoresis (PFGE) is considered the gold standard for molecular typing of STEC O157:H7 isolates (Liebana et al. 2003).

The presence of STEC O157:H7 in cattle (Kuyucuoglu et al. 2011), ground beef (Kalender 2013) and humans (Erdogan et al. 2008) has previously been reported in Turkey. However, to our

doi: 10.17221/45/2016-VETMED

knowledge, no study on PFGE typing of STEC O157:H7 isolates from cattle in Turkey has been published. The aim of this study was to determine the genotypic diversity of STEC O157:H7 isolates from slaughtered cattle in Turkey using PFGE.

MATERIAL AND METHODS

STEC O157:H7 isolates. STEC O157:H7 isolates were obtained from a study carried out in eastern Turkey (Kalender 2013). In brief, a total of 540 rectal swab samples were collected from slaughtered cattle at two abattoirs (coded A and B) located in eastern Turkey between December 2011 and June 2012. The swabs were placed in a modified tryptone soya broth (mTSB) (LAB165; Lab M) supplemented with novobiocin. The mTSB medium containing rectal swab samples was incubated at 41.5 °C for 24 h. Then, immunomagnetic separation (IMS) was performed according to the manufacturer's instructions (Captive O157, Lancashire, UK). The IMS samples were plated onto Sorbitol MacConkey Agar supplemented with cefixime and tellurite (CT-SMAC Agar) (LAB161; Lab M). The agar plates were incubated at 37 °C for 24 h. Colourless colonies on CT-SMAC Agar were considered presumptive *E. coli* O157. *E. coli* O157:H7 was identified by amplification of the genes encoding O157 somatic antigen (*rfbE*) and H7 flagella (*fliCh7*). The presence of identification genes (*rfbE* and *fliCh7*) and virulence genes (*stx1*, *stx2*, *hlyA*, and *eae*) was detected using the polymerase chain reaction (PCR). PCR amplification consisted of 30 cycles of template denaturation at 94 °C, primer annealing at 50 °C, and primer extension at 72 °C for 40 s (DebRoy and Roberts 2006).

Pulsed-field gel electrophoresis. The STEC O157:H7 isolates were genotyped using a pulsed-field gel electrophoresis technique described by Donaldson et al. (2006). Briefly, bacterial colonies grown overnight on Trypticase Soy Agar plates with 5% defibrinated sheep blood were suspended directly with cotton swabs in about 2 ml of TE buffer (100mM Tris and 100mM EDTA). The concentrations of cell suspensions were adjusted using a bioMe'rieux Vitek colorimeter. Aliquots of 100 µl of the cell suspensions were transferred to 1.5-ml microcentrifuge tubes. Lysozyme (10 mg/ml stock solution) and proteinase K (20 mg/ml stock solution) were added to a final concentration of 1 mg/ml each and mixed several times by pipetting up and down. The bacterial suspensions were incubated at 37 °C for 10–15 min. InCert agarose (FMC BioProducts, Rockland, Maine) was prepared in water to a final concentration of 1.2% and maintained at 55 °C in a water bath. Following the lysozyme-proteinase K incubation, 7 µl of 20% sodium dodecyl sulphate and 140 µl of 1.2% InCert agarose were mixed with each bacterial suspension with the help of a pipette. This bacterium-agarose mixture was immediately added to plug molds (Bio-Rad Laboratories). The plugs were allowed to solidify for 5–10 min at 4 °C and then transferred to 2-ml round-bottom tubes containing 1.5 ml of ESP buffer (0.5M EDTA, pH 9.0; 1% sodium lauryl sarcosine; 1 mg of proteinase K per ml). These were incubated in a water bath at 55 °C for 2 h. The plugs were transferred to 50-ml tubes containing 8–10 ml of sterile, preheated (50 °C) distilled water and incubated for 10 min at 50 °C with gentle mixing in a shaking water bath. Subsequently, four 50 °C washes were done for 15 min each with TE buffer (10mM Tris, pH 8.0; 1mM EDTA, pH 8.0). For restriction endonuclease digestion, a small slice of each plug was incubated at 37 °C for 3 h with 50 IU of *Xba*I enzyme. The plug slices of the samples were loaded and electrophoresed in 1% SeaKem Gold agarose with standard TBE running buffer. The CHEF Mapper XA PFGE system (Bio-Rad, Hercules, CA) was used to perform electrophoresis. The electrophoretic conditions used were as follows: initial switch time, 2.2 s; final switch time, 54.2 s; run time, 18 h; angle, 120°; gradient, 6.0 V/cm; temperature, 14 °C; ramping factor, linear. Following electrophoresis, the gels were stained with ethidium bromide and were visualised under ultraviolet light. Genotypic relatedness was determined using PFGE DNA fingerprint subtypes. Analysis of PFGE patterns was performed using Gel Doc 2000 Molecular Analyst Fingerprinting Plus software, version 6.1 (Bio-Rad, Hercules, CA). The analysis of the bands generated was carried out using the Dice coefficient and the unweighted pair group method with arithmetic averages (optimisation of 1.00% and position tolerance 1.0%).

RESULTS

Thirteen different PFGE patterns were observed among the 18 STEC O157:H7 isolates from 540 cattle. The most common PFGE pattern was X1, which

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Table 1. Virulence genes and PFGE patterns of the 18 STEC O157:H7 isolates from cattle

Isolate No.	Abattoir	*stx1*	*stx2*	*eae*	*hlyA*	PFGE pattern
1	B	–	+	+	+	X3
2	A	–	+	+	+	X2
3	B	–	+	+	+	X4
4	A	–	+	+	+	X1
5	A	–	+	+	+	X1
6	A	–	+	+	+	X1
7	A	–	+	+	+	X1
8	A	–	+	+	+	X1
9	A	–	+	+	+	X1
10	B	–	+	+	+	X11
11	B	–	+	+	+	X13
12	B	–	+	+	+	X5
13	B	–	+	+	+	X10
14	B	–	+	+	+	X12
15	B	+	+	+	+	X7
16	B	+	+	+	+	X9
17	B	+	+	+	+	X6
18	B	+	+	+	+	X8

was found in six (33.3%) of the STEC O157:H7 isolates. These six isolates contained the same virulence genes (*stx2*, *eae* and *hlyA*). Eleven different PFGE patterns were detected among the isolates from abattoir B, and two different PFGE patterns were detected among the isolates from abattoir A (Table 1). PFGE analysis revealed that the similarity of PFGE patterns ranged from 51% to 100% (Figure 1). Animals with the same PFGE and virulence types originated from different farms in the same region. Some isolates were closely related to each other (more than 85% similarity). However, two isolates (Isolate 11 and Isolate 14) which originated from abattoir B were only distantly related to other isolates (less than 55% similarity). These two isolates were obtained from farms in distant geographic areas. PFGE patterns of the isolates containing all four virulence genes had an 88.3% similarity.

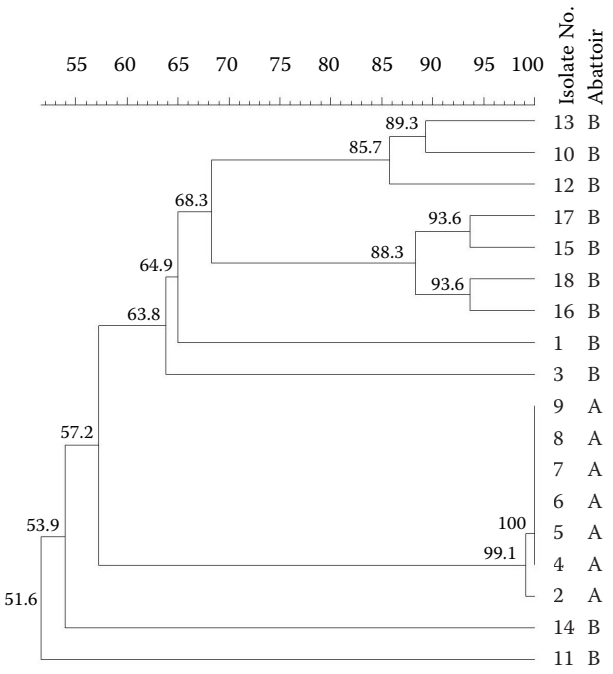

Figure 1. Dendrogram showing PFGE analysis of the 18 STEC O157:H7 isolates

DISCUSSION

PFGE has been reported to be a highly sensitive test to determine the genetic relatedness between STEC O157:H7 isolates (Gauntom 1997; Liebana et al. 2003). PFGE primarily detects insertions and deletions in genomic segments of STEC O157 (Lenehan et al. 2009). It has been used as the standard subtyping technique for STEC O157:H7 in many countries. In Spain, analysis of 108 STEC O157:H7 isolates from cattle, sheep and humans revealed 53 different PFGE patterns (Mora et al. 2004). PFGE analysis of cattle and human isolates in Japan, showed that over 95% of isolates were homologous (Kawano et al. 2012). In the USA, 15 different PFGE profiles were determined in 254 *E. coli* O157:H7 isolates from cattle (Jacob et al. 2011). In Argentina, 280 *E. coli* O157:H7 strains isolated from humans and cattle showed 148 PFGE patterns, and the strains were found to be 75% similar (D'Astek et al. 2012). In England and Wales, 57 PFGE patterns were found among 228 *E. coli* O157:H7 isolates from cattle (Liebana et al. 2003). In Sweden, 29 different PFGE patterns were identified among 45 *E. coli* O157:H7 cattle strains (Eriksson et al. 2010). In the present study, 13 different PFGE patterns were detected in the STEC O157:H7 isolates. The most common PFGE pattern (X1) was found in 33.3% of the STEC O157:H7 isolates. To our knowledge, this is first report of PFGE genotypes of STEC O157:H7 cattle isolates in Turkey. Because there is no previous study comparing PFGE genotypes of STEC O157:H7 isolates from humans, cattle, and food products in Turkey, we were not able to compare our PFGE results.

doi: 10.17221/45/2016-VETMED

In recent years, multi-locus variable number tandem repeat analysis (MLVA) typing has been used as an alternative to PFGE typing of STEC O157:H7. MLVA is more rapid and less labour-intensive than PFGE (Prendergast et al. 2011). In a study conducted by Eriksson et al. (2010), it was reported that MLVA produced similar results to PFGE typing with respect to the clustering of *E. coli* O157:H7 strains. Nucleotide polymorphism-derived genotyping and PFGE are highly effective in assessing the evolutionary relatedness of epidemiologically unrelated STEC O157 genetic subtypes (Bono et al. 2012). In the present study, we used the *Xba*I restriction enzyme for the digestion of DNA. When using a single restriction enzyme, PFGE might not determine the genetic relatedness between *E. coli* O157:H7 strains with apparently similar PFGE patterns (Lenahan et al. 2009).

In the present study, isolates showing different PFGE patterns were found to have the same virulence genes. This is in agreement with the findings of other researchers (Scott et al. 2006; Cobbaut et al. 2011). All of the isolates harboured the *stx2*, *eae* and *hlyA* genes and four of the isolates had all the virulence genes (*stx1*, *stx2*, *eae* and *hlyA*). The prevalence of *stx1* was found to be much higher than that of *stx2* in diarrhoeic calves (Wieler et al. 1996). *E. coli* strains producing Shiga toxin 2 were reportedly more effective in causing haemolytic-uremic syndrome infections in humans compared to *E. coli* strains producing Shiga toxin 1 (Chui et al. 2015). The co-existence of *stx*, *eae* and *hlyA* genes in an *E. coli* strain indicates higher pathogenicity for humans (Posse et al. 2007).

In the present study, we detected that six isolates (Isolates 4, 5, 6, 7, 8 and 9) were identical (100% similarity), sharing the same PFGE pattern and virulence type. These six isolates originated from different farms in the same region. The results of this study support the conclusion that a single strain of *E.coli* O157:H7 can be transmitted between farms. Some isolates shared more than 85% similarity. Our results show that these isolates were highly related and suggest that they are likely to be one and the same strain. High similarity between PFGE patterns was reported to result from horizontal transmission among animals (Sanchez et al. 2010). Variations in PFGE patterns among *E. coli* O157:H7 strains are thought to be primarily a result of insertions, deletions, or duplications of discrete genomic segments containing *XbaI* restriction sites (Lenahan et al. 2009). Minor genetic changes between *E. coli* O157:H7 isolates may occur due to the loss of a plasmid or a phage (Scott et al. 2006). On the other hand, PFGE patterns of two isolates originating from abattoir B were only distantly related to other isolates (less than 55% similarity). These two isolates originated from farms in distant geographic areas. This study presents data on the genetic diversity of only 18 STEC O157:H7 isolates from cattle in eastern Turkey. Hence, additional studies are needed in this area.

In the present study, although STEC O157:H7 isolates from animals slaughtered in abattoir A showed two PFGE patterns, STEC O157:H7 isolates from animals slaughtered in abattoir B showed 11 PFGE patterns. This difference may be due to the fact that animals slaughtered in abattoir B were raised in a range of different locations in the east of Turkey.

In conclusion, our study demonstrates that clinically healthy cattle are important carriers of STEC O157:H7, and that STEC O157:H7 isolates of cattle origin have high genetic diversity. During the slaughter of animals carrying STEC O157:H7 in abattoirs, animal carcasses may become contaminated. This creates a potential public health hazard. Therefore, hygiene must be maintained in farms, abattoirs, and the food industry, and also consumers must be educated. Comprehensive studies are needed to determine the genetic relatedness between human, animal and food isolates in Turkey.

Acknowledgement

The authors thank to Dr. Chitrita DebRoy (The Pennsylvania State University, USA) for her valuable help.

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Received: 2016–03–04
Accepted after corrections: 2016–10–14

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